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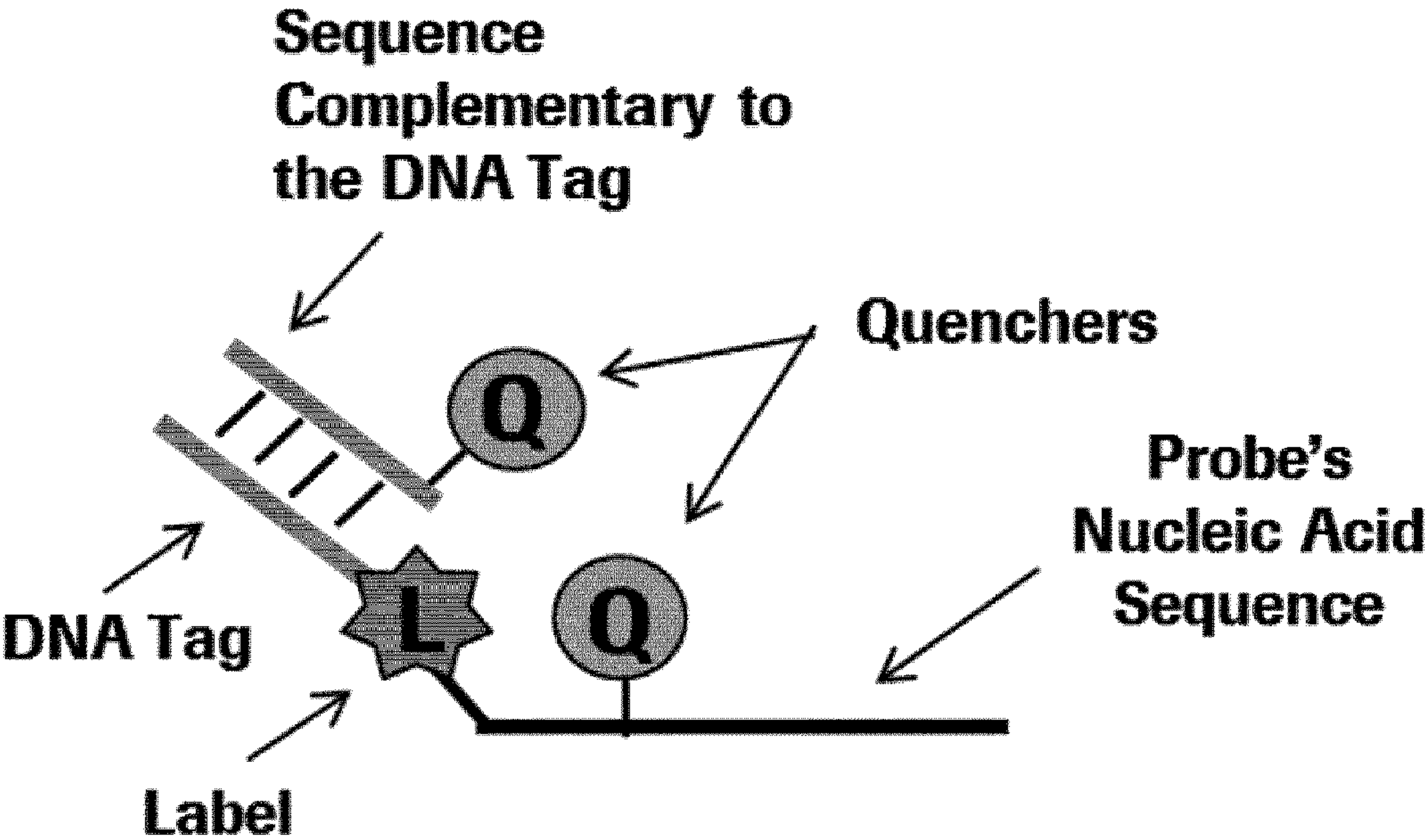


FIG. 1

(57) **Abrégé/Abstract:**
The present invention describes methods for performing higher multiplexed PCR for detection and quantitation of target nucleic acids using tagged hydrolysis probes.

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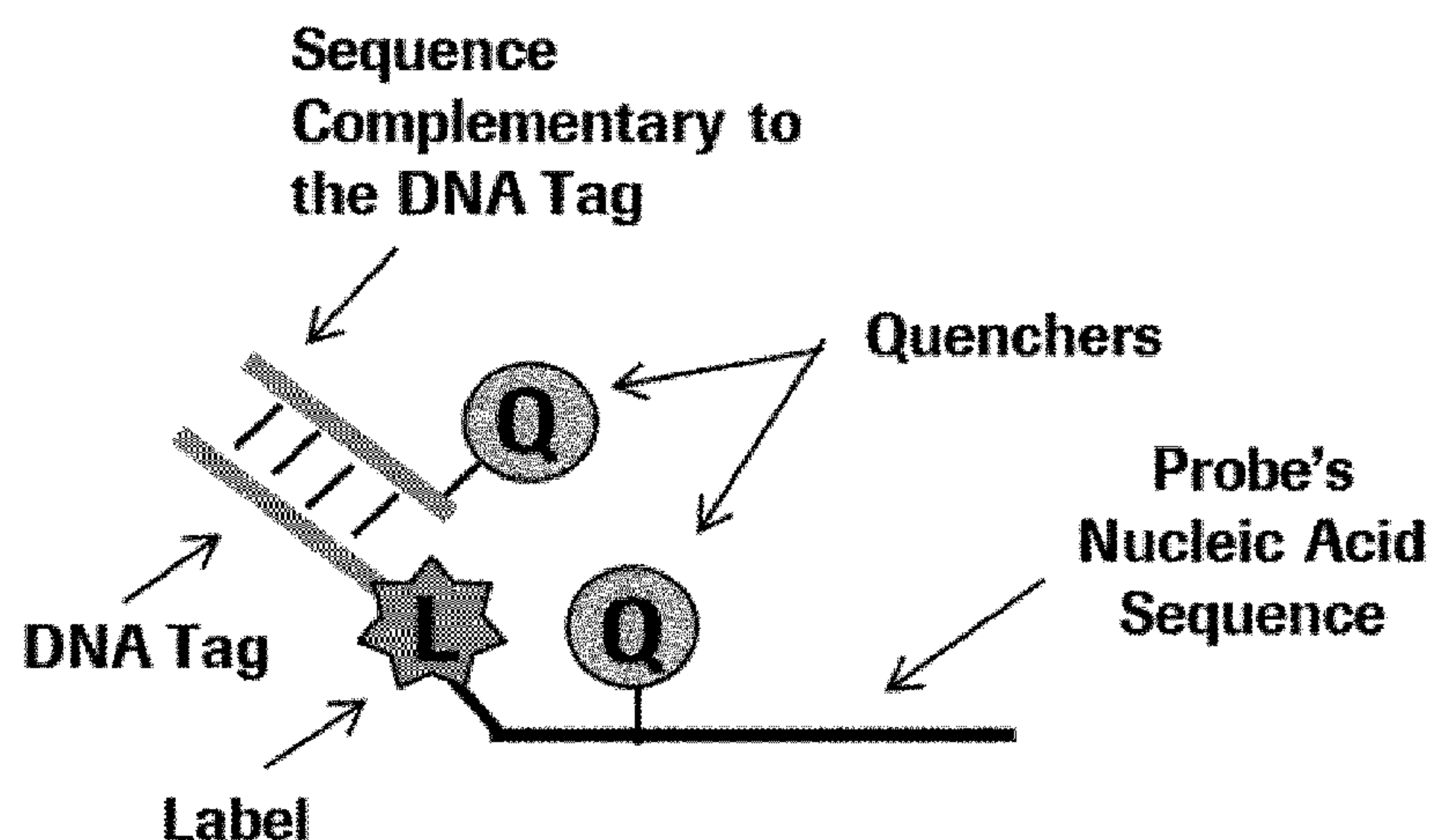


FIG. 1

(57) Abstract: The present invention describes methods for performing higher multiplexed PCR for detection and quantitation of target nucleic acids using tagged hydrolysis probes.

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METHODS FOR PERFORMING MULTIPLEXED PCR

FIELD OF THE INVENTION

The present invention relates to methods for polymerase chain reaction (PCR)
5 particularly to methods for performing multiplexed PCR.

BACKGROUND OF THE INVENTION

The polymerase chain reaction (PCR) has become a ubiquitous tool of biomedical research, disease monitoring and diagnostics. Amplification of nucleic acid sequences by PCR is described in U.S. Patent Nos. 4,683,195, 4,683,202, and 4,965,188. PCR is
10 now well known in the art and has been described extensively in the scientific literature. See PCR Applications, ((1999) Innis et al., eds., Academic Press, San Diego), PCR Strategies, ((1995) Innis et al., eds., Academic Press, San Diego); PCR Protocols, ((1990) Innis et al., eds., Academic Press, San Diego), and PCR Technology, ((1989) Erlich, ed., Stockton Press, New York).

15 A “real-time” PCR assay is able to simultaneously amplify and detect and quantify the starting amount of the target sequence. A typical real-time PCR protocol involves the use of a labeled probe, specific for each target sequence. The probe is preferably labeled with one or more fluorescent moieties, which absorb and emit light at specific wavelengths. Upon hybridizing to the target sequence or its amplicon, the probe
20 exhibits a detectable change in fluorescent emission as a result of probe hybridization or hydrolysis.

The major challenge of a PCR assay however remains the ability to analyze numerous targets in a single tube. In virtually every field of medicine and diagnostics, the number of loci of interest increases rapidly. For example, multiple loci must be analyzed in

forensic DNA profiling, pathogenic microorganism detection, multi-locus genetic disease screening and multi-gene expression studies, to name a few.

With the majority of current methods, the ability to multiplex an assay is limited by the detection instruments. Specifically, the use of multiple probes in the same reaction
5 requires the use of distinct fluorescent labels. To simultaneously detect multiple probes, an instrument must be able to discriminate among the light signals emitted by each probe. The majority of current technologies on the market do not permit detection of more than four to seven separate wavelengths in the same reaction vessel. Therefore, using one uniquely-labeled probe per target, no more than four to seven separate
10 targets can be detected in the same vessel. In practice, at least one target is usually a control nucleic acid. Accordingly, in practice, no more than three to six experimental targets can be detected in the same tube. The use of fluorescent dyes is also limited due to the spectral width where only about six or seven dyes can be fit within the visible spectrum without significant overlap interference. Thus the ability to multiplex an
15 assay will not keep pace with the clinical needs, unless radical changes in the amplification and detection strategy are made.

An additional ability to multiplex an amplification reaction is provided by a post-PCR melting assay. See U.S. patent application Serial No. 11/474,071, filed on June 23, 2006. In a melting assay, the amplified nucleic acid is identified by its unique melting profile.
20 A melting assay involves determining the melting temperature (melting point) of a double-stranded target, or a duplex between the labeled probe and the target. As described in U.S. Patent No. 5,871,908, to determine melting temperature using a fluorescently labeled probe, a duplex between the target nucleic acid and the probe is gradually heated (or cooled) in a controlled temperature program. The dissociation of
25 the duplex changes the distance between interacting fluorophores or between a fluorophore and a quencher. The interacting fluorophores may be conjugated to separate probe molecules, as described in U.S. Patent No. 6,174,670. Alternatively, one

fluorophore may be conjugated to a probe, while the other fluorophore may be intercalated into a nucleic acid duplex, as described in U.S. Patent No. 5,871,908. As yet another alternative, the fluorophores may be conjugated to a single probe oligonucleotide. Upon the melting of the duplex, the fluorescence is quenched as the
5 fluorophore to the quencher are brought together in the now single-stranded probe.

The melting of the nucleic acid duplex is monitored by measuring the associated change in fluorescence. The change in fluorescence may be represented on a graph referred to as “melting profile.” Because different probe-target duplexes may be designed to melt (or reanneal) at different temperatures, each probe will generate a unique melting
10 profile. Properly designed probes would have melting temperatures that are clearly distinguishable from those of the other probes in the same assay. Many existing software tools enable one to design probes for a same-tube multiplex assay with these goals in mind. For example, Visual OMP™ software (DNA Software, Inc., Ann Arbor, Mich.) enables one to determine melting temperatures of nucleic acid duplexes under
15 various reaction conditions.

The method of multiplex PCR using color detection and subsequent post-amplification melting assay is described in U.S. Patent No. 6,472,156. The number of targets detectable by such a method is a product of the number of detectable wavelengths and the number of distinguishable melting profiles. Therefore adding a melting assay to
20 color detection was a step forward in the ability to detect multiple targets.

The post-amplification melting assay is most commonly used for qualitative purposes, i.e. to identify target nucleic acids, see U.S. Patent Nos. 6,174,670, 6,427,156 and 5,871,908. It is known to obtain a melting peak by differentiating the melting curve function. Ririe et al. (“Product differentiation by analysis of DNA melting curves during
25 the polymerase chain reaction,” (1997) Anal. Biochem. 245:154-160) observed that differentiation helps resolve melting curves generated by mixtures of products. After differentiation, the melting peaks generated by each component of the mixture become

easily distinguishable. It was also previously known that the post-amplification melting signal, i.e. melting peak, is higher in proportion to the amount of the nucleic acid in the sample. For example, U.S. Patent No. 6,245,514 teaches a post-amplification melt assay using a duplex-intercalating dye, to generate a derivative melting peak, and then, using
5 proprietary software, to integrate the peak. The integration provides information about the efficiency of amplification and relative amount of the amplified nucleic acid.

In practice, it would be desirable to move beyond a qualitative assay and be able to quantify multiple targets in the same sample. See e.g. Sparano et al. "Development of the 21-gene assay and its application in clinical practice and clinical trials," J. Clin.
10 Oncol. (2008) 26(5):721-728. The ability to quantify the amount of target is useful in clinical applications, such as determination of viral load in a patient's serum, measuring the level of expression of a gene in response to drug therapy or determining the molecular signature of a tumor to predict its response to therapy.

Many methods for detection of target nucleic acids are known. Currently available
15 homogeneous assays for nucleic acid detection include the TaqMan®, Amplifluor®, dye-binding, allele-selective kinetic PCR and Scorpion® primer assays. These assay procedures are not readily multiplexed due to the requirement for a different dye for each target nucleic acid to be detected, and thus are limited in their potential for improvement. To overcome such limitations, several recent studies have disclosed the
20 use of oligonucleotide probes containing a cleavable "tag" portion which can be readily separated and detected (e.g. see Chenna et al, U.S. Patent Application Publication No. 2005/0053939; Van Den Boom, U.S. Patent No. 8,133,701). More recently, improved methods to perform multiplexed nucleic acid target identification by using structure based oligonucleotide probe cleavage have been described in U.S. Patent Application
25 Publication No. 2014/0272955, U.S. 2015/0176075, and U.S. 2015/0376681. Further methods to detect target nucleic acid sequence from DNA or a mixture of nucleic acid by the use of a combination of "Probing and Tagging Oligonucleotide" (PTO) and

“Capturing and Templating Oligonucleotide” (CTO) in a so-called PTO Cleavage and Extension assay have been described by Chun et al. in U.S. Patent No. 8,809,239.

However the need still exists for an accurate method to perform high throughput multiplex detection of target nucleic acids. It would particularly be an advantage to perform high throughput multiplex detection of target nucleic acids in an end-point PCR assay whereby detection of the amplified products need to occur only once, at the end (or nearly the end) of the PCR assay or after the completion of the PCR assay.

SUMMARY OF THE INVENTION

- 10 The present invention provides for novel methods for nucleic acid sequence detection, particularly detection of multiple target nucleic acids wherein a PCR reaction is run until it reaches an end-point beyond the log phase of amplification, (e.g. in the plateau phase). The methods are performed by the use of novel oligonucleotide probes having two unique features, a non-complementary tag portion and a quenching molecule.
- 15 Therefore in one aspect, the invention provides for a method for amplification and detection of a target nucleic acid in a sample comprising the steps of: (a) contacting the sample containing the target nucleic acid in a single reaction vessel with (i) one pair of oligonucleotide primers, each oligonucleotide primer capable of hybridizing to opposite strands of a subsequence of the target nucleic acid; (ii) an oligonucleotide probe that
- 20 comprises an annealing portion and a tag portion, wherein the tag portion comprises a nucleotide sequence non-complementary to the target nucleic acid sequence or a non-nucleotide molecule, wherein the annealing portion comprises a nucleotide sequence at least partially complementary to the target nucleic acid sequence and hybridizes to a region of the subsequence of the target nucleic acid that is bounded by the pair of
- 25 oligonucleotide primers, wherein the probe further comprises an interactive dual label comprising a reporter moiety located on the tag portion or on the annealing portion and a first quencher moiety located on the annealing portion and wherein the reporter

moiety is separated from the first quencher moiety by a nuclease susceptible cleavage site; and wherein the tag portion is reversibly bound in a temperature-dependent manner to a quenching molecule that comprises or is associated with one or more quencher moieties capable of quenching the reporter moiety when the quenching molecule is bound to the tag portion; (b) amplifying the target nucleic acid by PCR using a nucleic acid polymerase having 5' to 3' nuclease activity such that during an extension step of each PCR cycle, the nuclease activity of the polymerase allows cleavage and separation of the reporter moiety from the first quenching moiety on the annealing portion of the probe; (c) measuring one or more signals from the reporter moiety at a first temperature at which the quenching molecule is bound to the tag portion; (d) measuring one or more signals from the reporter moiety at a second temperature, which is higher than the first temperature, at which the quenching molecule is not bound to the tag portion; (e) obtaining a calculated signal value by subtracting a median or average of the one or more signals detected at the first temperature from a median or average of the one or more signal detected at the second temperature; whereby a calculated signal value that is higher than a threshold signal value allows determination of the presence of the target nucleic acid. In one embodiment, PCR amplification is allowed to reach an end-point beyond the log phase of amplification (e.g. in the plateau phase).

20 In one embodiment, the tag portion comprises a modification such that it is not capable of being extended by a nucleic acid polymerase. In one embodiment, the reporter moiety is on the tag portion of the oligonucleotide probe. In another embodiment, the reporter moiety is located on the annealing portion of the oligonucleotide probe and is able to interact in a temperature-dependent manner to the quenching molecule that

25 comprises the second quencher moiety. In one embodiment, the tag portion comprises a nucleotide sequence non-complementary to the target nucleic acid sequence and the quenching molecule is an oligonucleotide comprising a nucleotide sequence at least partially complementary to the tag portion of the oligonucleotide probe and binds to

the tag portion by hybridization. In another embodiment, the tag portion of the oligonucleotide probe or the quenching molecule or both the tag portion and the quenching molecule contain one or more nucleotide modifications. In yet another embodiment, the one or more nucleotide modifications is selected from the group
5 consisting of Locked Nucleic Acid (LNA), Peptide Nucleic Acid (PNA), Bridged Nucleic Acid (BNA), 2'-O alkyl substitution, L-enantiomeric nucleotide, or combinations thereof. In one embodiment, the reporter moiety is a fluorescent dye and the quencher moiety quenches a detectable signal from the fluorescent dye.

