36. The method of claim 35 wherein the resonance energy transfer pair comprises fluorescein as the donor and a member selected from the group consisting of Cy5 and Cy5.5 as the acceptor.

37. The method of claim 35 wherein the donor and the acceptor are coupled to the probes such that when both probes are hybridized to the amplified selected segment or the amplified corresponding segment, the donor and acceptor are separated by no more than about 25 nucleotide residues.

38. The method of claim 37 wherein the donor and the acceptor are coupled to probes such that when the probes are hybridized to the amplified selected segment or the amplified corresponding segment, the donor and acceptor are separated by about 0-5 nucleotide residues.

39. The method of claim 38 wherein the resonance energy transfer donor and the resonance energy transfer acceptor are coupled to the probes such that when the probes are hybridized to the amplified selected segment or the amplified corresponding segment the donor and acceptor are separated by about 0-2 nucleotide residues.

40. The method of claim 39 wherein the resonance energy transfer donor and the resonance energy transfer acceptor are coupled to the probes such that when the probes are hybridized to the amplified selected segment or the amplified corresponding segment the donor and acceptor are separated by 1 nucleotide residue.

41. A method of detecting a difference at a selected locus in a first nucleic acid as compared to a second nucleic acid, comprising the steps of:

(a) providing a pair of oligonucleotide primers configured for amplifying, by polymerase chain reaction, a selected segment of the first nucleic acid and a corresponding segment of the second nucleic acid, wherein the selected segment and corresponding segment each comprises the selected locus, to result in amplified products containing a copy of the selected locus;

(b) providing an oligonucleotide probe, wherein one of said primers and the probe are each labeled with one member of a fluorescence energy transfer pair comprising an donor fluorophore and an acceptor fluorophore, and wherein the labeled probe and labeled primer hybridize to the amplified products such that the donor and acceptor are in resonance energy transfer relationship, and wherein the probe is configured for hybridizing to the amplified products such that said probe spans the selected locus and exhibits a melting

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profile when the difference is present in the first nucleic acid that is distinguishable from a melting profile of the second nucleic acid;

(c) amplifying the selected segment of first nucleic acid and the corresponding segment of the second nucleic acid by polymerase chain reaction in the presence of effective amounts of primers and probe to result in an amplified selected segment and an amplified corresponding segment, at least a portion thereof having the labled primer and probe hybridized thereto with the fluorogenic resonance energy transfer pair in resonance energy transfer relationship;

(d) illuminating the amplified selected segment and the amplified corresponding segment with the labeled primer and probe hybridized thereto with a selected wavelength of light to elicit fluorescence by the fluorogenic resonance energy transfer pair;

(e) measuring fluorescence emission as a function of temperature to determine in a first melting profile of said probe melting from the amplified selected segment of first nucleic acid and a second melting profile of said probe melting from the amplified corresponding segment of second nucleic acid; and

(f) comparing the first melting profile to the second melting profile, wherein a difference therein indicates the presence of the difference in the sample nucleic acid.

42. The method of claim 41 wherein the resonance energy transfer pair comprises fluorescein as the donor and a member selected from the group consisting of Cy5 and Cy5.5 as the acceptor.

43. The method of claim 41 wherein the donor and the acceptor are coupled to the labeled primer and probe such that when both the labeled primer and probe are hybridized to the amplified selected segment or the amplified corresponding segment, the donor and acceptor are separated by no more than about 15 nucleotide residues.

44. The method of claim 43 wherein the donor and the acceptor are coupled to labeled primer and probe such that when the labeled primer and probe are hybridized to the amplified selected segment or the amplified corresponding segment, the donor and acceptor are separated by about 4-6 nucleotide residues.

45. A method of detecting heterozygosity at a selected locus in the genome of an individual, wherein the genome comprises a mutant allele and a corresponding reference allele, each comprising the selected locus, comprising the steps of:

(a) obtaining sample genomic DNA from the individual;

(b) providing a pair of oligonucleotide primers configured for amplifying, by polymerase chain reaction, a first selected

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segment of the mutant allele and a second selected segment of the corresponding reference allele wherein both the first and second selected segments comprise the selected locus;

(c) providing a pair of oligonucleotide probes, one of said probes being labeled with an acceptor fluorophore and the other probe being labeled with a donor fluorophore of a fluorogenic resonance energy transfer pair such that upon hybridization of the two probes with the amplified first and second selected segments one of the probes spans the selected locus and exhibits a first melting profile with the amplified first selected segment that is distinguishable from a second melting profile with the amplified second selected segment;

(d) amplifying the first and second selected segments of sample genomic DNA by polymerase chain reaction in the presence of effective amounts of probes to result in amplified first and second selected segments, at least a portion thereof having both the probes hybridized thereto with the fluorogenic resonance energy transfer pair in resonance energy transfer relationship;

(e) illuminating the amplified first and second selected segments having the probes hybridized thereto with a selected wavelength of light to elicit fluorescence by the donor and acceptor;

(f) measuring a fluorescence emission as a function of temperature to determine a first melting profile of said one of the probes melting from the amplified first selected segment and a second melting profile of said one of the probes melting from the amplified second selected segment; and

(g) comparing the first melting profile to the second melting profile, wherein distinguishable melting profiles indicate heterozygosity in the sample genomic DNA.

46. The method of claim 45 wherein the fluorogenic resonance energy transfer pair comprises fluorescein as the donor and Cy5 or Cy5.5 as the acceptor.

47. The method of claim 45 wherein the donor and the acceptor are coupled to the probes such that when the probe and reference oligonucleotide are hybridized to the amplified first selected segment or the amplified second selected segment, the donor and acceptor are separated by no more than about 25 nucleotide residues.

48. The method of claim 47 wherein the donor and the acceptor are coupled to the probes such that when the probes are hybridized to the amplified first selected segment or the amplified second

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selected segment, the donor and acceptor are separated by about 0-5 nucleotide residues.

49. The method of claim 48 wherein the donor and the acceptor are coupled to the probes such that when the probes are hybridized to the amplified first selected segment or the amplified second selected segment, the donor and acceptor are separated by about 0-2 nucleotide residues.

50. The method of claim 49 wherein the donor and the acceptor are coupled to the probes such that when the probes are hybridized to the amplified first selected segment or the amplified second selected segment, the donor and acceptor are separated by 1 nucleotide residue.

51. A method of detecting heterozygosity at a selected locus in the genome of an individual, wherein the genome comprises a mutant allele and a corresponding reference allele, each comprising the selected locus, comprising the steps of:

(a) obtaining sample genomic DNA from the individual;

(b) providing a pair of oligonucleotide primers configured for amplifying, by polymerase chain reaction, a first selected segment of the mutant allele and a second selected segment of the corresponding reference allele wherein both the first and second selected segments comprise the selected locus;

(c) providing an oligonucleotide probe, wherein one of said primers and the probe are each labeled with one member of a fluorescence energy transfer pair comprising an donor fluorophore and an acceptor fluorophore, and wherein the labeled probe and labeled primer hybridize to the amplified first and second selected segments such that one of the probes spans the selected locus and exhibits a first melting profile with the amplified first selected segment that is distinguishable from a second melting profile with the amplified second selected segment;

(d) amplifying the first and second selected segments of sample genomic DNA by polymerase chain reaction in the presence of effective amounts of primers and probe to result in amplified first and second selected segments, at least a portion thereof having both the labeled primer and probe hybridized thereto with the fluorogenic resonance energy transfer pair in resonance energy transfer relationship;

(e) illuminating the amplified first and second selected segments having the labeled primer and probe hybridized thereto with

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a selected wavelength of light to elicit fluorescence by the donor and acceptor;

(f) measuring a fluorescence emission as a function of temperature to determine a first melting profile of said probe melting from the amplified first selected segment and a second melting profile of said probe melting from the amplified second selected segment; and

(g) comparing the first melting profile to the second melting profile, wherein distinguishable melting profiles indicate heterozygosity in the sample genomic DNA.

52. The method of claim 51 wherein the resonance energy transfer pair comprises fluorescein as the donor and Cy5 or Cy5.5 as the acceptor.

53. The method of claim 51 wherein the donor and the acceptor are coupled to the labeled primer and probe such that when the labeled primer and probe are hybridized to the amplified first selected segment or the amplified second selected segment, the donor and acceptor are separated by no more than about 15 nucleotide residues.

54. The method of claim 53 wherein the donor and the acceptor are coupled to the labeled primer and probe such that when the labeled primer and probe are hybridized to the amplified first selected segment or the amplified second selected segment, the donor and acceptor are separated by about 4-6 nucleotide residues.

55. A method of determining completion of a polymerase chain reaction in a polymerase chain reaction mixture comprising (1) a nucleic acid wherein the nucleic acid or a polymerase-chain-reaction-amplified product thereof consists of two distinct complementary strands, (2) two oligonucleotide primers configured for amplifying by polymerase chain reaction a selected segment of the nucleic acid to result in an amplified product, and (3) a DNA polymerase for catalyzing the polymerase chain reaction, comprising the steps of:

(a) adding to the mixture (1) an effective amount of an oligonucleotide probe labeled with a resonance energy transfer donor or a resonance energy transfer acceptor of a fluorogenic resonance energy transfer pair, wherein the probe is configured for hybridizing to the amplified product under selected conditions of temperature and monovalent ionic strength, and (2) an effective amount of a reference oligonucleotide labeled with the donor or the acceptor, with the proviso that as between the probe and reference oligonucleotide one is labeled with the donor and the other is labeled with the acceptor,

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wherein the reference oligonucleotide is configured for hybridizing to the amplified product under the selected conditions of temperature and monovalent ionic strength such that the donor and the acceptor are in resonance energy transfer relationship when both the probe and the reference oligonucleotide hybridize to the amplified product;

(b) amplifying the selected segment of nucleic acid by polymerase chain reaction to result in the amplified product, at least a portion thereof having both the probe and the reference oligonucleotide hybridized thereto with the fluorogenic resonance energy transfer pair in resonance energy transfer relationship; and

(c) illuminating the amplified product having the probe and reference oligonucleotide hybridized thereto with a selected wavelength of light for eliciting fluorescence by the fluorogenic resonance energy pair and monitoring fluorescence emission and determining a cycle when the fluorescence emission reaches a plateau phase, indicating the completion of the reaction.

56. The method of claim 55 wherein the fluorogenic resonance energy transfer pair is fluorescein and a member selected from the group consisting of Cy5 and Cy5.5.

57. A method of determining completion of a polymerase chain reaction in a polymerase chain reaction mixture comprising (1) a nucleic acid wherein the nucleic acid or a polymerase-chain-reaction-amplified product thereof consists of two distinct complementary strands, (2) two oligonucleotide primers configured for amplifying by polymerase chain reaction a selected segment of the nucleic acid to result in an amplified product, and (3) a DNA polymerase for catalyzing the polymerase chain reaction, comprising the steps of:

(a) adding to the mixture an effective amount of a nucleic-acid-binding fluorescent dye;

(b) amplifying the selected segment of nucleic acid by polymerase chain reaction in the mixture to which the nucleic-acid-binding fluorescent dye has been added to result in the amplified product with nucleic-acid-binding fluorescent dye bound thereto; and

(c) illuminating amplified product with nucleic-acid-binding fluorescent dye bound thereto with a selected wavelength of light for eliciting fluorescence therefrom and monitoring fluorescence emission and determining a cycle when the fluorescence emission reaches a plateau phase, indicating the completion of the reaction.

58. The method of claim 57 wherein the nucleic-acid-binding fluorescent dye is a member selected from the group consisting of

SYBR™ GREEN I, ethidium bromide, pico green, acridine orange, thiazole orange, YO-PRO-1, and chromomycin A3.

59. The method of claim 58 wherein the double-strand-specific fluorescent dye is SYBR™ GREEN I.

60. A method of controlling temperature cycling parameters of a polymerase chain reaction comprising repeated cycles of annealing, extension, and denaturation phases of a polymerase chain reaction mixture comprising a double-strand-specific fluorescent dye, wherein the parameters comprise duration of the annealing phase, duration of the denaturation phase, and number of cycles, comprising

(a) illuminating the reaction with a selected wavelength of light for eliciting fluorescence from the fluorescent dye and continuously monitoring fluorescence during the repeated annealing, extension, and denaturation phases;

(b) determining at least

(i) duration for fluorescence to stop increasing during the extension phase, or

(ii) Duration for fluorescence to decrease to a baseline level during the denaturation phase, or

(iii)number of cycles for fluorescence to reach a preselected level during the extension phase; and

(c) adjusting the length of the extension phase according to the length of time for fluorescence to stop increasing during the extension phase, the length of the denaturation phase according to the length of time for fluorescence to decrease to the baseline level during the denaturation phase, or the number of cycles according to the number of cycles for fluorescence to reach the preselected level during the extension phase.

61. The method of claim 60 wherein the double-strand specific fluorescent dye is a member selected from the group consisting of SYBR™ GREEN I, ethidium bromide, pico green, acridine orange, thiazole orange, YO-PRO-1, and chromomycin A3.

62. The method of claim 61 wherein the double-strand-specific fluorescent dye is SYBR™ GREEN I.

63. A method of determining a concentration of an amplified product in a selected polymerase chain reaction mixture comprising

(a) determining a second order rate constant for the amplified product at a selected temperature and reaction conditions by monitoring rate of hybridization of a known concentration of the amplified product;

(b) determining rate of annealing for an unknown concentration of the amplified product; and

(c) calculating the concentration of the amplified product from the rate of annealing and the second order rate constant.

64. The method of claim 63 wherein the rate of annealing is determined after multiple cycles of amplification.

65. The method of claim 63 wherein determining the second oder rate constant comprises the steps of

raising the temperature of a first polymerase chain reaction mixture comprising a known concentration of the amplified product and an effective amount of a double-strand specific fluorescent dye, above the denaturation temperature of the amplified product to result in a denatured amplified product;

rapidly reducing the temperature of the first polymerase chain reaction mixture comprising the known amount of denatured amplified product to a selected temperature below the denaturation temperature of the amplified product while continuously monitoring the fluorescence of the first polymerase chain reaction mixture as a function of time;

plotting fluorescence as a function of time for determining maximum fluorescence, minimum fluorescence, the time at minimum fluorescence, and a second order rate constant for the known concentration of amplified product from the equation

$$F = F_{max} - \frac{F_{max} - F_{min}}{k(t-t_0)[DNA] + 1}$$

wherein F is fluorescence, F_{max} is maximum fluorescence, F_{min} is minimum fluorescence, k is the second order rate constant, t_0 is the time at F_{min}, and [DNA] is the known concentration of the amplified product.

66. The method of claim 65 wherein the double-strand specific fluorescent dye is a member selected from the group consisting of SYBR™ GREEN I, ethidium bromide, pico green, acridine orange, thiazole orange, YO-PRO-1, and chromomycin A3.

67. The method of claim 66 wherein the double-strand specific fluorescent dye is SYBR™ GREEN I.

68. A method of determining a concentration of a selected nucleic acid template by competitive quantitative polymerase chain reaction comprising the steps of:

(a) in a reaction mixture comprising:

(i) effective amounts of each of a pair of oligonucleotide primers configured for amplifying, in a polymerase chain reaction, a selected segment of the selected template and a corresponding selected segment of a competitive template to result in amplified products thereof,

(ii) an effective amount of an oligonucleotide probe labeled with a resonance energy transfer donor or a resonance energy transfer acceptor of a fluorogenic resonance energy transfer pair, wherein the probe is configured for hybridizing to the amplified products such that the probe melts from the amplified product of the selected template at a melting temperature that is distinguishable from the melting temperature at which the probe melts from the amplified product of the competitive template,

(iii) an effective amount of a reference oligonucleotide labeled with the donor or the acceptor, with the proviso that as between the probe and transfer oligonucleotide one is labeled with the donor and the other is labeled with the acceptor, wherein the reference oligonucleotide is configured for hybridizing to the amplified products such that the donor and the acceptor are in resonance energy transfer relationship when both the probe and the reference oligonucleotide hybridize to the amplified products;

amplifying, by polymerase chain reaction, an unknown amount of the selected template and a known amount of the competitive template to result in the amplified products thereof;

(b) illuminating the reaction mixture with a selected wavelength of light for eliciting fluorescence by the fluorogenic resonance energy transfer pair and determining a fluorescence emission as a function of temperature as the temperature of the reaction mixture is changed to result in a first melting curve of the probe melting from the amplified product of the selected template and a second melting curve of the probe melting from the competitive template;

(c) converting the first and second melting curves to first and second melting melting peaks and determining relative amounts of the selected template and the competitive template from such melting peaks; and

(d) calculating the concentration of the selected template based on the known amount of the competitive template and the relative amounts of selected template and competitive template.

69. The method of claim 68 wherein the reference oligonucleotide is one of the pair of oligonucleotide primers.

70. The method of claim 69 wherein the resonance energy transfer pair comprises fluorescein as the donor and Cy5 or Cy5.5 as the acceptor.

71. The method of claim 69 wherein the donor and the acceptor are coupled to the probe and reference oligonucleotide such that when the probe and reference oligonucleotide are hybridized to the amplified segment, the donor and acceptor are separated by no more than about 25 nucleotide residues.

72. The method of claim 71 wherein the donor and the acceptor are coupled to the probe and the reference oligonucleotide such that when the probe and reference oligonucleotide are hybridized to the amplified segment, the donor and acceptor are separated by about 0-5 nucleotide residues.

73. The method of claim 72 wherein the donor and the acceptor are coupled to the probe and reference oligonucleotide such that when the probe and transfer oligonucleotide are hybridized to the amplified segment, the donor and acceptor are separated by about 0-2 nucleotide residues.

74. The method of claim 73 wherein the donor and the acceptor are coupled to the probe and reference oligonucleotide such that when the probe and transfer oligonucleotide are hybridized to the amplified segment, the donor and acceptor are separated by 1 nucleotide residue.

75. The method of claim 68 wherein the reference oligonucleotide is not one of the oligonucleotide primers.

76. The method of claim 75 wherein the resonance energy transfer pair comprises fluorescein as the donor and Cy5 or Cy5.5 as the acceptor.

77. The method of claim 76 wherein the donor and the acceptor are coupled to the probe and the reference oligonucleotide such that when the probe and reference oligonucleotide are hybridized to the amplified segment, the donor and acceptor are separated by no more than about 15 nucleotide residues.

78. The method of claim 77 wherein the donor and the acceptor are coupled to the probe and the reference oligonucleotide such that when the probe and reference oligonucleotide are hybridized to the amplified segment, the donor and acceptor are separated by about 4-6 nucleotide residues.

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79. A fluorogenic resonance energy transfer pair consisting of fluorescein and Cy5.

80. A fluorogenic resonance energy transfer pair consisting of fluorescein and Cy5.5.

81. A method of determining a concentration of a selected nucleic acid template in a polymerase chain reaction comprising the steps of:

(a) in a reaction mixture comprising:

(i) effective amounts of each of a first pair of oligonucleotide primers configured for amplifying, in a polymerase chain reaction, a selected first segment of the selected template to result in an amplified first product thereof,

(ii) effective amounts of each of a second pair of oligonucleotide primers configured for amplifying, in a polymerase chain reaction, a selected second segment of a reference template to result in an amplified second product thereof,

(iii) an effective amount of a nucleic-acid-binding fluorescent dye;

amplifying, by polymerase chain reaction, an unknown amount of the selected template to result in the amplified first product and a known amount of the reference template to result in the amplified second product thereof;

(b) illuminating the reaction mixture with a selected wavelength of light for eliciting fluorescence by the nucleic-acid-binding fluorescent dye and continuously monitoring the fluorescence emitted as a function of temperature to result in a melting curve of the amplified products wherein the first product and second product melt at different temperatures;

(c) converting the melting curves to melting melting peaks and determining relative amounts of the selected template and the reference template from such melting peaks; and

(d) calculating the concentration of the selected template based on the known amount of the reference template and the relative amounts of selected template and reference template.

82. The method of claim 81 wherein the nucleic-acid-binding fluorescent dye is selected from the group consisting of of SYBR™ GREEN I, ethidium bromide, pico green, acridine orange, thiazole orange, YO-PRO-1, and chromomycin A3.

83. The method of claim 82 wherein the double-strand specific fluorescent dye is SYBR™ GREEN I.

84. A method of monitoring amplification of a selected template in a polymerase chain reaction that also comprises a positive control template comprising the steps of:

(a) in a reaction mixture comprising:

(i) effective amounts of each of a first pair of oligonucleotide primers configured for amplifying, in a polymerase chain reaction, a selected first segment of the selected template to result in an amplified first product thereof,

(ii) effective amounts of each of a second pair of oligonucleotide primers configured for amplifying, in a polymerase chain reaction, a selected second segment of the positive control template to result in an amplified second product thereof,

(iii) an effective amount of a nucleic-acid-binding fluorescent dye;

subjecting the selected template and the positive control template to conditions for amplifying the selected template and the positive control template by polymerase chain reaction; and

(b) illuminating the reaction mixture with a selected wavelength of light for eliciting fluorescence by the nucleic-acid-binding fluorescent dye and continuously monitoring the fluorescence emitted as a function of temperature during an amplification cycle of the polymerase chain reaction to result in a first melting peak of the amplified first product, if the selected template is amplified, and a second melting peak of the amplified second product, if the positive control template is amplified;

wherein obtaining of the second melting curve indicates that the polymerase chain reaction was operative, obtaining the first melting curve indicates that the selected first segment was amplifiable, and absence of the first melting curve indicates that the selected first segment was not amplifiable.

85. The method of claim 84 wherein the nucleic-acid-binding fluorescent dye is selected from the group consisting of of SYBR™ GREEN I, ethidium bromide, pico green, acridine orange, thiazole orange, YO-PRO-1, and chromomycin A3.

86. The method of claim 85 wherein the double-strand specific fluorescent dye is SYBR™ GREEN I.

87. A method of detecting the factor V Leiden mutation in an individual, wherein the factor V Leiden mutation consists of a single base change at the factor V Leiden mutation locus as compared to wild type, comprising the steps of:

(a) obtaining sample genomic DNA from the individual;

(b) providing wild type genomic DNA as a control;

(c) providing a pair of oligonucleotide primers configured for amplifying by polymerase chain reaction a selected segment of the sample genomic DNA and of the wild type genomic DNA wherein the selected segment comprises the factor V Leiden mutation locus to result in amplified products containing a copy of the factor V Leiden mutation locus;

(d) providing an oligonucleotide probe labeled with a resonance energy transfer donor or a resonance energy transfer acceptor of a fluorogenic resonance energy transfer pair, wherein the probe is configured for hybridizing to the amplified products such that the probe spans the mutation locus and exhibits a melting profile when the factor V Leiden mutation is present in the sample genomic DNA that is differentiable from a melting profile of the wild type genomic DNA;

(e) providing a transfer oligonucleotide labeled with the resonance energy transfer donor or the resonance energy transfer acceptor, with the proviso that as between the probe and transfer oligonucleotide one is labeled with the resonance energy transfer donor and the other is labeled with the resonance energy transfer acceptor, wherein the transfer oligonucleotide is configured for hybridizing to the amplified products such that the resonance energy transfer donor and the resonance energy transfer acceptor are in resonance energy transfer relationship when both the probe and the transfer oligonucleotide hybridize to the amplified products;

(f) amplifying the selected segment of sample genomic DNA and wild type genomic DNA by polymerase chain reaction in the presence of effective amounts of oligonucleotide probe and transfer oligonucleotide to result in amplified selected segments, at least a portion thereof having both the probe and the transfer oligonucleotide hybridized thereto with the fluorogenic resonance energy transfer pair in resonance energy transfer relationship;

(g) determining fluorescence as a function of temperature during an amplification cycle of the polymerase chain reaction to result in a melting profile of the probe melting from the amplified

segment of sample genomic DNA and a melting profile of the probe melting from the amplified segment of wild type genomic DNA; and

(h) comparing the melting profile for the sample genomic DNA to the melting profile for the wild type genomic DNA, wherein a difference therein indicates the presence of the factor V Leiden mutation in the sample genomic DNA.

88. The method of claim 87 wherein the transfer oligonucleotide is one of the pair of oligonucleotide primers.

89. The method of claim 88 wherein the resonance energy transfer pair comprises fluorescein and Cy5 or Cy5.5.

90. The method of claim 89 wherein the oligonucleotide primers are SEQ ID NO:11 and SEQ ID NO:12.

91. The method of claim 90 wherein SEQ ID NO:11 is labeled with Cy5 or Cy5.5.

92. The method of claim 90 wherein the oligonucleotide probe is SEQ ID NO:13.

93. The method of claim 90 wherein SEQ ID NO:13 is labeled with fluorescein.

94. The method of claim 87 wherein the resonance energy transfer donor and the resonance energy transfer acceptor are coupled to nucleotide residues, such that when the probe and transfer oligonucleotide are hybridized to the amplified segment, are separated by no more than about 15 nucleotide residues.

95. The method of claim 94 wherein the resonance energy transfer donor and the resonance energy transfer acceptor are coupled to nucleotide residues, such that when the probe and transfer oligonucleotide are hybridized to the amplified segment, are separated by about 4-6 nucleotide residues.

96. The method of claim 86 wherein the transfer oligonucleotide is not one of the oligonucleotide primers.

97. The method of claim 96 wherein the resonance energy transfer pair comprises fluorescein and Cy5 or Cy5.5.

98. The method of claim 97 wherein the oligonucleotide primers are SEQ ID NO:11 and SEQ ID NO:12.

99. The method of claim 98 wherein the oligonucleotide probe is SEQ ID NO:13.

100. The method of claim 99 wherein SEQ ID NO:13 is labeled with fluorescein.

101. The method of claim 100 wherein the transfer oligonucleotide is labeled with Cy5 or Cy5.5.

102. The method of claim 101 wherein the resonance energy transfer donor and the resonance energy transfer acceptor are coupled to nucleotide residues, such that when the probe and transfer oligonucleotide are hybridized to the amplified segment, are separated by no more than about 25 nucleotide residues.

103. The method of claim 102 wherein the resonance energy transfer donor and the resonance energy transfer acceptor are coupled to nucleotide residues, such that when the probe and transfer oligonucleotide are hybridized to the amplified segment, are separated by no more than about 0-5 nucleotide residues.

104. The method of claim 103 wherein the resonance energy transfer donor and the resonance energy transfer acceptor are coupled to nucleotide residues, such that when the probe and transfer oligonucleotide are hybridized to the amplified segment, are separated by no more than about 0-2 nucleotide residues.

105. The method of claim 104 wherein the resonance energy transfer donor and the resonance energy transfer acceptor are coupled to nucleotide residues, such that when the probe and transfer oligonucleotide are hybridized to the amplified segment, are separated by 1 nucleotide residue.

106. The method of claim 87 wherein the difference in melting profiles represents differences in thermal melting temperature of at least about 2°C.

107. An oligonucleotide having a nucleotide sequence as in SEQ ID NO:11.

108. An oligonucleotide having a nucleotide sequence as in SEQ ID NO:12.

109. An oligonucleotide having a nucleotide sequence as in SEQ ID NO:13.

110. A method of analyzing nucleic acid hybridization comprising the steps of

(a) providing a mixture comprising a nucleic acid sample to be analyzed and a nucleic acid binding fluorescent entity; and

(b) monitoring fluorescence while changing temperature at a rate of $\geq$ 0.1°C/second.

111. The method of claim 110 wherein the nucleic acid binding fluorescent entity is SYBR™ Green I.

112. The method of claim 110 wherein the nucleic acid binding fluorescent entity comprises a pair of oligonucleotide probes wherein one of the probes is labeled with a donor and the other probes is

labeled with an acceptor of a fluorogenic resonance energy transfer pair.

113. The method of claim 112 wherein one of the probes is a primer for polymerase chain reaction amplification.

114. A method of quantitating an initial copy number of a sample containing an unknown amount of nucleic acid comprising the steps of

(a) amplifying by polymerase chain reaction at least one standard of known concentration in a mixture comprising the standard and a nucleic acid binding fluorescent entity;

(b) measuring fluorescence as a function of cycle number to result in a set of data points;

(c) fitting the data points to a given predetermined equation describing fluorescence as a function of initial nucleic acid concentration and cycle number;

(d) amplifying the sample containing the unknown amount of nucleic acid in a mixture comprising the sample and the nucleic acid binding fluorescent entity and monitoring fluorescence thereof; and

(e) determining initial nucleic acid concentration from the equation determined in step (c).

115. The method of claim 114 wherein the nucleic acid binding fluorescent entity is SYBR™ Green I.

116. The method of claim 114 wherein the nucleic acid binding fluorescent entity comprises a pair of oligonucleotide probes, one of which is labeled with a donor and the other of which is labeled with an acceptor of a fluorogenic resonance energy transfer pair.

117. The method of claim 116 wherein one of the probe is a primer for amplification.

118. A fluorescence resonance energy transfer pair comprising a donor fluorophore having an emission spectrum and an acceptor fluorophore having an absorption spectrum and an extinction coefficient greater than 100,000 $M^{-1}cm^{-1}$, wherein the donor fluorophore's emission spectrum and the acceptor fluorophore's absorption spectrum overlap less than 25%

119. The fluorescence resonance energy transfer pair wherein the donor fluorophore is fluorescein and the acceptor fluorophore is Cy5 or Cy5.5.

120. A method for analyzing a target DNA sequence of a biological sample, said method comprising the steps of

amplifying the target sequence by polymerase chain reaction in the presence of a nucleic acid binding fluorescent

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entity, said polymerase chain reaction comprising the steps of adding a thermostable polymerase and primers for the targeted nucleic acid sequence to the biological sample and thermally cycling the biological sample between at least a denaturation temperature and an elongation temperature;

exciting the sample with light at a wavelength absorbed by the nucleic acid binding fluorescent entity; and

monitoring the temperature dependent fluorescence from the nucleic acid binding fluorescent entity as temperature of the sample is changed.

121. The method of claim 120 wherein said nucleic acid binding fluorescent entity comprises a double stranded nucleic acid binding fluorescent dye.

122. The method of claim 121 wherein the double stranded nucleic acid binding fluorescent dye is SYBR™ Green I.

123. The method of claim 122 wherein the temperature dependent fluorescence is used to identify the amplified products.

124. The method of claim 123 wherein the amplified products are identified by analysis of melting curves.

125. The method of claim 124 wherein the relative amounts fo two or more amplified products are determined by analysis of melting curves.

126. The method of claim 124 wherein areas under the melting curves are found by non-linear least squares regressionof the sum of multiple gaussians.

Figure 1.

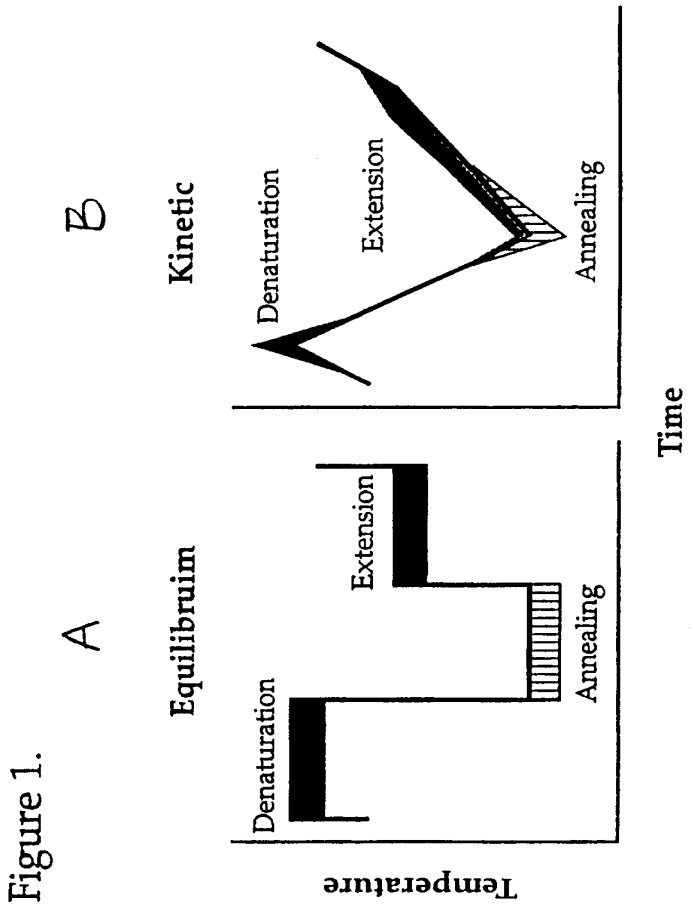

A
Equilibrium
Denaturation
Extension
Annealing
B
Kinetic
Denaturation
Extension
Annealing
Temperature
Time

Figure 2.

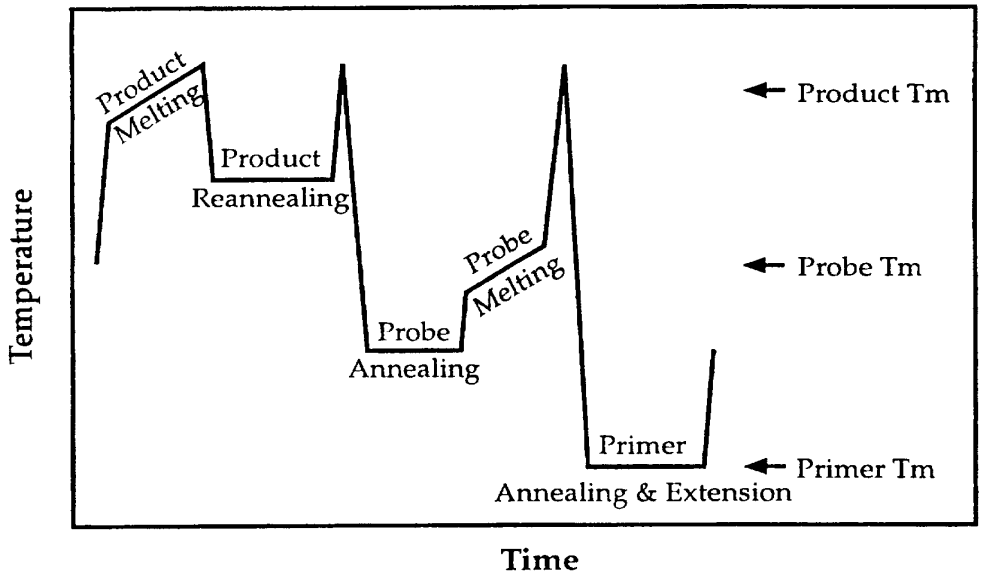

Product Melting
Product Reannealing
Probe Annealing
Probe Melting
Primer Annealing & Extension
Product Tm
Probe Tm
Primer Tm
Temperature
Time

FIGURE 3.

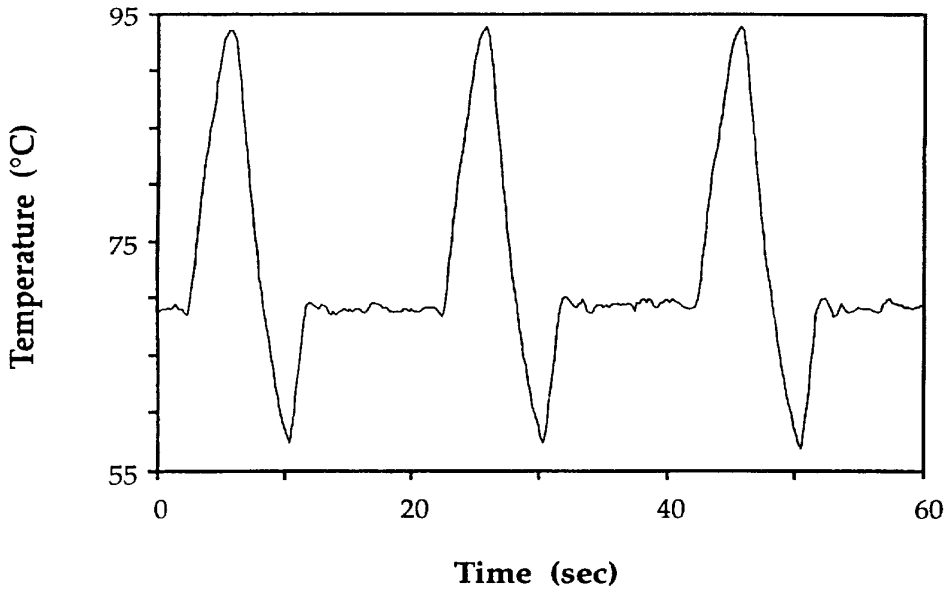


Figure 4.

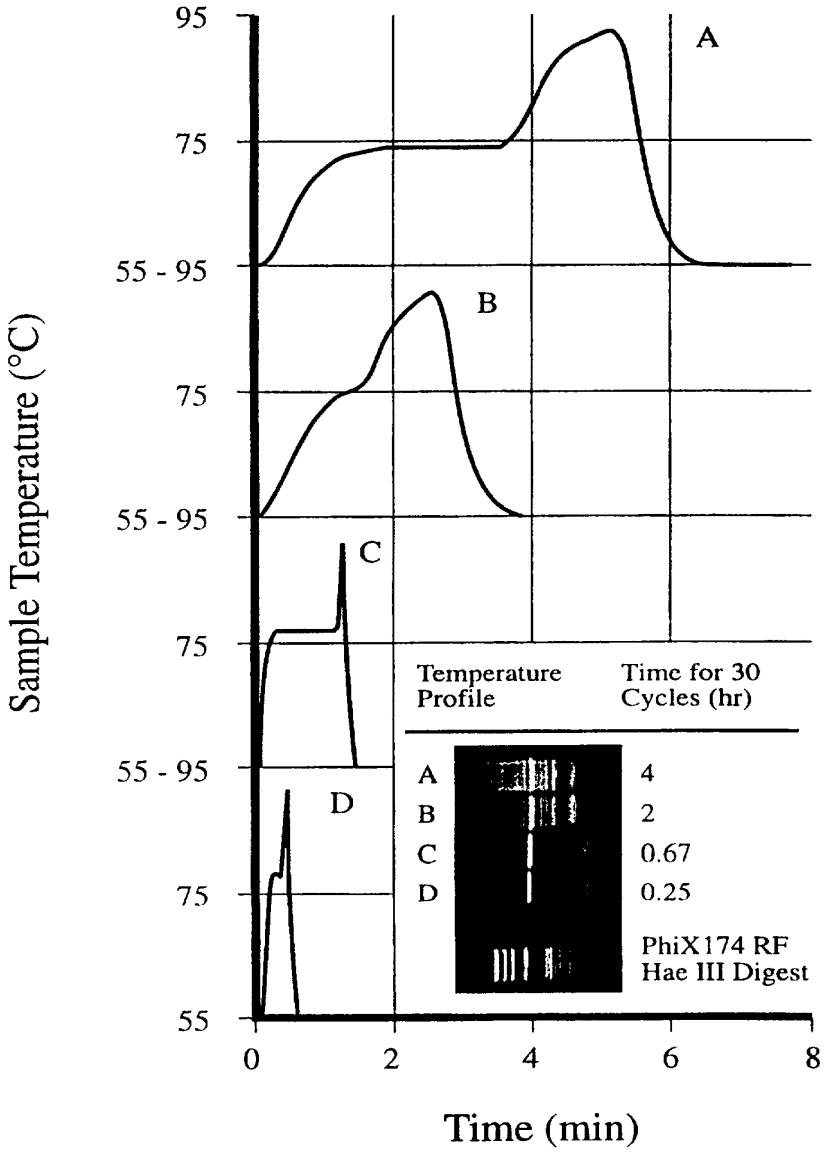
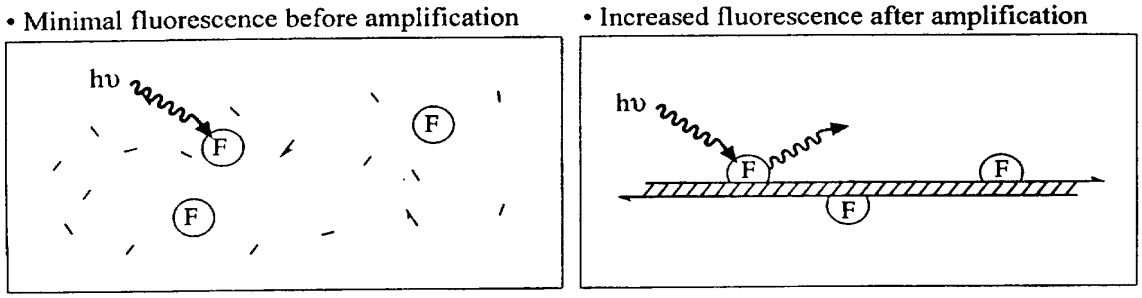
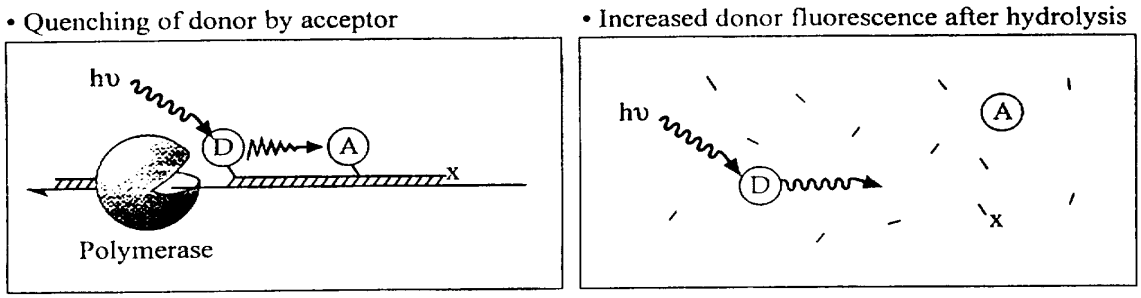

Sample Temperature (°C)
95
75
55 - 95
75
55 - 95
75
55 - 95
75
55
A
B
C
D
Temperature Profile
Time for 30 Cycles (hr)
A 4
B 2
C 0.67
D 0.25
PhiX174 RF Hae III Digest
0
2
4
6
8
Time (min)

Figure 5.

A. Increased fluorescence by binding double stranded DNA.



B. Release from quenching by hydrolysis.



C. Increased resonance energy transfer by hybridization.

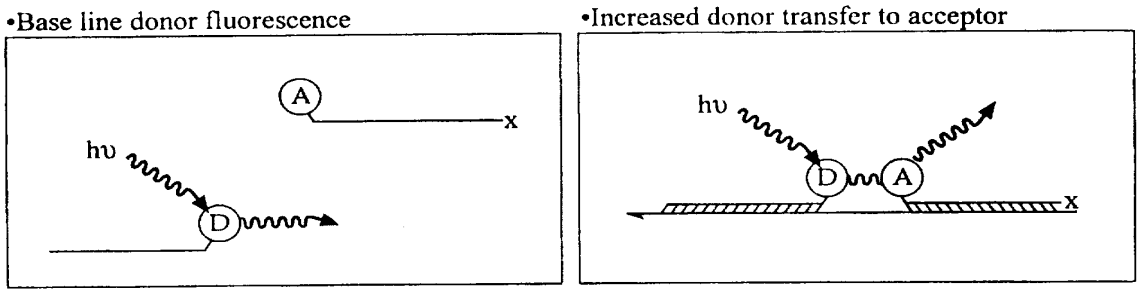


Figure 6.

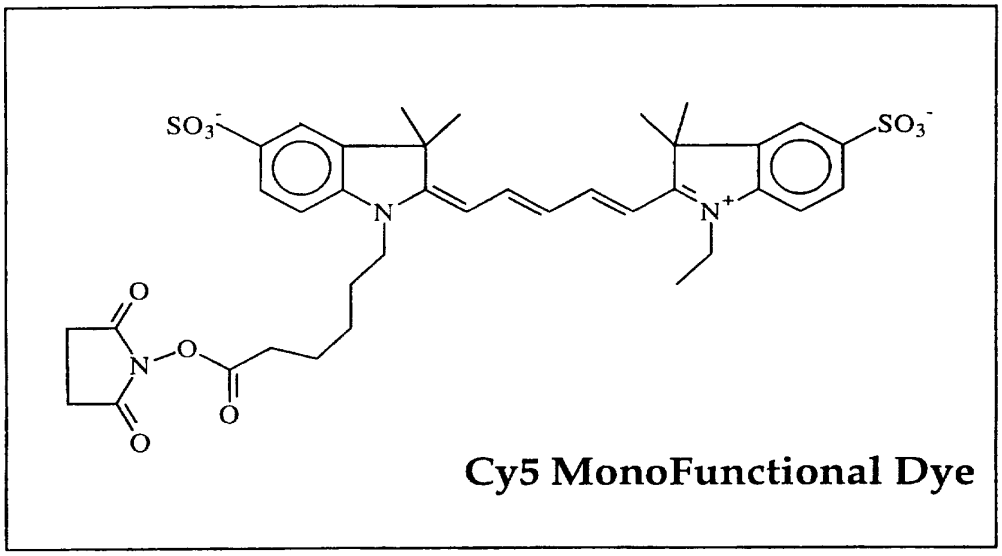


Figure 7.

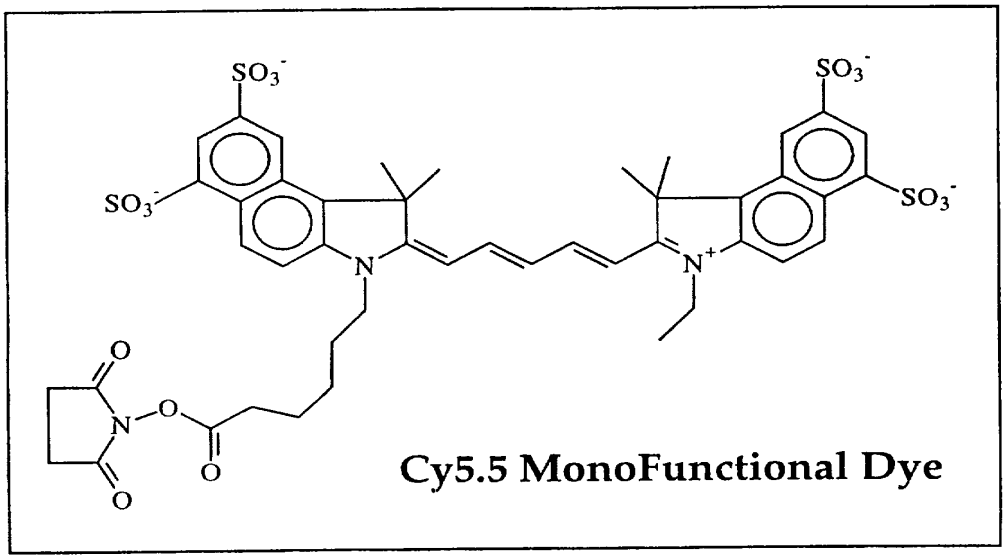
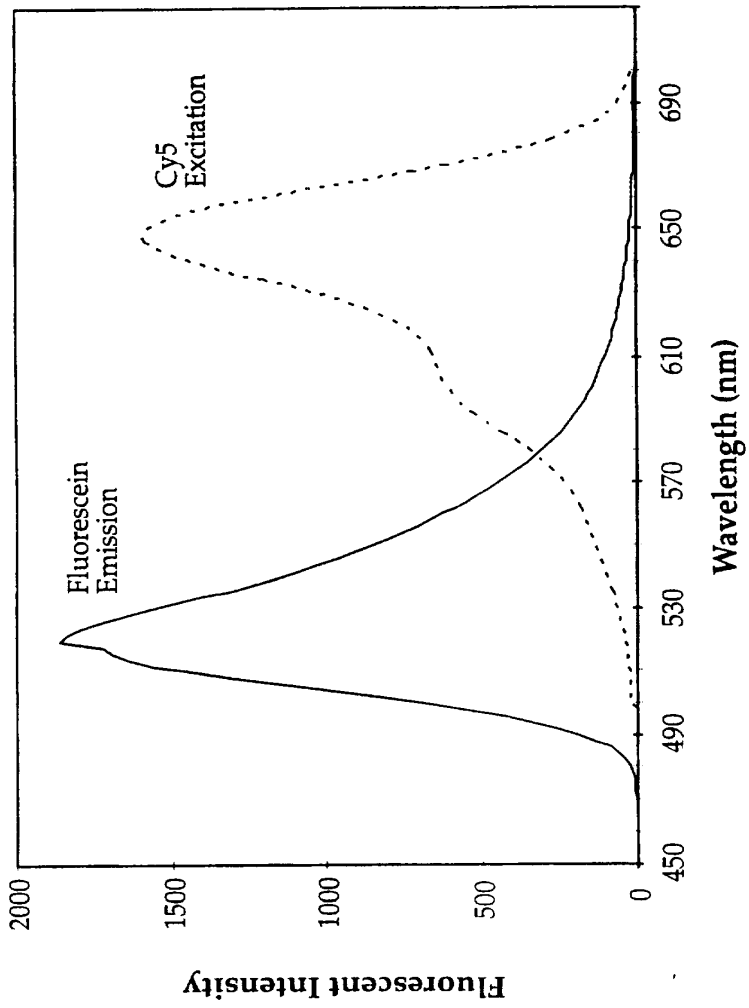


Figure 8.


Fluorescein
Emission
Cy5
Excitation
Fluorescent Intensity
2000
1500
1000
500
0
450
490
530
570
610
650
690
Wavelength (nm)

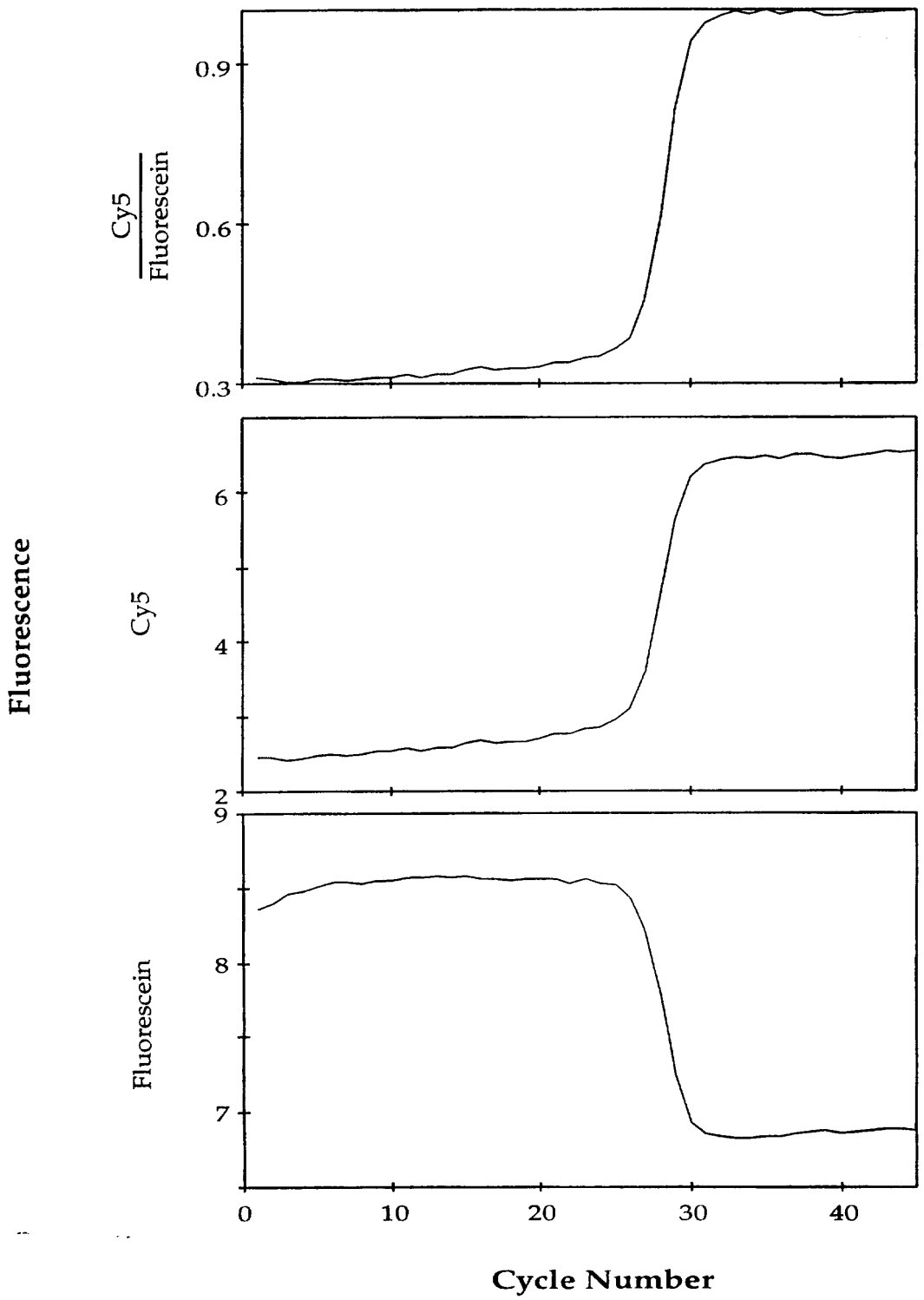

Fluorescence
Cy5 / Fluorescein
0.9
0.6
0.3
Cy5
6
4
2
Fluorescein
9
8
7
0
10
20
30
40
Cycle Number

Figure 10.

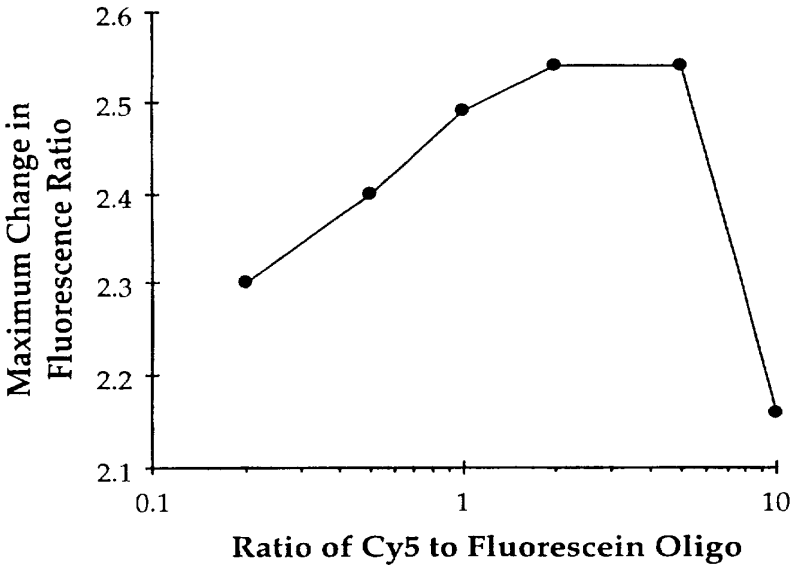

Maximum Change in Fluorescence Ratio
2.6
2.5
2.4
2.3
2.2
2.1
0.1
1
10
Ratio of Cy5 to Fluorescein Oligo

Figure 11.

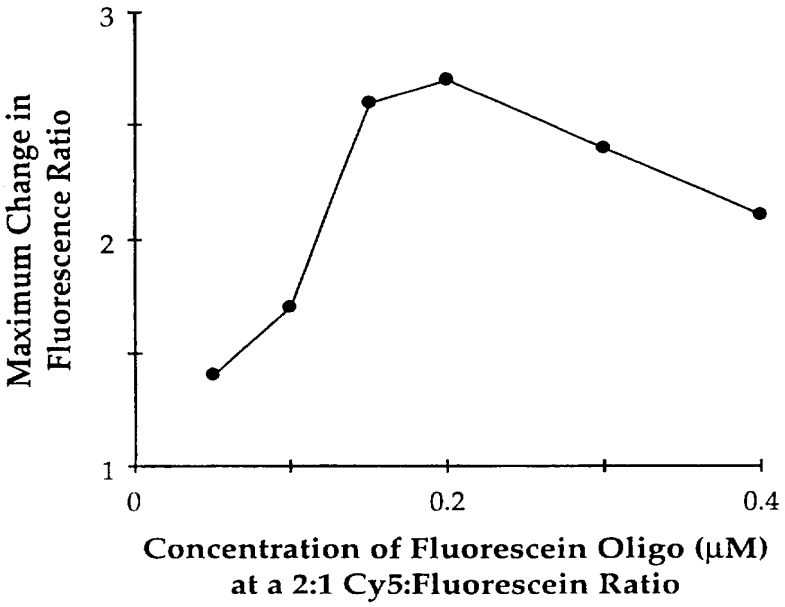
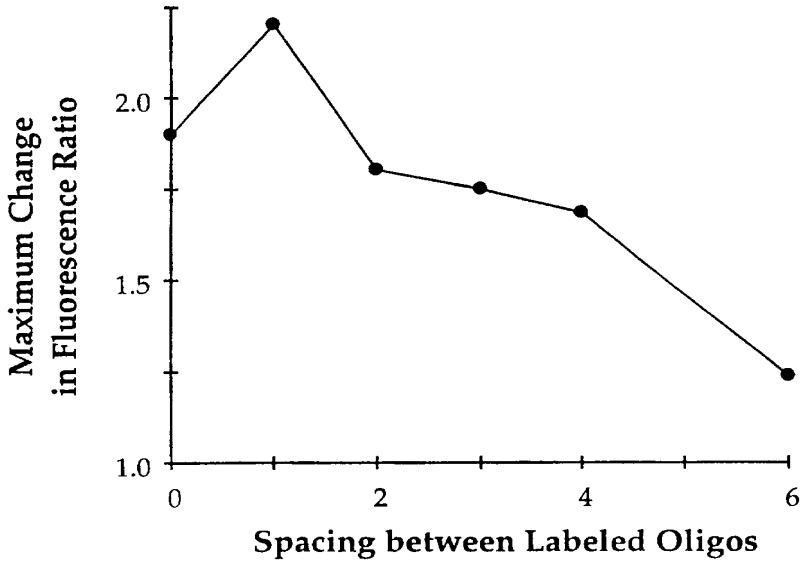

Maximum Change in Fluorescence Ratio
3
2
1
0
0.2
0.4
Concentration of Fluorescein Oligo (μM) at a 2:1 Cy5:Fluorescein Ratio

Figure 12.


Maximum Change
in Fluorescence Ratio
2.0
1.5
1.0
0
2
4
6
Spacing between Labeled Oligos

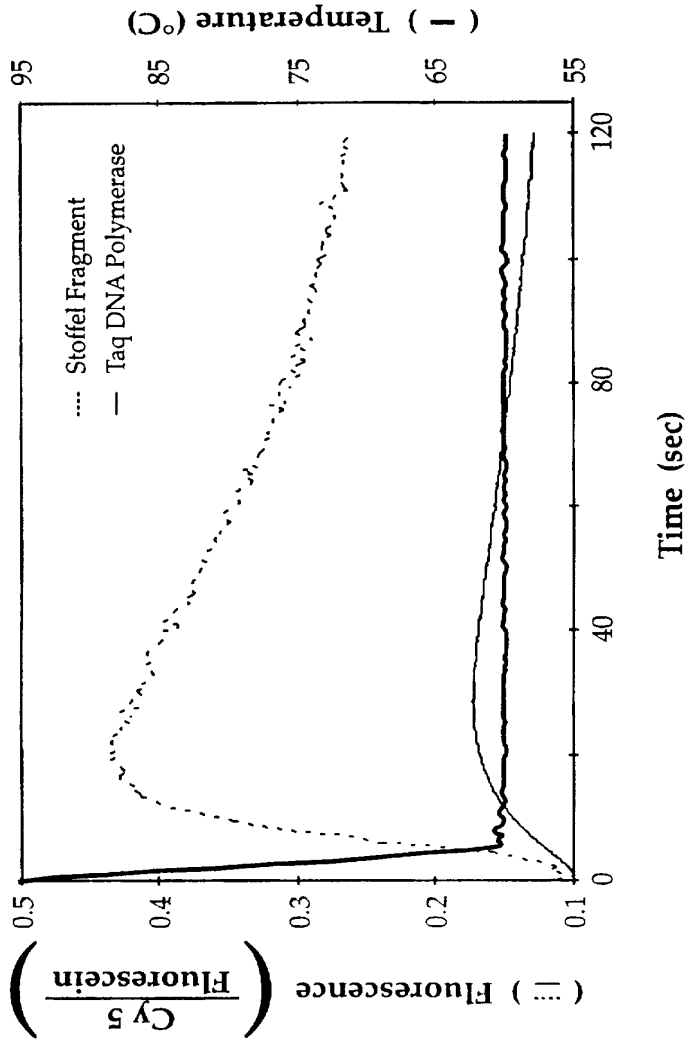
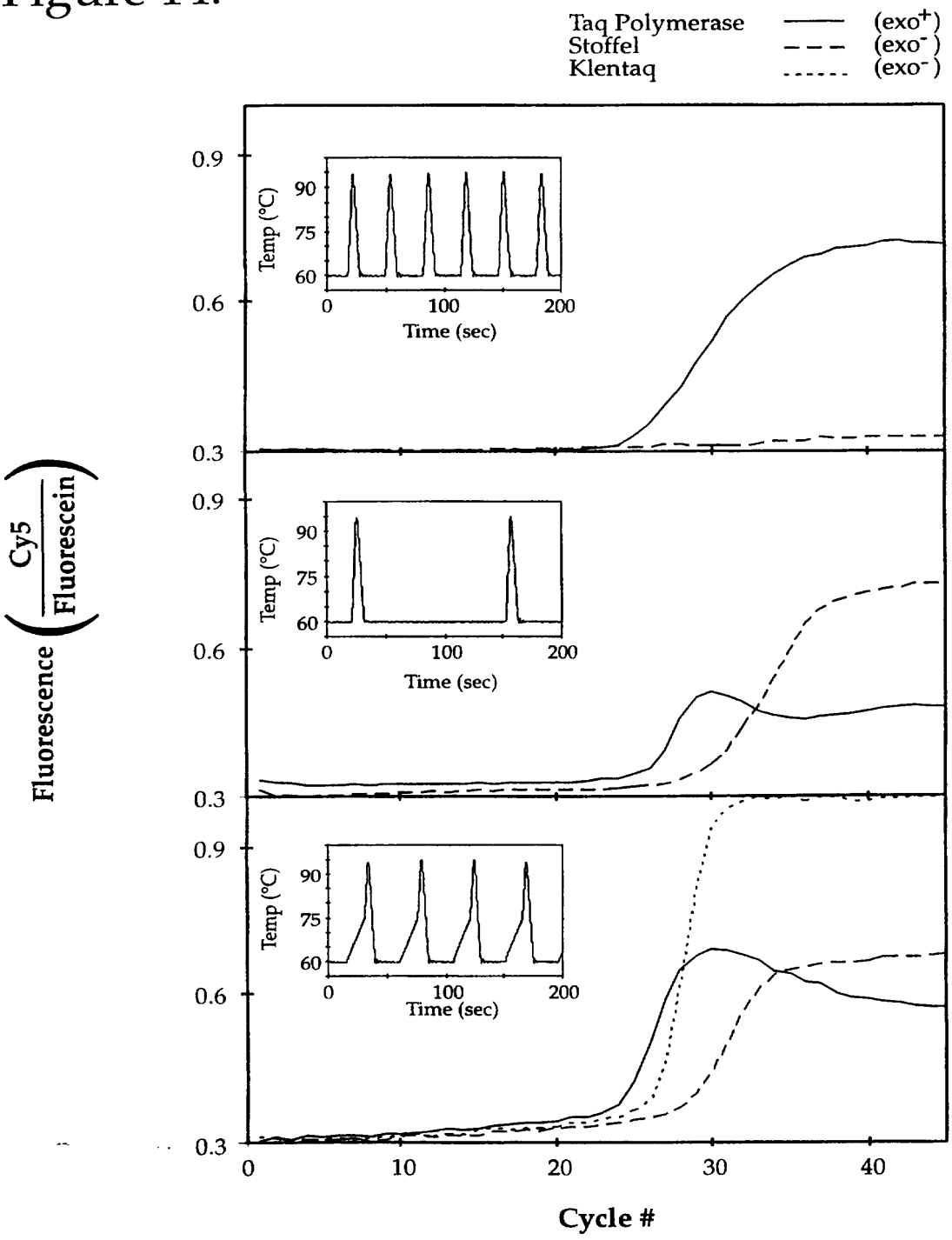

Temperature (°C)
95
85
75
65
55
Stoffel Fragment
Taq DNA Polymerase
Fluorescence (Cy 5 / Fluorescein)
0.5
0.4
0.3
0.2
0.1
0
40
80
120
Time (sec)

Figure 13.

Figure 14.


Taq Polymerase (exo+)
Stoffel (exo-)
Klentaq (exo-)
Fluorescence (Cy5 / Fluorescein)
Temp (°C)
Time (sec)
Cycle #

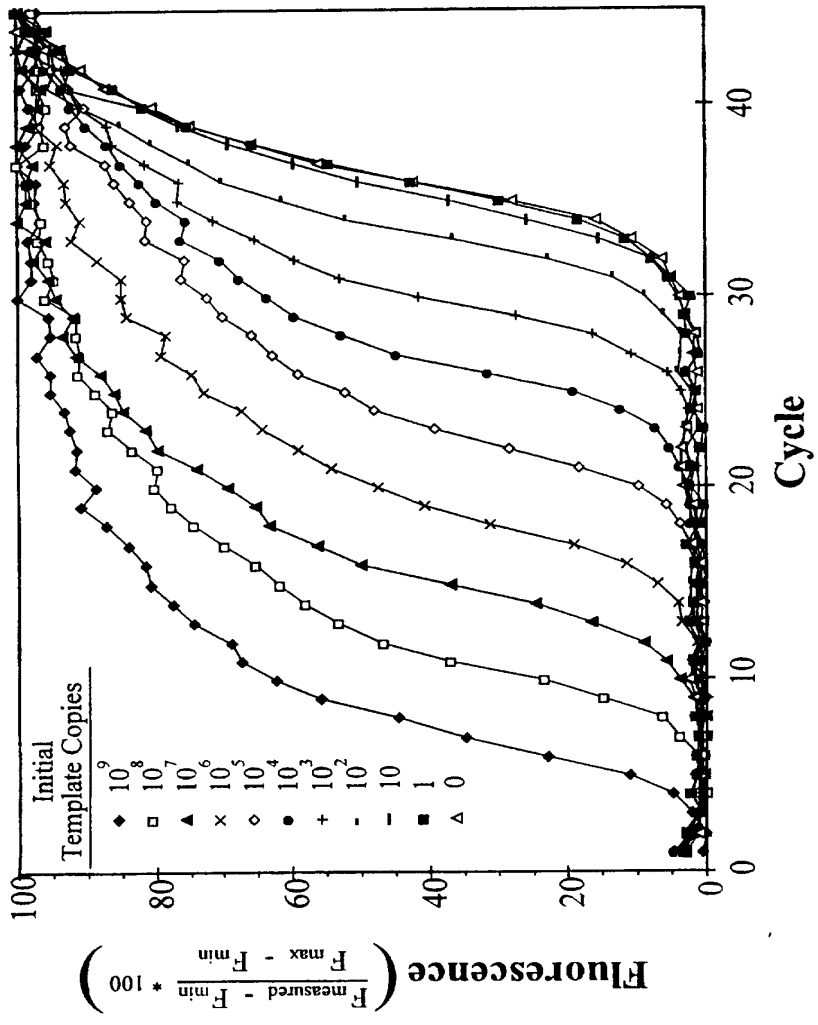

Fluorescence (F measured - F min / F max - F min * 100)
Cycle
Initial Template Copies
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10
1
0
100
80
60
40
20
0
0
10
20
30
40

Figure 15.

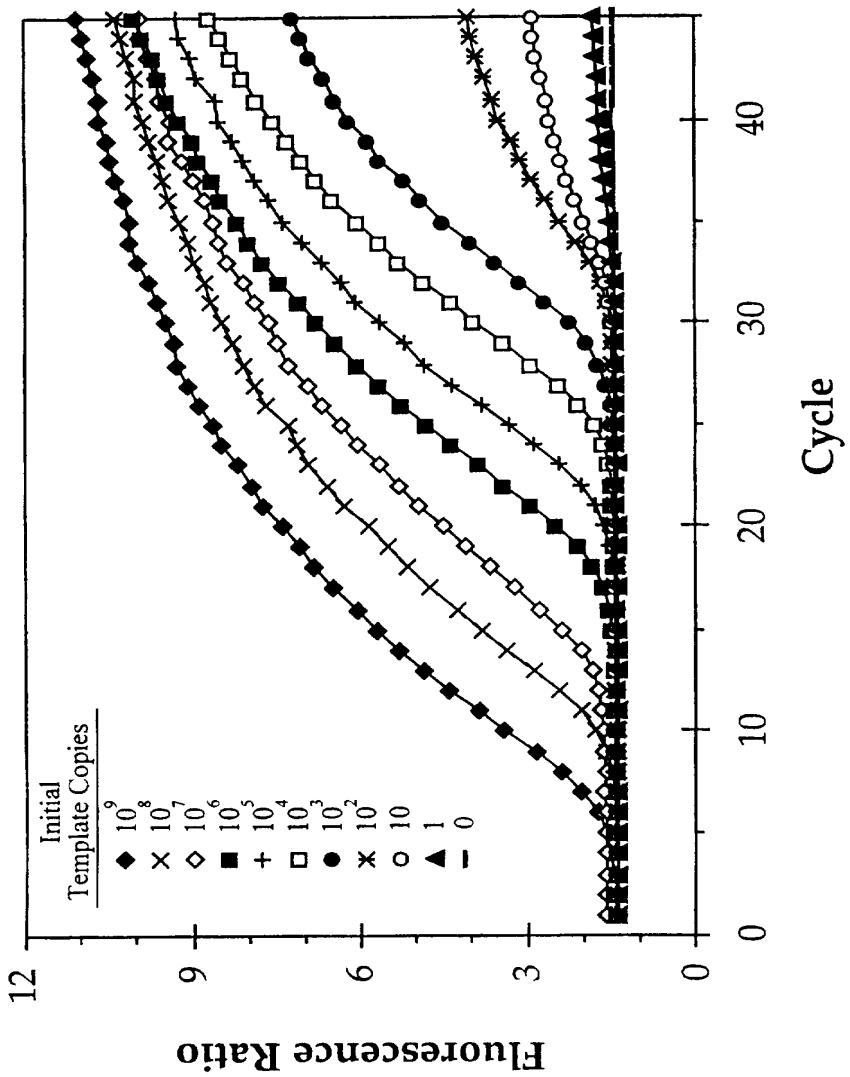

Fluorescence Ratio
Cycle
Initial Template Copies
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10
1
0
12
9
6
3
0
0
10
20
30
40

Figure 16.

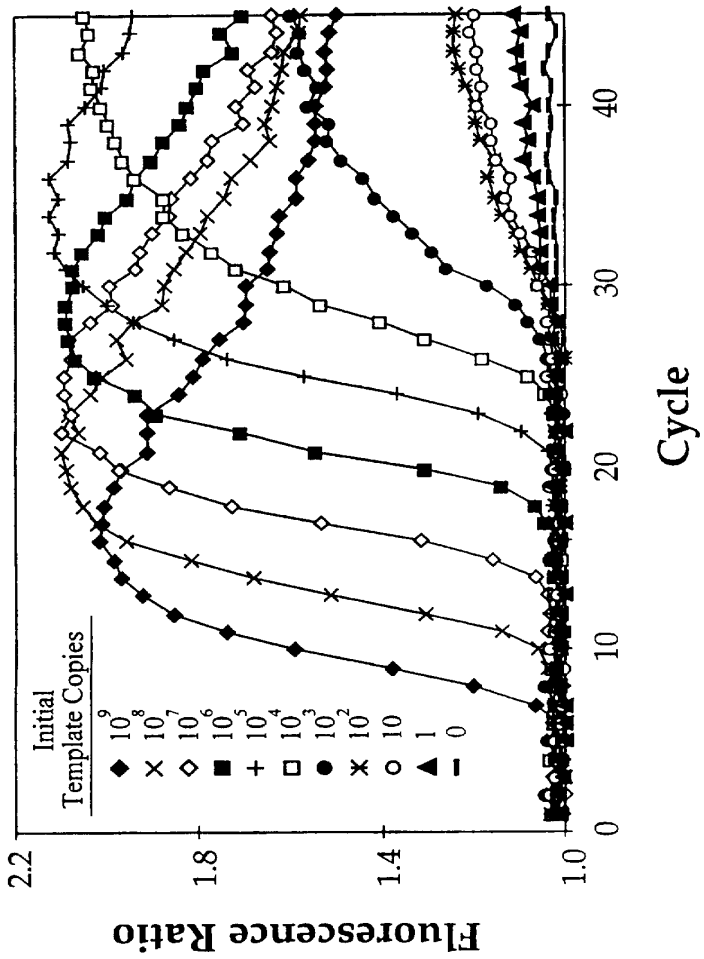
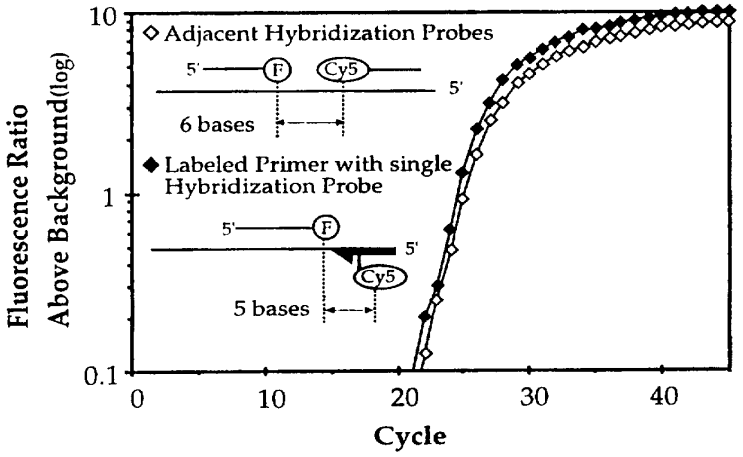

Fluorescence Ratio
2.2
1.8
1.4
1.0
0
10
20
30
40
Cycle
Initial Template Copies
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10
1
0

Figure 17.

