

In one aspect, the present invention refers to 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 reaction vessel with (i) one pair of oligonucleotide primers, each oligonucleotide primer
5 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 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
10 complementary to the target nucleic acid sequence and hybridizes to a region of said subsequence of said target nucleic acid that is 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
15 and wherein said reporter moiety is separated from said first quencher moiety by a nuclease susceptible cleavage 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 when said quenching molecule is bound to said tag portion;
20 (b) amplifying said 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 tag portion from the first quencher moiety on the annealing portion of the probe; (c) measuring a suppressed signal from the reporter moiety at a first
25 temperature at which the quenching molecule is bound to the tag portion; (d) increasing temperature to a second temperature at which the quenching molecule is not bound to the tag portion; (e) measuring a temperature corrected signal from the reporter moiety at the second temperature; (f) obtaining a calculated signal value by subtracting the suppressed signal detected at the first temperature from
30 the temperature corrected signal detected at the second temperature; (g) repeating steps (b) through (f) through multiple PCR cycles; (h) measuring the calculated signal values from the multiple PCR cycles to detect the presence of the target nucleic acid. In another aspect, the present invention refers to 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 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 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 from 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 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 a fluorescent signal at a first temperature at which the quenching oligonucleotide is hybridized to the tag portion from the second oligonucleotide probe; (d) increasing temperature to 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) measuring a fluorescent signal at the second temperature; (f) obtaining a calculated signal value by subtracting the fluorescent signal detected at the first temperature from the fluorescent signal detected at the second temperature; (g) repeating steps (b) through (f) in multiple PCR cycles to produce desired quantity of amplification products from the first and second target nucleic acid sequences; (h) determining the presence of the first target nucleic acid sequence from the fluorescent signals detected at the first temperature from the multiple PCR cycles and the presence of the second target nucleic acid sequence from the calculated signal values from the multiple PCR cycles. In another aspect, the present invention refers to 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 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 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

5 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

10 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)

15 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

20 extension step the second quenching oligonucleotide remains hybridized to the second tag portion; (c) increasing temperature to 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)

25 measuring a fluorescent signal at the first temperature; (e) increasing temperature to 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; (f) measuring a fluorescent signal at the second temperature; (g) obtaining a calculated signal value by subtracting

30 the fluorescent signal detected at the first temperature from the fluorescent signal detected at the second temperature; (h) repeating steps (b) through (g) in multiple PCR cycles to produce desired quantity of amplification products from

the first and second target nucleic acid sequences; (i) determining the presence of the first target nucleic acid sequence from the fluorescent signals detected at the first temperature from the multiple PCR cycles and the presence of the second target nucleic acid sequence from the calculated signal values from the multiple PCR cycles. In another aspect, the present invention provides a kit for detecting two or more target nucleic acid sequences in a sample comprising: (a) two or more pairs of oligonucleotide primers with sequences that are complementary to each strand of the two or more target nucleic acid sequences; (b) at least one oligonucleotide probe comprising two distinct portions, an annealing portion comprising a sequence at least partially complementary to one of the more than one target nucleic acid sequences and anneals within said one of the more than one target nucleic acid sequences, wherein the annealing portion comprises a first quencher moiety; and a 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 annealing portion, and comprising a nucleotide sequence that is non-complementary to the more than one target nucleic acid sequences, wherein the tag portion comprises a fluorescent moiety whose detectable signal is capable of being quenched by the first quencher moiety on the annealing portion, wherein said fluorescent moiety is separated from the first quenching moiety by a nuclease susceptible cleavage site; (c) at least one quenching oligonucleotide comprising a nucleotide sequence at least partially complementary to the tag portion of the oligonucleotide probe and hybridizes to the tag portion to form a duplex, wherein said quenching oligonucleotide comprises a second 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.

Therefore in one example, the disclosure 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 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

5 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

10 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

15 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 a suppressed signal from the reporter moiety at a first temperature at which the quenching molecule is bound to the tag portion; (d) increasing temperature to a second temperature at which the quenching molecule is not

20 bound to the tag portion; (e) measuring a temperature corrected signal from the reporter moiety at the second temperature; (f) obtaining a calculated signal value by subtracting the suppressed signal detected at the first temperature from the temperature corrected signal detected at the second temperature; (g) repeating steps (b) through (f) through multiple PCR cycles; (h) measuring the calculated

25 signal values from the multiple PCR cycles to detect the presence of the target nucleic acid.

In one example, the tag portion comprises a modification such that it is not capable of being extended by a nucleic acid polymerase. In one example, the reporter moiety is on the tag portion of the oligonucleotide probe. In another

30 example, 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 comprises the second quencher moiety. In one

example, 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.

5 In another example, 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 example, 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-
10 enantiomeric nucleotide, or combinations thereof. In one example, the reporter moiety is a fluorescent dye and the quencher moiety quenches a detectable signal from the fluorescent dye.