In another aspect, the invention provides for a method for detecting two or more target
10 nucleic acid sequences in a sample comprising the steps of: (a) contacting the sample suspected of containing the two or more target nucleic acid sequences in a single reaction vessel with (i) a first pair of oligonucleotide primers with nucleotide sequences that are complementary to each strand of a first target nucleic acid sequence, and a second pair of oligonucleotide primers with nucleotide sequences that are
15 complementary to each strand of a second target nucleic acid sequence; (ii) a first oligonucleotide probe comprising a nucleotide sequence at least partially complementary to the first target nucleic acid sequence and anneals within the first target nucleic acid sequence bounded by the first pair of oligonucleotide primers, wherein the first oligonucleotide probe comprises a fluorescent moiety capable of
20 generating a detectable signal and a first quencher moiety capable of quenching the detectable signal generated by the fluorescent moiety, wherein the fluorescent moiety is separated the first quencher moiety by a nuclease susceptible cleavage site ; (iii) a second oligonucleotide probe comprising two distinct portions, an annealing portion comprising a nucleotide sequence at least partially complementary to the second target
25 nucleic acid sequence and anneals within the second target nucleic acid sequence bounded by the second pair of oligonucleotide primers, wherein the annealing portion comprises a second quencher moiety; and a tag portion attached to the 5' terminus or to the 3' terminus of the annealing portion or attached via a linker to a region of the

annealing portion and comprising a nucleotide sequence that is non-complementary to the two or more target nucleic acid sequences, wherein the tag portion comprises a fluorescent moiety that is identical to the fluorescent moiety on the first oligonucleotide probe and whose detectable signal is capable of being quenched by the second quencher moiety on the annealing portion, wherein the fluorescent moiety is separated from the second quenching moiety by a nuclease susceptible cleavage site; (iv) a quenching oligonucleotide comprising a nucleotide sequence at least partially complementary to the tag portion of the second oligonucleotide probe and hybridizes to the tag portion to form a duplex, wherein the quenching oligonucleotide comprises a third quencher moiety which quenches the detectable signal generated by the fluorescent moiety on the tag portion when the quenching oligonucleotide is hybridized to the tag portion; (b) amplifying the first and second target nucleic acid sequences by polymerase chain reaction (PCR) using a nucleic acid polymerase having 5' to 3' nuclease activity such that during an extension step of each PCR cycle, the 5' to 3' nuclease activity of the nucleic acid polymerase allows cleavage and separation of the fluorescent moiety from the first quenching moiety on the first oligonucleotide probe, and cleavage and separation of the fluorescent moiety on the tag portion from the second quenching moiety on the annealing portion of the second oligonucleotide probe, wherein at the extension step the quenching oligonucleotide remains hybridized to the tag portion; (c) measuring one or more fluorescent signals at a first temperature at which the quenching oligonucleotide is hybridized to the tag portion from the second oligonucleotide probe; (d) measuring one or more fluorescent signals at a second temperature, which is higher than the first temperature, at which the quenching oligonucleotide is not hybridized to the tag portion from the second oligonucleotide probe; (e) obtaining a calculated signal value by subtracting a median or average of the one or more fluorescent signals detected at the first temperature from a median or average of the one or more fluorescent signal detected at the second temperature; whereby a value of the median or average of the one or more fluorescent signals

detected at the first temperature that is higher than a threshold value allows determination of the presence of the first target nucleic acid sequence and the calculated signal value that is higher than the threshold signal value allows determination of the presence of the second target nucleic acid. In one embodiment, PCR amplification is allowed to reach an end-point beyond the log phase of amplification (e.g. in the plateau phase).

In one embodiment, the tag portion comprises a modification such that it is not capable of being extended by a nucleic acid polymerase. In another embodiment, the tag portion is attached to the 5' terminus of the annealing portion. In yet another embodiment, the tag portion is attached to the 3' terminus of the annealing portion. In yet another embodiment, the tag portion is attached via a linker to a region of the annealing portion. In another embodiment, the tag portion of the second oligonucleotide probe or the quenching oligonucleotide or both the tag portion and the quenching oligonucleotide contain one or more nucleotide modifications. In yet another embodiment, the one or more nucleotide modifications is selected from the group consisting of Locked Nucleic Acid (LNA), Peptide Nucleic Acid (PNA), Bridged Nucleic Acid (BNA), 2'-O alkyl substitution, L-enantiomeric nucleotide, or combinations thereof.

In yet another aspect, the invention provides for a method for detecting two or more target nucleic acid sequences in a sample comprising the steps of: (a) contacting the sample suspected of containing the two or more target nucleic acid sequences in a single reaction vessel with (i) a first pair of oligonucleotide primers with sequences that are complementary to each strand of a first target nucleic acid sequence, and a second pair of oligonucleotide primers with sequences that are complementary to each strand of a second target nucleic acid sequence; (ii) a first oligonucleotide probe comprising two distinct portions, a first annealing portion comprising a sequence at least partially complementary to the first target nucleic acid sequence and anneals within the first

target nucleic acid sequence bounded by the first pair of oligonucleotide primers, wherein the first annealing portion comprises a first quencher moiety; and a first tag portion attached to the 5' terminus or to the 3' terminus of the first annealing portion or attached via a linker to a region of the first annealing portion and comprising a

5 nucleotide sequence that is non-complementary to the two or more target nucleic acid sequences, wherein the first tag portion comprises a fluorescent moiety whose detectable signal is capable of being quenched by the first quencher moiety on the first annealing portion, wherein the fluorescent moiety is separated from the first quenching moiety by a nuclease susceptible cleavage site; (iii) a first quenching oligonucleotide

10 comprising a sequence at least partially complementary to the first tag portion of the first oligonucleotide probe and hybridizes to the first tag portion to form a duplex, wherein the first quenching oligonucleotide comprises a second quenching moiety which quenches the detectable signal generated by the fluorescent moiety on the first tag portion when the first quenching oligonucleotide is hybridized to the first tag

15 portion; (iv) a second oligonucleotide probe comprising two distinct portions, a second annealing portion comprising a sequence at least partially complementary to the second target nucleic acid sequence and anneals within the second target nucleic acid sequence bounded by the second pair of oligonucleotide primers, wherein the second annealing portion comprises a third quencher moiety; and a second tag portion attached to the 5'

20 terminus or to the 3' terminus of the second annealing portion or attached via a linker to a region of the second annealing portion and comprising a nucleotide sequence that is non-complementary to the two or more target nucleic acid sequences, and has a different nucleic acid sequence or different nucleotide modifications compared to the nucleotide sequence of the first tag portion of the first oligonucleotide probe, wherein

25 the second tag portion comprises a fluorescent moiety that is identical to the fluorescent moiety on the first oligonucleotide probe and whose detectable signal is capable of being quenched by the third quencher moiety on the second annealing portion, wherein the fluorescent moiety is separated from the third quenching moiety by a nuclease

susceptible cleavage site; (v) a second quenching oligonucleotide comprising a sequence at least partially complementary to the second tag portion of the second oligonucleotide probe and hybridizes to the second tag portion to form a duplex, wherein the second quenching oligonucleotide comprises a fourth quenching moiety which quenches the detectable signal generated by the fluorescent moiety on the second tag portion when the second quenching oligonucleotide is hybridized to the second tag portion; wherein the duplex between the second quenching oligonucleotide and the second tag portion of the second oligonucleotide probe has a higher melting temperature (T_m) value than the duplex between the first quenching oligonucleotide and the first tag portion of the first oligonucleotide probe; (b) amplifying the first and second target nucleic acid sequences by polymerase chain reaction (PCR) using a nucleic acid polymerase having 5' to 3' nuclease activity such that during an extension step of each PCR cycle, the 5' to 3' nuclease activity of the nucleic acid polymerase allows (i) cleavage and separation of the fluorescent moiety on the first tag portion from the first quenching moiety on the first annealing portion of the first oligonucleotide probe, wherein at the extension step the first quenching oligonucleotide remains hybridized to the first tag portion, and (ii) cleavage and separation of the fluorescent moiety on the second tag portion from the third quenching moiety on the second annealing portion of the second oligonucleotide probe, wherein at the extension step the second quenching oligonucleotide remains hybridized to the second tag portion; (c) measuring one or more fluorescent signals at a first temperature at which the first quenching oligonucleotide is not hybridized to the first tag portion from the first oligonucleotide probe and the second quenching oligonucleotide remains hybridized to the second tag portion from the second oligonucleotide probe; (d) measuring one or more fluorescent signals at a second temperature, which is higher than the first temperature, at which the second quenching oligonucleotide is not hybridized to the second tag portion from the second oligonucleotide probe; (e) obtaining a calculated signal value by subtracting a median or average of the one or more fluorescent signals detected at the first temperature from

a median or average of the one or more fluorescent signals detected at the second temperature; whereby a value of the median or average of the one or more fluorescent signals detected at the first temperature that is higher than a threshold value allows determination of the presence of the first target nucleic acid sequence and the
5 calculated signal value that is higher than the threshold signal value allows determination of the presence of the second target nucleic acid. In one embodiment, PCR amplification is allowed to reach an end-point beyond the log phase of amplification (e.g. in the plateau phase).

In one embodiment, the first tag portion and the second tag portion both comprise a
10 modification such that both tag portions are not capable of being extended by a nucleic acid polymerase.

In one embodiment, the first tag portion is attached to the 3' terminus of the first annealing portion of the first oligonucleotide probe and the second tag portion is attached to the 3' terminus of the second annealing portion of the second
15 oligonucleotide probe. In another embodiment, the first tag portion is attached to the 3' terminus of the first annealing portion of the first oligonucleotide probe and the second tag portion is attached to the 5' terminus of the second annealing portion of the second oligonucleotide probe. In another embodiment, the first tag portion is attached to the 3' terminus of the first annealing portion of the first oligonucleotide probe and
20 the second tag portion is attached via a linker to a region of the second annealing portion of the second oligonucleotide probe.

In one embodiment, the first tag portion is attached to the 5' terminus of the first annealing portion of the first oligonucleotide probe and the second tag portion is attached to the 5' terminus of the second annealing portion of the second
25 oligonucleotide probe. In another embodiment, the first tag portion is attached to the 5' terminus of the first annealing portion of the first oligonucleotide probe and the second tag portion is attached to the 3' terminus of the second annealing portion of the

second oligonucleotide probe. In another embodiment, the first tag portion is attached to the 5' terminus of the first annealing portion of the first oligonucleotide probe and the second tag portion is attached via a linker to a region of the second annealing portion of the second oligonucleotide probe.

- 5 In one embodiment, the first tag portion is attached via a linker to a region of the first annealing portion of the first oligonucleotide probe and the second tag portion is attached to the 5' terminus of the second annealing portion of the second oligonucleotide probe. In another embodiment, the first tag portion is attached via a linker to a region of the first annealing portion of the first oligonucleotide probe and
- 10 the second tag portion is attached to the 3' terminus of the second annealing portion of the second oligonucleotide probe. In another embodiment, the first tag portion is attached via a linker to a region of the first annealing portion of the first oligonucleotide probe and the second tag portion is attached via a linker to a region of the second annealing portion of the second oligonucleotide probe.
- 15 In one embodiment, any of the first tag portion of the first oligonucleotide probe or the first quenching oligonucleotide or the second tag portion of the second oligonucleotide probe or the second quenching oligonucleotide or any combinations thereof contains one or more nucleotide modifications. In one embodiment, the one or more nucleotide modifications is selected from the group consisting of Locked Nucleic Acid (LNA),
- 20 Peptide Nucleic Acid (PNA), Bridged Nucleic Acid (BNA), 2'-O alkyl substitution, L-enantiomeric nucleotide, or combinations thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

- FIG. 1 is a graphical description of the oligonucleotide probe used to perform the
- 25 methods of the invention.

FIG. 2 is a graphical representation of the method of the invention that shows the separation of the tag portion and subsequent dissociation of the quenching oligonucleotide.

FIG. 3 is a description of one embodiment of the methods of the present invention.

5 FIG. 4 shows the signal detection temperatures using another embodiment of the methods of the present invention.

FIG. 5 shows different embodiments of the oligonucleotide probes used to practice the methods of the present invention.

FIG. 6 shows the results of the hybridization and dissociation at two temperatures
10 between a quenching oligonucleotide and a fluorescently labeled complementary oligonucleotide as described in Example 1.

FIG. 7 shows the detection of the IC target in increasing background of the HIV1-M target as described in Example 2.

FIG. 8 shows the detection of the HIV1-M gag target in increasing background of the
15 IC target as described in Example 2.

FIG. 9 shows the detection of the HIV1-M ltr target in increasing background of the IC target as described in Example 2.

FIG. 10 shows the results of detection of the IC sample in the experiment as described in Example 3. IC only detection in thermal channel 1 (58°C, FIG. 10A) versus thermal
20 channel 2 (83°C, FIG. 10B). Baseline subtraction was used where IC signal temperature correction and subtraction was applied in thermal channel 2. Thermal channel 1: threshold = 0.75, 84 copies IC detected, $p = 0.58$, $\lambda = 0.88$. Thermal channel 2: threshold = 1.00, 0 copies IC detected. $p = 0$, $\lambda = 0$.

FIG 11 shows the results of detection of the HIV1-M ltr sample in the experiment as described in Example 3. HIV-1 only detection in thermal channel 1 (58°C, FIG. 11A) versus thermal channel 2 (83°C, FIG. 11B) with baseline subtraction applied. Thermal channel 1: threshold = 0.75, 0 copies HIV1-M ltr detected, $p = 0$, $\lambda = 0$. Thermal channel 2:
 5 threshold = 1.00, 48 copies HIV1-M ltr detected, $p = 0.39$, $\lambda = 0.50$.

FIG 12 shows the results of dual target detection of IC and HIV1-M ltr samples in the experiment as described in Example 3. Both IC and HIV1-M were detected in thermal channel 1 (58°C, FIG. 12A) versus thermal channel 2 (83°C, FIG. 12B). Baseline subtraction, IC signal temperature correction and subtraction were applied in thermal
 10 channel 2. Thermal channel 1: threshold = 0.75, 82 copies IC detected, $p = 0.57$, $\lambda = 0.85$. Thermal channel 2: threshold = 1.00, 47 copies HIV1-M ltr detected, $p = 0.39$, $\lambda = 0.49$.

DETAILED DESCRIPTION OF THE INVENTION

DEFINITIONS

15 The term "sample" as used herein includes a specimen or culture (e.g., microbiological cultures) that includes nucleic acids. The term "sample" is also meant to include both biological and environmental samples. A sample may include a specimen of synthetic origin. Biological samples include whole blood, serum, plasma, umbilical cord blood, chorionic villi, amniotic fluid, cerebrospinal fluid, spinal fluid, lavage fluid (e.g.,
 20 bronchioalveolar, gastric, peritoneal, ductal, ear, arthroscopic), biopsy sample, urine, feces, sputum, saliva, nasal mucous, prostate fluid, semen, lymphatic fluid, bile, tears, sweat, breast milk, breast fluid, embryonic cells and fetal cells. In a preferred embodiment, the biological sample is blood, and more preferably plasma. As used herein, the term "blood" encompasses whole blood or any fractions of blood, such as
 25 serum and plasma as conventionally defined. Blood plasma refers to the fraction of whole blood resulting from centrifugation of blood treated with anticoagulants. Blood

serum refers to the watery portion of fluid remaining after a blood sample has coagulated. Environmental samples include environmental material such as surface matter, soil, water and industrial samples, as well as samples obtained from food and dairy processing instruments, apparatus, equipment, utensils, disposable and non-
5 disposable items. These examples are not to be construed as limiting the sample types applicable to the present invention.

The terms "target" or "target nucleic acid" as used herein are intended to mean any molecule whose presence is to be detected or measured or whose function, interactions or properties are to be studied. Therefore, a target includes essentially any molecule for
10 which a detectable probe (e.g., oligonucleotide probe) or assay exists, or can be produced by one skilled in the art. For example, a target may be a biomolecule, such as a nucleic acid molecule, a polypeptide, a lipid, or a carbohydrate, which is capable of binding with or otherwise coming in contact with a detectable probe (e.g., an antibody), wherein the detectable probe also comprises nucleic acids capable of being detected by
15 methods of the invention. As used herein, "detectable probe" refers to any molecule or agent capable of hybridizing or annealing to a target biomolecule of interest and allows for the specific detection of the target biomolecule as described herein. In one aspect of the invention, the target is a nucleic acid, and the detectable probe is an oligonucleotide. The terms "nucleic acid" and "nucleic acid molecule" may be used interchangeably
20 throughout the disclosure. The terms refer to oligonucleotides, oligos, polynucleotides, deoxyribonucleotide (DNA), genomic DNA, mitochondrial DNA (mtDNA), complementary DNA (cDNA), bacterial DNA, viral DNA, viral RNA, RNA, message RNA (mRNA), transfer RNA (tRNA), ribosomal RNA (rRNA), siRNA, catalytic RNA, clones, plasmids, M13, P1, cosmid, bacteria artificial chromosome (BAC), yeast
25 artificial chromosome (YAC), amplified nucleic acid, amplicon, PCR product and other types of amplified nucleic acid, RNA/DNA hybrids and polyamide nucleic acids (PNAs), all of which can be in either single- or double-stranded form, and unless otherwise limited, would encompass known analogs of natural nucleotides that can

function in a similar manner as naturally occurring nucleotides and combinations and/or mixtures thereof. Thus, the term "nucleotides" refers to both naturally-occurring and modified/nonnaturally-occurring nucleotides, including nucleoside tri, di, and monophosphates as well as monophosphate monomers present within polynucleic acid or oligonucleotide. A nucleotide may also be a ribo; 2'-deoxy; 2',3'-deoxy as well as a vast array of other nucleotide mimics that are well-known in the art. Mimics include chain-terminating nucleotides, such as 3'-O-methyl, halogenated base or sugar substitutions; alternative sugar structures including nonsugar, alkyl ring structures; alternative bases including inosine; deaza-modified; chi, and psi, linker-modified; mass label-modified; phosphodiester modifications or replacements including phosphorothioate, methylphosphonate, boranophosphate, amide, ester, ether; and a basic or complete internucleotide replacements, including cleavage linkages such a photocleavable nitrophenyl moieties.

The presence or absence of a target can be measured quantitatively or qualitatively. Targets can come in a variety of different forms including, for example, simple or complex mixtures, or in substantially purified forms. For example, a target can be part of a sample that contains other components or can be the sole or major component of the sample. Therefore, a target can be a component of a whole cell or tissue, a cell or tissue extract, a fractionated lysate thereof or a substantially purified molecule. Also a target can have either a known or unknown sequence or structure.

The term "amplification reaction" refers to any in vitro means for multiplying the copies of a target sequence of nucleic acid.

"Amplifying" refers to a step of submitting a solution to conditions sufficient to allow for amplification. Components of an amplification reaction may include, but are not limited to, e.g., primers, a polynucleotide template, polymerase, nucleotides, dNTPs and the like. The term "amplifying" typically refers to an "exponential" increase in target nucleic acid. However, "amplifying" as used herein can also refer to linear increases in

the numbers of a select target sequence of nucleic acid, but is different than a one-time, single primer extension step.

"Polymerase chain reaction" or "PCR" refers to a method whereby a specific segment or subsequence of a target double-stranded DNA, is amplified in a geometric progression.

