

regions designed to form secondary structure. The predicted melting temperature of the secondary structure was 51 °C, as determined by mFold (idtdna.com) using the PCR buffer conditions. The PCR probe was labeled with a 5' Cy3 fluorophore and a Black Hole Quencher 2 moiety on the 3' end.

[0128] The capture probe sequence attached to the bottom substrate of the PCR chamber was: NNN NNN NNN NNN N (SEQ ID NO:2) with a T_m of 42 °C using the PCR buffer conditions.

[0129] DNA plasmids comprising a target sequence were added to each PCR reaction at a concentration of 10^6 , 10^4 , and 10^2 copies/ μ L. The solution was then degassed by heating to 95 °C. After degassing, polymerase was added and the reaction was loaded into the PCR chamber using a pipette. Left over solution was loaded onto an Applied Biosystems 7500 for parallel analysis.

[0130] The cycling conditions for the array based PCR were as follows:

Temp	Time	Purpose
95 °C	120 sec	Fast Start Enzyme activation
95C	15 sec	denaturation
60C	60 sec	polymerase extension
30C	120 sec	Flap hybridization and optical reading

(denaturation and extension were performed for 5 cycles, and then denaturation/ extension/ flap hybridization and optical reading are repeated for 8 cycles).

[0131] The Cycling conditions for the ABI 7500 were as follows:

Temp	Time	Purpose
95 °C	120 sec	Fast Start Enzyme activation
95 °C	15 sec	denaturation
60 °C	60sec	polymerase extension and optical reading

Denaturation/ extension and reading were performed for 40 cycles.

[0132] The results for the copy number titrations are shown in **Figure 3** for the array based PCR and **Figure 4** for the solution phase PCR. As can be seen from the figures, the results are comparable, giving similar behavior for the titrations.

EXAMPLE 2: TWO STEP AMPLIFICATION REACTION

[0133] As with Example 1, above, the amplification reagent mix contained standard PCR reagents including two PCR primers (200nM) complementary to each target being amplified as well as a PCR probe (300nM) having a sequence complementary to each target being amplified. The structure of typical probe is shown in Figure 1B. As shown, the labeled probe again includes a nucleic acid fragment (A) that is complimentary to a target amplicon designed using the same rules as is typical for a traditional real time PCR probe (i.e. TaqMan). Also included is orthogonal nucleic acid "flap" sequence (B) that is complimentary to a corresponding capture probe on the capture array. The probe also includes a fluorescent label (C) coupled to the flap portion B and a quencher moiety (D) coupled to the target specific portion (A).

[0134] For the two step amplification, the orthogonal flap (B) comprises a sequence that was designed to have a T_m with its complement on the capture array of 70° C. Typically the sequence length is 25 to 27 bases. As with Example 1, above, the overall probe is designed so that the most stable secondary structure has a T_m no higher than 10° C below the extension and measurement temperature in the buffer conditions used for PCR. The oligo was designed using the unfold software available at www.idtdna.com. The following PCR probe sequence was used in this example:

NNN NNN NNN NNN NNN NNN N/Cy3/NN NNN NNN ATG GCC GTT AGC TTC AGT
CAA TTC AAC AG/BHQ_2/(SEQ ID NO:40)

[0135] Where the double underlined sequence constitutes the orthogonal flap and the non-underlined sequence is homologous to the amplicon. The most stable secondary structure of the probe has a melting temperature of 45° C. The T_m of the orthogonal flap is 71° C. The PCR probe was labeled with an internal Cy3 fluorophore C (available from GE Healthcare Biosciences, Piscataway, NJ) and a Black Hole Quencher 2 moiety D (available from Biosearch, Inc., Novato, CA) on the 3' end.

[0136] A capture probe was spotted that was homologous to the flap portion of the PCR

[0140] The PCR probe and capture probe sequences are shown below:

PCR Probes:

Flu A: NNNNNNNNNNNNNNCCCCAT GGAATGTTAT

CTCCCTTTTAAGCTTCTNNNNNNNN (SEQ ID NO:4) (T_m of 50.3°)

A/H1: NNNNNNNNNNNNNNACCTTGGC GCTATTAGAT TTCCATTTC CNNNNNNNN
(SEQ ID NO:5) (T_m of 51.2°)