Figure 18.


10
1
0.1
0
10
20
30
40
Cycle
Fluorescence Ratio
Above Background(log)
◇ Adjacent Hybridization Probes
5'
F
Cy5
5'
6 bases
◆ Labeled Primer with single
Hybridization Probe
5'
F
5'
Cy5
5 bases

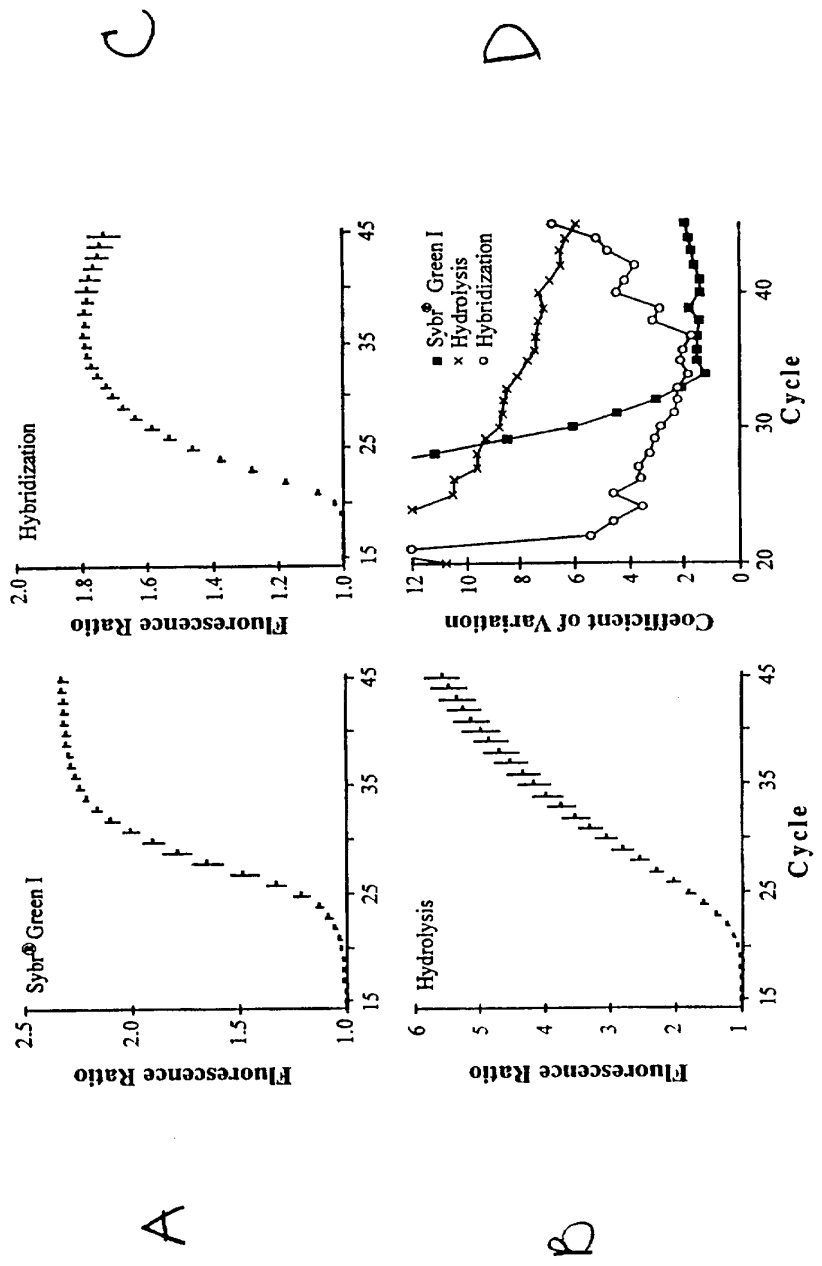

A
Sybr® Green I
Fluorescence Ratio
2.5
2.0
1.5
1.0
15
25
35
45
B
Hydrolysis
Fluorescence Ratio
6
5
4
3
2
1
15
25
35
45
Cycle
C
Hybridization
Fluorescence Ratio
2.0
1.8
1.6
1.4
1.2
1.0
15
25
35
45
D
Sybr® Green I
× Hydrolysis
o Hybridization
Coefficient of Variation
12
10
8
6
4
2
0
20
30
40
Cycle

Figure 19.

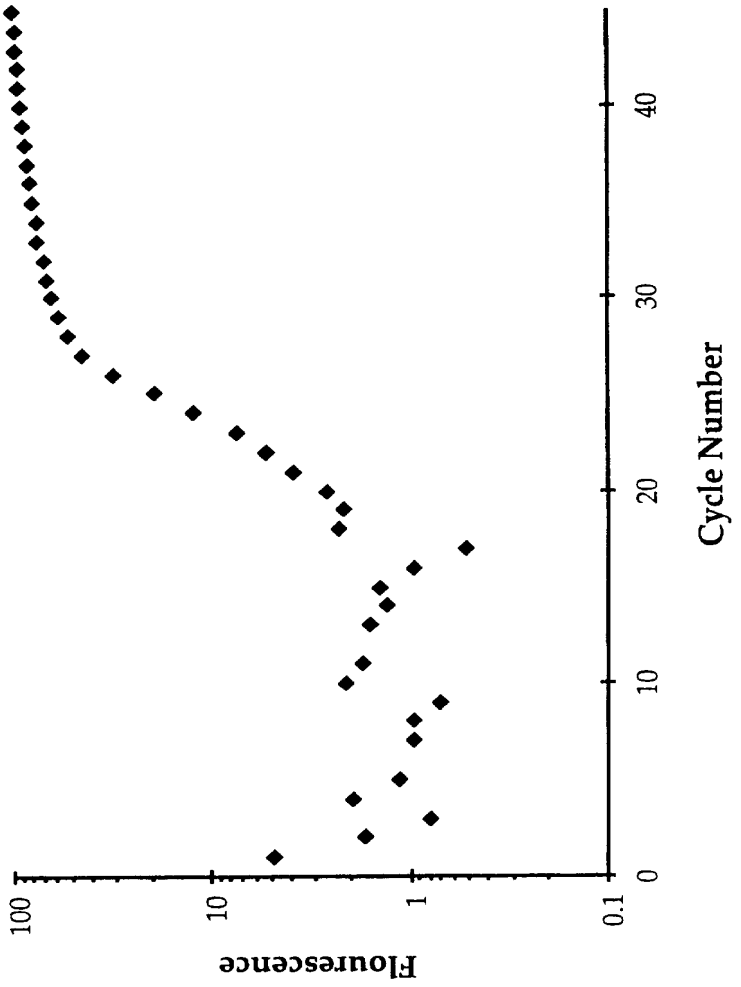


Figure 20.

Figure 21.

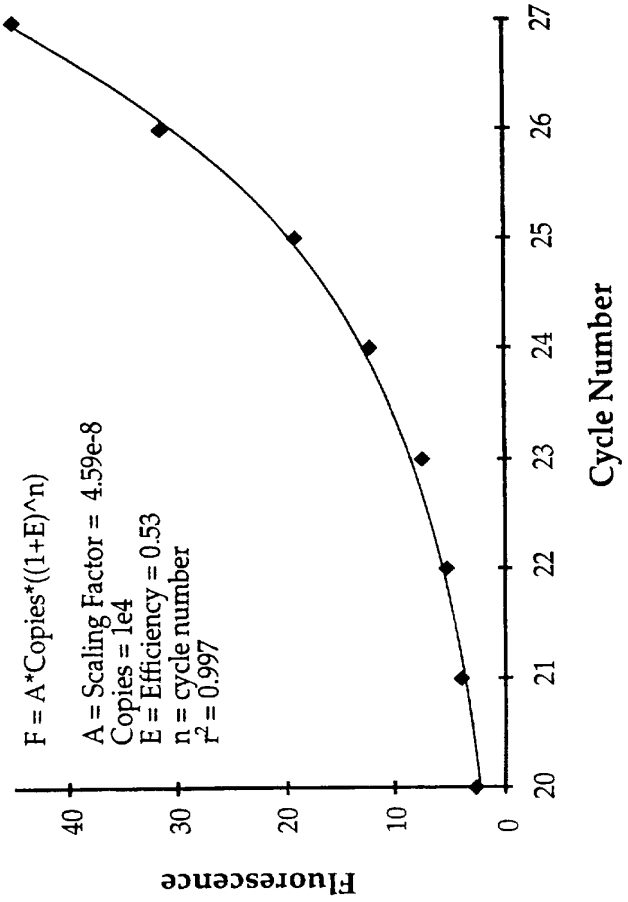

F = A*Copies*((1+E)^n)
A = Scaling Factor = 4.59e-8
Copies = 1e4
E = Efficiency = 0.53
n = cycle number
r2 = 0.997
Fluorescence
0
10
20
30
40
20
21
22
23
24
25
26
27
Cycle Number

Figure 22.

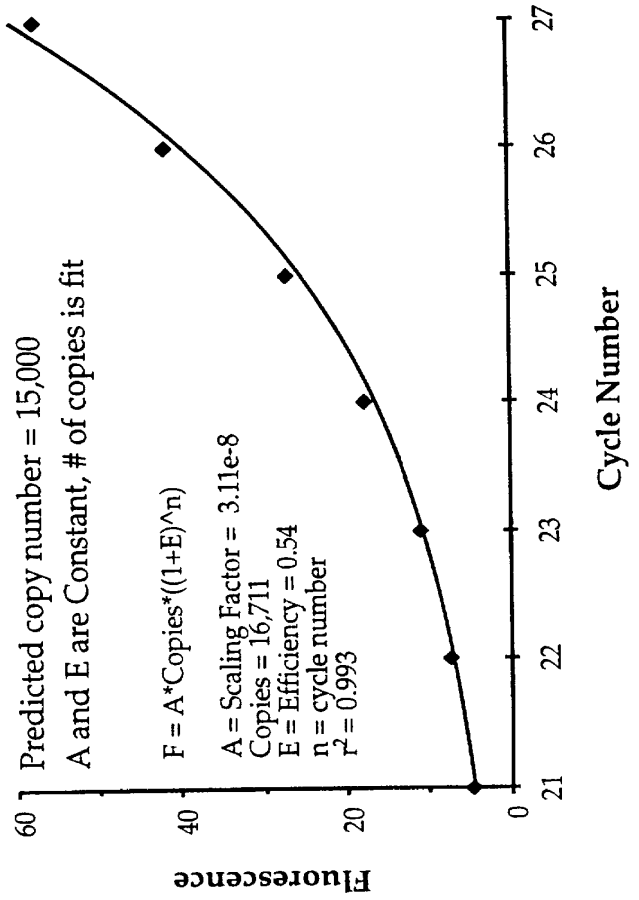

Predicted copy number = 15,000
A and E are Constant, # of copies is fit
F = A*Copies*((1+E)^n)
A = Scaling Factor = 3.11e-8
Copies = 16,711
E = Efficiency = 0.54
n = cycle number
r2 = 0.993
Fluorescence
Cycle Number
60
40
20
0
21
22
23
24
25
26
27

Figure 23.

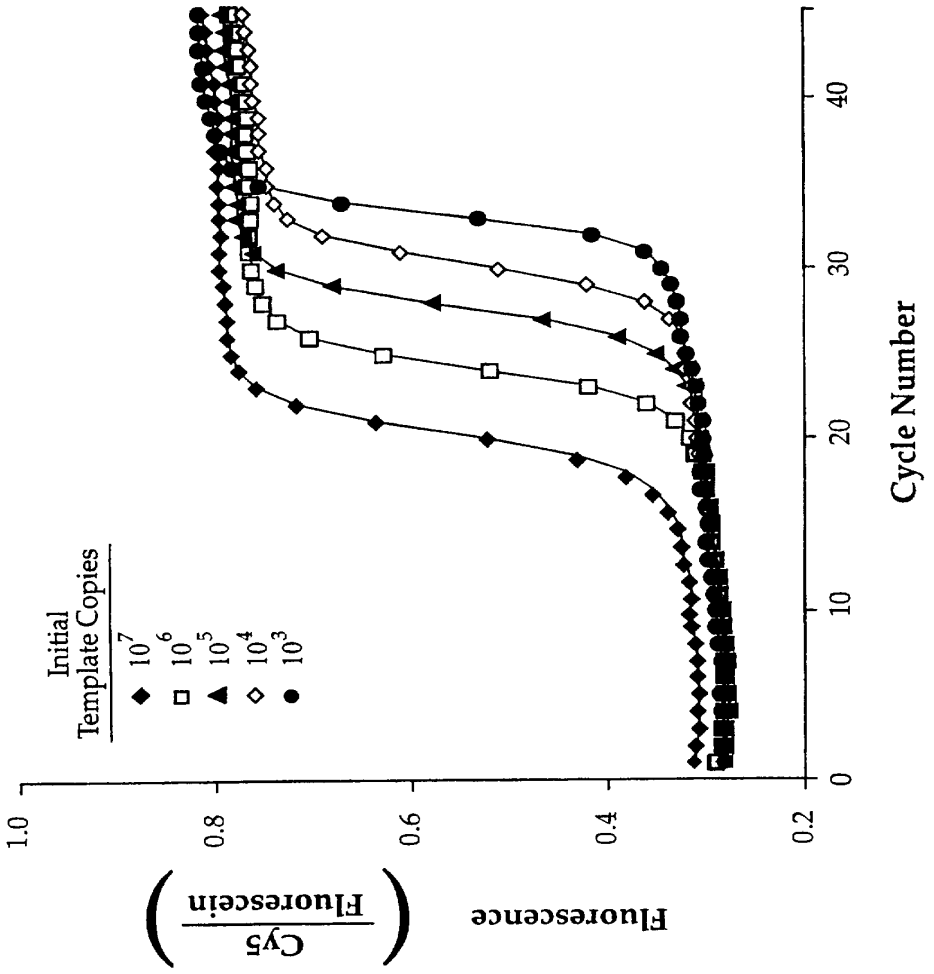
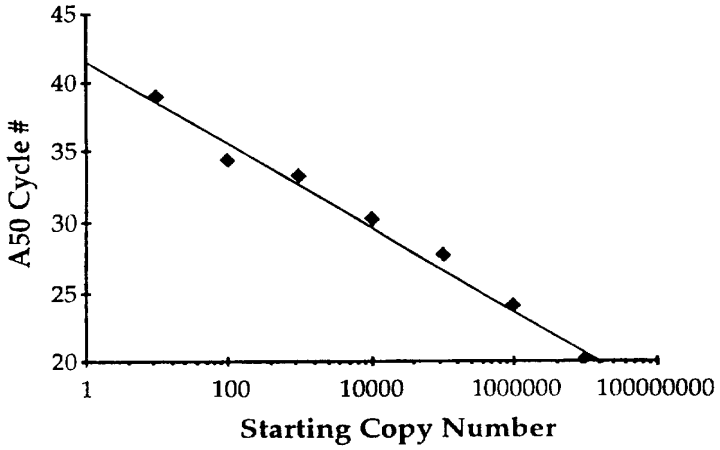

Initial
Template Copies
10^7
10^6
10^5
10^4
10^3
Fluorescence
$\left(\frac{Cy5}{Fluorescein}\right)$
1.0
0.8
0.6
0.4
0.2
0
10
20
30
40
Cycle Number

Figure 24.


45
40
35
30
25
20
A50 Cycle #
1
100
10000
1000000
100000000
Starting Copy Number

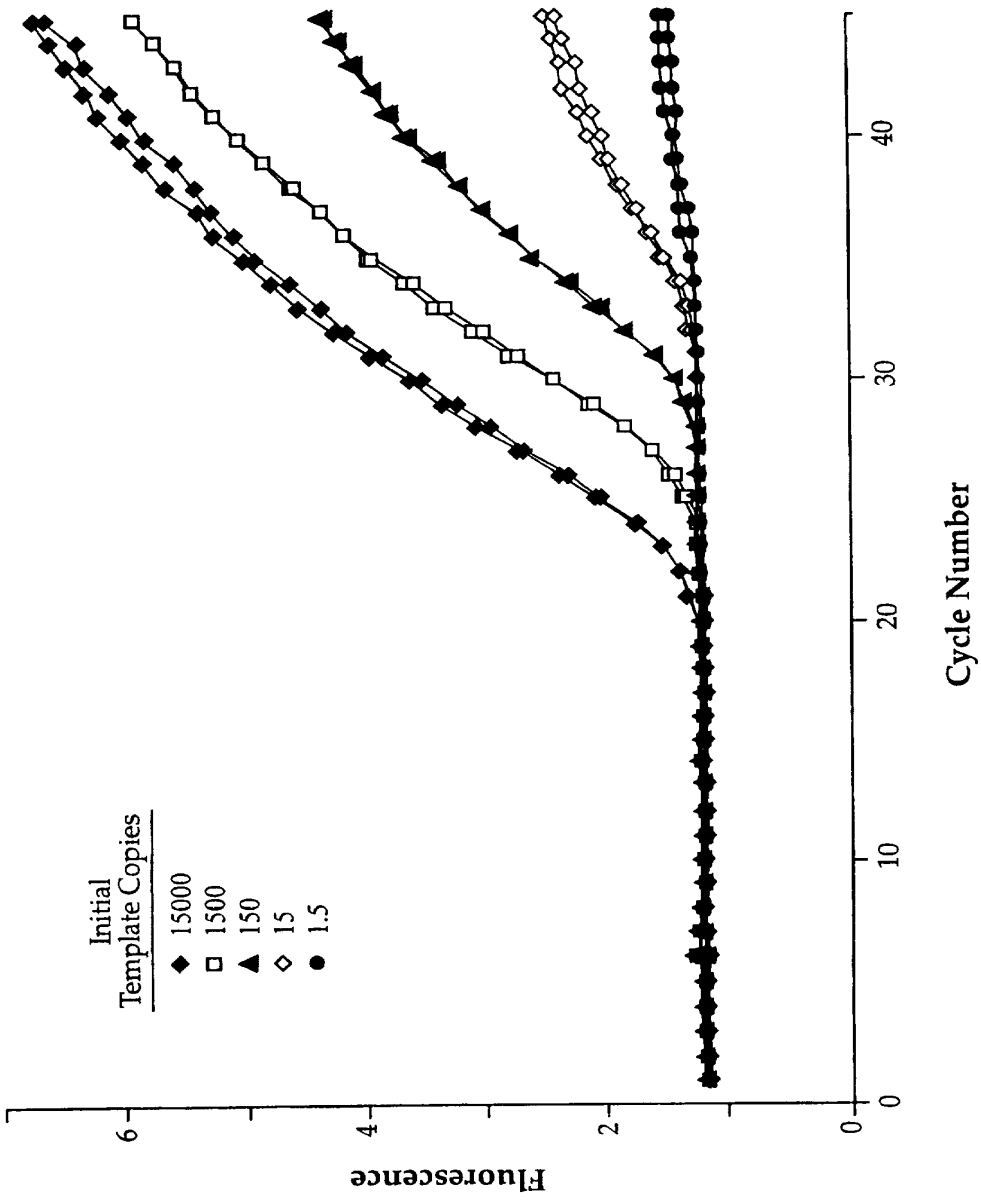

Initial
Template Copies
15000
1500
150
15
1.5
Fluorescence
0
2
4
6
0
10
20
30
40
Cycle Number

Figure 25.

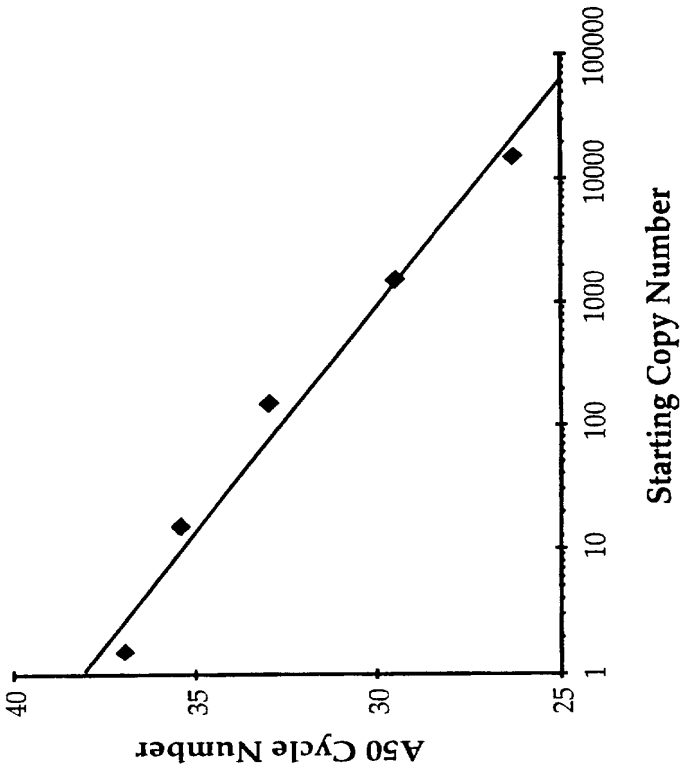

A50 Cycle Number
40
35
30
25
Starting Copy Number
1
10
100
1000
10000
100000

Figure 26.

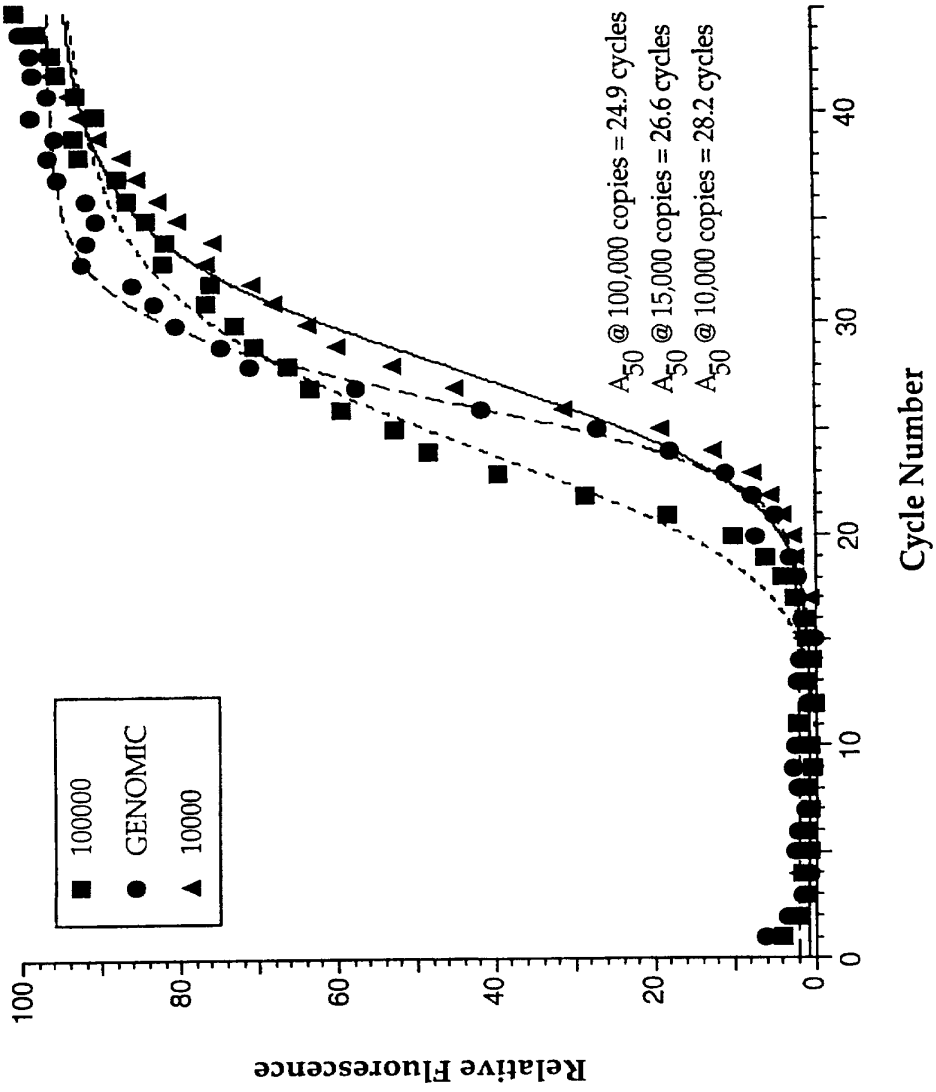

Relative Fluorescence
Cycle Number
100000
GENOMIC
10000
A_{50} @ 100,000 copies = 24.9 cycles
A_{50} @ 15,000 copies = 26.6 cycles
A_{50} @ 10,000 copies = 28.2 cycles
0
20
40
60
80
100
0
10
20
30
40

Figure 27.

Figure 28.

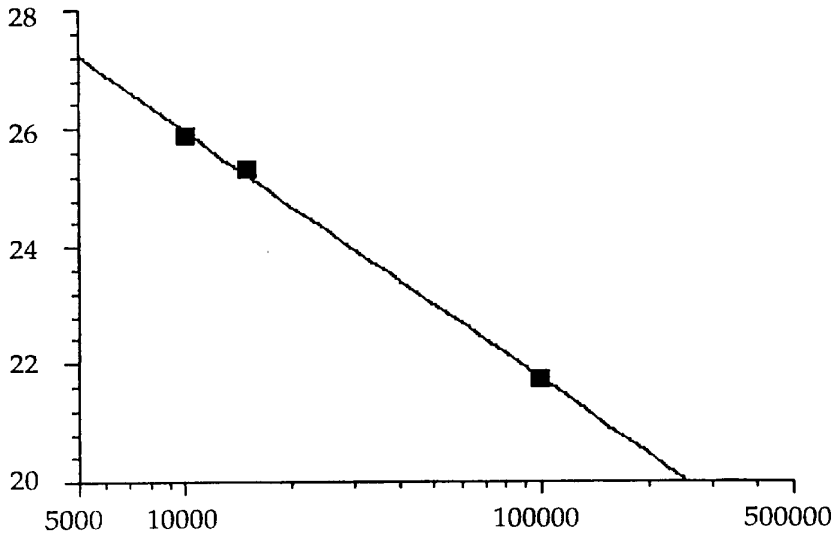


Figure 29.

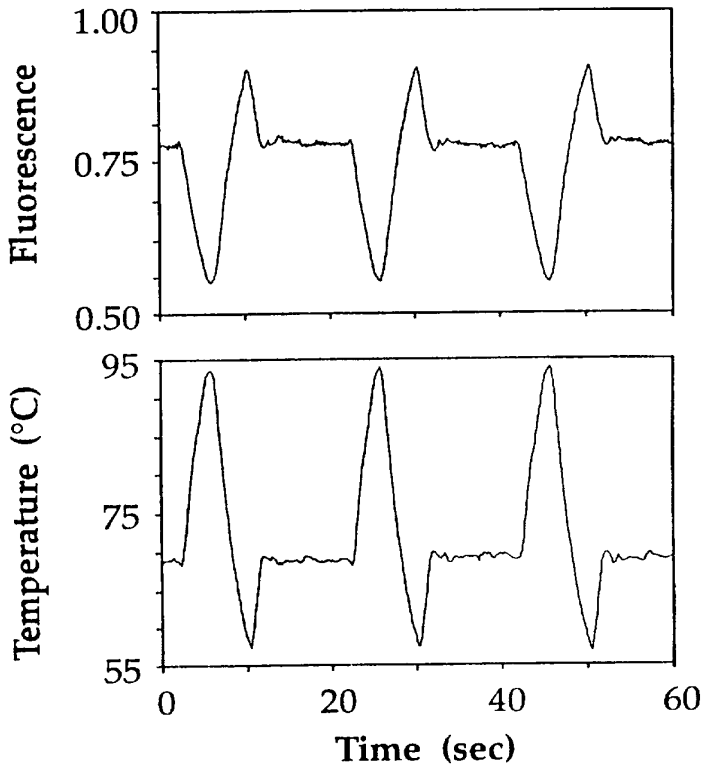
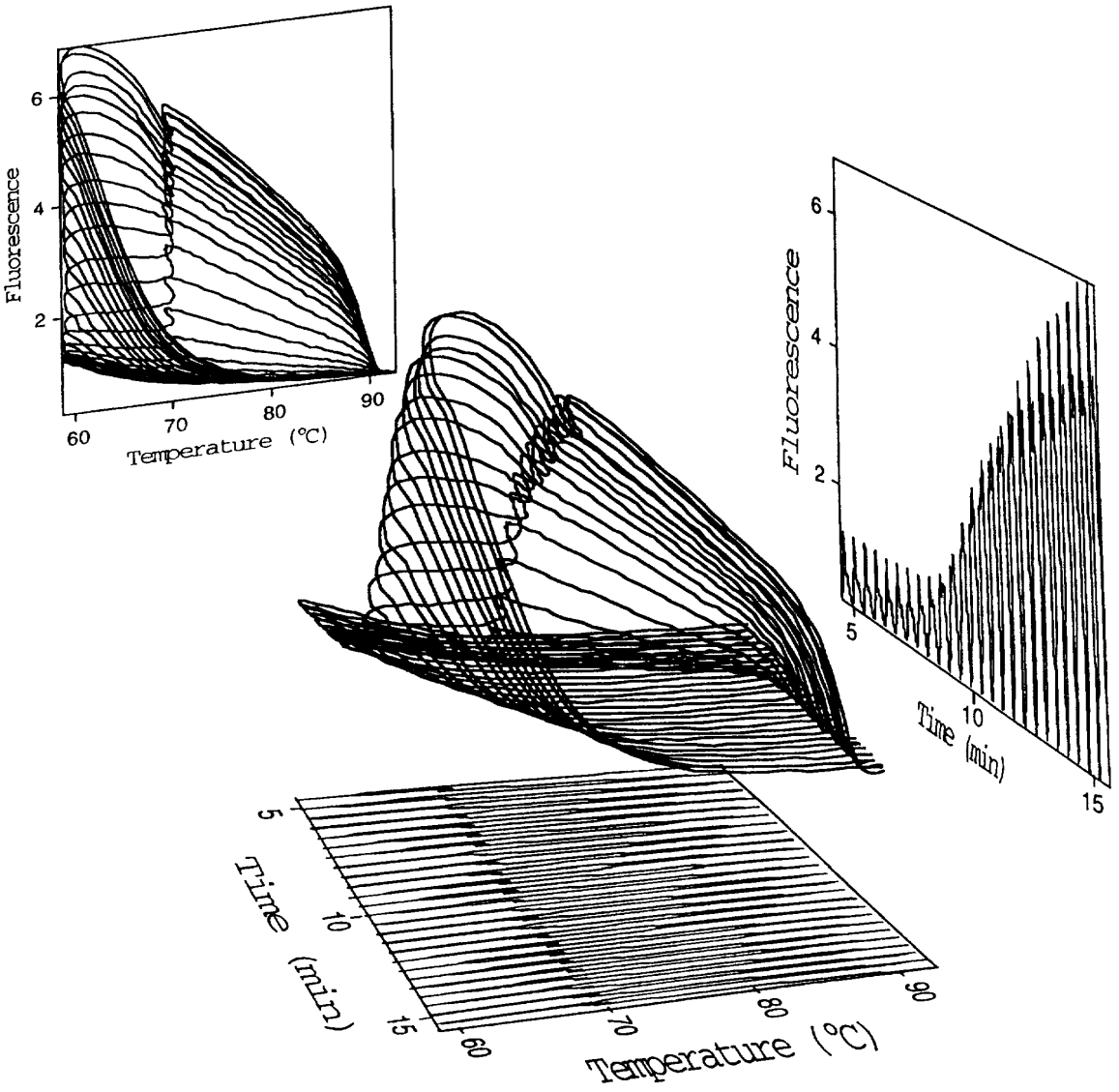

A
B
1.00
0.75
0.50
Fluorescence
95
75
55
Temperature (°C)
0
20
40
60
Time (sec)

Figure 30


Fluorescence
6
4
2
60
70
80
90
Temperature (°C)
Fluorescence
6
4
2
5
10
15
Time (min)
5
Time
10
(min)
15
60
70
80
90
Temperature (°C)

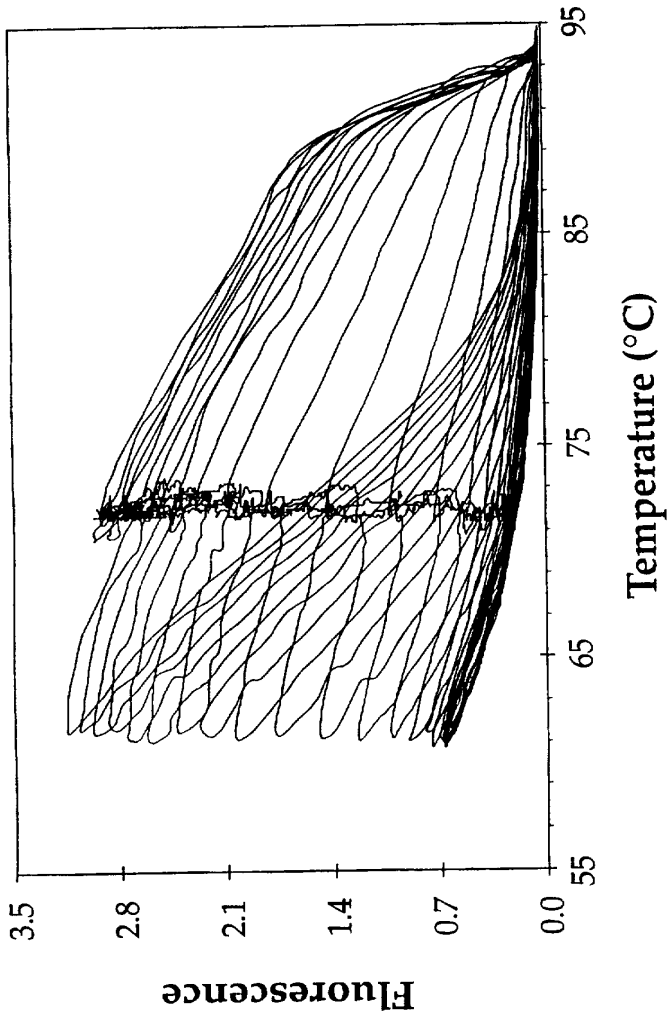
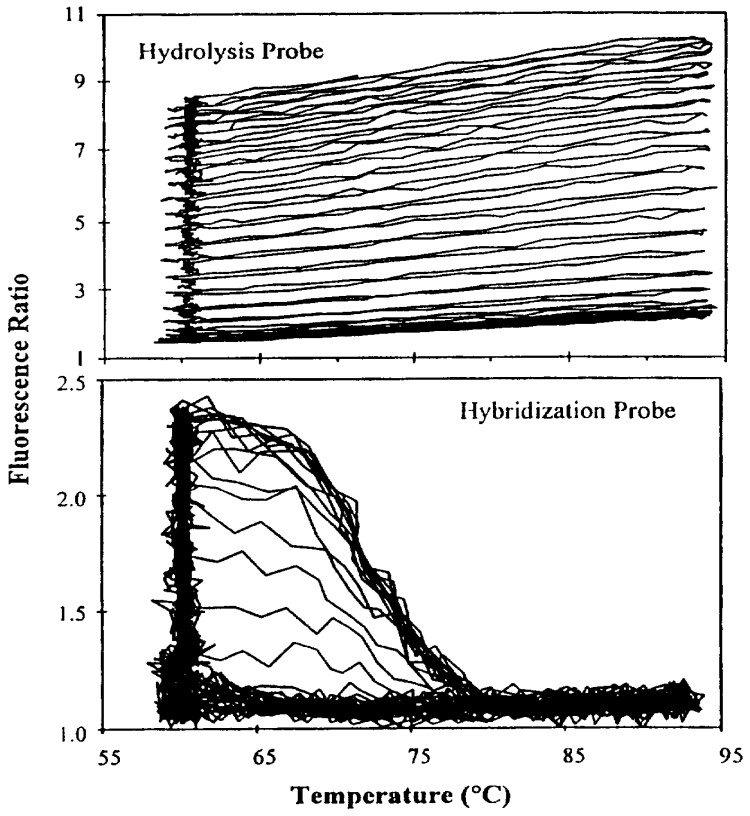


Figure 31.

Figure 32.

A

B


Hydrolysis Probe
Hybridization Probe
Fluorescence Ratio
Temperature (°C)
11
9
7
5
3
1
2.5
2.0
1.5
1.0
55
65
75
85
95

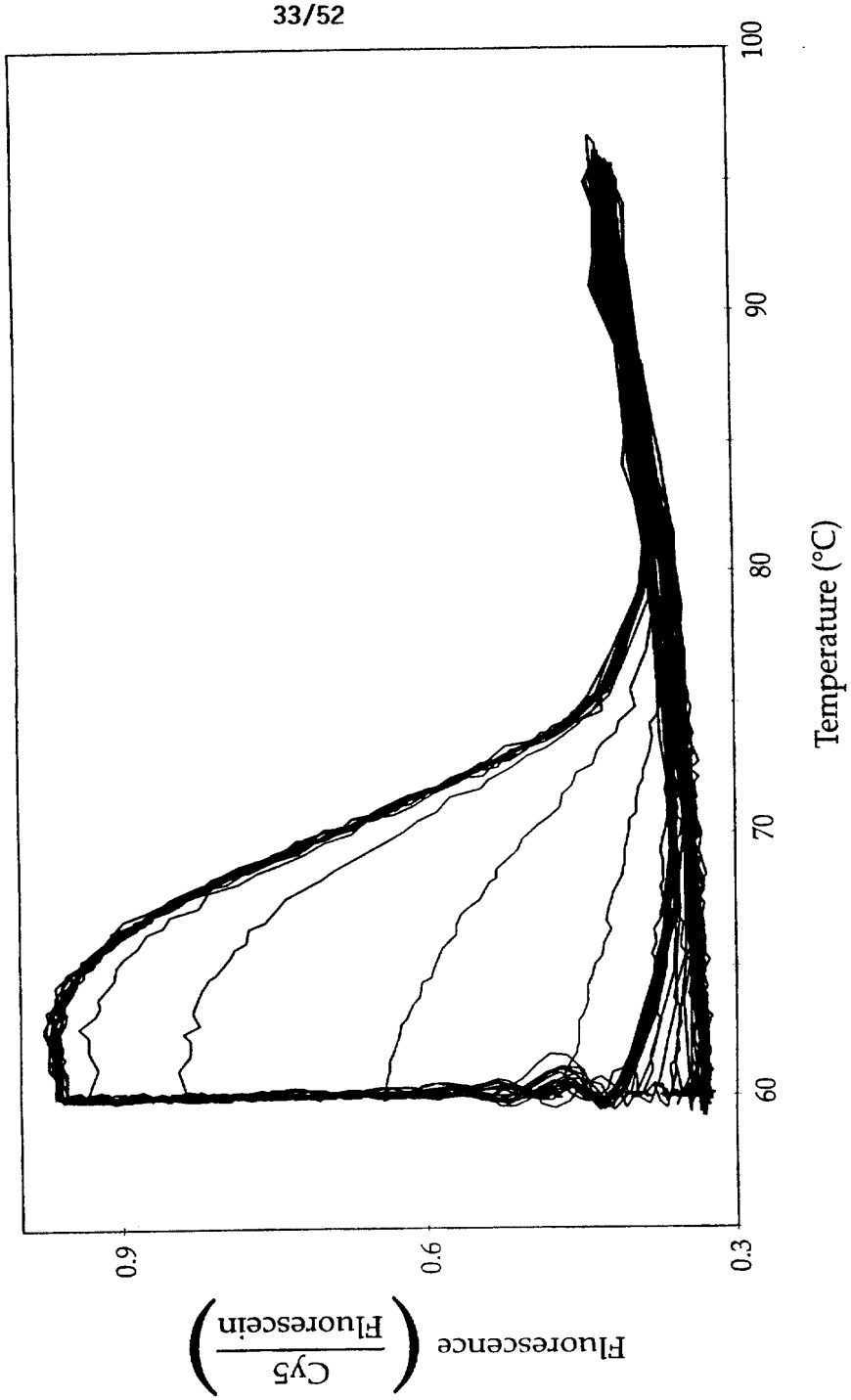

Fluorescence $\left(\frac{Cy5}{Fluorescein}\right)$
0.9
0.6
0.3
60
70
80
90
100
Temperature (°C)

Figure 33.

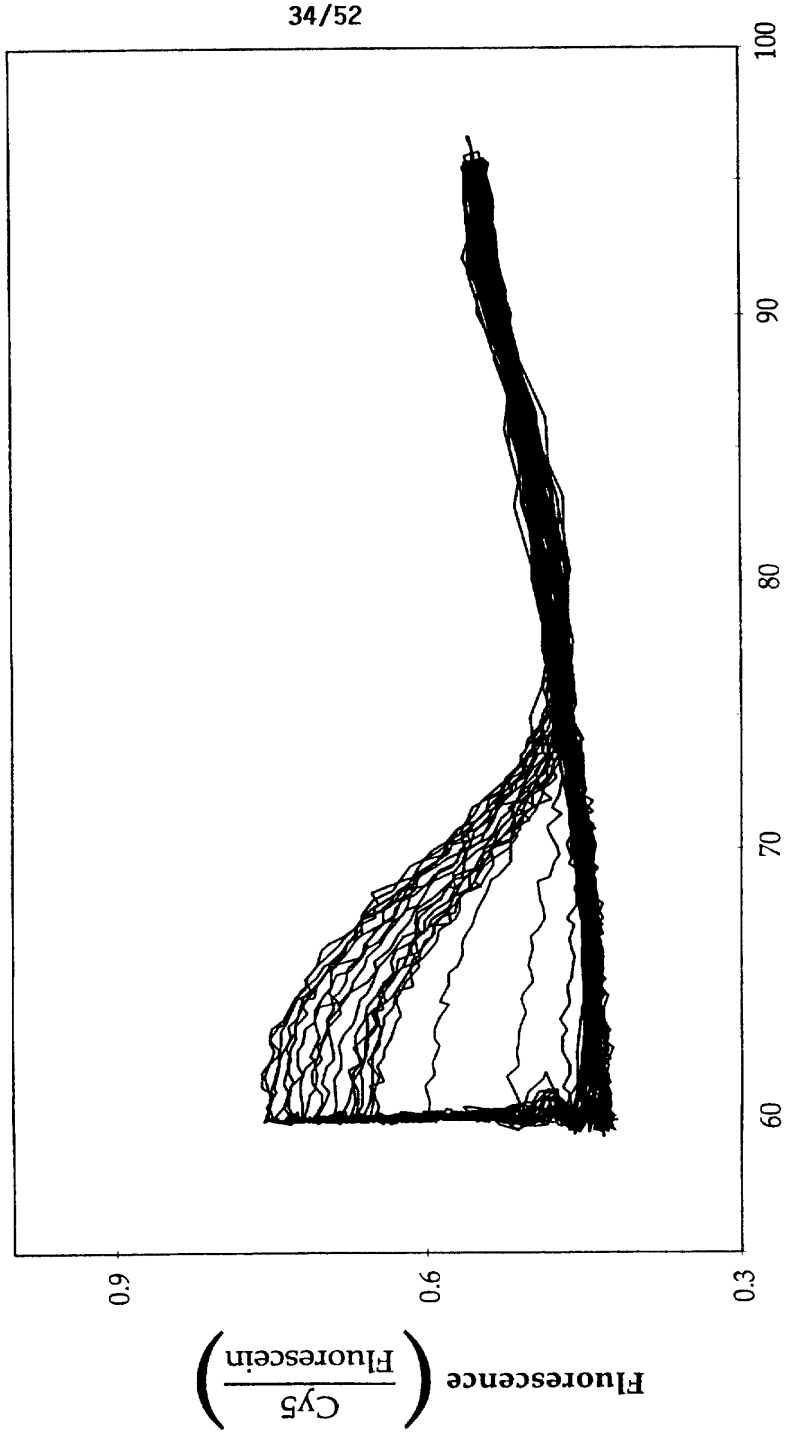

Fluorescence (Cy5/Fluorescein)
0.9
0.6
0.3
60
70
80
90
100
Temperature (°C)

Figure 34.

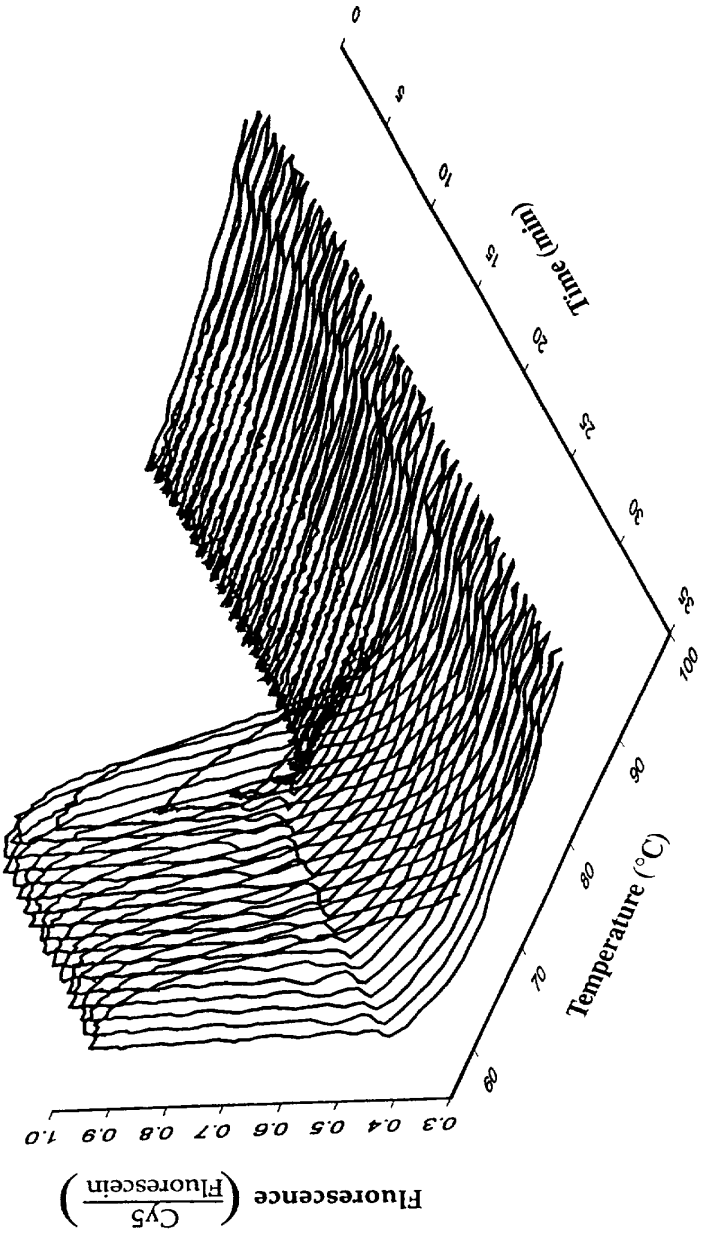

Fluorescence $\left(\frac{Cy5}{Fluorescein}\right)$
0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
Temperature (°C)
80 70 80 90 100
Time (min)
0 5 10 15 20 25 30 35

Figure 35.

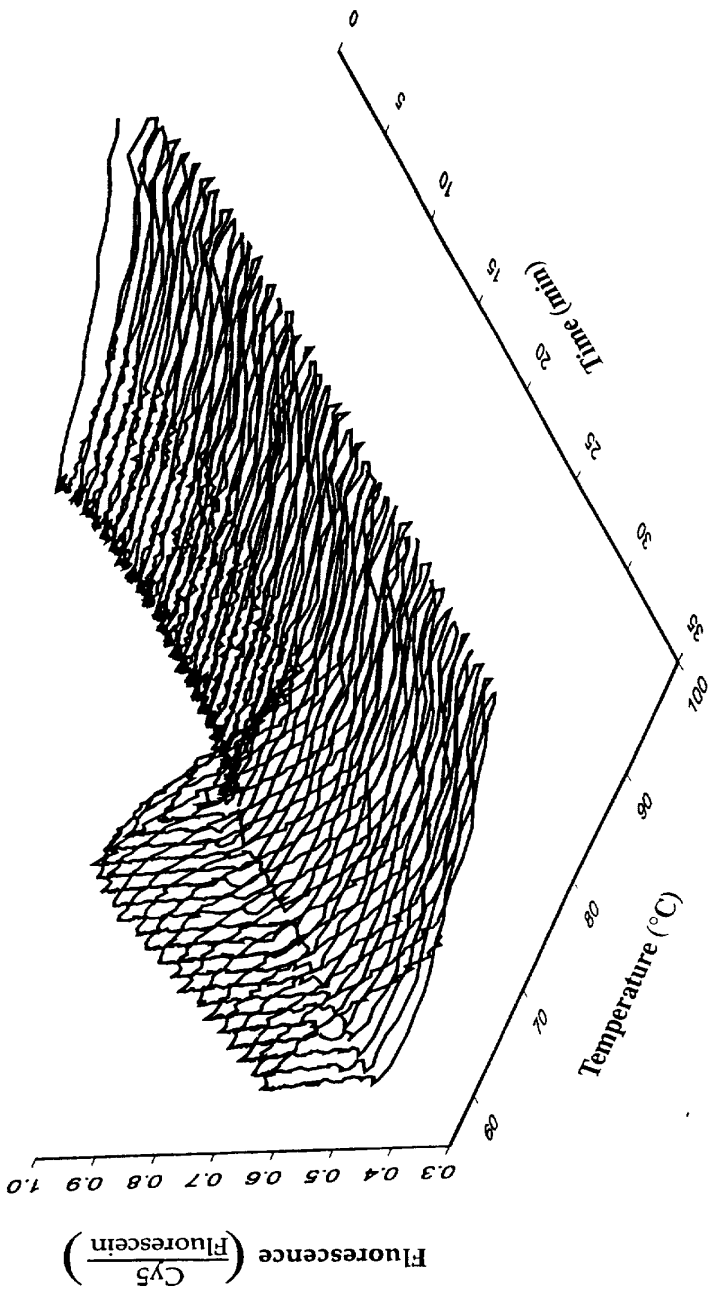

Fluorescence (Cy5 / Fluorescein)
0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
Temperature (°C)
60 70 80 90 100
Time (min)
0 5 10 15 20 25 30 35

Figure 36.

Figure 37

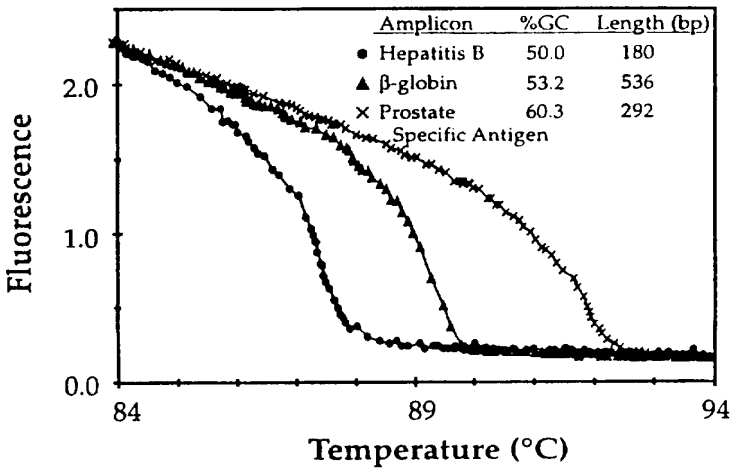

Amplicon %GC Length (bp)
Hepatitis B 50.0 180
β-globin 53.2 536
Prostate Specific Antigen 60.3 292
Fluorescence
2.0
1.0
0.0
84
89
94
Temperature (°C)

Figure 38

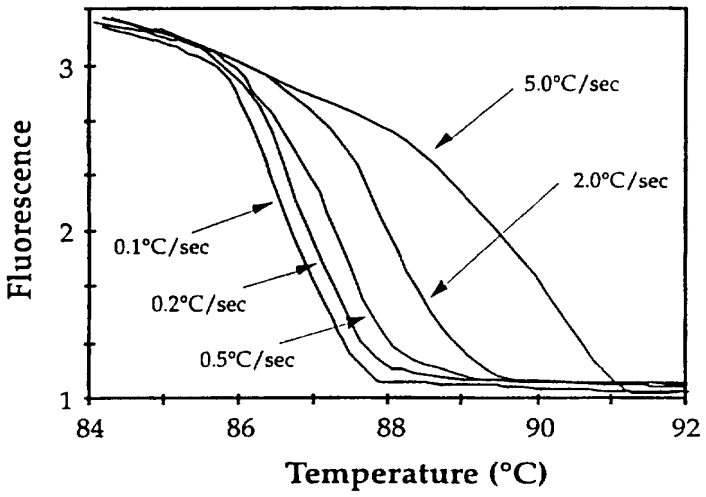

Fluorescence
3
2
1
84
86
88
90
92
Temperature (°C)
5.0°C/sec
2.0°C/sec
0.1°C/sec
0.2°C/sec
0.5°C/sec

Figure 39

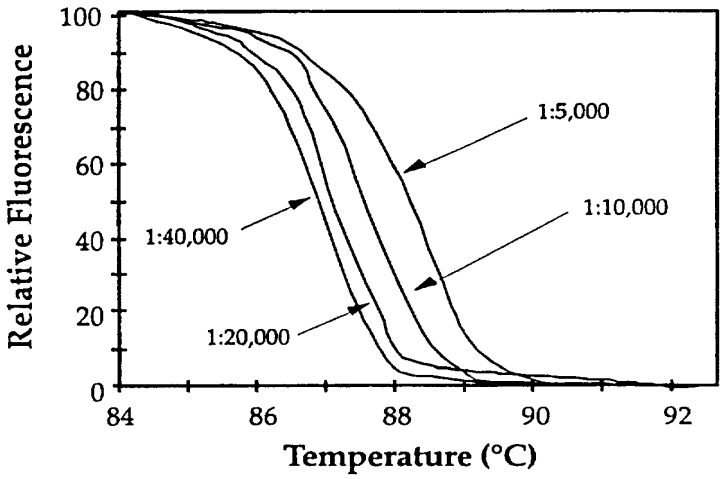
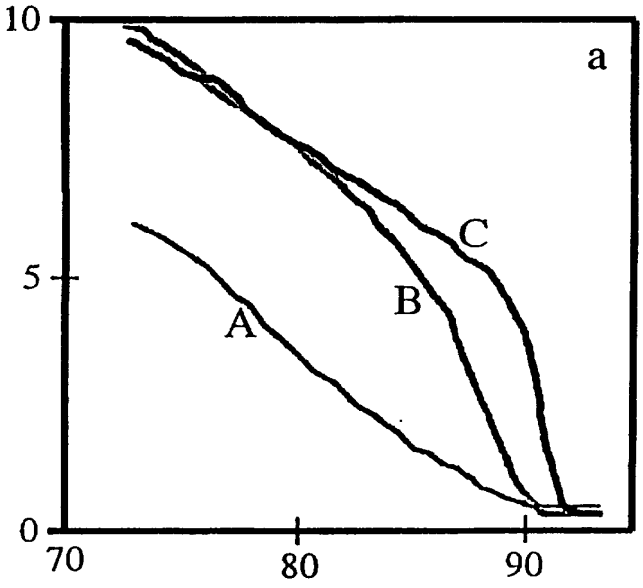

100
80
60
40
20
0
Relative Fluorescence
84
86
88
90
92
Temperature (°C)
1:5,000
1:10,000
1:40,000
1:20,000

Figure 40

A


10
5
0
70
80
90
a
A
B
C
Temperature (°C)

B

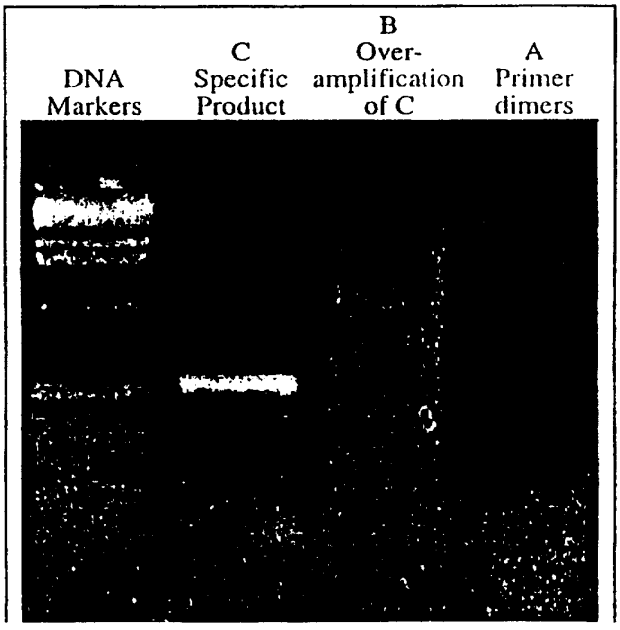
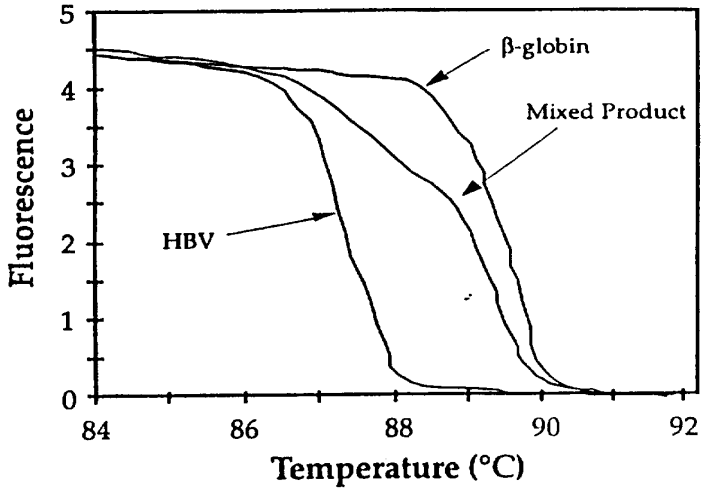

DNA Markers
C Specific Product
B Over-amplification of C
A Primer dimers

Figure 41

A


Fluorescence
Temperature (°C)
β-globin
Mixed Product
HBV
0
1
2
3
4
5
84
86
88
90
92

B

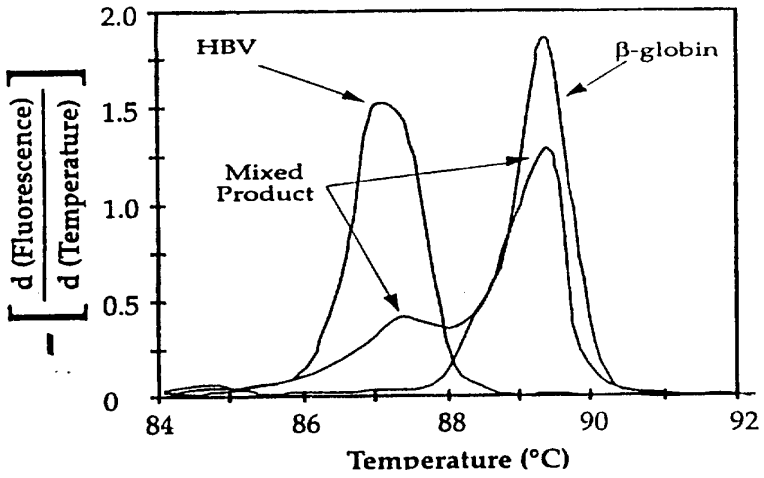

- [d (Fluorescence) / d (Temperature)]
Temperature (°C)
HBV
β-globin
Mixed Product
0
0.5
1.0
1.5
2.0
84
86
88
90
92

Figure 42

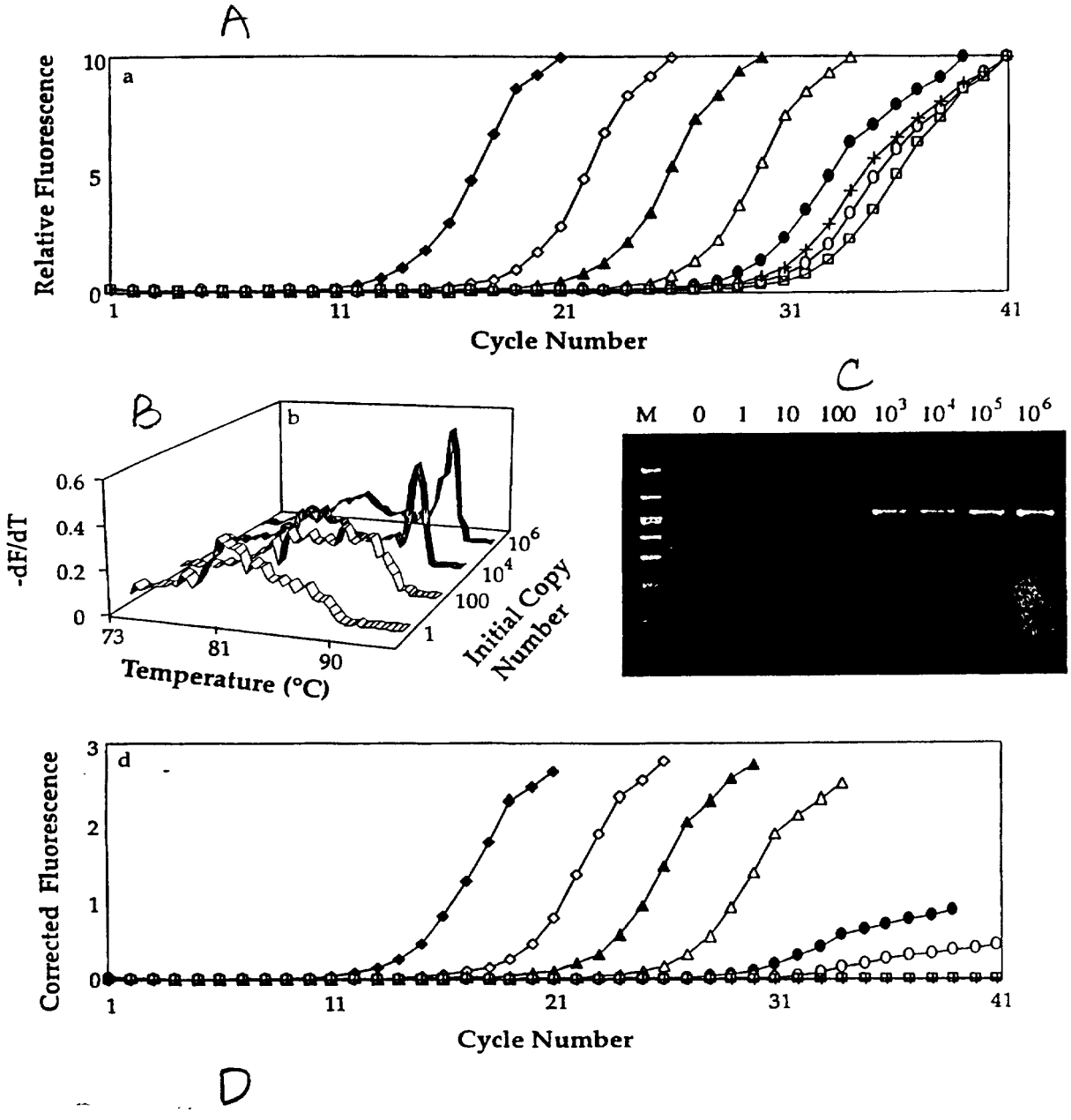

A
a
Relative Fluorescence
10
5
0
1
11
21
31
41
Cycle Number
B
b
-dF/dT
0.6
0.4
0.2
0
73
81
90
Temperature (°C)
10^6
10^4
100
1
Initial Copy Number
C
M 0 1 10 100 10^3 10^4 10^5 10^6
d
Corrected Fluorescence
3
2
1
0
1
11
21
31
41
Cycle Number
D

Figure 43

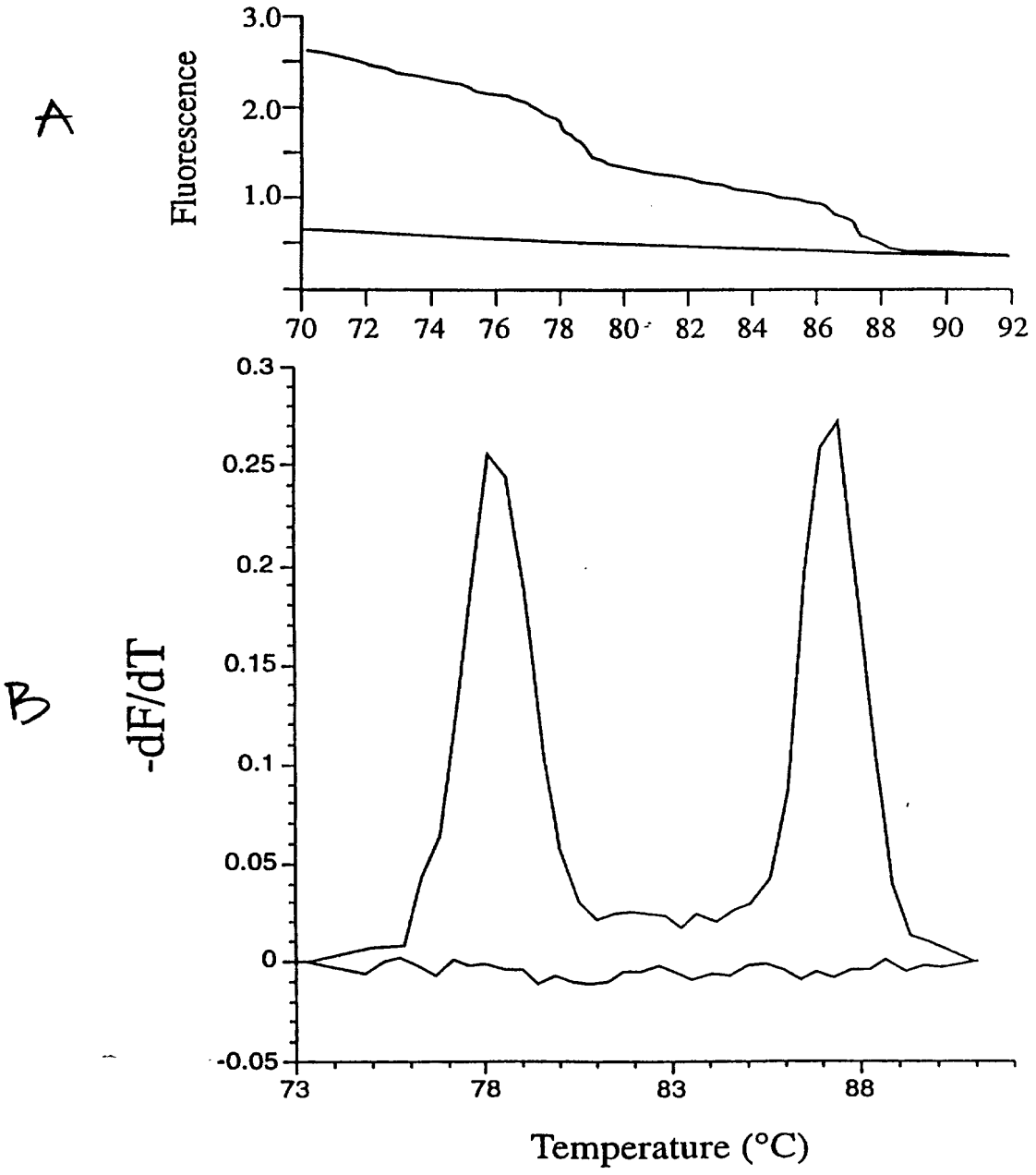

A
Fluorescence
3.0
2.0
1.0
70 72 74 76 78 80 82 84 86 88 90 92
B
-dF/dT
0.3
0.25
0.2
0.15
0.1
0.05
0
-0.05
73 78 83 88
Temperature (°C)

Figure 44

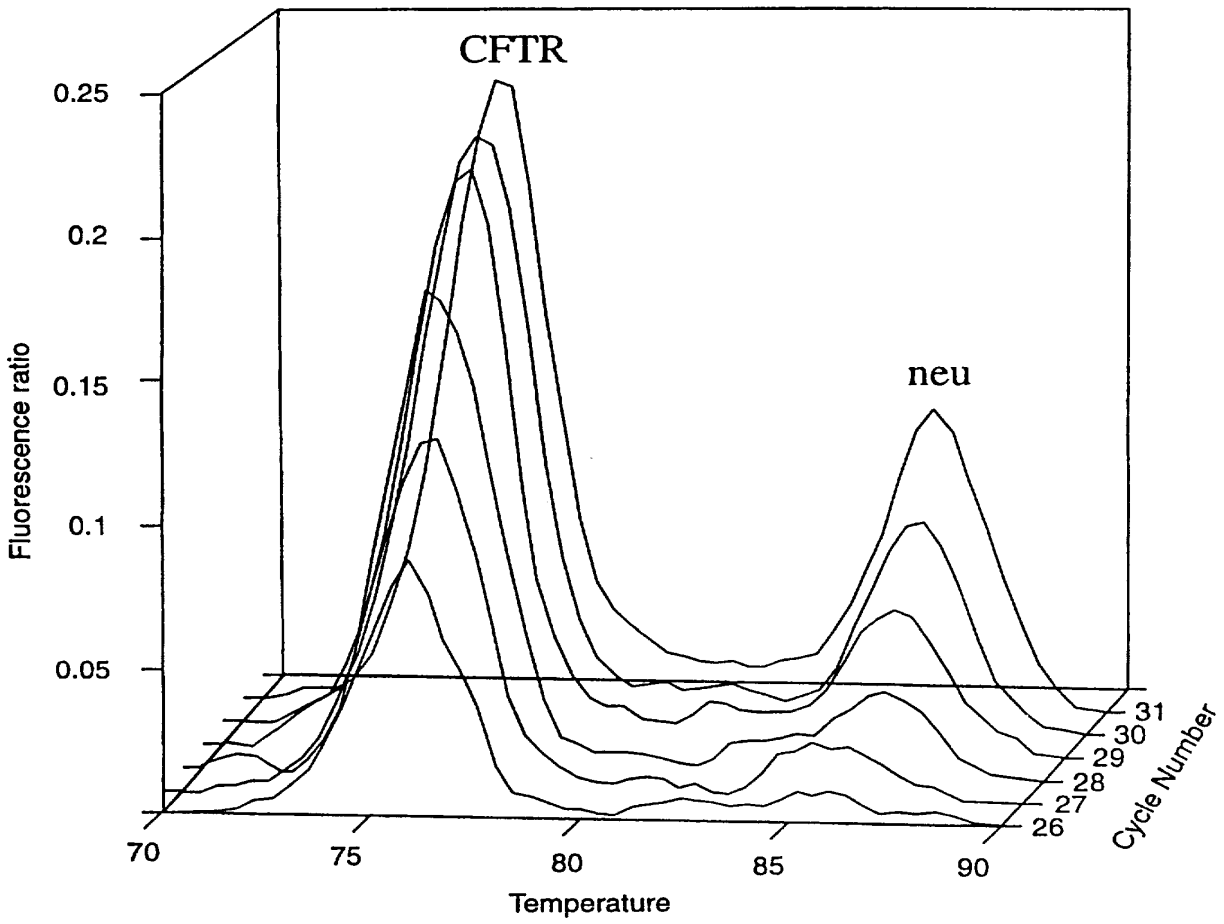

CFTR
neu
0.25
0.2
0.15
0.1
0.05
Fluorescence ratio
70
75
80
85
90
Temperature
31
30
29
28
27
26
Cycle Number

Figure 45

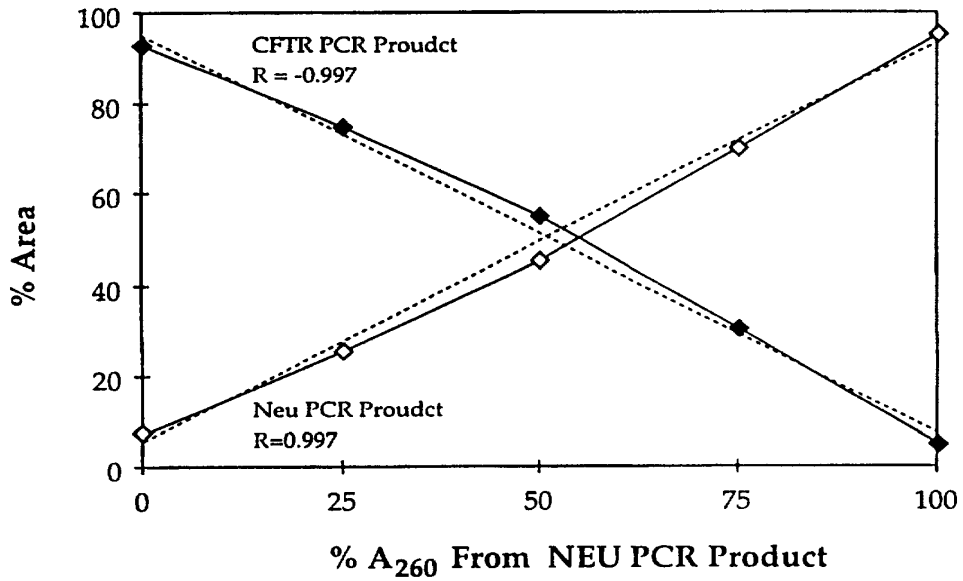

100
80
60
40
20
0
% Area
CFTR PCR Proudct
R = -0.997
Neu PCR Proudct
R=0.997
0
25
50
75
100
% A260 From NEU PCR Product