5 PCR is well known to those of skill in the art; see, e.g., U.S. Pat. Nos. 4,683,195 and 4,683,202; and PCR Protocols: A Guide to Methods and Applications, Innis et al., eds, 1990.

"Oligonucleotide" as used herein refers to linear oligomers of natural or modified nucleosidic monomers linked by phosphodiester bonds or analogs thereof.

10 Oligonucleotides include deoxyribonucleosides, ribonucleosides, anomeric forms thereof, peptide nucleic acids (PNAs), and the like, capable of specifically binding to a target nucleic acid. Usually monomers are linked by phosphodiester bonds or analogs thereof to form oligonucleotides ranging in size from a few monomeric units, e.g., 3-4, to several tens of monomeric units, e.g., 40-60. Whenever an oligonucleotide is
15 represented by a sequence of letters, such as "ATGCCTG," it will be understood that the nucleotides are in 5'-3' order from left to right and that "A" denotes deoxyadenosine, "C" denotes deoxycytidine, "G" denotes deoxyguanosine, "T" denotes deoxythymidine, and "U" denotes the ribonucleoside, uridine, unless otherwise noted. Usually oligonucleotides comprise the four natural deoxynucleotides; however, they may also
20 comprise ribonucleosides or non-natural nucleotide analogs. Where an enzyme has specific oligonucleotide or polynucleotide substrate requirements for activity, e.g., single stranded DNA, RNA/DNA duplex, or the like, then selection of appropriate composition for the oligonucleotide or polynucleotide substrates is well within the knowledge of one of ordinary skill.

25 As used herein "oligonucleotide primer", or simply "primer", refers to a polynucleotide sequence that hybridizes to a sequence on a target nucleic acid template and facilitates the detection of an oligonucleotide probe. In amplification embodiments of the

invention, an oligonucleotide primer serves as a point of initiation of nucleic acid synthesis. In non-amplification embodiments, an oligonucleotide primer may be used to create a structure that is capable of being cleaved by a cleavage agent. Primers can be of a variety of lengths and are often less than 50 nucleotides in length, for example 12-
5 25 nucleotides, in length. The length and sequences of primers for use in PCR can be designed based on principles known to those of skill in the art.

The term "oligonucleotide probe" as used herein refers to a polynucleotide sequence capable of hybridizing or annealing to a target nucleic acid of interest and allows for the specific detection of the target nucleic acid.

10 A "reporter moiety" or "reporter molecule" is a molecule that confers a detectable signal. The detectable phenotype can be colorimetric, fluorescent or luminescent, for example. A "quencher moiety" or "quencher molecule" is a molecule that is able to quench the detectable signal from the reporter moiety.

A "mismatched nucleotide" or a "mismatch" refers to a nucleotide that is not
15 complementary to the target sequence at that position or positions. An oligonucleotide probe may have at least one mismatch, but can also have 2, 3, 4, 5, 6 or 7 or more mismatched nucleotides.

The term "polymorphism" as used herein refers to an allelic variant. Polymorphisms can include single nucleotide polymorphisms (SNP's) as well as simple sequence length
20 polymorphisms. A polymorphism can be due to one or more nucleotide substitutions at one allele in comparison to another allele or can be due to an insertion or deletion, duplication, inversion and other alterations known to the art.

The term "modification" as used herein refers to alterations of the oligonucleotide probe at the molecular level (e.g., base moiety, sugar moiety or phosphate backbone).
25 Nucleoside modifications include, but are not limited to, the introduction of cleavage blockers or cleavage inducers, the introduction of minor groove binders, isotopic

enrichment, isotopic depletion, the introduction of deuterium, and halogen modifications. Nucleoside modifications may also include moieties that increase the stringency of hybridization or increase the melting temperature of the oligonucleotide probe. For example, a nucleotide molecule may be modified with an extra bridge
5 connecting the 2' and 4' carbons resulting in locked nucleic acid (LNA) nucleotide that is resistant to cleavage by a nuclease (as described in Imanishi et al., U.S. Patent No. 6,268,490 and in Wengel et al., U.S. Patent No. 6,794,499. The compositions of the tag portion of the oligonucleotide probe and of the quenching oligonucleotide molecule are only restricted by their ability to form stable duplexes. These oligonucleotides can
10 therefore comprise of DNA, L-DNA, RNA, L-RNA, LNA, L-LNA, PNA(peptide nucleic acid, as described in Nielsen et al., U.S. Patent No. 5,539,082), BNA (bridged nucleic acid, for example, 2',4'-BNA(NC) [2'-O,4'-C-aminomethylene bridged nucleic acid] as described in Rahman et al., J. Am. Chem. Soc. 2008;130(14):4886-96), L-BNA etc. (where the "L-XXX" refers to the L-enantiomer of the sugar unit of the nucleic acids) or
15 any other known variations and modifications on the nucleotide bases, sugars, or phosphodiester backbones.

Other examples of nucleoside modifications include various 2' substitutions such as halo, alkoxy and allyloxy groups that are introduced in the sugar moiety of oligonucleotides. Evidence has been presented that 2'-substituted-2'-deoxyadenosine
20 polynucleotides resemble double-stranded RNA rather than DNA. Ikehara et al., (Nucleic Acids Res., 1978, 5, 3315) have shown that a 2'-fluoro substituent in poly A, poly I, or poly C duplexed to its complement is significantly more stable than the ribonucleotide or deoxyribonucleotide poly duplex as determined by standard melting assays. Inoue et al., (Nucleic Acids Res., 1987, 15, 6131) have described the synthesis of
25 mixed oligonucleotide sequences containing 2'-OMe (O-Methyl) substituents on every nucleic nucleotide. The mixed 2'-OMe-substituted oligonucleotide hybridized to its RNA complement as strongly as the RNA-RNA duplex which is significantly stronger than the same sequence RNA-DNA heteroduplex. Therefore, examples of substitutions

at the 2' position of the sugar include F, CN, CF₃, OCF₃, OMe, OCN, O-alkyl, S-alkyl, SMe, SO₂Me, ONO₂, NO₂, NH₃, NH₂, NH-alkyl, OCH₃=CH₂ and OCCH.

The term "specific" or "specificity" in reference to the binding of one molecule to another molecule, such as a probe for a target polynucleotide, refers to the recognition, contact, and formation of a stable complex between the two molecules, together with substantially less recognition, contact, or complex formation of that molecule with other molecules. As used herein, the term "anneal" refers to the formation of a stable complex between two molecules.

A probe is "capable of annealing" to a nucleic acid sequence if at least one region of the probe shares substantial sequence identity with at least one region of the complement of the nucleic acid sequence. "Substantial sequence identity" is a sequence identity of at least about 80%, preferably at least about 85%, more preferably at least about 90%, 95% or 99%, and most preferably 100%. For the purpose of determining sequence identity of a DNA sequence and a RNA sequence, U and T often are considered the same nucleotide. For example, a probe comprising the sequence ATCAGC is capable of hybridizing to a target RNA sequence comprising the sequence GCUGAU.

The term "cleavage agent" as used herein refers to any means that is capable of cleaving an oligonucleotide probe to yield fragments, including but not limited to enzymes. For methods wherein amplification does not occur, the cleavage agent may serve solely to cleave, degrade or otherwise separate the second portion of the oligonucleotide probe or fragments thereof. The cleavage agent may be an enzyme. The cleavage agent may be natural, synthetic, unmodified or modified.

For methods wherein amplification occurs, the cleavage agent is preferably an enzyme that possesses synthetic (or polymerization) activity and nuclease activity. Such an enzyme is often a nucleic acid amplification enzyme. An example of a nucleic acid amplification enzyme is a nucleic acid polymerase enzyme such as *Thermus aquaticus*

(Taq) DNA polymerase (TaqMan®) or *E. coli* DNA polymerase I. The enzyme may be naturally occurring, unmodified or modified.

A “nucleic acid polymerase” refers to an enzyme that catalyzes the incorporation of nucleotides into a nucleic acid. Exemplary nucleic acid polymerases include DNA
5 polymerases, RNA polymerases, terminal transferases, reverse transcriptases, telomerases and the like.

A “thermostable DNA polymerase” refers to a DNA polymerase that is stable (i.e., resists breakdown or denaturation) and retains sufficient catalytic activity when subjected to elevated temperatures for selected periods of time. For example, a
10 thermostable DNA polymerase retains sufficient activity to effect subsequent primer extension reactions, when subjected to elevated temperatures for the time necessary to denature double-stranded nucleic acids. Heating conditions necessary for nucleic acid denaturation are well known in the art and are exemplified in U.S. Pat. Nos. 4,683,202 and 4,683,195. As used herein, a thermostable polymerase is typically suitable for use in
15 a temperature cycling reaction such as the polymerase chain reaction (“PCR”). The examples of thermostable nucleic acid polymerases include *Thermus aquaticus* Taq DNA polymerase, *Thermus* sp. Z05 polymerase, *Thermus flavus* polymerase, *Thermotoga maritima* polymerases, such as TMA-25 and TMA-30 polymerases, Tth DNA polymerase, and the like.

20 A “modified” polymerase refers to a polymerase in which at least one monomer differs from the reference sequence, such as a native or wild-type form of the polymerase or another modified form of the polymerase. Exemplary modifications include monomer insertions, deletions, and substitutions. Modified polymerases also include chimeric polymerases that have identifiable component sequences (e.g., structural or functional
25 domains, etc.) derived from two or more parents. Also included within the definition of modified polymerases are those comprising chemical modifications of the reference sequence. The examples of modified polymerases include G46E E678G CS5 DNA

polymerase, G46E L329A E678G CS5 DNA polymerase, G46E L329A D640G S671F CS5 DNA polymerase, G46E L329A D640G S671F E678G CS5 DNA polymerase, a G46E E678G CS6 DNA polymerase, Z05 DNA polymerase, Δ Z05 polymerase, Δ Z05-Gold polymerase, Δ Z05R polymerase, E615G Taq DNA polymerase, E678G TMA-25
 5 polymerase, E678G TMA-30 polymerase, and the like.

The term "5' to 3' nuclease activity" or "5'-3' nuclease activity" refers to an activity of a nucleic acid polymerase, typically associated with the nucleic acid strand synthesis, whereby nucleotides are removed from the 5' end of nucleic acid strand, e.g., *E. coli* DNA polymerase I has this activity, whereas the Klenow fragment does not. Some
 10 enzymes that have 5' to 3' nuclease activity are 5' to 3' exonucleases. Examples of such 5' to 3' exonucleases include: Exonuclease from *B. subtilis*, Phosphodiesterase from spleen, Lambda exonuclease, Exonuclease II from yeast, Exonuclease V from yeast, and Exonuclease from *Neurospora crassa*.

The term "propanediol" or "propanediol spacer" refers to 1,3-Propanediol and is
 15 synonymous with Propane-1,3-diol, 1,3-Dihydroxypropane, and Trimethylene glycol. The term "HEG" or "HEG spacer" refers to hexaethylene glycol, which is synonymous with 3,6,9,12,15-Pentaoxaheptadecane-1,17-diol.

Various aspects of the present invention are based on a special property of nucleic acid polymerases. Nucleic acid polymerases can possess several activities, among them, a 5'
 20 to 3' nuclease activity whereby the nucleic acid polymerase can cleave mononucleotides or small oligonucleotides from an oligonucleotide annealed to its larger, complementary polynucleotide. In order for cleavage to occur efficiently, an upstream oligonucleotide must also be annealed to the same larger polynucleotide.

The detection of a target nucleic acid utilizing the 5' to 3' nuclease activity can be
 25 performed by a "TaqMan[®]" or "5'-nuclease assay", as described in U.S. Pat. Nos. 5,210,015; 5,487,972; and 5,804,375; and Holland et al., 1988, Proc. Natl. Acad. Sci. USA

88:7276-7280. In the TaqMan® assay, labeled detection probes that hybridize within the amplified region are present during the amplification reaction. The probes are modified so as to prevent the probes from acting as primers for DNA synthesis. The amplification is performed using a DNA polymerase having 5' to 3' exonuclease activity. During each synthesis step of the amplification, any probe which hybridizes to the target nucleic acid downstream from the primer being extended is degraded by the 5' to 3' exonuclease activity of the DNA polymerase. Thus, the synthesis of a new target strand also results in the degradation of a probe, and the accumulation of degradation product provides a measure of the synthesis of target sequences.

10 Any method suitable for detecting degradation product can be used in a 5' nuclease assay. Often, the detection probe is labeled with two fluorescent dyes, one of which is capable of quenching the fluorescence of the other dye. The dyes are attached to the probe, typically with the reporter or detector dye attached to the 5' terminus and the quenching dye attached to an internal site, such that quenching occurs when the probe
15 is in an unhybridized state and such that cleavage of the probe by the 5' to 3' exonuclease activity of the DNA polymerase occurs in between the two dyes. Amplification results in cleavage of the probe between the dyes with a concomitant elimination of quenching and an increase in the fluorescence observable from the initially quenched dye. The accumulation of degradation product is monitored by
20 measuring the increase in reaction fluorescence. U.S. Pat. Nos. 5,491,063 and 5,571,673 describe alternative methods for detecting the degradation of probe which occurs concomitant with amplification.

A 5' nuclease assay for the detection of a target nucleic acid can employ any polymerase that has a 5' to 3' exonuclease activity. Thus, in some embodiments, the polymerases
25 with 5'-nuclease activity are thermostable and thermoactive nucleic acid polymerases. Such thermostable polymerases include, but are not limited to, native and recombinant forms of polymerases from a variety of species of the eubacterial genera *Thermus*,

Thermatoga, and *Thermosipho*, as well as chimeric forms thereof For example, *Thermus* species polymerases that can be used in the methods of the invention include *Thermus aquaticus* (*Taq*) DNA polymerase, *Thermus thermophilus* (*Tth*) DNA polymerase, *Thermus* species Z05 (Z05) DNA polymerase, *Thermus* species sps17 (sps17), and

5 *Thermus* species Z05 (e.g., described in U.S. Pat. Nos. 5,405,774; 5,352,600; 5,079,352; 4,889,818; 5,466,591; 5,618,711; 5,674,738, and 5,795,762. *Thermatoga* polymerases that can be used in the methods of the invention include, for example, *Thermatoga maritima* DNA polymerase and *Thermatoga neapolitana* DNA polymerase, while an example of a *Thermosipho* polymerase that can be used is *Thermosipho africanus* DNA polymerase.

10 The sequences of *Thermatoga maritima* and *Thermosipho africanus* DNA polymerases are published in International Patent Application No. PCT/US91/07035 with Publication No. WO 92/06200. The sequence of *Thermatoga neapolitana* may be found in International Patent Publication No. WO 97/09451.

In the 5' nuclease assay, the amplification detection is typically concurrent with

15 amplification (i.e., "real-time"). In some embodiments the amplification detection is quantitative, and the amplification detection is real-time. In some embodiments, the amplification detection is qualitative (e.g., end-point detection of the presence or absence of a target nucleic acid). In some embodiments, the amplification detection is subsequent to amplification. In some embodiments, the amplification detection is

20 qualitative, and the amplification detection is subsequent to amplification, i.e. when amplification reaches an end-point beyond the log phase of amplification.

In the present invention, PCR amplification and end-point detection of two or more target nucleic acid in a single reaction vessel (e.g. tube, well) may be performed using a standard TaqMan® oligonucleotide probe for detecting the presence of the first target

25 and one or more novel TaqMan® oligonucleotide probes for detecting the presence of the second, third or more targets, with all the probes containing the same fluorescent label. Alternatively, the novel TaqMan® oligonucleotide probes may be used for

detecting the presence of all the target nucleic acids. The novel probes have two distinguishing features.

The first feature of the novel probe is that it comprises two distinct portions. The first portion is referred as an annealing portion and comprises a sequence that is at least
5 partially complementary to a target nucleic acid sequence such that it is capable of being hybridized with the target sequence. The annealing portion also contains a quencher moiety. In one embodiment, the annealing portion contains a reporter moiety such as a fluorescent dye that is capable of being quenched the quencher moiety and is separated from the quencher moiety by a nuclease susceptible cleavage site. The second portion of
10 the oligonucleotide probe is referred as a tag portion. In one embodiment, the tag portion is attached to the 5' terminus of the annealing portion. In another embodiment, the tag portion is attached to the 3' terminus of the annealing portion. In another embodiment, the tag portion is attached anywhere between the 5' terminus and the 3' terminus of the annealing portion via a linker. The tag portion may comprise
15 a nucleotide sequence that is not complementary to the target nucleic acid sequence and forms a "flap" region that is not capable of binding to the target nucleic acid (see FIG. 1 for a graphical representation of a 5' flap probe). The tag portion may also be comprised of non-nucleotides such as any organic moieties, or repeat units (e.g. (CH₂-CH₂-O)_n, etc.) as long as it can be attached to the annealing portion and can interact
20 with a quenching molecule (as described in the following section). In one embodiment, the tag portion contains a reporter moiety such as a fluorescent dye that is capable of being quenched by the quencher moiety on the annealing portion. The annealing and tag portions of the oligonucleotide probe may optionally be separated by a non-nucleotide linker. This linker can be comprised of carbon, carbon and oxygen, carbon
25 and nitrogen, or any combination of these and can be of any length. Furthermore, the linker can be comprised of a linear moiety or a cyclic moiety. The linker may be derived from a single unit or from multiple identical or different units separated by phosphate linkages. The purpose of the linker is to create a region at the junction of the annealing

and tag portions of the oligonucleotide probe. When the tag portion is separated from the annealing portion, the linker also prevents it from being extended by a nucleic acid polymerase. Alternatively, another modification on the separated tag portion renders it non-extendible by the nucleic acid polymerase.

- 5 The second feature of the novel probe is that the tag portion binds to a quenching molecule. If the tag portion is a nucleotide sequence, the quenching molecule may be an oligonucleotide that is fully or partially complementary to the nucleotide sequence of the tag portion and hybridizes to the tag portion. The quenching molecule also contains or is associated with a quencher moiety, i.e. a second quencher moiety, which is also
- 10 capable of quenching the signal from the reporter moiety (e.g. fluorescent dye) on the tag portion. The quencher moiety on or associated with the quenching molecule (the second quencher moiety) can be the same as or different from the quencher moiety on the annealing portion (the first quencher moiety). Therefore, prior to performing PCR amplification, the reporter moiety on the tag portion is quenched by both the quencher
- 15 moiety on the annealing portion of the probe and by the quencher moiety on or associated with the quenching molecule (e.g. by a quenching oligonucleotide as shown in FIG. 1).

The general principle of using the novel probe to perform PCR amplification and end-point detection of target nucleic acid in a 5' nuclease assay is described below. First, a

20 sample suspected of containing the target nucleic acid is provided. The sample is then contacted inside a single reaction vessel (e.g. a single test tube or a single well in a multi-well microplate) with PCR reagents that contain both the oligonucleotide primers capable of generating amplicons of the target nucleic acid and the novel oligonucleotide probe. PCR amplification begins by using a nucleic acid polymerase having 5' to 3'

25 nuclease activity such that during the extension step of each PCR cycle, the nuclease activity of the polymerase allows cleavage and separation of the tag portion from the quenching moiety on the annealing portion of the probe. The separated tag portion may

optionally contain a modification (such as the non-nucleotide linker) such that it is not capable of being extended by a nucleic acid polymerase.