A/H3: NNNNNNNNNNNNNNNCCTGTT GCCAATTT CAGAGTGTT
TTGCTTAACNNNNNNNNNNNN (SEQ ID NO:6) (T_m of 51°)

FluB: NNNNNNNNNNNNNNNTCAAAGC CAATTCGAG CAGCTGAAAC TNNNNNNNN
(SEQ ID NO: 7) (T_m of 51°)

phiMS2: NNNNNNNNNNNNNNTCGCTGAA CAAGCAACC
GTTACCCNNNNNNNNNNNN (SEQ ID NO:8) (T_m of 52°)

Capture Probes

FluA: NNN NNN NNN NNN N (SEQ ID NO:8) (T_m of 46°)

A/H1: NNN NNN NNN NNN N (SEQ ID NO: 9) (T_m of 45°)

A/H3: NNN NNN NNN NNN N (SEQ ID NO: 10) (T_m of 42°)

FluB: NNN NNN NNN NNN N (SEQ ID NO: 11) (T_m of 46°)

phiMS2: NNN NNN NNN NNN N (SEQ ID NO: 12) (T_m of 43°)

[0141] 5 targets plasmids encompassing the sequences specific to the primers and PCR probes above were added to a 100uL PCR reaction and the solution was prepped and loaded as described in above. The resulting real time array based PCR data is shown in **Figure 6**.

EXAMPLE 5: HIGH LEVEL MULTIPLEXING

[0142] This example demonstrates single chamber multiplexing for detection of multiple targets that can be any of the ten potential targets included in the panel of this example. This level of multiplexing – a panel of more than five potential targets – cannot be achieved in traditional solution phase PCR.

[0143] The experimental materials and procedures were the same as that above except for the following: first, 10 Primer sets and PCR probes were incorporated into PCR reaction in the same concentrations as above. The sequences of the PCR probes and capture probes are given below:

PCR Probes:

FluA: NNNNNNNNNNNN NNCCCCATGG AATGTTATCT CCCTTTTAAG
CTTCTNNNNNNNN (SEQ ID NO: 13) (T_m of 50.3°)

A/H1: NNNNNNNNNNNNNNNN ACCTTGCGCT ATTAGATTTC
CA'TTGGCCNNNNNNNN (SEQ ID NO: 14) (T_m of 51.2°)

A/H3: NNNNNNNNNNNNNN CCTGTTGCCA ATTCAGAG TGTTTGTCT
TAACNNNNNNNNNNNN (SEQ ID NO: 15) (T_m of 51°)

FluB-v2: NNNNNNNNNNNNNN TCAAAGCC AATTCGAGCA GCTGAAAC TNNNNNNNN
(SEQ ID NO: 16) (T_m of 51°)

phiMS2: NNNNNNNNNN NNNNTCGCTG AACAAGCAA CCGTTACCC NNNNNNNN
(SEQ ID NO: 17) (T_m of 52°)

MPV: NNNNNNNNNNNNNN NATGG CCGTTAGCTT CAGTCAATTC AACAGNNNNNNNN
(SEQ ID NO: 18) (T_m of 48.4°)

PIV1: NNNNNNNNNNNNNNTTGGAATT GTCTCGACA ACAATCTTTG
GCCTNNNNNNNNNN (SEQ ID NO: 19) (T_m of 50.4°)

PIV2: NNNNNNNNNNNNNNCCATTT ACCTAAGTGA TGGAAATCAAT
CGCAAAGNNNNNNNN (SEQ ID NO: 20) (T_m of 48.8°)

PIV3: NNNNNNNNNNNNNNNACATAA GCTTTGATC AACCCATATG
CTGCACNNNNNNNNNN (SEQ ID NO: 21) (T_m of 49.9°)

RSV: NNNNNNNNNN NNNTTCGAAGGCTC CACATACACAG CTGCTGNNNNNNNNNN
(SEQ ID NO: 22) (T_m of 49.9°)

RSV-v2: NNNNNNNNNNNNNNTCGAAGGC TCCACATACA CAGCTGCTGNNNNNNNNNN
(SEQ ID NO: 23) (T_m of 51°)

OPC1: NNNNNNNNNNNNNNTTCGGCAT TTCCTGGATTGAGT
CGGTACTANNNNNNNNNN (SEQ ID NO: 24) (T_m of 48.7°)