FIG. 46A

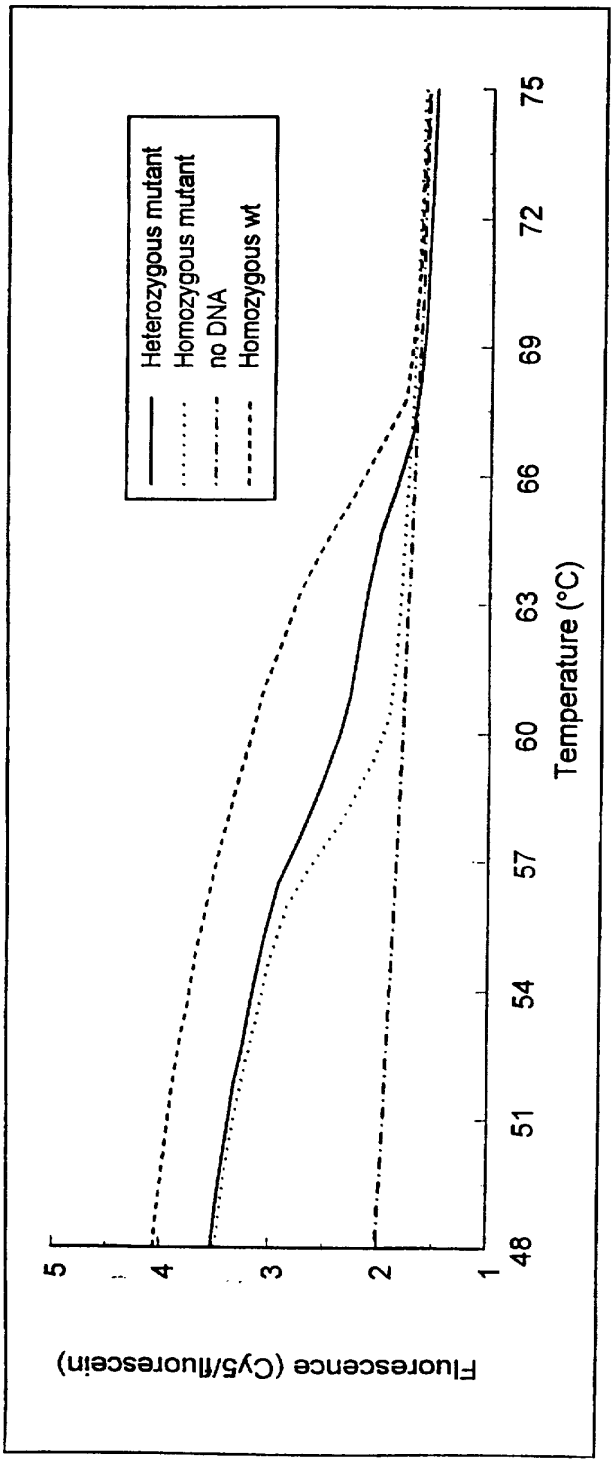
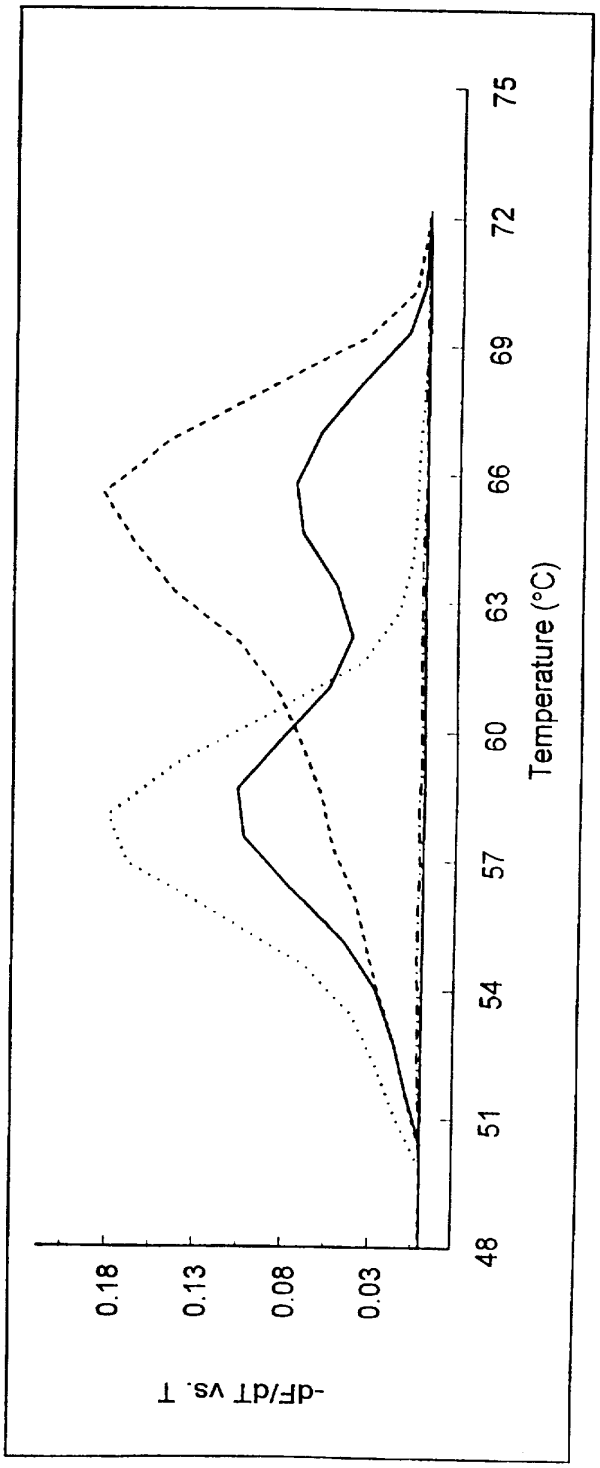


FIG. 46B



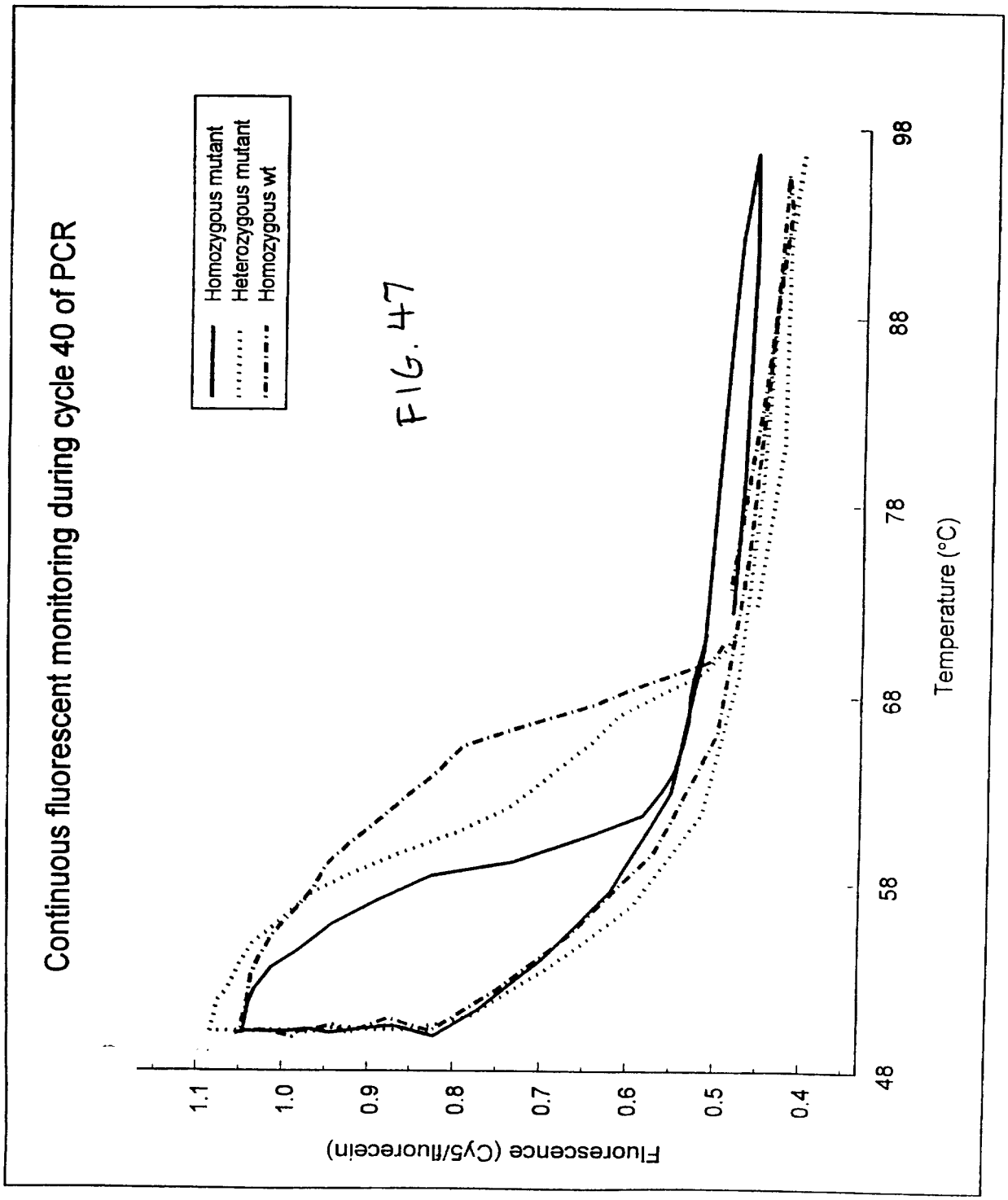

Continuous fluorescent monitoring during cycle 40 of PCR
Homozygous mutant
Heterozygous mutant
Homozygous wt
Fluorescence (Cy5/fluorescein)
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
48
58
68
78
88
98
Temperature (°C)

FIG. 47

Figure 48

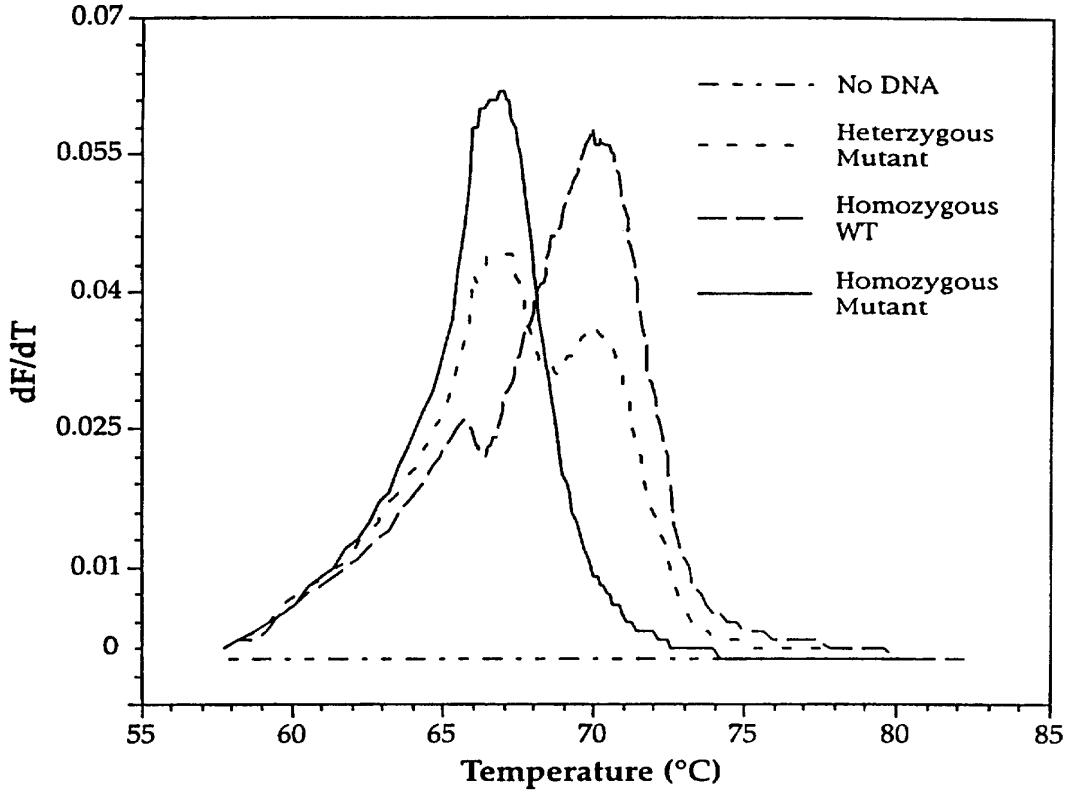

0.07
0.055
0.04
0.025
0.01
0
dF/dT
55
60
65
70
75
80
85
Temperature (°C)
No DNA
Heterzygous Mutant
Homozygous WT
Homozygous Mutant

Figure 49

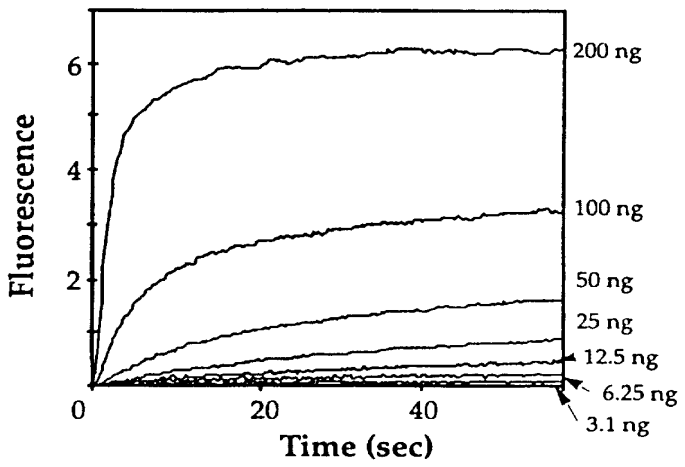
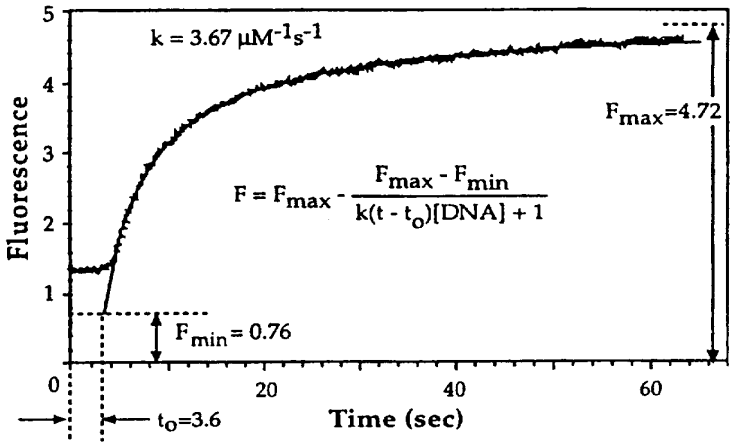

Fluorescence
6
4
2
0
20
40
Time (sec)
200 ng
100 ng
50 ng
25 ng
12.5 ng
6.25 ng
3.1 ng

FIGURE 50


k = 3.67 μM-1s-1
Fmax=4.72
F = Fmax - (Fmax - Fmin) / k(t - to)[DNA] + 1
Fmin = 0.76
Fluorescence
Time (sec)
to=3.6
0
1
2
3
4
5
20
40
60

LightCycler Functional Block Diagram

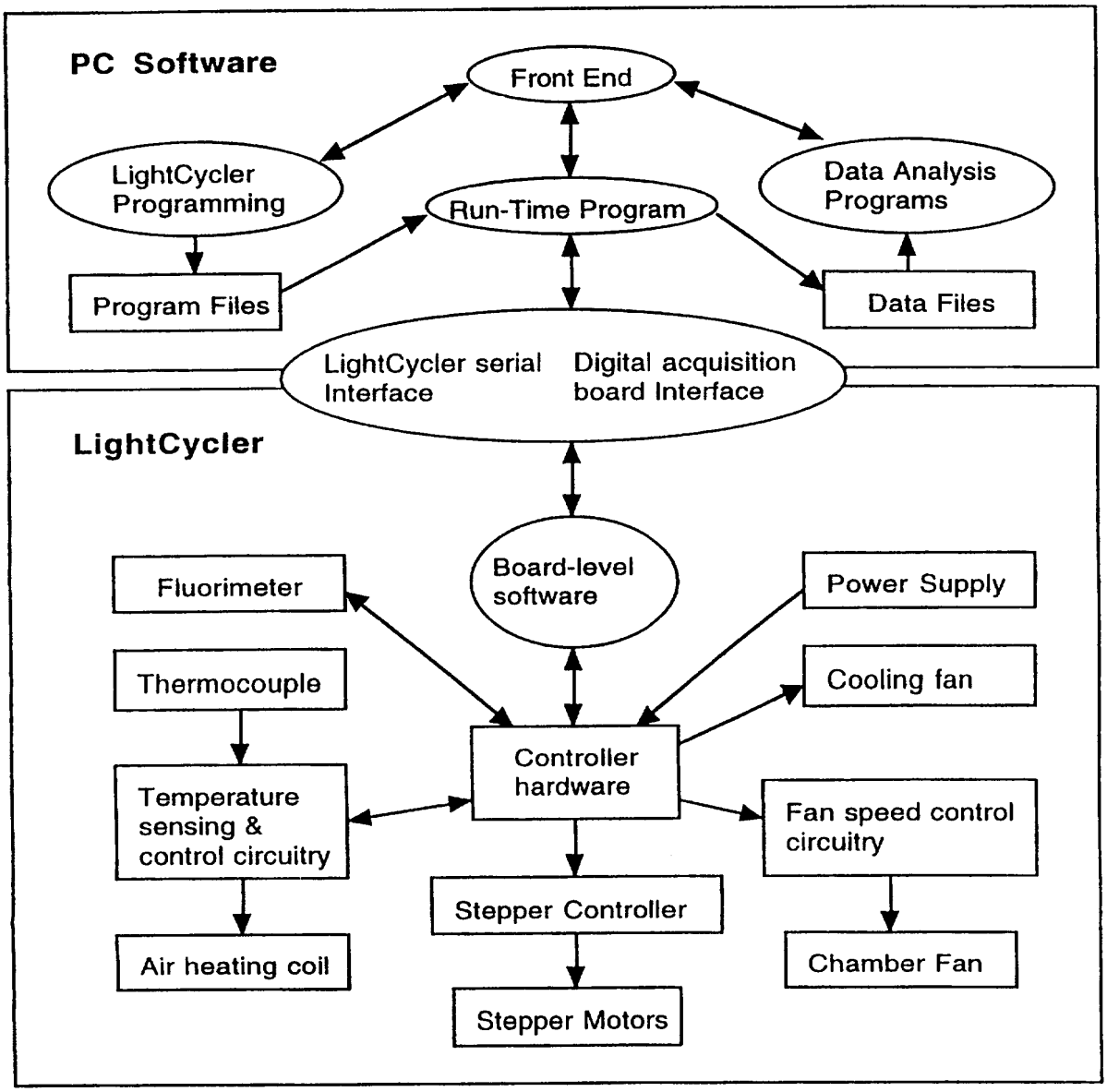
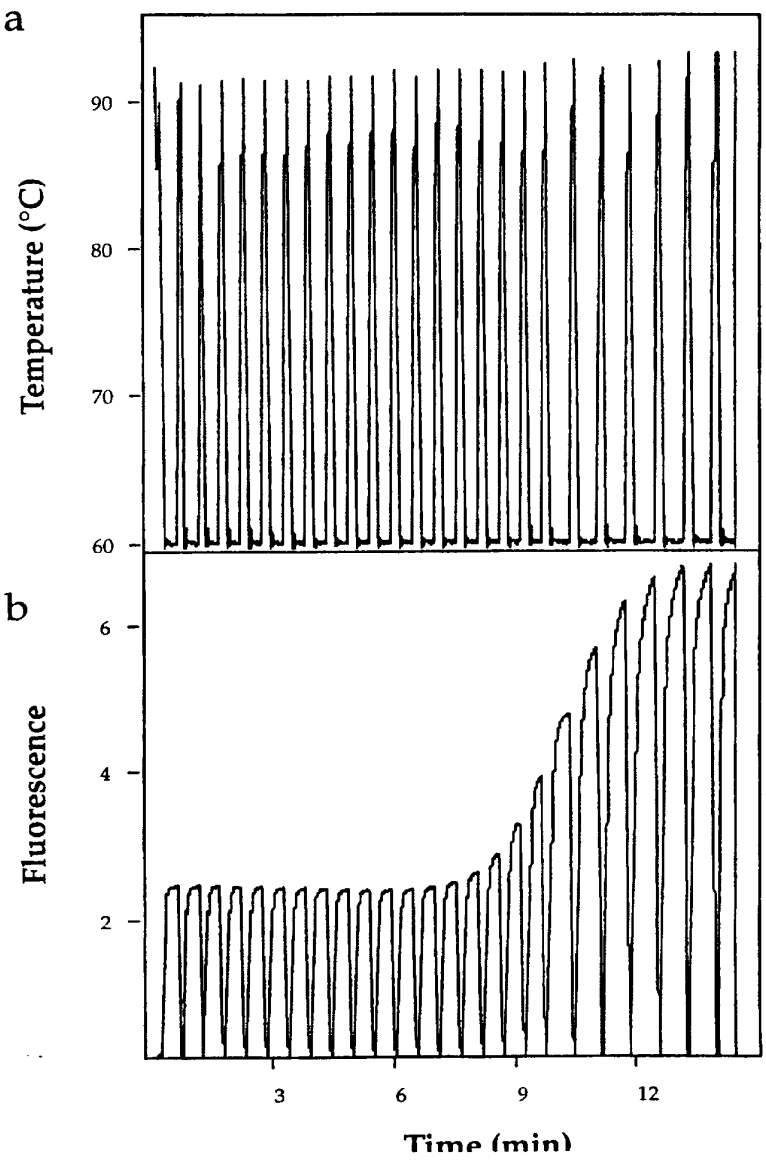


FIG. 51

Figure 52



INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 97/10008

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 G01N33/58

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	EP 0 566 751 A (HOFFMANN LA ROCHE) 27 October 1993 see the whole document ---	6,7,30, 34 1-5,22, 55,56
X Y	EP 0 229 943 A (MOLECULAR BIOSYSTEMS INC) 29 July 1987 see the whole document ---	15-21 1-5,22, 55,56
Y	WO 96 00901 A (UNIV CALIFORNIA) 11 January 1996 see the whole document ---	1-5,22, 55,56, 118,119
Y	WO 95 32306 A (BIOTRONICS CORP.) 30 November 1995 see the whole document --- -/--	1-5,22, 55,56

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 October 1997	18. 11. 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Osborne, H

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INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 97/10008

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	WO 95 30139 A (PERKIN ELMER CORP) 9 November 1995 see page 9, line 20 - page 14	1-5,22, 55,56, 118,119
X	EP 0 640 828 A (HOFFMANN LA ROCHE) 1 March 1995 cited in the application	28,29, 57-60, 114,115, 120-123
A	see the whole document	9-14, 63-67, 81-86, 110,111
X	EP 0 686 699 A (PERKIN ELMER CORP) 13 December 1995	28,29, 57-60, 114,115, 120-123
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X	EP 0 643 140 A (CANON KK) 15 March 1995	28,29, 57-60, 114,115, 120-123
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X	WITTWER C ET AL: "Rapid cycle amplification, pages 174-181; in PCR: Polymerase chain reaction, (ED.) Mullis K B., F. Ferre and R. A. Gibbs" 1994 , BIRKHAEUSER BOSTON, INC , NEW YORK, NEW YORK, USA XP002044531 see the whole document	9,10,28, 29, 57-60, 114,115, 120-123

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IPR2024-01178
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INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter ›nal Application No
PCT/US 97/10008

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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INTERNATIONAL SEARCH REPORT

Information on patent family members

Inten nal Application No
PCT/US 97/10008

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		US 5624798 A	29-04-97

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(71) Applicant *(for all designated States except US)*: **MOLECULAR PLANT BREEDING NOMINEES LTD** [AU/AU]; 1 Park Drive, Bundoora, Victoria 3083 (AU).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **HAYDEN, Matthew James** [AU/AU]; 37 Illuka Crescent, Mount Waverley, Victoria 3149 (AU). **TABONE, Tania** [AU/AU]; 116 Mt Alexander Road, Travancore, Victoria 3032 (AU).

(74) Agents: **OLIVE, Mark** et al.; c/- FB Rice & Co, Level 23, 44 Market Street, Sydney, New South Wales 2000 (AU).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

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Published:
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— *with sequence listing part of description published separately in electronic form and available upon request from the International Bureau*

(54) Title: METHOD OF AMPLIFYING NUCLEIC ACID

(57) Abstract: The present invention provides a method for detecting a polymorphism or mutation in nucleic acid comprising a first phase to amplify or enrich for a sequence comprising a polymorphism or mutation and a second phase for detecting the polymorphism or mutation, wherein both phases are performed in the same reaction vessel.

WO 2009/036514 A2

IPR2024-01178
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Method of amplifying nucleic acid

Cross-reference to related application

The present application claims priority from USSN 60/973,928 filed in the United States Patent and Trademark Office on September 20, 2007, the contents of which are incorporated by reference in their entirety.

Field of invention

The present invention relates to methods for detecting a polymorphism or a mutation, such as by polymerase chain reaction (PCR), and applications therefor.

Background of invention

Description of related art

Genetic variations between organisms, such as polymorphisms and mutations are detected in a variety of assays used in, for example, gene mapping, positional cloning, identification of individuals (e.g., for animal or plant marker-assisted breeding or for forensic identification, maternity testing, paternity testing), genotype/phenotype association, for determining a subject likely to develop a trait of interest or for determining a subject at risk of developing a genetic disorder.

Single nucleotide polymorphisms (SNPs) are the most common type of genetic variation within the genome of several organisms. For example, a SNP occurs on average once per 250-1000 base pairs (bp) and account for 90% of sequence variants in the human genome (Collins *et al., Genome Res., 8*: 1229-1231, 1998). As for plants, in maize a SNP occurs on average once every 104 base pairs (Tenaillon *et al., Proc. Natl. Acad. Sci USA, 98*: 9161-9166); soybean has about one SNP every 273 bp (Zhu *et al., Genetics, 163*: 1123-1134, 2003); wheat has one SNP about every 200 bp (Ravel *et al., In:* Vollman *et al* (Eds) Genetic variation for plant breeding, Eucarpia: Tulln, Austria, pp177-181); and rapeseed has one SNP about every 600 bp (Fourmann *et al., Theor. Appl. Genet. 105*: 1196-1206). The high density and mutational stability of SNPs make them particularly useful genetic markers for population genetics and for mapping genes associated with complex traits.

Nucleic acid amplification techniques have become key tools for detecting nucleotide sequence variations. Several of the most common techniques currently used for amplification and analysis of genetic markers use a polymerase (such as, for example, a

DNA polymerase and/or a RNA polymerase) to replicate a nucleic acid template using, for example, a polymerase chain reaction (PCR; e.g., Saiki *et al., Science 230*:1350, 1985). These methods are useful for amplifying nucleic acid from DNA, DNA/RNA hybrid or RNA and/or for determining the nucleotide sequence of a specific nucleic acid (e.g., by sequencing, allele specific PCR or primer extension). Generally, a polymerase-mediated replication technique uses a primer (e.g., a short oligonucleotide) capable of annealing selectively to a nucleic acid template to provide the binding site for the polymerase to initiate replication. By iteratively annealing the primer and replicating the nucleic acid template of interest, the nucleic acid is amplified.

A standard PCR is performed using two oligonucleotide primers designed to hybridize to opposite strands of a double stranded nucleic acid adjacent to the region of interest. Strands of nucleic acid in a sample are separated, typically by thermal denaturation, and the primers then allowed to anneal to the single strand templates. These primers provide the site of binding for a polymerase and initiate replication of the region of interest. Both the original nucleic acid and the newly synthesized nucleic acid are then be used as templates for further amplification cycles, thereby permitting exponential amplification of the nucleic acid region of interest.

An example of a method for detecting a polymorphism using PCR is the PCR-restriction fragment length polymorphism (RFLP) method, involving a combination of the polymerase chain reaction (PCR) method and cleavage with restriction enzymes (Olerup, *Tissue Antigens, 36*:83-87, 1990). In this method a nucleic acid comprising a polymorphism an allele of which modifies the binding site of a restriction endonuclease is amplified by PCR, and the resulting amplification product contacted with the restriction endonuclease under conditions sufficient for cleavage to occur in the presence of the correct binding site. By resolving the resulting nucleic acid fragments, e.g., using electrophoresis, the presence or absence of the restriction endonuclease binding site is determined, as is the sequence of the allele. However, this method is time consuming, because it requires both a PCR and treatment with a restriction enzyme for a sufficient time for cleavage to occur (typically, 3 to 24 hours).

Another method used for detection of a polymorphism is a single-strand conformation polymorphism (SSCP) detection method. SSCP detection is based on the principle that single-strand DNA and RNA having different sequences exhibit different electrophoretic mobility in polyacrylamide gels. This method involves amplifying a

sequence comprising a polymorphism using PCR and separating the resulting amplicons to thereby determine their electrophoretic mobility and, as a consequence, the sequence of the polymorphism. However, SSCP methods require that the experimental conditions are strictly controlled to detect subtle differences in electrophoretic mobility. Accordingly, the methods are extremely complicated. Moreover, such a method is not readily adapted to detection of polymorphisms in a polyploid organism or a specific gene in a well-conserved gene family. This is because, the method relies on amplification of short nucleic acid fragments (i.e., less than about 300bp). The size restrictions of SSCP methods mean that it may not be possible to selectively amplify a fragment comprising the polymorphism to be detected, e.g., a fragment from a homologous gene or homeologous genes in a polyploid organism may also be amplified, thereby confounding the results of the analysis.

Assays such as TaqMan® and Molecular Beacon® assays, have also been produced which amplify a sequence comprising a polymorphism using PCR and detecting an allele of the polymorphism with an oligonucleotide probe that selectively binds to one allele of the polymorphism. The probe is labeled with a fluorescent moiety and a quencher moiety. In the absence of binding to the allele, the quencher moiety prevents the fluorescent moiety from emitting a detectable signal. When bound to the allele, the fluorescent moiety and quencher moiety are separated, and the fluorescent moiety emits a detectable signal. A disadvantage of TaqMan® and Molecular Beacon® assays is that they are expensive since they require specialized probes to detect a polymorphism. These assays are also not readily adapted to detection of polymorphisms in polyploid species, e.g., wheat or in well conserved gene families (Giancola *et al., Theor. Appl. Genet., 112:* 1115-1124, 2006). This is because the assays require amplification of relatively short sequences, e.g., about 150bp, prior to detection. Accordingly, the methods may not be amenable to amplifying a sequence specific to the nucleic acid comprising the polymorphism of interest, e.g., they may amplify related genes in a gene family and/or homeologous genes in a polyploid organism.

Previous methods for detecting polymorphisms in polyploid organisms, such as plants have generally involved amplifying a sequence from one genome comprising a polymorphism of interest, isolating the amplification product and detecting the polymorphism in the amplification product. Accordingly, these methods are often complex, requiring multiple steps to amplify and isolate a nucleic acid specific to one genome from the polyploid organism. Moreover, the requirement for multiple steps,

often requiring additional handling of a sample increases the risk of contamination of a sample.

It will be apparent form the foregoing that notwithstanding the advances in methods for detecting polymorphisms, it is clear that these methods suffer from several disadvantages, such as, for example, lengthy assay time, increased expense, complicated assay format and inability to detect polymorphisms in genes from conserved gene families or in polyploid organisms. Accordingly, it is clear that there is a need in the art for a rapid and inexpensive assay that enables detection of a polymorphism in a sample, including a sample from a polyploid organism. Such an assay would have clear utility in, for example, diagnosis of a condition and/or the identification of an individual or group thereof.

Conventional techniques of molecular biology and recombinant DNA technology used in performance of the present invention are described, for example, in the following texts that are incorporated by reference:

i. Sambrook, Fritsch & Maniatis, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratories, New York, Second Edition (1989), whole of Vols I, II, and III;

ii. DNA Cloning: A Practical Approach, Vols. I and II (D. N. Glover, ed., 1985), IRL Press, Oxford, whole of text;

iii. Oligonucleotide Synthesis: A Practical Approach (M. J. Gait, ed., 1984) IRL Press, Oxford, whole of text, and particularly the papers therein by Gait, pp1-22; Atkinson *et al.,* pp35-81; Sproat *et al.,* pp 83-115; and Wu *et al.*, pp 135-151;

iv. Nucleic Acid Hybridization: A Practical Approach (B. D. Hames & S. J. Higgins, eds., 1985) IRL Press, Oxford, whole of text;

v. Perbal, B., A Practical Guide to Molecular Cloning (1984);

vi. Methods In Enzymology (S. Colowick and N. Kaplan, eds., Academic Press, Inc.), whole of series;

Summary of the invention

Introduction

In work leading up to the present invention, the present inventors sought to produce a simple and inexpensive method for detecting a polymorphism or mutation, and that was amenable to detecting a polymorphism in a specific gene from a conserved gene family

or in nucleic acid from a polyploid organism. The inventors also sought to produce a method for detecting a polymorphism or mutation that does not require multiple distinct reactions, thereby reducing costs and risks of contamination.

As exemplified herein, the inventors have produced a PCR-based method for detecting a polymorphism or mutation comprising multiple phases of amplification, i.e., a first phase to amplify or enrich for a sequence comprising a polymorphism or mutation, and a second phase for detecting the polymorphism or mutation. As exemplified herein, the method developed by the inventors comprises a first amplification phase in which a set of first primers is used to selectively amplify a nucleic acid comprising the polymorphism or mutation, i.e., to enrich for the sequence comprising the polymorphism or mutation. A second phase amplification is performed using one or more second primers comprising (i) an allele specific region comprising a sequence complementary to the template nucleic acid adjacent to the polymorphism or mutation and that has a lower Tm than the first primers; and (ii) a tag region comprising a sequence that does not anneal to the template nucleic acid, however increases the Tm of the second primer to about the Tm of the first primer. By reducing the annealing temperature in the second phase amplification, the allele specific region of the second primer(s) anneals to the amplification product of the first phase amplification, thereby permitting amplification with the second primer(s) and first primers. Following several amplification cycles, the sequence of the second primer(s) is incorporated into amplification products thereby permitting the annealing temperature to be increased, and for the entire second primer and the first primer to anneal to target sequences and prime amplification by PCR. By detecting one or more amplification products produced in this second phase of amplification a polymorphism or mutation is detected.

In one exemplified form of the present invention a second primer comprises one or more nucleotide(s) positioned at the 3' end of the allele specific region that is complementary to an allele of the polymorphism or mutation. The 3' end of the second primer only anneals in the presence of that allele, and permits amplification by PCR. Detection of the amplification product produced using this second primer and either another second primer or a first primer, an allele of a polymorphism or mutation is detected. On the other hand, failure to detect an amplification product produced using this primer may indicate the presence of a different allele. Use of two or more second primers complementary to different alleles permits positive detection of different alleles. In this respect, the two or more second primers may be used in the same

reaction if each primer is labeled so as to permit differentiation between amplification products produced by different primers, e.g., using tag regions having different molecular weights or different detectable markers. Alternatively, each second primer is used in a separate reaction.

The assay of the present invention can thus be configured utilizing a variety of primer combinations, including one or a plurality of locus-specific primers and one or a plurality of allele-specific primers for allele discrimination, wherein the primers bind to the same or opposite nucleic acid strands and/or are differentially labeled and wherein the products are resolved e.g., on a variety of size separation matrixes e.g., agarose gel, polyacrylamide, or using eGENE by end-point or real-time melting analysis using instrumentation such as the RotorGene6000 (Corbett Research). For example, data presented in example 1 hereof demonstrate allelic discrimination by differential product size using a pair of allele-specific primers designed for opposite DNA strands, to thereby permit codominant allelic discrimination in a single reaction, wherein the sizes of the resulting PCR products are resolved on a variety of size separation matrices e.g., agarose gel, polyacrylamide, or using eGENE by end-point or real-time melting analysis using instrumentation such as the RotorGene6000 (Corbett Research). In another example, data presented in example 2 hereof demonstrate allelic discrimination by differential product size using a pair of allele specific primers designed to the same DNA strand, to thereby permit codominant allelic discrimination using a size separation matrix such as that described in the preceding paragraph. In another example, data presented in example 3 hereof demonstrate allelic discrimination using a single allele-specific primer e.g., AS_1, wherein the number of reactions required for genotype determination is influenced by the size of the PCR fragment amplified by the locus specific (LS) primer pair. In another example, data presented in example 4 hereof demonstrate allelic discrimination by differential product labeling using a pair of allele-specific (AS) primers designed to the same DNA strand, wherein AS primers differ by having a detectable marker, such as a fluorescent dye, attached to their 5'-ends such that differential detection of the detectable marker attached to each AS primer facilitates codominant allelic discrimination. In another example, data presented in example 5 hereof demonstrate allelic discrimination by end point and/or real time high resolution melting analysis using a pair of AS primers designed to anneal to a region adjacent the allele being detected such that they amplify nucleic acid comprising the allele. In another example, data presented in example 6 hereof demonstrate allelic discrimination by end point and/or real time high resolution melting

analysis using a single AS primer designed to anneal to a region adjacent the allele being detected such that it amplifies nucleic acid comprising the allele. The present inventors have also demonstrated that, for some assay configurations, such as allelic discrimination by differential product detection e.g., as demonstrated in examples 5 and 6, the capture of sequence variation within the second phase PCR amplification product eliminates the requirement for AS primers to contain mismatched nucleotides that can cause primer annealing destabilization.

As exemplified herein, the method of the present invention is biphasic as demonstrated by real-time polymerase chain reaction (PCR) to monitor the accumulation of the first and second phase products e.g., in assays configured for allelic discrimination wherein differential product sizes are identified for each phase e.g., a first phase employing locus-specific (LS) primers e.g., L_1, L_2, L_3, etc., and a second phase employing allele-specific (AS) primers A_1, A_2, A_3, etc. Data presented in example 8 hereof affirm the reaction mechanism of the assay of the invention i.e., sequential enrichment of a target sequence harboring the SNP by the LS primers L_1 and L_2, followed by nested amplification of the interrogated allele by the AS primers A_1 and A_2.

The inventors have empirically determined parameters for minimizing the participation of AS primers in the first phase of amplification performed using LS primers, as shown in example 7 and example 13 hereof for two model genes, wherein examination of the PCR specificity and yield suggests that AS primers having melting temperatures below about 48°C, and preferably in the range of about 40°C to about 48°C, and still more preferably having melting temperatures of about 45°C, do not participate significantly in the first phase of amplification. Preferred AS primers should also be selected to be at least about 12-15 nucleotides in length and not exceeding about 36-40 nucleotides in length, preferably having a size range of about 15 nucleotides in length to about 36 nucleotides in length.

Alternatively, or in addition, the annealing efficiency of AS primers is normalized during the initial cycles of the second phase of TSP amplification e.g., by increasing the melting temperature on the complementary region to the AS forward primer, to thereby facilitate correct genotype determination for genomic loci producing mismatched product in samples.

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As exemplified herein, the separation of the amplification phases by using different annealing temperatures permits the method produced by the inventors to be performed in a single closed-tube reaction. Accordingly, the method produced by the inventors reduces the risk of contamination caused by sample handling, and is simple since all reagent required for PCR may be included in a single tube.

The inventors have shown that the exemplified method is useful for detecting a polymorphism or mutation in a sample. Such a method is useful for characterizing or identifying one or more individuals, isolates of an organism, cultivars of an organism, species or genera, e.g., based on one or more polymorphisms or mutations in the genome of said individuals, isolates of an organism, cultivars of an organism, species or genera. The method of the present invention can also be applied to identifying a subject having a trait or a disease or having a predisposition to developing a trait or disease, e.g., for marker assisted breeding.

In one example, e.g., example 12 hereof, the inventors have demonstrated sensitivity and accuracy of the assay of the invention for actual genotype determination in plants, wherein mapping populations, each comprising about 250 individuals, were screened independently for SNPs on chromosome 2H containing a frost tolerance QTL and on chromosome 5H containing a malting quality QTL, using cleaved amplified polymorphism (CAP) assays (Minamiyama *et al., Plant Breeding 124*: 288-291, 2005) and the assay of the present invention, and which demonstrates concordance between the two genotyping methods across all assays.

The inventors have also demonstrated that the exemplified method is useful for the detection of polymorphisms in polyploid organisms, e.g., wheat. This is because the first amplification phase of the method permits use of one or more primers that selectively anneal to one genome of the polyploid organism comprising a polymorphism or mutation to thereby enrich for that sequence prior to detection of the polymorphism or mutation in the second phase of amplification.

In another example, e.g., example 14 hereof, the inventors have demonstrated efficacy of the method of the invention for discrimination of alleles comprising cytosine or thymine at position 677 of the coding region of a gene encoding methylenetetrahydrofolate reductase (MTHFR) of humans. A C677T mutation results in substitution of valine for alanine at position 222 of the encoded protein, thereby

producing a thermolabile protein associated with folic acid deficiency, neural tube defects, arterial and venous thrombosis, cardiovascular disease and schizophrenia. Homozygotes carrying two 677T alleles have decreased risk of developing leukemia and/or colon cancer.

In another example, e.g., example 15 hereof, the inventors provide means for using the method of the present invention for detecting HSV-1 and HSV-2 in samples, and for discriminating between HSV-1 and HSV-2. In yet another example, e.g., example 16 hereof, the inventors provide means for using the method of the present invention for detecting HSV-1 in samples, and for discriminating between strains or isolates of HSV-1. In yet another example e.g., example 17 hereof, the inventors provide means for discriminating between *Staphylococcus aureus* and other bacteria. These examples demonstrate the broad applicability of the invention to clinical diagnoses of disease and infectious agents in animals and humans, and more particularly, for detecting pathogens such as pathogenic bacteria, viruses, fungi, protists, protozoa or parasites, in samples taken from subjects suspected of being infected. The present invention is clearly useful for detection of infection in early stages, such as before the onset of disease symptoms, for detecting outbreaks of infectious disease such as epidemics or pandemics, and more particularly, for discrimination between pathogenic strains of an organism, the identification of new strains of pathogenic organisms, and for the identification of non-cultivatable or slow-growing microorganisms such as mycobacteria, anaerobic bacteria and viruses. The biphasic reaction mechanism of the present invention permits PCR specificity to be introduced during the first and/or second phase of amplification. The biphasic TSP assay mechanism can be especially useful for diagnostic tests, since it allows for both the detection of the presence-absence of an infectious agent, as well as the identification of the particular species, ecotype, serotype or strain of an infectious agent in a single assay.

Specific embodiments

The scope of the invention will be apparent from the claims as filed with the application that follow the examples. The claims as filed with the application are hereby incorporated into the description. The scope of the invention will also be apparent from the following description of specific embodiments.

In one example, the present invention provides a method for detecting a polymorphism or mutation in nucleic acid, said method comprising:

(i) performing a polymerase chain reaction (PCR) under conditions sufficient to amplify a nucleic acid template comprising a polymorphism or mutation with one or more set(s) of first primers thereby producing a first amplification product, said set(s) of first primers capable of annealing selectively to a nucleic acid template comprising a polymorphism or mutation at a first temperature;

(ii) performing PCR under conditions sufficient to amplify the first amplification product with one or more second primer(s) or set(s) of second primers and/or with one or more of the primers from the set of first primers thereby producing a second amplification product comprising a sequence complementary to the allele-specific region and the tag region, said second primer(s) comprising an allele-specific region capable to annealing to the nucleic acid template and/or the first amplification product and a tag-region that does not anneal to the nucleic acid template, wherein said allele-specific region has a melting temperature (Tm) lower than the first primer and is not capable of annealing selectively to the template nucleic acid or the first amplification product at the first temperature and wherein the second primer is capable of annealing selectively to a nucleic acid comprising a sequence complementary to the allele-specific region and the tag region at similar to the first temperature, wherein said conditions comprise an annealing temperature suitable for annealing of the allele-specific region of the second primer(s) or set(s) of second primers to the first amplification product and/or the template nucleic acid and for the annealing of the first set of primers to the first amplification product and/or the template nucleic acid;

(iii) performing PCR under conditions sufficient to amplify the second amplification product to produce one or more third amplification product(s), said conditions comprising an annealing temperature suitable for annealing of the second primer(s) or set(s) of second primers to the second amplification product and for annealing of one or more primers from the set of first primers to the second amplification product but not for annealing of the allele specific region of the second primer(s) or set(s) of second primers to anneal selectively to the first amplification product at a detectable level, wherein the third amplification product(s) is/are amplified with the set(s) of second primers and/or a second primer and a first primer; and

(iv) detecting the third amplification product(s) with a detection means,

wherein detection of said third amplification product(s) is/are indicative of the polymorphism or mutation.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or

step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

As used herein, the term "polymorphism" shall be taken to mean a naturally-occurring variation in the nucleotide sequence of a specific site or region of the genome of a subject, or an expression product thereof that occurs in a population of subjects. Preferably, the polymorphism is a single nucleotide polymorphism (SNP).

As used herein, the term "mutation" shall be taken to mean a permanent, transmissible change in nucleotide sequence of the genome of a subject and optionally, an expression product thereof. Examples of mutations include an insertion of one or more new nucleotides or deletion of one or more nucleotides or substitute of one or more existing nucleotides with different nucleotides.

As used herein, the term "PCR" or "polymerase chain reaction" shall be taken to mean an amplification reaction employing multiple cycles of (i) denaturation of double-stranded nucleic acid such as a nucleic acid template to be amplified or a hybrid between a template and a complementary primer; (ii) annealing of a primer to its complementary sequence in the single-stranded "template"; and (iii) extension of the primer in the 5'- to 3'- direction by a polymerase activity e.g., an activity of a thermostable polymerase, such as, *Taq*, to thereby produce a double-stranded nucleic acid comprising a newly-synthesized strand complementary to the single-stranded template. By utilizing two primers capable of annealing to the complementary strands in the double-stranded template (i.e., to each denatured single-stranded template), multiple copies of the template are produced in each cycle, thereby amplifying the template. Many formats of PCR are known in the art including, for example, reverse-transcriptase mediated PCR (RT-PCR), nested PCR, touch-up and loop incorporated primers (TULIP) PCR, touch-down PCR, competitive PCR, rapid competitive PCR (RC-PCR), and multiplex PCR.

As used herein the term "template nucleic acid" includes DNA, RNA or RNA/DNA with or without any nucleotide analogs therein including single-stranded or double-stranded genomic DNA, mRNA or cDNA. The present invention is not limited by the nature or source of the template nucleic acid. The template nucleic acid can be derived directly or indirectly from an organism, a tissue or cellular sample obtained previously

from an organism, or can be present in an aqueous or non-aqueous extract of a tissue or cellular sample.

As will be known to the skilled artisan, a "primer" is a nucleic acid molecule comprising any combination of ribonucleotides, deoxyribonucleotides and analogs thereof such that it comprises DNA, RNA or DNA/RNA, optionally with one or more ribonucleotide or deoxyribonucleotide analogs contained therein, and capable of annealing to a nucleic acid template to act as a binding site for an enzyme, e.g., DNA or RNA polymerase, thereby providing a site for initiation of replication of a specific nucleic acid in the 5' to 3' direction. The nucleotide sequence of a primer is generally substantially complementary to the nucleotide sequence of a template nucleic acid to be amplified, or at least comprises a region of complementarity sufficient for annealing to occur and extension in the 5' to 3' direction therefrom. However, as will be apparent to the skilled artisan a degree of non-complementarity will not significantly adversely affect the ability of a primer to initiate extension. Suitable methods for designing and/or producing a primer suitable for use in the method of the present invention are known in the art and/or described herein. Primers are generally, but not necessarily, short synthetic nucleic acids of about 12-50 nucleotides in length. Preferably, each primer of the set of first primers and/or the allele-specific region of the second primer(s) or each primer of the set of second primer(s) comprises at least about 12-30 nucleotides in length capable of annealing to a strand of the nucleic acid template.

The term "set" with reference to a "set of first primers" or a "set of second primers" or more generally to a "set of primers" shall be taken to mean a number of primers having different, albeit not necessarily entirely different, sequences. A preferred set of primers will comprise primers that are capable of annealing to opposite DNA strands and priming the amplification of an amplification product from one or more template molecules.

By "amplification product" is meant an amplified sequence, which may be nucleic acid comprising a polymorphism or mutation.

In the present context, the term "annealing" or similar term shall be taken to mean that a primer and a nucleic acid to be amplified (i.e., template or amplification product) are base-paired to each other to form a double-stranded or partially double-stranded nucleic acid, using a temperature or other reaction condition known in the art to promote or

permit base-pairing between complementary nucleotide residues. As will be known to the skilled artisan, the ability to form a duplex and/or the stability of a formed duplex depends on one or more factors including the length of a region of complementarity between the primer and nucleic acid to be amplified, the percentage content of adenine and thymine in a region of complementarity (i.e., "A+T content"), the incubation temperature relative to the melting temperature (Tm) of a duplex, and the salt concentration of a buffer or other solution in which the amplification is performed. Generally, to promote annealing, the primers and nucleic acid to be amplified are incubated at a temperature that is at least about 1-5°C below a primer Tm that is predicted from its A+T content and length. Duplex formation can also be enhanced or stabilized by increasing the amount of a salt (e.g., NaCl, $MgCl_2$, KCl, sodium citrate, etc) in the reaction buffer, or by increasing the time period of the incubation, as described by Sambrook et al., Molecular Cloning: A Laboratory Manual , Cold Spring Harbor Laboratory Press ; Hames and Higgins, Nucleic Acid Hybridization: A Practical Approach, IRL Press, Oxford (1985); Berger and Kimmel, Guide to Molecular Cloning Techniques, *In:* Methods in Enzymology, Vol 152, Academic Press, San Diego CA (1987); or Ausubel *et al.,* Current Protocols in Molecular Biology, Wiley Interscience, ISBN 047150338 (1992).

As used herein, the term " anneals selectively" shall be taken to mean that a primer anneals to a target nucleic acid, e.g., a template nucleic acid and/or an amplification product more often than it anneals to another nucleic acid, e.g., to produce a signal that is significantly above background (i.e., a high signal-to-noise ratio). The level of specificity of annealing is determined, for example, by performing an amplification reaction using the primer and detecting the number of different amplification products produced. By "different amplification products" is meant that amplified nucleic acids of differing nucleotide sequence and/or molecular weight to the target nucleic acid. Clearly, amplification products that differ in molecular weight are readily identified, for example, using gel electrophoresis. A primer that selectively anneals to a target nucleic acid produces an amplification product from that target nucleic acid at a level greater than any other amplification product.

The absence of detectable annealing of the allele-specific region of the second primer or set of second primers to the template and/or the first amplification product is determined empirically e.g., by the appearance of a correct amplification product following a first round amplification or alternatively by the absence of detectable

amplification of template using a second primer or set thereof at the first temperature. This selectivity is partially attributed to the fact that the first primer or set thereof has a greater predicted melting temperature (Tm) than the allele-specific region of the second primer or set thereof. Preferably, the first primer or set thereof has a Tm at least about 10°C to about 21°C greater than that of the second primer or set thereof. More preferably, the first primer or set thereof has a Tm at least about 12°C or about 15°C or about 18°C or about 21°C greater than that of the second primer or set thereof. Methods for determining the Tm of a primer are known in the art and/or described herein.

In one example, the Tm of the first primer is between about 60°C and about 75°C, more preferably between about 60°C and about 65°C, even more preferably about 61°C or 62°C or 63°C or 64°C or 65°C. It is also preferred for first primers i.e., locus-specific primers to amplify a region in nucleic acid having a length greater than about 400 bp in length, preferably greater than about 450 bp or about 500 bp in length. Although not essential to the present invention, it is preferred for the locus-specific primer to comprise a 3'-nucleotide complementary to a SNP allele present at the locus of interest.

The second primer comprises an allele-specific region and a tag. In one example, the Tm of the allele specific region of the second primer is between about 35°C and about 50°C, more preferably between about 42°C and about 48°C, even more preferably about 42°C or 43°C or 44°C or 45°C or 46°C or 47°C or 48°C. Allele specific regions of the second primers are preferably between about 12 nucleotides and about 40 nucleotides in length, preferably between about 15 nucleotides in length and about 36 nucleotides in length, including about 18 nucleotides in length or about 21 nucleotides in length or about 24 nucleotides in length or about 27 nucleotides in length or about 30 nucleotides in length or about 33 nucleotides in length or about 36 nucleotides in length. It is also preferred for allele-specific regions of second primers to amplify a region in nucleic acid having a length between about 90 bp and about 300 bp in length, preferably between about 100 bp and about 250 bp in length or between about 100 bp and about 200 bp in length or between about 100 bp and about 150 bp in length.

The second primer should include a tag region at the 5'end thereof that is not complementary to the target DNA, and preferably increases the melting temperature of the primer relative to a second primer comprising an allele specific region and lacking a tag by about 5°C or 6°C or 7°C or 8°C or 9°C or 10°C or 11°C. This means that the

melting temperature of a second primer comprising an allele specific region and a tag is between about 40°C and about 59°C, more preferably between about 47°C and about 59°C, even more preferably about 47°C or 48°C or 49°C or 50°C or 51°C or 52°C or 53°C or 54°C or 55°C or 56°C or 57°C or 58°C or 59°C.

An alternative technique to determine the selective annealing of a primer of the invention comprises performing a search of known nucleotide sequences from the sample being assayed (e.g., a database of known sequences from an organism or cell from which the template nucleic acid is derived). Using this technique a sequence similar to or complementary to the sequence of the primer is identified. Whilst such a technique does not ensure selective annealing it is useful for determining a primer (or set of primers) capable of annealing to a plurality of sites in a nucleic acid and possibly producing multiple amplification products (i.e., non-selective annealing).

In one example of the invention, the first primer is a "locus-specific primer". As used herein, a locus-specific primer is a primer that binds to one or more closely related loci i.e., one or more members of a gene family, one or more homeologous genes or parologous genes, etc. For example, locus-specific primers selectively anneal to a template nucleic acid in a sample comprising a plurality of related nucleotide sequences.

In one example, the second primer is "allele-specific" i.e., able to distinguish between one or more related sequences amplified using locus-specific primer(s), by virtue of comprising an allele-specific region. In one example, an allele specific region anneals to a region of a template nucleic acid and/or a region of a first amplification product comprising a polymorphism or mutation. For example, the allele-specific region comprises a nucleotide complementary to the sequence of an allele of a polymorphism or mutation. Preferably, a nucleotide complementary to the sequence of an allele of a polymorphism or mutation is positioned at the 3' end of the allele specific region, e.g., to facilitate amplification by PCR when said nucleotide anneals to said allele. In an alternative example, the allele-specific region comprises a sequence complementary to a sequence adjacent to a polymorphism or mutation.

By "tag region" is meant a region of a second primer other than an allele-specific sequence that does not anneal to the template nucleic acid or first amplification product. The tag region also comprises a sequence that, in combination with the sequence of the allele-specific region has a Tm similar to the Tm of the first primer.

For example, the Tm of the allele specific region and the tag region combined is within about 4°C or 5°C or 6°C or 7°C of the Tm of the first primer. In one example, the tag region is at least about 4 nucleotides in length, for example about 5 nucleotides in length, preferably at least about 6 nucleotides in length, more preferably at least about 7 nucleotides in length and still more preferably at least about 8 nucleotides in length. It will be apparent tot he skilled artisan from the description herein that amplification from PCR is initiated from the 3' nucleotide of the allele-specific region of the second primer. Proceeding on this basis, the tag region is located 5' to the allele-specific region in the second primer.

In one example, amplification of a first and a second and a third amplification products is performed in a single reaction vessel, and reagents suitable for performing PCR are provided in said reaction vessel, said reagents comprising the first primer or set of first primers and said second primer or set of second primers.

The term "reaction vessel" shall be construed in its broadest context to include any standard vessel suitable for performing a PCR, such as, for example, a reaction tube (such as, for example, an Eppendorf tube, a polypropylene tube, a glass tube or a glass/plastic composite tube), capillary, microtitre well, or a solid substrate such as a glass slide, microarray matrix, or tissue slice.

The term "providing in a reaction vessel" shall be taken to include the supply of one or more reaction vessels with reagents therein, or alternatively, the provision of a reaction vessel with any number of reagents therein, and separately one or more reagents, with instructions for their combination. Preferably, at least the primers are provided in a reaction vessel, or alternatively, provided separately with instructions for their combination.

The skilled artisan will be aware of reagents suitable for performing PCR, such as, for example, primers, template nucleic acid, ribonucleotide triphosphates and/or deoxyribonucleotide triphosphates or analogs thereof, an appropriate reaction buffer, and a polymerase enzyme (e.g., a thermostable polymerase). Other reaction components known to the skilled artisan are not excluded.

Preferably, no additional components are added to the reaction vessel after amplification of the template has commenced and the reaction volume is not modified

by the addition or subtraction of any reagents after this point. This feature of the invention avoids or reduces contamination problems associated with excessive sample handling.

As discussed herein above, one example of the method of the present invention makes use of one or more second primer(s) comprising one or more 3' terminal nucleotide(s) of the allele-specific region complementary to an allele of a polymorphism or mutation, wherein said primer(s) detectably produce the second amplification product and third amplification product only when said 3' nucleotides anneal to the allele of said polymorphism or mutation. Accordingly, in the presence of an allele complementary to the 3' nucleotide of the allele-specific region an amplification product is produced. However, in the presence of an allele that is not complementary to the 3' nucleotide of the allele specific region, an amplification product is not detectably produced.

In one example, the second primer(s) additionally comprise a nucleotide positioned at the second or third nucleotide position from the 3' terminus of the allele-specific region that is non-complementary to the sequence of the template nucleic acid and the first amplification product. Such a mismatch destabilizes annealing of the 3' end of the allele specific region, thereby reducing the likelihood that amplification will be initiated from the 3' end of the allele-specific region when the 3' nucleotide of the allele-specific region is not complementary to the allele of the polymorphism or mutation.

In one example of the invention, the third amplification product is produced by PCR with a first primer and a second primer. Such a result indicates the presence of an allele of a polymorphism or mutation comprising a sequence complementary to the 3' end of the allele-specific region of the second primer. Such a method is useful for detecting the presence of an allele of a polymorphism or mutation using only a single second primer, since amplification with this primer also makes use of a first primer already present in the reaction.

In one example, the method of the present invention is performed with an additional second primer can be included in the reaction, wherein said additional second primer anneals to a sequence adjacent to the polymorphism or mutation. In accordance with this example, the method of the invention is performed with a set of second primers, said set of second primers comprising (i) a second primer comprising one or more 3'

terminal nucleotide(s) of the allele-specific region complementary to an allele of said polymorphism or mutation, wherein said primer only detectably produces the second amplification product and the third amplification product when said 3' nucleotides anneal to the allele of said polymorphism or mutation; and (ii) a second primer that anneals to nucleic acid adjacent to the polymorphism or mutation. In such a situation, detection of an amplification product produced with both second primers is indicative of an allele of a polymorphism or mutation comprising a sequence complementary to the 3' end of the allele-specific region of the second primer.

On the other hand, if the 3' terminal nucleotide(s) of the second primer at (i) do(es) not anneal(s) to the allele, a third amplification product is produced by PCR with the second primer at (ii) and a first primer, thereby indicating an allele of the polymorphism or mutation comprising a sequence non-complementary to the sequence of the 3' nucleotide of the allele specific region of the second primer at (i).

In a further example, a method as described herein according to any embodiment is performed with a plurality of second primers, wherein individual primers in said plurality comprise one or more 3' nucleotide(s) complementary to a different allele of the polymorphism or mutation wherein said primers only detectably produce a second amplification product and third amplification product when said 3' nucleotides anneal to the allele of said polymorphism or mutation, and wherein primers having different 3' complementary nucleotide(s) also comprise a tag region having different molecular weights. In accordance with this example of the present invention, detecting the molecular weight of the third amplification product indicates which second primer has been incorporated into the third amplification product and, as a consequence, the allele of the polymorphism or mutation.

In one example of the method as described herein according to any embodiment, the detection means comprises performing electrophoresis. The skilled artisan will be aware of methods of electrophoresis, such as, for example, polyacrylamide gel electrophoresis or capillary electrophoresis.

In another example of the method as described herein according to any embodiment, the detection means detects the melting temperature of the third amplification product. Examples of such detection means include for example, a LightCycler® (Perkin Elmer). In one example, melting temperature of a nucleic acid is determined by

contacting a nucleic acid with a compound that binds to double stranded nucleic acid and emits light, e.g., fluoresces when excited with light of a particular wavelength. The temperature of the nucleic acid is increased, and the temperature at which the amount of fluorescence detected is reduced as a result of the double stranded nucleic acid denaturing into single stranded nucleic acid is considered the melting temperature of the nucleic acid.

In a further example, a method as described herein according to any embodiment is performed with a plurality of second primers, wherein individual primers in said plurality comprise one or more 3' nucleotide(s) complementary to a different allele of the polymorphism or mutation wherein said primers only detectably produce the second amplification product and the third amplification product when said 3' nucleotides anneal to the allele of said polymorphism or mutation, and wherein primers comprising different 3' nucleotide(s) also comprise a different detectable marker. Preferably, the detectable marker is a fluorescent marker, such as, a fluorescent dye, for example, 6-carboxyfluorescein (FAM), VIC, 2,7',8'-benzo-5'-fluoro-2',4,7-trichloro-5-carboxyfluorescein (NED) or tetrachloro-6-carboxyfluorescein (TET). In accordance with this embodiment, detection of the detectable marker indicates which second primer has been incorporated into the third amplification product and, as a consequence, the allele of the polymorphism or mutation.

The present invention also provides a method in which the second primer(s) or set(s) of second primers comprise an allele-specific region capable to annealing to nucleic acid adjacent to the polymorphism or mutation. In accordance with this embodiment, neither primer anneals to the site of the polymorphism or mutation. Rather, the polymorphism or mutation is contained within the third amplification product. The polymorphism or mutation is then detected by determining the melting temperature of the third amplification product, wherein the melting temperature of the third amplification product is indicative of the polymorphism or mutation. Suitable methods for detecting melting temperature of a nucleic acid are described herein.

In one example, a method as described herein according to any embodiment additionally comprises providing the template nucleic acid. For example, the nucleic acid is in the form of a biological sample.

As discussed herein above, the present invention is useful for detecting a polymorphism in a polyploid organism. Accordingly, in one example of the present invention, the nucleic acid is from a polyploid organism or a sample comprising template nucleic acid is from a polyploid organism. In accordance with this example of the invention, it is preferred that the first set of primers is capable of annealing selectively to a genome of said polyploid organism comprising the polymorphism or mutation.

The present invention also provides a method as described herein according to any embodiment additionally comprising providing or obtaining or producing the first set of primers and/or providing the second primer(s) or set(s) of second primers. For example, the method additionally comprises synthesizing the first set of primers and/or providing the second primer(s) or set(s) of second primers. Methods for designing and producing a primer are described herein.

In one example, the present invention further comprises combining reagents suitable for performing PCR in a reaction vessel. For example, the method of the present invention comprises combining a first set of primers and one or more second primer(s) or set of second primer(s) in a reaction vessel. Additional suitable reagents will be apparent to the skilled artisan based on the description herein and include, for example, ribonucleotide triphosphates and/or deoxyribonucleotide triphosphates or analogs thereof, an appropriate reaction buffer, and a polymerase enzyme (e.g., a thermostable polymerase).

The present invention is not to be limited to the detection of a single polymorphism or mutation in a single reaction. Rather, the present invention also provides a method for detecting a plurality of polymorphisms or a plurality of mutations or one or more polymorphisms and one or more mutations in a single reaction, i.e., a multiplex reaction. In this respect, one or more of the polymorphisms and/or mutations can be amplified in the first amplification product. Alternatively, each polymorphism and/or mutation can be amplified in a separate first amplification product. The skilled artisan will be aware that for such a multiplex method each of the amplification products detected should have a different molecular weight and/or be labeled with a different detectable marker to thereby permit detection of each amplification product. Methods for predicting amplification products having sufficiently different molecular weight to permit detection in a single reaction will be apparent to the skilled artisan and are

described, for example, in International Patent Application No. PCT/AU2006/000318 (International Publication No. WO 2006/094360).

The skilled artisan will be aware that a method for detecting one or more polymorphisms and/or mutations is useful for, for example, determining relationships between one or more individuals, isolates of an organism, cultivars of an organism, species or genera. For example, the method of the present invention is used to detect one or more nucleic acids that are polymorphic between two or more individuals, isolates of an organism, cultivars of an organism, species or genera. Accordingly, the present invention additionally provides for characterizing or identifying one or more individuals, isolates of an organism, cultivars of an organism, species or genera said process comprising performing the method as described herein according to any embodiment to detect one or more polymorphisms and/or mutations in nucleic acid from one or more individuals, isolates of an organism, cultivars of an organism, species or genera, wherein the one or more polymorphisms or mutations is(are) characteristic of one or more individuals, isolates of an organism, cultivars of an organism, species or genera.

It will be apparent to the skilled artisan from the description herein that the present invention is useful for typing an organism within or between groups, or for differentiating between individuals or groups (e.g., for identification of a specific plant variety). The skilled artisan will appreciate that the method of the present invention is also applicable to, for example, the analysis of a sample (e.g., a food sample) to identify the presence of a foreign agent (e.g., a genetically modified plant).

The present invention additionally provides a process for detecting one or more polymorphisms and/or mutations associated with a trait, e.g., to select a subject having a trait or having a predisposition to a trait and/or for the purpose of marker-assisted breeding. For example, the present invention provides a process for screening an animal species to identify an animal having or having a predisposition to a trait of interest, e.g., for the purpose of animal husbandry, e.g., for the selection of a desired trait (e.g., marbled beef from cattle, or enhanced milk quality from cattle, enhanced speed or stamina in horses or enhanced meat quality from pigs). The present invention also provides a process for screening a plant species to identify a plant having a trait or having a predisposition to a trait, such as increased productivity, e.g., resistance to drought, resistance to a disease or a pest, resistance to pre-harvest sprouting, resistance

to frost and an increased nutritional quality. In accordance with this embodiment, the present invention provides a process for identifying a subject having a trait or having a predisposition to a trait, said method comprising performing a method as described herein according to any embodiment to detect one or more polymorphism(s) and/or mutation(s) associated with a trait or a predisposition to a trait, wherein detection of said polymorphisms and/or mutations is indicative of a subject having the trait or having a predisposition to developing the trait.

As used herein the term "subject" shall be understood to include a bacterium, virus, fungus, protist, plant, non-human animal or human, including any developmental stage of said bacterium, virus, fungus (e.g., endophytic fungi), protist, plant, non-human animal or human. Specific strains of HSV, and/or specific strains or species or races of brewer's yeast e.g., *Saccharomyces sp.*, and/or specific strains or species or races of *Escherichia coli, Staphylococcus aureus e.g., multi-resistant S. aureus (MRSA), or Mycobacterium sp.* are particularly contemplated herein.

As used herein, the term "associated with" shall be taken to mean that the presence of a specific genetic marker is significantly correlated with a trait of interest in an organism or a population of organisms. Preferably, the presence of the genetic marker is significantly correlated with the presence of the trait of interest in a population of unrelated organisms.

In one example, the method additionally comprises selecting a subject having the trait or a predisposition to a trait, based on the detection of one or more polymorphism(s) and/or mutation(s) associated with a trait or a predisposition to developing a trait. For example, the subject is selected from a population of subjects.

In one example, a method for identifying or selecting a subject as described herein according to any embodiment additionally comprises obtaining or providing a cell or a gamete or other reproductive material or an embryo or a fetus from the selected or identified subject.

In one example, a method of identifying or selecting a subject of the present invention additionally comprises breeding a subject identified or selected by a method described herein in any embodiment. Optionally, such a method of breeding additionally comprises performing a method described herein to identify and/or select an embryo or

a fetus or a plantlet or an offspring plant or an offspring non-human animal or an offspring human comprising one or more polymorphism(s) and/or mutation(s) associated with the trait or a predisposition to the trait.

The skilled artisan will also appreciate that the present invention is also useful for identifying a subject having a disease or disorder or a subject at risk of developing a disease or disorder. In this respect, the present invention also provides a process for identifying a subject having a disease or disorder or at risk of developing a disease or disorder, said process comprising performing a method as described herein according to any embodiment to detect one or more polymorphism(s) and/or mutation(s) that are associated with a disease or disorder, wherein detection of said polymorphism(s) and/or mutation(s) indicates that the subject suffers from the disease or disorder or has a predisposition to the disease or disorder.

The skilled artisan will be aware that this example of the invention relates to the diagnosis of a disease or disorder caused by a mutation, e.g., cystic fibrosis or sickle cell anemia or Tay Sachs disease or folic acid deficiency, and/or to determining a subject at risk of developing a disease or disorder that is associated with a mutation or polymorphism, e.g., Parkinson's disease or Alzheimer's disease or neural tube defect or arterial thrombosis or venous thrombosis or cardiovascular disease or schizophrenia or having increased or decreased risk of developing cancer e.g., leukemia and/or colon cancer.

Furthermore, the present invention is applicable to diagnosis of infection by virtue of detecting and/or identifying an infectious agent that causes an infection and/or for discriminating between strains, ecotypes, serotypes or species of an infectious agent. Clearly, this encompasses the diagnosis and/or prognosis of disease caused by the infectious agent. Accordingly, in another example, the present invention provides a process for identifying an infectious agent in a sample and/or for discriminating between infectious agents in a sample, said process comprising performing a method as described herein according to any embodiment on a sample obtained from a subject to thereby detect one or more nucleic acid sequences of one or more infectious agents, wherein detection of said one or more nucleic acid sequences in the sample indicates the presence of an infectious agent in the sample and/or discriminates between infectious agents in the sample.

The present invention also provides a kit comprising:

(i) one or more set(s) of first primers, said set(s) of first primers capable of annealing selectively to a nucleic acid template comprising a polymorphism or mutation at a first temperature;

(ii) one or more second primer(s) or set(s) of second primers, said second primer(s) comprising an allele-specific region capable of hybridizing to the nucleic acid template and a tag-region that does not anneal to the nucleic acid template, wherein said allele-specific region has a melting temperature (Tm) lower than the first primer and is not capable of annealing selectively to the nucleic acid template at the first temperature and wherein the second primer is capable of annealing selectively to a nucleic acid comprising a sequence complementary to the allele-specific region and the tag region at about the first temperature; and

(iii) optionally, instructions for performing the method as described herein according to any embodiment.

Preferably, the set(s) of first primers and the second primer(s) or set(s) of second primers are provided in a reaction vessel suitable for performing polymerase chain reaction (PCR).

The present invention also provides for the use of a kit as described herein in any embodiment in any method of the present invention.

The present invention also provides a method of producing a set of primers, said method comprising:

(i) producing one or more set(s) of first primers, said set(s) of first primers capable of annealing selectively to a nucleic acid template comprising a polymorphism or mutation at a first temperature; and

(ii) producing one or more second primer(s) or set(s) of second primers, said second primer(s) comprising an allele-specific region capable to hybridizing to the nucleic acid template and a tag-region that does not anneal to the nucleic acid template, wherein said allele-specific region has a melting temperature (Tm) lower than the first primer and is not capable of annealing selectively to the nucleic acid template at the first temperature and wherein the second primer is capable of annealing selectively to a nucleic acid comprising a sequence complementary to the allele-specific region and the tag region at about the first temperature.

In one example, the method further comprises analyzing nucleotide sequence data to thereby determine a panel of candidate primers for inclusion in said set.

In another example, the method of the present invention as described according to any embodiment hereof is performed using the panel of primers to thereby determine a panel of first primer(s) and/or second primer(s) that provide discrimination, more preferably optimum discrimination, between alleles in the nucleotide sequence analyzed.

In another example, the method further comprises selecting a panel of first primer(s) and/or second primer(s) that provide discrimination, more preferably optimum discrimination, between alleles in the nucleotide sequence analyzed.

In another example, the method further comprises providing the set of primers and/or the analyzed primers and/or selected primers.

In another example, the method further comprises providing information pertaining to the sequences of the set of primers and/or the analyzed primers and/or selected primers e.g., in a computer-readable form or by way of an electronic medium or paper medium.

In one example, the first primer comprises a sequence having a Tm between about 60°C and about 75°C, more preferably between about 60°C and about 65°C, even more preferably about 61°C or 62°C or 63°C or 64°C or 65°C. It is preferred for first primers i.e., locus-specific primers to be capable of annealing to a nucleic acid template at a distance that is separated by about 400 bp, preferably about 450 bp or about 500 bp. Alternatively, or in addition, locus-specific primer(s) comprise a 3'-nucleotide complementary to a SNP allele present in a nucleic acid template.