When PCR assay has reached a level whereby sufficient amplicons have been produced, (e.g. beyond the log phase of amplification or in the plateau phase) or after the PCR
5 assay has been completed, the signal from the reporter moiety on the separated tag portion is measured at a first temperature, usually, the annealing and/or extension temperature, at which the quenching molecule is still bound to the tag portion. Due to the presence of the quencher moiety on or associated with the quenching molecule, the signal from the reporter moiety (e.g. a fluorescent dye) on the tag portion is still
10 quenched. In some instances, it may be advantageous to measure the signal from the reporter moiety more than once at the first temperature and obtain an average or median signal value at the first temperature. Then, a second signal reading is performed at a temperature point at which the quenching molecule is no longer bound to the tag portion. If the quenching molecule is an oligonucleotide that has sequences
15 complementary to the nucleotide sequence of the tag portion, this dissociation occurs at the melting temperature (T_m) of the duplex formation between the quenching oligonucleotide molecule and the tag portion. Signal from the reporter moiety which is no longer quenched by the quencher moiety on or associated with the quenching oligonucleotide is then measured at a second temperature that is at or above the T_m
20 temperature of the duplex. In fact, it may be better that the second temperature is above the T_m temperature to ensure that close to 100% of the tag portion are in single-stranded form. However, it is also possible to measure the signal at a temperature below the T_m temperature. In some instances, it may be advantageous to measure the signal from the reporter moiety more than once at the second temperature and obtain an
25 average or median signal value at the second temperature. Then, a calculated signal value is determined by subtracting the signal (or the average or median signal value) detected at the first temperature when the quenching molecule is still bound to the tag

portion from the signal (or the average or median signal value) detected at the second temperature when the quenching molecule is not bound to the tag portion (see FIG. 2 and FIG. 3). The calculated signal value may optionally be normalized for correction of signals that may be affected by temperature. For example, fluorescent signals are known to decrease at higher temperatures, and therefore, standards can be used to normalize the signal values obtained at different temperatures.

These signal measurements and calculations are performed usually at the end (or nearly the end) of the PCR assay or after the completion of the PCR assay and the determined signal values can be used to determine the presence or absence of the target nucleic acid.

One example of an end-point PCR assay is digital PCR (dPCR). In dPCR, a sample is partitioned so that individual nucleic acid molecules within the sample are localized and concentrated within many separate regions. The quenching or isolation of individual nucleic acid molecules has been performed in micro well plates, capillaries, the dispersed phase of an emulsion, and arrays of miniaturized chambers, as well as on nucleic acid binding surfaces. The partitioning of the sample allows one to estimate the number of different molecules by assuming that the molecule population follows the Poisson distribution. As a result, each part will contain "0" or "1" molecules, or a negative or positive reaction, respectively. After PCR amplification, nucleic acids may be quantified by counting the regions that contain PCR end-product, positive reactions. In conventional PCR, the number of PCR amplification cycles is proportional to the starting copy number. dPCR, however, is not dependent on the number of amplification cycles to determine the initial sample amount, eliminating the reliance on uncertain exponential data to quantify target nucleic acids and therefore provides absolute quantification. Current digital PCR approaches are achieved by either a microfluidic based approach where a reaction is partitioned into nano-wells on an array or by a droplet based approach where the reaction is separated into micro-droplets.

Commercially available systems employing the microfluidic approach include the Biomark HD system from Fluidigm Corporation (South San Francisco, CA) and the QuantStudio 3D dPCR system from Thermo Fisher Scientific (Waltham, MA). Other competitors in the commercial digital PCR landscape include RainDance Technologies
5 (Billerica, MA) with their Raindrop system and Bio-Rad (Hercules, CA) with their QX200 droplet based systems.

Multiplex PCR assays using only one reporter moiety (e.g. one fluorescent dye) is possible by designing oligonucleotide probes that have tag portions hybridized to their respective quenching oligonucleotide molecules at various T_m temperatures. For
10 example, amplification and detection of three target nucleic acid in one reaction can be achieved by using three oligonucleotide probes all labeled with the same fluorophore. A standard TaqMan® oligonucleotide probe may be used to detect the first target by measuring the fluorescent signal at a first temperature (usually the annealing temperature of a PCR cycle). A first “tagged” probe with a low T_m tag-quenching
15 oligonucleotide duplex may be used to detect the second target by measuring the calculated fluorescent value at a second temperature at or above its T_m temperature and that is higher than the first temperature. A second “tagged” probe with a high T_m tag-quenching oligonucleotide duplex may be used to detect the third target by measuring the calculated fluorescent value at a third temperature at or above its T_m temperature
20 and that is higher than the second temperature. (see FIG. 4) Theoretically, it would be possible to use one TaqMan® probe and two different tagged probes with four to seven different reporter moieties (e.g. fluorescent dyes) to detect between 12 and 21 different target nucleic acids in one reaction or one TaqMan® probe and 3 different tagged probes to detect between 16 and 28 different target nucleic acids in one reaction.

25 Additionally, the novel probes of the present invention can be designed such that the tag portion is a nucleotide sequence and is connected to a quenching oligonucleotide to form a hairpin (i.e. a stem-loop structure). In this structure, the “stem” portion will

consist of the complementary regions between the tag portion and quenching oligonucleotide while the “loop” portion may be comprised of non-complementary nucleotides or non-nucleotides such as linkers as previously described.

Although the novel probes of the present invention have been described as having the reporter moiety located on the tag portion of the probes, it is also possible to position the reporter moiety on the annealing portion and place the first quencher moiety on the tag portion, as long as the reporter moiety can reversibly interact with the second quencher moiety on the quenching oligonucleotide. In a general sense, the reporter moiety is designed and positioned in the probe oligonucleotide in such a way that it is separated from the first quenching moiety on the annealing portion during the 5' nuclease (TaqMan®) assay and further designed to reversibly interact with the second quenching moiety on the quenching molecule. Some of these various alternate embodiments of the novel probes can be seen in FIG. 5.

In order to practice the methods of the present invention, certain features are necessary in the design of the tag portion of the probe oligonucleotide and of the quenching molecule. In one embodiment, both the tag portion and the quenching molecule are comprised of nucleotide sequences. In this situation, both the tag portion and the quenching oligonucleotide should not hybridize specifically to the target nucleic acid sequence but they should be fully or partially complementary to each other to allow hybridization at the desired temperatures. Both may include a modification at their 3' termini in order to not be extended by the nucleic acid polymerase during PCR amplification. Both the reporter moiety (e.g. fluorescent dye) on the tag portion and the quencher moiety on the quenching oligonucleotide can be located at the 5' terminus, the 3' terminus or at any position between the 5' and 3' termini but they must be located in proximity to each other when the tag portion is hybridized to the quenching oligonucleotide to allow the quenching moiety to quench the detectable signal from the reporter moiety.

With respect to different tag portions being hybridized to their respective quenching oligonucleotide molecules at various T_m temperatures, modified nucleotides can be introduced at all or some positions on either the tag portions, on the quenching oligonucleotides or on both the tag portions and the quenching oligonucleotides such that oligonucleotide length can be shortened. Examples of nucleotide modifications that serve to increase the melting temperature include LNA, PNA, G-clamp (9-(aminoethoxy)-phenoxazine-2'-deoxycytidine), propynyl deoxyuridine (pdU), propynyl deoxycytidine (pdC), and various 2' modifications at the sugar group, such as 2'-O-Methyl modifications. Another type of modification that may serve to prevent the unwanted binding of nucleic acid polymerase to the tag portion or to the quenching oligonucleotide would include the use of enantiomeric L-form of a nucleotide, such as L-DNA, L-RNA or L-LNA.

In another embodiment, the tag portion of the oligonucleotide probe and the quenching molecular are comprised of non-nucleotide molecules that reversibly interact with each other in a temperature-dependent manner. Examples of such non-nucleotide interactions include but are not limited to protein-protein interactions, protein-peptide interactions (e.g. peptide aptamers), protein-small molecule interactions, peptide-small molecule interactions, small molecule-small molecule interactions. In one example, the well-known interaction between biotin and avidin (or streptavidin) can be exploited by modifying either the biotin moiety (e.g. desthiobiotin) or the avidin moiety (see, Nordlund et al., J. Biol. Chem., 2003, 278 (4) 2479-2483) or both in order to make the interaction reversible and temperature dependent.

In yet another embodiment, the interaction between the tag portion and the quenching molecule may involve interaction between a nucleotide sequence (or nucleotide sequences) and a non-nucleotide molecule in a sequence specific manner. Examples of these types of interactions include but are not limited to nucleic acid aptamers, DNA binding proteins or peptides and DNA minor groove binders. The design and synthesis

of sequence-specific DNA-binding molecules have been described in several papers (see e.g. Dervan, Science, 1986, 232, 464-471; White et al., Nature, 1998, 391, 468-471) and these methods may be used to generate interactions between the tag portion and the quenching molecule that are temperature-dependent. Similarly, interactions between double stranded nucleotides and soluble quenchers can also be explored such that the quenching moiety does not need to be contained within the quenching molecule itself but may be in a soluble form that will interact with and quench the reporter moiety only when the tag portion is bound to the quenching molecule.

Embodiments of the present invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

EXAMPLES

Example 1 Verification of Quenching by the Quenching Oligonucleotide

An experiment was performed to verify that a quenching oligonucleotide containing a quencher moiety would be able to hybridize with the fluorescently-labeled released tag portion of an oligonucleotide probe and quench the fluorescent signal at a temperature below the melting temperature of the duplex but not at a temperature above the melting temperature in which the duplex has been dissociated. Table 1 contains the nucleotide sequences of the tag portion and the quenching oligonucleotide. Quenching oligonucleotide Q0 does not contain the quencher whereas quenching oligonucleotide Q1 contains a BHQ-2 quencher at its 5' terminus.

TABLE 1

Name	Sequence	Modifications	SEQ ID NO:
9FAM9TAG	CGTCGCCAGTCAGCTCCGG 9F9T	9= C9 spacer, F = FAM	1
Q0	CCGGAGCTGACTGGCGACG p	p = phosphate	2
Q1	Q CCGGAGCTGACTGGCGACG p	p= phosphate, Q = BHQ-2	3

The 9FAM9 TAG oligonucleotide was incubated without a quenching oligonucleotide (QX) or with the Q0 or Q1 quenching oligonucleotide at 1:5 molar ratio. The mixtures were then cycled in 50μL reactions that consisted of 60mM Tricine, 120mM potassium acetate, 5.4% DMSO, 0.027% Sodium Azide, 3% glycerol, 0.02% Tween 20, 43.9uM
5 EDTA, 0.2U/uL UNG, 0.1uM 19TAGC9FAMC9, 0.5uM Q0 or Q1, 400μM dATP, 400μM 4CTP, 400μM dGTP, 800μM dUTP, and 3.3mM Manganese Acetate. Cycle conditions resembling a typical PCR amplification reaction are shown on Table 2.

TABLE 2

Step	Description	Cycle #	Temperature (°C)	Time	Data acquisition
1	Sterilization/RT	1	50	2 min	none
			94	5 sec	none
			55	2 min	none
			60	6 min	none
			65	4 min	none
2	Dark Cycles (no data acquisition)	5	95	5 sec	none
			55	30 sec	none
3	TaqMan Cycles	55	95	5 sec	none
			58	25 sec	fluorescence read
			80	5 sec	fluorescence read

10

The results of the experiment are shown on FIG. 6. When the signal from the FAM dye was measured at 58°C, fluorescence was detected with no quenching oligonucleotide (QX) or with a quenching oligonucleotide with no quenching moiety (Q0) but no signal was detected at any of the cycles in the presence of the Q1 quenching oligonucleotide.
15 In contrast, when fluorescence was measured at 80°C, signals could be detected in all cycles even in the presence of the Q1 quenching oligonucleotide, which demonstrates

that at the higher temperature, the Q1 quenching oligonucleotide was no longer hybridized with the TAG, and no quenching was observed.

Example 2 PCR with Tagged Probe and Quenching Oligonucleotide with End-Point Detection

5 A matrix experiment was performed wherein 0, 100, or 1,000 copies of an internal control (IC) template was amplified in the presence of 0, 10, or 100 copies of HIV1-M template over 50 cycles of PCR. Detection of three unique targets in a single fluorescence channel using fluorescein (FAM) was achieved through a combination of TaqMan® chemistry and thermal assisted generation of signal (TAGS) using tagged probes and their respective quenching oligonucleotides. Endpoint fluorescence signal (average fluorescence signal for last four cycles) was measured in the FAM fluorescence channel for all three targets post TaqMan® PCR. Table 3 shows the sequences of the probes and quenching oligonucleotides used in this experiment.

TABLE 3

Name	Sequence	Modifications	SEQ ID NO:
IC	QTGCGCGTCCCGTTTTGATACTTCGTAAC GGTGCFp	F = FAM, Q= BHQ-2, p= phosphate	4
HIV1-M gag	QMLMGLAGLMMLLMLAMMGAMGGMA MLMMMMMAALF <u>ΦE ΦE ΦEIIHH ΦE ΦΦH Φ</u> <u>ΦHI ΦIP</u>	F = FAM, Q= BHQ-2, p = phosphate, L=5-methyl-dC, M=5-propynyl-dU, E=L-DNA-A, Φ=L-DNA-C, H=L-DNA-G, I=L-DNA-T, <u>tag underlined</u>	5
HIV1-M ltr	QTCTCTAGCAGTGGCGCCCGAACAGGGA CKF <u>CACGTCTCGGTGGTGGCGTTTP</u>	F = FAM, Q = BHQ-2, p= phosphate, K = spacer-C9, <u>tag underlined</u>	6
Q-Tag gag	EHEΦHHΦHHIHΦΦEEIHHIHQp	Q= BHQ-2, p= phosphate, E=L-DNA-A, Φ=L-DNA-C, H=L-DNA-G, I=L-DNA-T	7
Q-Tag ltr	JNNNOROONOONOORNRRNORSRJQ	Q=BHQ-2, J=lysine, N=PNA-A, O=PNA-C, R=PNA-G, S=PNA-T	8

- Each 50 μ L reaction contained 3% glycerol, 60mM Tricine, 120mM potassium acetate, 400 μ M each dATP, dCTP and dGTP, 800 μ M dUTP, 0.3 μ M of each forward primer, 0.3 μ M of each reverse primer, 0.3 μ M of detection probe (sequence 1-3), 0.6 μ M of each Q-tag (Sequence 4-5) 5.4% DMSO, 0.02% Tween 20, 43.9 μ M EDTA, 0.03% sodium azide, 0.2U/ μ L uracil-N-glycosylase (UNG), 0.67 μ M NTQ21-46A aptamer, 0.9U/ μ L Z05D polymerase, and 3.3mM manganese acetate (pH 6.3). Amplification was done on a Roche LightCycler® 480 instrument and the reactions were subjected to the temperature profile as shown in Table 2 except 50 instead of 55 cycles were performed and data acquisition was performed only during the last four cycles (see below).
- 10 The results of the experiment are seen in FIGs 7-9 with endpoint absolute fluorescence plotted on the Y-axis with various samples across the X-axis. Fluorescence signal was observed at 58°C (FIG. 7, thermal channel 1) in the presence of IC target due to the cleavage of the conventional TaqMan® probe (IC, SEQ ID NO: 4) during repeated cycles of amplification. Additionally, fluorescence signal was observed at 80°C (FIG. 8,
- 15 thermal channel 2) when the HIV1-M gag target was present due to the tagged probe (HIV-1M gag, SEQ ID NO: 5) cleavage and increased fluorescence from melting of the probe cleavage fragment from the complementary quenching oligonucleotide (Q-Tag gag, SEQ ID NO: 7). Furthermore, fluorescence signal was observed at 95°C (FIG. 9,
- 20 thermal channel 3) when the HIV1-M ltr target was present due to cleavage of the second tagged probe (HIV-1M ltr, SEQ ID NO: 6) and increased fluorescence from melting of the probe cleavage fragment from the second complementary quenching oligonucleotide (Q-Tag ltr, SEQ ID NO: 8). Any samples with fluorescence signals below the defined fluorescence threshold signal per thermal channel (dotted lines) were considered as negative.
- 25 Temperature corrected thermal channel 1 (58°C) fluorescence was subtracted from thermal channel 2 (80°C) fluorescence to achieve specific signal for HIV1-M gag. Similarly, temperature corrected thermal channel 2 fluorescence was subtracted from

thermal channel 3 (95°C) fluorescence to achieve specific signal for HIV1-M ltr.

Therefore, it was observed that amplification of the IC target was specifically detected at 58°C (thermal channel 1), HIV1-M gag target was specifically detected at 80°C (thermal channel 2), and HIV1-M ltr target was specifically detected at 95°C (thermal channel 3).

5 *Example 3 Digital PCR with Tagged Probe and Quenching Oligonucleotide with End Point Detection*

An experiment was conducted in which approximately 47 copies of HIV1-M RNA or 83 copies of internal control RNA was amplified and detected individually or together across 96 wells on a 384 well plate. Two fluorescein labeled TaqMan® probes were
 10 present in each reaction. A first probe (TABLE 4, SEQ ID NO: 9) binds specifically to the internal control target and detects amplification at or below 58°C (thermal channel 1). A second probe (TABLE 4, SEQ ID NO: 10), with a 5' non-target binding tag sequence, binds specifically to HIV1-M ltr target and detects amplification in a second thermal channel defined at 83°C.

15 TABLE 4

Description	Sequence	Modifications	SEQ ID NO:
IC probe	FTGCGCGTCCCGTTTTGATA CTTCGTAACGGTGCQP	Q=BHQ-2, F=threoninol-Fam, P=phosphate	9
HIV1-M ltr probe	QTCTCTAGCAGTGGCGCCC GAACAGGGACFCACACATT <u>GGCACCGCCGTCTP</u>	Q=BHQ-2, F=threoninol-Fam, P=phosphate Underline= L-DNA modified bases	10
Q-tag	<u>AGACGGCGGTGCCAATGTG</u> <u>TGQP</u>	Q=BHQ-2, P=phosphate, Underline= L-DNA modified bases	11

Each 16 μ L reaction contained 3% glycerol, 60mM Tricine, 120mM potassium acetate, 400 μ M each dATP, dCTP and dGTP, 800 μ M dUTP, 0.3 μ M of each forward primer, 0.3 μ M of each reverse primer, 0.3 μ M of detection probe (SEQ ID NO: 9 & 10), 0.6 μ M of Q-tag (SEQ ID NO: 11) 5.4% DMSO, 0.02% Tween 20, 43.9 μ M EDTA, 0.03% sodium azide, 0.2U/ μ L uracil-N-glycosylase (UNG), 0.67 μ M NTQ21-46A aptamer, 0.9U/ μ L Z05D polymerase, and 3.3mM manganese acetate (pH 6.3).