Capture Probes

	Capture Probe T_m	
FluA	NNN NNN NNN NNN N	SEQ ID NO:26 (T_m of 46°)
A/H1	NNN NNN NNN NNN N	SEQ ID NO:27 (T_m of 45°)
A/H3	NNN NNN NNN NNN N	SEQ ID NO:28 (T_m of 42°)
FluB-v2	NNN NNN NNN NNN N	SEQ ID NO:29 (T_m of 46°)
phiMS2	NNN NNN NNN NNN N	SEQ ID NO:30 (T_m of 43°)
MPV	NNN NNN NNN NNN N	SEQ ID NO:31
PIV1	NNN NNN NNN NNN N	SEQ ID NO:32
PIV2	NNN NNN NNN NNN N	SEQ ID NO:33

PIV3	NNN NNN NNN NNN N	SEQ ID NO:34
RSV	NNN NNN NNN NNN N	SEQ ID NO:35
RSV-v2	NNN NNN NNN NNN N	SEQ ID NO:36
OPC1	NNN NNN NNN NNN N	SEQ ID NO:37

[0144] **Figure 7** shows the resulting real time array-based PCR curves when no target was added to the PCR reactions (No Template Control). As can be seen from the figure, no signal was obtained from a solution containing all PCR components except target. **Figure 8** shows the same experiment with 3 plasmid targets (MPV, OPC-1, PIV2) added at 10,000 copies/uL.

EXAMPLE 6: DEMONSTRATION OF FAST HYBRIDIZATION KINETICS

[0145] A PCR chamber was constructed as described above. The following amine, pegylated capture probe sequence was deposited on the bottom substrate: NNN NNN NNN NNN N (SEQ ID NO:38).

[0146] A solution containing the PCR buffer described above was prepared containing the following oligo sequence (100 nM) which mimics the 5' flap portion of the PCR probe, labeled with a Cy3 fluorophore on the 5' end and complimentary to the capture probe: NNN NNN NNN NNN N (SEQ ID NO:39).

[0147] The solution was loaded into the PCR chamber and the chamber was heated to 60 °C (15 degrees above the T_m of the duplex) and then cooled back down to 30 °C. This mimics the conditions during the hybridization step of the PCR protocol. An optical reading was taken every 20 sec for 2 min. The resulting data is shown in **Figure 9**.

[0148] One interesting aspect of the data shows that there is already significant hybridization occurring at the instant that the internal temperature reaches 30 °C.

EXAMPLE 7: DARK ASSAY CONFIGURATION

[0149] The three step amplification assay described in Example 1, above, was repeated using a flap portion of the probe that included an Iowa Black RQ quencher group available from Integrated DNA Technologies, coupled to its 5' end, and without any other labeling or

WHAT IS CLAIMED IS:

1. A method of detecting a target nucleic acid sequence in a sample, comprising:
performing an amplification reaction on the sample with a polymerase enzyme that possesses nuclease activity, in the presence of a reagent comprising first probes that comprise a first portion complementary to the target nucleic acid sequence and a second portion not complementary to the first target nucleic acid sequence, the second portion comprising a first quencher moiety coupled to the second portion at a first position, such that the second portion is cleaved from the first portion as a first probe fragment, when the target nucleic acid sequence is amplified;

hybridizing the first probe fragment to capture probes immobilized upon a substrate, wherein the capture probes comprise a fluorophore that is at least partially quenched by the first quencher moiety, the fluorophore coupled to a second position on the capture probes such that upon hybridization of the probe fragments to the capture probes, the fluorophore is at least partially quenched by the quencher; and

detecting the presence of the target sequence based upon the quenching of the fluorophore on the capture probes.

2. The method of claim 1, wherein:
the amplification reagent comprises a plurality of different probes having a plurality of different first portions complementary to different target nucleic acid sequences, and different second portions not complementary to the plurality of target nucleic acid sequences, the second portions each comprising a quencher moiety coupled to the first position, the plurality of second portions being cleaved as probe fragments upon amplification of the plurality of target nucleic acid sequences; and

wherein the substrate comprises a plurality of different capture probe regions arrayed on the substrate, each capture probe region comprising capture probes complementary to a different second portion of the plurality of different probes, and each capture probe comprising a fluorophore quenched by the quencher moiety.