In one example, the Tm of the allele specific region of the second primer is between about 35°C and about 50°C, more preferably between about 42°C and about 48°C, even more preferably about 42°C or 43°C or 44°C or 45°C or 46°C or 47°C or 48°C.

Allele specific regions of the second primers are preferably between about 12 nucleotides and about 40 nucleotides in length, preferably between about 15 nucleotides in length and about 36 nucleotides in length, including about 18 nucleotides in length or about 21 nucleotides in length or about 24 nucleotides in length or about 27

nucleotides in length or about 30 nucleotides in length or about 33 nucleotides in length or about 36 nucleotides in length. It is also preferred for allele-specific regions of second primers to amplify a region in nucleic acid having a length between about 90 bp and about 300 bp in length, preferably between about 100 bp and about 250 bp in length or between about 100 bp and about 200 bp in length or between about 100 bp and about 150 bp in length.

The tag region is not complementary to a target DNA, and preferably comprises a sequence having a melting temperature of about 5°C or 6°C or 7°C or 8°C or 9°C or 10°C or 11°C.

The tag region is between about 2 nucleotides in length and about 9 nucleotides in length and has a melting temperature of about 5°C or 6°C or 7°C or 8°C or 9°C or 10°C or 11°C. Preferred forms of the tag region have a length between 2 nucleotides in length and about 8 nucleotides in length or between 2 nucleotides in length and about 7 nucleotides in length or between 2 nucleotides in length and about 6 nucleotides in length or between 2 nucleotides in length and about 5 nucleotides in length or between 2 nucleotides in length and about 4 nucleotides in length or 2 or 3 nucleotides in length.

The present invention also provides a computer-readable medium comprising information pertaining to the sequences of a panel of first primer(s) and/or second primer(s) that provide discrimination between alleles in nucleic acid comprising a sequence homologous to the nucleic acid template, wherein said information is obtained by a method of the invention described with reference to any embodiment or example hereof.

General information and definitions

This specification contains nucleotide and amino acid sequence information prepared using PatentIn Version 3.4, presented herein after the claims. Each nucleotide sequence is identified in the sequence listing by the numeric indicator <210> followed by the sequence identifier (e.g. <210>1, <210>2, <210>3, etc). The length and type of sequence (DNA, protein (PRT), etc), and source organism for each nucleotide sequence are indicated by information provided in the numeric indicator fields <211>, <212> and <213>, respectively. Nucleotide sequences referred to in the specification are defined by the term "SEQ ID NO:", followed by the sequence identifier (e.g. SEQ ID NO: 1 refers to the sequence in the sequence listing designated as <400>1).

The designation of nucleotide residues referred to herein are those recommended by the IUPAC-IUB Biochemical Nomenclature Commission, wherein A represents Adenine, C represents Cytosine, G represents Guanine, T represents thymine, Y represents a pyrimidine residue, R represents a purine residue, M represents Adenine or Cytosine, K represents Guanine or Thymine, S represents Guanine or Cytosine, W represents Adenine or Thymine, H represents a nucleotide other than Guanine, B represents a nucleotide other than Adenine, V represents a nucleotide other than Thymine, D represents a nucleotide other than Cytosine and N represents any nucleotide residue.

Throughout this specification, unless specifically stated otherwise or the context requires otherwise, reference to a single step, composition of matter, group of steps or group of compositions of matter shall be taken to encompass one and a plurality (i.e. one or more) of those steps, compositions of matter, groups of steps or group of compositions of matter.

Each embodiment described herein is to be applied *mutatis mutandis* to each and every other embodiment unless specifically stated otherwise.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and/or all combinations or any two or more of said steps or features.

The present invention is not to be limited in scope by the specific embodiments described herein, which are intended for the purpose of exemplification only. Functionally-equivalent products, compositions and methods are clearly within the scope of the invention, as described herein.

Brief description of the drawings

Figure 1 is a diagrammatic representation of a temperature switch PCR method of the present invention for allelic discrimination by differential amplification product size using a pair of allele specific (AS) primers designed to anneal to different DNA strands. Locus specific (LS) primers are labeled L_1 and L_2, and AS primers are labeled A1 and

A2. AS primer A_1 is designed to anneal to the B allele of the polymorphism N. Expected PCR products for each genotype are also shown at the bottom of the figure.

Figure 2 is a diagrammatic representation of a temperature switch PCR method of the present invention for allelic discrimination by differential product size using a pair of AS primers that anneal to the same DNA strand. LS primers are labeled L_1 and L_2, and AS primers are labeled A_1 and A_2. AS primer A_1 anneals to the A allele of polymorphism N, and AS primer A_2 anneals to the B allele of polymorphism B. AS primer A_1 has a longer tag region than AS primer A_2 thereby producing a longer PCR product than produced. Expected PCR products for each genotype are also shown at the bottom of the Figure.

Figure 3a is a diagrammatic representation showing a temperature switch PCR method of the present invention for allelic discrimination by different product size using a single AS primer. LS primers are labeled L_1 and L_2, and the AS primer is labeled A_1. AS primer A_1 anneals to the B allele of polymorphism N. Expected PCR products for each genotype are also shown at the bottom of the Figure.

Figure 3b is a diagrammatic representation showing a temperature switch PCR of the present invention for allelic discrimination using a single AS primer. LS primers are labeled LS_1 and LS_2 and AS primers are labeled AS_1 and AS_2. AS_1 anneals to allele A of polymorphism N and AS_2 anneals to allele B of polymorphism N. Two separate PCRs are performed, one with AS_1 to detect allele A and the other with AS_2 to detect allele B. Expected PCR products are also shown at the bottom of the figure.

Figure 4 is a diagrammatic representation showing a temperature switch PCR of the present invention for allelic discrimination by differential product labeling using a pair of AS primers designed to the same DNA strand. LS primers are labeled L_1 and L_2, and AS primers are labeled A_1 and A_2. AS primer A_1 anneals to the A allele of polymorphism N, and AS primer A_2 anneals to the B allele of polymorphism B. AS primer A_1 comprises a detectable marker X that is different to the detectable marker Y linked to AS primer A_2. Expected PCR products and label(s) linked thereto for each genotype are also shown at the bottom of the Figure.

Figure 5 is a graphical representation showing a temperature switch PCR of the present invention for allelic discrimination by differential product detection using a pair of AS

primers designed to opposite DNA strands. LS primers are labeled L_1 and L_2, and AS primers are labeled A_1 and A_2. Allele specific primers amplify an amplification product and the allele at polymorphism N is detected using, for example, melting curve analysis. Expected PCR products are also shown at the bottom of the figure.

Figure 6 is a diagrammatic representation showing a temperature switch PCR of the present invention for allelic discrimination by differential product detection using a single AS primer. LS primers are labeled L_1 and L_2, and the AS primer is labeled A_1. Amplification product from L_1 and A_1 are analyzed using, for example, melting curve analysis to detect the allele at polymorphism N. Expected PCR products are also shown at the bottom of the figure.

Figure 7 is a copy of photographic representations showing PCR products amplified from eight barley lines using AS primers with the complementary region having a melting temperature of 40°C, 45°C and 50°C, respectively (as indicated). The AS primers were designed for validated SNPs having allele A and allele B in a (**a**) putative gene located on chromosome 5H, and (**b**) nicotinate phosphoribosyltransferase-like gene. Solid arrows indicate the size of the expected PCR product.

Figure 8 is a copy of a photographic representation showing amplicons produced using primer combinations set forth in Table 1 in reactions performed using temperature switch PCR of the present invention (TSP cycling) and standard PCR cycling conditions (standard cycling). PCR products are shown for reactions using primers designed for a putative gene located on chromosome 5H in barley. The barley lines tested (wells A-D) had the genotypes AA, BB, AB and AB, respectively. Numbers correspond to numbers in Table 1.

Figure 9a is a graphical representation showing a temperature switch PCR of the present invention allelic discrimination by differential product size using a pair of AS primers designed to opposite DNA strands. The assays were performed in barley (*Hordeum vulgare*) using samples with the following zygosity in lanes 1-8 BB, BB, AA, AB, BB, AB, BB and BB. AS primers have a complementary region melting temperature of 40°C. Two reactions were performed for each sample, one with AS forward primers specific for allele A, and the other with AS forward primer specific for allele B. Primers were designed to assay SNPs in a putative gene located on chromosome 5H.

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Figure 9b is a copy of a photographic representation showing a temperature switch PCR of the present invention allelic discrimination by differential product size using a pair of AS primers designed to opposite DNA strands. The assays were performed in barley (*Hordeum vulgare*) using samples with the following zygosity in lanes 1-8 BB, AA, AA, AB, BB, AB, BB, AB. AS primers have a complementary region melting temperature of 40°C. Two reactions were performed for each sample, one with AS forward primers specific for allele A, and the other with AS forward primer specific for allele B. Primers were designed to assay SNPs in a nicotinate phosphoribosyltransferase-like gene.

Figure 9b is a copy of a photographic representation showing a temperature switch PCR of the present invention allelic discrimination by differential product size using a pair of AS primers designed to opposite DNA strands. The assays were performed in barley (*Hordeum vulgare*) using samples with the following zygosity in lanes 1-8 AA, BB, BB, AB, AA, AB, AA, AB. AS primers have a complementary region melting temperature of 40°C. Two reactions were performed for each sample, one with AS forward primers specific for allele A, and the other with AS forward primer specific for allele B. Primers were designed to assay SNPs in a nicotinate phosphoribosyltransferase-like gene.

Figure 10 is of a copy of a photographic representation showing results of a temperature switch PCR of the present invention configured for allelic discrimination by differential product size using a pair of AS primers designed to opposite DNA strands. The assay was performed using genomic DNA from bread wheat (*Triticum aestivum*) using samples with known zygosity. The AS forward and reverse primers A_1 and A_2 had complementary region melting temperatures of 50°C and 40°C, respectively. Two reactions were performed for each sample, one using the AS forward primer specific for allele A, and the other using AS forward primer specific for allele B. Primers were designed to assay a SNP located in a putative nodulin gene on the chromosome 3B.

Figure 11 is a copy of a photographic representation showing results of a temperature switch PCR of the present invention configured for allelic discrimination by differential product detection using a pair of AS primers designed to opposite DNA strands. The assay was performed in barley (*Hordeum vulgare*) using samples with known zygosity.

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The AS primers have a complementary region melting temperature of 40°C. Primers were designed to assay a SNP in a nicotinate phosphoribosyltransferase-like gene.

Figure 12 provides graphical representations showing biphasic accumulation of reaction product in TSP assays performed using real-time PCR during the final 45 cycles of amplification. TSP assays were performed in barley (*Hordeum vulgare*) using samples with known zygosity. Allele specific (AS) primers have a complementary region melting temperature of 45°C, a 3'-nucleotide complementary to the SNP allele present at the locus, and a non-complementary 5'-tag designed to increase the melting temperature of the AS primer to 53°C once the non-complementary sequence is incorporated into PCR product. Primers were designed to assay SNPs in genes encoding (a) putative Rieske Fe-S precursor protein, (b) fructose-6-phosphate 2-kinase, (c), unnamed protein product from rice, and (d) cytosolic aldehyde dehydrogenase. Numbers for each curve in each panel represent different reactions obtained using the following primer combinations: 1 is primer Combination L1 and L2; 2 is primer combination L1 L2 A1 and A2; 3 is primer combination A1 and A2; and 4 is no primer, wherein L1 is an LS forward primer; L2 is LS reverse primer; A1 is AS forward primer specific for allele A and A2 is AS reverse primer. Data demonstrate efficient transition from the amplification of LS product to the accumulation of AS product in the second phase of the reaction and efficient annealing of AS primers to the enriched target sequence (LS product) at the second phase annealing temperature, allowing for highly efficient self-amplification of AS product in subsequent cycles due to incorporation of the non-complementary 5'-tail, and therefore, out-competing of the accumulation of LS product.

Figure 13 is a copy of a photographic representation showing results of TSP amplificatin of methylenetetrahydrofolate reductase alleles comprising 677C or 677T resolved using 2% (w/v) agarose gel electrophoresis and stained using ehtidium bromide. Lanes from left to right are as follows: Lane 1, a 1.1 kb plus ladder (Invitrogen; 100 bp increment bands); lanes 2-13 show alleles in the MTHFR gene, wherein lanes 2, 7, 9, 11 and 12 show two copies of the 677C allele, lanes 4, 6, 8 show two copies of the 677T allele, and lanes 3, 5, 10 and 13 show one copy of the 677C allele and one copy of the 677T allele in the sample DNA.

Detailed description of the preferred embodiments

Primer design

In one example of the present invention, a first primer or an allele-specific region of a second primer is designed such that it comprises a sequence having at least about 80% identity overall to a strand of a template nucleic acid. More preferably, the degree of sequence identity is at least about 85% or 90% or 95% or 98% or 99%. For example, the primer or a region of a primer may comprise a sequence having at least about 80% identity to a strand of a locus of interest.

Clearly, the specific composition of a primer of the invention (or more specifically, a first primer or an allele-specific region of a second primer) will depend upon the sequence of the template nucleic acid of interest. Accordingly, the sequence of a first primer or an allele-specific region of a second primer is not to be taken to be limited to a particular sequence. Rather the sequence need only be sufficient to allow for annealing of the first primer or allele-specific region of a second primer to a template nucleic acid and initiation of an amplification reaction.

For a first primer of the invention, as a primer is generally extended in the 5'- to 3'-direction it is preferred that at least the 3'-terminal nucleotide is complementary to the relevant nucleotide in the template nucleic acid. More preferably, at least the 3 or 4 or 6 or 8 or 10 contiguous nucleotides at the 3'- terminus of the primer are complementary to the relevant nucleotides in the template nucleic acid. The complementarity of the 3' terminus of the primer ensures that the extending end of the primer is capable of initiating amplification of the template nucleic acid, for example, by a polymerase.

As for an allele-specific primer, in some methods described herein a 3' nucleotide of said region is complementary to an allele of a polymorphism or a mutation. Accordingly, the 3' nucleotide will also be non-complementary to another allele of the polymorphism or mutation. Such a primer is useful for only amplifying nucleic acid to a detectable level in the presence of the allele complementary to the 3' nucleotide of the allele-specific region.

In some embodiments of the present invention, an allele specific region additionally comprises a nucleotide that is non-complementary to a template nucleic acid or first amplification product, said non-complementary nucleotide being positioned at nucleotide position -2 or -3 from the 3' terminus of the allele specific region. Suitable

non-complementary nucleotides will be apparent to the skilled artisan and/or are described, for example, in Little *et al., In:* Taylor (ed) *Laboratory Methods for the Detection of Mutations and Polymorphisms in DNA*, CRC Press, Boca Raton, Florida, USA, pp. 45-51. For example, in the case of a strong 'mismatch' (non-complementary nucleotide) (G/A or C/T mismatch) at the 3' terminus of an allele specific region the additional non-complementary nucleotide can be a 'weak' mismatch (C/A or G/T), and *vice versa*. In the presence of a 'medium' mismatch (A/A, C/C/ G/G or T/T) at the 3' terminal nucleotide of the allele-specific region, the additional non-complementary nucleotide can also be a 'medium' mismatch.

As regions of non-complementarity reduce the predicted Tm of a primer and may be associated with amplification of non-template nucleic acid it is preferred that a primer of the invention does not comprise multiple contiguous nucleotides that are not identical to a strand of the template nucleic acid. Preferably, the primer comprises no more than 6 or 5 or 4 or 3 or 2 contiguous nucleotides that are not identical to a strand of the template nucleic acid. More preferably, any nucleotides that are not identical to a strand of the template nucleic acid are non-contiguous.

To determine whether or not two nucleotide sequences fall within a particular percentage identity limitation recited herein, those skilled in the art will be aware that it is necessary to conduct a side-by-side comparison or multiple alignment of sequences. In such comparisons or alignments, differences may arise in the positioning of non-identical residues, depending upon the algorithm used to perform the alignment. In the present context, reference to a percentage identity between two or more nucleotide sequences shall be taken to refer to the number of identical residues between said sequences as determined using any standard algorithm known to those skilled in the art. For example, nucleotide sequences may be aligned and their identity calculated using the BESTFIT program or other appropriate program of the Computer Genetics Group, Inc., University Research Park, Madison, Wisconsin, United States of America (Devereaux et al, Nucl. Acids Res. 12, 387-395, 1984).

Alternatively, a suite of commonly used and freely available sequence comparison algorithms is provided by the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) (Altschul *et al. J. Mol. Biol. 215*: 403-410, 1990), which is available from several sources, including the NCBI, Bethesda, Md.. The BLAST software suite includes various sequence analysis programs including

"blastn," that is used to align a known nucleotide sequence with other polynucleotide sequences from a variety of databases. Also available is a tool called "BLAST 2 Sequences" that is used for direct pairwise comparison of two nucleotide sequences.

As used herein the term "NCBI" shall be taken to mean the database of the National Center for Biotechnology Information at the National Library of Medicine at the National Institutes of Health of the Government of the United States of America, Bethesda, MD, 20894.

Generally, a primer comprises or consists of at least about 10 nucleotides, more preferably at least about 12 nucleotides or at least about 15 or 20 nucleotides that anneal to a nucleic acid template or are complementary to the nucleic acid template. However, longer primers are also used in PCR reactions, for example, reactions in which a long region of nucleic acid (e.g., greater than 1000bp) is amplified. Accordingly, the present invention additionally contemplates a primer comprising at least about 25 or 30 or 35 nucleotides that anneal to a nucleic acid template or are complementary to the nucleic acid template.

Alternatively, a primer comprising one or modified bases, such as, for example, locked nucleic acid (LNA) or peptide nucleic acid (PNA) need only comprise a region of at least about 8 nucleotides that anneal to a nucleic acid template or are complementary to the nucleic acid template. Preferably, the complementary nucleotides are contiguous.

As will be apparent to the skilled artisan, the number of nucleotides capable of annealing to a nucleic acid template is related to the stringency under which the primer will anneal. Preferably, a primer of the invention anneals to a nucleic acid template under moderate to high stringency conditions.

In one embodiment, the stringency under which a primer of the invention anneals to a template nucleic acid is determined empirically. Generally, such a method requires performance of an amplification reaction using one or more primers under various conditions and determining the level of specific amplification produced.

Alternatively, a primer of the invention is labeled with a detectable marker (e.g., a radionucleotide or a fluorescent marker) and the level of primer that has annealed to a target nucleic acid under suitably stringent conditions is determined.

For the purposes of defining the level of stringency, a moderate stringency annealing conditions will generally be achieved using a condition selected from the group consisting of:

(i) an incubation temperature between about 42°C and about 55°C;

(ii) an incubation temperature between about 15°C and 10°C less than the predicted Tm for a primer; and

(iii) a Mg^{2+} concentration of between about 2mM and 3mM.

High stringency annealing conditions will generally be achieved using a condition selected from the group consisting of:

(i) an incubation temperature above about 55°C and preferably above about 65°C;

(ii) an incubation temperature between about 10°C and 1°C less than the predicted Tm for a primer; and

(iii) a Mg^{2+} concentration of between about 1mM and 1.9mM.

Alternative or additional conditions for enhancing stringency of annealing will be apparent to the skilled artisan. For example, a reagent such as, for example, glycerol (5-10%), DMSO (2-10%), formamide (1 - 5%), Betaine (0.5 - 2M) or tetramethylammonium chloride (TMAC, >50mM) are known to alter the annealing temperature of a primer and a nucleic acid template(Sarkar *et al., Nucl. Acids Res. 18*: 7465; 1990, Baskaran *et al. Genome Res. 6*: 633-638, 1996; and Frackman *et al., Promega Notes 65*: 27, 1998).

Conditions for altering the stringency of a PCR reaction are understood by those skilled in the art. For the purposes of further clarification only, reference to the parameters affecting annealing between nucleic acid molecules is found in Ausubel *et al.* (Current Protocols in Molecular Biology, Wiley Interscience, ISBN 047150338, 1992), which is herein incorporated by reference.

Alternatively, the conditions under which a primer anneals to a nucleic acid template are determined *in silico*. For example, methods for determining the predicted melting temperature (or Tm) of a primer (or the temperature at which a primer denatures from a specific nucleic acid) are known in the art.

For example, the method of Wallace *et al.,* (*Nucleic Acids Res. 6*, 3543, 1979) estimates the Tm of a primer based on the G, C, T and A content. In particular, the described method uses the formula 2(A + G) + 4(G + C) to estimate the Tm of a probe or primer.

Alternatively, the nearest neighbor method described by Breslauer *et al., Proc. Natl. Acad. Sci. USA, 83*:3746-3750, 1986 is useful for determining the Tm of a primer. The nearest neighbour method uses the formula:

$$T_m \text{ (calc)} = \Sigma\Delta H^0/(\mathbf{R}\ln(\mathbf{C_t}/\mathbf{n}) + \Sigma\Delta S^0) \quad ,$$

wherein ΔH^0 is standard enthalpy for helix formation, ΔS^0 is standard entropy for helix formation, C_t is the total strand concentration, n reflects the symmetry factor, which is 1 in the case of self-complementary strands and 4 in the case of non-self-complementary strands and R is the gas constant (1.987).

Ryuchlik *et al., Nucl. Acids Res. 18*: 6409-6412, 1990 described an alternative formula for determining Tm of an oligonucleotide:

$$Tm^{primer} = \frac{dH}{dS + R\ln(c/4)} + 16.6\lg\frac{[K^+]}{1 + 0.7[K^+]} - 273.15 \quad ,$$

wherein, dH is enthalpy for helix formation, dS is entropy for helix formation, R is molar gas constant (1.987cal/°C mol), "c" is the nucleic acid molar concentration (determined empirically, W.Rychlik *et.al., supra*), (default value is 0.2 μM for unified thermodynamic parameters), $[K^+]$ is salt molar concentration (default value is 50 mM).

Suitable software for determining the Tm of an oligonucleotide using the nearest neighbor method is known in the art and available from, for example, US Department of Commerce, Northwest Fisheries Service Center and Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine.

Alternatively, for longer primers (i.e., a primer comprising at least about 200 nucleotides), the method of Meinkoth and Wahl (In: *Anal Biochem, 138*: 267-284, 1984), is useful for determining the Tm of the primer. This method uses the formula:

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81.5 + 16.6($\log_{10}$M) + 0.41(% GC) - 0.61(% form) - 500 / Length in bp,

wherein M is the molarity of Na+ and % form is the percentage of formamide (set to 50%)

For a primer that comprises or consists of PNA the Tm is determined using the formula (described by Giesen *et al., Nucl. Acids Res., 26*: 5004-5006):

$$T_{mpred} = c_0 + c_1 * T_{mnnDNA} + c_2 * f_{pyr} + c_3 * \text{length},$$

wherein, in which T_{mnnDNA} is the melting temperature as calculated using a nearest neighbor model for the corresponding DNA/DNA duplex applying ΔH^0 and ΔS^0 values as described by SantaLucia *et al. Biochemistry, 35*: 3555-3562, 1995. f_{pyr} denotes the fractional pyrimidine content, and length is the PNA sequence length in bases. The constants are $c_0 = 20.79$, $c_1 = 0.83$, $c_2 = -26.13$ and $c_3 = 0.44$

To determine the Tm of a primer comprising one or more LNA residues a modified form of the formula of SantaLucia *et al. Biochemistry, 35*: 3555-3562, 1995 is used:

$$Tm = \frac{\Delta H}{\Delta S + ln([Na]^{0.368} * (C/4)^{1.987})},$$

A suitable program for determining the Tm of a primer comprising LNA is available from, for example, Exiqon, Vedbaek, Germany.

A temperature that is similar to (e.g., within 5°C or within 10°C) or equal to the proposed/estimated temperature at which a primer denatures from a template nucleic acid is considered to be high stringency. Medium stringency is to be considered to be within 10°C to 20°C or 10°C to 15°C of the calculated Tm of the probe or primer.

A primers or primer sequence that is predicted to be or shown to be capable of selectively annealing to a nucleic acid template is also optionally analyzed for one or more additional characteristics that make it suitable for use as a primer in the method of the invention. For example, a primer is analyzed to ensure that it is unlikely to form

secondary structures (i.e., the primer does not comprise regions of self-complementarity).

Furthermore, should the primer be proposed to be used in a reaction with one or more other primers (e.g., a PCR reaction and/or a multiplex reaction) all primers may be assessed to determine their ability to anneal to one another and form "primer dimers". Methods for determining a primer that is capable of self-dimerization and/or primer dimer formation are known in the art and/or described *supra*.

Methods for designing and/or selecting a primer suitable for use in an amplification reaction are known in the art and described, for example, in Innis and Gelfand (1990) (In: Optimization of PCRs. pp. 3-12 in: PCR Protocols (Innis, Gelfand, Sninsky and White, eds.); Academic Press, New York) and Dieffenbach and Dveksler (Eds) (In: PCR Primer: A Laboratory Manual, Cold Spring Harbor Laboratories, NY, 1995). Such methods are particularly suited, for example, for designing a locus specific sequence of a primer of the invention.

Generally, it is recommended that a primer satisfies the following criteria:

(i). the primer comprises a region that is to anneal to a target sequence having at least about 17-28 bases in length;

(ii). the primer comprises about 50-60% (G+C);

(iii) the 3'-terminus of the primer is a G or C, or CG or GC (this prevents "breathing" of ends and increases efficiency of initiation of amplification);

(iv) preferably, the primer has a Tm between about 55 and about 80°C;

(v) the primer does not comprise three or more contiguous Cs and/or Gs at the 3'-ends of primers (as this may promote mispriming at G or C-rich sequences due to the stability of annealing);

(vi) the 3'-end of a primer should not be complementary with another primer in a reaction; and

(vii) the primer does not comprise a region of self-complementarity.

Several software programs are available that enable the design of one or more primers, or a region of a primer (e.g., a locus specific sequence of a first primer of the invention). For example, a program selected from the group consisting of:

(i) Primer3, available from the Center for Genome Research, Cambridge, MA, USA, designs one or more primers for use in an amplification reaction based upon a known template sequence;

(ii) Primer Premier 5, available from Biosoft International, Palo Alto, CA, USA, designs and/or analyzes primers;

(iii) CODEHOP, available from Fred Hutchinson Cancer Research Centre, Seattle, Washington, USA, designs primers based on multiple protein alignments; and

(iv) FastPCR, available from Institute of Biotechnology, University of Helsinki, Finland, designs multiple primers, including primers for use in a multiplex reaction, based on one or more known sequences.

When designing a primer of the invention, the composition of the template nucleic acid is considered (i.e. the nucleotide sequence) as is the type of amplification reaction to be used. For example, should allele specific PCR be used, the 3' nucleotide of one of the primers used in such a reaction corresponds to the site of an allele of interest, such as, for example a SNP. In this manner only in the presence of a nucleotide that is complementary to that in the primer does annealing occur and amplification achieved.

Furthermore, should the primer be used in a multiplex reaction it is preferred that the amplification product produced is not similar in molecular weight to that produced using another primer or set thereof thereby rendering detection difficult. Accordingly, it is preferred that there is sufficient difference in molecular weight in amplified products to enable detection using a technique known in the art, such as, for example, gel electrophoresis or mass spectrometry.

While it is preferable to produce amplification products of distinct molecular weights, by using differential labeling with different detectable markers, products of similar length are resolved. Accordingly, it is not essential that each of the nucleic acids amplified using the method of the invention is different molecular weight.

Tag regions

The tag region in a second primer of the invention serves the dual purpose of enhancing the specificity annealing of the second primer and increasing the temperature at which a second primer anneals to a nucleic acid following incorporation of the tag region into a nucleic acid.

The length and/or nucleotide composition of the tag region depends, in part, on the temperature at which an allele specific region anneals to a nucleic acid and the temperature at which a first primer anneals to a nucleic acid and/or the temperature at which a PCR is performed to produce a third amplification product.

A tag region that is unable to anneal to the template nucleic acid is selected to ensure that it does not cause non-specific annealing of the first primer in the first amplification reaction and the amplification of non-template nucleic acid. Preferably, the tag region is unable to anneal to a nucleic acid in a sample being assayed to such a degree as to amplify nucleic acid to a detectable level (i.e. background amplification).

As will be apparent to the skilled artisan, the requirement that the tag region not anneal to a template nucleic acid does not require that the tag region not anneal under any conditions. Rather, it is preferred that the tag region is not capable of annealing to the template nucleic acid under conditions sufficient for annealing of the locus specific sequence to the template nucleic acid. For example, the tag region may anneal to the template nucleic acid under low stringency conditions.

In one embodiment, it is preferred that the tag comprises a sequence of nucleotides that does not naturally occur in a sample being assayed. Methods for determining a sequence that is not present in a sample being assayed will be apparent to the skilled artisan. For example, the nucleotide sequence of the tag region is analyzed using a program, such as, for example, BLAST to determine whether or not that sequence (or its complement) occurs naturally in an organism being assayed.

Preferred tags comprise a high G+C content. Such a high G+C content means that a short tag region is required to sufficiently increase the Tm of a second primer. For example, a tag region comprises at least about 70% G and/or C, or at least about 80% G and/or C, or at least about 90% G and/or C, or at least about 100% G and/or C.

Examples of sequences of suitable tag regions include, for example:

GG

GC

CG

CC

GCG

CGC
GGC
CCG
GCC
GCGG
GGCG
GCGC
GGGC
GCCG
GGCC
GCGG
CGCG
CCGC
CGGC
CCCG
CGCC
CCGG
GCCCGCG
GGCGGCGG
CCCGCG
GGCGC
GCGCCG
GCCCG
CCGCCC
CCCG
GGCCG
GGGGCGGGG

Preferred tags are 2 to about 9 nucleotides in length or from 2 to about 8 nucleotides in length or from 2 to about 7 nucleotides in length or from 2 to about 6 nucleotides in length or from 2 to about 5 nucleotides in length or from 2 to about 4 nucleotides in length or 2 or 3 nucleotides in length.

However, the present invention is not to be limited to a tag region comprising any specific sequence.

Primer synthesis

Following primer design and or analysis, a specific the primer is produced and/or synthesized. Methods for producing/synthesizing a primer are known in the art. For example, oligonucleotide synthesis is described, in Gait (Ed) (*In*: Oligonucleotide Synthesis: A Practical Approach, IRL Press, Oxford, 1984). For example, a probe or primer may be obtained by biological synthesis (e.g. by digestion of a nucleic acid with a restriction endonuclease) or by chemical synthesis. For short sequences (up to about 100 nucleotides) chemical synthesis is preferable.

In one embodiment, a primer comprising deoxynucleotides (e.g., a DNA based oligonucleotide) is produced using standard solid-phase phosphoramidite chemistry. Essentially, this method uses protected nucleoside phosphoramidites to produce an oligonucleotide of up to about 80 nucleotides. Typically, an initial 5'-protected nucleoside is attached to a polymer resin by its 3'-hydroxy group. The 5' hydroxyl group is then de-protected and the subsequent nucleoside-3'-phophoramidite in the sequence is coupled to the de-protected group. An internucleotide bond is then formed by oxidizing the linked nucleosides to form a phosphotriester. By repeating the steps of de-protection, coupling and oxidation an oligonucleotide of desired length and sequence is obtained. Suitable methods of oligonucleotide synthesis are described, for example, in Caruthers, M. H., *et al*., "Methods in Enzymology," Vol. 154, pp. 287-314 (1988).

Other methods for oligonucleotide synthesis include, for example, phosphotriester and phosphodiester methods (Narang, *et al. Meth. Enzymol 68:* 90, 1979) and synthesis on a support (Beaucage, *et al Tetrahedron Letters 22:* 1859-1862, 1981), and others described in "Synthesis and Applications of DNA and RNA," S. A. Narang, editor, Academic Press, New York, 1987, and the references contained therein.

For longer sequences standard replication methods employed in molecular biology are useful, such as, for example, the use of M13 for single stranded DNA as described by J. Messing (1983) *Methods Enzymol*, 101, 20-78.

Alternatively, a plurality of primers are produced using standard techniques, each primer comprising a portion of a desired primer and a region that allows for annealing to another primer. The primers are then used in an overlap extension method that comprises allowing the primers to anneal and synthesizing copies of a complete primer

using a polymerase. Such a method is described, for example, by Stemmer *et al.*, *Gene 164*, 49-53, 1995.

As discussed *supra* a primer of the invention may also include one or more nucleic acid analogs. For example, a primer comprises a phosphate ester analog and/or a pentose sugar analog. Alternatively, or in addition, a primer of the invention comprises polynucleotide in which the phosphate ester and/or sugar phosphate ester linkages are replaced with other types of linkages, such as N-(2-aminoethyl)-glycine amides and other amides (see, e.g., Nielsen *et al., Science 254*: 1497-1500, 1991; WO 92/20702; and USSN 5,719,262); morpholinos (see, for example, USSN 5,698,685); carbamates (for example, as described in Stirchak & Summerton, *J. Org. Chem. 52*: 4202, 1987); methylene(methylimino) (as described, for example, in Vasseur *et al., J. Am. Chem. Soc. 114*: 4006, 1992); 3'-thioformacetals (see, for example, Jones *et al., J. Org. Chem. 58*: 2983, 1993); sulfamates (as described, for example in, USSN 5,470,967); 2-aminoethylglycine, commonly referred to as PNA (see, for example, WO 92/20702). Phosphate ester analogs include, but are not limited to, (i) C_1-C_4 alkylphosphonate, e.g. methylphosphonate; (ii) phosphoramidate; (iii) C_1-C_6 alkyl-phosphotriester; (iv) phosphorothioate; and (v) phosphorodithioate. Methods for the production of a primer comprising such a modified nucleotide or nucleotide linkage are known in the art and discussed in the documents referred to *supra*.

For example, a primer of the invention comprises one or more LNA and/or PNA residues. Primers comprising one or more LNA or PNA residues have been previously shown to anneal to nucleic acid template at a higher temperature than a primer that comprises substantially the same sequence but does not comprise the LNA or PNA residues.

Methods for the synthesis of an oligonucleotide comprising LNA are described, for example, in Nielsen *et al, J. Chem. Soc. Perkin Trans., 1:* 3423, 1997; Singh and Wengel, *Chem. Commun.* 1247, 1998. Methods for the synthesis of an oligonucleotide comprising are described, for example, in Egholm *et al., Am. Chem. Soc., 114:* 1895, 1992; Egholm *et al., Nature, 365:* 566, 1993; and Orum *et al., Nucl. Acids Res., 21:* 5332, 1993.

As described herein, a second primer can additionally comprise a detectable marker (for example, a fluorescent dye) to enable detection of an amplification product

produced using the method of the invention. Accordingly, in one embodiment, at least one primer of the invention comprises or is conjugated to a detectable marker. As used herein, the term "detectable marker" refers to any moiety which can be attached to a primer of the invention and: (i) provides a detectable signal; (ii) interacts with a second detectable marker to modify the detectable signal provided by the second detectable marker, e.g. FRET (Fluorescent Resonance Energy Transfer); (iii) stabilize annealing, e.g., duplex formation; or (iv) provide a member of a binding complex or affinity set, e.g., affinity, antibody/antigen, ionic complexation, hapten/ligand, e.g. biotin/avidin.

Labeling of a primer is accomplished using any one of a large number of known techniques employing known detectable markers, linkages, linking groups, reagents, reaction conditions, and analysis and purification methods. Detectable markers include, but are not limited to, light-emitting or light-absorbing compounds which generate or quench a detectable fluorescent, chemiluminescent, or bioluminescent signal (for example, as described in Kricka, L. in Nonisotopic DNA Probe Techniques (1992), Academic Press, San Diego, pp. 3-28). Fluorescent reporter dyes useful for labeling biomolecules include, but are not limited to, fluoresceins (see, for example USSN 5,188,934; 6,008,379; or USSN 6,020,481), rhodamines (as described, for example, in USSN 5,366,860; USSN 5,847,162; USSN 5,936,087; or USSN 6,051,719), benzophenoxazines (for example, as described in USSN U.S. Pat. No. 6,140,500), energy-transfer fluorescent dyes, comprising pairs of donors and acceptors (as described in USSN 5,863,727; USSN 5,800,996; or 5,945,526), or a cyanine (as described, for example, in WO 97/45539). Exemplary fluorescein dyes include, but are not limited to, 6-carboxyfluorescein; 2',4',1,4,-tetrachlorofluorescein; and 2',4',5',7',1,4-hexachlorofluorescein. Detectable markers also include, but are not limited to, semiconductor nanocrystals, or Quantum Dots® (as described, for example in US Pat. No. 5,990,479 or US Pat. No. 6,207,392). Suitable methods for linking a detectable marker to a primer (or labeling a primer) are also described in the references *supra*.

Alternatively, or in addition, a primer is produced with a fluorescent nucleotide analog to facilitate detection. For example, coupling allylamine-dUTP to the succinimidyl-ester derivatives of a fluorescent dye or a hapten (such as biotin or digoxigenin) enables preparation of many common fluorescent nucleotides. Such a method is described in, for example Henegariu, *Nat. Biotechnol. 18*:345-348, 2000. Other fluorescent nucleotide analogs are also known in the art and described, for example, Jameson, *Methods Enzymol. 278*:363-390, 1997 or USSN 6,268,132. Such nucleotide analogs

are incorporated into nucleic acids, e.g., DNA and/or RNA, or oligonucleotides, via either enzymatic or chemical synthesis (e.g., a method described *supra*).

In one preferred example of the present invention, a primer is labeled with a fluorescent dye, such as, for example, 6-carboxyfluorescein (FAM), VIC, NED or PET. To label a primer with a fluorescent dye a simple two-step process is used. In the first step, an amine-modified nucleotide, 5-(3-aminoallyl)-dUTP, is incorporated into DNA using conventional enzymatic labeling methods. This step ensures relatively uniform labeling of the probe with primary amine groups. In the second step, the amine-modified DNA is chemically labeled using an amine-reactive fluorescent dye. Various commercial kits for labeling a primer are known in the art and available from, for example, Molecular Probes (Invitrogen detection Technology) (Eugene, OR, USA) or Applied Biosystems (Foster City, CA, USA).

Commercial sources for the production of a labeled probe or primer or for a suitable label will be known to the skilled artisan, e.g., Sigma-Genosys, Sydney, Australia or Applied Biosystems, Foster City, CA, USA.

Using any method for oligonucleotide synthesis described herein and/or known in the art a set of first primers and/or a second primer or set thereof is synthesized.

In another example, a second primer is produced by coupling an oligonucleotide comprising a tag region to an oligonucleotide comprising an allele-specific region. For example, an oligonucleotide comprising a tag region is linked to another oligonucleotide using a RNA ligase, such as, for example T4 RNA ligase (as available from New England Biolabs). An RNA ligase catalyzes ligation of a 5' phosphoryl-terminated nucleic acid donor to a 3' hydroxyl-terminated nucleic acid acceptor through the formation of a 3'-5' phosphodiester bond, with hydrolysis of ATP to AMP and PP_i. Suitable methods for the ligation of DNA and/or RNA molecules using a RNA ligase are known in the art and/or described in Ausubel *et al* (In: Current Protocols in Molecular Biology. Wiley Interscience, ISBN 047 150338, 1987) and Sambrook *et al* (In: Molecular Cloning: Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratories, New York, Third Edition 2001).

The present invention additionally provides a first and/or second primer of the invention, for example, as produced using a method known in the art and/or described herein.

Clearly the present invention additionally contemplates a kit comprising one or more first primers and/or one or more second primers. The kit optionally comprises reagents suitable for amplification of a nucleic acid using the method of the invention (e.g., a buffer and/or one or more deoxynucleotides and/or a polymerase). Optionally, the kit is packaged with instructions for use.

Nucleic acid amplification

The method of the present invention is based on the amplification of a template nucleic acid using multiple rounds of PCR in a single reaction vessel. Accordingly, this single reaction vessel contains all of the components required for the performance of the multiple PCRs. Reagents required for a PCR are known in the art and include for example, one or more primers (described herein), a suitable polymerase, deoxynucleotides and/or ribonucleotides, a buffer. Suitable reagents are described for example, in Dieffenbach (ed) and Dveksler (ed) (*In*: PCR Primer: A Laboratory Manual, Cold Spring Harbor Laboratories, NY, 1995).

For example, a suitable polymerase for use in the method of the invention include, a DNA polymerase, a RNA polymerase, a reverse transcriptase, a T7 polymerase, a SP6 polymerase, a T3 polymerase, Sequenase™, a Klenow fragment, a Taq polymerase, a Taq polymerase derivative, a Taq polymerase variant, a Pfu polymerase, a Pfx polymerase, a Tfi polymerase, an AmpliTaq™ FS polymerase, a thermostable DNA polymerase with minimal or no 3'-5' exonuclease activity, or an enzymatically active variant or fragment of any of the above polymerases. Preferably, a polymerase used in the method of the invention is a thermostable polymerase.

In one example, a mixture of two or more polymerases is used. For example, the mixture of a Pfx or Pfu polymerase and a Taq polymerase has been previously shown to be useful for amplifying templates comprising a high GC content or for amplifying a large template.

Suitable commercial sources for a polymerase useful for the performance of the invention will be apparent to the skilled artisan and include, for example, Stratagene

(La Jolla, CA, USA), Promega (Madison, WI, USA), Invitrogen (Carlsbad, CA, USA), Applied Biosystems (Foster City, CA, USA) and New England Biolabs (Beverly, MA, USA).

Methods of PCR are known in the art and described, for example, in Dieffenbach (ed) and Dveksler (ed) (*In*: PCR Primer: A Laboratory Manual, Cold Spring Harbor Laboratories, NY, 1995). Generally, for PCR two non-complementary nucleic acid primers comprising at least about 8, more preferably, at least about 15 or 20 nucleotides are annealed to different strands of a template nucleic acid, and amplicons of the template are amplified enzymatically using a polymerase, preferably, a thermostable DNA polymerase.

The present invention additionally contemplates RT-PCR. For RT-PCR, RNA is reverse transcribed using a reverse transcriptase (such as, for example, Moloney Murine Leukemia Virus) to produce cDNA. In this regard, the reverse transcription of the RNA is primed using, for example, a random primer (e.g., a hexa-nucleotide random primer) or oligo-dT (that binds to a poly-adenylation signal in mRNA). Alternatively, a locus-specific primer is used to prime the reverse transcription (e.g., a first primer of the invention). A sample is heated to ensure production of single stranded nucleic acid and then cooled to enable annealing of the primer. The sample is then incubated under conditions sufficient for reverse-transcription of the nucleic acid adjacent to an annealed primer by a reverse transcriptase. Following reverse transcription, the cDNA is used as a template nucleic acid for a PCR reaction, e.g., as described *supra*.

3. Detecting amplified nucleic acid

In one example, an amplification product, e.g., a third amplification product produced using the method of the present invention is/are separated using gel electrophoresis. The separated amplification product(s) is(are) then detected using a detectable marker that selectively binds nucleic acid, such as, for example, ethidium bromide, 4'-6-diamidino-2-phenylinodole (DAPI), methylene blue or SYBR® green I or II (available from Sigma Aldrich). Suitable methods for detection of a nucleic acid using gel electrophoresis are known in the art and described, for example, in Ausubel *et al* (In: Current Protocols in Molecular Biology. Wiley Interscience, ISBN 047 150338, 1987) and Sambrook *et al* (In: Molecular Cloning: Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratories, New York, Third Edition 2001).

In one example, the nucleic acid is separated using one dimensional agarose, agarose-acrylamide or polyacrylamide gel electrophoresis. Such separation techniques separate nucleic acids on the basis of molecular weight.

Alternatively, an amplification product is separated using two dimensional electrophoresis and detected using a detectable marker (e.g., as described *supra*). Two dimensional agarose gel electrophoresis is adapted from the procedure by Bell and Byers *Anal. Biochem. 130*:527, 1983. The first dimension gel is run at low voltage in low percentage agarose to separate DNA molecules in proportion to their mass. The second dimension is run at high voltage in a gel of higher agarose concentration in the presence of ethidium bromide so that the mobility of a non-linear molecule is drastically influenced by its shape.

Alternatively, or in addition, an amplification product is characterized or isolated using capillary electrophoresis. Capillary electrophoresis is reviewed in, for example, Heller, *Electrophoresis 22*:629-43, 2001; Dovichi *et al., Methods Mol Biol 167*:225-39, 2001; Mitchelson, *Methods Mol Biol 162*:3-26, 2001; or Dolnik, *J Biochem Biophys Methods 41*:103-19, 1999. Capillary electrophoresis uses high voltage to separate molecules according to their size and charge. A voltage gradient is produced in a column (i.e. a capillary) and this gradient drives molecules of different sizes and charges through the tube at different rates.

In another example, an amplification product is detected using an automated system, such as, for example, as produced by eGene, Inc. An example of such a system is described, for example, in Szantai *et al., Clinical Chemistry 52:* 1756-1762, 2006.

Alternatively, or in addition, one or more amplification products is detected using a microplate array diagonal gel electrophoresis (MADGE) method, e.g., as described in US Pat. No. 6,071,396.

Alternatively, an amplification product is identified and/or isolated using chromatography. For example, ion pair-reversed phase HPLC has been shown to be useful for isolating a PCR product (Shaw-Bruha and Lamb, *Biotechniques. 28*:794-7, 2000.

Rather than contacting amplified nucleic acid with a detectable marker, the present invention additionally contemplates using a primer that comprises a detectable marker to facilitate detection of an amplification product. For example, a second primer of the invention is labeled with a detectable marker using a method known in the art and/or described herein and used in the method of the invention.

Amplified nucleic acid is then readily detected by detecting the label. In the case of a radiolabeled primer, the detection technique may comprise, for example, the use of a photographic film. In the case of a fluorescently labeled primer, the nucleic acid is detected, for example, by exposing a gel on which an amplification product has been resolved to light of a suitable wavelength to excite the label and detecting the fluorescence produced therefrom.

In another embodiment, the amplified nucleic acid is detected using, for example, mass spectrometry (e.g., MALDI-TOF). For example, a sample comprising nucleic acid amplified using the method of the invention is incorporated into a matrix, such as for example 3-hydroxypropionic acid, α-cyano-4-hydroxycinnamic acid, 3,5 dimethoxy-4-hydroxycinnamic acid (Sinapinic acid) or 2,5 dihydroxybenzoic acid (Gentisic acid). The sample and matrix are then spotted onto a metal plate and subjected to irradiation by a laser, promoting the formation of molecular ions. The mass of the produced molecular ion is analyzed by its time of flight (TOF), essentially as described by Yates, *J. Mass Spectrom. 33*, 1-19, 1998 and references cited therein. A time of flight instrument measures the mass to charge ratio (m/z) ratio of an ion by determining the time required for it to traverse the length of a flight tube. Optionally, such a TOF mass analyzer includes an ion mirror at one end of the flight tube that reflects said ion back through the flight tube to a detector. Accordingly, an ion mirror serves to increase the length of a flight tube, increasing the accuracy of this form of analysis. By determining the time of flight of the ion, the molecular weight of an amplified nucleic acid is determined.

The advantage of this form of technique is that an amplification product is detected and characterized without the requirement for labeling of the nucleic acid.

Variations of MALDI-TOF are available in the art and will be apparent to the skilled artisan.

In another example, a third amplification product is detected by determining the melting temperature of the third amplification product. In one example, a melting temperature assay takes advantage of the different absorption properties of double stranded and single stranded DNA, that is, double stranded DNA absorbs less light than single stranded DNA at a wavelength of 260nm, as determined by spectrophotometric measurement. This is because heterocyclic rings of nucleotides adsorb light strongly in the ultraviolet range (with a maximum close to 260 nm that is characteristic for each base). However, the adsorption of DNA is approximately 40% less than would be displayed by a mixture of free nucleotides of the same composition. This effect is called hyperchromism and results from interactions between the electron systems of the bases, made possible by their stacking in the parallel array of the double helix. Any departure from the duplex state is immediately reflected by a decline in this effect (that is, by an increase in optical density toward the value characteristic of free bases. Denaturation of double stranded DNA is detectable by this change in optical density. The midpoint of the temperature range over which the strands of DNA separate is called the melting temperature, denoted T_m. Moreover, the sequence of a nucleic acid affects the temperature at which the nucleic acid denatures. Accordingly, two sequences amplified with the same primers that differ by even a single nucleotide can be detected by a change in melting temperature.

Melting temperature assays can also make use of a dye that binds to double-stranded nucleic acid and emit a detectable fluorescent signal at a wavelength that is characteristic of the particular dye (see, e.g., Zhang *et al., Hepatology 36*:723-728, 2002). A dissociation or melting curve can be obtained during or following an amplification reaction by monitoring the nucleic acid dye fluorescence as the reaction temperatures pass through the melting temperature of an amplification product. The dissociation of a double-stranded amplification product is observed as a rapid decrease in fluorescence at the emission wavelength characteristic of the dye. In this manner, it is possible to detect the melting temperature of multiple amplification products comprising different sequences is determined.

Characterization of an individual or group of individuals

As the present invention is useful for detecting a polymorphism or mutation, the invention has clear application in determining relationships between one or more individuals, isolates of an organism, cultivars of an organism, species or genera.

Furthermore, the present invention is useful for identifying an individual, isolate of an organism, cultivar of an organism, species or genus.

For example, the method of the invention is useful for a form of genetic mapping, such as, for example bulked segregant analysis (BSA). In its simplest form this form of analysis uses nucleic acid from a plurality of organisms (preferably, plants) that only differ in one trait (e.g., as a result of mutation or introgression). Nucleic acid from organisms with one phenotype is pooled, as is nucleic acid from organisms with the other phenotype. Using the method of the invention, a region of nucleic acid in which the two pools of nucleic acid differ is determined. Such a method is particularly useful for, for example, mapping of a gene responsible for a monogenic trait or a quantitative trait. Suitable methods for BSA are described, for example, in Wang and Paterson *Theor. Appl. Genet. 88*:355-361, 1994 and Mackay and Caligari *Crop Science 40*:626-630, 2000.

Clearly, the present invention contemplates performing a multiplex reaction to identify or characterize an individual, isolate of an organism, cultivar of an organism, species or genus, for example, the detection of a plurality of polymorphisms and/or mutations.

Clearly, the method of the present invention has broad reaching application in any assay that detects one or more polymorphisms or mutations. Accordingly, the present invention is useful for, for example, marker assisted breeding programs (e.g., animal husbandry), gene mapping, the identification of specific strains, races, isolates, serotypes or serogroups of microorganisms, the identification of cultivars, species or genera of plants, and for identification of organisms likely to have a trait of interest.

Genetic markers in plants

Genetic markers are used for a variety of purposes in association with plants. For example, one or more genetic markers is (are) used to identify a specific plant variety. For instance, a plant that is protected by an intellectual property right is characterized to determine one or more genetic markers that are specific to said plant. This then enables simple and rapid characterization of similar plants to determine whether or not an intellectual property right has been infringed.

In another embodiment, the present invention is used to determine a plant that is likely to comprise a trait of interest. Examples of suitable primers for detection of

polymorphisms associated with a trait are described herein, or in, for example, Chiapparino *et al., Genome. 47:*414-420, 2004 (e.g. SNPs in sucrose synthase of Barley); Hayashi *et al., Theor Appl Genet. 108*:1212-1220, 2004 (blast resistance in rice); and Schwarz *et al., J Agric Food Chem. 51*:4263-4267, 2003 (SNPs associated with HMW glutenin expression in wheat).

Genetic markers in humans and animals

The method of the present invention for detecting one or more genetic markers is also useful for, for example, marker assisted breeding of animals and/or to select for those animals with one or more desired traits.

For example, the assay is used to screen animals for enhanced commercial properties, such as, for example, food quality for human consumption. Such an assay is performed to detect one or more markers that is (are) associated with increased marbling in beef. Marbled beef is of commercial importance as consumers in several countries pay a premium price for beef with a high level of marbling. Recently, several markers have been reported that are associated with an increased level of marbling.

For example, Barendse *et al.*, Beef Quality CRC Marbling Symposium, Coffs Harbour pp. 52-57, 2001 describe a SNP in the calpastatin gene (detected using primers comprising the sequence GGGGATGACTACGAGTATGACTG and GTGAAAATCTTGTGGAGGCTGTA.

Furthermore, researchers have reported markers in the leptin gene are associated with an increased marbling score in cattle (Buchanan *et al., Genet Sel Evol. 34:*105-16, 2002). Accordingly, by producing primers used by Barendse *et al.* and/or Buchanan *et al.,* tagged with a tag region described herein a multiplex reaction is performed to amplify the respective markers. The amplified nucleic acid is then further amplified using the relevant second set of primers. By subsequently detecting the presence or absence of the described markers cattle with increased marbling scores are identified.

Ciobanu *et al., J. Anim. Sci. 82*: 2829-2839, 2004 and Chang *et al., Vet. J. 165*: 157-163, 2003 describe markers useful for determining an increased pork quality from a pig. The marker described by Ciobanu *et al.,* occurs in the calpastatin gene, while the marker described by Chang *et al.,* polymorphism in the desmin gene.

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A method described herein according to any embodiment is also applicable to, for example, selecting enhanced race horses (e.g., with enhanced speed and/or endurance), selecting sheep that produce superior wool, or selecting a mammal (e.g., a cow) that produces superior quality milk.

Diagnostics

As the present invention is useful for the detection of genetic differences, it is particularly useful for the diagnosis of a disease or disorder or the presence of one or more infectious agents in a sample. For example, the method of the invention is useful for detecting a genetic change that is associated with a disease or disorder in a human or a non-human animal.

Exemplary common genetic diseases or disorders in humans associated with a polymorphism or mutation include, for example, cystic fibrosis, sickle cell anemia, β-thalasemia, or muscular dystrophy. Exemplary common diseases in sheep include, for example, Menkes disease or Scrapie. Examples of common genetic diseases in goats include, for example, gynecomastia and anotia-microtia complex. Exemplary genetic diseases in horses include, for example, hyperkalemic periodic paralysis (HYPP), combined immune deficiency syndrome (CID), overo-lethal white syndrome and epitheliogenesis.

The mutations that cause these disorders are now known and, as a consequence, a screen may be developed using the method of the invention to screen for any or all of these disorders in a specific organism.

The present invention is described further in the following non-limiting examples.

EXAMPLE 1

Allelic discrimination by differential product size using a pair of allele specific primers designed to opposite DNA strands

Figure 1 depicts a method of the present invention for detecting an allele in which allele specificity is conferred by an allele specific (AS) primer that anneals to a locus of interest such that a nucleotide complementary to the allele is positioned at or near the 3' end of the primer. Accordingly, in the presence of an allele of interest (allele B in Figure 1) the 3' end of the primer will anneal to the nucleic acid, however in the presence of an alternate allele (allele A in Figure 1) the 3' end of the primer will not anneal or will anneal at a reduced level compared to the level when allele B is present.

As depicted in Figure 1, the assay is performed using both locus specific (LS) primers (L_1 and L_2) and allele specific (AS) primers (A_1 and A_2). The locus specific primers anneal to nucleic acid in the sample at a first temperature (e.g., from about 63°C to about 74°C). These primers amplify the nucleic acid or locus comprising the allele of interest, e.g., in the case of a polyploid organism the LS primers amplify a nucleic acid specific to a genome comprising the allele of interest.

The AS primers comprise a first region that anneals to the nucleic acid comprising the allele of interest at a lower temperature than the annealing temperature of the LS primers. The AS primers also comprise second region, which is a 5'-tail that is not complementary to the nucleic acid comprising the allele. Following amplification of a sequence using an AS primer, the second region is incorporated into the amplification product. Accordingly, the entire AS primer may then anneal to the amplification product at a higher temperature than that of the first region of the AS primer, and preferably at about the same temperature at which a LS primer anneals to a target sequence.

A single PCR is performed using both LS and AS primers. In a first phase, the reaction is performed using an annealing temperature at which the LS primers anneal to a target sequence, however the first region of the AS primers do not substantially anneal to a target sequence. This enriches or amplifies the locus of interest. In a second phase, the reaction is performed using an annealing temperature at which the first region the AS primers anneal to a target sequence. Following several rounds of amplification, the

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annealing temperature is increased to approximately the same temperature used in the first phase.

In the assay depicted in Figure 1, the presence of allele B is detected by a PCR fragment that is the product of the AS forward and reverse primer pair (A_1A_2), and hereafter referred to as matched product. The presence of allele A is detected by a PCR product resulting from the LS forward primer and AS reverse primer (L_1A_2), and hereafter referred to as mismatched product. The mismatched product also acts as a positive control against a failed PCR assay. In heterozygous samples, where alleles A and B are present, both matched and mismatched products are amplified.

This assay configuration permits codominant allelic discrimination in a single reaction, and enables the size of the resulting PCR products (matched and mismatched) to be readily adjusted, e.g., within the range of about 60-bp to about 500-bp to suit end-point detection on a variety of size separation matrixes such as agarose gel, and a range of dedicated instruments such as eGENE (eGENE Inc.). Alternatively, allelic discrimination is achieved by end-point or real-time melting analysis using instrumentation such as the RotorGene6000 (Corbett Research), since the matched and mismatched products have different melting temperatures.

EXAMPLE 2

Allelic discrimination by differential product size using a pair of allele specific primers designed to the same DNA strand.

Figure 2 depicts a method of the present invention for detecting an allele in which specificity for allele A or allele B is conferred by allele specific primers designed to anneal to the same DNA strand. LS and AS primers are produced essentially as described in Example 1, and assays are performed essentially as described in Example 1. In the assay depicted in Figure 2 both AS primers (A_1 and A_2 in Figure 2) are designed to anneal to the target locus such that a nucleotide complementary to one allele is positioned at or near the 3' end of one primer (e.g., A_1), and a nucleotide complementary to the other allele is positioned at or near the 3' end of the other primer (e.g., A_2). The AS primers differ by having 5' non-complementary tails of different length.

A single reaction is performed for genotype determination using LS primers L_1 and L_2, and AS primers A_1 and A_2. Depending on the sample genotype, either one or both AS primers anneal to the nucleic acid and amplify to generate an amplification product that is the product of the LS forward primer (L_1) and one AS reverse primer. Unequal length of the PCR products for allele A and allele B enables codominant allelic discrimination using a size separation matrix such as polyacrylamide gel, or a dedicated instrument such as eGENE (eGENE Inc.). Alternatively, allelic discrimination is achieved by end-point or real-time melting analysis using an instrument such as the RotorGene6000 (Corbett Research), since each allele-specific PCR product has a distinct melting temperature that depends on which of the two AS primers is responsible for amplification.

EXAMPLE 3

Allelic discrimination by differential product size using a single AS primer

In the assay depicted in Figures 3a and 3b, allele specificity is conferred by the AS reverse primer. LS and AS primers are produced essentially as described in Example 1, and assays are performed essentially as described in Example 1. The number of reactions required for genotype determination will be influenced by the size of the PCR fragment amplified by the LS primer pair.

The assay depicted in Figure 3a is an example in which a PCR fragment amplified by the LS primer pair is relatively short, for example less than about 500-bp. In this case, a single reaction is performed for genotype determination using the LS primers L_1 and L_2 and AS reverse primer A_1. In the assay depicted in Figure 3a allele specificity is conferred by the AS primer that anneals to a locus of interest such that a nucleotide complementary to the an allele (e.g., the B allele) is positioned at or near the 3' end of the primer. The presence of allele B is detected by a PCR fragment that is the product of the LS forward primer and AS reverse primers (L_1A_1; matched product), whereas the presence of allele A is detected by a PCR fragment that is the product of the LS primer pair (L_1L_2; mismatched product). Samples heterozygous for the allele are detected by the presence of both matched and mismatched product.

The assay depicted in Figure 3b is an example of an assay in which a PCR fragment amplified by the LS primer is relatively large, for example, longer than 500-bp. In this situation, two separate reactions are performed for genotype determination, one using

the LS primers L_1 and L_2 and AS reverse primer A_1 specific for the allele A (i.e., comprising a sequence complementary to the sequence of allele A), and the other with the LS primers L_1 and L_2 and AS primer reverse primer A_2 specific for allele B (i.e., comprising a sequence complementary to the sequence of allele B). The presence of the allele of interest in each assay is detected by a PCR fragment that is the product of the LS forward primer and AS reverse primer (L_1A_1 or L_1A_2). Samples homozygous at the site of the allele are detected by the presence of matched product in only one reaction, while samples heterozygous at the site of the allele are detected by the presence of matched product in both reactions.

EXAMPLE 4

Allelic discrimination by differential product labeling using a pair of AS primers designed to the same DNA strand

Figure 4 depicts a method of the present invention for detecting an allele in which specificity for allele A or allele B is conferred by allele specific primers designed to anneal to the same DNA strand. LS and AS primers are produced essentially as described in Example 1, and assays are performed essentially as described in Example 1. In the assay depicted in Figure 2 both AS primers (A_1 and A_2 in Figure 2) are designed to anneal to the target locus such that a nucleotide complementary to one allele is positioned at or near the 3' end of one primer (e.g., A_1), and a nucleotide complementary to the other allele is positioned at or near the 3' end of the other primer (e.g., A_2). The AS primers differ by having a detectable marker, such as a fluorescent dye attached to their 5'-end.

A single reaction is performed for genotype determination using LS primers L_1 and L_2, and AS primers A_1 and A_2. Depending on the sample genotype, either one or both AS primers anneal to the nucleic acid and amplify to generate an amplification product that is the product of the LS forward primer (L_1) and one AS reverse primer. Differential detection of the detectable marker attached to each AS primer by methods such as fluorescence detection facilitates codominant allelic discrimination.

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EXAMPLE 5

Allelic discrimination by differential product detection using a pair of AS primers designed to opposite DNA strands.

Figure 5 depicts a method of the present invention for detecting an allele in which allelic discrimination between allele A or allele B is determined using high resolution melting analysis. LS and AS primers are produced similar those described in Example 1, however the AS primers do not anneal to the site of the allele. Rather, the AS primers are adjacent to the allele and, when used in a PCR reaction amplify nucleic acid comprising the allele. A single reaction is performed for genotype determination using the LS primers L_1 and L_2 and AS primers A_1 and A_2, essentially as described in Example 1. Allelic discrimination is determined by end-point and/or real-time high resolution melting analysis due to a difference in the melting temperature between the PCR fragments comprising allele A or allele B, which is the product of the AS primers (A_1A_2; Diagram 5). An advantage of this assay configuration is that the size of the second phase PCR amplification product can be readily adjusted to maximize allele discrimination by high resolution melting analysis.

EXAMPLE 6

Allelic discrimination by differential product detection using a single AS primer

Figure 6 depicts an alternative method to that described in Example 5 for detecting an allele in which allelic discrimination between allele A or allele B is determined using high resolution melting analysis. A single AS primer and a pair of LS primers are produced similar those described in Example 1, however the AS primer does not anneal to the site of the allele. Rather, the AS primer is adjacent to the allele and, when used in a PCR reaction in combination with a suitable LS primer, amplifies nucleic acid comprising the allele. A single reaction is performed for genotype determination using the LS primers L_1 and L_2 and AS primer A_1. Allelic discrimination is determined by end-point and/or real-time high-resolution melting analysis due to a difference in the melting temperature between the PCR fragments for allele A and allele B, which is the product of the LS forward primer and AS reverse primer (L_1A_1).

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EXAMPLE 7

Selection and design of low-melting allele specific primers

To minimize the participation of allele specific primers in the first phase of amplification performed using locus specific primers, a series of allele specific primers were tested for amplification yield and specificity under first phase PCR conditions. Specifically, allele specific primers were synthesized for genomic loci harboring known SNPs in barley (*Hordeum vulgare*) and bread wheat (*Triticum aestivum*). Each allele specific primer was composed of two parts: a region complementary to sequence flanking the SNP and designed with a melting temperature in the range of 40 to 55°C, and a 5'-tail that was non-complementary to the DNA template, which increased the melting temperature of the AS primer to about 67°C once the non-complementary tail was incorporated into PCR product. For each target locus, three allele specific primers were synthesized with the complementary region having a melting temperature of 40°C, 45°C, 50°C and 55°C, respectively. The three allele specific primers for each melting temperature comprise two forward primers and one reverse primer. The two forward primers were designed adjacent to the SNP of interest with a 3'-nucleotide of each primer corresponding to one of the alleles present at the target locus respectively, and a deliberate nucleotide mismatch at the -1, -2 or -3 position from the 3'-terminus, according to Ye *et al. Nucl. Acids. Res., 29*: e88, 2001. The reverse primer was designed with complete complementarity to the opposite DNA strand at a position 100 to 150-bp downstream of the polymorphism.

The sequences of each of the primers is set forth below:

Primers sequences for the putative gene located on chromosome 5H gene were:

Complementary region melting temperature 40°C

(i) AS forward primer specific for allele A: ***GCCCGCG***TCATCACTAGTAAATCTT**G** (SEQ ID NO: 1)

(ii) AS forward primer specific for allele B: ***GCCCGCG***TCATCACTAGTAAATCTT**A** (SEQ ID NO: 2)

(iii) AS reverse primer: ***GGCGGCGG***AGAAAAAGTAATGGT (SEQ ID NO: 3)

Complementary region melting temperature 45°C

(i) AS forward primer specific for allele A: ***CCCGCG***AAATCATCACTAGTAAATCTT**G** (SEQ ID NO: 4)

(ii) AS forward primer specific for allele B: ***CCCGCG***AAATCATCACTAGTAAATCTTA (SEQ ID NO: 5)

(iii) AS reverse primer: ***GCGGC***GGGAGAAAAAGTAATGGT (SEQ ID NO: 6)

Complementary region melting temperature 50°C

(i) AS forward primer specific for allele A: ***GGCGC***AGTAAATCATCACTAGTAAATCTTG (SEQ ID NO: 7)

(ii) AS forward primer specific for allele B: ***GGCGC***AGTAAATCATCACTAGTAAATCTTA (SEQ ID NO: 8)

(iii) AS reverse primer: ***CCTGCC***TTGTTCTGGACGTTTTCAT (SEQ ID NO: 9)

Primers sequences for the nicotinate phosphoribosyltransferase-like gene were:

Complementary region melting temperature 40°C

(i) AS forward primer specific for allele A: ***GCGCCG***GCCGAATCAGTTTAC (SEQ ID NO: 10)

(ii) AS forward primer specific for allele B: ***GCGCCG***GCCGAATCAGTTTAG (SEQ ID NO: 11)

(iii) AS reverse primer: ***GGCGGC***TGAATTCACAGGCTG (SEQ ID NO: 12)

Complementary region melting temperature 45°C

(i) AS forward primer specific for allele A: ***GCCCG***CGCCGAATCAGTTTAC (SEQ ID NO: 13)

(ii) AS forward primer specific for allele B: ***GCCCG***CGCCGAATCAGTTTAG (SEQ ID NO: 14)

(iii) AS reverse primer: ***GCGGC***ACTGAATTCACAGGCTG (SEQ ID NO: 15)

Complementary region melting temperature 50°C

(i) AS forward primer specific for allele A: ***CCGCCC***GCCGAATCAGTTTAC (SEQ ID NO: 16)

(ii) AS forward primer specific for allele B: ***CCGCCC***GCCGAATCAGTTTAG (SEQ ID NO: 17)

(iii) AS reverse primer: ***CCGC***AACTGAATTCACAGGCTGA (SEQ ID NO: 18)

The non-complementary 5'tail of the AS primers is shown in bold and italics font, the nucleotide corresponding to the SNP is shown in bold, and deliberate mismatches at the -1 or -3 position are underlined.

PCR assays were performed using 1 μM of allele specific forward and reverse primer in a 4 μl reaction mixture containing 0.2 mM dNTP, 1 x PCR buffer, 1.5 mM $MgCl_2$, 100 ng/μl bovine serum albumin Fraction V, between 25 and 50 ng genomic DNA and 0.15 U Platinum *Tfi* DNA polymerase. Two reactions were performed for each DNA sample, one using an allele specific forward primer specific for one allele, the other

with an allele specific forward primer specific for the other allele present at the site of the SNP. Following an initial denaturation step of 2 min at 94°C, PCR was performed for a total of 35 cycles with the profile: 30 s at 92°C, 30s at 58°C, 2 min at 72°C. The reaction products were separated by electrophoresis in a 1.5% agarose gel and visualized by ethidium bromide staining (Sambrook and Russell 2001, Molecular Cloning: a laboratory manual. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York).

Examination of the PCR specificity and yield revealed that allele specific primers having a complementary region with a melting temperature below 45°C amplified essentially no PCR product, or product of unexpected size (as shown in Figure 7). Accordingly, AS primers designed with a complementary region having a melting temperature below 45°C are expected not to participate significantly in the first phase of TSP amplification. In subsequent experiments, AS primers were designed with the complementary region to have a melting temperature of 40°C.

A deliberate nucleotide mismatch at the -1, -2 or -3 position from the 3' terminus of allele-specific primers (according to Ye *et al.* 2000) is not essential to the invention. Nor is this preferred, as it may reduce TSP genotyping accuracy in some cases.

EXAMPLE 8

Detection of SNPs using Temperature Switch PCR and comparison to standard PCR conditions.

Introduction

Without being bound by any theory or mode of action, it is expected that under standard PCR cycling conditions employing a high annealing temperature the LS primers will anneal with high efficiency to the genomic template, resulting in the efficient accumulation of LS product. In contrast, minimal, or no, annealing is expected for the AS primers. However, as the reaction progresses the accumulation of LS product may lead to conditions under which the AS primer can anneal, since the amplification product produced by amplification with LS primers contains sequence complementary to the AS primers. This is expected as the melting temperature of an oligonucleotide primer is related to the concentration of complementary template (Panjkovich and Francisco, *Bioinformatics 21*: 711-722, 2005). Once LS product has sufficiently accumulated to allow the AS primers to anneal, AS product is produced at high efficiency because of self-amplification. Highly efficient self-amplification occurs, despite the high PCR annealing temperature, because the non-complementary

tail of the AS primers is incorporated into the product to provide a much longer region of complementarity. Therefore, the final reaction product is expected to contain a mixture of LS and AS products.

Under cycling conditions of the method of the present invention, it is also expected that LS product will accumulate with high efficiency at the high PCR annealing temperature used in the first phase of the reaction. However, a lowering of the annealing temperature after 15 cycles of first phase amplification enables the AS primers to participate in PCR amplification before their participation might otherwise be expected. Efficient annealing of the AS primers to the enriched target sequence (amplification product produced by amplification with LS primers) at the second phase annealing temperature allows for highly efficient self-amplification of AS product in subsequent cycles due to incorporation of the non-complementary 5'-tail. Accordingly, the accumulation of AS product during the second phase of TSP amplification is expected to out-compete the accumulation of LS product, resulting in a predominance of AS product.

To demonstrate the TSP mechanism, the accumulation of amplicons produced from amplifications with LS or AS primers was monitored. In the assays, AS primers were designed to opposite DNA strands (see Figure 1). Assays were performed using samples with known zygosity and different combinations of the four LS and AS primers to show the contribution of each primer to the accumulation of the expected PCR products. For comparison, each reaction was also performed under standard PCR cycling conditions with a high annealing temperature and the same number of cycles for amplification.

PCR assays were performed using 0.1 μM of LS primer and 1 μM of AS primer in a 4 μl reaction mixture containing 0.2 mM dNTP, 1 x PCR buffer, 1.5 mM $MgCl_2$, 100 ng/μl bovine serum albumin Fraction V, between 25 and 50 ng genomic DNA and 0.15 U Platinum *Tfi* DNA polymerase. The composition of LS and AS primer in each reaction is described in Table 1. Assays performed using standard PCR cycling conditions were performed with an initial denaturation step of 2 min at 94□C, followed by 35 cycles of 30 s at 92°C, 30s at 58°C, 2 min at 72°C.

Reaction products were separated by electrophoresis in a 1.5% agarose gel and visualized by ethidium bromide staining.

For the assay of the invention, amplification reactions were performed using the following reaction mixture: 0.2 mM dNTP, 1 x PCR buffer (16 mM $(NH_4)_2SO_4$, 0.01% Tween-20, 100 mM Tris-HCl, and pH 8.3), 1.5 mM $MgCl_2$, 100 ng/µl bovine serum albumin Fraction V (Sigma Aldrich), 0.1 µM each of each locus specific forward and reverse primer, 1 µM each of each allele specific forward and reverse primer, between 25 and 50 ng genomic DNA and 0.15 U Platinum *Tfi* DNA polymerase (Invitrogen) (total reaction volume 4 µl). Amplification reactions were performed under the following conditions:

(i) initial denaturation, 2 min at 94°C;

(ii) 35 cycles with the profile: 30 s at 92°C, 30s at 58°C, 2 min at 72°C for 15 cycles (hereafter referred to as the first reaction phase);

(iii) 5 cycles were with 10 sec at 92°C and 30 sec at 35°C;

(iv) 15 cycles with 10 sec at 92°C, 30 sec at 58°C (hereafter referred to as the second reaction phase);

(v) 10 min at 72°C; and

(vi) indefinite hold at 15°C.

Reaction products were separated by electrophoresis in a 1.5% agarose gel and visualized by ethidium bromide staining.

Table 1. LS and AS primers present in each set of reactions.