PCR amplification was performed on a Roche LightCycler[®] 480 instrument. Reactions were subjected to the temperature profile as described in TABLE 2 in Example 1. Fluorescence was captured at each thermal channel prior to PCR cycling (to define baseline fluorescence) and post-PCR. Baseline subtracted fluorescence data was plotted and a threshold was defined to determine negative (no target present) versus positive (one or more targets present) wells at each thermal channel. When IC was present, fluorescence signal was observed in thermal channel 1 due to cleavage of the conventional IC probe and separation of reporter dye from quencher during repeated cycles of amplification (FIG. 10A). When HIV1-M ltr was present, fluorescence signal was observed in thermal channel 2 (FIG. 11B) due to the tagged HIV1-M ltr probe cleavage and thermal activation of signal due to the melting of the reporter dye labeled probe cleavage fragment from the complementary Q-tag. When both targets were present, signal was observed in both thermal channels (FIG. 12A and 12B). Accordingly, IC crossover signal was removed from thermal channel 2 via temperature correction and fluorescence subtraction, enabling detection of only HIV1-M in thermal channel 2 (FIG 12B).

The ratio of negative reactions for each set of 96 wells was applied to a Poisson calculation to determine total copies of each target across those wells. The poisson equation is based on $\lambda = -\ln(1-p)$, where λ is the copies calculated per well and p is the positive fraction of all wells. The total copies across 96 wells are calculated by multiplying λ by 96. Results are shown on TABLE 5 and demonstrate the unique

detection of the IC template versus the HIV1-M ltr template in a single fluorescence channel and two thermal channels at endpoint detection.

TABLE 5

Sample	Thermal Channel 1, 58°C	Thermal Channel 2, 83°C
IC only	84 copies	Not detected
HIV1-M only	Not detected	48 copies
IC + HIV1-M	82 copies	47 copies

5 INFORMAL SEQUENCE LISTING

SEQ ID NO 1: 9FAM9TAG oligonucleotide sequence

CGTCGCCAGTCAGCTCCGGT

SEQ ID NO 2: Q0 quenching oligonucleotide sequence (no quencher)

CCGGAGCTGACTGGCGACG

10 SEQ ID NO 3: Q1 quenching oligonucleotide sequence (BHQ-1 quencher on 5' terminus)

CCGGAGCTGACTGGCGACG

SEQ ID NO 4: IC TaqMan probe oligonucleotide sequence (FAM/BHQ/phosphate)

TGCGCGTCCCGTTTTGATACTTCGTAACGGTGC

15 SEQ ID NO 5: HIV1-M gag tagged probe oligonucleotide sequence (BHQ/FAM/phosphate)

TCTGCAGCTTCCTCATTGATGGTATCTTTTAACCACACATTGGCACCGCCGTCT

SEQ ID NO 6: HIV1-M ltr tagged probe oligonucleotide sequence (BHQ/FAM/phosphate)

20 TCTCTAGCAGTGGCGCCCGAACAGGGACCACGTCTCGGTGGTGGCGTTT

SEQ ID NO 7: HIV1-M gag quenching oligonucleotide sequence (BHQ-1 quencher on 3' terminus)

AGACGGCGGTGCCAATGTGTG

5 SEQ ID NO 8: HIV1-M ltr quenching oligonucleotide sequence (BHQ-1 quencher on 3' terminus)

AAACGCCACCACCGAGACGTG

SEQ ID NO 9: IC probe oligonucleotide sequence (from Example 3)

TGCGCGTCCCGTTTTGATACTTCGTAACGGTGC

SEQ ID NO 10: HIV1-M ltr probe oligonucleotide sequence (from Example 3)

10 TCTCTAGCAGTGGCGCCCGAACAGGGACCACACATTGGCACCGCCGTCT

SEQ ID NO 11: Q-Tag oligonucleotide sequence (from Example 3)

AGACGGCGGTGCCAATGTGTG

CLAIMS

1. A method for amplification and detection of a target nucleic acid in a sample comprising the steps of:
 - (a) contacting said sample containing said target nucleic acid in a single
5 reaction vessel with
 - (i) one pair of oligonucleotide primers, each oligonucleotide primer capable of hybridizing to opposite strands of a subsequence of said target nucleic acid;
 - (ii) an oligonucleotide probe that comprises an annealing portion and a tag
10 portion, wherein the tag portion comprises a nucleotide sequence non-complementary to the target nucleic acid sequence or a non-nucleotide molecule, wherein the annealing portion comprises a nucleotide sequence at least partially complementary to the target nucleic acid sequence and hybridizes to a region of said subsequence of said target nucleic acid that is
15 bounded by said pair of oligonucleotide primers, wherein said probe further comprises an interactive dual label comprising a reporter moiety located on said tag portion or on said annealing portion and a first quencher moiety located on said annealing portion and wherein said reporter moiety is separated from said first quencher moiety by a nuclease susceptible cleavage
20 site; and wherein said tag portion is reversibly bound in a temperature-dependent manner to a quenching molecule that comprises or associates with one or more quencher moieties capable of quenching said reporter moiety or on the annealing portion when said quenching molecule is bound to said tag portion;
 - (b) amplifying said target nucleic acid by PCR using a nucleic acid polymerase
25 having 5' to 3' nuclease activity such that during an extension step of each

PCR cycle, the nuclease activity of the polymerase allows cleavage and separation of the tag portion from the first quencher moiety on the annealing portion of the probe;

- 5 (c) measuring one or more signals from the reporter moiety at a first temperature at which the quenching molecule is bound to the tag portion;
- (d) measuring one or more signals from the reporter moiety at a second temperature, which is higher than the first temperature, at which the quenching molecule is not bound to the tag portion;
- 10 (e) obtaining a calculated signal value by subtracting a median or average of the one or more signals detected at the first temperature from a median or average of the one or more signals detected at the second temperature; whereby a calculated signal value that is higher than a threshold signal value allows determination of the presence of the target nucleic acid.
2. The method of claim 1 wherein PCR amplification of step (b) is allowed to reach
15 an end-point beyond the log phase of amplification.
3. The method of claim 1 wherein the reporter moiety is on the tag portion of the oligonucleotide probe or on the annealing portion of the oligonucleotide probe.
4. The method of any of claims 1 - 3 wherein the tag portion comprises a nucleotide sequence non-complementary to the target nucleic acid sequence and the
20 quenching molecule is an oligonucleotide comprising a nucleotide sequence at least partially complementary to the tag portion of the oligonucleotide probe and binds to the tag portion by hybridization.
5. The method of any of the preceding claims wherein the tag portion comprises a modification such that it is not capable of being extended by a nucleic acid
25 polymerase.

6. The method of any of the preceding claims wherein the tag portion of the oligonucleotide probe or the quenching molecule or both the tag portion and the quenching molecule contain one or more nucleotide modifications.
7. The method of any of the preceding claims wherein the one or more nucleotide
5 modifications is selected from the group consisting of Locked Nucleic Acid (LNA), Peptide Nucleic Acid (PNA), Bridged Nucleic Acid (BNA), 2'-O alkyl substitution, L-enantiomeric nucleotide, or combinations thereof.
8. The method of any of the preceding claims wherein the reporter moiety is a
10 fluorescent dye and the quencher moiety quenches a detectable signal from the fluorescent dye.
9. A method for detecting two or more target nucleic acid sequences in a sample comprising the steps of:
 - (a) contacting said sample suspected of containing said two or more target
nucleic acid sequences in a single reaction vessel with
 - 15 (i) a first pair of oligonucleotide primers with nucleotide sequences that are complementary to each strand of a first target nucleic acid sequence, and a second pair of oligonucleotide primers with nucleotide sequences that are complementary to each strand of a second target nucleic acid sequence;
 - (ii) a first oligonucleotide probe comprising a nucleotide sequence at least
20 partially complementary to the first target nucleic acid sequence and anneals within the first target nucleic acid sequence bounded by the first pair of oligonucleotide primers, wherein said first oligonucleotide probe comprises a fluorescent moiety capable of generating a detectable signal and a first quencher moiety capable of quenching the detectable signal generated

by the fluorescent moiety, wherein the fluorescent moiety is separated the first quencher moiety by a nuclease susceptible cleavage site ;

(iii) a second oligonucleotide probe comprising two distinct portions, an annealing portion comprising a nucleotide sequence at least partially complementary to the second target nucleic acid sequence and anneals within the second target nucleic acid sequence bounded by the second pair of oligonucleotide primers, wherein the annealing portion comprises a second quencher moiety; and a tag portion attached to the 5' terminus or to the 3' terminus of the annealing portion or attached via a linker to a region of the annealing portion and comprising a nucleotide sequence that is non-complementary to the two or more target nucleic acid sequences, wherein the tag portion comprises a fluorescent moiety that is identical to the fluorescent moiety on the first oligonucleotide probe and whose detectable signal is capable of being quenched by the second quencher moiety on the annealing portion, wherein said fluorescent moiety is separated from the second quenching moiety by a nuclease susceptible cleavage site;

(iv) a quenching oligonucleotide comprising a nucleotide sequence at least partially complementary to the tag portion of the second oligonucleotide probe and hybridizes to the tag portion to form a duplex, wherein said quenching oligonucleotide comprises a third quencher moiety which quenches the detectable signal generated by the fluorescent moiety on the tag portion when the quenching oligonucleotide is hybridized to the tag portion;

(b) amplifying the first and second target nucleic acid sequences by polymerase chain reaction (PCR) using a nucleic acid polymerase having 5' to 3' nuclease activity such that during an extension step of each PCR cycle, the 5' to 3' nuclease activity of the nucleic acid polymerase allows cleavage and

5 separation of the fluorescent moiety from the first quenching moiety on the first oligonucleotide probe, and cleavage and separation of the fluorescent moiety on the tag portion from the second quenching moiety on the annealing portion of the second oligonucleotide probe, wherein at the extension step the quenching oligonucleotide remains hybridized to the tag portion;

- (c) measuring one or more fluorescent signals at a first temperature at which the quenching oligonucleotide is hybridized to the tag portion from the second oligonucleotide probe;
- 10 (d) measuring one or more fluorescent signals at a second temperature, which is higher than the first temperature, at which the quenching oligonucleotide is not hybridized to the tag portion from the second oligonucleotide probe;
- (e) obtaining a calculated signal value by subtracting a median or average of the one or more fluorescent signals detected at the first temperature from a median or average of the one or more fluorescent signals detected at the
- 15 second temperature; whereby a value of the median or average of the one or more fluorescent signals detected at the first temperature that is higher than a threshold value allows determination of the presence of the first target nucleic acid sequence and the calculated signal value that is higher than the
- 20 threshold signal value allows determination of the presence of the second target nucleic acid.

10. The method of claim 9 wherein PCR amplification of step (b) is allowed to reach an end-point beyond the log phase of amplification.
11. The method of claim 10 wherein the tag portion is attached to the 5' terminus of
- 25 the annealing portion or to the 3' terminus of the annealing portion.

12. The method of any of claims 9 - 11 wherein the tag portion comprises a modification such that it is not capable of being extended by a nucleic acid polymerase.
13. The method of any of claims 9 - 12 wherein the quenching oligonucleotide is
5 connected to the tag portion of the second oligonucleotide probe via a stem-loop structure.
14. The method of any of claims 9 - 13 wherein the tag portion of the second oligonucleotide probe or the quenching oligonucleotide or both the tag portion and the quenching oligonucleotide contains one or more nucleotide
10 modifications.
15. The method of any of claims 9 - 14 wherein the one or more nucleotide modifications is selected from the group consisting of Locked Nucleic Acid (LNA), Peptide Nucleic Acid (PNA), Bridged Nucleic Acid (BNA), 2'-O alkyl substitution, L-enantiomeric nucleotide, or combinations thereof.
- 15 16. A method for detecting two or more target nucleic acid sequences in a sample comprising the steps of:
 - (a) contacting said sample suspected of containing said two or more target nucleic acid sequences in a single reaction vessel with
 - (i) a first pair of oligonucleotide primers with sequences that are
20 complementary to each strand of a first target nucleic acid sequence, and a second pair of oligonucleotide primers with sequences that are complementary to each strand of a second target nucleic acid sequence;
 - (ii) a first oligonucleotide probe comprising two distinct portions, a first annealing portion comprising a sequence at least partially complementary
25 to the first target nucleic acid sequence and anneals within the first target

nucleic acid sequence bounded by the first pair of oligonucleotide primers, wherein the first annealing portion comprises a first quencher moiety and is blocked at the 3' terminus to prohibit extension by a nucleic acid polymerase; and a first tag portion attached to the 5' terminus or to the 3' terminus of the first annealing portion and comprising a nucleotide sequence that is non-complementary to the two or more target nucleic acid sequences, wherein the first tag portion comprises a fluorescent moiety whose detectable signal is capable of being quenched by the first quencher moiety on the first annealing portion, wherein said fluorescent moiety is separated from the first quenching moiety by a nuclease susceptible cleavage site;

(iii) a first quenching oligonucleotide comprising a sequence at least partially complementary to the first tag portion of the first oligonucleotide probe and hybridizes to the first tag portion to form a duplex, wherein said first quenching oligonucleotide comprises a second quenching moiety which quenches the detectable signal generated by the fluorescent moiety on the first tag portion when the first quenching oligonucleotide is hybridized to the first tag portion;

(iv) a second oligonucleotide probe comprising two distinct portions, a second annealing portion comprising a sequence at least partially complementary to the second target nucleic acid sequence and anneals within the second target nucleic acid sequence bounded by the second pair of oligonucleotide primers, wherein the second annealing portion comprises a third quencher moiety and is blocked at the 3' terminus to prohibit extension by a nucleic acid polymerase; and a second tag portion attached to the 5' terminus or to the 3' terminus of the second annealing portion and comprising a nucleotide sequence that is non-complementary

to the two or more target nucleic acid sequences, and has a different nucleic acid sequence or different nucleotide modifications compared to the nucleotide sequence of the first tag portion of the first oligonucleotide probe, wherein the second tag portion comprises a fluorescent moiety that is identical to the fluorescent moiety on the first oligonucleotide probe and whose detectable signal is capable of being quenched by the third quencher moiety on the second annealing portion, wherein said fluorescent moiety is separated from the third quenching moiety by a nuclease susceptible cleavage site;

(v) a second quenching oligonucleotide comprising a sequence at least partially complementary to the second tag portion of the second oligonucleotide probe and hybridizes to the second tag portion to form a duplex, wherein said second quenching oligonucleotide comprises a fourth quenching moiety which quenches the detectable signal generated by the fluorescent moiety on the second tag portion when the second quenching oligonucleotide is hybridized to the second tag portion;

wherein the duplex between the second quenching oligonucleotide and the second tag portion of the second oligonucleotide probe has a higher melting temperature (T_m) value than the duplex between the first quenching oligonucleotide and the first tag portion of the first oligonucleotide probe;

(b) amplifying the first and second target nucleic acid sequences by polymerase chain reaction (PCR) using a nucleic acid polymerase having 5' to 3' nuclease activity such that during an extension step of each PCR cycle, the 5' to 3' nuclease activity of the nucleic acid polymerase allows

(i) cleavage and separation of the fluorescent moiety on the first tag portion from the first quenching moiety on the first annealing portion of the first

oligonucleotide probe, wherein at the extension step the first quenching oligonucleotide remains hybridized to the first tag portion, and

(ii) cleavage and separation of the fluorescent moiety on the second tag portion from the third quenching moiety on the second annealing portion of the second oligonucleotide probe, wherein at the extension step the second quenching oligonucleotide remains hybridized to the second tag portion;

(c) measuring one or more fluorescent signals at a first temperature at which the first quenching oligonucleotide is not hybridized to the first tag portion from the first oligonucleotide probe and the second quenching oligonucleotide remains hybridized to the second tag portion from the second oligonucleotide probe;

(d) measuring one or more fluorescent signals at a second temperature, which is higher than the first temperature, at which the second quenching oligonucleotide is not hybridized to the second tag portion from the second oligonucleotide probe;

(e) obtaining a calculated signal value by subtracting a median or average of the one or more fluorescent signals detected at the first temperature from a median or average of the one or more fluorescent signals detected at the second temperature; whereby a value of the median or average of the one or more fluorescent signals detected at the first temperature that is higher than a threshold value allows determination of the presence of the first target nucleic acid sequence and the calculated signal value that is higher than the threshold signal value allows determination of the presence of the second target nucleic acid.

17. The method of claim 16 wherein PCR amplification of step (b) is allowed to reach an end-point beyond the log phase of amplification.
18. The method of claims 16 or 17 wherein the first tag portion is attached to the 3' terminus of the first annealing portion of the first oligonucleotide probe and the second tag portion is attached to the 3' terminus of the second annealing portion of the second oligonucleotide probe or the first tag portion is attached to the 5' terminus of the first annealing portion of the first oligonucleotide probe and the second tag portion is attached to the 5' terminus of the second annealing portion of the second oligonucleotide probe.
19. The method of any of claims 16 - 18 wherein the first quenching oligonucleotide is connected to the first tag portion of the first oligonucleotide probe via a stem-loop structure.
20. The method of any of claims 16 - 19 wherein the second quenching oligonucleotide is connected to the second tag portion of the second oligonucleotide probe via a stem-loop structure.
21. The method of any of claims 16 - 20 wherein the released first tag portion and the released second tag portion both comprise a modification such that both released tag portions are not capable of being extended by a nucleic acid polymerase.
22. The method of any of claims 16 - 21 whereby amplification and detection of the target nucleic acid is performed by digital PCR.
23. The method of any of claims 16 - 22 whereby detection of two or more target nucleic acid sequences is performed by digital PCR.
24. The method of any of claims 16 - 23 whereby detection of two or more target nucleic acid sequences is performed by digital PCR.

25. The method of any of claims 16 - 24 wherein any of the first tag portion of the first oligonucleotide probe or the first quenching oligonucleotide or the second tag portion of the second oligonucleotide probe or the second quenching oligonucleotide or any combinations thereof contains one or more nucleotide
5 modifications.
26. The method of any of claims 16 - 25 wherein the one or more nucleotide modifications is selected from the group consisting of Locked Nucleic Acid (LNA), Peptide Nucleic Acid (PNA), Bridged Nucleic Acid (BNA), 2'-O alkyl substitution, L-enantiomeric nucleotide, or combinations thereof.