3. The method of claim 1, wherein the first position comprises the 5' end of the probe fragment, and the second position comprises the 3' end of the capture probe.
4. The method of claim 1, wherein each capture probe region comprises a non-rate limiting number of capture nucleic acids that hybridize to the probe fragment.
5. The method of claim 1, wherein the first probe fragment that hybridizes to the capture probes is less than about 30 nucleotides in length.
6. The method of claim 1, wherein the first probe fragment that hybridizes to the capture probes is less than about 20 nucleotides in length.
7. The method of claim 1, wherein the first probe fragment that hybridizes to the capture probes is less than about 15 nucleotides or less in length.
8. The method of claim 1, wherein the target nucleic acid is amplified for at least 5 amplification cycles prior to said detecting.
9. The method of claim 1, wherein the target nucleic acid is amplified in a plurality of amplification cycles prior to said detecting, wherein the target nucleic acid portion is additionally amplified after said detecting, in the presence of additional copies of the probe, with resulting released first probe fragments being subsequently hybridized to the array and detected, wherein detected signal intensity is correlated to a quantity of the target nucleic acid present in the sample.
10. The method of claim 1, wherein the hybridization temperature is less than the temperature of the amplification reaction.

11. The method of claim 1, comprising detecting local background for one or more regions of the array, and normalizing signal intensity measurements by correcting for said background.
12. The method of claim 2, wherein the sample comprises a plurality of target nucleic acids.
13. The method of claim 2, wherein the plurality of different capture probes are spatially separated on the substrate.
14. The method of claim 2, wherein there are between about 5 and about 100 different capture probes, and between about 5 and about 100 corresponding probes in the amplification reaction, wherein up to 5 to 100 different signals can be detected based upon positioning of the signals in the array.
15. The method of claim 2, wherein the capture probes are arrayed on the substrate at a density of about between about 350 fmoles/cm² and about 5,000 fmoles/cm² or greater.
16. The method of claim 2, wherein the capture probes are arrayed on the substrate at a density greater than 2000 fmoles/cm².
17. The method of claim 2, wherein the plurality of different capture probes comprise the same label moiety, and the plurality of different probe fragments comprise the same quencher moiety.
18. The method of claim 1, wherein the amplifying step and the step of hybridizing the first probe fragment to the substrate are carried out at the same temperature.

regions designed to form secondary structure. The predicted melting temperature of the secondary structure was 51 °C, as determined by mFold (idtdna.com) using the PCR buffer conditions. The PCR probe was labeled with a 5' Cy3 fluorophore and a Black Hole Quencher 2 moiety on the 3' end.

[0128] The capture probe sequence attached to the bottom substrate of the PCR chamber was: NNN NNN NNN NNN N with a T_m of 42 °C using the PCR buffer conditions.

[0129] DNA plasmids comprising a target sequence were added to each PCR reaction at a concentration of 10^6 , 10^4 , and 10^2 copies/uL. The solution was then degassed by heating to 95 °C. After degassing, polymerase was added and the reaction was loaded into the PCR chamber using a pipette. Left over solution was loaded onto an Applied Biosystems 7500 for parallel analysis.

[0130] The cycling conditions for the array based PCR were as follows:

Temp	Time	Purpose
95 °C	120 sec	Fast Start Enzyme activation
95C	15 sec	denaturation
60C	60 sec	polymerase extension
30C	120 sec	Flap hybridization and optical reading

(denaturation and extension were performed for 5 cycles, and then denaturation/ extension/ flap hybridization and optical reading are repeated for 8 cycles).

[0131] The Cycling conditions for the ABI 7500 were as follows:

Temp	Time	Purpose
95 °C	120 sec	Fast Start Enzyme activation
95 °C	15 sec	denaturation
60 °C	60sec	polymerase extension and optical reading

Denaturation/ extension and reading were performed for 40 cycles.

[0132] The results for the copy number titrations are shown in **Figure 3** for the array based PCR and **Figure 4** for the solution phase PCR. As can be seen from the figures, the results are comparable, giving similar behavior for the titrations.

EXAMPLE 2: TWO STEP AMPLIFICATION REACTION

[0133] As with Example 1, above, the amplification reagent mix contained standard PCR reagents including two PCR primers (200nM) complementary to each target being amplified as well as a PCR probe (300nM) having a sequence complementary to each target being amplified. The structure of typical probe is shown in Figure 1B. As shown, the labeled probe again includes a nucleic acid fragment (A) that is complimentary to a target amplicon designed using the same rules as is typical for a traditional real time PCR probe (i.e. TaqMan). Also included is orthogonal nucleic acid "flap" sequence (B) that is complimentary to a corresponding capture probe on the capture array. The probe also includes a fluorescent label (C) coupled to the flap portion B and a quencher moiety (D) coupled to the target specific portion (A).