Reaction	Primer Combination
1	L_1 L_2
2	A_{1a} A_2
3	A_{1b} A_2
4	L_1 A_{1a} A_2
5	L_1 A_{1b} A_2
6	L_2 A_{1a} A_2
7	L_2 A_{1b} A_2
8	L_1 L_2 A_{1a}
9	L_1 L_2 A_{1b}
10	L_1 L_2 A_2
11	L_1 L_2 A_{1a} A_2
12	L_1 L_2 A_{1b} A_2
13	L_1 A_2
14	L_2 A_{1a}
15	L_2 A_{1b}

where, L_1 is LS forward primer
L_2 is LS reverse primer
A_{1a} is AS forward primer specific for allele A
A_{1b} is AS forward primer specific for allele B
A_2 is AS reverse primer

as depicted in Figure 1 and described in Example 1

Primers used in one example of the assay are as follows:

(i) LS forward primer, L_1: TGTGTCTGAACTTGCATTTGATGACG

(ii) LS reverse primer, L_2: CCTCTCTTTGTGCTCTCAACTTGTCCA

(iii) AS forward primer specific for allele A, A_{1a}: ***GCCCGCG***TCATCACTAGTAAATC<u>T</u>TG

(iv) AS forward primer specific for allele B, A_{1b}: ***GCCCGCG***TCATCACTAGTAAATC<u>C</u>TTA

(v) AS reverse primer, A_2: ***GGCGGCGG***AGAAAAAGTAATGGT

The non-complementary 5'tail of the AS primers is in highlighted in bold italic font, and deliberate mismatches at the -3 position are underlined.

If the TSP assay mechanism functions as predicted, correct genotype determination should only be achieved for reactions performed under TSP cycling conditions in the presence of all four LS and AS primers (reactions 11 and 12, Table 1). All other reactions should produce no amplification product, or PCR product the size of which depends on the interactions among the primers present.

Inspection of the PCR fragments amplified across multiple DNA samples and genomic loci revealed that genotype determination was more accurate for assays of the invention in which the four LS and AS primers were present (reactions 11 and 12, Figure 8). All other primer combinations tested under TSP cycling conditions gave no amplification, incorrect genotypes or inconsistent genotyping accuracy. For example, assays performed using only the LS forward primer L_1 and the pair of AS primers A_1 and A_2 often produced the expected genotype (reactions 4 and 5, Figure 8), but were not as reliable for genotype determination across larger numbers of samples. Correct genotype determination was not achieved for any of the reactions performed using standard PCR cycling conditions (Figures 8). The results demonstrate that accurate genotype determination for the assay format tested was achieved best under cycling conditions of the assay of the invention, in the presence of all four LS and AS primers. These results affirm that the reaction mechanism of the assay of the invention involves sequential enrichment of a target sequence harboring the SNP by the LS primers L_1 and L_2, followed by nested amplification of the interrogated allele by the AS primers A_1 and A_2.

The presence of mismatched product resulting from the LS forward primer L_1 and AS reverse primer A_2 in samples homozygous for the reference allele, in which only matched product resulting from the AS forward and reverse primer pair A_1 and A_2 was expected, indicates that interactions among primers affect both the yield and specificity

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of TSP reactions (reactions 11 and 12, Figure 8). In general, the amount of mismatched product observed in samples homozygous for the reference allele varied between different genomic loci, and ranged from almost absent (i.e. only matched product was observed) to having sufficient yield to suggest that the sample was heterozygous. It is likely that the presence of mismatched product in these sample results from a destabilization of the annealing efficiency of the AS forward primer A_1, compared to the AS reverse primer A_2, due to the presence of the secondary mismatch at the -1 or -2 position from the 3'-terminus. Primer destabilization may lead to more efficient formation of mismatched product during the initial cycles of second phase TSP amplification. Assays to ensure correct genotype are described below.

EXAMPLE 9
Separate TSP assays to detect each form of an allele

Assays were configured for allelic discrimination by differential product size using a pair of AS primers designed to opposite DNA strands (as shown in Diagram 1). The assays were performed in barley (*Hordeum vulgare*) using samples with known zygosity, using the following assay reaction mixture: 0.2 mM dNTP, 1 x PCR buffer (16 mM $(NH_4)_2SO_4$, 0.01% Tween-20, 100 mM Tris-HCl, and pH 8.3), 1.5 mM $MgCl_2$, 100 ng/µl bovine serum albumin Fraction V (Sigma Aldrich), 0.1 µM each of each locus specific forward and reverse primer, 1 µM each of each allele specific forward and reverse primer, between 25 and 50 ng genomic DNA and 0.15 U Platinum *Tfi* DNA polymerase (Invitrogen) (total reaction volume 4 µl). Amplification reactions were performed under the following conditions:

(i) initial denaturation, 2 min at 94°C;
(ii) 35 cycles with the profile: 30 s at 92°C, 30s at 58°C, 2 min at 72°C for 15 cycles (hereafter referred to as the first reaction phase);
(iii) 5 cycles were with 10 sec at 92°C and 30 sec at 35°C;
(iv) 15 cycles with 10 sec at 92°C, 30 sec at 58°C (hereafter referred to as the second reaction phase);
(v) 10 min at 72°C; and
(vi) indefinite hold at 15°C.

AS primers used have a complementary region melting temperature of 40°C. Two reactions were performed for each sample, one with AS forward primers specific for allele A, and the other with AS forward primer specific for allele B. Primers were

designed to assay SNPs in putative gene located on chromosome 5H (Figure 9a), and a nicotinate phosphoribosyltransferase-like gene (Figure 9b and 9c). Primer sequences are as follows:

Primers sequences for the putative gene located on chromosome 5H gene were:

(i) LS forward primer, L_1: TGTGTCTGAACTTGCATTTGATGACG (SEQ ID NO: 24)

(ii) LS reverse primer, L_2: CCTCTCTTTGTGCTCTCAACTTGTCCA (SEQ ID NO: 25)

(iii) AS forward primer specific for allele A, A_{1a}: ***GCCCGCG***TCATCACTAGTAAATC<u>T</u>T**G** (SEQ ID NO: 26)

(iv) AS forward primer specific for allele B, A_{1b}: ***GCCCGCG***TCATCACTAGTAAATC<u>T</u>TA (SEQ ID NO: 27)

(v) AS reverse primer: ***GGCGGCGG***AGAAAAAGTAATGGT (SEQ ID NO: 28)

Primers sequences for the nicotinate phosphoribosyltransferase-like gene (Figure 9b) were:

(i) LS forward primer, L_1: CTACTGGAAGGCCGGCAAGC (SEQ ID NO: 29)

(ii) LS reverse primer, L_2: CGCATAAACCTCAACATCTGAGCA (SEQ ID NO: 30)

(iii) AS forward primer specific for allele A, A_{1a}: ***GCGCCG***GCCGAATCAGTTT<u>A</u>**C** (SEQ ID NO: 31)

(iv) AS forward primer specific for allele B, A_{1b}: ***GCGCCG***GCCGAATCAGTTT<u>A</u>**G** (SEQ ID NO: 32)

(v) AS reverse primer: ***GGCGGC***TGAATTCACAGGCTG (SEQ ID NO: 33)

Primers sequences for the nicotinate phosphoribosyltransferase-like gene (Figure 9c) were:

(i) LS forward primer, L_1: CTACTGGAAGGCCGGCAAGC (SEQ ID NO: 34)

(ii) LS reverse primer, L_2: CGCATAAACCTCAACATCTGAGCA (SEQ ID NO: 35)

(iii) AS forward primer specific for allele A, A_{1a}: ***CCCG***TCGCGTGACAACTAAAATTATAC<u>A</u> (SEQ ID NO: 36)

(iv) AS forward primer specific for allele B, A_{1b}: ***CCCG***TCGCGTGACAACTAAAATTATAC<u>T</u> (SEQ ID NO: 37)

(v) AS reverse primer: ***GGCCG***TCGCTCATACAAGTGGAA (SEQ ID NO: 38)

The non-complementary 5'tail of the AS primers is in highlighted in bold italic font, and deliberate mismatches at the -1, -2 or -3 position are underlined.

As shown in Figures 9a-9c one manner in which to achieve correct genotype determination for genomic loci producing mismatched product in samples homozygous for a reference allele is to perform two assays, one specific for the reference allele and the other specific for the alternate allele.

EXAMPLE 10
Altering melting temperature of allele specific primers to normalize annealing efficiency

Assays were configured for allelic discrimination by differential product size using a pair of AS primers designed to opposite DNA strands (see Figure 1 and Example 1). The assay was performed using genomic DNA from bread wheat (*Triticum aestivum*) using samples with known zygosity. The AS forward and reverse primers A_1 and A_2 had complementary region melting temperatures of 50°C and 40°C, respectively. Two reactions were performed for each sample, one using the AS forward primer specific for allele A, and the other using AS forward primer specific for allele B. Assay conditions were essentially as described in Example 9. Primers were designed to assay a SNP located in a putative nodulin gene on the chromosome 3B.

Primer sequences for the putative nodulin gene were as follows:

(i) LS forward primer, L_1: TACTTCCTCGAGAAGTACGCCG (SEQ ID NO: 39)

(ii) LS reverse primer, L_2: GTAGAGCGTGATCACCGTGG (SEQ ID NO: 40)

(iii) AS forward primer specific for allele A, A_{1a}: ***GCGCCAAA***GCTTCTGCCAGTC**TC** (SEQ ID NO: 41)

(iv) AS forward primer specific for allele B, A_{1b}: ***GCGCCAAA***GCTTCTGCCAGTGA**G** (SEQ ID NO: 42)

(v) AS reverse primer, A_2: ***GCGTGC***CAGCGAGAAGGTGAG (SEQ ID NO: 43)

The non-complementary 5'tail of the AS primers is in highlighted in red font, and deliberate mismatches at the -2 or -3 positions are underlined.

As shown in Figure 10, increasing the melting temperature of the complementary region in the AS forward primer A_1, relative to the melting temperature of the AS reverse primer A_2, normalizes the annealing efficiency of the AS primer pair during the initial cycles of second phase of TSP amplification. Such normalization facilitates achieve correct genotype determination for genomic loci producing mismatched product in samples.

EXAMPLE 11
Discrimination by differential product detection

For some assay configurations, such as allelic discrimination by differential product detection (see Figures 5 and 6 and Examples 5 and 6), the capture of sequence variation within the second phase PCR amplification product eliminates the requirement for AS primers to contain mismatched nucleotides that can cause primer annealing destabilization.

Results of such an assay are shown in Figure 11. The assay was performed using genomic DNA from barley (*Hordeum vulgare*) using samples with known zygosity. The AS primers have a complementary region melting temperature of 40°C. Assay conditions were essentially as described in Example 9. Primers were designed to assay a SNP in a nicotinate phosphoribosyltransferase-like gene as follows

(i) LS forward primer, L_1: CTACTGGAAGGCCGGCAAGC (SEQ ID NO: 44)

(ii) LS reverse primer, L_2: CGCATAAACCTCAACATCTGAGCA (SEQ ID NO: 45)

(iii) AS forward primer, A_1: ***GCGCCG***GCCGAATCAGTTTG (SEQ ID NO: 46)

(iv) AS reverse primer, A_2: ***GGCGGC***TGAATTCACAGGCTG (SEQ ID NO: 47)

The non-complementary 5'tail of the AS primers is in highlighted in bold italic font.

These assay configurations result in the efficient accumulation of the expected PCR fragment (as shown in Figure 10).

EXAMPLE 12
Blinded Analysis of results

To test the sensitivity and accuracy of the assay of the invention for actual genotype determination, a blinded study was performed using F_4 progeny derived from crosses between the barley lines Chebec and Harrington, Amagi Nijo and WI2585, and Haruna Nijo and Galleon. Assays were developed for 28 SNPs identified by Sanger sequencing in 23 genes located in a region on chromosome 2H containing a frost tolerance QTL, and chromosome 5H containing a malting quality QTL. The mapping populations, each comprising about 250 individuals, were screened independently for each SNP using cleaved amplified polymorphism (CAP) assays (Minamiyama *et al. Plant Breeding*

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124: 288-291, 2005) and the assay of the present invention. For each SNP, two separate assays of the invention were performed, one specific for allele A, and the other specific for allele B. Complete concordance between the two genotyping methods across all assays demonstrated that the assay of the present invention achieves high genotyping accuracy.

EXAMPLE 13
Further evidence of biphasic nature of TSP amplification

To further demonstrate the reaction mechanism for biphasic PCR amplification of TSP genotyping products, real-time PCR assays were performed to monitor the accumulation of LS and AS product in TSP assays configured for allelic discrimination by differential product size using a pair of AS primers designed to opposite DNA strands e.g., as shown in Figure 1. TSP assays were performed using DNA samples with known zygosity and different combinations of the four LS and AS primers (Table S1) to show the contribution of each primer to the accumulation of the expected PCR products.

Real-time PCR assays were performed on a RotorGene6000 thermocycler (Corbett Research) using SYBR Green detection in a 12 µl reaction mixture containing 0.2 mM dNTP, 1x PCR buffer (16 mM $(NH_4)_2SO_2$, 0.01% Tween-20, 100 mM Tris-HCl, pH 8.3), 1.5 mM $MgCl_2$, 100 ng/µl bovine serum albumin Fraction V, 0.1 µM LS primer, 0.5 µM AS primer, 0.45 U Platinum Tfi DNA polymerase (Invitrogen) and 20 ng genomic DNA. Following an initial denaturation step of 2 min at 94°C to heat activate the DNA polymerase, PCR was performed for a total of 65 cycles with the profile: 30 s at 92°C, 30 s at 58°C, 2 min at 72°C for 15 cycles (hereafter referred to as the first reaction phase). The next five cycles were with 10 s at 92°C, 30 s at 45°C, followed by 45 cycles with 30 s at 92°C, 30 s at 53°C, 5 s at 72°C (hereafter referred to as the second reaction phase). The accumulation of reaction products was monitored during each PCR cycle by measuring changes in SYBR Green fluorescence.

Primer combinations used in each assay were as described in the legend to Figure 12.

Primers sequences for gene encoding a putative Rieske Fe-S precursor protein:
LS forward primer, L1: CGAGGATTGGCTCAAGACGC (SEQ ID NO: 78);
LS reverse primer, L2: GCAGCGTTCTTAGGACTGGCA (SEQ ID NO: 79);
AS forward primer, A1: CGAATGGATTCTTCAGAAAAG (SEQ ID NO: 80);

AS reverse primer, A2: GCGTTCCTCTGCCCTTG (SEQ ID NO: 81).

Primers sequences for gene encoding fructose-6-phosphate 2-kinase:
LS forward primer, L1: GCGTCGCAAAGACAAGCTGA (SEQ ID NO: 82);
LS reverse primer, L2: CCGCAGGCGAACCTTTACAT (SEQ ID NO: 83);
AS forward primer, A1: CGTGCATACTGCACAAAAT (SEQ ID NO: 84);
AS reverse primer, A2: GCACCTCATAAAGAATGGTTC (SEQ ID NO: 85).

Primers sequences for gene encoding an unnamed protein product from rice:
LS forward primer, L1: GAAGTCGACGCTGATGGCAA (SEQ ID NO: 86);
LS reverse primer, L2: TCGTGCGATCCGTTTTAGCA (SEQ ID NO: 87);
AS forward primer, A1: GGGTCTTCGGAGCACGA (SEQ ID NO: 88);
AS reverse primer, A2: GCAATCTCGGCGAGAAG (SEQ ID NO: 89).

Primers sequences for gene encoding cytosolic aldehyde dehydrogenase:
LS forward primer, L1: CGGAGATCCTTTCAACCCGA (SEQ ID NO: 90);
LS reverse primer, L2: TCGGATGTCCGTCCAGATCA (SEQ ID NO: 91);
AS forward primer, A1: GGCATTTTGTAACATGTTCAG (SEQ ID NO: 92);
AS reverse primer, A2: CGGTCGGTAAGAGCGAAG (SEQ ID NO: 93).

The non-complementary 5'tail of the AS primers is underlined in each case.

Data shown in Figure 12 indicate that the TSP assay mechanism functions as predicted, because accumulation of PCR product occurs earlier in reactions containing LS primer (Reactions 1 and 2, Figure 12) by virtue of only those primers efficiently hybridizing to genomic template at the high PCR annealing temperature used in the first phase of the reaction. As expected, the accumulation of PCR product was more rapid for reactions containing LS primer (Reactions 1 and 2, Figure 12), compared to reactions containing only AS primer (Reaction 3, Figure 12). These data indicate that the amplification of PCR product in reactions with only AS primer is efficient only after the PCR annealing temperature is lowered in the second stage of the reaction. These results demonstrate an effective partitioning in TSP assays of the participation of LS and AS primers in the first and second reaction stages, respectively.

Furthermore, the real-time PCR data demonstrates an efficient transition from the amplification of LS product to the accumulation of AS product in the second phase of

the reaction. Reactions containing both LS and AS primers (Reaction 2, Figure 12) consistently had lower relative fluorescence at each PCR cycle, compared to reactions containing only LS primer (Reaction 1, Figure 12). Reduced fluorescence corresponds to the transition from amplification of LS product to that of AS product, and is observed because AS product is significantly shorter than LS product (typically by more than 100-bp). SYBR Green dye binds only to double-stranded DNA, producing an increase in fluorescence that is proportional to both the total amount of, and length of the PCR product.

Thus, data in Figure 12 demonstrate the efficient annealing of AS primers to the enriched target sequence (LS product) at the second phase annealing temperature, allowing for highly efficient self-amplification of AS product in subsequent cycles due to incorporation of the non-complementary 5'-tail, and therefore, out-competing of the accumulation of LS product.

EXAMPLE 14
TSP amplification to discriminate alleles in a methylenetetrahydrofolate reductase (MTHFR) gene of humans

This example demonstrates the application of the method of the present invention to discriminating between alleles of a clinically significant diseases and disorders in humans. The methylenetetrahydrofolate reductase gene (MTHFR; GenBank Accession No. NM 005957) is located on human chromosome 1 p36.3. The gene encodes the enzyme, methylenetetrahydrofolate reductase (EC 1.5.1.20), which catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, a co-substrate for homocysteine remethylation to methionine. The most widely studies polymorphism in this gene (C677T; rs1801133) results in an alanine to valine substitution at position 222 resulting in a thermolable enzyme with reduced activity that has been implicated in folic acid deficiency. This polymorphism has also been associated with neural tube defects, arterial and venous thrombosis, cardiovascular disease and schizophrenia. Homozygous mutant (677TT) individuals are at a decreased risk of certain leukemias and colon cancer. The rs1801133 SNP is presented in the following sequence:

TGAAGGAGAAGGTGTCTGCGGGAG[C/T]CGATTTCATCATCACGCAGCTTT (SEQ ID NO: 94).

To produce template nucleic acids for TSP amplification, 48 genomic DNA samples were obtained and purified from human brain tissue and concentration adjusted to 20 ng/µl.

Primers were as follows:

F-LS.MTHFR: TCTTCATCCCTCGCCTTGAA (SEQ ID NO: 95);
R-LS.MTHFR: GCCTGCCGTTTTCTCCTCTT (SEQ ID NO: 96);
F-AS.MTHFR_REF: GCGTGTCTGCGGGAGC (SEQ ID NO: 97); and
R-AS.MTHFR CGGATGGGGCAAGTGAT (SEQ ID NO: 98),

wherein F-LS and R-LS are locus-specific primers LS1 and LS2, respectively, and wherein F-AS and R-AS indicate allele-specific primers AS1 and AS2, respectively.

The non-complementary 5'tail of the AS primers is underlined in each case.

Amplifications were performed in 96-well PCR plates in a 15 µl final reaction volume consisting of 20 ng genomic DNA, 1x PCR buffer (Invitrogen), 100 ng/µl bovine serum albumin Fraction V (Sigma-Aldrich), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.1 µM each locus-specific primer, 0.5 µM each allele-specific primer and 0.375 U Taq DNA polymerase (Invitrogen).

Thermal amplification was performed in a 225-PTC thermal cycler (MJ Research, Bio-Rad) using the following conditions: 94°C for 3 mins, followed by 15 cycles of 92°C for 30 s, 58°C for 30 s and 72°C for 60 s, 5 cycles consisting of 92°C for 10 s and 45°C for 30 s and 15 cycles consisting of 92°C for 10 s, 53°C for 30 s and 72°C for 5 s with a final extension step of 72°C for 10 mins.

Data presented in Figure 13 indicate specific amplification of both alleles which are successfully resolved using 2% (w/v) agarose by virtue of the smaller size and more rapid mobility of the 677T allele relative to the 677C allele. Homozygotes for both alleles, and heterozygotes, are readily identified using this method.

EXAMPLE 15

TSP amplification to identify HSV and discriminate between HSV-1 and HSV-2

This example demonstrates the application of the method of the present invention to detect HSV in a sample and discriminate between HSV-1 and HSV-2.

Herpes simplex virus is a viral infectious agent of humans. There are two infectious forms of the virus: herpes simplex 1 (HSV-1) and herpes simplex 2 (HSV-2). HSV-1 infection is contracted through direct contact with an active lesion or bodily fluid of an infected person. It is generally acquired during childhood and adolescence, and primarily affects the face and mouth. HSV-2 infection is a sexually-transmitted disease and primarily affects the genitalia. Although gential herpes is largely caused by HSV-2, gential HSV-1 infections are common. Similarly, cases of orofacial herpes caused by infection of HSV-2 are known. Diagnostic tests to distinguish HSV-1 and HSV-2 infection types are therefore important.

PCR primers for the TSP assay are based on the nucleotide sequence of the virion glycoprotein B (UL27) gene of HSV-1 (Genbank Accession No. AB252863) and HSV-2 (Genbank Accession No. AB442016). The forward locus-specific (F-LS) and reverse locus-specific (R-LS) primers are designed to nucleic acid sequence conserved between HSV-1 and HSV-2. A single forward allele-specific (F-AS) primer is designed to assay a single nucleotide polymorphism (SNP) distinguishing HSV-1 and HSV-2.

Primers are as follows:

F-LS.HSV: GCCACCGCTACTCCCAGTTT (SEQ ID NO: 99);
R-LS.HSV: CCTCCTCGACGATGCAGTT (SEQ ID NO: 100);
F-AS.HSV-1: CACGACATGGAGCT**G**AA**A** (SEQ ID NO: 101),

wherein F-LS and R-LS are locus-specific primers LS1 and LS2, respectively, and wherein F-AS indicates an allele-specific primer AS1 specific for HSV-1.

The non-complementary 5'tail of the AS primers is underlined in the allele-specific primer, and nucleotides specific for HSV-1 are shown in bold font.

Amplifications are performed in 96-well PCR plates in a 15 µl final reaction volume consisting of 20 ng DNA, 1x PCR buffer (Invitrogen), 100 ng/µl bovine serum albumin Fraction V (Sigma-Aldrich), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.1 µM each locus-specific primer, 0.5 µM allele-specific primer and 0.375 U Taq DNA polymerase (Invitrogen).

Thermal amplification is performed in a 225-PTC thermal cycler (MJ Research, Bio-Rad) using the following conditions: 94°C for 3 mins, followed by 15 cycles of 92°C

for 30 s, 58°C for 30 s and 72°C for 60 s, 5 cycles consisting of 92°C for 10 s and 45°C for 30 s and 15 cycles consisting of 92°C for 10 s, 53°C for 30 s and 72°C for 5 s with a final extension step of 72°C for 10 mins.

HSV-1 in a sample produces a 139-bp amplification product of the F-AS and R-LS primers, whereas HSV-2 in a sample produces a 300-bp amplification product of the same F-LS and R-LS primers. Accordingly, the presence of both the 139 and 300-bp amplification products indicates the presence of both types of herpes simplex virus. The absence of PCR product indicates the absence of both HSV-1 and HSV-2.

EXAMPLE 16
TSP amplification to identify HSV and discriminate between HSV-1 strains

This example demonstrates the application of the method of the present invention to detect HSV-1 in a sample and discriminate between HSV-1 strains MP-S and gC-39-R6.

PCR primers for the TSP assay are based on the nucleotide sequence of the virion glycoprotein B (UL27) gene of HSV-1 (Genbank Accession No. EF177454) and HSV-2 (Genbank Accession No. EF177453). The forward locus-specific (F-LS) and reverse locus-specific (R-LS) primers are designed to nucleic acid sequence specific to HSV-1 such that HSV-2 sequences are not amplified. A single forward allele-specific (F-AS) primer is designed to assay a single nucleotide polymorphism (SNP) distinguishing HSV-1 strain MP-S from HSV-1 gC-39-R6.

Primers are as follows:

F-LS.HSV: CAGCGCCATGTCAACGATATGT (SEQ ID NO: 102);
R-LS.HSV: CGCATCGAGTTTTGGACGAT (SEQ ID NO: 103);
F-AS.HSV (MP-S): TGCATCGCCTCGGC (SEQ ID NO: 104),

wherein F-LS and R-LS are locus-specific primers LS1 and LS2, respectively, and wherein F-AS indicates an allele-specific primer AS1 specific for HSV-1.

The non-complementary 5'tail of the AS primers is underlined in the allele-specific primer, and the nucleotide specific for HSV-1 are shown in bold font.

Amplifications are performed in 96-well PCR plates in a 15 µl final reaction volume consisting of 20 ng DNA, 1x PCR buffer (Invitrogen), 100 ng/µl bovine serum albumin Fraction V (Sigma-Aldrich), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.1 µM each locus-specific primer, 0.5 µM allele-specific primer and 0.375 U Taq DNA polymerase (Invitrogen).

Thermal amplification is performed in a 225-PTC thermal cycler (MJ Research, Bio-Rad) using the following conditions: 94°C for 3 mins, followed by 15 cycles of 92°C for 30 s, 58°C for 30 s and 72°C for 60 s, 5 cycles consisting of 92°C for 10 s and 45°C for 30 s and 15 cycles consisting of 92°C for 10 s, 53°C for 30 s and 72°C for 5 s with a final extension step of 72°C for 10 mins.

The presence of HSV-1 viral strain MP-S produces a 119-bp PCR product resulting from the F-AS and R-LS primers, while the presence of HSV-1 viral strain gC-39-R6 produces a 224-bp PCR product resulting from the F-LS and R-LS primers. Amplification of both the 119 and 224-bp PCR products indicates the presence of both HSV-1 viral strains. The absence of PCR product indicates the absence of both HSV-1 viral strains.

EXAMPLE 17
TSP amplification to identify *Staphylococcus aureus*

This example demonstrates the application of the method of the present invention to detect *S. aureus* in a sample.

S. aureus is a common cause of infections and a major public health threat causing a range of illnesses from minor skin infections to life threatening diseases such as pneumonia, toxic shock syndrome, acut respiratory distress syndrome (ARDS) and septicemia. Methicillin-resistant *S. aureus* (MRSA) strains, which are commonly multidrug resistant, present both a treatment and infection control challenge in hospital settings. Diagnostic tests to distinguish *S. aureus* infection are therefore important.

PCR primers for the TSP assay are based on the nucleotide sequence of the 16S rRNA gene of *S. aureus* (Genbank Accession No. AP009351).

Primers are as follows:

F-LS.SA: TGGAGCATGTGGTTTAATTCGA (SEQ ID NO: 105);

R-LS.SA: TGCGGGACTTAACCCAACA (SEQ ID NO: 106);

F-AS.SA: CGCTTACCAAATCTTGACAT (SEQ ID NO: 107),

wherein F-LS and R-LS are locus-specific primers LS1 and LS2, respectively, and wherein F-AS indicates an allele-specific primer AS1 specific for HSV-1.

The non-complementary 5'tail of the AS primers is underlined in the allele-specific primer.

Amplifications are performed in 96-well PCR plates in a 15 µl final reaction volume consisting of 20 ng DNA, 1x PCR buffer (Invitrogen), 100 ng/µl bovine serum albumin Fraction V (Sigma-Aldrich), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.1 µM each locus-specific primer, 0.5 µM allele-specific primer and 0.375 U Taq DNA polymerase (Invitrogen).

Thermal amplification is performed in a 225-PTC thermal cycler (MJ Research, Bio-Rad) using the following conditions: 94°C for 3 mins, followed by 15 cycles of 92°C for 30 s, 58°C for 30 s and 72°C for 60 s, 5 cycles consisting of 92°C for 10 s and 45°C for 30 s and 15 cycles consisting of 92°C for 10 s, 53°C for 30 s and 72°C for 5 s with a final extension step of 72°C for 10 mins.

The primers used in the first phase of amplification are directed to sequences conserved in all bacteria, whereas the second phase allele-speicifc primer is specific to *S. aureus*. As the 16S ribosomal gene is present in all bacteria, the presence of a 161-bp PCR product resulting from the F-LS and R-LS primers serves as a positive PCR internal control. Absence of this PCR product indicates a failed PCR assay, or the absence of nucleic acid from bacteria in the sample assayed. The presence of bacterium from the *Staphylococcus* genus is detected by the presence of a 124-bp PCR product resulting from the F-AS and R-LS primers, as well as the presence of the 161-bp PCR product.

WE CLAIM:

1. A method for detecting a polymorphism or mutation in nucleic acid, said method comprising:

(i) performing a polymerase chain reaction (PCR) under conditions sufficient to amplify a nucleic acid template comprising a polymorphism or mutation with one or more set(s) of first primers thereby producing a first amplification product, said set(s) of first primers capable of annealing selectively to a nucleic acid template comprising a polymorphism or mutation at a first temperature;

(ii) performing PCR under conditions sufficient to amplify the first amplification product with one or more second primer(s) or set(s) of second primers and/or with one or more of the primers from the set of first primers thereby producing a second amplification product comprising a sequence complementary to the allele-specific region and the tag region, said second primer(s) comprising an allele-specific region capable to annealing to the nucleic acid template and/or the first amplification product and a tag-region that does not anneal to the nucleic acid template, wherein said allele-specific region has a melting temperature (Tm) lower than the first primer and is not capable of annealing selectively to the template nucleic acid or the first amplification product at the first temperature and wherein the second primer is capable of annealing selectively to a nucleic acid comprising a sequence complementary to the allele-specific region and the tag region at about the first temperature, wherein said conditions comprise an annealing temperature suitable for annealing of the allele-specific region of the second primer(s) or set(s) of second primers to the first amplification product and/or the template nucleic acid and for the annealing of the first set of primers to the first amplification product and/or the template nucleic acid;

(iii) performing PCR under conditions sufficient to amplify the second amplification product to produce one or more third amplification product(s), said conditions comprising an annealing temperature suitable for annealing of the second primer(s) or set(s) of second primers to the second amplification product and for annealing of one or more primers from the set of first primers to the second amplification product but not for annealing of the allele specific region of the second primer(s) or set(s) of second primers to anneal selectively to the first amplification product at a detectable level, wherein the third amplification product(s) is/are amplified with the set(s) of second primers and/or a second primer and a first primer; and

(iv) detecting the third amplification product(s) with a detection means,

wherein detection of said third amplification product(s) is/are indicative of the polymorphism or mutation.

2. The method according to claim 1 wherein (i), (ii) and (iii) are performed in a single reaction vessel, and reagents suitable for performing PCR are provided in said reaction vessel, said reagents comprising the first primer or set of first primers and said second primer or set of second primers.

3. The method according to claim 1 wherein the conditions at (i) comprise an annealing temperature suitable for the set(s) of first primers to anneal selectively to the nucleic acid template but not for the allele-specific region of said second primer(s) or said set(s) of second primers to anneal selectively at a detectable level.

4. The method according to claim 1 wherein the second primer(s) comprise one or more 3' terminal nucleotide(s) of the allele-specific region complementary to an allele of said polymorphism or mutation, wherein said primer(s) detectably produce the second amplification product and third amplification product only when said 3' nucleotides anneal to the allele of said polymorphism or mutation.

5. The method according to claim 1 wherein the third amplification product is produced by PCR with a first primer and a second primer.

6. The method according to claim 1 additionally comprising detecting the first amplification product.

7. The method according to claim 1, wherein detection of the third amplification product produced by PCR with a first primer and a second primer homozygous for an allele of the polymorphism or mutation.

8. The method according to claim 6 wherein detection of the third amplification product produced by PCR with a first primer and a second primer and detection of the first amplification product is indicative of a nucleic acid heterozygous for an allele of the polymorphism or mutation.

9. The method according to claim 1 comprising performing a PCR at (ii) with a set of second primers, said set of second primers comprising (i) a second primer comprising one or more 3' terminal nucleotide(s) of the allele-specific region complementary to an allele of said polymorphism or mutation, wherein said primer

only detectably produces the second amplification product and the third amplification product when said 3' nucleotides anneal to the allele of said polymorphism or mutation; and (ii) a second primer that anneals to nucleic acid adjacent to the polymorphism or mutation.

10. The method according to claim 9, wherein the 3' terminal nucleotide(s) of the second primer at (i) anneal(s) to the allele and the third amplification product is produced by a PCR with the set of second primers, thereby indicating an allele of the polymorphism or mutation.

11. The method according to claim 9, wherein the 3' terminal nucleotide(s) of the second primer at (i) do(es) not anneal(s) to the allele and the third amplification product is produced by PCR with the second primer at (ii) and a first primer, thereby indicating an allele of the polymorphism or mutation.

12. The method according to claim 1 comprising performing a PCR at (ii) with a plurality of second primers, wherein individual primers in said plurality comprise one or more 3' nucleotide(s) complementary to a different allele of the polymorphism or mutation wherein said primers only detectably produce a second amplification product and third amplification product when said 3' nucleotides anneal to the allele of said polymorphism or mutation, and wherein primers having different 3' complementary nucleotide(s) also comprise a tag region having different molecular weights.

13. The method according to claim 12 comprising detecting the molecular weight of the third amplification product, wherein said molecular weight is indicative of an allele of the polymorphism or mutation.

14. The method according to claim 1 wherein the detection means comprises performing electrophoresis.

15. The method according to claim 14 wherein the electrophoresis is polyacrylamide gel electrophoresis or capillary electrophoresis.

16. The method according to claim 1 wherein the detection means detects the melting temperature of the third amplification product.

17. The method according to claim 1 comprising performing a PCR at (ii) with a plurality of second primers, wherein individual primers in said plurality comprise one or more 3' nucleotide(s) complementary to a different allele of the polymorphism or mutation wherein said primers only detectably produce the second amplification product and the third amplification product when said 3' nucleotides anneal to the allele of said polymorphism or mutation, and wherein primers comprising different 3' nucleotide(s) also comprise a different detectable marker.

18. The method according to claim 17 comprising detecting the detectable marker, wherein detection of the detectable marker is indicative of the third amplification product.

19. The method according to claim 17 wherein the detectable marker is a fluorescent marker.

20. The method according to claim 1 comprising performing a PCR at (ii) with one or more second primer(s) or set(s) of second primers, said second primer(s) comprising an allele-specific region capable to annealing to nucleic acid adjacent to the polymorphism or mutation, and detecting the third amplification product comprises determining the melting temperature of the third amplification product, wherein the melting temperature of the third amplification product is indicative of the polymorphism or mutation.

21. The method according to claim 1 wherein the Tm of the allele-specific region of the second primer is at least about 10°C less than the Tm of the first primer and/or the second primer.

22. The method according to claim 1 wherein the Tm of the first primer and Tm of the second primer is between about 60°C and about 75°C.

23. The method according to claim 1 wherein the Tm of the allele specific region of the second primer is between about 35°C and about 50°C.

24. The method according to claim 1 additionally comprising providing the nucleic acid.

25. The method according to claim 24 comprising providing the nucleic acid in a biological sample.

26. The method according to claim 1 additionally comprising providing a first set of primers and/or providing a second primer(s) or set(s) of second primers.

27. The method according to claim 1 wherein a first set of first primers is capable of annealing selectively to a genome of a polyploid organism to thereby detect a polymorphism or mutation in that genome.

28. A process for characterizing or identifying one or more individuals, isolates of an organism, cultivars of an organism, species or genera said process comprising performing the method according to claim 1 to detect one or more polymorphisms or mutations, wherein the one or more polymorphisms or mutations is(are) characteristic of the one or more individuals, isolates of an organism, cultivars of an organism, species or genera.

29. A process for identifying an infectious agent in a sample and/or for discriminating between infectious agents in a sample, said process comprising performing the method according to claim 1 to thereby detect one or more nucleic acid sequences of one or more infectious agents, wherein detection of said one or more nucleic acid sequences in the sample indicates the presence of an infectious agent in the sample and/or discriminates between infectious agents in the sample.

30. The process of claim 29, wherein the infectious agent is a virus, bacterium, fungus, protist, protozoan or parasite.

31. A process for identifying a subject having a trait or a disease or having a predisposition to developing a trait or disease, said process comprising performing the method according to claim 1, wherein the polymorphism or mutation is associated with said trait or disease and detection of said third amplification product is indicative of a subject having a trait or a disease or having a predisposition to developing a trait or disease.

32. The process of claim 31, wherein the polymorphism or mutation in is a methylenetetrahydrofolate reductase (MTHFR) gene of humans.

33. The process according to claim 31, wherein the polymorphism or mutation is in a plant gene associated with resistance of a plant to drought, frost, disease or a pest, or a plant gene associated with pre-harvest sprouting or nutritional quality of grain.

34. The process of claim 31 additionally comprising selecting a subject having the trait or a predisposition to developing the trait.

35. The process of claim 34 additionally comprising breeding a non-human subject having the trait or a predisposition to developing the trait.

36. A kit comprising:

(i) one or more set(s) of first primers, said set(s) of first primers capable of annealing selectively to a nucleic acid template comprising a polymorphism or mutation at a first temperature;

(ii) one or more second primer(s) or set(s) of second primers, said second primer(s) comprising an allele-specific region capable to hybridizing to the nucleic acid template and a tag-region that does not anneal to the nucleic acid template, wherein said allele-specific region has a melting temperature (Tm) lower than the first primer and is not capable of annealing selectively to the nucleic acid template at the first temperature and wherein the second primer is capable of annealing selectively to a nucleic acid comprising a sequence complementary to the allele-specific region and the tag region at about the first temperature; and

(iii) optionally, instructions for performing the method according to claim 1.

37. The kit according to claim 36 wherein the set(s) of second primers and the second primer(s) or set(s) of second primers are provided in a reaction vessel suitable for performing polymerase chain reaction (PCR).

38. A method of producing a set of primers, said method comprising:

(i) producing one or more set(s) of first primers, said set(s) of first primers capable of annealing selectively to a nucleic acid template comprising a polymorphism or mutation at a first temperature; and

(ii) producing one or more second primer(s) or set(s) of second primers, said second primer(s) comprising an allele-specific region capable to hybridizing to the nucleic acid template and a tag-region that does not anneal to the nucleic acid template, wherein said allele-specific region has a melting temperature (Tm) lower than the first primer

and is not capable of annealing selectively to the nucleic acid template at the first temperature and wherein the second primer is capable of annealing selectively to a nucleic acid comprising a sequence complementary to the allele-specific region and the tag region at about the first temperature.

39. The method of claim 38 further comprises analyzing nucleotide sequence data to thereby determine a panel of candidate primers for inclusion in a set of primers.

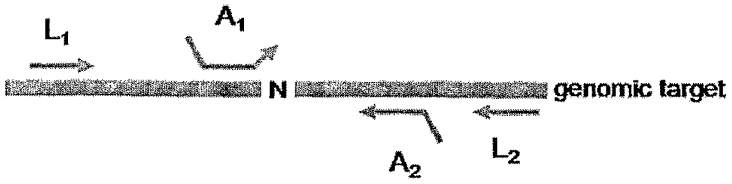
40. The method of claim 38 further comprising determining a panel of first primer(s) and/or second primer(s) that provide discrimination between alleles in nucleic acid comprising a sequence homologous to the nucleic acid template.

41. The method of claim 38 further comprising selecting a panel of first primer(s) and/or second primer(s) that provide discrimination between alleles in nucleic acid comprising a sequence homologous to the nucleic acid template.

42. The method of claim 38 further comprising providing a panel of first primer(s) and/or second primer(s) that provide discrimination between alleles in nucleic acid comprising a sequence homologous to the nucleic acid template.

43. The method of claim 38 further comprising providing information pertaining to the sequences of a panel of first primer(s) and/or second primer(s) that provide discrimination between alleles in nucleic acid comprising a sequence homologous to the nucleic acid template.

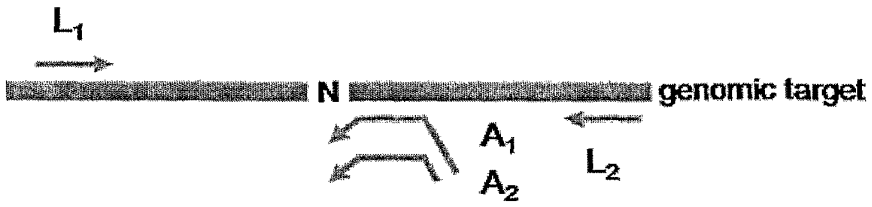
44. A computer-readable medium comprising information pertaining to the sequences of a panel of first primer(s) and/or second primer(s) that provide discrimination between alleles in nucleic acid comprising a sequence homologous to the nucleic acid template, wherein said information is obtained by the method of claim 43.



Predicted TSP amplification pattern:

Genotype	AA	AB	BB
PCR product	L_1A_2	L_1A_2 A_1A_2	A_1A_2

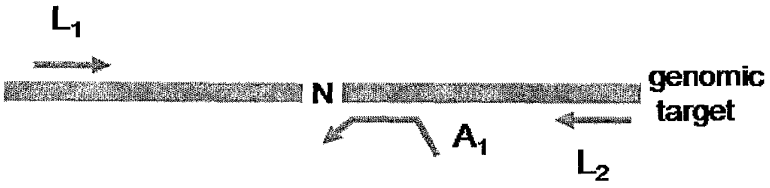
Figure 1



Predicted TSP amplification pattern:

Genotype	AA	AB	BB
PCR product	L_1A_1	L_1A_1 L_1A_2	L_1A_2

Figure 2



Predicted TSP amplification pattern:

Genotype	AA	AB	BB
PCR product	L_1L_2	L_1L_2 L_1A_1	L_1A_1

Figure 3a

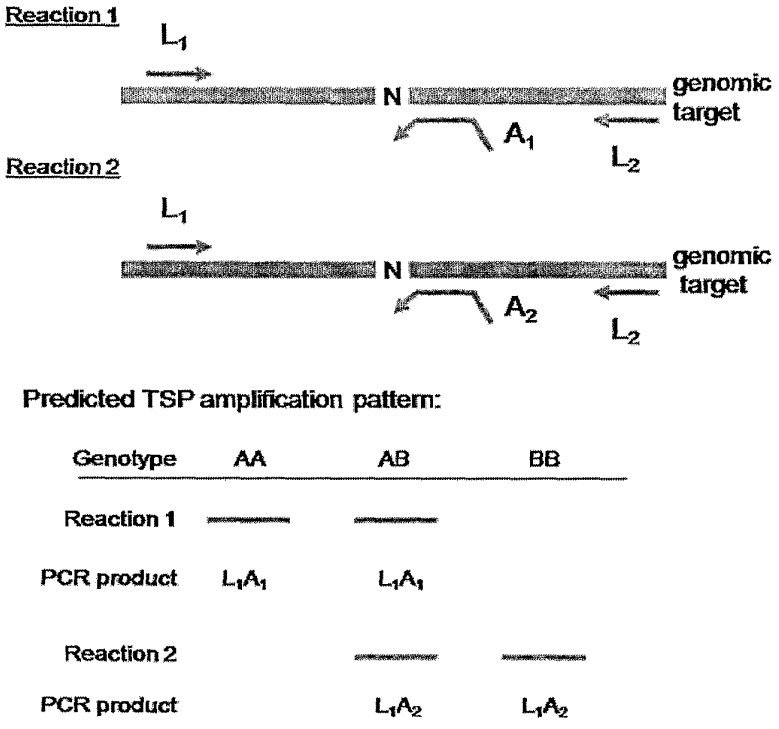
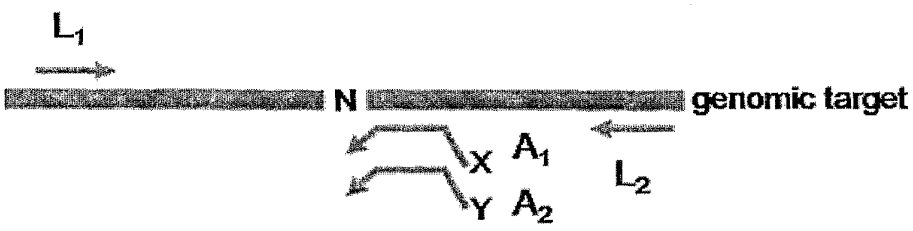

Reaction 1
L1
N
genomic target
A1
L2
Reaction 2
L1
N
genomic target
A2
L2
Predicted TSP amplification pattern:
Genotype
AA
AB
BB
Reaction 1
PCR product
L1A1
L1A1
Reaction 2
PCR product
L1A2
L1A2

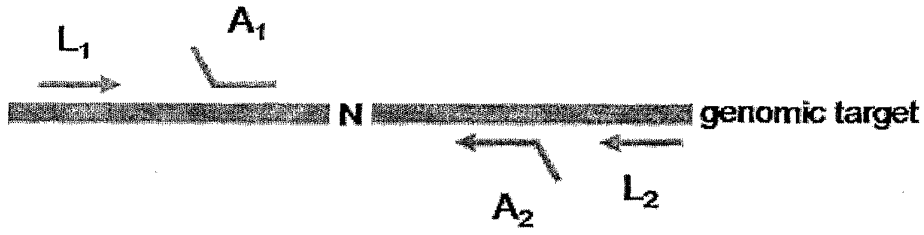
Figure 3b



Predicted TSP amplification pattern:

Genotype	AA	AB	BB
	X	X/Y	Y
PCR product	L_1A_1	L_1A_1 L_1A_2	L_1A_2

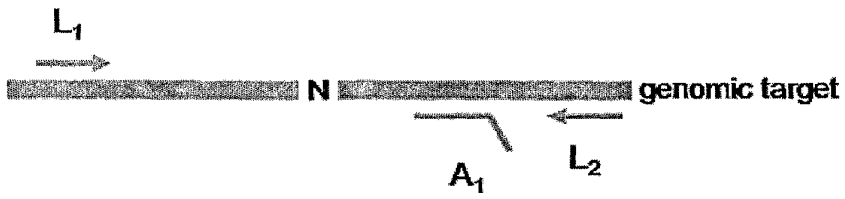
Figure 4



Predicted TSP amplification pattern:

Genotype	AA	AB	BB
PCR product	A_1A_2	A_1A_2	A_1A_2

Figure 5



Predicted TSP amplification pattern:

Genotype	AA	AB	BB
PCR product	L_1A_1	L_1A_1	L_1A_1

Figure 6

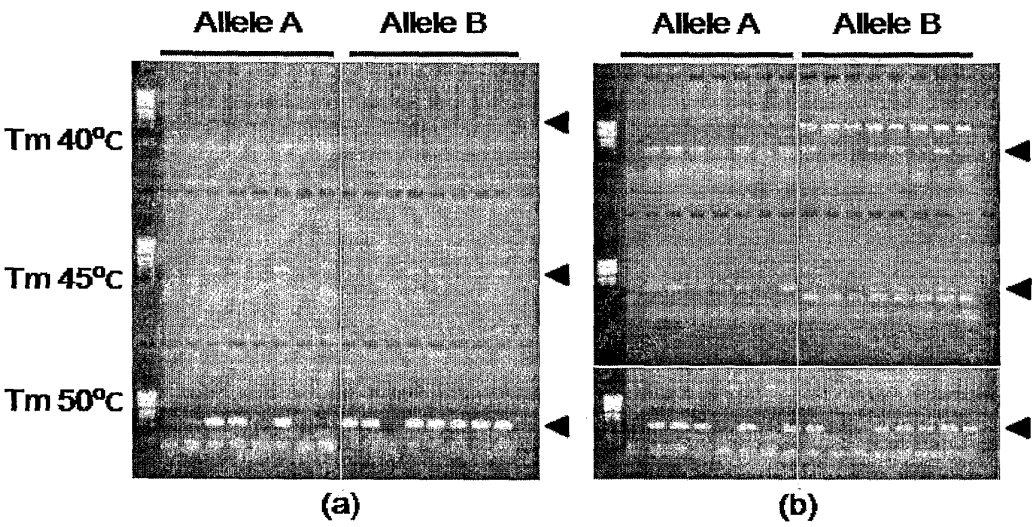


Figure 7

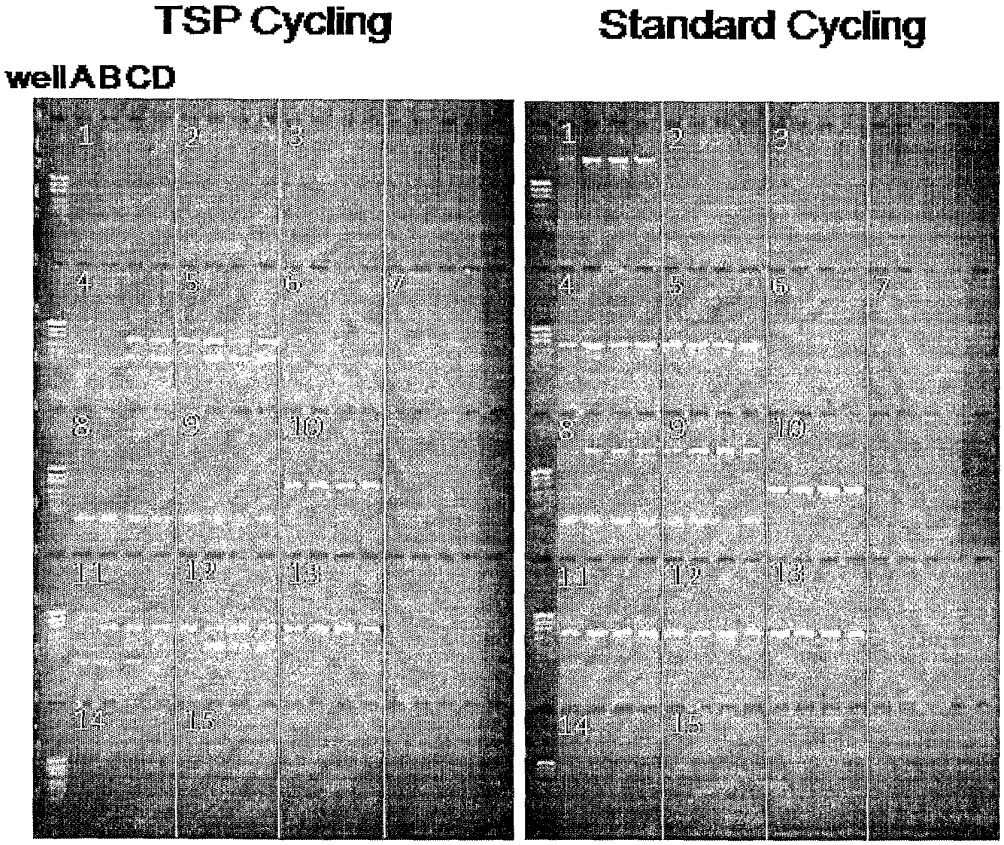

TSP Cycling
Standard Cycling
well A B C D
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15

Figure 8

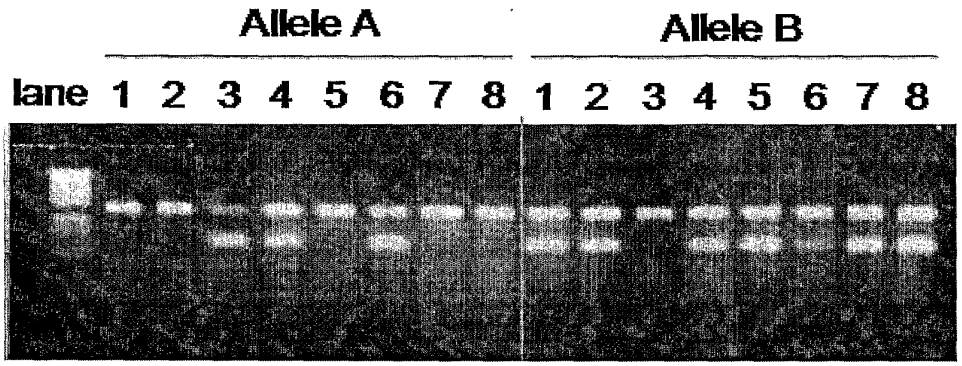


Figure 9a

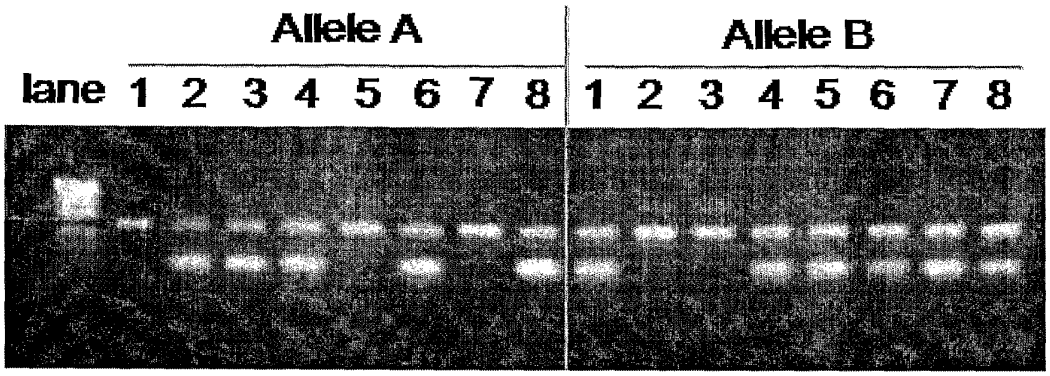


Figure 9b

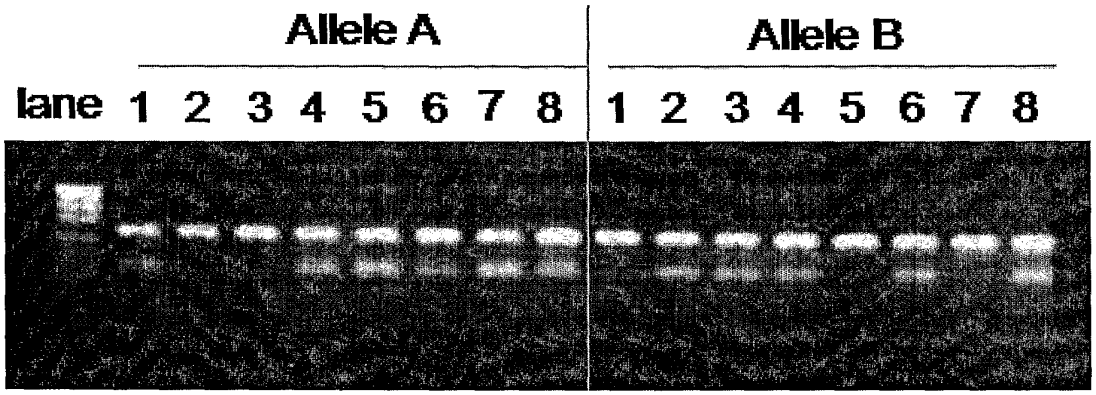


Figure 9c

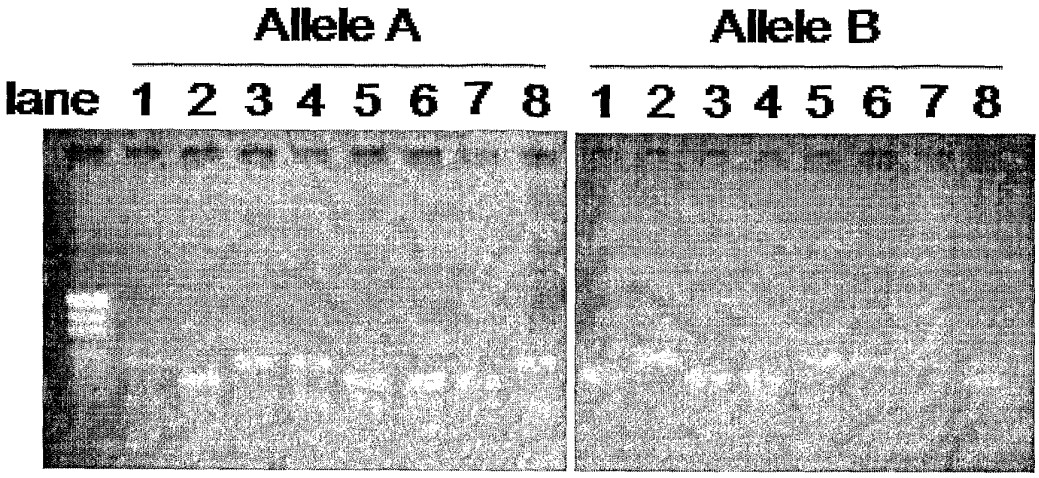


Figure 10

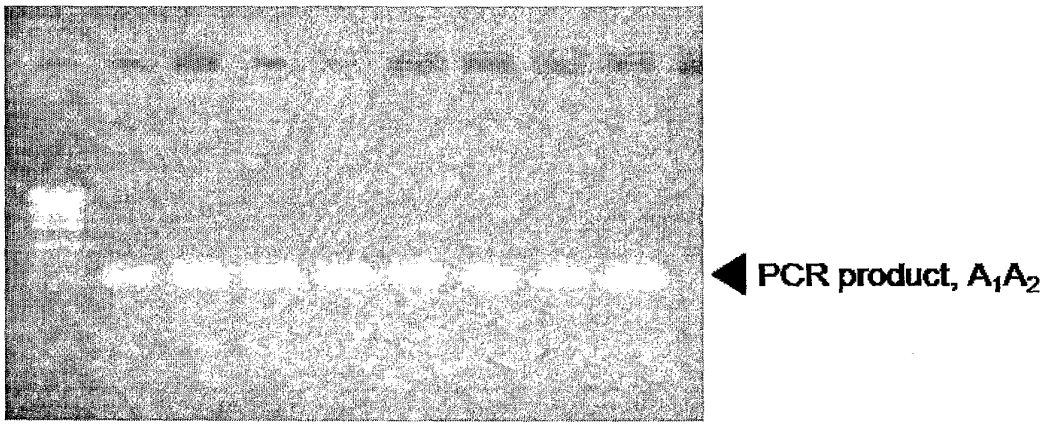


Figure 11

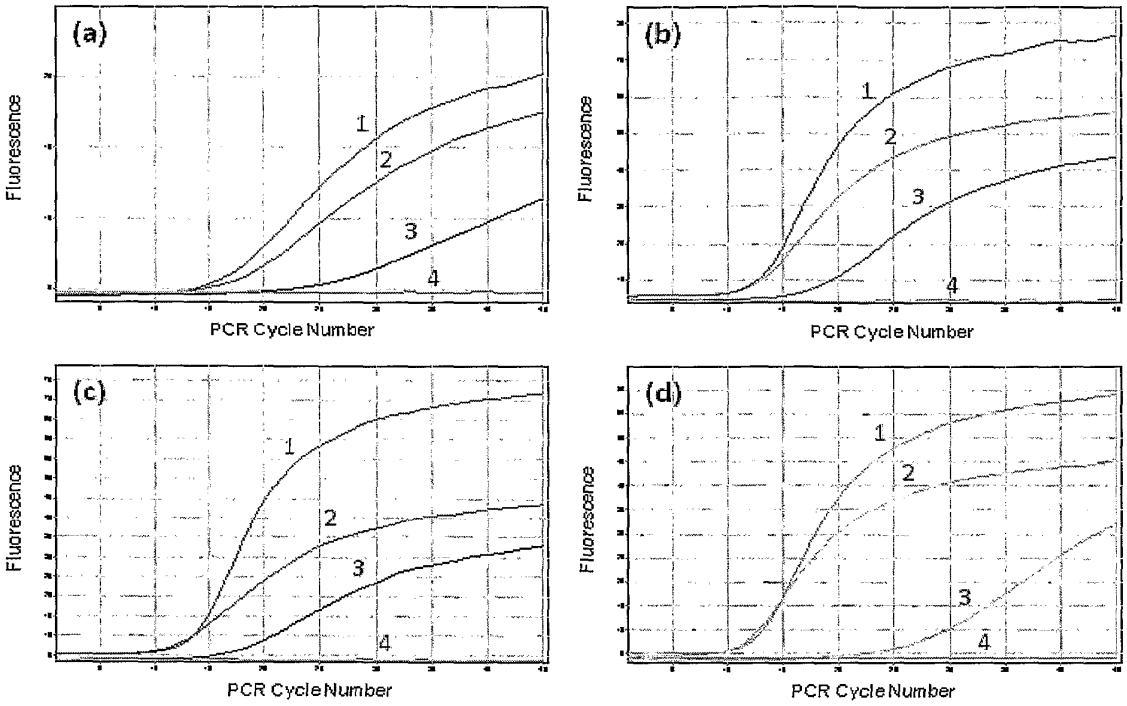


Figure 12

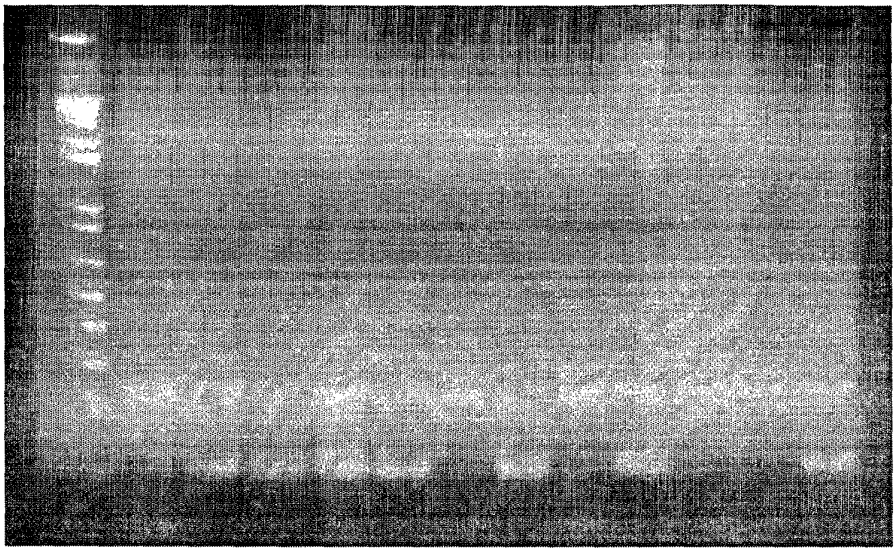


Figure 13

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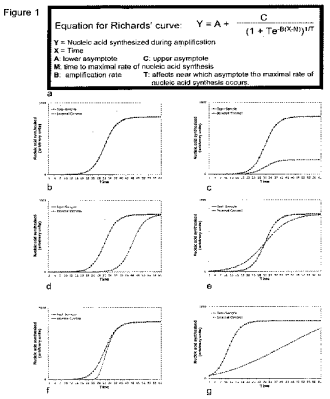
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(54) **Title**: NUCLEIC ACID AMPLIFICATION

Figure 1



(57) **Abstract**: A method for determining the presence and/or amount of a first polynucleic acid in a sample comprising subjecting the sample to nucleic acid amplification in which product is detectable by the presence of a signal generated by polynucleic acid formation from the first polynucleotide characterised in that the nucleic acid amplification reaction is conducted, in the same reaction vessel, with a predetermined amount of a second polynucleic acid which is subjected to nucleic acid amplification, the product of which is detectable by the presence of the same signal generated by polynucleic acid formation from the second polynucleotide as that generated by polynucleic acid formation from the first polynucleotide and wherein the product of the second polynucleic acid is produced with different reaction kinetics from the product of the first polynucleic acid such that the second polynucleic acid acts as an internal control for the method.

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NUCLEIC ACID AMPLIFICATION

FIELD OF THE INVENTION

The present invention relates to the field of nucleic acid amplification. In particular, it relates to a method for specific amplification and detection of a test sample and an internal control in the same reaction vessel.

BACKGROUND

Nucleic acid amplification technology (NAAT) is an invaluable and powerful tool in many areas of research and diagnosis. Such NAAT techniques allow detection and quantitation of a nucleic acid in a sample with high sensitivity and specificity as well as quantitative analysis of nucleic acids in a sample.

Nucleic acid amplification may be used to determine the presence of a particular template nucleic acid in a sample, as indicated by the presence of an amplification product following the implementation of a particular NAAT. Conversely, the absence of any amplification product indicates the absence of template nucleic acid in the sample. Such techniques are of great importance in diagnostic applications, for example, for determining whether a pathogen is present in a sample.

The prior art has described a variety of thermocycling and isothermal techniques for amplification of nucleic acids. Thermocycling techniques, such as the polymerase chain reaction (PCR), use temperature cycling to drive repeated cycles of DNA synthesis leading to large amounts of new DNA being synthesised in proportion to the original amount of template DNA. A number of isothermal techniques have also been developed that do not rely on thermocycling to drive the amplification reaction. Isothermal techniques, which utilise DNA polymerases with strand-displacement activity, have been developed for amplification reactions that do not involve an RNA-synthesis step. Similarly, for amplification reactions that do involve an RNA-synthesis step, isothermal techniques have been developed that may use reverse transcriptase, RNase H and/or a DNA-dependent RNA polymerase (see for example, Nucleic Acid Isothermal Amplification Technologies - A Review. Nucleosides, Nucleotides and Nucleic Acids, Volume 27, Issue 3 March 2008, pages 224 – 243).

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One thing all NAAT techniques have in common is that it is often essential that the reaction is monitored by suitable controls in order to ensure that a negative result is actually due to the absence of the nucleic acid rather than due to other factors, for example the presence of inhibitors in a sample. The prior art describes several methods to achieve this.

One method is to perform two amplification reactions in separate vessels in parallel. One vessel contains the test sample and the other contains a nucleic acid of a known sequence, which serves as a positive control, in addition to the test sample. If no amplification is detected in the test sample but an amplification product can be detected in the control sample, the test can be considered true negative. Likewise, if no amplification product is present in the control, an inhibitor must be present in the sample.

The use of an internal control, i.e. a control which is amplified in the same reaction vessel, has some advantages over using two vessels to establish whether inhibitors are present. Firstly, fewer tubes and reagents are required, thereby reducing the unit cost per test where an inhibitor control is absolutely required. Secondly, fewer manipulations are required. Thirdly, since fewer tubes are required, more samples can be analysed per unit capacity of the hardware used to run the reaction. For example, using a standard 96 well detection system, 96 samples can be analysed using an internal control whereas only 48 samples can be analysed where two vessels must be used per test.

The internal control method presents the technical challenge of differentiating between the signal resulting from the target polynucleotide in the sample and the signal resulting from the control nucleic acid. To detect the signals from a sample and the control nucleic acid in the same vessel, it is necessary that the sequence of the control nucleic acid has some associated difference from the target nucleic acid to allow a detection system to differentiate between the two amplified products. Further still, there needs to be some means to differentiate the respective signals from the two amplification processes. This has been achieved by the use of separate reporter systems for the test-sample and control respectively. For example, two fluorescent probes may be employed of different

emission maxima (or different enough for their respective signals to be differentiated) one which only gives a signal on binding to the products of the test-sample amplification process, and one which only gives a signal when binding to the products of the control products. In this way, by detecting two independent signals from a sample, it is possible to follow the respective amplification processes in the same reaction vessel.

Alternatively, where appropriate, a melt-curve analysis of the results of an amplification reaction can be performed to assess if there is signal from the test-sample and control. This may or may not encompass the use of fluorescent probes (see, for example EP1109934). The test-sample and the control are thereby amplified with the same reaction kinetics. The disadvantage of the melt-curve analysis is that it requires an additional step after the amplification reaction in order to detect the control which does not only elongate the process but also adds significant complexity to the hardware which is required to detect the control and the amplified polynucleic acid.
Where two or more reporter systems must be employed, the hardware used to detect the reporter must be sophisticated enough to perform measurements and differentiate at least two reporter signals. Further, for practical applications in diagnostics, these readings must be performed on a small sample, or multiple small samples: typically reactions volumes are between 10-100µl to avoid high reagent costs per test. This requires very sophisticated hardware which is very expensive.

Hence, there is a need in the art for improved detection methods for detection of nucleic acids and internal controls in the same reaction vessel. In particular, a method whereby an internal control could be monitored without the need for two separate reporter signals to be independently measured, would be of great benefit as this would omit the necessity for expensive hardware that can measure multiple signals following NAAT. Furthermore there are some NAAT techniques which utilize reporter technologies whereby it is not possible to measure more than one type of signal from a sample. Such methods include reporter systems based on bioluminescence (published International patent applications WO 2004/062338 & WO 2006/010948), turbidity (published International patent application WO 01/83817) or certain electrochemical methodologies.

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SUMMARY OF THE INVENTION

The invention provides a method for specific amplification and detection of a polynucleic acid and an internal control in the same reaction vessel.

Therefore, in one embodiment the present invention provides a method for determining the presence and/or amount of a first polynucleic acid in a sample comprising subjecting the sample to nucleic acid amplification in which product is detectable by the presence of a signal generated by polynucleic acid formation from the first polynucleotide characterised in that the nucleic acid amplification reaction is conducted, in the same reaction vessel, with a predetermined amount of a second polynucleic acid, the product of which is detectable by the presence of the same signal generated by nucleic acid formation from the second polynucleotide as that generated by polynucleic acid formation from the first polynucleotide and wherein the product of the second polynucleic acid is produced with different reaction kinetics from the product of the first polynucleic acid such that the second polynucleic acid acts as an internal control for the method.

In this specification the term "first polynucleic acid" is used interchangeably with "test-sample" and "target polynucleotide".

The nucleic acid amplification product is detectable by the presence of a signal generated by polynucleic acid formation. The signal may be generated from the product of amplification directly (e.g. by detecting polynucleic product generated by nucleic acid amplification) or the signal may be generated from by-products of the amplification (e.g. by detecting products which are produced during the process of amplification).

The underlying principle of the invention is the recognition that an internal control does not necessarily need to be involved in exactly the same amplification process as that of the test-sample. Rather, both the test-sample and standard need only share the same vessel and amplification reagents but not necessarily exactly equivalent amplification processes. Thus, it is possible to distinguish the amplification products from the target nucleic acid and the control.

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In this way, it is possible to combine in a single vessel two amplification reactions which have different kinetics of amplification. Where the two amplification methods have sufficiently differing reaction kinetics it is possible, from a set of readings of signal against time, to resolve signals resulting from amplification of the test sample, from amplification resulting from the internal control. The respective signals from the two amplification processes may vary, for example, in respect of their maximal signal magnitude. In this way, an effective internal control is accomplished that can be analysed on hardware far simpler than fluorescent methods which require two signals to be measured from the vessel.

A method according to the invention may be practised with any NAAT known in the art in which the amplification product is detectable by the presence of a signal generated by polynucleic acid formation.

Some NAAT techniques require that the sample temperature is cycled between different temperatures in order to achieve amplification of a target nucleic acid sequence. Examples of such methods are Polymerase Chain Reaction (PCR, US 4683202) and Ligase Chain Reaction (LCR; US5185243). Other NAATs may operate substantially at a single temperature. Of these some are dependent on transcription as part of the amplification process, for example Nucleic Acid Sequence Based Amplification (NASBA; US5409818) and Transcription Mediated Amplification (TMA; US5399491) while others are dependent on the action of a Helicase or Recombinase for example Helicase Dependent Amplification (HDA; WO2004027025) and Recombinase polymerase amplification (RPA; WO03072805) respectively, others still are dependent on the strand displacement activity of certain DNA polymerases, for example Strand Displacement Amplification (SDA; US5455166), Loop-mediated Isothermal Amplification (LAMP; WO 00/28082, WO 01/34790), Chimeric Displacement Reaction (RDC) (WO9794126), Rolling Circle Amplification (RCA; Lizardi, P. M. et al. Nature Genetics, (1998) 19.225-231), Isothermal Chimeric Amplification of Nucleic Acids (ICAN; WO0216639), SMart Amplification Process (SMAP; WO2005063977).

The methods used for amplification of the target nucleic acid and the control may be the same or different.

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In one embodiment of the invention the internal control is amplified using an exponential nucleic acid amplification whilst the test-sample is amplified using a non-exponential, amplification process. However, in a preferred embodiment it is the internal control that is amplified using a non-exponential nucleic acid amplification and the test-sample using an exponential nucleic acid amplification. This is preferred because there is less chance of the amplification associated with the internal control, out-competing that of the test-sample.

Examples of exponential NAATs which can be used in accordance with the embodiments of the present invention include, but are not limited to, polymerase chain reaction, SDA, LAMP, ICAN, SMAP, RDC, (exponential)-RCA, NASBA, TMA, HDA and RPA.

Examples of non-exponential NAATs which can be used in accordance with the embodiments of the present invention include, but are not limited to Rolling circle amplification, asymmetric PCR, asymmetric LAMP (Isolation of single-stranded DNA from Loop-Mediated Isothermal Amplification (LAMP) products. Kentaro Nagamine, Yoko Kuzuhara et al. Biochemical and Biophysical Research Communications, Vol.290, No.4, 1195-1198, 2002) or any of the aforementioned exponential NAATs performed under conditions that affect the ability of the amplification process to successfully re-copy copies of the original template polynucleic acid.