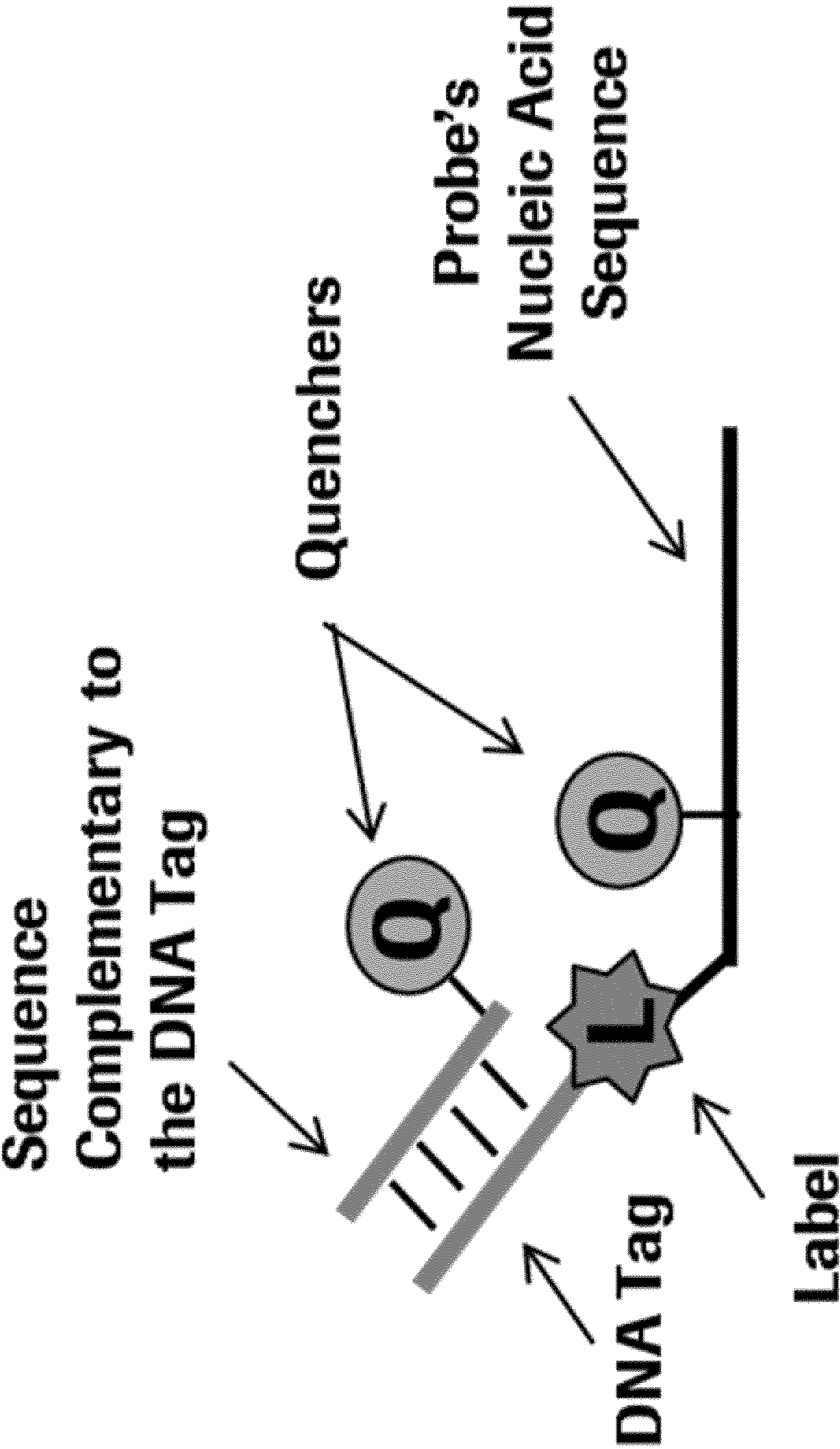


FIG. 1

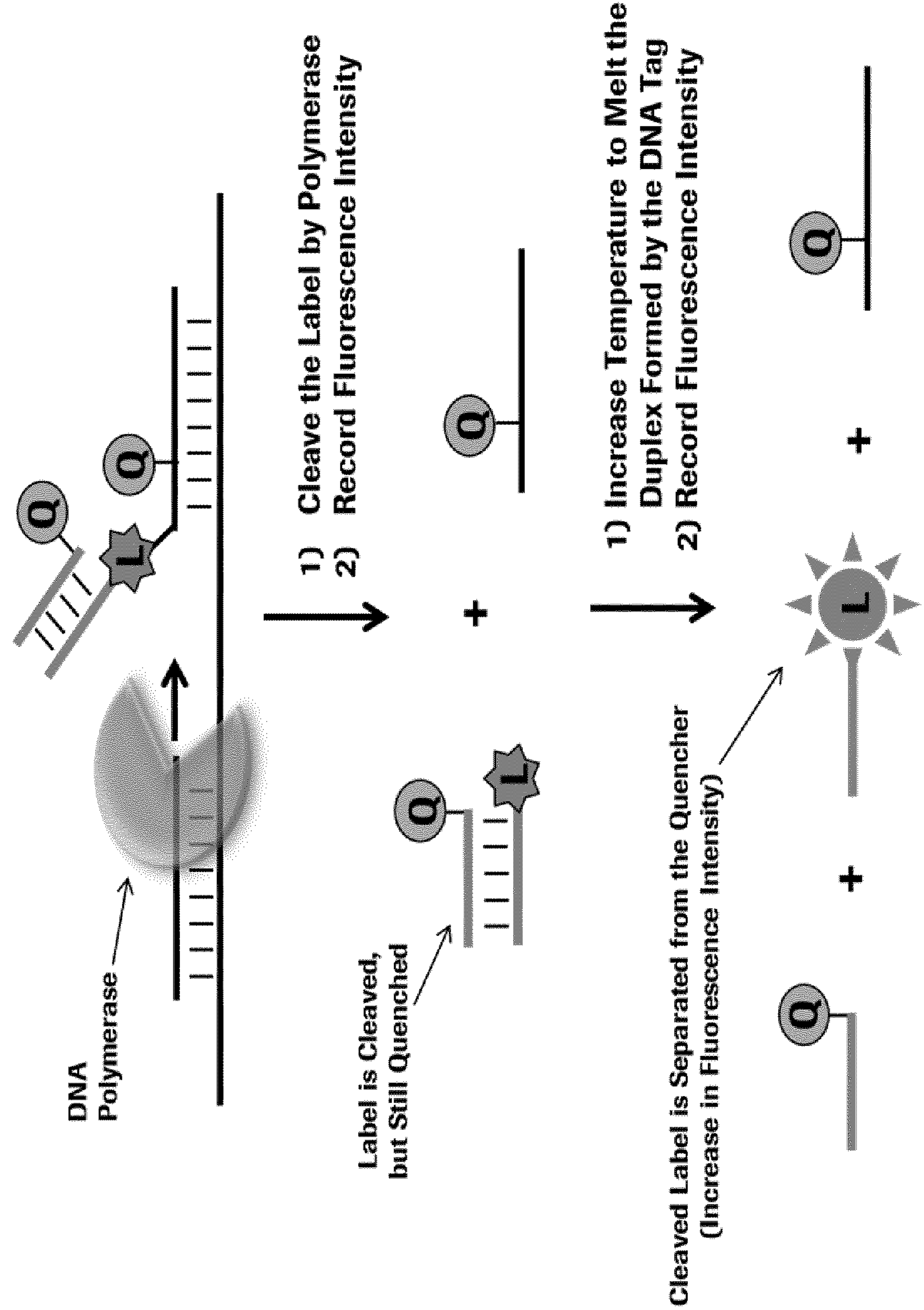


FIG. 2

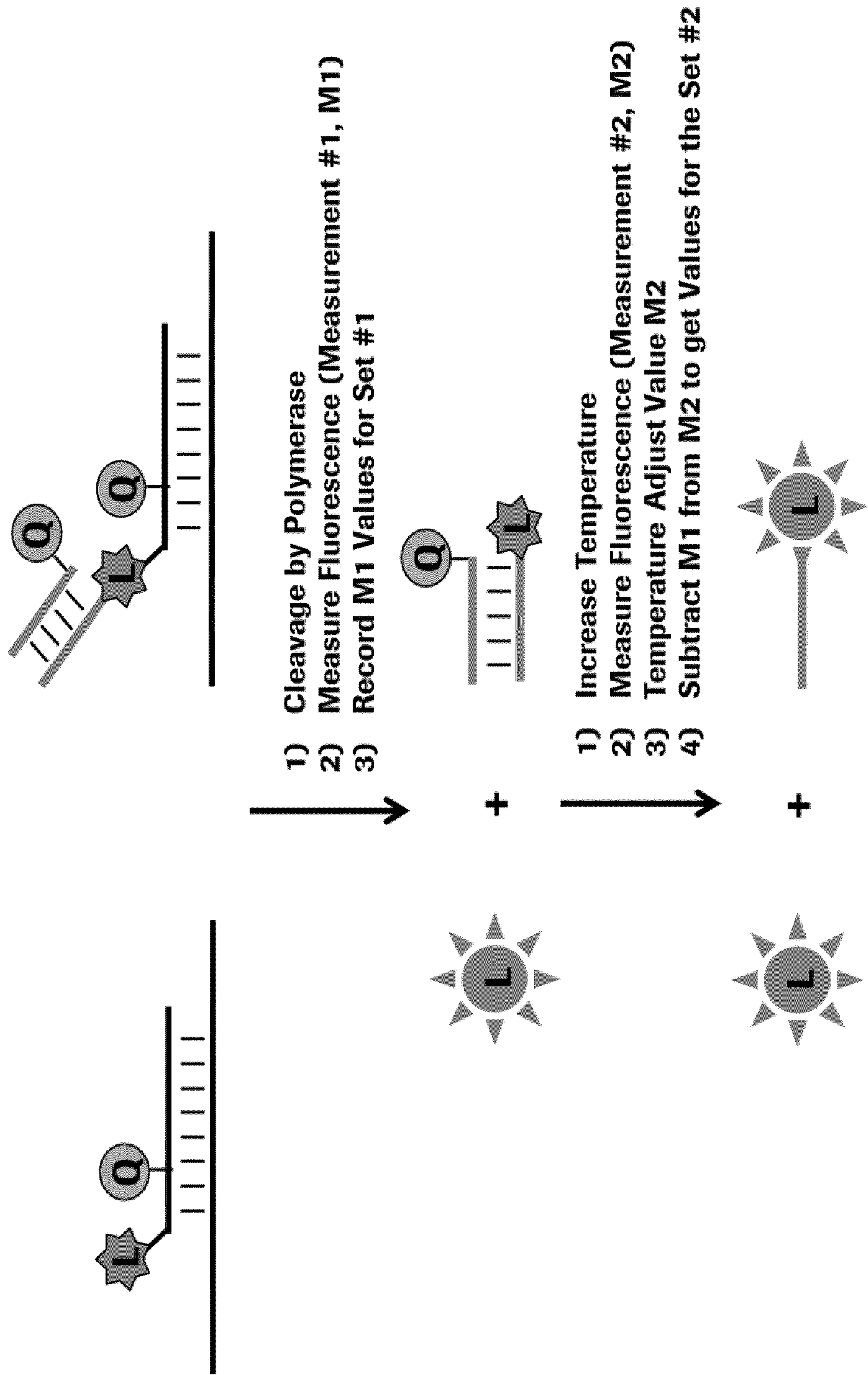


FIG. 3

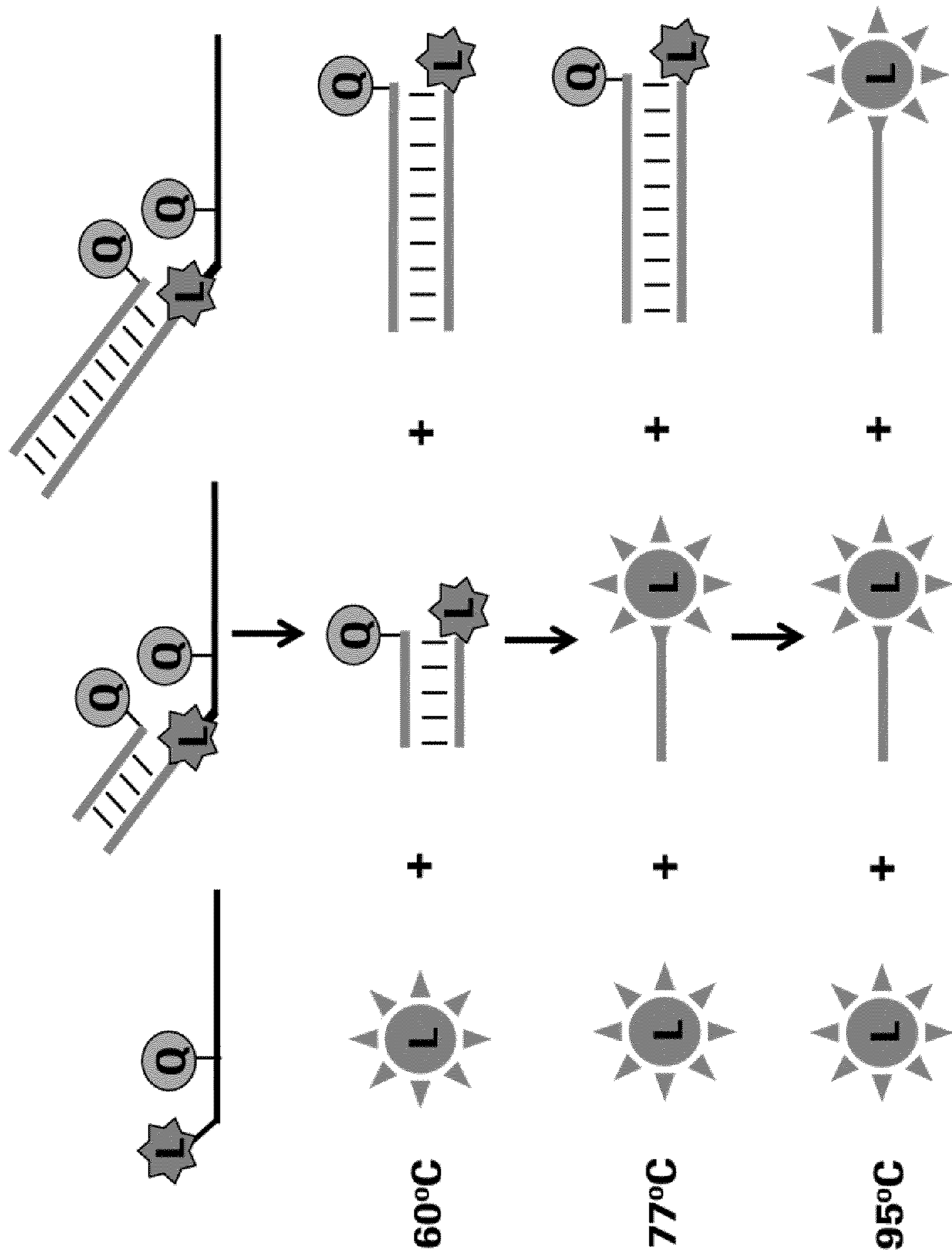


FIG. 4

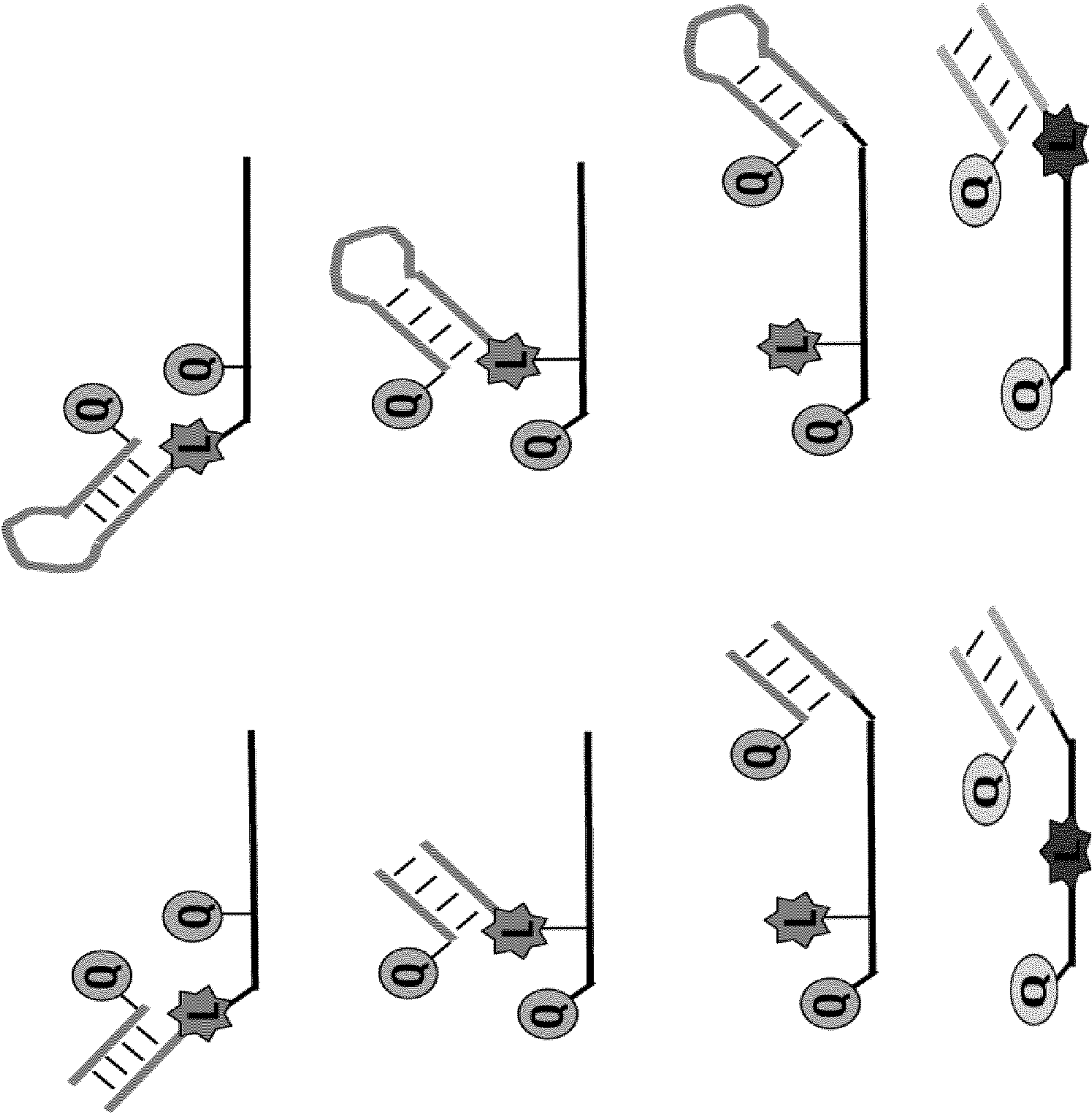


FIG. 5