[0134] For the two step amplification, the orthogonal flap (B) comprises a sequence that was designed to have a T_m with its complement on the capture array of 70° C. Typically the sequence length is 25 to 27 bases. As with Example 1, above, the overall probe is designed so that the most stable secondary structure has a T_m no higher than 10° C below the extension and measurement temperature in the buffer conditions used for PCR. The oligo was designed using the unfold software available at www.idtdna.com. The following PCR probe sequence was used in this example:

NNN NNN NNN NNN NNN NNN N/Cy3/NN NNN NNN ATG GCC GTT AGC TTC
AGT CAA TTC AAC AG/BHQ 2/ (SEQ ID NO:2)

[0135] Where the double underlined sequence constitutes the orthogonal flap and the non-underlined sequence is homologous to the amplicon. The most stable secondary structure of the probe has a melting temperature of 45° C. The T_m of the orthogonal flap is 71° C. The PCR probe was labeled with an internal Cy3 fluorophore C (available from GE Healthcare Biosciences, Piscataway, NJ) and a Black Hole Quencher 2 moiety D (available from Biosearch, Inc., Novato, CA) on the 3' end.

[0136] A capture probe was spotted that was homologous to the flap portion of the PCR

[0140] The PCR probe and capture probe sequences are shown below:

PCR Probes:

Flu A: NNNNNNNNNNNNNNCCCAT GGAATGTTAT

CTCCCTTTTAAGCTTCTNNNNNNNN (SEQ ID NO:4) (T_m of 50.3°)

A/H1: NNNNNNNNNNNNNNACCTTGGC GCTATTAGAT TTCCATTGTC CNNNNNNNN

(SEQ ID NO:5) (T_m of 51.2°)

A/H3: NNNNNNNNNNNNNNCCTGTT GCCAATTT CAGAGTGTT

TTGCTTAACNNNNNNNNNNNN (SEQ ID NO:6) (T_m of 51°)

FluB: NNNNNNNNNNNNNNNTCAAAGC CAATTCGAG CAGCTGAAAC TNNNNNNNN

(SEQ ID NO: 7) (T_m of 51°)

phiMS2: NNNNNNNNNNNNNNNTCGCTGAA CAAGCAACC

GTTACCCNNNNNNNNNNNN (SEQ ID NO:8) (T_m of 52°)

Capture Probes

FluA:	NNN NNN NNN NNN N	(T_m of 46°)
A/H1:	NNN NNN NNN NNN N	(T_m of 45°)
A/H3:	NNN NNN NNN NNN N	(T_m of 42°)
FluB:	NNN NNN NNN NNN N	(T_m of 46°)
phiMS2:	NNN NNN NNN NNN N	(T_m of 43°)

[0141] 5 targets plasmids encompassing the sequences specific to the primers and PCR probes above were added to a 100uL PCR reaction and the solution was prepped and loaded as described in above. The resulting real time array based PCR data is shown in **Figure 6**.

EXAMPLE 5: HIGH LEVEL MULTIPLEXING

[0142] This example demonstrates single chamber multiplexing for detection of multiple targets that can be any of the ten potential targets included in the panel of this example. This level of multiplexing – a panel of more than five potential targets – cannot be achieved in traditional solution phase PCR.

[0143] The experimental materials and procedures were the same as that above except for the following: first, 10 Primer sets and PCR probes were incorporated into PCR reaction in the same concentrations as above. The sequences of the PCR probes and capture probes are given below:

PCR Probes:

FluA: NNNNNNNNNNNN NNCCCCATGG AATGTTATCT CCCTTTTAAG
CTTCTNNNNNNNN (SEQ ID NO:9) (T_m of 50.3°)

A/H1: NNNNNNNNNNNNNNNN ACCTTGCGCT ATTAGATTTC
CATTGCGCNNNNNNNN (SEQ ID NO:10) (T_m of 51.2°)

A/H3: NNNNNNNNNNNNNNNN CCTGTTGCCA ATTTCAAGAG TGTTTTGCT
TAACNNNNNNNNNN (SEQ ID NO:11) (T_m of 51°)