In another example, the disclosure provides for a method for detecting two or more target nucleic acid sequences in a sample comprising the steps of: (a)
15 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 complementary to each strand of a second target
20 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 generating a detectable signal and a first quencher
25 moiety capable of quenching the detectable signal generated by the fluorescent moiety, wherein the fluorescent moiety is separated from 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
30 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

5 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

10 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)

15 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

20 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 a fluorescent signal at a first temperature at which the quenching oligonucleotide is hybridized to the tag portion from the second oligonucleotide probe; (d) increasing temperature to a second temperature, which

25 is higher than the first temperature, at which the quenching oligonucleotide is not hybridized to the tag portion from the second oligonucleotide probe; (e) measuring a fluorescent signal at the second temperature; (f) obtaining a calculated signal value by subtracting the fluorescent signal detected at the first temperature from the fluorescent signal detected at the second temperature; (g)

30 repeating steps (b) through (f) in multiple PCR cycles to produce desired quantity of amplification products from the first and second target nucleic acid sequences; (h) determining the presence of the first target nucleic acid sequence from the

fluorescent signals detected at the first temperature from the multiple PCR cycles and the presence of the second target nucleic acid sequence from the calculated signal values from the multiple PCR cycles.

In one example, the tag portion comprises a modification such that it is not
5 capable of being extended by a nucleic acid polymerase. In another example, the tag portion is attached to the 5' terminus of the annealing portion. In yet another example, the tag portion is attached to the 3' terminus of the annealing portion. In yet another example, the tag portion is attached via a linker to a region of the annealing portion. In another example, the tag portion of the second
10 oligonucleotide probe or the quenching oligonucleotide or both the tag portion and the quenching oligonucleotide contain one or more nucleotide modifications. In yet another example, 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
15 combinations thereof.

In yet another example, the disclosure 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
20 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
25 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
30 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 comprising a sequence at least partially

5 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 portion; (iv)

10 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

15 portion attached to the 5' 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 sequence or different nucleotide modifications compared to the nucleotide sequence of the first tag

20 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 the fluorescent moiety is separated from the third quenching moiety by a nuclease

25 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

30 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) increasing temperature to 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 a fluorescent signal at the first temperature; (e) increasing temperature to a second temperature at which the second quenching oligonucleotide is not hybridized to the second tag portion from the second oligonucleotide probe; (f) measuring a fluorescent signal at the second temperature; (g) obtaining a calculated signal value by subtracting the fluorescent signal detected at the first temperature from the fluorescent signal detected at the second temperature; (h) repeating steps (b) through (g) in multiple PCR cycles to produce desired quantity of amplification products from the first and second target nucleic acid sequences; (I) determining the presence of the first target nucleic acid sequence from the fluorescent signals detected at the first temperature from the multiple PCR cycles and the presence of the second target nucleic acid sequence from the calculated signal values from the multiple PCR cycles.

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

5 In one example, 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. In another example, 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
10 of the second oligonucleotide probe. In another example, 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 via a linker to a region of the second annealing portion of the second oligonucleotide probe.

15 In one example, 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. In another example, 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
20 of the second oligonucleotide probe. In another example, 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.

25 In one example, 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 example, 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 3' terminus of the second annealing
30 portion of the second oligonucleotide probe. In another

example, 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.

- 5 In one example, 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 example, the one or more nucleotide modifications is selected from the group
- 10 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 example, the disclosure provides for a kit for detecting two or more target nucleic acid sequences in a sample comprising:
- 15 (a) two or more pairs of oligonucleotide primers with sequences that are complementary to each strand of the two or more target nucleic acid sequences; (b) at least one oligonucleotide probe comprising two distinct portions, an annealing portion comprising a sequence at least partially complementary to one of the more than one target nucleic acid sequences and anneals within said one of the more than one target nucleic acid sequences, wherein the annealing portion
- 20 comprises a first quencher moiety; and a 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 annealing portion, and comprising a nucleotide sequence that is non-complementary to the more than one target nucleic acid sequences, wherein the tag portion comprises a fluorescent moiety whose detectable signal is capable of
- 25 being quenched by the first quencher moiety on the annealing portion, wherein said fluorescent moiety is separated from the first quenching moiety by a nuclease susceptible cleavage site; (c) at least one quenching oligonucleotide comprising a nucleotide sequence at least partially complementary to the tag portion of the oligonucleotide probe and hybridizes to the tag portion to form a duplex, wherein
- 30 the quenching oligonucleotide comprises a second 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. In one example, the

- tag portion of the oligonucleotide probe or the quenching oligonucleotide or both the tag portion of the oligonucleotide probe and the quenching oligonucleotide contains one more nucleotide modifications wherein the one or more nucleotide modifications is selected from the group consisting of Locked Nucleic Acid (LNA),
- 5 Peptide Nucleic Acid (PNA), Bridged Nucleic Acid (BNA), 2'-O alkyl substitution, L-enantiomeric nucleotide, or combinations thereof.