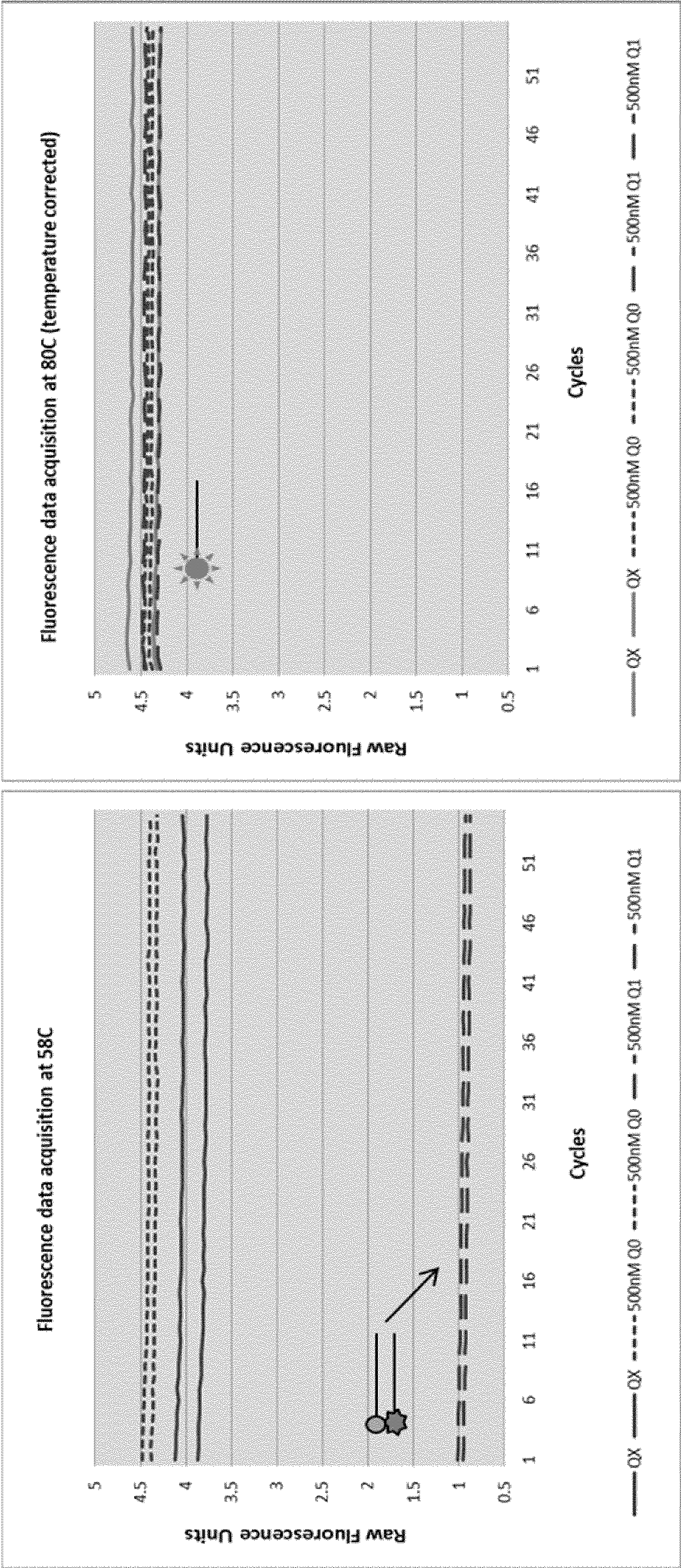


FIG. 6

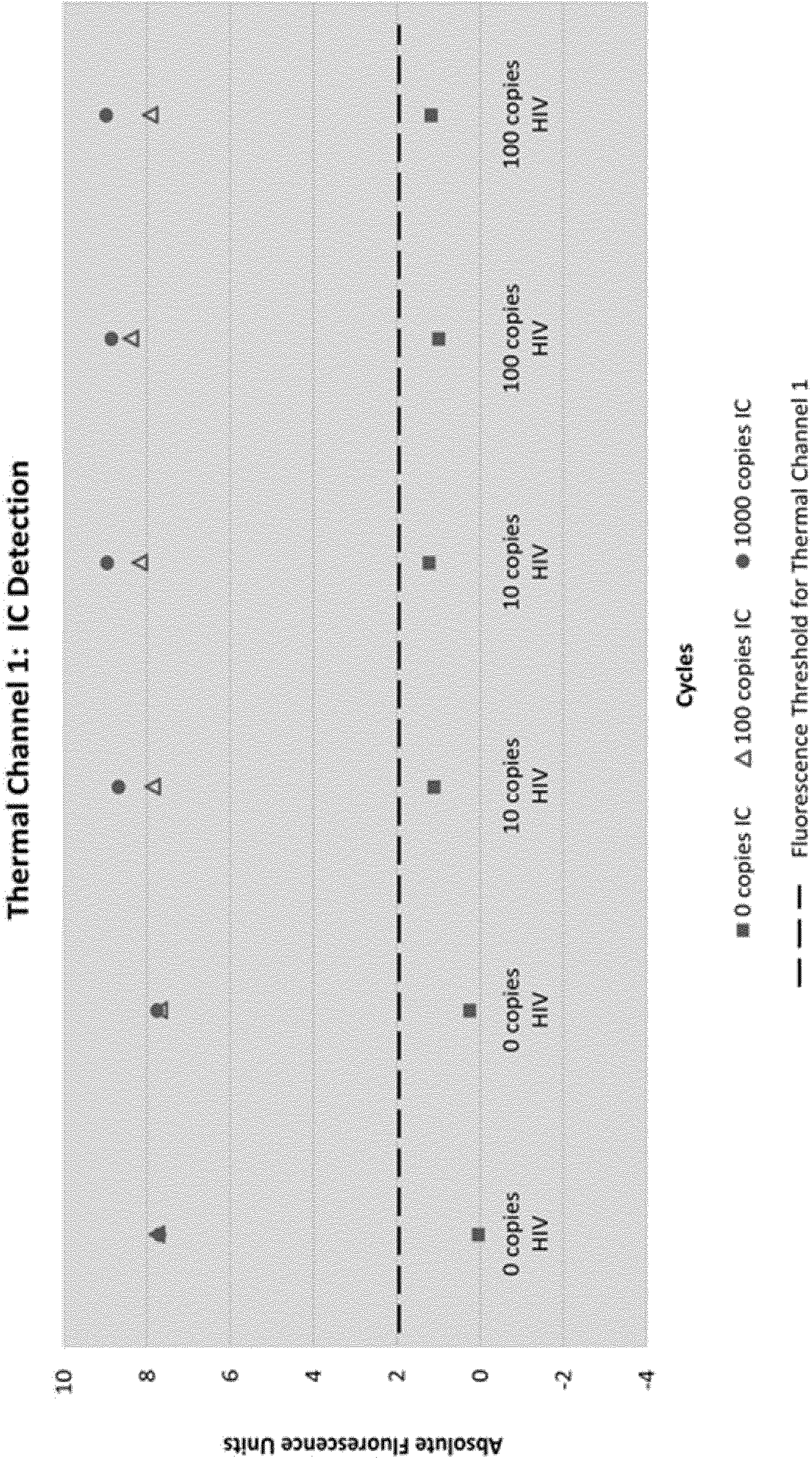


FIG. 7

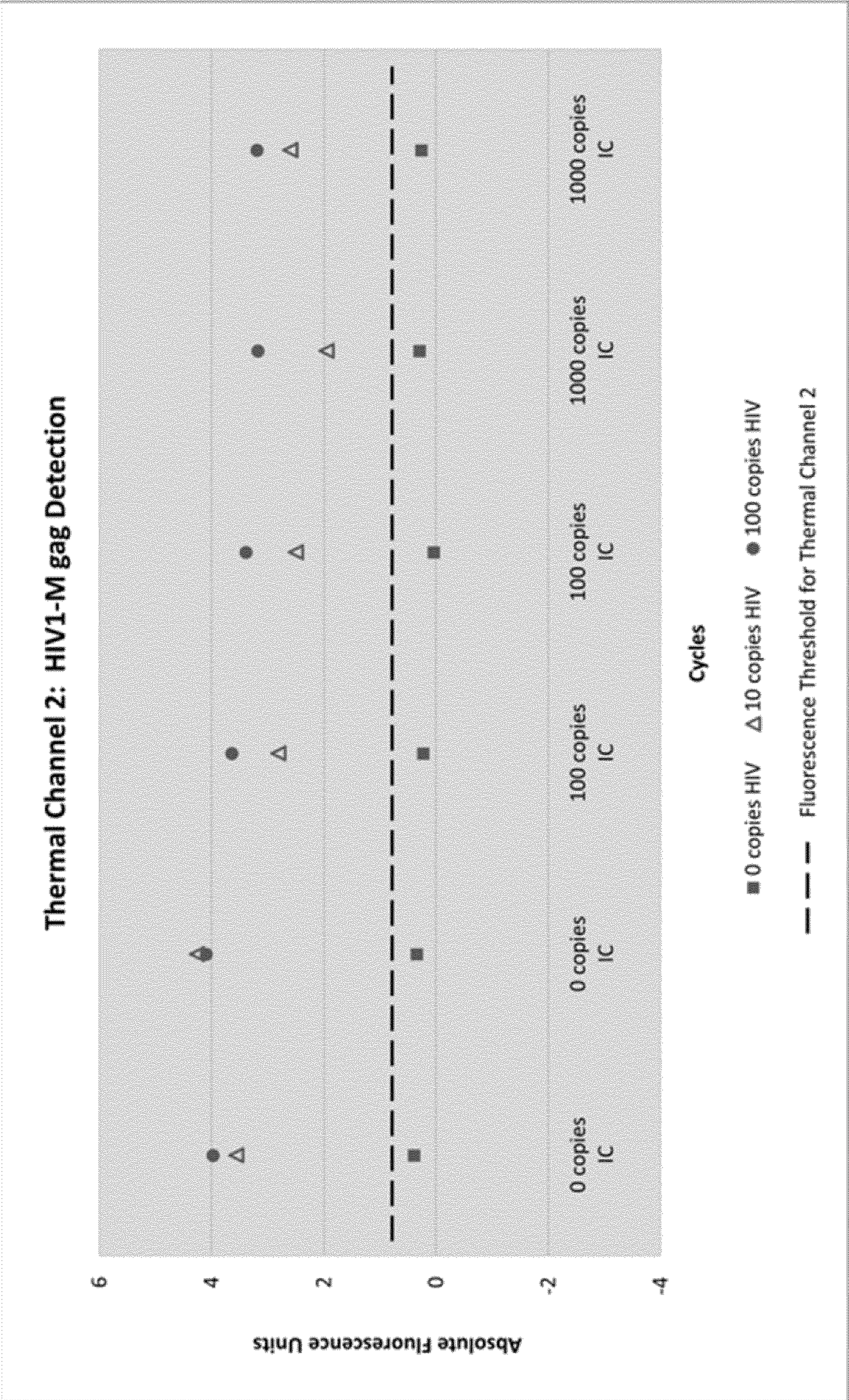


FIG. 8

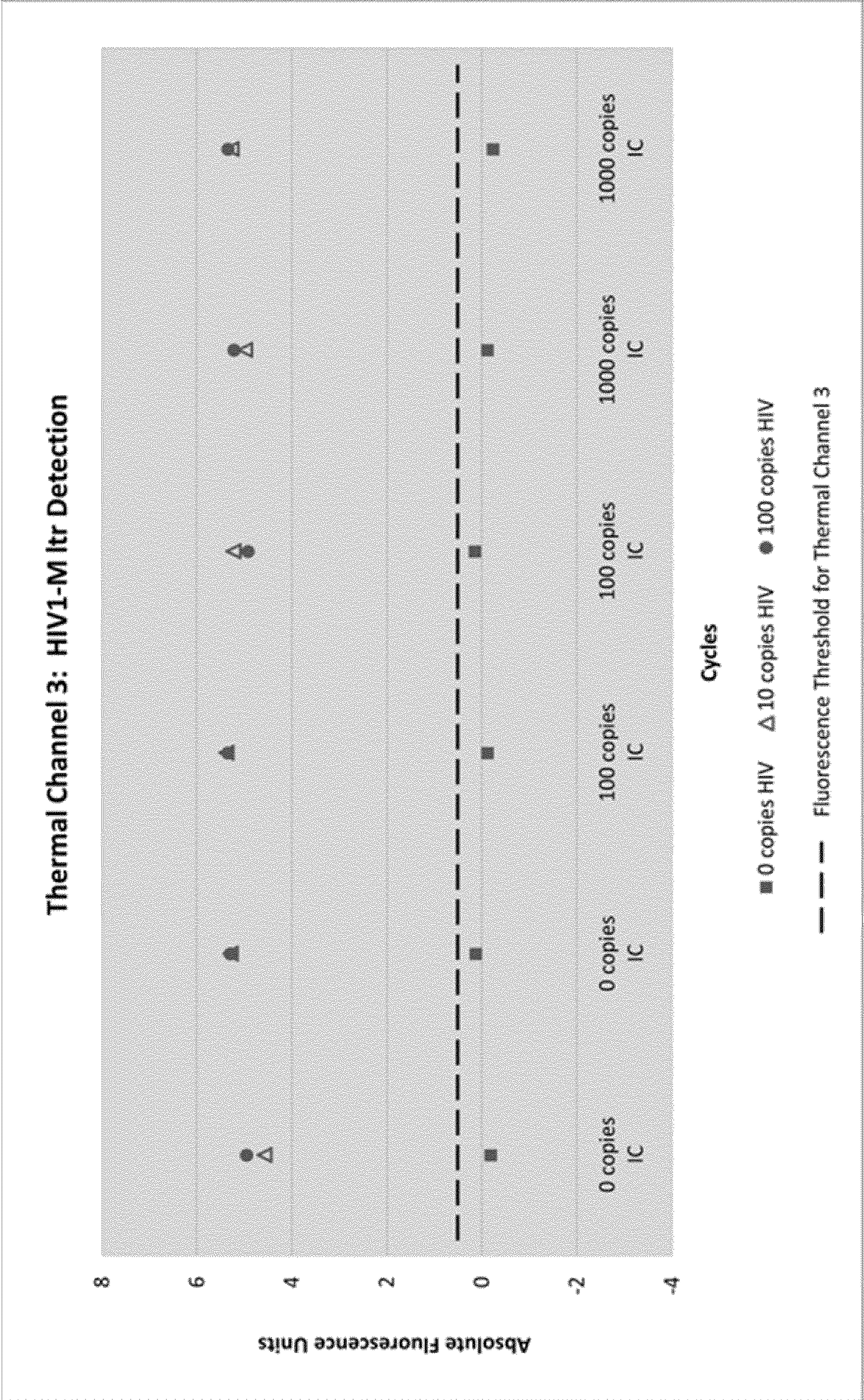
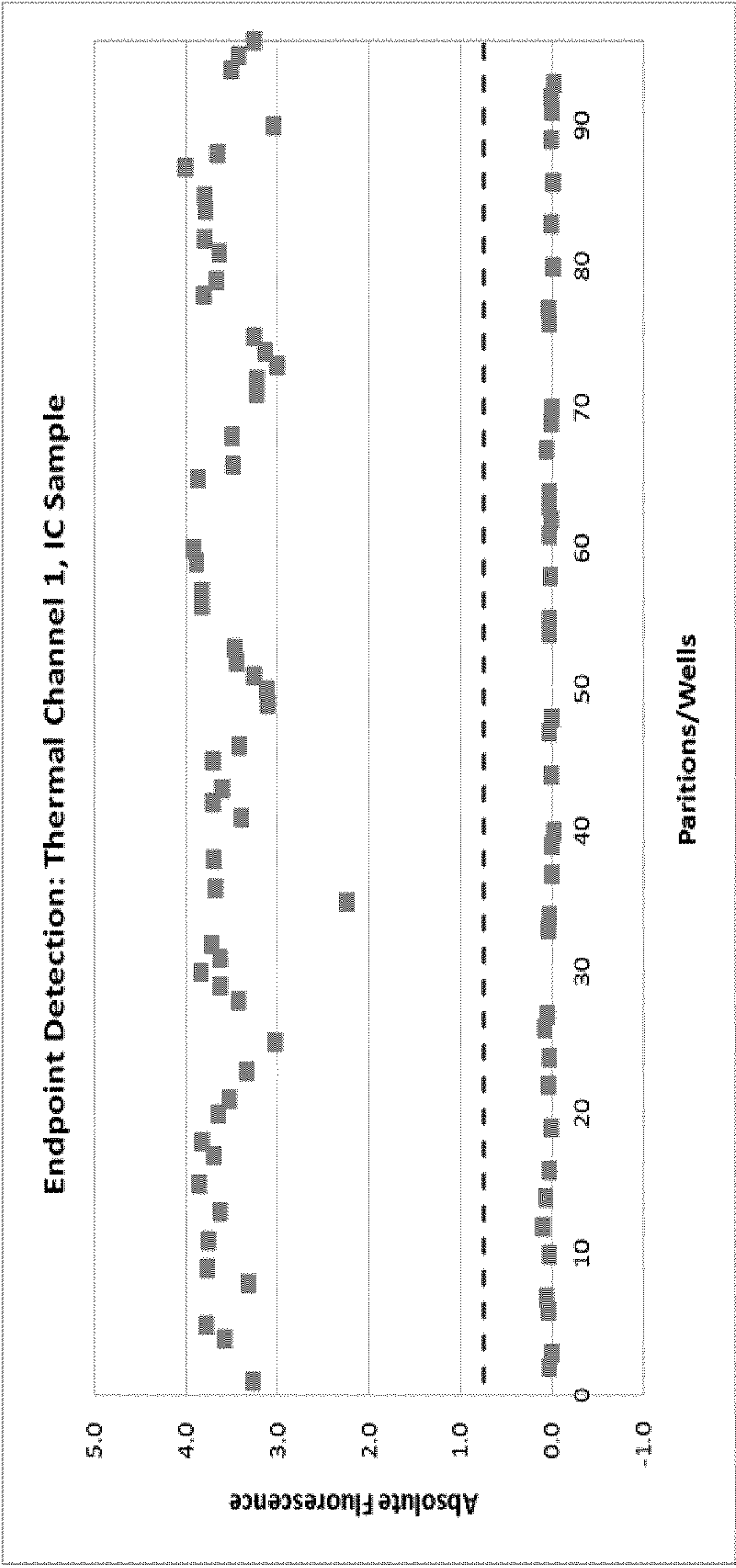
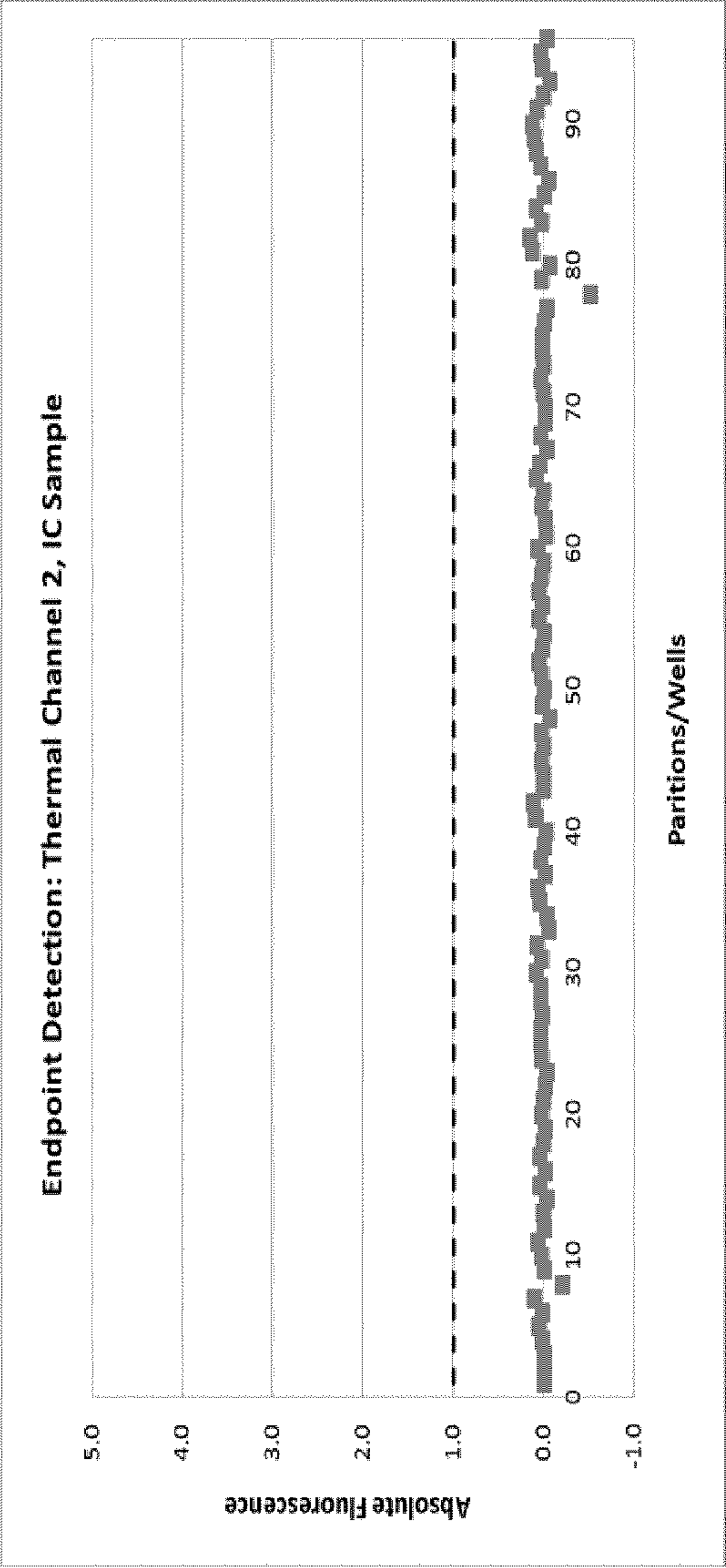