FluB-v2: NNNNNNNNNNNNNNNN TCAAAGCC AATTCGAGCA GCTGAAAC
TNNNNNNNN (SEQ ID NO:12) (T_m of 51°)

phiMS2: NNNNNNNNNN NNNNTCGCTG AACAAGCAA CCGTTACCC NNNNNNNN
(SEQ ID NO:13) (T_m of 52°)

MPV: NNNNNNNNNNNNNNNN ATGG CCGTTAGCTT CAGTCAATTC
AACAGNNNNNNNN (SEQ ID NO:14) (T_m of 48.4°)

PIV1: NNNNNNNNNNNNNNNTTGGAATT GTCTCGACA ACAATCTTTG
GCCTNNNNNNNNNN (SEQ ID NO:15) (T_m of 50.4°)

PIV2: NNNNNNNNNNNNNNNCCATTT ACCTAAGTGA TGGAATCAAT
CGCAAAAGNNNNNNNNNN (SEQ ID NO:16) (T_m of 48.8°)

PIV3: NNNNNNNNNNNNNNNNNACATAA GCTTTGATC AACCTATG
CTGCACNNNNNNNNNN (SEQ ID NO:17) (T_m of 49.9°)

RSV: NNNNNNNNNN NNNTTCGAAGGCTC CACATACACAG
CTGCTGNNNNNNNNNN (SEQ ID NO:18) (T_m of 49.9°)

RSV-v2: NNNNNNNNNNNNNNNTCGAAGGC TCCACATACA
CAGCTGCTGNNNNNNNNNN (SEQ ID NO:19) (T_m of 51°)

OPC1: NNNNNNNNNNNNNNNTTCGGCAT TTCCTGGATTGAGT
CGGTACTANNNNNNNNNN (SEQ ID NO:20) (T_m of 48.7°)

Capture Probes

	Capture Probe T _m	
FluA	NNN NNN NNN NNN N	(T _m of 46°)
A/H1	NNN NNN NNN NNN N	(T _m of 45°)
A/H3	NNN NNN NNN NNN N	(T _m of 42°)
FluB-v2	NNN NNN NNN NNN N	(T _m of 46°)
phiMS2	NNN NNN NNN NNN N	(T _m of 43°)
MPV	NNN NNN NNN NNN N	
PIV1	NNN NNN NNN NNN N	
PIV2	NNN NNN NNN NNN N	

PIV3	NNN NNN NNN NNN N
RSV	NNN NNN NNN NNN N
RSV-v2	NNN NNN NNN NNN N
OPC1	NNN NNN NNN NNN N

[0144] **Figure 7** shows the resulting real time array-based PCR curves when no target was added to the PCR reactions (No Template Control). As can be seen from the figure, no signal was obtained from a solution containing all PCR components except target. **Figure 8** shows the same experiment with 3 plasmid targets (MPV, OPC-1, PIV2) added at 10,000 copies/uL.

EXAMPLE 6: DEMONSTRATION OF FAST HYBRIDIZATION KINETICS

[0145] A PCR chamber was constructed as described above. The following amine, pegylated capture probe sequence was deposited on the bottom substrate: NNN NNN NNN NNN N.

[0146] A solution containing the PCR buffer described above was prepared containing the following oligo sequence (100 nM) which mimics the 5' flap portion of the PCR probe, labeled with a Cy3 fluorophore on the 5' end and complimentary to the capture probe: NNN NNN NNN NNN N.

[0147] The solution was loaded into the PCR chamber and the chamber was heated to 60 °C (15 degrees above the T_m of the duplex) and then cooled back down to 30 °C. This mimics the conditions during the hybridization step of the PCR protocol. An optical reading was taken every 20 sec for 2 min. The resulting data is shown in **Figure 9**.

[0148] One interesting aspect of the data shows that there is already significant hybridization occurring at the instant that the internal temperature reaches 30 °C.