The invention does not strictly require that the internal control be amplified by a non-exponential (e.g. linear) amplification process and the test-sample by an exponential process. Rather, it is only necessary that the kinetics and/or signal amplitudes associated with the respective amplification reactions differ significantly. Therefore, in a further embodiment, the invention provides a method wherein the first nucleic acid and the internal control are amplified using exponential nucleic acid amplification and wherein the two amplification reactions have different reaction kinetics, i.e. where the two reactions have fundamentally different kinetic description (i.e. where a different mathematical description is necessary to describe the internal control kinetics compared to the test-sample, for example where one process is linear and the other is exponential).

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In this embodiment, it is necessary to be able to ascertain, from a negative sample, that the signal from the internal control is not, in fact, a signal from the test-sample. For example, these different reaction kinetics may be achieved by virtue of the signal from the internal control

a) having a smaller or bigger amplitude than the test sample

b) having a longer or shorter lag-time before maximal amplification

c) having a slower or faster intrinsic rate of amplification, or

The above features allow mathematical algorithms to analyse a set of signal readings against time to ascertain whether the signal encompasses that of positive sample, a signal from the internal control alone or no signal from either the test-sample or the internal control (as would happen in the presence of an inhibitor).

For example, with reference to i) above, an algorithm could be employed to check whether the signal ever reached an amplitude of A_{IS}, associated with the expected amplitude from the internal control. If the amplitude failed to achieve an amplitude of A_{IS} this would then be associated with inhibition of the sample. If the amplitude was greater than A_{IS}, the signal would be expected to stem from the test sample. "Greater than X" thereby means that the amplitude is at least 1.2times, 1.5times, 1.8times, 2times, 2.5 times, 3times, 3.5times, 4times, 4.5times, 5times, 6times, 7times, 8, times, 9times, 10times, 20times, 30times, 40times, 50times or even 100timess bigger than the amplitude of the internal control. The value of A_{IS} can thereby be easily determined by performing the method of the invention without the first polynucleic acid.

Similarly, with reference to ii) above, an algorithm could be employed to check whether a signal of particular amplitude was achieved at time T_{IS}, which would be solely associated with a signal from the internal control, or whether a signal of a particular amplitude was not achieved until after time T_{IS}, which would be associated with an inhibited sample. If a signal of a particular amplitude was achieved before time T_{IS}, this would be associated with a signal from the test sample.

Further, with reference to iii) above, an algorithm could be employed to check whether the rate of change of a signal achieved a predetermined amount ΔS_{IS} associated solely with signal from an internal control, whether the rate of change of signal failed to exceed

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ΔS_{IS} associated with an inhibited sample or whether the signal was greater than ΔS_{IS}, which would then be associated with a signal from a positive sample. "Greater than ΔS_{IS}" thereby means that the rate of change of a signal is at least 1.2times, 1.5times, 1.8times, 2times, 2.5 times, 3times, 3.5times, 4times, 4.5times, 5times, 6times, 7times, 8, times, 9times, 10times, 20times, 30times, 40times, 50times or even 100times greater than the rate of change of the internal control. The value of ΔS_{IS} can thereby be easily determined by performing the method of the invention without the first polynucleic acid.

Further, an algorithm could be employed to fit the signal generated from a sample to particular kinetic models to ascertain which kinetic description best fitted the data. For example, if the rate of change of signal fitted to an exponential rate equation with a better fit than a linear rate equation then the signal may be assigned to the amplification of target polynucleic acid in the test-sample rather than the internal control, whereas if the signal had been best fitted to a linear rate equation then the signal could be assigned to the internal control, if the signal approximates a straight line of zero gradient, then the signal could be assigned to be that from an inhibited sample. Suitable algorithms will be evident to those of skill in the art.

There is a variety of ways in which the kinetics of amplification processes can vary. For example, the kinetics of amplification can be described by a) the rate constant of amplification b) the time to reach maximal amplification c) the starting and final amplitudes of the measured parameter (i.e. the upper and lower asymptotes of the curve) and d) whether the amplitude of the signal at the time of maximal amplification is nearer the upper or lower asymptote. Such a description of a kinetic curve is known as Richards' Curve (see Figure 1). However, a variety of other means to describe and differentiate between two different kinetic processes can also be envisaged other than that described by Richards' Curve.

Different kinetics of amplification can be achieved in various ways. In one embodiment of the invention, an internal control is provided wherein the binding sites for the primers used to exponentially amplify the polynucleotide sequence of interest are either partially absent or non-optimal in the control. Various examples of how such an internal control can be designed are shown in Figure 10. For example in methods which require two

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primer binding sites (A and B) for exponential amplification (for example polymerase chain reaction, strand displacement amplification and Isothermal Chimeric Amplification of Nucleic Acids) different kinetics can be achieved by the following means:

- only one of the primer binding sites (A or B) is present on the internal control
- both primer binding sites are present but more separated in sequence compared to the target polynucleotide. "More separated" thereby means that the distance between the two primers is bigger by at least 10 nucleotides, 50 nucleotides, 100 nucleotides, 500 nucleotides, 1000 nucleotides or even 5000 nucleotides.
- both primer binding sites are present but separated by a region which is slower for a polymerase to copy compared to the target polynucleotide.
- both primer binding sites are present but either one or both of the primer sites contain miss-matches that cause amplification to occur less efficiently
- one or both primer binding sites are present on a circular polynucleic acid molecule but in an orientation that prevents exponential amplification

All these examples could result in amplification kinetics for the internal control that are distinguishable from the exponential process amplifying the target polynucleic acid and are therefore an embodiment of the present invention. This approach does not require any additional reagents to be present in the amplification mix other than the internal control polynucleic acid; therefore it is facile to envisage how it is possible to accommodate both the amplification of the test sample and the amplification of the internal control in the same reaction mix where both processes are subject to the same concentrations of polymerase, nucleotide precursors, pH, salts and other additives.

Where an amplification method uses more than two primer binding sites to enable exponential amplification of a target polynucleic acid (for example Loop-mediated Isothermal Amplification (LAMP)), some of these primer sites are absolutely required for exponential amplification, whereby some merely affect the rate of amplification. As such, by varying which primer sites are present in the internal control, one can modulate amplification kinetics. This method forms one embodiment of the invention.

The generation of internal control polynucleotide sequences containing only a subset of desired primer binding sites and interspersed with sequences of choice is facile using

modern recombinant DNA technologies. Further the generation of circular templates is known in the art via the use of DNA ligases.

In a further embodiment the internal control is amplified by different primers, or partially different primers, compared to the target polynucleotide sequence of interest so offering further means to amplify the internal control polynucleotide via a significantly different kinetic profile (Figure 11). The word "different" thereby means that the primers used for amplification of the target polynucleic acid and the internal control have less than 80%, 75%, 70%, 65%, 60%, 55%, 50%, 45%, 40%, 35%, 30% or even less than 25% homology. Further, by limiting the amount of the primers used for amplification of the internal control, it is possible to limit the total amount of amplicon produced from the internal control polynucleic acid: this would mean that the signal from the internal control would be of lower amplitude than that of the test sample. "Limiting the amount" thereby means that the primers used for amplification of the internal control are added at concentrations which are less than 80%, 75%, 70%, 65%, 60%, 55%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10% or even 5% than the concentrations of primers used for amplification of the target polynucleotide. It is only necessary that the mechanism of amplification afforded by these different primers is compatible with the amplification process associated with amplifying the test sample for the two processes to occur in the same vessel. The skilled person, armed with the teachings of the present invention, will be able to ascertain whether primers are suitable for use in the chosen amplification process.

In a further embodiment, an internal control is effected by taking advantage of the activity of certain proteins acting on polynucleotide sequences used as the internal control, whereby the protein generates priming sites for the polymerase used within the control polynucleotide, without the need for the binding of extraneous primers in the amplification process in such a way that *de novo* polynucleotides resulting from copying the supplied polynucleotide is generated at a linear (or slower) rate than that of polynucleotide of interest from the test-sample. As long as said protein can function under the amplification conditions then it is possible to combine both amplification reactions in the same vessel. Examples of such proteins include RNase Hs, Nickases, restriction endonucleases, & priming proteins (e.g. adenovirus priming protein pTP).

One way to achieve this (Figure 12a) is to add to the reaction mixture a polynucleic acid (which is not similar to the sequence of the target polynucleic acid) which can be acted on by some additive to generate priming sites for nucleic acid synthesis. Preferably the additive is a protein that generates priming sites either via enzymatic action on the internal control polynucleotide or via some ability to act as a primer itself. For example, the inventors have found that TLi RNaseH can ellicit linear amplification of polynucleotides in a linear-like fashion under conditions used to perform methods such as Loop-mediated Isothermal Amplification (LAMP) or RDC. In this way an internal control can be achieved by adding to the amplification mixture the internal control polynucleotide (defined as a polynucleotide having no homology to the target polynucleotide and no deliberate primer binding sites for any of the primers used that can be acted on by the additive) and e.g. Tli RNaseH.

Alternatively, a further means to elicit amplification of an internal control without primer binding sites or additional reagents/proteins/enzymes, is to use as an internal control a polynucleic acid capable of self-replication under the amplification conditions used to amplify any target nucleic acid from the test sample (Figure 12b). Examples of polynucleic acid sequences capable of self replication include circular templates capable of undergoing rolling circle amplification (as in linear RCA) or linear templates whereby hairpin structures generated at the 3' end cause the template to recopy it-self (this is an inherent feature of the LAMP technology for example). This forms a further embodiment of the invention.

Some nucleic acid amplification processes have been described as "linear" rather than "exponential" in the art, due to the fact that the kinetics of amplicon production appear to fit to a straight-line equation. In many cases, "linear" or "close to linear" kinetics have been sought to improve, for example, quantification methods or means to amplify various nucleic acid targets without biasing the amplicon to a particular population of sequences (something exponential amplification methods are more prone to do). Examples of "linear" or "close to linear" amplification methods include the aforementioned RCA (where only a single primer is used), asymmetric PCR (WO03040397) and also "Linear amplification of specific nucleic acid sequences" (US 6743605), also the novel isothermal method described in WO/2007/030505, to name a

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few. In general, such "linear" methods describe amplifications processes whereby the target nucleic acid molecule is copied (perhaps repeatedly) but the copies are not themselves copied.

However, it is possible for an amplification processes to differ from another by virtue of the copies being only partially copied, or copied less efficiently or copied only under certain conditions.

In another embodiment of the present invention, different reaction kinetics are achieved by amplifying the first polynucleic acid and the internal control with the same or two similar amplification processes but in such a way that each amplification process can be independently controlled such as to affect the kinetics of amplification. The inventors have recognised that within a single vessel where the reagents for two distinct amplification processes are mixed, that one of the amplification processes can be favoured by virtue of the conditions chosen. In this way it is possible to affect the 'time to maximal amplification' as described in Richards' curve, in particular. This can allow kinetic differentiation of the two amplification processes and thus enable the present invention. Hence, in one embodiment amplification of the first polynucleic acid and the internal control is performed with the same amplification technique, wherein amplification of the first polynucleic acid and the internal control can be controlled by extrinsic conditions.

In one embodiment, the two amplification reactions are performed at different temperatures. In either amplification reactions where the temperature is repeatedly cycled or amplifications that are performed largely isothermally, the temperature thereby refers to the temperature at which the polynucleic acid is amplified and/or the temperature at which the primers bind to the primer binding sites on the polynucleic acid. In this embodiment it is possible to perform the amplification reaction at a temperature whereby one of the two amplification processes is favoured and hence occurs at a faster rate than the other amplification process then to later change the temperature such that the other amplification process is able to occur at a higher rate than before the temperature change. This could be achieved by careful selection of primers such that the melting temperature (Tm) of the primers associated with the

respective processes were significantly different. Two primers are thereby considered to have a different Tm when the Tm of the primers differs by at least 1°C, 2°C, 3°C, 4°C, 5°C, 6°C, 7°C, 8°C, 9°C or 10°C. A method wherein primers with different Tm values are used to amplify the first polynucleic acid and the internal control is an embodiment of the present invention.

Another way to achieve this, more applicable to PCR, is to choose two amplification processes whereby the extension time required for amplification is substantially different. The reaction may be started under conditions where one of the amplification reactions can occur given a particularly extension time but that the other is completely or substantially inhibited; following an appropriate period of time the extension time could, e.g. be increased to better suit the other amplification process. In this way it would be possible to ascertain whether amplification was observed (via a single signal type) from two separate amplification processes. Hence, a method wherein the amplification time is varied in order to achieve different reaction kinetics forms one embodiment of the invention.

An example of a means by which to produce amplification of an internal control of noticeably different kinetics to that used on the test-sample is shown in Figure 5a. The BART-LAMP (Bioluminescent Assay in Real-Time-Loop-mediated Isothermal Amplification) technique which is used in this example is described in further detail below. Here is shown the BART output from the amplification of a target nucleic acid molecular where two of the six primers normally used in LAMP are omitted from the amplification mix. As a consequence, for a given amount of target DNA, the lag-time to reach maximal amplification is far longer than that for the full amplification mix containing all six primers. Further, the breadth of the BART light peak (a reflection of intrinsic amplification rate) is far broader than that normally observed with the full amplification mix containing all six primers. Therefore one means to implement the present invention is to have the internal control amplified via the LAMP method but omitting, for example, two of the six primers normally employed for amplification.

A further example of a means by which to produce amplification of an internal control of noticeably different kinetics to that used on the test-sample is shown in Figure 5b. In

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this case, an amplification process which is dependent on Tli RNaseH and Bst Polymerase is shown acting on a DNA target consisting of DNA co-purified with Tli RHaseH during purification of Tli RNase H from recombinant E.Coli. The amplification produces very high molecular weight amplicon in a close-to linear fashion. As a result, the BART light peak appears very different (much broader) than light peaks resulting from, e.g. LAMP. As such, the type of amplification process demonstrated here exemplifies the present invention.

Verification that the Tli RNase H dependent amplification can act as an internal control is shown in Figure 6. Here LAMP and the Tli RNase dependent amplification process are combined in the same tube. When the test is positive a sharp light peak from LAMP is seen at approximately 30 minutes, when the test is negative, the slow amplification from the Tli RNase dependent amplification process is observed instead. This reflects the model data shown in figures 3a and 3b (remembering that BART reports the instantaneous rate of amplification whereas figure 3 shows the accumulation of nucleic acid). As such, it is possible to ascertain from a sample, whether or not the sample is positive, negative or inhibited by combining a LAMP reaction with the Tli RNase dependent amplification process.

Another important advantage of the present invention is that both amplification reactions can be detected with the same signal. Therefore, the method of the present invention obviates the need to purchase and maintain specialised equipment that would be necessary if more than one signal would need to be detected.

Various signals which can be used to detect polynucleic acid amplification are known in the art. These include electrochemical signals, turbidity, bioluminescent signals and fluorescent signals. Detection by the same signal thereby means that both amplification reactions are detected with the same kind of signal, for example bioluminescence.

In one embodiment the BART-LAMP reporter system is used to detect the signals. This system has been explained in detail in WO2004/062338 and WO2006/010948, which are hereby incorporated by reference. BART is an example of a reporter system designed for isothermal NAATs which gives a single type of signal from a sample: a bioluminescent

signal. BART utilises the firefly luciferase-dependent detection of inorganic pyrophosphate: this is produced in large quantifies when 'target' sequences are detected using a NAAT. As such, molecular diagnostics can be achieved with BART simply by measuring the light emitted from closed tubes, in a homogeneous phase assay. BART is proven with several different NAATs, operating between 50-63°C. The BART reporter is a particularly effective means to follow the rate of amplification of a NAAT since the light output represents a measure of the instantaneous rate of amplification (whereas e.g. fluorescent outputs show the accumulation of a signal and hence the measurements have to be differentiated to obtain the amplification rates). By way of example, Figure 4 shows BART being used in conjunction with LAMP to detect a dilution series of a particular target DNA molecule. Note that as the amount of target DNA in the sample decreases, the lag-phase to reach the time of maximal light increase (which is proportional to the lag-phase to reach maximal amplification) increases. Put differently, the time to reach the characteristic light peak associated with positive samples in BART increases in inverse proportion to the amount of target nucleic acid in the sample. It is stressed that whilst further examples make use of the BART reporter system, the present invention is not limited to the use of BART and is equally applicable to methods such as fluorescence, turbidity, other spectroscopic techniques or electrochemical measurement methods.

The second polynucleic acid, which acts as an internal control, needs to be chosen such that its sequence is sufficiently different from the sequence of the first polynucleotide. Two polynucleic acids are thereby deemed sufficiently different when they are less than 100%, 95%, 90%, 85%, 80%, 75%, 70%, 65%, 60%, 55%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, or even 5% homologous. The amount of nucleic acid which needs to be added to the reaction will be evident to the person skilled in the art and can be easily determined, for example by testing various concentrations of the polynucleic acid in the NAAT which is to be used. However, it is expected that the amount of polynucleic acid which will need to be added lies between 10μg and 100 ag, 100ng and 100fg or even 100pg and 100fg.

The term "internal control", as used herein, refers to any nucleic acid which is known to be amplified under certain conditions provided in the method of the invention. The

internal control is thereby not restricted to polynucleic acids which were obtained from the same source as the test polynucleic acid, but rather encompasses any polynucleic acids given that such a polynucleic acid satisfies the criteria set out for the second polynucleic acid. The internal control may used as a control of the reaction itself and can also be used as a standard to quantitate the test-sample. Importantly, the inventors have shown that amplification of an internal control is inhibited by the presence of inhibitors in a sample, thereby confirming the suitability of the internal control as a control for amplification of the test sample (see figure 7).

Preferably, the method of the invention is performed in a sealed vessel. This is of great utility since it reduces or even prevents the possibility of the sample becoming contaminated. Moreover, it reduces or even prevents the possibility of the laboratory becoming contaminated. This is particularly important as if even one copy of the template nucleic acid were to escape into the laboratory, this could potentially contaminate other samples to be tested and give false- positive results. Thus, the ability to prevent contamination is of particular importance where a method of the invention is used in a diagnostic application. Methods by which to seal the reaction vessel will be evident to the person skilled in the art and include, but are not limited, to plastic films, wax, oil and foil covers.

A method according to the invention may be used in diagnostic applications. In particular the method allows identification of organisms in a patient sample and other samples. The organism may be any microorganisms, such as viruses, bacteria and fungi. The microorganism can be pathogenic but it may also be a non-pathogenic microorganism.

"Patient sample" refers to any sample taken from a patient and can include blood, stool, swabs, tissue samples, urine or spinal fluids. Other suitable patient samples and methods of extracting them are well known to those of skill in the art. A "patient" or "subject" from whom the sample is taken may be a human or a non-human animal. When a sample is not specifically referred to as a patient sample, the term also comprises samples taken from other sources. Examples include swabs from surfaces, water samples (for example waste water, marine water, lake water, drinking water), any other environmental samples

(for example air) food samples, cosmetic products, pharmaceutical products, fermentation products, cell and microorganism cultures and other samples in which the detection of a microorganism is desirable.

In a further aspect, there is provided a kit for use in a method according to the invention. Preferably such a kit comprises all the components necessary to practise the method of the invention, except the target polynucleic acid which is to be tested, except where the target polynucleic acid forms part of a supplied positive control or in quantitative testing where the known amount of target is added for reference.

A kit for use in a method according to the invention preferably comprises a nucleic acid polymerase, the substrates for the nucleic acid polymerase and primers for amplification of the target polynucleic acid and the internal control. More preferably, the kit further comprises buffer reagents, such as a source of magnesium ions. Alternatively, a kit for use in a method according to the invention may comprise only some of these components and/or additional components. The sample and any other components that have been omitted from the kit may then be added to the kit during use.

When BART is used for detection of the polynucleic acids a thermostable luciferase, luciferin and an enzyme that converts PPi to ATP, such as ATP sulphurylase, and any other required substrates or cofactors of the enzyme that converts PPi to ATP, such as adenosine 5' phosphosulphate, may be included in the kit. Thus in one embodiment a kit for use with BART comprises nucleic acid polymerase, b) the internal standard, c) at least two primers suitable for amplification of the test-sample and the internal standard, d) a thermostable luciferase, e) luciferin, f) ATP sulphurylase, and g) adenosine 5'phosphosulphate.

Preferably, at least one of the components of the kit is lyophilised or is in another form which is suitable for storage in the kit. More preferably, all of the components of the kit are lyophilised or in one or more other forms suitable for storage. Such other forms include components to which stabilising factors have been added and/or a refrigerated or frozen mastermix that contains the components of the kit.

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A further application of a method according to the invention is for determining whether a particular nucleic acid sequence is present in an organism's genetic code. For example, it could be used for determining whether the nucleic acid to which the template nucleic acid originates has been genetically modified, for detection of DNA associated with a particular non-genetically modified breed of plant or a genetically modified plant, for detection of DNA associated with pedigree breeds of animal or for medical or veterinary diagnostic applications such as genetic testing or forensic.

Various aspects and embodiments of the present invention will now be described in more detail by way of example. It will be appreciated that modification of detail may be made without departing from the scope of the invention.

DESCRIPTION OF FIGURES

Figure 1. Example of a mathematical model for a growth/amplification curve known as Richards' Curve. a) version of the Richards' curve equation adapted for nucleic acid amplification; b) two amplification processes with identical kinetics c) two amplification processes which differ in the amount of amplicon they can finally produce d) two amplification processes which differ in the time it takes to reach maximal amplification e) two amplification processes with differing amplification rates f) two amplification processes where, at the time to reach maximal amplification, the signal differs between how close they are to the respective asymptotes g) two amplification processes which are both described by Richards' curve: N.B. one of the processes appears at first inspection to be linear (i.e. a straight line). As such, in practice, it is not always easy (or necessary) to be sure whether a process is truly exponential or linear from a particular set of measurements; the issue with respect to the present invention is whether the separate processes can be unambiguously deconvoluted from the shared signal.

Figure 2. Mathematical model of the invention. This demonstrates the general principle that it is possible to follow two separate processes, in a closed tube, using a single signal so long as the two processes give signal outputs that are sufficiently different by way of their kinetic description or amplitude.

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Figure 3. Mathematical model of the invention demonstrating that, even when the two processes are occurring simultaneously, as opposed to sequentially, as shown in Figure 1, it remains possible to differentiate amplification from the test sample compared to amplification from the control by virtue of differences in the intrinsic rate of amplification.

Figure 4. Exponential amplification of a dilution series of a target nucleic acid sequence using the LAMP amplification technology in combination with the BART reporter technology. The BART technology is a bioluminescent reporter system whereby only a single type of signal can be emitted from the sample. BART, unlike other reporter systems, gives both an increase and rapid decrease during amplification: the time to the peak of light is inversely proportional to the amount of target nucleic acid in the sample. Further, the width of the peak of light is a function of the inherent rate of amplification of the NAAT being monitored.

Figure 5a. Delayed exponential amplification of a target nucleic acid;

Figure 5b. Reduced rate of change of bioluminescent output observed in isothermal amplification of nucleic acids via a Tli-RNaseH dependent process that shows a much slower inherent rate of amplification than compared to e.g. LAMP. In fact the process appears to be close to a linear rate of amplification. The precise mechanism of this Tli-RNaseH dependent process is unclear but appears to be caused by the action of Tli-RNaseH on DNA containing small amounts of RNA-DNA heteroduplexes originating from either or both transcription or DNA Primase..

Figure 6. Exponential and non-exponential amplification recorded from a single-tube assay in the presence (1) or absence (2) of target nucleic acid.

Figure 7. Inhibition of genomic DNA amplification in Lamp-BART with different concentrations of Buffered Peptone Water (BPW), a known inhibitor of NAATs, or CTP. 7a. Inhibition of target-containing sample (1 - no BPW, 2 - 5x diluted BPW, 3 - 2x diluted, 4 - non-diluted BPW); 7b. Inhibition of no-template control (1 - no BPW, 2 - 5x

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diluted BPW, 3 - 2x diluted, 4 - non-diluted BPW); 7c. Inhibition of target-containing sample (1-3) and no-template control with different concentrations of CTP (1 and 4 – no CTP, 2 and 5 – 2 mM CTP, 3 and 6 – 3 mM CTP).

Figure 8. Mathematical model of the invention demonstrating two processes occurring simultaneously in the absence and presence of inhibitors. a) Two types of kinetics in the absence of inhibitors; b) Real-time monitoring of positive and negative samples with internal inhibition control.

Figure 9. Amplification of a target DNA molecule, via the NAAT known as RDC, in the presence of an internal control based on RNaseH Tli-based amplification as followed by BART. In dotted light grey is shown the successful amplification of the target DNA molecule as evidenced by the rapid increase and then more rapid still decrease in light from the sample. This amplification takes place in the presence of the internal control. In solid black is shown the result of amplification when no target DNA is present and the only amplification detected is from the internal control. In this case, the light peak from the BART reporter system is visibly broader than that of the target DNA molecule, in particular, it lacks the rapid decrease in light after maximal light emissions, associated with exponential amplification processes. As such, in regarding the black trace, it is facile for an observer to establish that a) the amplification reagents are not inhibited, since amplification has occurred, but that b) amplification is from the internal control and not the test-sample as the shape of the BART output is not that associated with rapid exponential amplification. A computer algorithm would be able to distinguish between the two curves on the basis that the positive result has a far faster negative rate of change following the light peak compared to the internal control.

Figure 10 shows a variety of methods to achieve, in a single vessel, two amplification processes of different kinetics.

Figure 11 reflects all the features shown in figure 10 except in this case the internal control polynucleotide may contain primer binding sites for primers not used to amplify the target polynucleic acid.

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Figure 12 shows two alternatives to using internal control polynucleotides which have primer binding sites either for the primers used to amplify the target polynucleic acid or any further primers to help amplify the internal control.

EXAMPLES

Example 1

LAMP-BART of genomic Salmonella DNA purified by ChargeSwitch® direct gDNA Kit (Invitrogen) was amplified in Lamp-BART at 55°C on Lucy, bespoke imaging hardware (Lumora). The reaction mixture contained: 0.8 μM LampB primer (AACCTTGTGGAGCATATTCGTGGTTTTCCGCCATTGGCGAATTTATG), 0.8 μM LampF primer (TCTCTTGGCGCCCACAATGTTTTTAAGCGAACGTGTTTCCG), 0.4 μM LoopB primer (CAATGGCGCGTTATATTTG), 0.4 μM LoopF primer (GAGCGCTTCCATAATTAATTTC), 0.2 μM DisplB primer (CATTACTGCTCGTAATTC), 0.2 μM DisplF primer (ATATCTGAAGTTTTGCAGC) (MWG), 1.6 mM dNTPs (total) (Invitrogen), 0.16 U/μl Bst (NEW ENGLAND BIOLABS), 0.1 mg/ml luciferin (Europa Bioproducts), 0.5 mM adenosine 5'-phosphosulphate (Biolog), 5.6 μg/μl firefly luciferase (Ultra Glow, Promega), 0.125 U/ml ATP-sulphurylase (Sigma) in 1x Thermopol buffer (New England Biolabs) with some stabilisers and additives. The total volume of each reaction was 20 μl. Observed BART signals responded to the exponential DNA amplification by an increase in light followed by a prompt decrease in light for positive samples containing different starting target copy number and by a steady gradual decay for negative sample that did not contain any target DNA (Figure 4).

Example 2

Delayed exponential amplification of a target nucleic acid was observed under the conditions identical to those in Example 1 but omitting LoopB and LoopF primers. The lag-time preceding the flash signal was detected even for high copy number of target nucleic acid and became significantly longer; the exponential rise was much slower (Figure 5a). The half-width of the flash observed in this example was three times bigger than in the exponential amplification described in Example 1.

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Reduced rate of amplification was demonstrated by a non-specific, linear-like isothermal amplification of nucleic acids via a Tli-RNaseH driven process at 55°C on Lucy, bespoke imaging hardware (Lumora). The reaction mixture contained: 0.32 U/µl Tli-RNaseH (Takara), 1.6 mM dNTPs (total) (Invitrogen), 0.16 U/µl Bst (New England Biolabs), 0.1 mg/ml luciferin (Europa Bioproducts), 0.5 mM adenosine 5'-phosphosulphate (Biolog), 5.6 µg/µl firefly luciferase (Ultra Glow, Promega), 0.125 U/ml ATP-sulphurylase (Sigma) in 1x Thermopol buffer (New England Biolabs) with some stabilisers and additives, DNA target for the Tli-RNaseH driven process. The total volume of each reaction was 20 µl. Observed BART signals responded to the nucleic acid amplification in a significantly slowed down, almost linear, gradual rise followed by a decrease in light output (Figure 5b). The half-width of these signals was even bigger than in the previous case.

Example 3

Exponential amplification of genomic Salmonella DNA by LAMP was combined in a single tube assay with the aforementioned near-linear Tli-RNaseH dependent amplification of nucleic acids and monitored by BART at 55°C on Lucy, bespoke imaging hardware (Lumora). The reaction mixture contained: 0.8 µM LampB primer, 0.8 µM LampF primer, 0.4 µM LoopB primer, 0.4 µM LoopF primer, 0.2 µM DisplB primer, 0.2 µM DisplF primer (MWG), 0.32 U/µl Tli-RNaseH (Takara), 1.6 mM dNTPs (total) (Invitrogen), 0.16 U/µl Bst (New England Biolabs), 0.1 mg/ml luciferin (Europa Bioproducts), 0.5 mM adenosine 5'-phosphosulphate (Biolog), 5.6 µg/µl firefly luciferase (Ultra Glow, Promega), 0.125 U/ml ATP-sulphurylase (Sigma) in 1x Thermopol buffer (New England Biolabs) with some stabilisers and additives, DNA target for Tli-RNaseH driven amplification. Total volume of each reaction was 20 µl. Sample containing target sequence responded with a sharp flash reflecting fast exponential production of DNA. Sample without the target sequence but with the DNA target for Tli-RNaseH driven amplification responded (i.e. the internal control) with a slow rise of the light signal (Figure 6)

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Example 4

Inhibition of amplification of genomic DNA from Salmonella (2 ng per assay) with buffered peptone water (BPW) or CTP was demonstrated in LAMP-BART under the conditions described in Example 3. 5 µl of BPW or CTP pre-diluted to different concentrations was added into 15 µl of the assay mixture either containing target DNA, positive samples, or no-target DNA negative samples. The results observed in positive and negative samples with BPW are shown in Fig. 7a and Fig. 7b and with CTP in Fig. 7c, respectively. Addition of non-diluted and 2x pre-diluted BPW completely inhibited non-exponential amplification in negative samples and significantly slowed down exponential amplification in positive samples. 5x diluted BPW had only a slight slowing down effect on both amplifications. Similarly addition of 3 mM CTP completely inhibited non-exponential amplification in negative samples and slowed down significantly exponential amplification in positive sample, while 2 mM CTP just slowed down both amplifications. This shows that the near-linear, Tli-RNaseH driven amplification acted as an effective internal control for the detection of inhibitors.

Example 5

The results of mathematic modelling of exponential and slow non-exponential kinetics required for internal inhibition control in NAAT in a single-tube assay are shown in Figure 8a. The results of mathematic modelling of the effect of inhibitor on the single parameter in real-time detection of NAAT with an internal inhibition control are shown in Figure 8b.

Example 6

A reaction mixture of the following constituents was made up at 4°C: 20 mM Tris-HCl pH 8.8, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 4.8 mM $MgSO_4$, 0.1% Triton X-100, 500 µM of each dNTP, 10 U RNaseOut, 25 pmol Lm-InlA-RDCus3r2d (tgactgaaccagctaagcctgUAAaa), 25 pmol Lm-InlA-RDCus3r2d (cgttgctgtgtagctgttaatacUAAat), 6.25 pmol InlA Df-v2 (ataatctactgtttgagatg), 6.25 pmol InlA Db-v2 (taatgctaagtttcatgtg), 0.1 mg/ml LH2, 0.5 mM APS, 140 µg rLUC, 3.125 U

ATP sulphurylase, 4 U Bst polymerase large fragment, 1 U Tli RNase H II. 20 µl of the above mixture was added to two separate tubes containing 5 µl of either 10 or 0 copies of a plasmid harbouring the InlA gene. The reactions were placed from 4°C to a BART imaging system and ran for 260 minutes at 60°C with 240 image acquisitions.

In dotted light grey is shown the successful amplification of the target DNA molecule as evidenced by the rapid increase and then more rapid still decrease in light from the sample. This amplification takes place in the presence of the internal control. In solid black is shown the result of amplification when no target DNA is present and the only amplification detected is from the internal control.

Claims

1. A method for determining the presence and/or amount of a first polynucleic acid in a sample comprising subjecting the sample to nucleic acid amplification in which product is detectable by the presence of a signal generated by polynucleic acid formation from the first polynucleotide characterised in that the nucleic acid amplification reaction is conducted, in the same reaction vessel, with a predetermined amount of a second polynucleic acid which is subjected to nucleic acid amplification, the product of which is detectable by the presence of the same signal generated by polynucleic acid formation from the second polynucleotide as that generated by polynucleic acid formation from the first polynucleotide and wherein the product of the second polynucleic acid is produced with different reaction kinetics from the product of the first polynucleic acid such that the second polynucleic acid acts as an internal control for the method.
2. The method of claim 1 wherein the first polynucleic acid is amplified in exponential nucleic acid amplification and the internal control is amplified in non-exponential nucleic amplification.
3. The method of claim 1 wherein the first polynucleic acid is amplified using non-exponential nucleic acid amplification and the internal control is amplified using exponential nucleic amplification.
4. The method of claims 1-3, wherein the exponential nucleic amplification is selected from the group consisting of polymerase chain reaction, Strand Displacement Amplification (SDA), Loop-mediated Isothermal Amplification (LAMP), Isothermal Chimeric Amplification of Nucleic Acids (ICAN), SMart Amplification Process (SMAP), Chimeric Displacement Reaction (RDC), (exponential)-rolling circle amplification (exponential-RCA), Nucleic Acid Sequence Based Amplification (NASBA), Transcription Mediated Amplification (TMA), Helicase Dependent Amplification (HAD) and Recombinase polymerase amplification (RPA).
5. The method of claims 1-4, wherein the linear nucleic amplification is selected from the group consisting of rolling circle amplification, asymmetric

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polymerase chain reaction (asymmetric PCR), Rolling circle amplification, asymmetric PCR and asymmetric LAMP.

6. The method of claim 1 wherein the first polynucleic acid is amplified using exponential nucleic acid amplification and the internal control is also amplified using exponential nucleic acid amplification, and wherein the two nucleic acid amplifications have different reaction kinetics.

7. The method of claim 6, wherein the nucleic acid amplification of the first polynucleic acid and the internal control differ in one or more of the following parameters:

a) amplitude

b) lag-time before maximal amplification or

c) intrinsic rate of amplification

8. The method of any one of claims 1, 2 and 4-7, wherein the nucleic acid amplification involves the use of one or more primers binding to primer binding sites on the polynucleic acid and at least one of the primer binding sites used to exponentially amplify the first polynucleotide sequence is either partially absent or non-optimal in the internal control in order to achieve different reaction kinetics.

9. The method of claim 7, wherein one of the primer binding sites present on the first polynucleic acid is absent on the internal control and wherein the nucleic acid amplification requires two primer binding sites.

10. The method of claim 6, wherein the nucleic acid amplification involves the use of primers binding to two primer binding sites on the polynucleic acid and wherein the primer binding sites are more separated on the internal control compared to the first polynucleic acid.

11. The method of claim 6, wherein the nucleic acid amplification involves the use of primers binding to two primer binding sites on the polynucleic acid and wherein the primer binding sites are separated by a region which is slower for a polymerase to copy compared to the target polynucleotide.

12. The method of claim 6, wherein the nucleic acid amplification involves the use of primers binding to two primer binding sites on the polynucleic acid and wherein one or both of the primer sites on the internal control contain

miss-matches that cause amplification to occur less efficiently in the internal control compared to amplification of the first polynucleic acid.

13. The method of claim 8, wherein one or both primer binding sites are present on a circular polynucleic acid molecule but in an orientation that prevents exponential amplification and wherein the amplification reaction requires two primer binding sites.

14. The method of claim 1, wherein the first polynucleic acid and the internal control are amplified by different nucleic acid amplification techniques.

15. The method of claims 1-13, wherein the first polynucleic acid and the internal control have less than 100% homology.

16. The method of claims 1-15, wherein the reaction is performed in a sealed vessel.

17. The method of claims 1-16, wherein the signal used for detection of the amplification products is selected from the group consisting of: a fluorescent signal, an electrochemical signal, a bioluminescent signal and turbidity.

18. The method of claim 17, wherein the bioluminescent signal is detected by a real time bioluminescent assay.

19. The method of claims 1-18, wherein amplification of the first polynucleic acid and the internal control is performed with the same amplification technique but wherein amplification of the first polynucleic acid and the internal control can be controlled by extrinsic conditions.

20. The method of claim 19, wherein amplification of the first polynucleic acid and the internal control is performed at different temperatures.

21. The method of claim 20, wherein the primers used for amplification of the first polynucleic acid and the internal control have different Tm values.

22. The method of claims 1-21 for use in diagnostic applications.

23. The method of claims 1-22 for use in detecting an organism in a sample.

24. The method of claims 1-23 for use in detecting a microorganism in a sample.

25. A kit for performing the method of claims 1-24 comprising a polynucleic acid for use as an internal control and primers which allow for amplification of the first polynucleic acid and the internal control with different reaction kinetics.

26. The kit of claim 25, comprising a thermostable luciferase, luciferin and an enzyme that converts PPi to ATP and any other required substrates or cofactors of the enzyme that converts PPi to ATP.

27. The kit of claim 25 or 26, comprising a control polynucleic acid containing at least two primer binding sites which are separated by a region which is slower for a polymerase to copy compared to the target polynucleic acid and two primers which can bind to said primer binding sites on the control polynucleic acid.

28. The kit of claim 25 or 26, comprising at least two primers binding to at least two primer binding sites on the target polynucleic acid and on the control polynuleic acid and a control polynucleic acid containing miss-matches in the primer sites that cause amplification to occur less efficiently in the control compared to amplification of the target polynucleic acid.

29. The kit of claim 25 or 26, comprising a control polynucleic acid and primers that result in the control polynucleic acid to be amplified by a different nucleic acid amplification technique compared to the target polynucleic acid.

30. The kit of claim 25 or 26, comprising a control polynucleic acid which is a circular polynucleic acid and which contains two primer binding sites and primers which bind to the control polynucleic acid in an orientation that prevents exponential amplification of the control polynucleic acid.

31. The kit of claim 25 or 26, comprising one or more primers that bind to primer binding sites on the polynucleic acid, wherein at least one of the primer binding sites used to exponentially amplify the first polynucleotide sequence is either partially absent or non-optimal in the internal control in order to achieve different reaction kinetics.

32. The kit of claim 25 or 26, wherein the nucleic acid amplification is exponential and requires two primer binding sites and one of the primer binding sites present on the first polynucleotide sequence is absent on the internal control, such that the nucleic acid amplification of the first polynucleic acid and the internal control differ in one or more of the following parameters:

a) amplitude;

b) lag-time before maximal amplification; or

c) intrinsic rate of amplification.

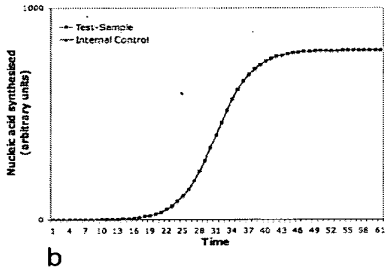
33. The kit of claim 25 or 26, wherein the primers bind to two primer binding sites on the polynucleic acid and wherein the primer binding sites are more separated on the internal control compared to the first polynucleic acid, such that the two nucleic acid amplifications are exponential and have different reaction kinetics.

Figure 1

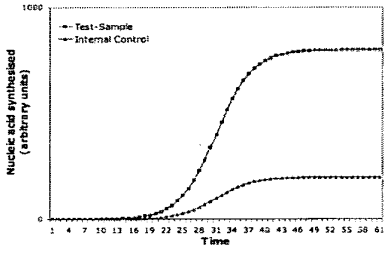
Equation for Richards' curve: $Y = A + \frac{C}{(1 + Te^{-B(X-M)})^{1/T}}$

Y = Nucleic acid synthesized during amplification
X = Time
A: lower asymptote **C**: upper asymptote
M: time to maximal rate of nucleic acid synthesis
B: amplification rate **T**: affects near which asymptote the maximal rate of nucleic acid synthesis occurs.

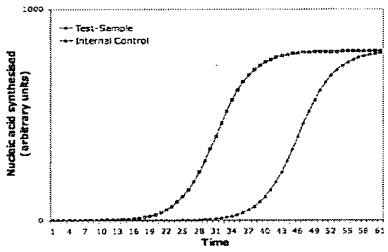
a



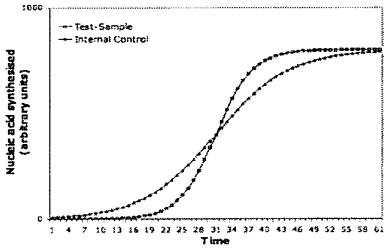
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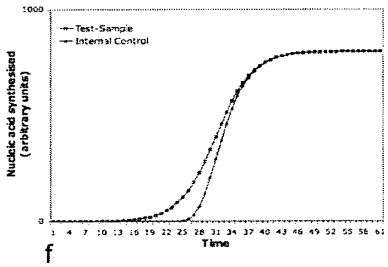
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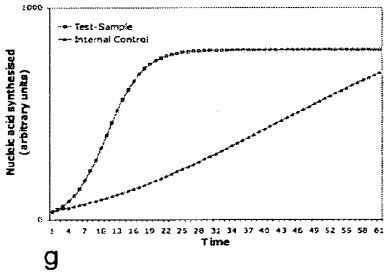
d



e

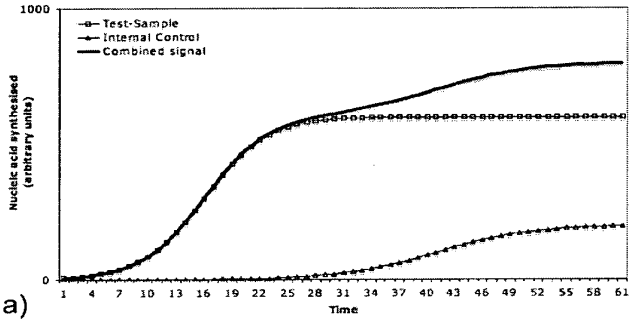


f



g

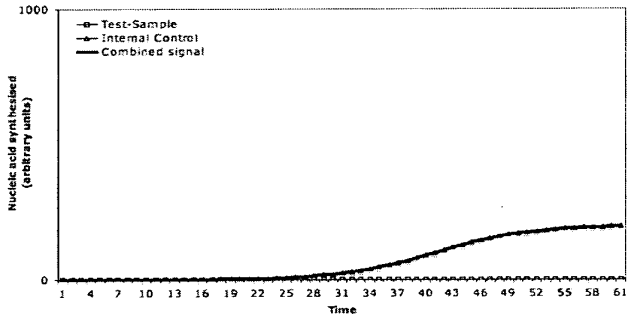
Figure 2



a)

Positive assay
The target sequence is rapidly amplified from test sample; the inhibitor control is also amplified, but more slowly.

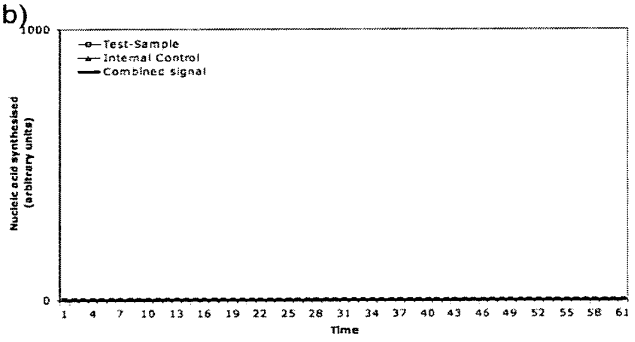
It can be seen that, In principle, the observed signal can indicate the amplification of both the target and control since it is clearly a component of two processes.



b)

Negative assay
The target sequence is not amplified from test sample; the inhibitor control is amplified.

N.B. since the signal from the control shows slower kinetics, It is possible from the observed combined signal actually measured, to deduce that this signal must be from the control and not the test sample.



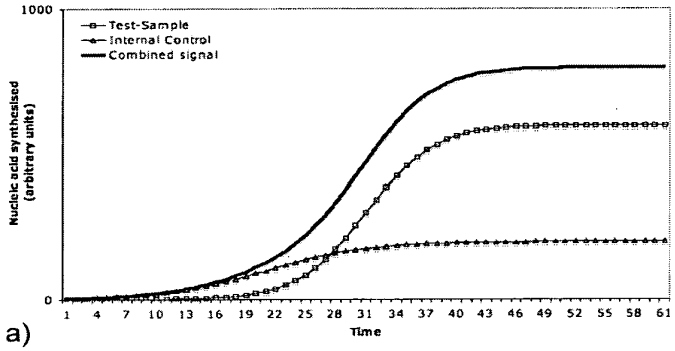
Inhibited assay
Neither the test sample nor the internal control are amplified.

No diagnostic conclusion can be made from the assay.

N.B. in all the above the 'Combined signal' is the signal actually measured during the assay.

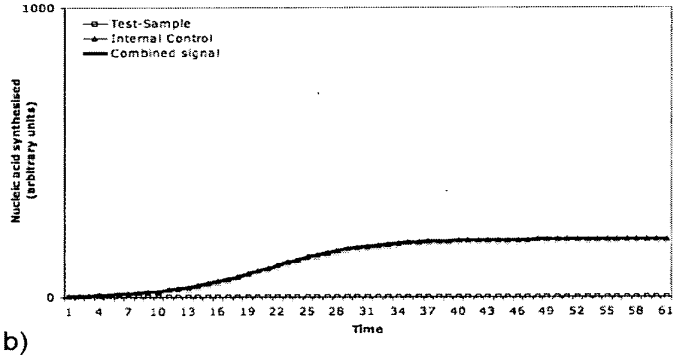
c)

Figure 3



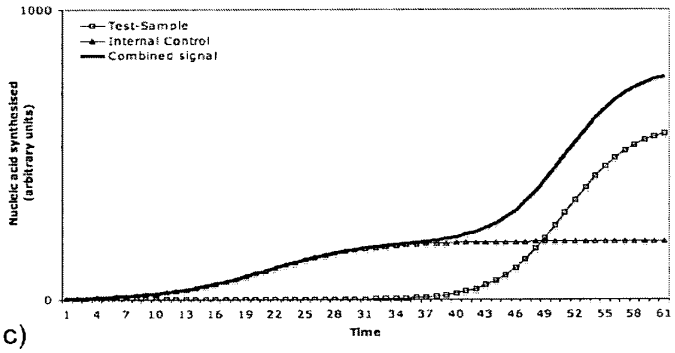
a)

As before but where the internal control has an earlier 'time to maximum amplification rate' than in figure 2a, but a slower intrinsic rate of amplification.



b)

Ditto



c)

In this example, the signal from the positive sample comes very late in the assay, *after* the signal from the control starts to appear.

However, it is still very clear from the observed signal (i.e. the combined signal) that amplification of the test sample has occurred.

Figure 4

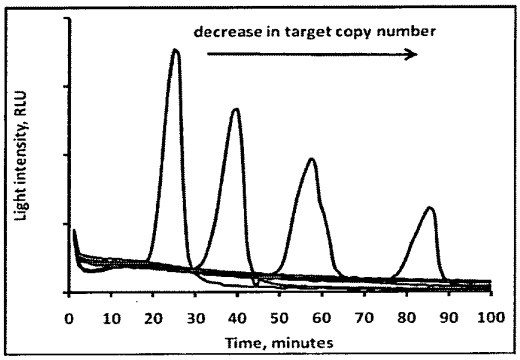
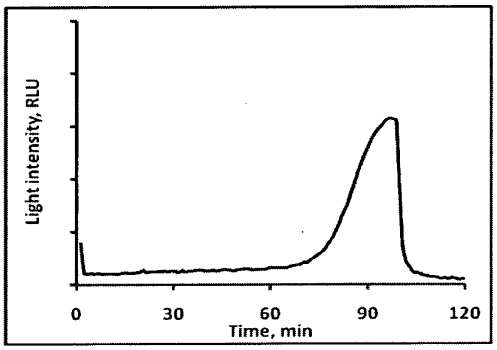


Figure 5

a)



b)

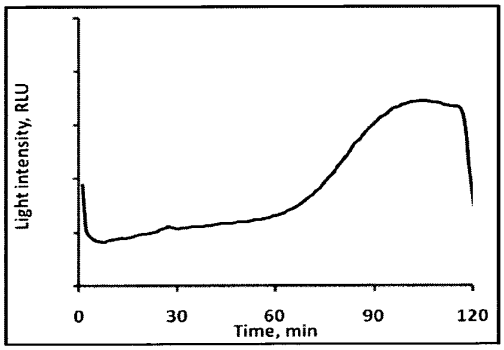


Figure 6

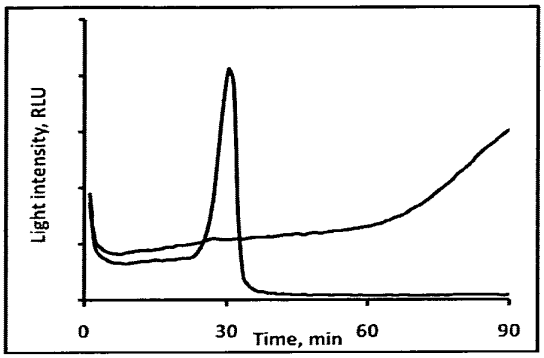
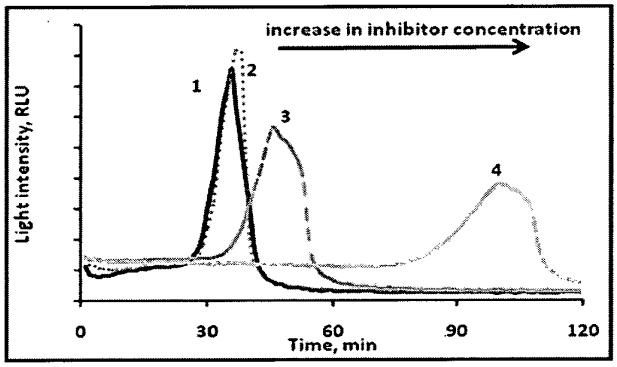
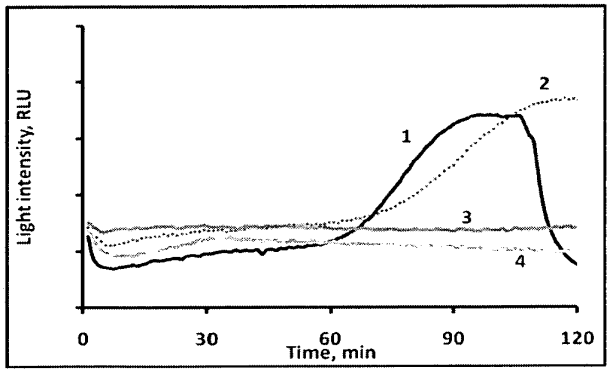


Figure 7



a)



b)

Figure 7

c)

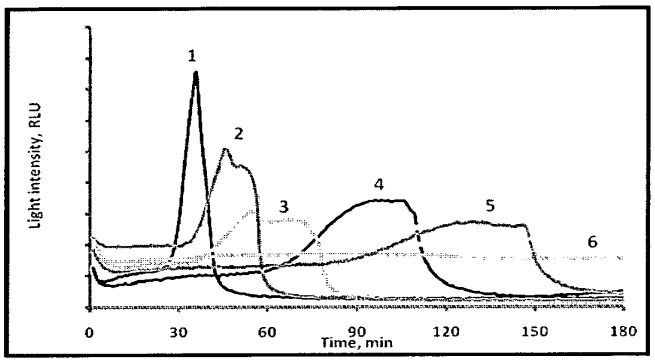


Figure 8

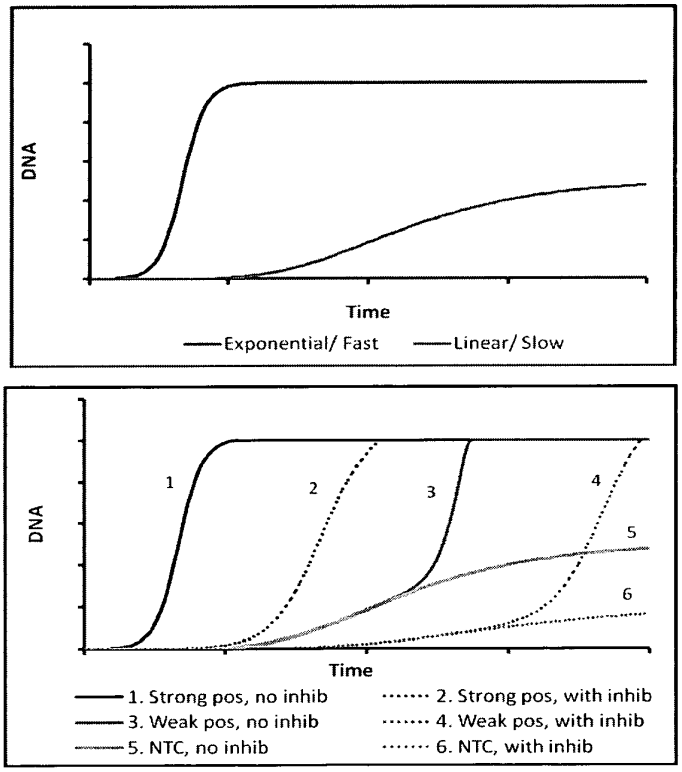


Figure 9

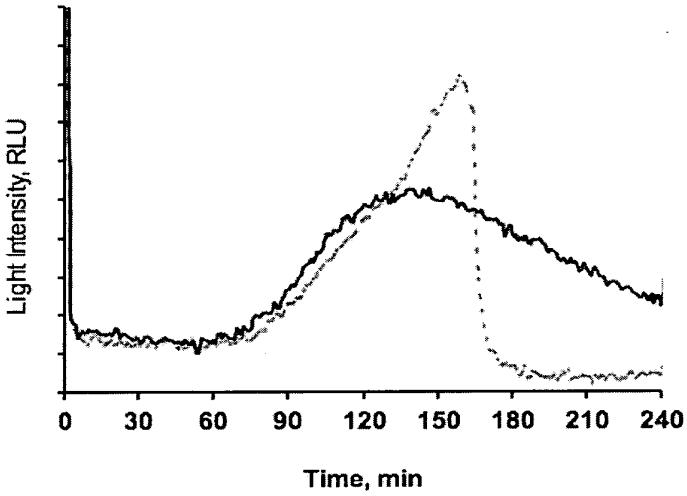


Figure 10

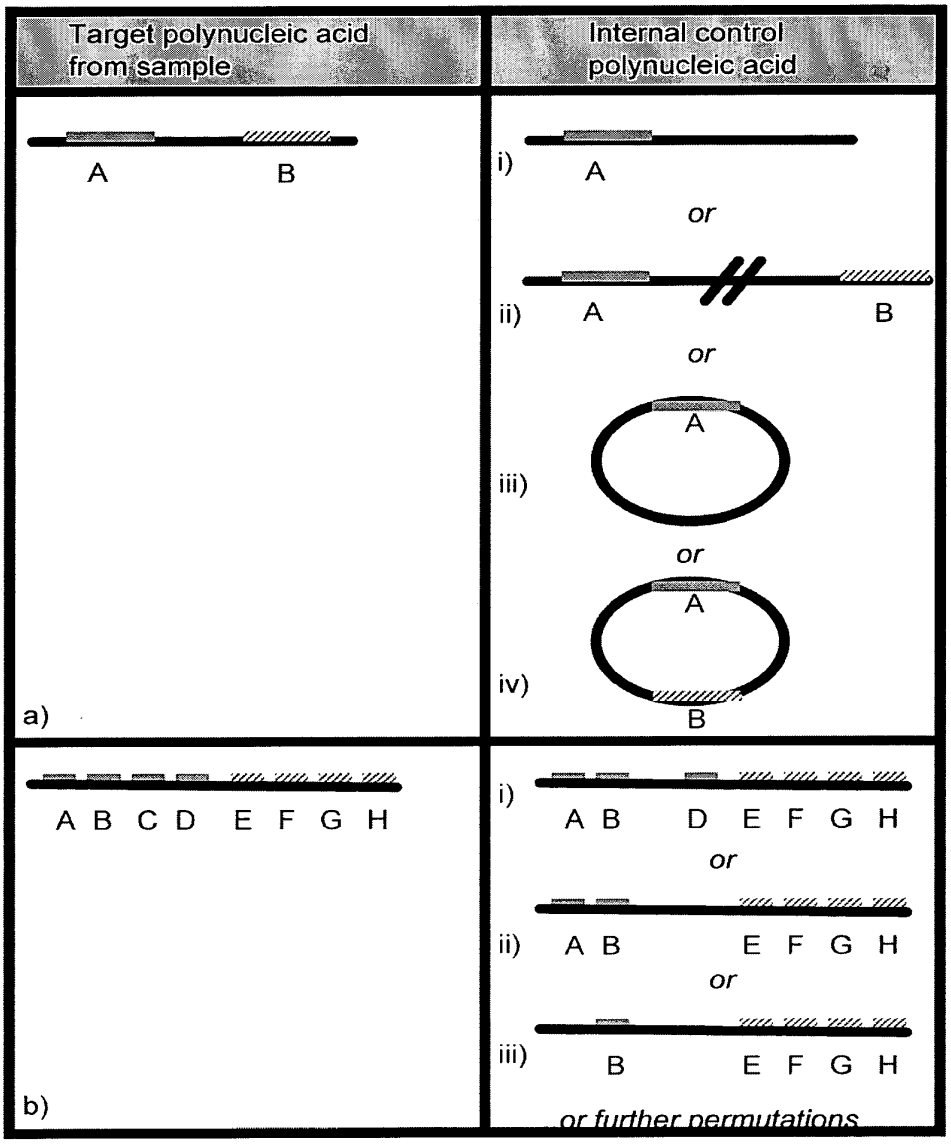

Target polynucleic acid from sample
Internal control polynucleic acid
A
B
i)
A
or
ii)
A
B
or
iii)
A
or
iv)
A
B
a)
A B C D E F G H
i)
A B D E F G H
or
ii)
A B E F G H
or
iii)
B E F G H
or further permutations
b)

Figure 11

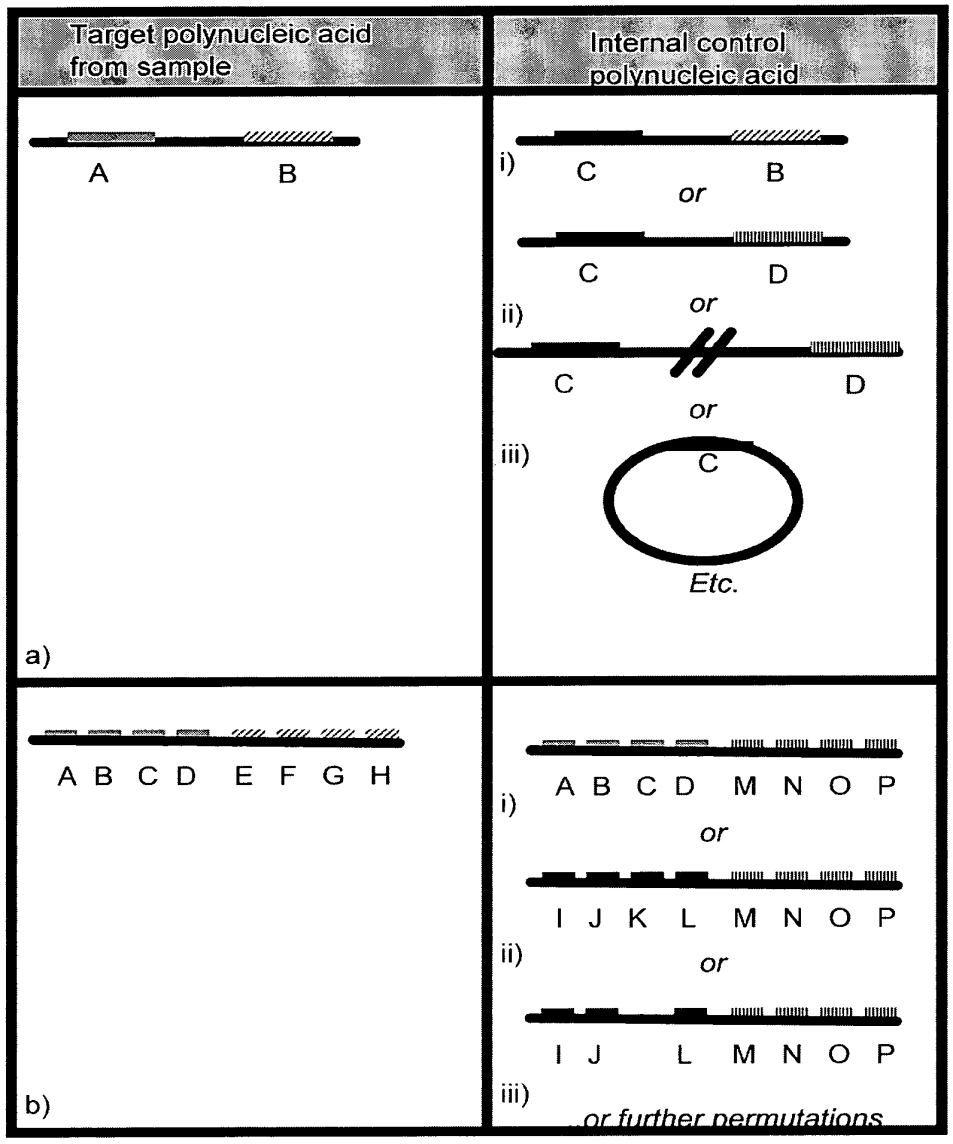
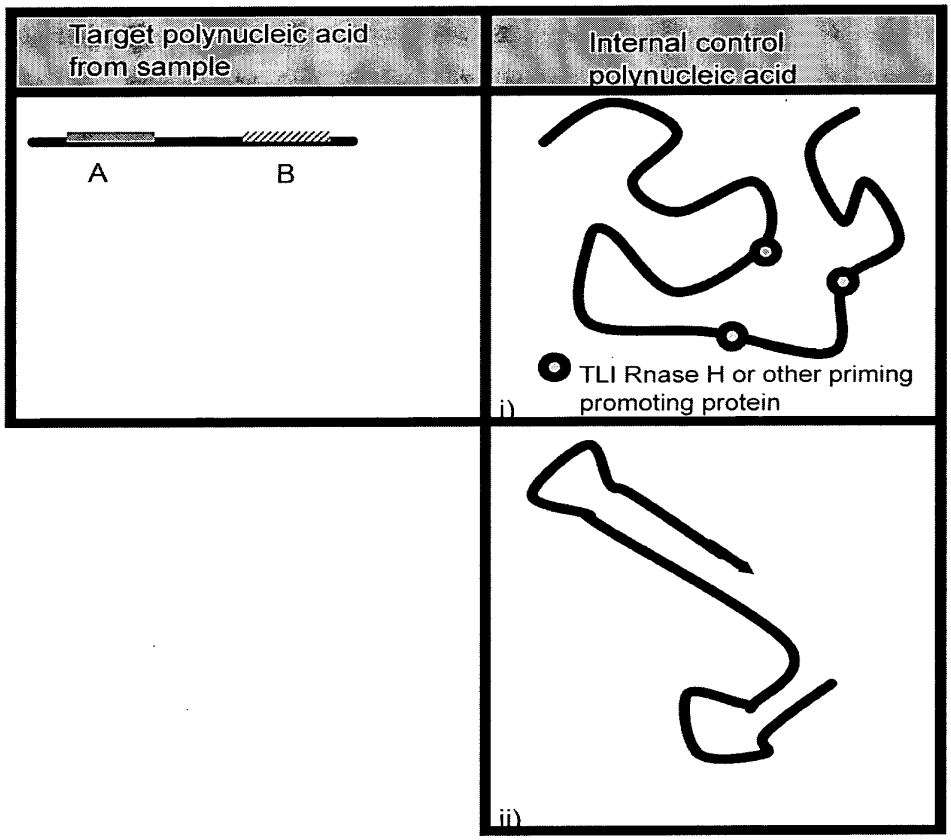

Target polynucleic acid from sample
Internal control polynucleic acid
A
B
i)
C
B
or
C
D
ii)
or
C
D
or
iii)
C
Etc.
a)
A B C D E F G H
i)
A B C D M N O P
or
I J K L M N O P
ii)
or
I J L M N O P
iii)
or further permutations
b)

Figure 12


Target polynucleic acid from sample
Internal control polynucleic acid
A
B
TLI Rnase H or other priming promoting protein
i)
ii)

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2009/001728

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2006/292571 A1 (BABIEL REINER [DE] ET AL) 28 December 2006 (2006-12-28) paragraphs [0004], [0096] - [0098]; claim 18; figure 16	1-33
A	SANCHEZ J AQUILES ET AL: "Linear-After-The-Exponential (LATE)-PCR: An advanced method of asymmetric PCR and its uses in quantitative real-time analysis" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE, WASHINGTON, DC, US, vol. 101, no. 7, 17 February 2004 (2004-02-17), pages 1933-1938, XP002397153 ISSN: 0027-8424 the whole document	2-3,9

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
1 December 2009	07/12/2009

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Aslund, Fredrik

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/GB2009/001728

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2006292571 A1	28-12-2006	NONE	

Form PCT/ISA/210 (patent family annex) (April 2005)

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0817706.5	26 September 2008 (26.09.2008)	GB
0909333.7	1 June 2009 (01.06.2009)	GB

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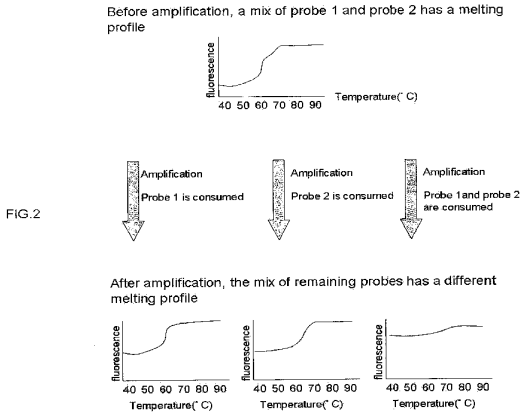


FIG.2

(57) Abstract: The invention relates to the field of multiplex amplification. In particular, the invention relates to methods for assaying a sample for one or more nucleic acid targets in a single reaction based on the distinct melting temperatures or melting profiles of primers and/or probes. The invention also provides probes and kits for use in such methods.

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MULTIPLEX AMPLIFICATION AND DETECTION

The present invention relates to the field of multiplex detection. In particular, the invention relates to methods for assaying a sample for one or more nucleic acid targets in a single reaction based on the distinct melting temperatures or melting profiles of probes. The invention also provides probes and kits for use in such methods.

Multiplex PCR, which uses multiple pairs of primers to simultaneously amplify multiple target sequences in a single PCR reaction, is a more efficient approach to PCR than standard single primer-pair PCR. The simultaneous amplification of various targets reduces both the cost and turn-around time of PCR analysis, minimizes experimental variations and the risk of cross-contamination, and increases the reliability of end results. Multiplex PCR has been used in many areas of DNA testing including identification of micro-organisms, gene expression analysis, mutation and polymorphism analysis, genotyping and DNA array analysis, and RNA detection.

Real-time PCR has been developed to quantify amplified products during PCR reactions. Real-time PCR is based on the principles that emission of fluorescence from dyes directly or indirectly associated with the formation of newly-synthesized amplicons or the annealing of primers with DNA templates can be detected and is proportional to the amount of amplicons in each PCR cycle. Real-time PCR is carried out in a closed-tube format and it is quantitative. Several methods are currently available for performing real-time PCR, such as utilising TaqMan probes (U.S. Pat. Nos. 5,210,015 and 5,487,972, and Lee et al., Nucleic Acids Res. 21:3761-6, 1993), molecular beacons (U.S. Pat. Nos. 5,925,517 and 6,103,476, and Tyagi and Kramer, Nat. Biotechnol. 14:303-8, 1996), self-probing amplicons (scorpions) (U.S. Pat. No. 6,326,145, and Whitcombe et al., Nat. Biotechnol. 17:804-7, 1999), Amplisensor (Chen et al., Appl. Environ. Microbiol. 64:4210-6, 1998), Amplifluor (U.S. Pat. No. 6,117,635, and Nazarenko et al., Nucleic Acids Res. 25:2516-21, 1997, displacement hybridization probes (Li et al., Nucleic Acids Res. 30:E5, 2002); DzyNA-PCR (Todd et al., Clin. Chem. 46:625-30, 2000), fluorescent restriction enzyme detection (Cairns et al. Biochem. Biophys. Res. Commun. 318:684-90, 2004) and adjacent hybridization probes (U.S. Pat. No. 6,174,670 and Wittwer et al., Biotechniques 22:130-1, 134-8, 1997). Most of these probes consist of a pair of dyes (a reporter dye and an acceptor dye) that are involved in fluorescence resonance energy transfer (FRET), whereby the acceptor dye quenches the emission of the reporter dye. In general, the fluorescence-labelled probes increase the specificity of amplicon quantification.

Another form of probe used in PCR is a double-stranded linear probe which has two complementary oligonucleotides. The probes described in the prior art have been of equal length, in which at least one of the oligonucleotides acts as a probe for a target sequence in a single-stranded conformation. The 5' end of one of the oligonucleotides is labelled with a fluorophore and the 3' end of the other oligonucleotide is labelled with a quencher, e.g., an acceptor fluorophore, or vice versa. When these two oligonucleotides are annealed to each other, the two labels are close to one another, thereby quenching fluorescence. Target nucleic acids, however, compete for binding to the probe, resulting in a less than proportional increase of probe fluorescence with increasing target nucleic acid concentration (Morrison L. et al., Anal. Biochem., Vol. 183, pages 231-244 (1989); US 5,928,862).

Double-stranded linear probes modified by shortening one of the two complementary oligonucleotides by a few bases to make a partially double-stranded linear probe, are also known in the art. In such double-stranded linear probes in the prior art, the longer oligonucleotide has been end-labelled with a fluorophore and the slightly shorter oligonucleotide has been end-labelled with a quencher. In the double-stranded form, the probe is less fluorescent due to the close proximity of the fluorophore and the quencher. In the presence of a target, however, the shorter quencher oligonucleotide is displaced by the target. As a result, the longer oligonucleotide (in the form of probe-target hybrid) becomes substantially more fluorescent (Li et al., Nucleic Acids Research, Vol. 30, No. 2, e5 (2002)).

US 2005/0227257 describes a slightly modified double stranded linear nucleic acid probe. The probe described in this patent application is modified by shortening one of the two complementary oligonucleotides by more bases, compared to the above, to make a partially double-stranded linear probe.

Fluorescent hybridisation probes have also been used in other fields. For example, methods for multiplex genotyping using fluorescent hybridisation probes have been described (e.g. US 6,140,054) which use the melting temperature of fluorescent hybridization probes that hybridize to a PCR amplified targeted region of genome/nucleic acid sequence to identify mutations and polymorphisms.

The advent of high-throughput genetic testing has necessitated both qualitative and quantitative analysis of multiple genes and has led to the convergence of multiplex PCR and real-time PCR into multiplex real-time PCR. Since double-stranded DNA intercalating dyes are not suitable for multiplexing due to their non-specificity, fluorescence-labelled probes have

made multiplex real-time PCR possible. However, multiplex real-time PCR is limited by the availability of fluorescence dye combinations. Currently, only up to four or five fluorescence dyes can be detected and quantified simultaneously in real-time PCR.

US2005/0053950 describes a protocol for quantifying multiplex real-time polymerase chain reactions (PCR). The methods quantify multiple PCR products or amplicons in a single real-time PCR reaction based on the different melting temperatures (T_m) of each amplicon and the emission changes of double-stranded DNA dyes such as SYBR Green I when amplicons are in duplex or in separation. For a specific amplicon with a T_m, the emission difference between the emission reading taken at a temperature below the T_m and the emission reading taken at a temperature above the T_m corresponds to the emission value of the amplicon in duplex. Accordingly, the emission difference of each amplicon in a single PCR reaction can be used to quantify each amplicon. However, the multiplexity and sensitivity of such methods can be relatively low. For example, the difference in melting temperatures between amplicons of the order of 100-150 nucleotides in length is small. Therefore, these techniques require the use of amplicons with large differences in their sizes in order to be able to distinguish between them.

There is a need, however, to develop other methods of amplifying and quantifying multiple target sequences in a single PCR reaction for multiplex real-time PCR with greater levels of multiplexity and sensitivity.

The method of the present invention differs from prior art technologies. Firstly, the method is based on the different melting properties (T_m or melting profile) of each probe and the emission changes of labels on the probe when the probe's internal double-stranded portions are in duplex or in separation. The probe of present invention comprises a double-stranded portion which can be formed by a first oligonucleotide and a second oligonucleotide; the double stranded portion has a distinct T_m for each probe which distinguishes different probes within a set of probes comprising the same or similar labels. Secondly, the first oligonucleotide may or may not comprise one or more labels and it is the first oligonucleotide which is consumed during the amplification reaction. The emission difference between the emission readings at two different temperatures corresponds to the emission value of the probe after some probe being consumed. Thirdly, measuring melting profiles of unconsumed probes provide an indication of the presence or amount of target nucleic acids presented in a sample.

To facilitate understanding of the invention, a number of terms are defined below.