FIG. 9

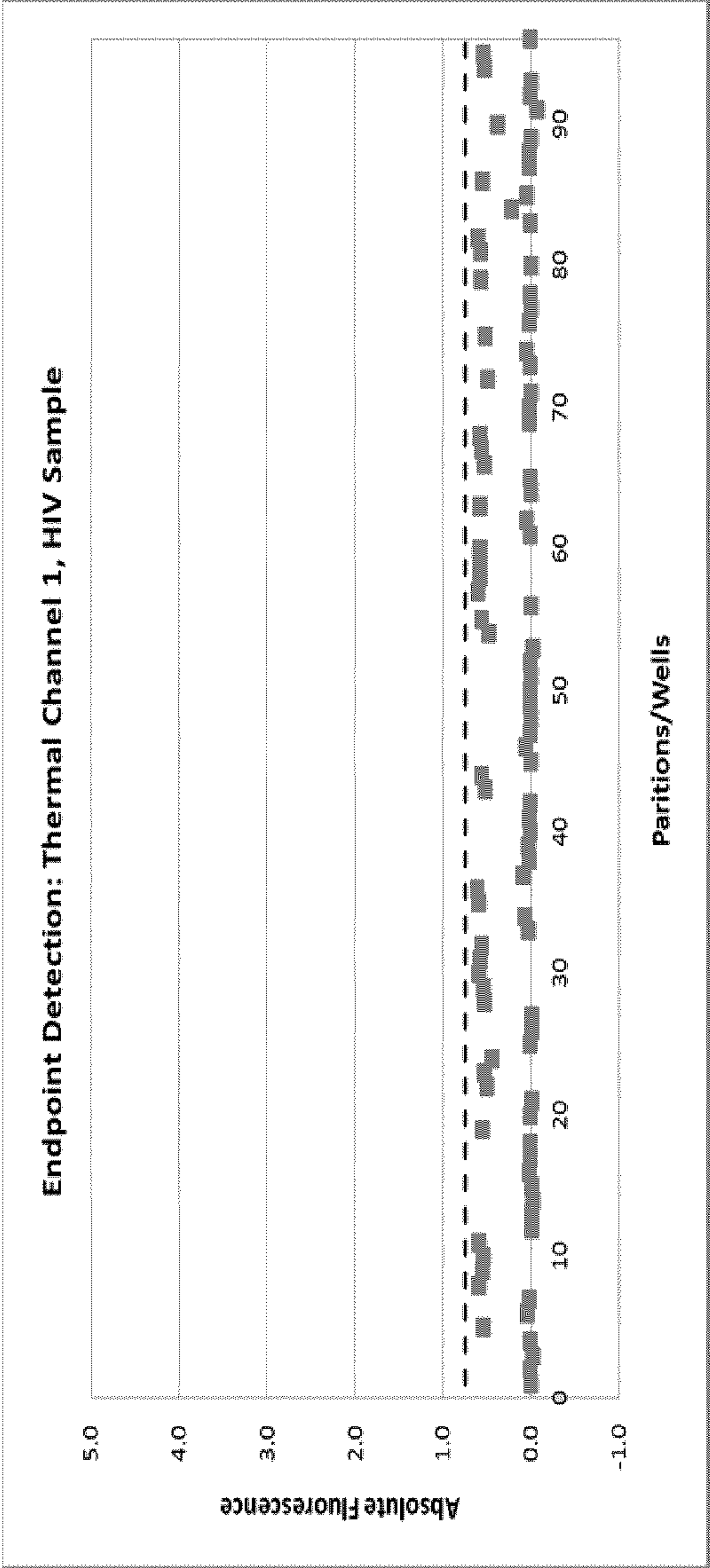


10A

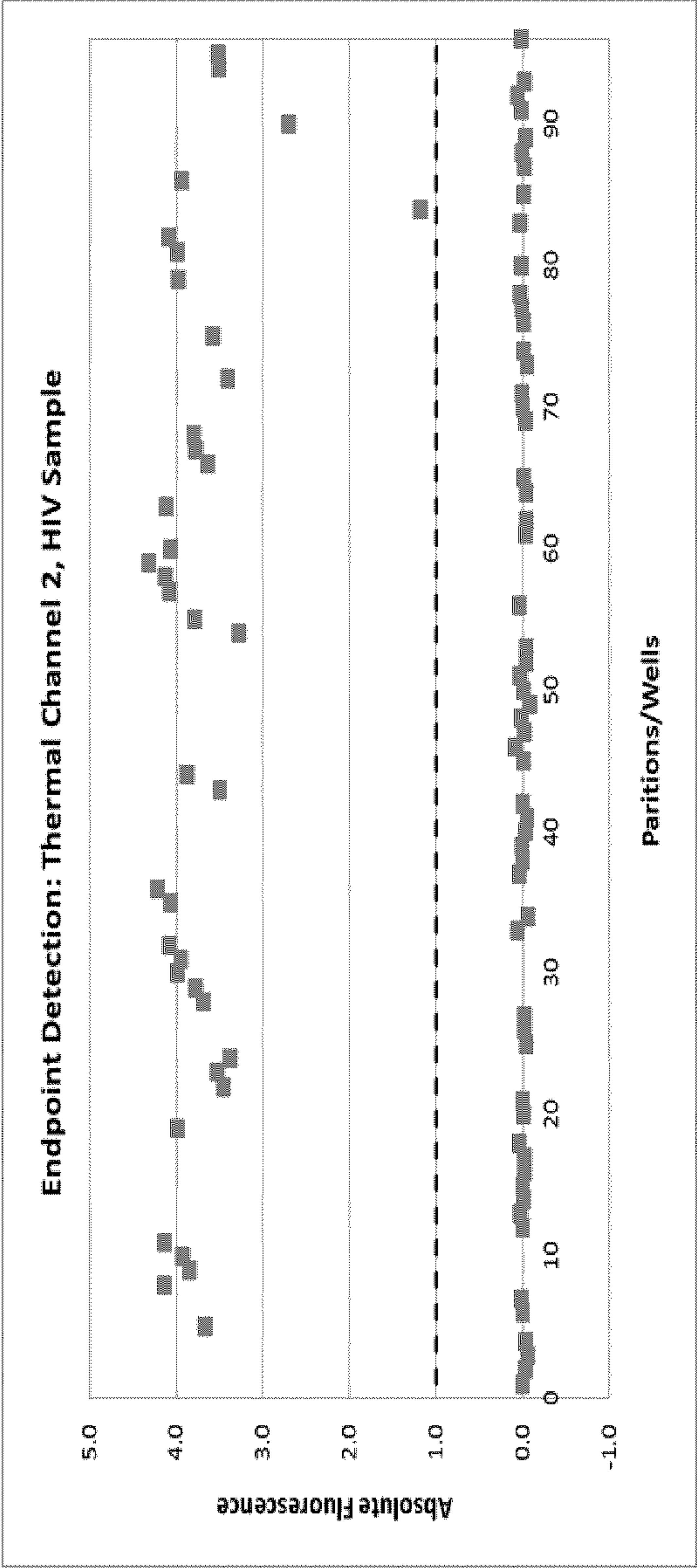


10B

FIG. 10

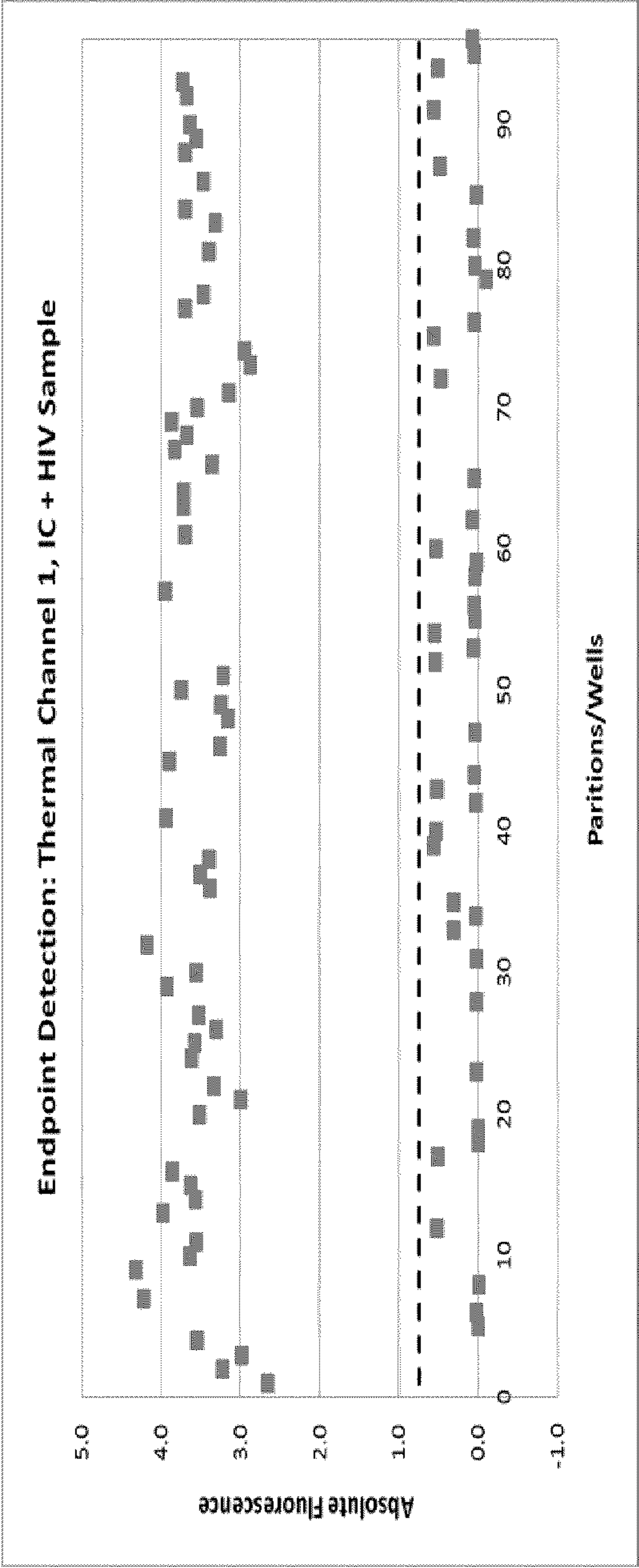


11A

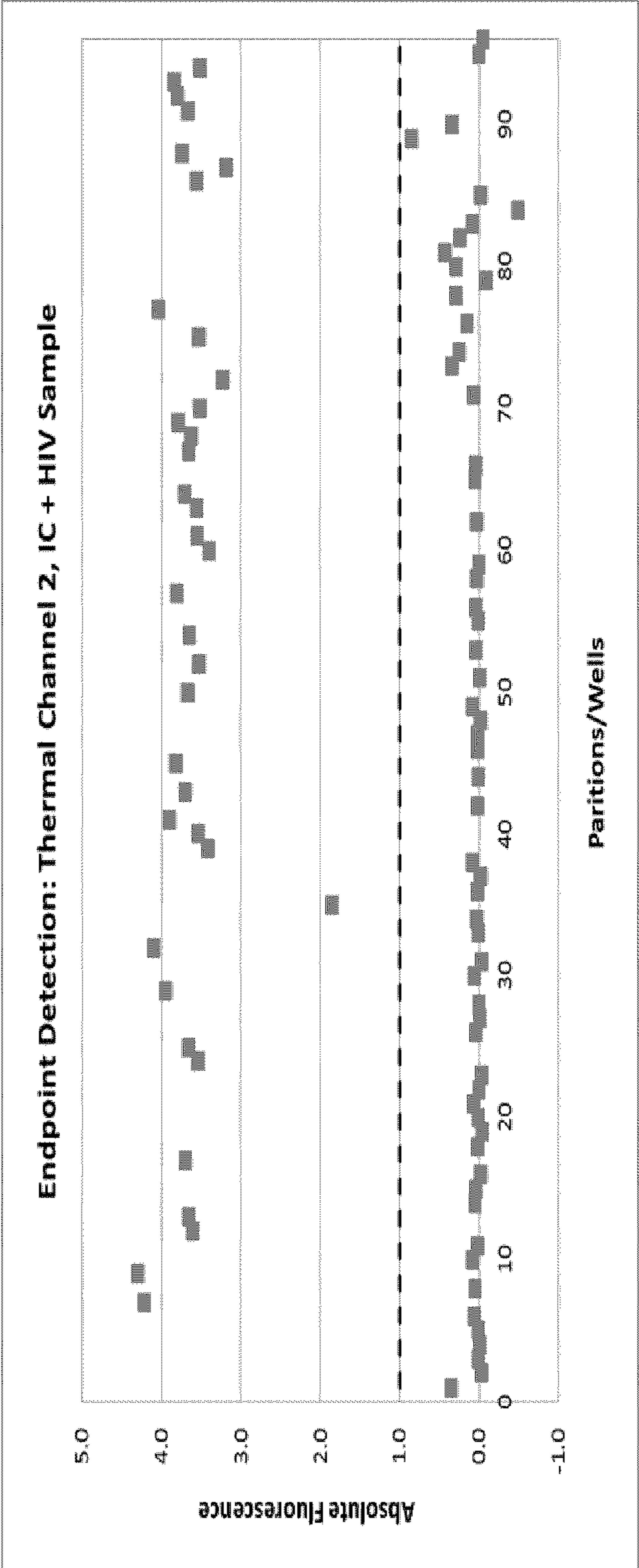


11B

FIG. 11



12A



12B

FIG. 12

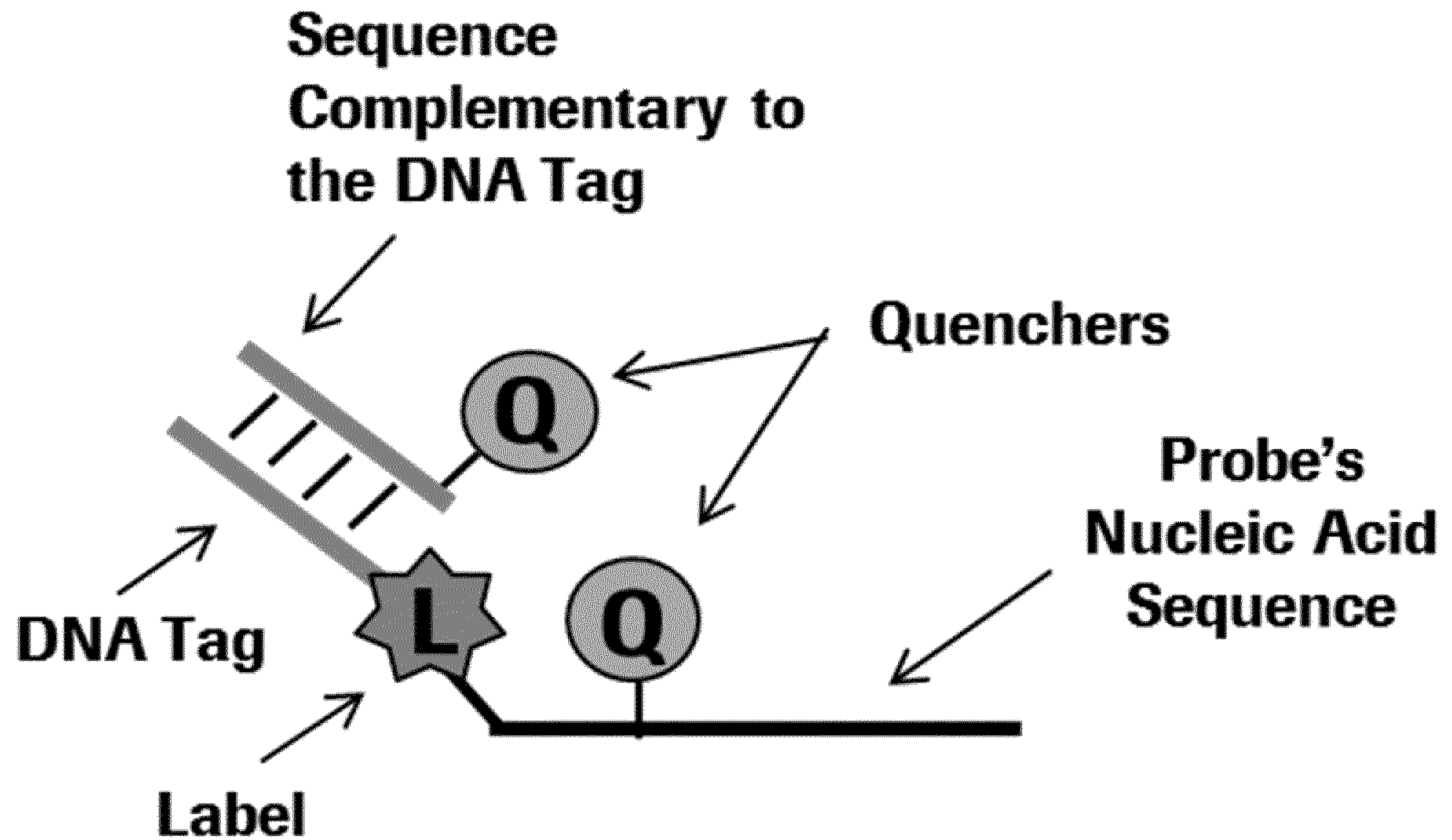


FIG. 1