EXAMPLE 7: DARK ASSAY CONFIGURATION

[0149] The three step amplification assay described in Example 1, above, was repeated using a flap portion of the probe that included an Iowa Black RQ quencher group available from Integrated DNA Technologies, coupled to its 5' end, and without any other labeling or

WHAT IS CLAIMED IS:

1. A method of detecting a target nucleic acid sequence in a sample, comprising:
performing an amplification reaction on the sample with a polymerase enzyme that possesses nuclease activity, in the presence of a reagent comprising first probes that comprise a first portion complementary to the target nucleic acid sequence and a second portion not complementary to the first target nucleic acid sequence, the second portion comprising a first quencher moiety coupled to the second portion at a first position, such that the second portion is cleaved from the first portion as a first probe fragment, when the target nucleic acid sequence is amplified;

hybridizing the first probe fragment to capture probes immobilized upon a substrate, wherein the capture probes comprise a fluorophore that is at least partially quenched by the first quencher moiety, the fluorophore coupled to a second position on the capture probes such that upon hybridization of the probe fragments to the capture probes, the fluorophore is at least partially quenched by the quencher; and

detecting the presence of the target sequence based upon the quenching of the fluorophore on the capture probes.

2. The method of claim 1, wherein:

the amplification reagent comprises a plurality of different probes each different probe having a different first portion complementary to a different target nucleic acid sequence, and each different probe having a different second portion not complementary to a target nucleic acid sequence, the second portion of each different probe comprising a quencher moiety coupled to the first position, the second portion being cleaved as a probe fragment upon amplification of the plurality of target nucleic acid sequences; and

wherein the substrate comprises a plurality of different capture probe regions arrayed on the substrate, each capture probe region comprising capture probes complementary to a different second portion of the plurality of different probes, and each capture probe comprising a fluorophore quenched by the quencher moiety.

3. The method of claim 1, wherein the first position comprises the 5' end of the probe fragment, and the second position comprises the 3' end of the capture probe.
4. The method of claim 2, wherein each capture probe region comprises a non-rate limiting number of capture nucleic acids that hybridize to the probe fragment.
5. The method of claim 1, wherein the first probe fragment that hybridizes to the capture probes is less than about 30 nucleotides in length.
6. The method of claim 1, wherein the first probe fragment that hybridizes to the capture probes is less than about 20 nucleotides in length.
7. The method of claim 1, wherein the first probe fragment that hybridizes to the capture probes is less than about 15 nucleotides or less in length.
8. The method of claim 1, wherein the target nucleic acid is amplified for at least 5 amplification cycles prior to said detecting.
9. The method of claim 1, wherein the target nucleic acid is amplified in a plurality of amplification cycles prior to said detecting, wherein the target nucleic acid portion is additionally amplified after said detecting, in the presence of additional copies of the probe, with resulting released first probe fragments being subsequently hybridized to the array and detected, wherein detected signal intensity is correlated to a quantity of the target nucleic acid present in the sample.
10. The method of claim 1, wherein a temperature of the hybridizing step is less than a temperature of the amplification reaction.
11. The method of claim 1, comprising detecting local background for one or more regions of the array, and normalizing signal intensity measurements by correcting for said background.

12. The method of claim 2, wherein the sample comprises a plurality of target nucleic acids.
13. The method of claim 2, wherein the plurality of different capture probes are spatially separated on the substrate.
14. The method of claim 2, wherein there are between about 5 and about 100 different capture probes, and between about 5 and about 100 corresponding probes in the amplification reaction, wherein up to 5 to 100 different signals can be detected based upon positioning of the signals in the array.
15. The method of claim 2, wherein the capture probes are arrayed on the substrate at a density of about between about 350 fmoles/cm² and about 5,000 fmoles/cm² or greater.
16. The method of claim 2, wherein the capture probes are arrayed on the substrate at a density greater than 2000 fmoles/cm².
17. The method of claim 2, wherein the plurality of different capture probes comprise the same fluorophore, and the plurality of different probe fragments comprise the same quencher moiety.
18. The method of claim 1, wherein the amplifying step and the step of hybridizing the first probe fragment to the substrate are carried out at the same temperature.

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SEQUENCE LISTING

<110> NVS Technologies, Inc.
Scaboo, Kris
Martin, Patrick
Taft, Brad
La, Jason

<120> QUANTITATIVE, HIGHLY MULTIPLEXED DETECTION OF NUCLEIC ACIDS

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 <223> n is a, c, g, or t

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45

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54

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49

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51

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<400> 12
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<400> 13
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<400> 16
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<223> n is a, c, g, or t

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<400> 18

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<210> 19

<211> 48

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<220>

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<223> n is a, c, g, or t

<400> 19

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48

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<222> (44)..(51)

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51