A "nucleic acid", as used herein, is a covalently linked sequence of nucleotides in which the 3' position of the pentose of one nucleotide is joined by a phosphodiester group to the 5' position of the pentose of the next, and in which the nucleotide residues (bases) are linked in specific sequence; i.e., a linear order of nucleotides. A "polynucleotide", as used herein, is a nucleic acid containing a sequence that is greater than about 200 nucleotides in length. An "oligonucleotide", as used herein, is a short polynucleotide or a portion of a polynucleotide. An oligonucleotide typically contains a sequence of about two to about one hundred bases. "Nucleic acid", "DNA" and similar terms also include nucleic acid analogs, i.e. analogs having other than a phosphodiester backbone. For example, the so-called "peptide nucleic acids," which are known in the art and have peptide bonds instead of phosphodiester bonds in the backbone, are considered within the scope of the present invention.

As used herein, the terms "target sequence", "target nucleic acid", "target nucleic acid sequence" and "nucleic acids of interest" are used interchangeably and refer to a desired region which is to be either amplified, detected or both. The target sequence, which is the object of amplification and detection, can be any nucleic acid. The target sequence can be RNA, cDNA, genomic DNA, or DNA or RNA, for example from a disease-causing micro-organism or virus. The target sequence can also be DNA treated by chemical reagents, various enzymes and physical exposure. A target nucleic acid sequence of interest in a sample may appear as single-stranded DNA or RNA such as cDNA, mRNA, other RNA or as separated complementary strands. Separating complementary strands of target nucleic acid may be accomplished by physical, chemical or enzymatic means. For the ease of description and understanding, references to nucleic acids of interest or targets refer both to these moieties as found in a test sample and to amplified copies of portions of theses nucleic acids, unless specifically noted to the contrary.

"Primer" as used herein refers to an oligonucleotide, whether occurring naturally or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product which is complementary to a nucleic acid strand is induced i.e., in the presence of nucleotides and an agent for polymerization such as DNA polymerase and at a suitable temperature and buffer. The primers herein are selected to be substantially complementary to the different strands of each specific sequence to be amplified. This means that the primers must be sufficiently complementary to hybridize with their respective strands. A non-complementary nucleotide fragment may be attached to the 5'-end of the primer, with the remainder of the primer

sequence being complementary to the diagnostic section of the target base sequence. Commonly, the primers are complementary except when non-complementary nucleotides may be present at a predetermined primer terminus as described.

The term "complementary to" is used herein in relation to a nucleotide that will base pair with another specific nucleotide. Thus adenosine is complementary to uridine or thymidine and guanosine is complementary to cytidine. It is appreciated that whilst thymidine and guanosine may base pair under certain circumstances they are not regarded as complementary for the purposes of this specification. For purposes of the present invention, the term "substantially complementary" means that equal or more than 70%, preferably more than 80%, more preferably more than 90% and most preferably more than 95% or 99% of nucleobases on one strand of the probe finds its Watson-Crick binding partner on the other strand of the probe (or in the nucleic acid of interest) in an alignment such that the corresponding nucleotides can hybridize to each other. Methods to determine identity and similarity are codified in publicly available computer programs. Preferred computer program methods to determine identity and similarity between two sequences include, but are not limited to: the GCG Pileup program found in the GCG program package, using the Needleman and Wunsch algorithm with their standard default values of gap creation penalty=12 and gap extension penalty=4 (Devereux et al., Nucleic Acids Res. 12: 387-395 (1984)), BLASTP, BLASTN, and FASTA (Pearson et al., Proc. Natl. Acad. Sci. USA 85: 2444-2448 (1988)). The BLASTX program is publicly available from NCBI and other sources (BLAST Manual, Altschul et al., Natl. Cent. Biotechnol.Inf., Natl. Library Med. (NCBI NLM) NIH, Bethesda, MD; Altschul et al., J. Mol. Biol. 215: 403-410 (1990); Altschul et al., Nucleic Acids Res. 25: 3389-3402 (1997)).

The terms “duplex” and “double-stranded” are interchangeable, mean one strand of oligo-poly-nucleotides hybridises to the complementary oligo-poly-nucleotides.

The term "identical" means that two nucleic acid sequences have the same sequence or a complementary sequence.

The term "homologous" means that one single-stranded nucleic acid sequence may hybridize to a complementary single-stranded nucleic acid sequence. The degree of hybridization may depend on a number of factors including the amount of identity between the sequences and the hybridization conditions such as temperature and salt concentration. Preferably the region of identity is greater than about 5 bp, more preferably the region of identity is greater than 10 bp.

As used herein, "continuous monitoring" and similar terms refer to monitoring multiple times during a cycle of PCR, preferably during temperature transitions, and more preferably obtaining at least one data point in each temperature transition.

As used herein, "cycle-by-cycle" monitoring means monitoring the PCR reaction once each cycle.

The term "Actual Consumed Amount" (ACA) means the amount of probe being consumed in a reaction as reflected by fluorescence measuring.

"Amplification" as used herein denotes the use of any amplification procedures to increase the concentration of a particular nucleic acid sequence within a mixture of nucleic acid sequences.

The term "sample" as used herein is used in its broadest sense. A biological sample suspected of containing nucleic acid can comprise, but is not limited to, genomic DNA, cDNA (in solution or bound to a solid support), and the like.

The term "label" as used herein refers to any atom or molecule which can be used to provide or aid to provided a detectable (preferably quantifiable) signal, and which can be attached to a nucleic acid or protein. Labels may provide signals detectable by fluorescence, radioactivity, colorimetry, gravimetry, magnetism, enzymatic activity and the like.

The term "adjacent" or "substantially adjacent" as used herein refers to the positioning of two oligonucleotides on its complementary strand of the template nucleic acid. The two template regions hybridised by oligonucleotides may be contiguous, i.e. there is no gap between the two template regions. Alternatively, the two template regions hybridised by the oligonucleotides may be separated by 1 to about 40 nucleotides, more preferably, about 1 to 10 nucleotides.

The term "thermally cycling," "thermal cycling", "thermal cycles" or "thermal cycle" refers to repeated cycles of temperature changes from a total denaturing temperature, to an annealing (or hybridising) temperature, to an extension temperature and back to the total denaturing temperature. The terms also refer to repeated cycles of a denaturing temperature and an extension temperature, where the annealing and extension temperatures are combined into one temperature. A total denaturing temperature unwinds all double stranded fragments into single strands. An annealing temperature allows a primer to hybridize or anneal to the complementary sequence of a separated strand of a nucleic acid template. The extension temperature allows the synthesis of a nascent DNA strand of the amplicon.

The term "reaction" as used herein refers to hybridisation reaction, extension reaction or amplification reaction or other biological, chemical reactions.

The terms "amplification mixture" or "PCR mixture" as used herein refer to a mixture of components necessary to detect target nucleic acid from nucleic acid templates. The mixture may comprise nucleotides (dNTPs), probes, a thermostable polymerase, primers, and a plurality of nucleic acid templates. The mixture may further comprise a Tris buffer, a monovalent salt, and Mg^{2+}. The concentration of each component is well known in the art and can be further optimized by an ordinary skilled artisan.

The terms "amplified product" or "amplicon" refers to a fragment of DNA amplified by a polymerase using a pair of primers in an amplification method such as PCR

The term "melting profile" refers to a collection of measurements of an oligo(or poly)nucleotide and its complement which indicate the oligo(or poly)nucleotide molecule's transition from double-stranded to single-stranded nucleic acid (or vice-versa). The transition of a nucleic acid from double-stranded to single-stranded form is often described in the art as the "melting" of that nucleic acid molecule. The transition may also be described as the "denaturation" or "dissociation" of the nucleic acid. Accordingly, a melting profile of the present invention may also be referred to as a "dissociation profile", a "denaturation profile", a "melting curve", a "dissociation curve", a "hybridisation/dissociation profile" etc.

The "melting temperature" or "T_m" of a nucleic acid molecule generally refers to the temperature at which a polynucleotide dissociates from its complementary sequence. Generally, the T_m may be defined as the temperature at which one-half of the Watson-Crick base pairs in duplex nucleic acid molecules are broken or dissociated (i.e., are "melted") while the other half of the Watson-Crick base pairs remain intact in a double stranded conformation. In preferred embodiments where duplex nucleic acid molecules are oligonucleotides and in other embodiments where the duplex nucleic acids dissociate in a two-state fashion, the T_m of a nucleic acid may also be defined as the temperature at which one-half of the nucleic acid molecules in a sample are in a single-stranded conformation while the other half of the nucleic acid molecules in that sample are in a double-stranded conformation. T_m, therefore defines a midpoint in the transition from double-stranded to single-stranded nucleic acid molecules (or, conversely, in the transition from single-stranded to double-stranded nucleic acid molecules). It is well appreciated in the art that the transition from double-stranded to single-stranded nucleic acid molecules does not occur at a single temperature but, rather, over a range of temperatures.

Nevertheless, the T_m provides a convenient measurement for approximating whether nucleic acid molecules in a sample exist in a single-stranded or double-stranded conformation. As such, the melting temperature of a nucleic acid sample may be readily obtained by simply evaluating a melting profile for that sample.

The term "consumed" or "consumption" means that the amount of free labeled probes is decreased at a temperature at which the labeled probe is normally intact, in other words at particular temperatures the probe's double-stranded portion remains double-stranded. In particular, the decrease of the amount of free labeled probe may be result of no availability of at least one strand of the probe, which is either the first oligonucleotide of the probe or second oligonucleotide of the probe or both. The no availability of at least one strand of the probe means the first oligonucleotide of the probe, the second oligonucleotide of the probe or both oligonucleotides of the probe are hybridised with target nucleic acid. The hybridisation of the first oligonucleotide of the probe, the second oligonucleotide of the probe or both oligonucleotides of the probe with the target nucleic acid may be followed by extension of the oligonucleotides of the probe which act as primer, or by degradation of the oligonucleotides of the probe.

The present invention describes methods that allow the essentially simultaneous amplification and detection of a large number of different target nucleic acid sequences.

In a first aspect, the invention provides a method for assaying a sample for one or more target nucleic acids, said method comprising:

(a) contacting a sample comprising one or more target nucleic acids with a reaction mixture comprising:

two or more probes, wherein each probe comprises

a first oligonucleotide which comprises a first region which is substantially complementary to part of one target nucleic acid and a second region, and

at least one second oligonucleotide which comprises a region which is substantially complementary to the second region of the first oligonucleotide, such that the first and second oligonucleotides are capable of forming a double-stranded portion,

wherein each probe comprises a detectable label or detectable combination of labels which is/are capable of producing a changeable signal which is characteristic of the presence or absence of a double-stranded portion between the first and second oligonucleotides of that probe, and

wherein at least two of the probes comprise the same detectable label or different detectable labels with undistinguishable emission spectra and wherein the melting characteristics (melting temperature T_m) of the double-stranded portions between the first and second oligonucleotides of each of such probes are different, and are distinguishable in a melting profile analysis;

(b) performing the reaction on the sample/reaction mixture, wherein the reaction is a primer extension reaction under extension conditions, wherein, when a target nucleic acid is present, the first oligonucleotides of the corresponding probe which are extendable primers, are hybridised with target sequence, therefore are consumed during the primer extension reaction, wherein the consumed oligonucleotides of probes are no longer available to participate in forming double stranded portion (the duplex) of the probe; and

(c) measuring, at least once, the melting profile of the double-stranded portions between the first and second oligonucleotides of the unconsumed probes in the reaction mixture by detecting the signal(s) from the labels in those probes as a function of temperature, wherein the melting profile provides an indication of whether or not at least one target nucleic acid is present in said sample.

In this embodiment, the first oligonucleotides of probes act as primers. In a primer extension reaction, a mixture of probes is added into the reaction mixture containing all ingredients for extension under extension conditions. If a particular target nucleic acid is present in the reaction, the oligonucleotides of the corresponding probe are hybridised with target sequence, followed by extension and incorporation into the primer extension product, therefore are consumed. The consumed oligonucleotides are no longer available to participate in forming the double stranded portion of the probe. In the melting profile analysis, the consumed probe can be seen as a peak reduced or missing.

In another aspect, the invention provides a method for assaying a sample for one or more target nucleic acids, said method comprising:

(a) contacting a sample comprising one or more target nucleic acids with a hybridisation reaction mixture comprising:

two or more probes, wherein each probe comprises

a first oligonucleotide which comprises a first region which is substantially complementary to part of one target nucleic acid and a second region, and

at least one second oligonucleotide which comprises a region which is substantially complementary to the second region of the first oligonucleotide,

such that the first and second oligonucleotides are capable of forming a double-stranded portion,

wherein each probe comprises a detectable label or detectable combination of labels which is/are capable of producing a changeable signal which is characteristic of the presence or absence of a double-stranded portion between the first and second oligonucleotides of that probe, and

wherein at least two of the probes comprise the same detectable label or different detectable labels with undistinguishable emission spectra and wherein the melting characteristics of the double-stranded portions between the first and second oligonucleotides of each of such probes are different and are distinguishable in a melting profile analysis;

(b) performing the hybridisation reaction on the sample/reaction mixture under hybridisation conditions, wherein, when a target nucleic acid is present, the first oligonucleotides of probes which are substantially complementary to part of that target nucleic acid are hybridised with target sequence, therefore are consumed during the reaction, wherein the consumed oligonucleotides of probes are no longer available to participate in forming double stranded portion (the duplex) of the probe; and

(c) measuring, at least once, the melting profile of the double-stranded portions between the first and second oligonucleotides of unconsumed probes in the reaction mixture by detecting the signal(s) from the labels in those probes as a function of temperature,

wherein the melting profile provides an indication of whether or not at least one target nucleic acid is present in said sample.

In this embodiment, the first oligonucleotides of probes may act as hybridisation probe. In a hybridisation reaction, a mixture of probes is added into the reaction mixture containing all hybridisation ingredients under hybridisation conditions. If a particular target nucleic acid is present in the reaction, the oligonucleotides of the corresponding probe are hybridised to the target sequence, therefore are consumed. The consumed oligonucleotides are no longer available to participate in forming the double stranded portion of the probe. In the melting profile analysis, the consumed probe can be seen as a peak reduced or missing.

In another aspect, the invention provides a method for assaying a sample for one or more target nucleic acids, said method comprising:

(a) contacting a sample comprising one or more target nucleic acids with an amplification reaction mixture comprising:

(i) one or more pairs of forward/reverse oligonucleotide primers, wherein the primer pairs are capable of amplifying one or more target nucleic acids, if present in the sample,

(ii) two or more probes, wherein each probe comprises

a first oligonucleotide which comprises a first region which is substantially complementary to part of one target nucleic acid and a second region, and at least one second oligonucleotide which comprises a region which is substantially complementary to the second region of the first oligonucleotide, such that the first and second oligonucleotides are capable of forming a double-stranded portion,

wherein each probe comprises a detectable label or detectable combination of labels which is/are capable of producing a changeable signal which is characteristic of the presence or absence of a double-stranded portion between the first and second oligonucleotides of that probe, and

wherein at least two of the probes comprise the same detectable label or different detectable labels with undistinguishable emissions spectra

wherein the melting characteristics of the double-stranded portions between the first and second oligonucleotides of each of such probes are different, and are distinguishable in a melting profile analysis;

(b) performing an amplification reaction on the sample/amplification reaction mixture wherein, when a target nucleic acid is present, the first oligonucleotides which are substantially complementary to part of that target nucleic acid are hybridised with target sequence, therefore are consumed during the amplification reaction;

(c) measuring, at least once, the melting profile of the double-stranded portions between the first and second oligonucleotides of unconsumed probes by detecting the signal(s) from the labels in those probes as a function of temperature,

wherein the melting profile provides an indication of whether or not at least one target nucleic acid has been amplified in said sample/amplification reaction mixture.

wherein a first probe of said at least two of the probes has a melting temperature T_m1 in terms of its double-stranded portion,

wherein a second probe of said at least two of the probes has a melting temperature T_m2 in terms of its double-stranded portion,

wherein $T_m1 > T_m2$,

wherein the same labels are independently attached to the first and second probes,

wherein a reduction of any melting peak at T_m1 and/or T_m2 provides indication of consumption of the first and/or second probe(s).

Preferably, the above method includes step (d):

(i) comparing at least two melting profiles obtained in (c)

and/or

(ii) comparing a melting profile obtained in step (c)

with a previously-obtained melting profile of the same probes or

with a melting profile of the same probes obtained in parallel at the same time in control reactions, or

with a theoretical melting profile of the same probes

wherein a change in the melting profile provides an indication of whether or not at least one target nucleic acid has been amplified in said sample/amplification reaction mixture.

The amplification reaction can be any amplification method, such as PCR, SDA, NASBA, LAMP, 3SR, ICAN, TMA, Helicase-dependent isothermal DNA amplification and the like. PCR is a preferred amplification method.

The amplification reaction mixture will comprise standard amplification reagents. Amplification reagents can conveniently be classified into four classes of components: (i) an aqueous buffer, often including without limitation a magnesium salt, (ii) amplification substrates, such as DNA or RNA, (iii) one or more oligonucleotide primers (normally two primers for each target sequence, the sequences defining the 5' ends of the two complementary strands of the double-stranded target sequence when PCR is employed), and (iv) an amplification enzyme such as a polynucleotide polymerase (for example, Taq polymerase for PCR or RNA polymerase for TMA), or a ligase. Appropriate nucleoside triphosphates will also generally be required. Additional reagents or additives can also be included at the discretion of the skilled artisan and selection of these reagents is within the skill of the ordinary artisan. Of course, when the amplification reagents are used to cause both reverse transcription and amplification, then reverse transcription reagents are also included in the amplification reagents. Selection of amplification reagents, according to the method of amplification reaction used, is within the skill of the ordinary artisan.

In the methods described herein, a sample is provided which is suspected to contain the target nucleic acid or the nucleotide variant of interest. The target nucleic acid contained in the sample may be double-stranded genomic DNA or cDNA if necessary, which is then denatured,

using any suitable denaturing method including physical, chemical, or enzymatic means that are known to those of skill in the art. A preferred physical means for strand separation involves heating the nucleic acid until it is completely (>99%) denatured. Typical heat denaturation involves temperatures ranging from about 80°C to about 105°C, for times ranging from a few seconds to minutes. As an alternative to denaturation, the target nucleic acid may exist in a single-stranded form in the sample, such as single-stranded RNA or DNA viruses.

The denatured nucleic acid strands are then incubated with oligonucleotide primers and probes under hybridisation conditions, i.e. conditions that enable the binding of the primers or probes to the single nucleic acid strands. In some embodiments of the invention the annealed primers and/or probes are extended by a polymerizing agent. Template-dependent extension of the oligonucleotide primer(s) is catalyzed by a polymerizing agent in the presence of adequate amounts of the four deoxyribonucleoside triphosphates (dATP, dGTP, dCTP, and dTTP), or analogues of these as discussed above, in a reaction medium comprised of the appropriate salts, metal cations and pH buffering system. Suitable polymerizing agents are enzymes known to catalyze primer- and template-dependent DNA synthesis. The reaction conditions for catalyzing DNA synthesis with these DNA polymerases are well known in the art. Probes are consumed during amplification.

An amplification primer can be a target-specific primer, which comprises a 3' priming portion which is complementary to a desired region of target nucleic acid. An amplification primer can also be a universal primer, which has a sequence which is identical or substantially identical to a 5' universal portion of target-specific primers. A reaction may contain multiple primers for amplification of multiple target sequences. The 5' universal portions of the multiple primers may have essentially the same sequence composition which is identical or substantially identical to the 3' priming portion of the universal amplification primer. Preferably, the primers are DNA primers, particularly those suitable for PCR amplification.

For SNP genotyping or detecting variant nucleotides, the amplification primer may be an allele-specific primer, wherein a terminal nucleotide of the primer is selected to be either complementary to the suspected variant nucleotide or to the corresponding normal nucleotide such that an extension product of the primer is synthesised when the primer anneals to the diagnostic region containing a particular nucleotide, but no such extension product is synthesised when the primer anneals to the diagnostic region containing no particular nucleotide of the target nucleic acid sequence.

Primer pairs of forward and reverse primers are included in the amplification reaction mixture such that, if a target nucleic acid is present in the sample, the primer pairs are capable of amplifying that target nucleic acid, preferably in an exponential manner.

In some embodiments, there will be 1-50, 1-25, 1-20 or 1-10 primer pairs in the reaction mixture. In other embodiments there will be 5-50, 5-25, 5-20 or 5-10 primer pairs in the reaction mixture. As mentioned above, the forward or reverse primer in a particular primer pair might be a universal primer which is common to more than one primer pair.

The amplification reaction mixture comprises two or more probes. Each probe comprises

a first oligonucleotide which comprises a first region which is substantially complementary to part of one target nucleic acid and a second region, and

a second oligonucleotide which comprises a region which is substantially complementary to the second region of the first oligonucleotide,

such that the first and second oligonucleotides are capable of forming a double-stranded portion.

The first oligonucleotide must be capable of binding, under appropriate hybridisation conditions, to part of at least one of the target nucleic acids. Preferably, each first oligonucleotide is specific to part of only one of the target nucleic acids. The first oligonucleotide will have a first region whose nucleotide sequence is complementary or substantially complementary to the nucleotide sequence of part of one of the target nucleic acids. The length of this first complementary region is preferably 6-100 nucleotides, more preferably 15-30 nucleotides.

The overall length of the first oligonucleotide is preferably 15-150 nucleotides, more preferably 17 to 100 nucleotides, and most preferably 20-80 nucleotides.

In some embodiments, where the reaction involves primer extension or amplification, the part of the target nucleic acid to which the first oligonucleotide is complementary must fall within or overlap with the sequence to be amplified by the forward and reverse primers. Alternatively, the first oligonucleotide can be one of the amplification primers, for example, either the forward or reverse primer. In some embodiments, the first and/or second oligonucleotide is not a forward or reverse primer.

The second oligonucleotide comprises a region which is substantially complementary to a second region of the first oligonucleotide. The length of this second region is preferably 4-

100 nucleotides, more preferably 15-30 nucleotides. The second region of the first oligonucleotide may or may not overlap with the first region of the first oligonucleotide.

The overall length of the second oligonucleotide is preferably 6-150 nucleotides, more preferably 10 to 100 nucleotides, and most preferably 12-80 nucleotides.

The first and second oligonucleotides may comprise 1-5 or 1-10 or more nucleotides that are not complementary to the target nucleic acid or to the first oligonucleotide, respectively, at the 5' or the 3' end.

The oligonucleotide probe may comprise nucleotides, nucleotide derivatives, nucleotide analogs, and/or non-nucleotide chemical moieties. Modifications of the probe that may facilitate probe binding include, but are not limited to, the incorporation of positively charged or neutral phosphodiester linkages in the probe to decrease the repulsion of the polyanionic backbones of the probe and target (see Letsinger et al., 1988, J. Amer. Chem. Soc. 110:4470); the incorporation of alkylated or halogenated bases, such as 5-bromouridine, in the probe to increase base stacking; the incorporation of ribonucleotides into the probe to force the probe:target duplex into an "A" structure, which has increased base stacking; and the substitution of 2,6-diaminopurine (amino adenosine) for some or all of the adenosines in the probe; the incorporation of nucleotide derivatives such as LNA (locked nucleic acid), PNA (peptide nucleic acid) or the like.

Generally the 3' terminus of the probe will be "blocked" to prohibit incorporation of the probe into a primer extension product. But in some preferred embodiments of the present invention, some probes are also working as primers and therefore are not blocked at the 3' terminus. "Blocking" can be achieved by using non-complementary bases or by adding a chemical moiety such as biotin or a phosphate group to the 3' hydroxyl of the last nucleotide, which may, depending upon the selected moiety, serve a dual purpose by also acting as a label for subsequent detection or capture of the nucleic acid attached to the label. Blocking can also be achieved by removing the 3'-OH or by using a nucleotide that lacks a 3'-OH such as a dideoxynucleotide.

It will be readily understood that the term "probe" refers to a plurality of that type of probes, i.e. the reaction mixture does not comprise merely a single molecule of that probe.

In some embodiments of the invention, the first region of said first oligonucleotide does not overlap or does not substantially overlap with the second region of said first oligonucleotide.

In other embodiments of the invention, the first region of the first oligonucleotide is substantially overlapping with the second region of said first oligonucleotide or the second region is embedded within the first region. In such embodiments, the T_m of the duplex of said first oligonucleotide hybridised with the target sequence is preferably higher than the T_m of the duplex of said first oligonucleotide hybridised with the second oligonucleotide such that if a target is present, the first oligonucleotide forms stronger hybrids with the target and consequently melts at a higher temperature than the first/second oligonucleotide duplex.

Preferably, said T_m of the duplex of said first oligonucleotide hybridised with the target sequence is at least 2 degrees or at least 5 degrees higher than the T_m of the duplex of said first oligonucleotide hybridised with the second oligonucleotide.

In other embodiments, the first oligonucleotide may comprise a third region which is identical or substantially identical to the sequence of a primer which is used in the amplification.

The probe of the present invention is capable of forming a double-stranded portion. Because of this double-stranded portion, the probe has a melting temperatures T_m and a signature melting profile. In particular, a mixture of multiple probes of the present invention also has a signature melting profile.

The melting temperature (T_m) is affected by a number of factors, including but not limited to, salt concentration, DNA concentration, and the presence of denaturants, nucleic acid sequence, GC content, and length. Typically, each probe of double stranded nucleic acids has a unique T_m. At a temperature below a given T_m at least 50% of nucleic acid duplex remains in duplex form. By contrast, at a temperature above a given T_m, over 50% of nucleic acid duplexes are expected to unwind into two single stranded oligonucleotide chains.

The T_m of any given DNA fragment can be determined by methods well known in the art. For example, one method in the art to determine a T_m of a DNA fragment is to use a thermostatic cell in an ultraviolet spectrophotometer and to measure absorbance at 268 nm as the temperature slowly rises. The absorbance versus temperature is plotted, presenting an S-shape curve with two plateaus. (See Figure 1, for example). The absorbance reading half way between the two plateaus corresponds to the T_m of the fragment. Alternatively, the first negative derivative of the absorbance versus temperature is plotted, presenting a normal distribution curve. The peak of the normal curve corresponds to the T_m of the fragment.

The T_m of a probe or T_ms of a mixture of multiple probes can also be determined by the nearest neighbour method and fine-tuned or accurately determined in the presence of a double stranded DNA dye or labels on the probe in a single reaction. For example, a reaction mixture containing a probe and appropriate buffer is heated from a hybridising temperature to the total denaturing temperature at a rate of 0.01°C to 3°C per second. At the same time, the mixture is illuminated with light at a wavelength absorbed by the dye (label) and the dye's (label's) emission is detected and recorded as an emission reading. The first negative derivative of the emission reading with respect to temperature is plotted against temperature to form a number of normal curves, and each peak of the curve corresponds to the actual T_m of the probe. The curve is also know as "melting profile" or "hybridisation/dissociation profile". The T_m or melting profile of a probe can also be estimated by a computer program based on theory well known in the art.

For a multiplex detection, sets of multiple probes for multiple target sequences are included in a reaction. In one embodiment, the different probes in a set of probes can comprise the same labels or labels with undistinguishable emission spectra. Each probe in such a set should have different T_ms, therefore enabling the individual melting profiles to be distinguished from one another. While the individual probe has a melting profile, the mixture of the multiple probes in the set also has melting profile which is characteristics for the set of the probes.

In accordance with the present invention, multiple loci of a target nucleic acid sequence can be analyzed in a single vessel by designing sets of probes that hybridize to different genetic loci and probes have different melting temperatures in terms of the probe's internal duplexes. If a target sequence is present, its corresponding probe is consumed. The sequence of the target loci can then be determined based on the comparison of the melting profile of the remaining probes with the melting profile of probes before the reaction. Advantageously, the different probes in a set can be attached with the same label, allowing for monitoring at a single emission wavelength. In one embodiment each probe in the set is attached with the same labels, for example a fluorescent energy transfer pair or contact quenching pair, and more particularly, a first label which is a fluorophore and a second label which is a quencher. On the other hand, the multiple sets of probes can be attached with different label pairs so that the sets of probes can be distinguished from one another based on the distinguishable emission spectra.

In accordance with one embodiment, the method of analysing multiple targets uses a mixture of probes that are attached with different labels that have distinguishable emission

spectra as well as probes that are attached with labels that have overlapping emission spectra, but are distinguishable based on differences in melting temperatures of the internal double stranded portions of the probes.

The probe comprises a first oligonucleotide, wherein said first oligonucleotide comprises a first region substantially complementary to a target nucleic acid (see for example, FIG. 4). In one embodiment, the probe comprises a first oligonucleotide and at least one second oligonucleotide (see for example, FIG. 4A-J). The first oligonucleotide comprises a first region which is substantially complementary to a target nucleic acid, and second region which is substantially complementary to a second oligonucleotide(s) such that the first oligonucleotide and second oligonucleotide(s) can bind together to form a double-stranded portion. The first region and second region can arranged in any order, such as 5' to 3' or 3' to 5' (see for example FIG. 4A) or one embodied within one another (see for example FIG. 4B). It is preferred that if the first oligonucleotide serves as primer, the first region and second region are arranged in an order as 3' to 5' (see for example FIG. 4A).

In one aspect, the first region of said first oligonucleotide is not overlapping or not substantially overlapping with second region of said first oligonucleotide (see for example FIG. 4A). In other words, the first region is complementary to a target sequence, while the second region may not be complementary to the target sequence. When the second region is not complementary to the target sequence, different probes may have the identical or substantially identical second region sequence and the same second oligonucleotide may be shared between different probes in the set of probes. While the second oligonucleotides may be the same among the set of probes, the second regions of first oligonucleotides of different probes in the set may have the length and/or nucleotide sequence difference such that the T_m and the melting profile of the probes are different.

In another aspect, the first region of said first oligonucleotide is substantially overlapping with the second region of said first oligonucleotide or the second region is embedded in the first region (see for example, FIG. 4B), wherein T_m of the duplex of said first oligonucleotide hybridised with the target sequence is higher than the T_m of the duplex of said first oligonucleotide hybridised with the second oligonucleotide such that if a target is present, the target forms stronger hybrids with the first oligonucleotide of the probe and consequently the duplex of the target/first oligonucleotide melts at a higher temperature than the duplex of second oligonucleotide/first oligonucleotide. In this aspect, the first region may be longer than the second region or the second region may comprise mismatch nucleotides when hybridised

with the second oligonucleotide. Binding of the first oligonucleotide to the target nucleic acid prevents the second oligonucleotide from binding to the first oligonucleotide of the probe. It is preferred that the T_m of the hybrid of said first oligonucleotide and the target sequence is at least 2 degrees higher than the T_m of the hybrid of said first oligonucleotide and the second oligonucleotide. It is more preferred that T_m of the hybrid of said first oligonucleotide and the target sequence is at least 5 degrees higher than the T_m of the hybrid of said first oligonucleotide and the second oligonucleotide.

In yet another aspect, the first oligonucleotide comprises a third region which is identical or substantially identical to a primer sequence (see for example, FIG. 4C and E). The third region may be complementary or not complementary to the target sequence. Multiple probes in a set of probes may comprise the same third region sequence. The primer identical to the third region sequence may act as a universal amplification primer. When the target specific probe (acting as primer) is running low at several cycles of amplification, the universal primer can take over and proceed to following cycles of amplification.

In some embodiments of the invention, the first and second oligonucleotides are linked by a linker moiety. This linker moiety may comprise nucleotides, nucleotide derivatives, nucleotide analogs or a non-nucleotide chemical linkage, i.e. the first and second oligonucleotides might be a single stretch of contiguous oligonucleotides (FIG. 4K). In this embodiment, the probe can be understood as comprises a first oligonucleotide only (see for example, FIG. 4K), which comprises self-complementary regions capable of forming stem-loop structure, wherein said self-complementary regions are substantially complementary to each other which form the double-stranded portion of the probe. The stem part can be located any part of oligonucleotide and has a length of 4 to 20 nucleotides. The 3' part of the oligonucleotide is preferably complementary to the target sequence. It can have a blunt end, or 3' protruding end or 5' protruding end. Blunt end, or 3' protruding end is preferred form.

The first oligonucleotide of the probe is capable of being consumed during amplification. Alternatively, both the first and second oligonucleotides are capable of being consumed during amplification. It is preferred that the first oligonucleotide is designed to be consumed, while the second oligonucleotide may remain unchanged in a reaction.

The first oligonucleotide of the probe may be extendable, thereby acting as a primer. Alternatively, the first oligonucleotide is blocked at the 3' end and second oligonucleotide is blocked at the 3' end, thereby being non-extendable.

Each probe comprises a detectable label which is capable of producing a changeable signal which is characteristic of the presence or absence of a double-stranded portion between the first and second oligonucleotides of that probe.

Furthermore, at least two of the probes comprise the same detectable label(s) or different detectable labels(s) with undistinguishable emission spectra.

The label on the probe may be a fluorophore, or the probe may comprise an interactive pair of labels, for example fluorophores and/or non-fluorophore dyes. One example of such interactive labels is a fluorophore-quencher pair. The label on the probe can be located anywhere as long as it interacts with other labels or other entities such as G nucleotides.

In some embodiments, the first oligonucleotide comprises a first label and the second oligonucleotide comprises second label. Preferably, the first label is a fluorophore and the second label is a quencher, or vice versa.

In other embodiments, the probe comprises two labels, the labels being a FRET pair. Preferably one label is on the first oligonucleotide and the second label is on the second oligonucleotide.

Additionally, both the first and second oligonucleotides can also comprise a plurality of label moieties. For example, both the first oligonucleotide and the second oligonucleotide may comprise both a fluorophore and a quencher.

Typically, the fluorophore and the quencher are attached to the oligonucleotides such that when the first oligonucleotide is bound to an unlabelled template sequence (e.g., a target), the fluorophore and the quencher are separated.

Alternatively, the fluorophore and the quencher are attached to the oligonucleotides such that when the first oligonucleotide is bound to an unlabelled template sequence (e.g., a target), the fluorophore and the quencher are brought into close proximity and hence the fluorophore is quenched.

"Fluorophore" as used herein to refer to a moiety that absorbs light energy at a defined excitation wavelength and emits light energy at a different defined wavelength.

Examples of fluorescence labels include, but are not limited to: Alexa Fluor dyes (Alexa Fluor 350, Alexa Fluor 488, Alexa Fluor 532, Alexa Fluor 546, Alexa Fluor 568, Alexa Fluor 594, Alexa Fluor 633, Alexa Fluor 660 and Alexa Fluor 680), AMCA, AMCA-S, BODIPY dyes (BODIPY FL, BODIPY R6G, BODIPY TMR, BODIPY TR, BODIPY 530/550, BODIPY 558/568, BODIPY 564/570, BODIPY 576/589, BODIPY 581/591, BODIPY 630/650, BODIPY 650/665), Carboxyrhodamine 6G, carboxy-X-rhodamine (ROX), Cascade

Blue, Cascade Yellow, Cyanine dyes (Cy3, Cy5, Cy3.5, Cy5.5), Dansyl, Dapoxyl, Dialkylaminocoumarin, 4 ', 5'-Dichloro-2 ',7'-dimethoxy-fluorescein, DM-NERF, Eosin, Erythrosin, Fluorescein, FAM, Hydroxycoumarin, IRDyes (IRD40, IRD 700, IRD 800), JOE, Lissamine rhodamine B, Marina Blue, Methoxycoumarin, Naphthofluorescein, Oregon Green 488, Oregon Green 500, Oregon Green 514, Pacific Blue, PyMPO, Pyrene, Rhodamine 6G, Rhodamine Green, Rhodamine Red, Rhodol Green, 2 ', 4 ', 5 ', 7'-Tetra-bromosulfone-fluorescein, Tetramethyl-rhodamine (TMR), Carboxytetramethylrhodamine (TAMRA), Texas Red and Texas Red-X.

As used herein, the term "quencher" includes any moiety that is capable of absorbing the energy of an excited fluorescent label when it is located in close proximity to the fluorescent label and capable of dissipating that energy. A quencher can be a fluorescent quencher or a non-fluorescent quencher, which is also referred to as a dark quencher. The fluorophores listed above can play a quencher role if brought into proximity to another fluorophore, wherein either FRET quenching or contact quenching can occur. It is preferred that a dark quencher which does not emit any visible light is used. Examples of dark quenchers include, but are not limited to, DABCYL (4-(4'-dimethylaminophenylazo) benzoic acid) succinimidyl ester, diarylrhodamine carboxylic acid, succinimidyl ester (QSY-7), and 4 ',5'-dinitrofluorescein carboxylic acid, succinirnidyl ester (QSY-33), quencherl, or "Black hole quenchers" (BHQ-1, BHQ-2 and BHQ-3), nucleotide analogs, nucleotide G residues, nanoparticles, and gold particles.

The interactive label pair can form either FRET or a contact quenching relationship. It is preferred that for effective contact quenching, the fluorophores and quencher are at a distance of about 0-10 nucleotides. It is more preferred that the fluorophores and quencher are at a distance of about 0-5 nucleotides. It is most preferred that the fluorophores and quencher are at a distance of about 0-2 nucleotides. The quencher is preferably a non-fluorescent entity. The quencher may be a nanoparticle. A nanoparticle may be a gold nanoparticle. It is also possible that the quencher is a G residue or multiple G residues.

The label or combination of labels on each probe are capable of producing a changeable signal which is characteristic of the presence or absence of a double-stranded portion between the first and second oligonucleotides of that probe. It can be seen therefore that the function of the label is to facilitate the determination of whether the first oligonucleotide is bound to the second oligonucleotide or to the target nucleic acid, and in particular whether or not the first oligonucleotide is bound to the second oligonucleotide.

At least one label is attached to the first oligonucleotide or to the second oligonucleotide. The label either increases or decreases fluorescence emission when the first oligonucleotide is bound to the second oligonucleotide.

Preferably, the probe comprises a first label and a second label, wherein at least one label is capable of producing a detectable signal and wherein the signal strength is affected by the proximity of the two labels.

In some embodiments of the invention, the first label is attached to one strand of the double stranded portion and the second label is attached to the opposite strand of the double stranded portion of the probe such that said first label and second label are in close proximity when the probe's internal duplex is formed.

In other embodiments, the first label is attached to the first oligonucleotide, and the second label is attached to the second oligonucleotide such that said first label and second label are in close proximity when the probe's internal duplex is formed.

In some embodiments of the invention, the first oligonucleotide does not comprise a label. In other embodiments, the second oligonucleotide comprises a single label which is capable of changing fluorescence emission when hybridised with the first oligonucleotide.

In some embodiments, the first label is attached to the first oligonucleotide and the second label is attached to the second oligonucleotide such that said first label and second label are in close proximity when the probe's internal duplex is formed. Preferably, the first label is attached to the second region of the first oligonucleotide and the second label is attached to the region of the second oligonucleotide which is complementary to the second region of the first oligonucleotide such that the first and second labels are brought into close proximity upon formation of the probe's internal duplex. Examples of such embodiments are shown in Figures 4A, 4B and 4C.

In some aspects of the invention, the first oligonucleotide of the probe does not comprise a label, but the second oligonucleotide of the probe comprises at least one, preferably two, labels.

In one embodiment of this aspect, the second oligonucleotide comprises a first label and a second label. The first label is attached at or near one end of second oligonucleotide and the second label is attached at or near the other end of the second oligonucleotide, whereby when the second oligonucleotide is not hybridised with the first oligonucleotide, the second oligonucleotide is in a random-coiled or a stem-loop structure which brings the first label and

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second label in close proximity. When the second oligonucleotide is hybridised with the first oligonucleotide, the two labels are held away from each other. Examples of such embodiments are shown in Figures 4D, 4E and 4F.

As is known in the prior art, a double-labelled oligonucleotide (equivalent to the second oligonucleotide in this invention) can form a random-coiled structure when it is in single-stranded form and at certain permissive temperatures. This kind of linear oligonucleotide probes in solution behaves like a random coil: its two ends occasionally come close to one another, resulting in a measurable change in energy transfer. However, when the probe binds to its template, the probe-template hybrid forces the two ends of the probe apart, disrupting the interaction between the two terminal moieties, and thus causing a fluorescence emission change.

A double-labelled oligonucleotide can also form a stem-loop structure known as molecular beacon. Molecular beacon probes are single-stranded oligonucleic acid probes that can form a hairpin structure in which a fluorophore and a quencher are usually placed on the opposite ends of the oligonucleotide. At either end of the probe short complementary sequences allow for the formation of an intramolecular stem, which enables the fluorophore and the quencher to come into close proximity. The loop portion of the molecular beacon is complementary to a target nucleic acid of interest. Binding of this probe to its target nucleic acid of interest forms a hybrid that forces the stem apart. This causes a conformation change that moves the fluorophore and the quencher away from each other and leads to a more intense fluorescent signal(Tyagi S. and Kramer F. R., Nature Biotechnology, Vol. 14, pages 303-308 (1996); Tyagi et al., Nature Biotechnology, Vol. 16, pages 49-53(1998); Piatek et al., Nature Biotechnology, Vol. 16, pages 359-363 (1998); Marras S. et al., Genetic Analysis: Biomolecular Engineering, Vol. 14, pages 151-156 (1999); Tpp I. et al, BioTechniques, Vol 28, pages 732-738 (2000)).

In the present invention, one type of the second oligonucleotides, which can be a molecular beacon-like oligonucleotide, is part of a double-stranded portion of a probe. The differences of the present invention from the prior art technologies are that the second oligonucleotide may not hybridise to the target sequence, but to the second region of the first oligonucleotide which may be unrelated to the target sequence. The second oligonucleotide may be capable of hybridising to the target sequence, but it is designed that it may not be able to hybridise to the target sequence in a real amplification reaction. The second oligonucleotide may contain sequence which is incapable of binding a target sequence strongly. During an

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extension and annealing step of amplification, the temperature may be too high for the second oligonucleotide to hybridise to the target sequence. While during the signal collection step, which often is carried out after an extension step, the temperature may be low, but the target sequence may be unavailable for the second oligonucleotide to hybridise, as the target sequence to be amplified may become double-stranded due to the extension of amplification primer already take place. Therefore, during signal collection step, the second oligonucleotide may only be able to hybridise to the unconsumed first oligonucleotide of the probe.

In another aspect, the first oligonucleotide does not comprise a label and the second oligonucleotide comprises a label. When the second oligonucleotide hybridises to the first oligonucleotide to form the double-stranded portion of the probe, the label changes its detectable signal emission relative to the emission of the label when in the single-stranded form of the second oligonucleotide. This may be because either the label is brought into close proximity with a nucleotide or nucleotides in the first oligonucleotide, or the label is held away with a nucleotide or nucleotides in the second oligonucleotide.

It is known in the prior art that the emission of a fluorescence dye can be changed when in close proximity to certain nucleotides, for example a G nucleotide.

In other embodiments, the first oligonucleotide of the probe does not comprises a label and the probe comprises two second oligonucleotides which are capable of hybridising adjacently or substantially adjacently to different parts of the second region of the first oligonucleotide, wherein one of the second oligonucleotides is attached with a first label, and the other second oligonucleotide is attached with a second label, such that when the two second oligonucleotides are hybridised to the first oligonucleotide, the two labels are brought in close proximity and one label affects the signal from the other.

This close proximity may, for example, cause either FRET or contact quenching relationship. The two second oligonucleotides that hybridise to the first oligonucleotide are part of the probe. The two labelled second oligonucleotides are designed to not hybridise to amplified target sequence, but to hybridise to the unconsumed first oligonucleotide. Examples of such embodiments are given in Figures 4I and 4J.

In yet other embodiments, the first and second oligonucleotides of a probe are joined by a linker moiety which comprises nucleotides or a non-nucleotide chemical linker, allowing the first oligonucleotide and second oligonucleotide to form a stem-loop structure, wherein the first and second oligonucleotides are each labelled such that, when the probe forms an internal

stem-loop structure, the labels are brought into close proximity and one label affects the signal from the other.

The linker may, for example, be a simple moiety of formula $(CH_2)_n$ or a linker which is functionally equivalent thereto. (n is preferably 1-100 or 1-50). Preferably, the linker is an oligonucleotide which is contiguous with the first and second oligonucleotides.

At least two of the probes in the amplification reaction mixture comprise the same detectable label or a different detectable label with undistinguishable emission spectra.

In multiplex reactions, two or more probes are used to assay for the presence of two or more target nucleic acids. This does not necessary mean, however, that different distinguishable labels are needed for each of the different probes. Each probe will have a characteristic melting profile which will be dependent on the features of its internal duplex. Therefore, provided that two or more probes have distinguishable melting characteristics, the same label(s) can be used for those probes. In other words, different probes which are labelled with the same labels or labels having undistinguishable emission spectra must have different melting characteristics, preferably different melting temperatures (T_m). These different melting characteristics will allow the individual probes to be identified and/or quantified in step (c).

As used herein, the term "melting characteristics" includes the melting profile of a probe (preferably measured by detecting the signal from the label(s) on that probe as function of temperature) and/or the melting temperature (T_m) of a probe.

When the melting characteristics of probes are said to be "different", it will be understood that the differences are to be measured under the same or control conditions.

In some embodiments of the invention, two or more probes are labelled with the same detectable label. For example, the first oligonucleotides of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 20 or 30 or more different probes may all be labelled with the same label and/or the second oligonucleotides of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 20 or 30 or more different probes may all be labelled with the same label.

In step (b), an amplification reaction is performed on the sample/amplification reaction mixture wherein, when a target nucleic acid is present, the first oligonucleotides which are substantially complementary to part of that target nucleic acid are consumed during the amplification reaction.

The amplification reaction can be carried out under conditions known in the art such that the first oligonucleotides which are substantially complementary to part of the target nucleic acid are consumed.

Preferably, the amplification comprises at least one denaturing step, at least one annealing step and at least one primer extension step.

More preferably, the amplification is a thermal cycling amplification comprising two or more denaturing, annealing, and primer extension steps.

Preferred amplification reactions include PCR, SDA, NASBA, LAMP, 3SR, ICAN, TMA, Helicase-dependent isothermal DNA amplification and the like. PCR is a preferred amplification method. If the amplification is PCR, the conditions comprise thermally cycling the reaction.

When a target nucleic is present in the sample, at least some of the first oligonucleotides which are complementary to part of the target nucleic acids will hybridise thereto under appropriate reaction conditions. The first oligonucleotides will therefore be consumed.

The consumption of the probes is achieved through hybridisation of the probe to the target sequence, which may be followed by incorporation of the probe into the amplified product or/and degradation of the probe during the amplification step. In other words, after consumption, the first oligonucleotide is no longer able to reconstitute the probe of which it previously formed a part.

In an amplification reaction, the probe of the present invention can be constituted by adding the first and second oligonucleotides in the reaction at any ratio of the second oligonucleotide to the first oligonucleotide, for example preferably more than 1, or may be more than 0.1 and less than 1. Thus the first and second oligonucleotides may be added to the amplification reaction mixture independently.

Depending on the type of assay and the type of label that is actually used, the signal from the probe (e.g. fluorescence emission) may either increase or decrease when the first oligonucleotide is consumed. Reference is made, for example, to the embodiments shown in Figure 4. In Figures 4A-4C, the consumption of the probe leads to an increase in fluorescence because the quencher attached to the first oligonucleotide is consumed, thus allowing the fluorophore which is attached to the second oligonucleotide to emit its signal. On the contrary, in Figures 4D-4F, the consumption of the first oligonucleotide releases the dual end-labelled

second oligonucleotide allowing the fluorophore and quencher to juxtapose one another, thus leading to a reduction in signal from the fluorophore.

In one embodiment, the first oligonucleotide of the probe is extendable and acts as the forward or reverse primer. The first oligonucleotide of the probe may be one of the amplification primers, which is capable of being incorporated into an amplified product, thereby being consumed (see for example FIG. 6). In a PCR, the first oligonucleotide of the probe pairs with another amplification primer for the opposite strand to perform the amplification. During signal collection step, or the step of measuring the melting profile of the probe, the incorporated first oligonucleotide of the probe is not available to form the duplex of the probe's internal double stranded portion. The amount of unconsumed first oligonucleotide of the probe which is able to form the probe duplex can be measured and determined, and the signal can be transformed to the amount of the first oligonucleotide being consumed, thereby determining which or how much of the target sequence is present in a sample. When the first oligonucleotide acts as a primer, the second oligonucleotide preferably does not act as a primer.

In another embodiment of the present invention, the first oligonucleotide of the probe is degraded during amplification. The first oligonucleotide may, for example, comprise nucleotides or non-nucleotide chemicals which are sensitive to a digestion agent. In a further example, upon hybridising to the target sequence the first oligonucleotide can be degraded by the digestion agent. For example, the first oligonucleotide can comprise RNA nucleotides. When the first oligonucleotide hybridises to the target sequence, it can be degraded by enzymes with the RNase H activity. It can be designed such that the first oligonucleotide is not able to be degraded when hybridised to the second oligonucleotide to form the double stranded portion of the probe. The first oligonucleotide can also be degraded by an exonuclease. For example, the 3' nucleotide of the first oligonucleotide can be degraded by the 3' exonuclease activity of a polymerase. The first oligonucleotide can also be degraded by an endonuclease, for example by a restriction enzyme upon hybridisation to a target nucleic acid.

It is preferred that the first oligonucleotide of the probe is degraded by the 5' exonuclease activity of a DNA polymerase, such as Taq DNA polymerase. In this embodiment, the first oligonucleotide of the probe may be blocked at the 3' end, and hence is therefore non-extendable. The first oligonucleotide hybridises to the target sequence in the region bounded by the forward and reverse primers and can be degraded by a nuclease activity, such as 5' exonuclease activity of Taq polymerase during PCR amplification (see for example FIG. 8). Alternatively, the first oligonucleotide of the probe is not blocked at the 3' end, and hence is

therefore extendable. The first oligonucleotide hybridises to the target sequence and can be extended by a polymerase. An amplification primer upstream of the first oligonucleotide is also extended. When the extension of the amplification primer encounter the extension strand of the first oligonucleotide, the entire extension strand of the first oligonucleotide can be degraded by a nuclease activity, such as 5' exonuclease activity of Taq polymerase during PCR amplification (see for example, FIG. 7).

In yet another embodiment of the present invention, the consumption of the first oligonucleotide of the probe can simply be the hybridisation of the first oligonucleotide to the target sequence, whereby the hybridised first oligonucleotide is not available to form double stranded duplex of the probe (see, for example, FIG. 5). It is preferred that amplification is designed to produce a single-stranded product so that the first oligonucleotide of the probe can hybridise to the target sequence during the annealing step and/or after the extension step. The methods to produce single-stranded products can be asymmetric PCR, or a method described in PCDR (PCT/GB2007/003793). The single-stranded amplification strand can form a strong hybrid with the first oligonucleotide; therefore the first oligonucleotide can be regarded as consumed, as it is not readily available to form the internal probe hybrid.

In yet another embodiment of the present invention, the first oligonucleotide of the probe plays a role as a nested inner amplification primer. The first oligonucleotide and an outer amplification primer anneal to the same strand of the target nucleic acid. The outer amplification primer and the first oligonucleotide may be both extended upon hybridisation to the target nucleic acid. The extension strand of the first oligonucleotide may be displaced during the extension of the outer primer if the DNA polymerase comprises a displacement activity. The extension strand of the first oligonucleotide may be degraded during the extension of the outer primer if the DNA polymerase comprises a 5' exonuclease activity.

In some embodiments, an amplification condition can be designed such that at some stages of the amplification, even when the target is present, the probe is not consumed, whereas at other stages of the amplification the probe is consumed. For example, if the amplification is PCR, in the some thermal cycles of amplification, the probe may not be consumed, because the annealing and extension temperature are set too high for the probe to bind. The amplification thermal condition can be designed such that the probes can be consumed at some stages, or the last cycle of the amplification. For example, after thermal cycling the PCR vessels are incubated at a temperature which is set lower than either annealing and extension temperatures

of the thermal cycling. This low temperature allows the probe to hybridise to the target nucleic acids, resulting in either extension or degradation of the hybridised probe.

Step (b) may further comprise the step (b1) of measuring cycle by cycle fluorescence emissions (FEs) at various measuring temperatures (MTs). The measuring temperatures (MTs) refers to the temperature at which an emission reading of the label in the probe is taken cycle by cycle to determine the emission amount of a probe.

The emission of a label on the probe is preferably obtained, detected and/or recorded in each cycle in a reaction after the reaction mixture is illuminated or excited by light with a wavelength absorbed by the label. The term "cycle by cycle" refers to measurement in each cycle. The emission reading at a measuring temperature is taken to calculate the emission amount of a remaining probe in a cycle. Emission can be detected, recorded, or obtained continuously or intermittently.

In a continuous recording process, the emission of the probe is monitored and recorded, for example, every 50 ms, every 100 ms, every 200 ms or every 1 s, in each cycle of, for example, a PCR reaction. A three dimensional plot of time, temperature and emission can be formed. In any given cycle, the emission reading at a time point that corresponds to a desired MT is taken to determine the emission amount of the probe in the cycle. In an intermittent recording process, the emission reading is taken only when the reaction temperature reaches a desired MT in each cycle.

For the first oligonucleotides of the probe which are consumed by incorporating into the amplified product or being degraded by a digestion agent, the obtaining of the cycle by cycle fluorescence emissions (FE) is preferably performed after the completion of the extension step at each cycle. For the first oligonucleotides of the probe which are consumed by being hybridised to the target sequence, the obtaining of the cycle by cycle fluorescence emissions (FE) may be performed before the completion of the extension step at each cycle.

Fluorescence emissions (FE) is used herein to refer to baseline corrected fluorescence (dR). Normally, for each well (reaction) and each optical path the raw fluorescence data are fitted over the specified range of cycles using a linear least mean squares algorithm (or other such algorithm) to produce a baseline. The value of the baseline function is calculated for every cycle and subtracted from the raw fluorescence to produce the baseline corrected fluorescence (dR).

The fluorescence intensity data (amplification plots) can be described as a two-component function: a linear component or background and an exponential component that

contains the relevant information. To isolate the exponential component, the linear contribution to the fluorescence can be estimated and subtracted. It is a three-step process that is carried out for each amplification plot (i.e. each reaction and each label):

1. Identify the range of cycles during which all contributions to the fluorescence are strictly linear (no exponential increase in fluorescence).
2. Using the fluorescence intensity values during the cycles determined above, fit the data to a straight line (a function predicting the contribution of the linear components throughout the reaction).
3. Subtract the predicted background fluorescence intensity during each cycle. The resulting curve corresponds to the change in fluorescence due to DNA amplification.

When the amplification reaction mixture comprises "n" probes for multiplex detection of "n" nucleic acid targets, first probe has a melting temperature of T_m1, the second probe has a melting temperature of T_m2, the third probe has a melting temperature of T_m3, the k-th probe has a melting temperature T_mk, and the n-th probe has a melting temperature of T_mn, wherein $T_m1>T_m2>T_m3> \ldots T_mk \ldots >T_mn$, wherein n and k are positive integers, $1 \leqslant k \leqslant n$, and $n \geqslant 2$.

The percentage of a probe's double-stranded form out of the total amount of probes at a particular temperature or at series temperatures may be determined experimentally or predicted which can be done by a computer program. Since the first negative derivative of a probe's melting emission with respect to temperature is plotted to form a normal distribution curve, an ordinary person skilled in the field of statistics could readily define a MT at which a percentage of the total number of a given probe is in duplex form or in single-stranded (i.e. separated) form. Accordingly, a measuring temperature is a temperature at which no more than 20%, for example, of a probe is in single-stranded form. A table may be created listing percentages of double-stranded (ds) and single-stranded forms(s) of each probe at each temperature.

A first fluorescence emission FEa can be obtained at a measuring temperature MTa, at which more than 50% of first probe is in duplex form; second fluorescence emission FEb can be obtained at a measuring temperature MTb, at which more than 50% of second probe is in duplex form; k-th fluorescence emission FEk can be obtained at a measuring temperature MTk, at which more than 50% of k-th probe is in duplex form; n-1 fluorescence emissions FE(n-1) can be obtained at a measuring temperature MT(n-1), at which more than 50% of (n-1)-th probe is in duplex form, n-th fluorescence emission FEn can be obtained at a measuring temperature MTn, at which more than 50% of n-th probe is in duplex form, and optionally a

fluorescence emission FE0 can be obtained at a measuring temperature MT0, at which no more than 10 % of first probe is in duplex form.

Although the above-mentioned 50% is a preferred amount of probe in the duplex form, it should be appreciated that any percentage can be used, such as 40%, 55%, 70% or 80%. It is preferred that in the step obtaining cycle by cycle a fluorescence emission FEk at a measuring temperature MTk, at which no more than 30% of (k-1)-th probe is in the probe's internal duplex form. It is even preferred that in the step obtaining cycle by cycle a fluorescence emission FEk at a measuring temperature MTk, at which no more than 20% of (k-1)-th probe is in the probe's internal duplex form.

The step (b) may further comprise the step (b2) determining cycle by cycle Actual Consumed Amount of fluorescence emission from consumed probe for each probe, wherein the Actual Consumed Amount of fluorescence emission t of k-th probe is depicted as ACA_k. At a particular measuring temperature (MTa), where a probe has certain percentage (dska)% in ds (double-strand) form, the fluorescence emission FE at this measuring temperature MTa contributed by first probe will be (ds1a)% * (ACA_1), contributed by the second probe will be (ds2a)% * (ACA_2), contributed by the k-th probe will be (dska)% * (ACA_k). For example, at 60°C 70% of probe 1 is in ds form; at 50°C 80% in ds. The FE contributed by the probe 1 at 60°C will be 70% * (ACA_1); FE contributed by the probe 1 at 50°C will be 80% * (ACA_1). If multiple probes are present, the FE will be the total amount contributed by consumed probes of all probes. The calculation of Actual Consumed Amount (ACA) can use the following formula:

At temperature a, the total fluorescence emission will be

FEa=(ACA1)*(ds1a)%+ (ACA2)*(ds2a)% +(ACA3)*(ds3a)% ...+(ACAn)*(dsna)%

At temperature b, the total fluorescence emission will be

FEb=(ACA1)*(ds1b)%+ (ACA2)*(ds2b)% +(ACA3)*(ds3b)% ...+(ACAn)*(dsna)%

At temperature c, the total fluorescence emission will be

FEc=(ACA1)*(ds1c)%+ (ACA2)*(ds2c)% +(ACA3)*(ds3c)% ...+(ACAn)*(dsna)%

And so on. The individual ACA can be calculated from the above formulas.

"*" denotes "multiply by".

It is preferred that the Actual Consumed Amount of each probe is obtained though a computer program which performs the above calculation. Alternatively, the calculation can be done manually.

For example, in an amplification reaction, there are three probes for three target sequences. At temperature 65°C, 5% of the first probe is in duplex form, 0% of the second probe and 0% of third probe is in duplex form. At 60°C, 60% of the first probe is in duplex form, 5% of second probe is in duplex form, 0% of the third probe is in duplex form. At 55°C, 90% of the first probe is in duplex form, 55% of second probe is in duplex form, 5% of third probe is in duplex form. At 45°C, more than 95% of all probes are in duplex form. The first fluorescence emission is collected at 60°C, which is FE60, the second fluorescence emission is collected at 55°C, which is FE55, the third fluorescence emission is collected at 45°C, which is FE45. Optionally before the first fluorescence emission is collected, a fluorescence emission is collected at 65°C or above, which is FE65. The FE contributed by the actual consumed amount ACA from individual probe is calculated as ds%*(ACA). See the table below:

	probe1		probe2		probe3		fluorescence emission
	ds%	ACA1	ds%	ACA2	ds%	ACA3	
65°C	5	5%ACA1	0	0%ACA2	0	0%ACA3	FE65
60°C	60	60%ACA1	5	5%ACA2	0	0%ACA3	FE60
55°C	90	90%ACA1	55	55%ACA2	5	5%ACA3	FE55
45°C	95	95%ACA1	95	95%ACA2	95	95%ACA3	FE45

$$FE65=5\%*ACA1 + 0\%*ACA2 + 0\%*ACA3 \approx 0$$

$$FE60=60\%*ACA1 + 5\%*ACA2 + 0\%*ACA3 \quad (1)$$

$$FE55=90\%*ACA1 + 55\%*ACA2 + 5\%*ACA3 \quad (2)$$

$$FE45=95\%*ACA1 + 95\%*ACA2 + 95\%*ACA3 \quad (3)$$

The individual ACA can be calculated from the above formulas. If we assume 5%*ACA can be neglected. The approximate ACA can be calculated from (1), (2) and (3), where

$$ACA1= (EF60)/0.6$$

$$ACA2= ((EF55)-0.9/0.6*(EF60))/0.55$$

$$ACA3= (EF45)/0.95-(EF60)/0.6-(EF55-(0.9/0.6)*(EF60))/0.55$$

As a PCR mixture undergoes thermal cycling, the fluorescence emission and actual consumed amount (ACA) are recorded (calculated) and plotted over the number of cycles to form an emission versus cycle plot. In the initial cycles, there is little change in the emission amount which appears as a baseline or a plateau in the plot. As thermal cycling continues, an increase in emission amount above the baseline may be expected to be observed, which indicates that the amplified product (or consumed probes) has accumulated to the extent that

fluorescence emission of a probe in the presence of the amplified product (or consumed probe) exceeds the detection threshold of a PCR instrument. An exponential increase in emission amount initiates the exponential phase and eventually reaches another plateau when one of the components in the PCR mixture becomes limiting. The plot usually produces an S-shape curve with two plateaus at both ends and an exponential phase in the middle. In the exponential phase, the emission amount of the probe is increasing by (1+E) fold over the previous amount of each cycle, wherein E is the efficiency of amplification, which ideally should be 100% or 1. It is commonly known that the higher the starting amount of the nucleic acid template from which a product is amplified, the earlier an increase over baseline is observed. As is well known in the art, the emission versus cycle plot provides significant information for attaining the initial copy number or amount of the nucleic acid template.

As is known in the field of real-time PCR, the unknown amount of a nucleic acid template may be quantified by comparing the emission versus cycle plot of the template with standardized plots.

In one embodiment of the present invention, when a plurality of nucleic acid templates are amplified to form a plurality of amplified products, each product is preferably compared with a standard curve formed by the same product. A single product per dilution per PCR mixture can be used to form the standard curve. Preferably, at each dilution, a plurality of products are placed in a single PCR mixture and emission readings of each probe can be measured and plotted to form a standard curve based on methods described in the present invention.

The starting amount of a nucleic acid template in a sample can also be determined by normalizing the template to a house-keeping gene or a normalizer in relative relationship to a calibrator without using a standard curve. For example, GAPDH (glyceraldehyde 3-phosphate dehydrogenase) and β-actin are commonly regarded a suitable house-keeping nucleic acid templates or normalizer templates due to their abundance and constant levels of expression in cells. The calibrator can be an untreated sample or a specific cell, tissue or organ used for the normalization of treated samples or targeted cells.

In one embodiment of the present invention, a plurality of nucleic acid templates of interest are amplified and quantified in a single PCR mixture. The starting amount of each nucleic acid template can simultaneously be calculated and normalized to a normalizer. It is also contemplated that a plurality of nucleic acid templates and a normalizer template can be monitored and amplified in the same PCR reaction. It is further contemplated that more than

one housekeeping template or normalizer can be amplified along with multiple nucleic acid templates in a single PCR reaction. It is further contemplated that the relative amount among these templates or the ratios between or among these templates can be determined from a single PCR mixture.

Step (c) comprises measuring, at least once, the melting profile of the double-stranded portions between the first and second oligonucleotides of unconsumed probes by detecting the signals from the labels in those probes as a function of temperature,
wherein the melting profile provides an indication of whether or not at least one target nucleic acid has been amplified in said sample/amplification reaction mixture..

To determine the melting (profile) curve for each probe or each set of probes, the reaction mixture is illuminated with light that is absorbed by the labels of the probes and the fluorescence of the reaction is monitored as a function of temperature. More particularly, the fluorescence of the labels is measured as the temperature of the sample is raised (or decreased) until a baseline level of fluorescence is achieved.

The data may be presented as fluorescence vs. temperature plots or as first derivative plots of fluorescence vs. temperature, for example. The two plots are interchangeable, but each focuses the viewer's attention on different aspects of the data. The melting peak (or T_m) is best viewed on derivative plots. However, the broadening of the transition and appearance of low melting transitions are easier to observe on fluorescence vs. temperature plots. The point at which there is a shift in the rate of decrease or increase of fluorescence can be more easily identified by viewing a plot of the first derivative of the fluorescence vs. temperature. The point of maximum rate of change is considered the melting temperature of the probe duplex. If one probe has a higher T_m, it forms a stronger probe duplex and will consequently melt at a higher temperature than another probe. The distinctly different melting temperatures of different probes allow identification of which probe is consumed during amplification (if the probes have the same label).

In some methods of this invention, fluorescence is monitored as a function of a denaturing gradient. Independent from the type of gradient, however, what is actually monitored is the change in fluorescence caused by the dissociation of the two strands of the double-stranded portion of the probe. The denaturing gradient may be a thermal gradient. In other words, the invention is illustratively directed to a method, characterized in that during or subsequent to the (preferably PCR) amplification, temperature dependent fluorescence is monitored. It is often desirable, however, if the monitoring of temperature-dependent

fluorescence is part of a homogeneous assay format such that (PCR) amplification and monitoring temperature dependent fluorescence are carried out in the same reaction vessel without intermediate opening of the reaction chamber.

Melting profile analysis may be obtained by monitoring temperature-dependent fluorescence during melting or hybridisation. Usually, melting curve analyses are performed as slowly as possible in order to generate precise and highly reproducible data, in order to obtain an exact determination of the melting point, which is defined as the maximum of the first derivative of a temperature versus fluorescence plot. However, if the selected time parameters are comparatively short, certain advantages may be seen.

A melting profile may be measured after completion of amplification (post-amplification melting profile), and/or may be measured during amplification at each cycle or at selected cycles (mid-amplification melting profile).

In one embodiment, the temperature-dependent fluorescence is monitored after completion of a PCR reaction. In an alternative embodiment, the temperature-dependent fluorescence is monitored in real-time during a PCR reaction.

In one embodiment during said measuring, the amplified product (amplicon) remains double stranded, thereby involving little, if any, or no detectable change of signals. While in other embodiment, the measuring melting profile may be performed when some of the amplified product is in single-stranded form.

The skilled person will be able to determine, merely from the melting profile after amplification (and without comparing the melting profile with any other melting profiles) whether or not at least one target nucleic acid has been amplified. For example, Figures 1 and 2 illustrate the "flatter" curves obtained after amplification, as compared to the more "S" shaped curves which are characteristic of unconsumed probes.

The method of the invention may further comprise a step (d)

(i) comparing at least two melting profiles obtained in (c)

and/or

(ii) comparing a melting profile obtained in step (c)

with a previously-obtained melting profile of the same probes or

with a melting profile of the same probes obtained in parallel at the same time in control reactions, or

with a theoretical melting profile of the same probes,

wherein a change in the melting profile provides an indication of whether or not at least one target nucleic acid has been amplified in said sample/amplification reaction mixture.

The melting profile may be measured before amplification takes place (pre-amplification melting profile), and/or is measured after completion of amplification (post-amplification melting profile), and/or is measured during amplification at each cycle or selected cycles (mid-amplification melting profile),

A pre-amplification melting profile of the probes may be measured in the same reaction vessel before the start of amplification, or may be measured in a separate reaction vessel where no amplification takes place due to that the reaction mixture lacking one or more ingredients necessary for the amplification. Examples of such ingredients include a dNTP, a polymerase or a target nucleic acid.

It should be noted that the method of the invention does not necessarily require the pre-amplification melting profile to be measured as part of the method of the invention. This profile might be one which is characteristic of the probe or combination of probes in question and which might have been measured previously and/or stored in a retrievable manner, for example in a computer-readable format.

Each probe in the reaction mixture will be specific for a particular target nucleic acid. Different probes are used which are labelled with the same label or labels having undistinguishable emission spectra, and the probes are selected so as to have different melting characteristics, for example different melting profiles or different melting temperatures (T_ms).

The melting profile which is obtained in step (c) will be an amalgamation of the melting profiles of the probes which are present in the amplification reaction mixture. Due to the fact that different probes are selected so as to have different melting profiles, the individual contributions made by each probe to the combined melting profile will be separable by those skilled in the art, either manually or preferably by computer-implemented means. In this way, the presence or absence of particular probes, and hence the presence or absence of particular target nucleic acids in the sample, can be distinguished from one another.

Comparison of the pre- and post-amplification melting profiles of each probe may identify which feature of the curve that is a signature for a particular probe has disappeared or has been reduced, therefore indicating that that particular probe is consumed during amplification and further indicating that the corresponding target is present in the sample.

It is preferred that the comparison of the melting profiles of probes before amplification taking place and after completion of amplification or after some cycles of amplification is done by a computer program. The computer program determines which probe or how much the probe is consumed, which is indicative of which target or how much of the corresponding target is present in a sample.

The melting temperatures (T_m) of probes with the same label(s) or different label(s) with indistinguishable emission spectra will generally be different from the melting temperatures of each other similarly-labelled probe. In one embodiment, multiple probes in a set are each labelled with the same label (or different label(s) with indistinguishable emission spectra) and each probe has a distinct melting temperature range. In a multiplex assay, when a reaction temperature rises from the hybridising temperature to a denaturing temperature, the probe duplex with the lowest T_m unwinds first, the probe duplex with the next highest T_m separates next, and the probe duplex with the highest T_m denatures last. Concurrently, the fluorescent emission of the label attached to the probe changes in proportion to the rising reaction temperature due to the incremental melting of the probe duplex, hence allowing each probe to be distinguished in the combined melting profile. The shape and position of a melting curve is a function of GC/AT ratio, length, and the sequence of the double-stranded portion of the probe.

Preferably, the T_ms of probes with the same label(s) or different label(s) with indistinguishable emission spectra are at least 2°C, preferably at least 3°C, 4°C or 5°C different from other similarly-labelled probes.

In one aspect of the present invention, real time fluorescence monitoring of a PCR reaction is used to acquire a probe's melting curves during the PCR reaction. The temperature cycles of PCR that drive amplification alternately denature the accumulating product and the labelled probes at a high temperature, and anneal the primers and at least one strand of the probe to the product at a lower temperature, thereby consuming some probes. Plotting fluorescence as a function of temperature as the sample is heated through the dissociation temperature of probe's internal double stranded portion of the remaining (unconsumed) probe gives a probe's melting curve. Thus continuous monitoring of fluorescence during a PCR reaction provides a system for detecting changes of probe concentrations by probe melting profiles. Such a system, particularly a HRM-PCR system, can be used to differentiate the remaining probes separated by less than 2°C in melting temperature.

The invention also provides a method for monitoring a PCR amplification of at least two nucleic acid targets, said method comprising:

(a) contacting a sample comprising one or more target nucleic acids with an amplification reaction mixture comprising:

(i) one or more pairs of forward/reverse oligonucleotide primers, wherein the primer pairs are capable of amplifying one or more target nucleic acids, if present in the sample, and a nucleic acid polymerase,

(ii) two or more probes, wherein each probe comprises

a first oligonucleotide which comprises a first region which is substantially complementary to part of one target nucleic acid and a second region, and at least one second oligonucleotide which comprises a region which is substantially complementary to a second region of the first oligonucleotide, such that the first and second oligonucleotides are capable of forming a double-stranded portion,

wherein each probe comprises a detectable label which is capable of producing a changeable signal which is characteristic of the presence or absence of a double-stranded portion between the first and second oligonucleotides of that probe, and wherein the at least two of the probes comprise the same detectable label or different detectable labels with undistinguishable emissions spectra

wherein the melting characteristics of the double-stranded portions between the first and second oligonucleotides of each of such probes are different;

(b) performing an amplification reaction on the sample/amplification reaction mixture wherein, when a target nucleic acid is present, the first oligonucleotides which are substantially complementary to part of that target nucleic acid are consumed during the amplification reaction; and

wherein the step (b) further comprises the step (b1) obtaining cycle by cycle fluorescence emissions (FE) at various measuring temperatures (MT), wherein said fluorescence emissions (FE) are baseline corrected fluorescence (dR),

wherein when said amplification reaction mixture comprises "n" probes for multiplex detection of "n" nucleic acid targets, wherein first probe has a melting temperature of T_m1, second probe has a melting temperature of T_m2, third probe has a melting temperature of T_m3, n-th probe has a melting temperature of T_mn, wherein $T_m1>T_m2>T_m3 \ldots >T_mn$, wherein the percentages of the double-stranded form of each probe at a particular temperature or different

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temperatures are determined experimentally or are calculated in theory by a computer program, wherein a first fluorescence emission FEa is obtained at a measuring temperature MTa, at which more than 50 % of first probe is in duplex form, second fluorescence emission FEb is obtained at a measuring temperature MTb, at which more than 50 % of second probe is in duplex form, n-1 fluorescence emission FE(n-1) is obtained at a measuring temperature MT(n-1), at which more than 50 % of (n-1)th probe is in duplex form, n-th fluorescence emission FEn is obtained at a measuring temperature MTn, at which more than 80 % of n-th probe is in duplex form, and optionally a fluorescence emission FE0 is obtained at a measuring temperature MT0, at which no more than 10 % of first probe is in duplex form, wherein n is a positive integer and $n \geqslant 2$,

wherein the step (b) may further comprise the step (b2) determining cycle by cycle Actual Consumed Amount of fluorescence emission from consumed probe for each probe, wherein the Actual Consumed Amount of fluorescence emission t of k-th probe is depicted as ACA_k. At a particular measuring temperature (MTa), wherein a probe has certain percentage (dska)% in ds (double-strand) form, the fluorescence emission FE at this measuring temperature MT contributed by first probe will be (ds1)% * (ACA_k), contributed by the second probe will be (ds2a)% * (ACA_2), contributed by the k-th probe will be (dska)% * (ACA_k). For example, at 60°C 70% of probe 1 is in ds form; at 50°C 80% in ds form. At 60°C the FE contributed by the probe 1 will be 70% * (ACA_1); at 50°C FE contributed by the probe 1 will be 80% * (ACA_1). If multiple probes are present, the FE will be the total amount contributed by consumed probes of all probes. The calculation of Actual Consumed Amount (ACA) can use the following formula:

At temperature "a", the total fluorescence emission will be

FEa=(ACA1)*(ds1a)%+ (ACA2)*(ds2a)% +(ACA3)*(ds3a)% ...+(ACAn)*(dsna)%

At temperature "b", the total fluorescence emission will be

FEb=(ACA1)*(ds1b)%+ (ACA2)*(ds2b)% +(ACA3)*(ds3b)% ...+(ACAn)*(dsna)%

At temperature "c", the total fluorescence emission will be

FEc=(ACA1)*(ds1c)%+ (ACA2)*(ds2c)% +(ACA3)*(ds3c)% ...+(ACAn)*(dsna)%

And so on. The individual ACA can be calculated from the above formulas.

wherein the emission amount of each probe is obtained though a computer program or is done manually.

The invention also provides a computer software product for use with the method of the invention adapted, when run on suitable data processing means, for comparing melting profiles of probes and/or quantifying a real time PCR amplification of multiplex targets which performs the calculation of the florescence emission and Actual Consumed Amount (ACA).

Generally, ACA can be calculated manually once the emission values are acquired through a PCR instrument and the percentages of the double stranded form of each probe at each temperature are known. However, it is frequently desirable to automate the calculation through the use of a computer system.

In a further embodiment, the invention relates to a computer system comprising a computer memory having a computer software program stored therein, wherein the computer software program, when executed by a processor or in a computer, performs methods according to the present invention. In a preferred embodiment, a computer program product comprises a computer memory having a computer software program stored therein, wherein the computer software program performs a method comprising the step of calculating the ACA and/or determination of features of melting profiles during amplification or at the end amplification.

As will be appreciated by those skilled in the art, a computer program product of the present invention, or a computer software program of the present invention, may be stored on and/or executed in a PCR instrument and used to calculate the amount of each probe.

The invention further provides a kit for assaying for one or more nucleic acid targets, which kit comprises a probe comprising:

a first oligonucleotide of 15-150 nucleotides which comprises a first region which is substantially complementary to part of one target nucleic acid and a second region, and at least one second oligonucleotide of 4-150 nucleotides which comprises a region which is substantially complementary to the second region of the first oligonucleotide, such that the first and second oligonucleotides are capable of forming a double-stranded portion,

wherein each probe comprises a detectable label or detectable combination of labels which is/are capable of producing a changeable signal which is characteristic of the presence or absence of a double-stranded portion between the first and second oligonucleotides of that probe,

and wherein

(a) the first oligonucleotide of the probe does not comprise a label, the second oligonucleotide comprises a first label and a second label, wherein the first label is attached at or near one end of second oligonucleotide and the second label is attached at or near the other end of the second oligonucleotide, whereby when the second oligonucleotide is not hybridised with the first oligonucleotide, the second oligonucleotide is in a random-coiled or a stem-loop structure which brings the first label and second label in close proximity and wherein when the second oligonucleotide is hybridised with the first oligonucleotide, the two labels are held away from each other; or

(b) the first oligonucleotide does not comprise a label and the second oligonucleotide comprises a label, wherein when the second oligonucleotide hybridises to the first oligonucleotide to form the double-stranded portion of the probe, the label is capable of changing its detectable signal emission relative to the emission of the label when in the single-stranded form of the second oligonucleotide; or

(c) the first oligonucleotide of the probe does not comprises a label, the probe comprises two second oligonucleotides which are capable of hybridising adjacently or substantially adjacently to different parts of the second region of the first oligonucleotide, wherein one of the second oligonucleotides is attached with a first label, and the other second oligonucleotide is attached with a second label, such that when the two second oligonucleotides are hybridised to the first oligonucleotide, the two labels are brought in close proximity and one label affects the signal from the other.

The invention also provides the use of a probe as defined in (a)-(d) above in a method of the invention.

The invention also provides the use of a probe comprising

a first oligonucleotide of 15-150 nucleotides which comprises a first region which is substantially complementary to part of one target nucleic acid and a second region, and at least one second oligonucleotide of 4-150 nucleotides which comprises a region which is substantially complementary to the second region of the first oligonucleotide, such that the first and second oligonucleotides are capable of forming a double-stranded portion,

wherein each probe comprises a detectable label or detectable combination of labels which is/are capable of producing a changeable signal which is characteristic of the presence or absence of a double-stranded portion between the first and second oligonucleotides of that probe,

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and wherein

(a) the first label is attached to the second region of the first oligonucleotide and the second label is attached to the region of the second oligonucleotide which is complementary to the second region of the first oligonucleotide such that the first and second labels are brought into close proximity upon formation of the probe's internal duplex, or

(b) the first and second oligonucleotides of the probe are joined by a linker moiety which comprises nucleotides or a non-nucleotide chemical linker, allowing the first oligonucleotide and second oligonucleotide to form a stem-loop structure, wherein the first and second oligonucleotides are each labelled such that, when the probe forms an internal stem-loop structure, the labels are brought into close proximity and one label affects the signal from the other,

in a method as disclosed herein.

Another aspect of the invention is directed to a method for assaying a sample for one or more variant nucleotides on the target nucleic acids, said method comprising:

(a) contacting a sample comprising target nucleic acids with an amplification reaction mixture comprising:

(i) one or more pairs of forward/reverse oligonucleotide primers, wherein the primer pairs are capable of amplifying one or more target nucleic acids, if present in the sample,

(ii) at least one pair of probes, wherein first probe in the pair comprises sequence complementary to the wild-type target nucleic acid sequence (the normal sequence), second probe in the pair comprises sequence complementary to the target nucleic acid sequence containing variant nucleotides (for example, SNP, mutated nucleotides etc), wherein each probe in the pair comprises

a first oligonucleotide which comprises a first region which is substantially complementary to part of one target nucleic acid and a second region, and at least one second oligonucleotide which comprises a region which is substantially complementary to the second region of the first oligonucleotide, such that the first and second oligonucleotides are capable of forming a double-stranded portion,

wherein each probe in the pair comprise the same second oligonucleotide,

wherein each probe comprises a detectable label or detectable combination of labels which is/are capable of producing a changeable signal which is characteristic of the presence or absence of a double-stranded portion between the first and second oligonucleotides of that probe, and

wherein at least two of the probes comprise the same detectable label or different detectable labels with undistinguishable emission spectra and wherein the melting characteristics of the double-stranded portions between the first and second oligonucleotides of each of such probes are different;

(b) performing an amplification reaction on the sample/amplification reaction mixture wherein, when a target nucleic acid is present, the first oligonucleotides of probes which are substantially complementary to part of that target nucleic acid are consumed during the amplification reaction; and

(c) measuring, at least once, the melting profile of any double-stranded portions between the first and second oligonucleotides of any unconsumed probes by detecting the signal(s) from the labels in those probes as a function of temperature,

wherein the melting profile provides an indication of whether or not at least one target nucleic acid has been amplified in said sample/amplification reaction mixture.

The same second oligonucleotide in the pair of the probe may comprise universal base or Inosine which corresponds to the variant nucleotide in the target nucleic acid sequence. The universal base may be 3-nitropyrrole 2'-deoxynucleoside, 5-nitroindole, pyrimidine analog or purine analog. Inosine occurs naturally in the wobble position of the anticodon of some transfer RNAs and is known to form base pairs with A, C and U during the translation process (Fig. 17).

For scanning multiple mutations or SNPs in a target sequence, multiple first oligonucleotides of different probes hybridising to different sites of the same amplified product may be included in a reaction. The probes may contain a competing pair of probes, the first probe in the pair hybridises to the wild-type (normal nucleotide) sequence; the second probe in the pair hybridises to target sequence containing the variant (mutated) nucleotides. When the wild-type target sequence is present, the probe complementary to the wild-type target sequence is consumed. When the target sequence having the variant nucleotide is present, the probe complementary to the variant target sequence is consumed. The multiple first oligonucleotides may hybridise to the same strand of target nucleic acid sequence adjacent to each other or with some overlapping region between adjacent first oligonucleotides (Fig. 17).

Another aspect of the invention is directed to a combination of primers/probes for monitoring, detecting or quantifying a plurality of nucleic acid templates in a single amplification reaction based on the properties of the melting profile of the primer or probe hybridised with the primer extension product which are amplified from the nucleic acid templates (Fig. 14).

The combination of primers and probes may comprise: first primers, second primers and first probes (Fig. 14A),

wherein said second primer comprises, in 3' to 5' order, a 3' target specific priming portion substantially complementary to a sequence of a target nucleic acid, a probe-complementary portion comprising a sequence capable of hybridising to the first probe, and 5' universal portion comprising sequence identical or substantially identical to the sequence of the first primer,

wherein the first probe capable of hybridising to the second primer comprises a first label, the first primer comprises a second label such that the first label and second label are capable of interacting when the two labels are brought into proximity,

wherein the first primer is capable of extending on the template complementary to the extension strand of the second primer,

wherein upon hybridisation of the first probe to the first primer extension product, the two labels are brought into proximity, thereby generating detectable signal.

In one embodiment, in the combination of primers and probes, the first primer is an universal primer, the first probe is an universal probe, the second primers constitute a set of primers in which each second primer is attached with the same label or labels with undistinguishable emission spectra. In the set of second primers, the 3' target specific priming portion of each second primer is capable of hybridising to each target specifically, wherein the first probe hybridises to each second primer or the extension strands of the first primer with different T_m or melting profile specific for each target. The different T_ms or different melting profiles of the first probe for each target are distinguishable from one another such that the distinct T_m or melting profile will provide the information if a target is present in a sample (Fig. 14B).

In another embodiment, the probe-complementary portion and the 3' target specific priming portion of the second primer are not substantially overlapping, wherein the probe-complementary portion comprises sequence which may not be related to the target sequence.

In a further embodiment, the probe-complementary portion is substantially overlapping with the 3' target specific priming portion of the second primer, or the probe-complementary portion is embedded in the 3' target specific priming portion of the second primer (Fig. 14A), wherein T_m of the duplex of said second primer hybridised with the target sequence is higher than the T_m of the duplex of said second primer hybridised with the first probe such that if a target is present, the second primer forms stronger hybrids with the target.

One of the labels can be either a quencher or a fluorophore. The hybridisation of the first probe with the extension strand of the first primer brings two labels in contact quencher relationship or FRET relationship.

Another aspect of the invention is directed to a method for monitoring, detecting or quantifying a plurality of nucleic acid templates in a single amplification reaction.

The method for detecting/quantifying a target nucleic acid or multiple target nucleic acids in a nucleic acid population, said method comprising:

(a) treating the nucleic acid population with a combination of primers and probes, or a set of combinations of primers and probes, as described above, under amplification conditions, wherein second primer anneal to the target nucleic acid, and is extended, the extension strand of the second primer is separated from template and participates in the another cycle of primer annealing, and extension,

wherein the concentration of second primer is lower than the concentration of first primer in the reaction mixture, the first primer participates in the amplification when the second primer is running low or running out, whereby the first primer is incorporated into the amplification product;

(b) detecting signal emissions from the hybridisation of the first probe with the extension product of the first primers at each cycle;

(c) measuring melting profile of the duplex of the first probe and the extension product of the first primer, wherein said melting profile is measured by illuminating the reaction mixture and monitoring the fluorescence as a function of temperature, wherein a signature Tm or melting profile is an indicative of the presence of a target sequence.

It is preferred that the amplification is an asymmetric amplification, wherein the single stranded first primer extension strands are accumulated during amplification. This can be achieved by using different ratio of forward primer and reverse primer.

Another aspect of the invention is directed to a kit for monitoring, detecting or quantifying a plurality of nucleic acid templates in a single amplification reaction.

The kit for detecting/quantifying a target nucleic acid or multiple target nucleic acids in a nucleic acid population, said kit comprises: a combination of primers and probes, or a set of combinations of primers and probes, as described above, wherein said combination of primers and probes comprises: first primers, second primers and first probes,

wherein said second primer comprises, in 3' to 5' order, a 3' target specific priming portion substantially complementary to a sequence of a target nucleic acid, a probe-complementary portion comprising a sequence capable of hybridising to the first probe, and 5' universal portion comprising sequence identical or substantially identical to the sequence of the first primer,

wherein the first probe capable of hybridising to the second primer comprises a first label, the first primer comprises a second label such that the first label and second label are capable of interacting when the two labels are brought into proximity,

wherein the first primer is capable of extending on the template complementary to the extension strand of the second primer,

wherein upon hybridisation of the first probe to the first primer extension product, the two labels are brought into proximity, thereby generating detectable signal.

The first probe capable of hybridising to the extension strand of the first primer can contain target specific sequence or arbitrary sequence and can have any length. Generally, it may be 4 to 60 nucleotides long, preferably 5 to 25 nucleotides long.

The probe-complementary portion of the second primer comprises sequence capable of hybridising to the first probe. It is designed that the duplex of probe-complementary portion and the first probe has distinct Tm or melting profile for each target sequence.

The 5' universal portion of the second primer comprises sequence identical or substantially identical to the 3' priming portion of the first primer or the whole sequence of the first primer. It is designed that the first primer can anneal to the template generated by the second primer replication product and initiate prime extension.

In one embodiment, the first primer is a target specific primer, which comprises the 3' priming portion which is complementary to a region of target nucleic acid. In this embodiment, the first probe included in a reaction comprises sequence capable of hybridising to the extension strand of the first primer and being adjacent to the first primer.

In a preferred embodiment, the first primer is an universal primer, the first probe is an universal probe, the second primers constitute a set of multiple primers, in which the 3' target specific priming portion of each second primer is capable of hybridising to each target specifically and priming amplification of each target, wherein the probe-complementary portion of each second primer comprises sequence capable of hybridising with the first probe with distinct hybridisation/dissociation property for each second primer.

For SNP genotyping or detecting variant nucleotide, the second primer may be allele-specific primer, wherein a terminal nucleotide of the second primer is selected to be either complementary to the suspected variant nucleotide or to the corresponding normal nucleotide such that an extension product of the second primer is synthesised when the second primer anneals to the diagnostic region containing a particular nucleotide, but no such extension product is synthesised when the second primer anneals to the diagnostic region containing no particular nucleotide of the target nucleic acid sequence. The allele specific second primers may differ by the probe-complementary portions so that the hybridisation/dissociation profiles can be distinguished among different alleles.

Alternatively, the first primer and first probe may be linked by a linker (Fig. 16). The linked first primer/probe may be arranged as, in 5' to 3' order, first probe – linker - first primer, wherein the first probe part may be referred to as 5' tail portion of the first primer. The linker may be natural nucleotides or any other chemical moiety. The linker may contain blocking moiety. If the blocking moiety is present, the replication of the first probe part is blocked. The blocking moiety may be hydrocarbon arm, an HEG, non-nucleotide linkage, abasic ribose, nucleotide derivatives or a dye.

In some embodiments of the invention, the set of multiple second primers used in amplification may be nested primers. Nested primers for use in the amplification are oligonucleotides having sequence complementary to a region on a target sequence between priming sites of the outside primer pair.

The first probe may be attached with first label. When the first probe hybridise to the extended part of the first primer extension product, the hybridisation/dissociation of the first probe generate detectable signal and/or distinct melting profile. More preferably, the 3' priming portion of the first primer may be attached with second label. The first label and second label are interactive label pair. When the first probe hybridises to the extended part of the first primer extension product, the hybridisation bring two labels in close proximity,

whereby the melting profile of the first probe is indicative of presence or amount of target nucleic acid in a sample (Fig. 15 and 16) .

Upon hybridisation of labelled primer/probe to the first primer extension product, the two labels are in fluorescence resonance energy transfer relationship (FRET) or contact quenching relationship. The label on the primer (or probe) can be located anywhere as long as it interacts with the label on the other probe (or primer).

A method for assaying a sample for a target nucleic acid or multiple target nucleic acids in a nucleic acid population, said method comprising:

(a) providing template nucleic acids which are derived from target nucleic acids in a nucleic acid population, wherein said template nucleic acids are capable of hybridising with first primers;

(b) treating the template nucleic acids with a first primer or multiple first primers, wherein each first primer anneals to its nucleic acid template under hybridisation conditions, wherein each first primer comprise a 3' priming portion substantially complementary to the first region of the template nucleic acid and 5' tail portion capable of hybridising to an extended part of an extension product of the first primer,

alternatively, treating the template nucleic acids with a first primer or multiple first primers, and first probe(s), wherein each first primer anneals to its nucleic acid template under hybridisation conditions, wherein each first primer comprise a 3' priming portion substantially complementary to the first region of the template nucleic acid, wherein the first probe is capable of hybridising to an extended part of the first primer extension product adjacent to the first primer;

(c) maintaining the mixture of step (a) under extension conditions, which comprise appropriate nucleoside triphosphates and a nucleic acid polymerase to extend the annealed first primers to synthesize extension products of the first primers;

(d) separating the extension products from the templates; and

(e) maintaining the mixture of step (c) under appropriate conditions, which comprises buffer and an appropriate temperature or a range of temperatures, wherein the first probe or the 5' tail portion of the first primer hybridise to or dissociate from the extended part of the first primer extension product, wherein the process of hybridisation and/or dissociation of the 5' tail portion or the first probe generate detectable signal or distinct melting profile.

The method may further comprise repeating steps (a) and (e) in an amplification method. Any amplification system can be used to include these steps, such as PCR, SDA, LAMP, 3SR and the like. PCR is a preferred amplification method to incorporate these steps.

In the method described herein, a sample is provided which is suspected to contain the target nucleic acid or the nucleotide variant of interest. The target nucleic acid contained in the sample may be double-stranded genomic DNA or cDNA if necessary, which is then denatured, using any suitable denaturing method including physical, chemical, or enzymatic means that are known to those of skill in the art. A preferred physical means for strand separation involves heating the nucleic acid until it is completely (>99%) denatured. Typical heat denaturation involves temperatures ranging from about 80°C to about 105°C, for times ranging from a few seconds to minutes. As an alternative to denaturation, the target nucleic acid may exist in a single-stranded form in the sample, such as single-stranded RNA or DNA viruses.

The denatured nucleic acid strands are then incubated with oligonucleotide primers under hybridisation conditions; conditions that enable the binding of the primers to the single nucleic acid strands. In one embodiment, the template nucleic acid is provided as the denatured target nucleic acids which are capable of hybridising with first primers. In this embodiment, the first primers are target specific primers, wherein each first primer in a set of multiple primers comprises a 3' priming portion capable of hybridising each target specifically and priming extension. In one instance, each first primer comprises a 5' tail portion capable of hybridising to an extended part of the extension product of the first primer. In another instance, the first primer does not comprise a 5' tail portion, but the reaction comprises a first probe which is capable of hybridising to an extended part of the first primer extension product adjacent to the first primer.

The annealed first primers are extended by a polymerizing agent. Template-dependent extension of the oligonucleotide primer(s) is catalyzed by a polymerizing agent in the presence of adequate amounts of the four deoxyribonucleoside triphosphates (dATP, dGTP, dCTP, and dTTP), or analogues of these as discussed above, in a reaction medium comprised of the appropriate salts, metal cations and pH buffering system. Suitable polymerizing agents are enzymes known to catalyze primer- and template-dependent DNA synthesis. The reaction conditions for catalyzing DNA synthesis with these DNA polymerases are well known in the art.

Separating the extension products from the templates can be carried out by heating. For example, extension products can be separated by heating at 95°C. Alternatively, extension products can be separated from their respective template by strand displacement.

After the first extension product is separated from templates, the reaction mixture is maintained under appropriate conditions, which comprises buffer and an appropriate temperature or a range of temperatures, wherein the first probe or the 5' tail portion of the first primer hybridise to or dissociate from the extended part of the first primer extension product, wherein the process of hybridisation and/or dissociation of the first probe or the 5' tail portion generate detectable signal or distinct profile.

Measuring Tm or monitoring melting curve profile of amplicon or probe duplex are known in the art. In this invention, the detection of multiple targets can be achieved through using label pair and hybridisation/dissociation profile analysis of the first probe hybridising to the extended part of the first primer extension product.

In another embodiment, the first primer is a universal primer; the first probe is a universal probe. Multiple targets can be detected via melting profile analysis of a universal first probe hybridised with multiple different targets through a single fluorescence detection channel.

In this embodiment, the step (a) providing template nucleic acids comprises: i) treating the nucleic acid population with second primers, which are a set of target specific primers, under hybridisation conditions, wherein each second primer anneals to its target nucleic acid, wherein a second primer comprise, in 3' to 5' order, a 3' target specific portion substantially complementary to a region of the target nucleic acid; a tail-probe complementary portion capable of hybridising to the first probe or the 5' tail sequence of the first primer, and 5' universal portion comprising sequence identical or substantially identical to the 3' priming portion of the first primer; ii) maintaining the above mixture under extension conditions, which comprise appropriate nucleoside triphosphates and a nucleic acid polymerase to extend the annealed second primers to synthesize extension products; iii) separating the extension products from the templates; iv) annealing another amplification primer (third primer) or a set of third amplification primers to the single-stranded extension products above and extending the annealed third primers; and v) separating the double-stranded extension products to single-stranded form which includes template nucleic acids, whereby the single-stranded template nucleic acids are capable of hybridising to the first primer (Fig. 15).

The third primer may be one of the amplification primers. The amplification primers are selected so that their relative positions along a duplex sequence are such that an extension product synthesized from the one amplification primer, when the extension product is separated from its template (complement), serves as a template for the extension of the another amplification primer.

The universal first primer may be present in the reaction at a concentration of least 3 times more than the concentration of the second primers. Preferably, the first primer may be present in the reaction at a concentration of at least 6 times more than the concentration of the second primers.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 graphically presents the melting profile of a nucleic acid probe of the present invention before amplification and after amplification in which the probe is consumed.

FIG. 2 graphically presents a melting profile of a mixture of nucleic acid probes (probe 1 and 2) of the present invention before amplification and after amplification in which one or both of the probes are consumed.

FIG. 3 graphically presents a real-time measurement of an amplicon synthesis at different temperatures.

FIG. 4 illustrates examples of different probes which may be used in the present invention.

FIG. 5 illustrates an example of a method of the present invention, where the first oligonucleotide of the probe is hybridised with a target nucleic acid sequence.

FIG. 6 illustrates an example of a method of the present invention, where the first oligonucleotide of the probe is incorporated into the amplified product.

FIG. 7 illustrates an example of a method of the present invention, where the first oligonucleotide of the probe is extended and degraded during the amplification.

FIG. 8 illustrates an example of a method of the present invention, where the first oligonucleotide of the probe is degraded during the amplification.

FIG. 9A graphically presents a melting profile of probe 1 and 2 of the present invention before amplification. Figure 9B presents a melting profile of a mixture of probes 1 and 2 at different ratios.

FIG. 10 graphically presents a melting profile of mixture of probes 1 and 2 in the 4 reactions before amplification.

FIG. 11 graphically presents a real-time measurement of amplicon synthesis at different temperatures in the Example.

FIG. 12 graphically presents a melting profile of a mixture of probes 1 and 2 in the 4 reactions after amplification.

FIG. 13 illustrates the amplification plot where the emission amount of the first probe (K10) is drawn as FE1. The calculated FE1 ×P plot is drawn as FE1xP. The amplification plot for the emission amount of the second probe (SV40) is shown as FE2-(FE1 x P).

FIG. 14 illustrates a combination of primers and probes (A) and a set of combinations of primers and probes (B).

FIG. 15 illustrates a method for detecting and quantifying 3 target nucleic acids using a set of combinations of primers and probes.

FIG. 16 illustrates a modified form of the combination of primers and probes, wherein the first primer and probe are linked together.

FIG. 17 A set of probes labeled with Fam contain sequences complementary to the wild-type sequence, which have a different Tm; another set of probes labeled with Hex contains sequences complementary to the variant sequence of the same target nucleic acid sequence.

FIG. 18 illustrates a method using the set probes described in FIG. 17.

FIG. 19 shows results of an example of triplex amplification. Three probes with different Tms are labeled with Fam dye. (A) shows a melting profile generated in a tube without adding target DNA. The following figures show comparison of melting profile generated in a tube without adding target DNA and melting profile generated in a tube with adding target DNA. (B) melting profiles generated in a tube with target 2 present in the sample. (C) melting profile generated in a tube with target 3 present in the sample.(D) melting profile generated in a tube with targets 2 and 3 present in the sample. (E) melting profile generated in a tube with target 1 present in the sample. (F) melting profile generated in a tube with targets 1 and 2 present in the sample. (G) melting profile generated in a tube with target1 and 3 present in the sample. (H) melting profile generated in a tube with targets 1, 2 and 3 present in the sample.

FIG. 20 (A) shows the melting temperature and collection setting up profile. (B) is melting profiles of probe 1, 2 and the mix of probe 1 and 2. (C) is the multicomponent view of the dissociation curve showing how to estimate of the percentage of the double-stranded form of a probe.

FIG. 21 is the graphic presentations of the amplification plots for Actual Consumed amount and standard curves (Example 4).

FIG. 22 results of an example of quadruplex amplification. Various combinations of targets present in the reaction were used: reaction (A) contains no template; reaction (B) contains target 3 template; reaction (C) contains target 2 template; reaction (D) contains target 4; reaction (E) contains target 1 and 3; reaction (F) contains targets 1, 3 and 4.

EXAMPLES

The present invention is further defined in the following Examples, in which parts and percentages are by weight and degrees are Celsius, unless otherwise stated. It should be understood that these Examples, while indicating preferred embodiments of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions. Thus, various modifications of the invention in addition to those shown and described herein will be apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

The disclosure of each reference set forth herein is incorporated herein by reference in its entirety.

Example 1

All primers used in the subsequent experiments were synthesized by EUROGENTEC.

Amplification primers and probe are:

K10R266Fam	GttcaATTGGGTTTCACCGCGCTTAGTTACA (SEQ ID NO: 1);
K10R266Dab	GCGCGGTGAAACCCAATTGAAC (SEQ ID NO: 2);
SV40RlF3FAM	ATCAGCCATACCACATTTGTAGAGGTTTTAC (SEQ ID NO: 3);
SV40RlF3Dab	CAAATGTGGTATGGCTGAT (SEQ ID NO: 4);
K10F155	CTCTGCTGACTTCAAAACGAGAAGAG (SEQ ID NO: 5);
SV40RealR	CCATTATAAGCTGCAATAAACAAGTTAACAAC(SEQ ID NO: 6)

Primer/probe K10R266Fam (the first oligonucleotide) is labelled at the 5' end with FAM. K10R266Dab (the second oligonucleotide) contains DABCYL at the 3'end. K10R266Fam and K10R266Dab can form double-stranded portion as:

Fam- 5'GTTCAATTGGGTTTCACCGCGCTTAGTTACA3' (SEQ ID NO: 1)

DABCYL- 3'CAAGTTAACCCAAAGTGGCGCG5' (SEQ ID NO: 2)

The above hybrid is referred to as probe K10R266. Primer/probe SV40RlF3FAM (the first oligonucleotide) is labelled at the 5' end with FAM. SV40RlF3Dab (the second oligonucleotide) contains DABCYL at the 3'end. SV40RlF3FAM and SV40RlF3Dab can form double-stranded portion as:

Fam- 5'ATCAGCCATACCACATTTGTAGAGGTTTTAC3' (SEQ ID NO: 3)

DABCYL- 3'TAGTCGGTATGGTGTAAAC5' (SEQ ID NO: 4)

This hybrid is referred to as probe SV40RlF3.

The first oligonucleotide and the second oligonucleotide were combined at various ratios, typically 1:3 to form a partially double-stranded linear DNA probe. In the absence of target, the formation of the first and the second oligonucleotide hybrid brings the quencher and the fluorophore into close proximity, efficiently quenching the fluorescent signal. In the presence of the target, the first oligonucleotide preferentially hybridises to the target sequence and incorporates into the amplicon. As a result, the quencher is separated from the fluorophore resulting in an increase in fluorescence emission.

Primer pair K10R266Fam and K10F155 amplifies a 110bp product in the presence of a K10 target sequence. SV40RlF3FAM and SV40RealR amplifies a 125bp product in the presence of an SV40 target sequence.

Example 2

Melting profile experiments were performed as follows: The thermal stability of the probes was characterized in melting profile experiments where fluorescence emission was measured at temperatures ranging from 40 to 90°C. Melting temperature T_m is defined as the characteristic temperature where the first and the second oligonucleotide duplex dissociate.

Each melting profile was measured in a tube in a 20 µl reaction containing PCR buffer (1xThermoPol reaction buffer, New England BioLabs). Thermal cycling was performed in an Mx3005p quantitative PCR system (Stratagene) with the following cycling conditions: 1 cycle of denaturation at 90°C for 3 min; 50 cycles of 30 second holding at a range of temperatures

from 40 to 90°C with a 1°C increment per cycle. Fluorescence measurements were recorded during each 30 second hold of the 50 cycles.

Melting profiles were determined for probe K10R266 (probe 1) and probe SV40RlF3 (probe 2) by monitoring fluorescence at temperatures ranging from 90 to 40°C. K10R266 has a T_m of 72°C; SV40RlF3 has a T_m of 62°C. (FIG. 9A).

Melting profiles were determined for a series of mixtures of probe K10R266 and probe SV40RlF3:

Sample 1 contains 0.5μM of K10R266 and 0.5μM of SV40RlF3.

Sample 2 contains 0.5μM of K10R266 and 0.25μM of SV40RlF3.

Sample 3 contains 0.5μM of K10R266 and 0.125μM of SV40RlF3.

Sample 4 contains 0.5μM of K10R266 and 0.0625μM of SV40RlF3.

Sample 5 contains 0.5μM of K10R266 and 0.003125μM of SV40RlF3.

The profiles are shown in Fig. 9B.

A combination of probes K10R266 and SV40RlF3 were tested in real-time PCR assays using plasmids DNA containing K10 and SV40 sequence as templates.

A master reaction mixture was made containing 0.5μM of K10R266 and 0.5 μM of SV40RlF3 (combination of first oligonucleotide and second nucleotide in 1:3 ratio mix) and standard PCR ingredients (NEB). Amplification reactions were performed in Stratagene Mx3005 real-time PCR system with the following cycling conditions:

1. Before amplification, melting profile: 50 cycles of 30 second holding at a range of temperatures from 90 to 40°C with a 1°C decrement per cycle, fluorescence measurements were recorded during each 30 second hold of the 50 cycles.
2. Amplification: 30 cycles of 94°C 20s; 63°C 30s; 51°C 30s; 72°C 30s; fluorescence measurements were recoded during the read steps 63°C, 51°C and 72°C.
3. Post-amplification melting profile: 50 cycles of 30 second holding at a range of temperatures from 40 to 90°C with a 1°C increment per cycle, fluorescence measurements were recorded during each 30 second hold of the 50 cycles.

Four reactions were set up: reaction 1 contains K10 template; reaction 2 contains SV40 template; reaction 3 contains both K10 and SV40 templates; reaction 4 contains no template.

The pre-amplification melting profiles for four reactions are shown in Fig. 10. Fluorescence emission was collected at 63°C during amplification is shown in Fig. 11A. Fluorescence emission was collected at 51°C during amplification is shown in Fig. 11B. Fluorescence emission was collected at 72°C during amplification is shown in Fig. 11C.

The post-amplification melting profiles for four reactions are shown in Fig. 12.

Comparison of the pre-amplification and post-amplification melting profiles indicated the following:

In reaction 1 the K10R266 probe is consumed and the profile is the signature of SV40RlF3 probe.

In reaction 2 the SV40RlF3 probe is consumed.

In reaction 3, both K10R266 and SV40RlF3 probes are consumed.

In reaction 4, no probe is consumed and the pre- and post-amplification profiles are similar.

Cycle by cycle fluorescence emissions FE were obtained at three measuring temperatures: MT 72°C, 63°C and 51°C.

A first fluorescence emission FE1 is obtained at a measuring temperature 63°C, at which no more than 10 % of second probe (SV40RlF3) is in duplex form (the internal double-stranded form of the probe); second fluorescence emission FE2 is obtained at a measuring temperature 51°C, at which more than 95 % of two probes are in duplex form, and optionally a fluorescence emission FE0 is obtained at a measuring temperature 72°C, at which no more than 10 % of first probe (K10R266) is in duplex form.

In the amplification reactions there are two probes for two target sequences K10 and SV40. At temperature 72°C, 10% of the K10R266 probe is in duplex form, 0% of the SV40RlF3 probe is in duplex form. At 63°C, 90% of the K10R266 probe is in duplex form, 5% of SV40RlF3 probe is in duplex form. At 51°C, more than 98% of all probes are in duplex form.

The first fluorescence emission is collected at 63°C, which is FE1; the second fluorescence emission is collected at 51°C, which is FE2.

	Probe1(K10)		Probe2(SV40)		fluorescence emission
	ds%	ACA1	ds%	ACA2	
72°C	10	10%ACA1	0	0%ACA2	
63°C	90	90%ACA1	5	5%ACA2	FE1
51°C	98	98%ACA1	98	98%ACA2	FE2

FE1=90%*ACA1 + 5%*ACA2

FE2=98%*ACA1 + 98%*ACA2

Assuming 5%*ACA2 is negletable, ACA1=FE1/90%; ACA2= FE2/98%-FE1/90%. ACA1 is the actual consumed amount of probe 1; ACA2 is the actual consumed amount of probe 2.

The amplification plot for the actual consumed amount of the first probe (K10) is drawn as ACA1. The amplification plot for the actual consumed amount of the second probe (SV40R1F3) is shown as ACA2 (FIG. 13).

The calculation of the actual consumed amount can be performed manually. The calculation can also be performed through a computer program or software. To expedite the quantification, software was designed to manage emission data from the multiplex real-time PCR and perform appropriate calculations. The software had other functions, such as manual selection of the Ct and subtraction of blanks.

The software was implemented in Visual Basic for applications (VBA) as an Addin for Microsoft Excel. The source code was organized in two main modules. One module contained all the "utility" functions such as mathematical functions, functions to generate arrays from emission data present in the Excel sheets, functions to print result data and labels, functions to handle errors or template and functions to generate charts of a certain types. The second module contained the functions to control the flow of the program. This module contained all the functions making possible the interaction with the user, such as menu selections, bar slicing, inclusion/exclusion of data in the standard curve.

Example 3

Amplification primers and probe are:

For target 1 (K10)

K10R266Fam GttcaATTGGGTTTCACCGCGCTTAGTTACA (SEQ ID NO: 1);

K10R266Dab GCGCGGTGAAACCCAATTGAAC (SEQ ID NO: 2);

K10F155 CTCTGCTGACTTCAAAACGAGAAGAG (SEQ ID NO: 5);

For target 2 (SV40)

SV40RlF3FAM ATCAGCCATACCACATTTGTAGAGGTTTTAC (SEQ ID NO: 3);

SV40RlF3Dab CAAATGTGGTATGGCTGAT (SEQ ID NO: 4);

SV40RealR CCATTATAAGCTGCAATAAACAAGTTAACAAC(SEQ ID NO: 6);

For target 3 (Jak2)

JKR3Fam AACAGATGCTCTGAGAAAGGCATTAGA (SEQ ID NO: 11);

JKR3FDabF CTCAGAGCATCTGTT (SEQ ID NO: 12);

JKF2 GCATCTTTATTATGGCAGAGAGAA (SEQ ID NO: 13).

The oligonucleotide K10R266Fam is an amplification primer, in the same time the oligonucleotide K10R266Fam is the first oligonucleotide of the probe 1. K10R266Fam (the first oligonucleotide) is labelled at the 5' end with FAM. K10R266Dab (the second oligonucleotide) contains DABCYL at the 3'end. K10R266Fam and K10R266Dab can form double-stranded portion. The hybrid of K10R266Fam and K10R266Dab is referred to as probe 1.

The oligonucleotide SV40RlF3FAM is an amplification primer, in the same time the oligonucleotide SV40RlF3FAM is the first oligonucleotide of the probe 2. SV40RlF3FAM (the first oligonucleotide) is labelled at the 5' end with FAM. SV40RlF3Dab (the second oligonucleotide) contains DABCYL at the 3'end. SV40RlF3FAM and SV40RlF3Dab can form double-stranded portion. The hybrid of SV40RlF3FAM and SV40RlF3Dab is referred to as probe 2.

The oligonucleotide JKR3Fam is an amplification primer, in the same time the oligonucleotide JKR3Fam is the first oligonucleotide of the probe 3. JKR3Fam (the first oligonucleotide) is labelled at the 5' end with FAM. JKR3FDabF (the second oligonucleotide) contains DABCYL at the 3'end. JKR3Fam and JKR3FDabF can form double-stranded portion. The hybrid of JKR3Fam and JKR3FDabF is referred to as probe 3.

The first oligonucleotide and the second oligonucleotide were combined at various ratios, typically 1:2-1:4 to form a partially double-stranded linear DNA probe. In the absence of target, the formation of the first and the second oligonucleotide hybrid brings the quencher and the fluorophore into close proximity, efficiently quenching the fluorescent signal. In the presence of the target, the first oligonucleotide preferentially hybridises to the target sequence and incorporates into the amplicon. As a result, the quencher is separated from the fluorophore resulting in an increase in fluorescence emission.

Primer pair K10R266Fam and K10F155 amplifies a 110bp product in the presence of a K10 target sequence. SV40RlF3FAM and SV40RealR amplifies a 125bp product in the presence of an SV40 target sequence. Primers JKR3Fam and JKF2 amplifies a 222bp product in the presence of Jak2 target sequence.

Melting profile analysis of probe 1, probe2, probe 3 and mixed probe 1, 2 and 3 were performed using the Stratagene Mx3005 real-time PCR machine (Fig. 19). The thermal profile was based on the dissociation curve analysis software parameters: heat at 70°C for 30 sec, cool to 40 °C hold for 30 second, and then slowly increase the temperature to 94 °C, the fluorescence emission data is continually collected during the rising temperatures. The first

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negative derivative of the emission reading with respect to temperature is plotted against temperature to form curves, and each peak of the curve corresponds to the actual T_m of the probe.

Singleplex, doubleplex and triplex amplifications were performed using the same reaction master mix, but in the presence of one target, or two targets or all three targets.

A master reaction mixture was made containing 0.1μM of first oligonucleotides of each of the three probes, 0.4 μM of second oligonucleotides of each of the three probes, 0.2 μM of primers (not the first oligonucleotide of probe) of each target, and standard PCR ingredients (NEB). Amplification reactions were performed in Stratagene Mx3005 real-time PCR system with the following cycling conditions:

1. Amplification: 40 cycles of 94°C 15s; 63°C 20s; 50°C 20s; 55°C 20s 63°C 20s; 68°C 20s; 72°C 20s; fluorescence measurements were recorded during the read steps 50°C, 55°C, 63°C, 68°C, and 72°C.

3. Post-amplification melting profile: after last cycle at 72°C for 20 sec, cool to 40 °C hold for 30 second, and then slowly increase the temperature to 78°C, the fluorescence emission data is continually collected during the rising temperatures.

Eight reactions were set up: reaction (A) contains no template; reaction (B) contains target 2 template; reaction (C) contains target 3 template; reaction (D) contains target 2 and 3; reaction (E) contains target 1; reaction (F) contains targets 1 and 2; reaction (G) contains targets 1 and 3; reaction (H) contains targets 1, 2 and 3. The post-amplification melting profiles for eight reactions are shown in Fig. 19.

Comparison of the post-amplification melting profiles in the presence of targets and without target indicated the following (Fig.19):

In reaction A the no probe is consumed and the melting profile is the signature of the mixture of all three probes.

In reaction B the probe 2 is consumed.

In reaction C, probe 3 is consumed.

In reaction D, probes 2 and 3 are consumed.

In reaction E, probe 1 consumed.

In reaction F, probes 1 and 2 are consumed.

In reaction G, probes 1 and 3 are consumed.

In reaction D, probes 1, 2 and 3 are consumed.

Cycle by cycle fluorescence emissions FE were obtained at five measuring temperatures: MT 50°C, 55°C, 63°C, 68°C, and 72°C. The Actual Consumed Amount for each probe may be calculated by the formula FEa=(ACA1)*(ds1a)%+ (ACA2)*(ds2a)% +(ACA3)*(ds3a)% ...+(ACAn)*(dsna)%.

Example 4

Amplification primers for target 1 (K10):

K10F155 CTCTGCTGACTTCAAAACGAGAAGAG (SEQ ID NO: 5);

K10R14 CCTGAGGGTTAAATCTTCCCCATTGA (SEQ ID NO: 21)

Probe for target 1 (referred to as probe1) includes: first oligonucleotide

K10R266Famph GTTCAATTGGGTTTCACCGCGCTTAGTTACA (SEQ ID NO: 7), which 5' end is attached with Fam; 3'end is attached with a phosphate group instead of 3' –OH; and second oligonucleotide

K10R266Dab GCGCGGTGAAACCCAATTGAAC (SEQ ID NO: 2), which 3' end is attached with DABCYL.

Amplification primers for target 2 (SV40):

dsredendF2 GTAAGATCCACCGGATCTAGATAAC (SEQ ID NO: 8);

sv40testR GGGAGGTGTGGGAGGTTTTTTAAAG (SEQ ID NO: 9).

Probe for target 2 (referred to as probe2) includes: first oligonucleotide

SV40RlF3FAPh ATCAGCCATACCACATTTGTAGAGGTTTTAC (SEQ ID NO: 10), which 5' end is attached with Fam; 3'end is attached with a phosphate group instead of 3' –OH; and second oligonucleotide

SV40RlF3Dab CAAATGTGGTATGGCTGAT (SEQ ID NO: 4), which 3' end is attached with DABCYL.

The first oligonucleotide and the second oligonucleotide were combined at various ratios, typically 1:2-1:4 to form a partially double-stranded linear DNA probe. In the absence of target, the formation of the first and the second oligonucleotide hybrid brings the quencher and the fluorophore into close proximity, efficiently quenching the fluorescent signal. In the presence of the target, the first oligonucleotide preferentially hybridises to the target sequence. As a result, the quencher is separated from the fluorophore resulting in an increase in fluorescence emission.

The first oligonucleotides in probe 1 and 2 are modified to contain blocked 3' end, so that it cannot be extended. However, when the first oligonucleotide binds to the target sequence, it can be degraded by the 5' nuclease activity of a polymerase. The degradation of the first oligonucleotide of the probe (the consumed probes) results in decrease of the number of first oligonucleotide available to bind with the second oligonucleotides of probe, thereby increasing the fluorescence signal when measured at appropriate temperature.

Melting profile analysis of probe 1, probe2, and mixed probe 1 and 2 was performed using the Stratagene Mx3005 real-time PCR machine (Fig. 20). The thermal profile was based on the dissociation curve analysis software parameters (Fig. 20A): heat at 72°C for 30 sec, cool to 40 °C hold for 30 second, and then slowly increase the temperature to 94 °C, the fluorescence emission data is continually collected during the rising temperatures. The first negative derivative of the emission reading with respect to temperature is plotted against temperature to form curves, and each peak of the curve corresponds to the actual T_m of the probe. The probe 1 has a peak at 67°C as Tm; the probe2 has a peak at 59°C as Tm (Fig. 20B).

The percentages of double stranded form and single stranded form of each probe were estimated in the following table. The actual calculation can also be done by computer software.

	probe 1		probe 2	
	ds %	ss %	ds %	ss %
71°C	30	70	0	100
69°C	40	60	0	100
67°C	50	50	1	99
65°C	65	35	3	97
62°C	75	25	5	95
61°C	85	15	25	75
59°C	95	5	50	50
57°C	97	3	65	35
55°C	98	2	75	25
54°C	100	0	85	15
52°C	100	0	100	0

The estimation (calculation) can be done based on the multi-component view of the dissociation curve (the Fluorescence R versus temperature, Figure 20C). The base line was assumed as 100% double-stranded, which has a R as 9000. The R at a temperature (100% single-stranded) is 24500. At temperature 62°C, the R is 13000. The difference of the fluorescence values between 62°C and base line dR=13000-9000=4000. The percentage of double-stranded probe at 62°C is estimated as 4000/(24500-9000) which is 25.8%.

Multiplex real-time PCR and standard curve analysis

Primer-probe master mix was set up as follows: the primers and probes were mixed to a final concentration 0.4 μM of probes and 0.6 μM of primers, which creates a 2X primer-probe master mix.

The reaction mix was created by combining equal amount of 2Xprimer-probe master mix and 2X TaqMan® Gene Expression Master Mix (Applied Biosystem, cat. No 4369514).

Template DNAs containing target 1, target 2, and mixed target 1 and 2 were serially diluted as follows: 1, 0.1, 0.01, 0.001, 0.0001, 0.00001, 0.000001.

The singleplex PCRs were performed using DNA sample containing target 1 (k10) or target 2 (SV40). Doubleplex PCR was performed using DNA sample containing mixture of target 1 (k10) and target 2 (SV40).

The thermal profile was: 95°C for 8 min 30 sec; 40 cycles of 94°C 10s; 66°C 20s; 63°C 20 sec; 54°C 30s; 52°C 20s; 61°C 20s; 62°C 20s; 68°C 20s; fluorescence measurements were recoded during the read steps 66°C, 63°C, 54°C; 52°C; 61°C; 62°C; 68°C.

Fluorescence emission (dR) at 62°C was chosen as FE1; Fluorescence emission (dR) at 52°C was chosen as FE2. Based on the table above and the formula for calculation of Actual Consumed Amount (ACA) in the description and claims, we had followings:

At 62°C, FE1=0.75*(ACA1)+0.05*ACA2 (1)

At 52°C, FE2=100%*ACA1+100%*ACA2 (2)

According to (1) and (2),

ACA1=(FE1-0.05*FE2)/0.7

ACA2=(0.75*FE2-FE1)/0.7

For the doubleplex reaction where both targets are present, The FE1 and FE2 were obtained as follows:

FE1:

DNA Cycles	1 Fluorescence (dR)	0.1 Fluorescence (dR)	0.01 Fluorescence (dR)	0.001 Fluorescence (dR)	0.0001 Fluorescence (dR)	0.00001 Fluorescence (dR)	0.000001 Fluorescence (dR)
1	221.8519	592.1771	466.8143	570.415	633.3002	1036.42	421.2163
2	78.16296	358.3926	225.5527	329.063	377.0944	719.6661	242.6808
3	-92.1926	86.34888	14.51325	45.78511	74.59224	416.5415	-6.11397
4	-50.1037	-12.7813	41.21457	-10.1348	-30.6753	276.2934	-85.6618
5	64.13333	14.05972	-1.8372	-40.602	15.13533	123.0042	-63.1274
6	201.4198	10.22446	-41.4733	-53.2517	82.6387	36.36786	44.4345
7	377.3893	-11.5029	-35.6376	-28.9621	-14.2937	-1.71745	48.67219
8	603.2532	-2.52759	-35.6445	72.64048	-95.038	-19.6191	10.80182
9	937.7486	37.68192	4.40094	56.68076	-41.7196	-22.7929	55.5621
10	1438.121	267.6362	54.46389	-16.4664	-0.047	5.60935	69.53259
11	1912.119	515.172	80.866	-8.3427	25.41037	-23.7965	73.90648
12	2478.326	746.9017	174.0478	-19.1287	-25.8707	-48.8049	9.74818
13	3092.269	1166.696	420.1562	1.78202	-86.7312	9.31902	-13.5875
14	3609.791	1705.178	759.2402	28.25835	-59.7849	10.1538	-2.64903
15	4192.506	2276.224	1071.983	81.25654	5.76368	57.22552	-0.2858
16	4766.951	2836.79	1433.611	167.0954	87.84637	69.04288	-7.78099
17	5317.307	3430.196	1859.535	343.2144	62.77375	-24.8912	-85.5623
18	5864.633	4030.883	2451.891	642.0935	29.9827	-35.4091	-71.1057
19	6371.949	4558.996	3146.391	1035.559	102.6188	4.87837	-54.5698
20	6808.262	5074.252	3798.272	1409.887	256.3974	-15.5657	-64.6741
21	7188.24	5633.888	4425.947	1861.169	432.2234	-59.9202	16.67492
22	7615.441	6064.984	5029.553	2489.103	739.0652	28.08837	39.50831
23	8014.048	6434.568	5640.136	3107.587	1285.579	186.218	25.16984
24	8365.791	6845.646	6207.045	3699.587	1822.317	301.388	61.10741
25	8736.58	7257.224	6784.729	4176.094	2328.129	483.2382	96.137
26	9026.05	7652.967	7387.671	4613.769	2908.966	836.6483	145.8639
27	9241.081	7934.433	7890.366	5104.5	3553.812	1367.912	282.49
28	9454.632	8185.806	8268.313	5596.249	4202.327	1961.793	465.7491
29	9667.69	8492.481	8646.676	6044.339	4731.732	2518.214	688.2191
30	9851.583	8824.257	8953.845	6525.541	5146.767	3114.147	997.4262
31	10081.42	9017.401	9232.615	6913.781	5628.011	3785.585	1400.212
32	10292.91	9159.333	9552.92	7172.034	6098.659	4475.191	1954.525
33	10403.28	9320.528	9898.736	7505.291	6576.109	5118.187	2599.346
34	10514.61	9401.811	10201.06	7778.55	7015.158	5748.646	3245.67
35	10611.93	9523.123	10353.21	7965.142	7308.741	6347.592	3873.495
36	10640.24	9682.444	10468.31	8154.512	7596.835	6862.701	4481.487
37	10704.55	9664.769	10654.39	8367.142	7799.1	7291.197	5006.868
38	10781.86	9608.762	10868.13	8504.858	7997.755	7619.823	5543.046
39	10833.84	9651.977	10982.09	8637.936	8213.206	7987.825	6035.822
40	10911.49	9675.693	11063.72	8819.347	8397.157	8380.493	6459.098

FE2:

DNA Cycles	1 Fluorescence (dR)	0.1 Fluorescence (dR)	0.01 Fluorescence (dR)	0.001 Fluorescence (dR)	0.0001 Fluorescence (dR)	0.00001 Fluorescence (dR)	0.000001 Fluorescence (dR)
1	251.4671	205.968	310.1105	43.54564	382.2339	312.4356	256.8311
2	55.8251	87.68861	218.6788	27.16047	275.7769	193.1356	172.3411
3	-49.5206	-4.1834	109.6545	25.47901	225.5422	99.35408	102.8511
4	-68.4342	-5.91962	23.09933	-18.3012	166.0481	38.41212	46.69446
5	62.12963	24.38944	-16.2994	-56.781	108.1344	46.41665	10.31559
6	415.1859	-14.2864	-25.6461	-9.82739	101.7474	35.07002	22.19599
7	903.4063	3.04275	6.69132	35.93742	40.86928	-2.06033	18.49614
8	1567.348	170.0403	13.25673	20.63928	-8.83917	6.54807	-22.3971
9	2529.197	501.5939	-1.10185	-22.6798	7.50892	-11.5973	-44.0215
10	3752.015	982.6662	29.5649	-23.006	-13.4575	-55.6605	15.77702
11	5171.823	1695.911	138.5734	-8.00106	-23.5287	-74.0298	26.04988
12	6558.96	2707.548	399.3626	-8.88584	-13.6349	-86.1676	-25.1858
13	7836.874	3920.648	860.0785	38.26616	-16.4193	-67.5617	-18.591
14	9129.714	5321.902	1511.437	144.7638	-20.7633	-61.3746	-20.3861
15	10335.2	6915.875	2471.009	310.3765	-5.96032	-51.9937	-13.3111
16	11419.56	8566.42	3724.986	633.3611	54.22491	-27.5482	-0.61273
17	12463.55	10027.49	5220.098	1150.136	114.5376	-31.0812	-9.03993
18	13343.41	11328.73	6908.922	1950.175	243.226	-26.9403	-27.509
19	14056.57	12656.7	8570.649	3099.635	520.0398	20.75852	-12.992
20	14700.82	13829.58	10210.68	4441.901	985.5619	84.30999	-1.81296
21	15160.44	14812.09	11791.47	5899.104	1636.987	165.1457	-30.7466
22	15500.18	15700.82	13229.19	7446.952	2533.379	292.7428	-35.3845
23	15882.3	16474.95	14480.55	8935.681	3752.761	531.2603	4.74292
24	16224.54	17109.88	15546.79	10263.7	5190.473	992.7513	27.12541
25	16511.15	17676.08	16537.66	11545.83	6745.627	1711.567	90.59292
26	16779.23	18230.03	17359.4	12765.31	8268.93	2691.491	222.7554
27	16974.12	18690.57	18075.1	13756.26	9666.282	4042.45	514.8163
28	16980.95	19057.31	18772.45	14598.35	11033.65	5713.422	1022.177
29	17028.43	19398.44	19303.02	15325.5	12286.02	7498.065	1731.637
30	17115.79	19590.37	19730.33	15961.32	13248.73	9328.264	2795.13
31	17137.45	19764.9	20228.55	16577.71	14156.22	11036.98	4243.635
32	17161.87	20071.3	20558.75	17061.62	15000.3	12520.54	5867.809
33	17179.21	20321.65	20749.6	17509.04	15713.24	13969.38	7541.208
34	17161.53	20504.66	21095.34	18035.63	16353.48	15241.98	9240.014
35	17091.17	20524.22	21345.03	18399.94	16909.47	16322.83	10855.29
36	17034.91	20487.29	21421.39	18585.82	17293.39	17336.1	12288.72
37	16877.36	20519.2	21450.29	18810.89	17550.28	18050.5	13425.21
38	16677.05	20515.39	21532.38	19083.03	17903.49	18608.29	14431.37
39	16547.15	20532.68	21645.86	19238.86	18119.48	19175.54	15425.44
40	16436.08	20571.96	21712.84	19343.85	18238.97	19721.29	16388.67

The calculated ACAs for the target 1 are listed in the following table:

ACA1=(FE1-0.05*FE2)/0.7

DNA Cycles	1 Fluorescence (dR)	0.1 Fluorescence (dR)	0.01 Fluorescence (dR)	0.001 Fluorescence (dR)	0.0001 Fluorescence (dR)	0.00001 Fluorescence (dR)	0.000001 Fluorescence (dR)
1	298.9693	831.2553	644.7269	811.7682	877.4121	1458.284	583.3925
2	107.6739	505.726	306.5982	468.15	519.0079	1014.299	334.3768
3	-128.167	123.6544	12.90075	63.58737	90.45019	587.9625	-16.0808
4	-66.6886	-17.8362	57.22801	-13.171	-55.6825	391.9611	-125.709
5	87.18121	18.34321	-1.46033	-53.9471	13.89802	172.4047	-90.9188
6	258.0864	15.62683	-57.4158	-75.3718	110.7876	49.44908	61.89243
7	474.5986	-16.65	-51.3888	-43.9414	-23.3388	-2.30633	68.21055
8	749.8369	-15.7566	-51.8677	102.2979	-135.137	-28.495	17.03097
9	1158.984	18.00318	6.365761	82.5925	-60.1358	-31.7328	82.51882
10	1786.458	312.1469	75.69378	-21.8801	0.894105	11.98911	98.20534
11	2362.183	614.8234	105.6248	-11.3466	37.98115	-28.7071	103.72
12	3071.969	873.6061	220.1138	-26.692	-35.9842	-63.5665	15.72496
13	3857.751	1386.662	538.789	-0.18755	-122.729	18.13872	-18.0828
14	4504.722	2055.833	976.669	30.0288	-83.9239	18.88933	-2.32818
15	5251.066	2757.757	1354.903	93.91102	8.659566	85.46458	0.542506
16	5994.248	3440.67	1781.946	193.4676	121.6216	100.6004	-11.0719
17	6705.9	4184.031	2283.615	408.1537	81.49553	-33.3388	-121.586
18	7424.947	4949.209	3009.207	777.9782	25.45914	-48.6602	-99.6146
19	8098.744	5608.801	3882.655	1257.968	109.4526	5.486349	-77.0288
20	8676.03	6261.104	4696.768	1696.846	295.8847	-28.2588	-92.262
21	9186.026	6990.405	5480.533	2237.449	500.5343	-97.3964	26.0175
22	9772.045	7542.776	6240.133	3023.936	874.8518	19.21605	58.96791
23	10314.19	8015.457	7023.012	3801.147	1568.487	228.0786	35.61813
24	10792.23	8557.361	7756.722	4552.003	2232.562	359.6435	85.35877
25	11301.46	9104.886	8511.208	5141.146	2844.068	568.0855	130.8676
26	11695.84	9630.665	9313.859	5679.29	3565.028	1002.963	192.4659
27	11989.11	9999.863	9980.874	6309.553	4386.426	1665.413	366.7845
28	12293.69	10332.77	10470.99	6951.903	5215.206	2394.46	592.3432
29	12594.67	10746.51	10973.61	7540.091	5882.044	3061.872	859.4819
30	12851.13	11206.77	11381.9	8182.107	6406.186	3782.477	1225.242
31	13177.93	11470.22	11744.55	8692.708	7028.858	4619.623	1697.187
32	13478.31	11651.1	12178.55	9027.076	7640.921	5498.806	2373.049
33	13634.74	11863.49	12658.94	9471.199	8272.066	6313.883	3174.694
34	13795.05	11966.54	13066.13	9823.955	8853.549	7123.638	3976.671
35	13939.1	12138.45	13265.66	10064.49	9233.239	7902.072	4758.187
36	13983.56	12368.69	13424.63	10321.74	9617.38	8565.566	5524.359
37	14086.69	12341.16	13688.39	10609.42	9887.98	9126.674	6193.726
38	14211.44	12261.42	13987.88	10786.72	10146.54	9556.297	6887.825
39	14294.98	12321.92	14142.57	10965.7	10438.9	10041.5	7520.786
40	14413.83	12352.99	14254.4	11217.36	10693.16	10563.47	8056.664

The graphic presentation of the amplification plots for ACA1 is shown in Figure 21A. The standard curve is shown in Figure 21B.

The calculated ACAs for the target 2 are listed in the following table:

ACA2=(0.75*FE2-FE1)/0.7

DNA Cycles	1 Fluorescence (dR)	0.1 Fluorescence (dR)	0.01 Fluorescence (dR)	0.001 Fluorescence (dR)	0.0001 Fluorescence (dR)	0.00001 Fluorescence (dR)	0.000001 Fluorescence (dR)
1	-47.5022	-625.287	-334.616	-768.223	-495.178	-1145.85	-326.561
2	-51.8488	-418.037	-87.9194	-440.99	-243.231	-821.163	-162.036
3	78.64594	-127.838	96.75373	-38.1084	135.092	-488.608	118.9319
4	-1.7456	11.91655	-34.1287	-5.13019	221.7306	-353.549	172.4038
5	-25.0516	6.046229	-14.8391	-2.83399	94.23638	-125.988	101.2344
6	157.0995	-29.9133	31.76967	65.54444	-9.04024	-14.3791	-39.6964
7	428.8077	19.69278	58.0801	79.87884	64.20807	0.246004	-49.7144
8	817.5112	185.7968	65.12441	-81.6586	126.298	35.04308	-39.4281
9	1370.213	483.5907	-7.46761	-105.272	67.64471	20.13557	-126.54
10	1965.557	670.5193	-46.1289	-1.1258	-14.3516	-67.6496	-82.4283
11	2809.639	1081.088	32.94868	3.345579	-61.5099	-45.3226	-77.6701
12	3486.991	1833.942	179.2487	17.8062	22.34935	-22.6011	-40.9108
13	3979.123	2533.985	321.2895	38.45371	106.3096	-85.7005	-0.50822
14	4624.992	3266.069	534.7678	114.7349	63.16068	-80.2639	-18.0579
15	5084.13	4158.117	1116.106	216.4655	-14.6199	-137.458	-13.8536
16	5425.31	5125.75	1943.041	439.8935	-67.3967	-128.149	10.4592
17	5757.647	5843.458	2936.484	741.9825	33.04203	2.257639	112.5462
18	5918.465	6379.525	3899.715	1172.197	217.7669	21.71986	72.10566
19	5957.824	7047.902	4687.995	1841.667	410.5871	15.27217	64.03682
20	6024.792	7568.477	5513.91	2745.055	689.6772	112.5688	90.44904
21	5974.415	7821.69	6310.941	3661.655	1136.453	262.5421	-56.7641
22	5728.138	8158.044	6989.059	4423.016	1658.528	273.5267	-94.3524
23	5568.108	8459.492	7457.54	5134.535	2184.274	303.1817	-30.8752
24	5432.303	8552.52	7790.069	5711.702	2957.911	633.1078	-58.2334
25	5209.693	8571.193	8026.448	6404.68	3901.559	1143.481	-40.2747
26	5083.386	8599.367	8045.539	7086.024	4703.901	1688.528	30.2895
27	4985.012	8690.708	8094.225	7446.704	5279.856	2377.037	148.0318
28	4687.26	8724.533	8301.466	7646.448	5818.443	3318.962	429.8334
29	4433.763	8651.925	8329.415	7785.405	6403.98	4436.192	872.1549
30	4264.659	8383.6	8348.434	7779.217	6842.547	5545.787	1569.888
31	3959.521	8294.68	8484.001	7885.006	7127.362	6417.359	2546.448
32	3683.561	8420.204	8380.202	8034.549	7359.379	7021.734	3494.76
33	3544.47	8458.161	8090.662	8037.842	7441.177	7655.495	4366.514
34	3366.479	8538.121	8029.209	8211.672	7499.927	8118.338	5263.343
35	3152.068	8385.771	8079.379	8335.443	7676.232	8420.755	6097.102
36	3051.351	8118.604	7996.758	8264.075	7676.007	8770.53	6764.362
37	2790.675	8178.046	7761.896	8201.47	7662.296	8923.83	7231.479
38	2465.607	8253.976	7544.503	8296.31	7756.948	9051.994	7543.548
39	2252.171	8210.76	7503.288	8273.155	7680.577	9134.043	7904.65
40	2022.247	8218.972	7458.443	8126.489	7545.813	9157.817	8332.001

The graphic presentation of the amplification plots for ACA2 is shown in Figure 21C. The standard curve is shown in Figure 21D.

If only target 1 is present in the reaction, the graphic presentation of the amplification plots for ACA1 is shown in Figure 21E, the amplification plots for ACA2 is shown in Figure 21G. The results show that because only target 1 is present in the reaction, the ACA1 reveal the normal amplification curve and normal standard curve(Figure 21 F), whereas the ACA2 reveal the background curve which demonstrates there is no signal for target 2.

If only target 2 is present in the reaction, the graphic presentation of the amplification plots for ACA1 is shown in Figure 21H, the amplification plots for ACA2 is shown in Figure 21I. The results show that because only target 2 is present in the reaction, the ACA2 reveal the normal amplification curve and normal standard curve (Figure 21J), whereas the ACA1 reveal the background curve which demonstrates there is no signal for target 1.

Example 5

Amplification primers for target 1 (K10):

K10F155 CTCTGCTGACTTCAAAACGAGAAGAG (SEQ ID NO: 5);

K10R14 CCTGAGGGTTAAATCTTCCCCATTGA (SEQ ID NO: 21)

Probe for target 1 (referred to as probe1) includes: first oligonucleotide

K10R266Famph GTTCAATTGGGTTTCACCGCGCTTAGTTACA (SEQ ID NO: 7), which 5' end is attached with Fam; 3'end is attached with a phosphate group instead of 3' –OH; and second oligonucleotide

K10R266Dab GCGCGGTGAAACCCAATTGAAC (SEQ ID NO: 2), which 3' end is attached with DABCYL.

Amplification primers for target 2 (SV40):

dsredendF2 GTAAGATCCACCGGATCTAGATAAC (SEQ ID NO: 8);

sv40testR GGGAGGTGTGGGAGGTTTTTTAAAG (SEQ ID NO: 9).

Probe for target 2 (referred to as probe2) includes: first oligonucleotide

SV40RlF3FAPh ATCAGCCATACCACATTTGTAGAGGTTTTAC (SEQ ID NO: 10), which 5' end is attached with Fam; 3'end is attached with a phosphate group instead of 3' –OH; and second oligonucleotide

SV40RlF3Dab CAAATGTGGTATGGCTGAT (SEQ ID NO: 4), which 3' end is attached with DABCYL.

Amplification primers for target 3 (Jak2):

JknewF8 GTGGAGACGAGAGTAAGTAAAACTACA (SEQ ID NO: 14);

JKnewR8 CTCCTGTTAAATTATAGTTTACACTGACA (SEQ ID NO: 15);

Probe for target 3 (referred to as probe 3) includes: first oligonucleotide JKR3FamPh AACAGATGCTCTGAGAAAGGCATTAGA (SEQ ID NO: 16), which 5' end is attached with Fam; 3'end is attached with a phosphate group instead of 3' –OH; and second oligonucleotide

JKR3FDabF CTCAGAGCATCTGTT (SEQ ID NO: 12), which 3' end is attached with DABCYL.

Amplification primers for target 4 (Kras):

KR12GVF1B GTCACATTTTCATTATTTTTATTATAAGGCCTGC (SEQ ID NO: 17);

KR12GVR12As GATCATATTCGTCCACAAAATGATTC (SEQ ID NO: 18).

Probe for target 4 (referred to as probe 4) includes: first oligonucleotide KR12GVFamPh GAATATAAACTTGTGGTAGTTGGAGCTGT (SEQ ID NO: 19), which 5' end is attached with Fam; 3'end is attached with a phosphate group instead of 3' –OH; and second oligonucleotide

KR12GVFamDab CACAAGTTTATATTC (SEQ ID NO: 20), which 3' end is attached with DABCYL.

The first oligonucleotide and the second oligonucleotide were combined at various ratios, typically 1:2-1:4 to form a partially double-stranded linear DNA probe. In the absence of target, the formation of the first and the second oligonucleotide hybrid brings the quencher and the fluorophore into close proximity, efficiently quenching the fluorescent signal. In the presence of the target, the first oligonucleotide preferentially hybridises to the target sequence. As a result, the quencher is separated from the fluorophore resulting in an increase in fluorescence emission.

The first oligonucleotides in all probes are modified to contain blocked 3' end, so that it cannot be extended. However, when the first oligonucleotide binds to the target sequence, it can be degraded by the 5' nuclease activity of a polymerase. The degradation of the first oligonucleotide of the probe (the consumed probes) results in decrease of the number of first oligonucleotide available to bind with the second oligonucleotides of probe, thereby increasing the fluorescence signal when measured at appropriate temperature.

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Multiplex real-time PCR and standard curve analysis

Primer-probe master mix was set up as follows: the primers and probes were mixed to a final concentration 0.4 μM of probes and 0.6 μM of primers, which creates a 2X primer-probe master mix.

The reaction mix was created by combining equal amount of 2Xprimer-probe master mix and 2X TaqMan® Gene Expression Master Mix (Applied Biosystem, cat. No 4369514).

The thermal profile was: 95°C for 8 min 30 sec; 40 cycles of 94°C 10s; 66°C 20s; 63°C 20 sec; 54°C 30s; 49°C 20s; 55°C 20s; 61°C 20s; 68°C 20s; fluorescence measurements were recoded during the read steps 66°C, 63°C, 54°C; 49°C; 55°C; 61°C; 68°C.

Various combinations of targets present in the reaction were used. Figure 22 show some of the results: reaction (A) contains no template; reaction (B) contains target 3 template; reaction (C) contains target 2 template; reaction (D) contains target 4; reaction (E) contains target 1 and 3; reaction (F) contains targets 1, 3 and 4.

SEQUENCE LISTING FREE TEXT

SEQ ID NOs: 1, 2, 5, 7 and 21	<223> K10 derived PCR primer
SEQ ID NOs: 3, 4, 6 and 8-10	<223> SV40 derived PCR primer
SEQ ID NOs: 11-16	<223> Jak2 derived PCR primer
SEQ ID NOs: 17-20	<223> Kras derived PCR primer

CLAIMS

1. A method for assaying a sample for one or more target nucleic acids, said method comprising:

(a) contacting a sample comprising one or more target nucleic acids with an amplification reaction mixture comprising:

(i) one or more pairs of forward/reverse oligonucleotide primers, wherein the primer pairs are capable of amplifying one or more target nucleic acids, if present in the sample,

(ii) two or more probes, wherein each probe comprises

a first oligonucleotide which comprises a first region which is substantially complementary to part of one target nucleic acid and a second region, and

at least one second oligonucleotide which comprises a region which is substantially complementary to the second region of the first oligonucleotide,

such that the first and second oligonucleotides are capable of forming a double-stranded portion,

wherein each probe comprises a detectable label or detectable combination of labels which is/are capable of producing a changeable signal which is characteristic of the presence or absence of a double-stranded portion between the first and second oligonucleotides of that probe, and

wherein at least two of the probes comprise the same detectable label or different detectable labels with undistinguishable emission spectra and wherein the melting characteristics of the double-stranded portions between the first and second oligonucleotides of each of such probes are different;

(b) performing an amplification reaction on the sample/reaction mixture under amplification conditions, wherein, when a target nucleic acid is present, the first oligonucleotides of probes which are substantially complementary to part of that target nucleic acid are hybridised with the target nucleic acid, therefore being consumed, wherein the consumed oligonucleotides of probes are no longer able to participate in forming double stranded portion (the duplex) of the probe; and

(c) measuring, at least once, the melting profile of the double-stranded portions between the first and second oligonucleotides of unconsumed probes in the reaction mixture by detecting the signal(s) from the labels in those probes as a function of temperature,

wherein the melting profile provides an indication of whether or not at least one target nucleic acid is present in said sample,

wherein a first probe of said at least two of the probes has a melting temperature T_m1 in terms of its double-stranded portion,

wherein a second probe of said at least two of the probes has a melting temperature T_m2 in terms of its double-stranded portion,

wherein $T_m1>T_m2$,

wherein the same labels are independently attached to the first and second probes,

wherein a reduction of any melting peak at T_m1 and/or T_m2 provides indication of consumption of the first and/or second probe(s).

2. A method according to claim1, wherein said melting profile is measured before reaction/amplification takes place (pre-amplification melting profile), and/or is measured after completion of reaction/amplification (post-amplification melting profile), and/or is measured during reaction/amplification at each cycle or selected cycles (mid-amplification melting profile),

wherein said method additionally comprises step (d)

(i) comparing at least two melting profiles obtained in (c)

and/or

(ii) comparing a melting profile obtained in step (c)

with a previously-obtained melting profile of the same probes or

with a melting profile of the same probes obtained in parallel at the same time in control reactions, or

with a theoretical melting profile of the same probes

wherein a change in the melting profile provides an indication of whether or not at least one target nucleic acid is present in said sample/reaction mixture,

wherein said the pre-amplification melting profile is measured in the same reaction vessel before the start of reaction/amplification, or is measured in a separate reaction vessel where no amplification takes place due to that reaction mixture lacking one or more ingredients necessary for the reaction/amplification,

wherein in step (d) the post-amplification or mid-amplification melting profile is compared with the pre-amplification melting profile of the duplex of probes to determine whether a particular probe is consumed, this being indicative of the presence of the corresponding target in the sample.

3. A method according to any one of the preceding claims, wherein at least one detectable label is a fluorescent label, wherein step (b) further comprises the step (b1) obtaining cycle by cycle fluorescence emissions (FE) at various measuring temperatures (MT), wherein said fluorescence emissions (FE) is a baseline corrected fluorescence (dR).

4. A method according to claim 3, wherein when said amplification reaction mixture comprises "n" probes for multiplex detection of "n" nucleic acid targets, wherein first probe has a melting temperature T_m1, second probe has a melting temperature T_m2, third probe has a melting temperature of T_m3, n-th probe has a melting temperature T_mn, wherein $T_m1>T_m2>T_m3 \ldots >T_mn$, the percentages of the double-stranded form of each probe at a particular temperature or different temperatures are determined experimentally or are calculated in theory by a computer program, wherein a first fluorescence emission FEa is obtained at a measuring temperature MTa, at which more than 50% of first probe is in duplex form, second fluorescence emission FEb is obtained at a measuring temperature MTb, at which more than 50% of second probe is in duplex form, n-1 fluorescence emission FE(n-1) is obtained at a measuring temperature MT(n-1), at which more than 50% of (n-1)th probe is in duplex form, n-th fluorescence emission FEn is obtained at a measuring temperature MTn, at which more than 80 % of n-th probe is in duplex form, and optionally a fluorescence emission FE0 is obtained at a measuring temperature MT0, at which no more than 10 % of first probe is in duplex form, wherein n is a positive integer and $n \geqslant 2$.

5. A method according to claim 3, wherein the step (b) further comprises the step (b2) determining cycle by cycle the Actual Consumed Amount (ACA) of fluorescence emission for each probe, wherein the Actual Consumed Amount of fluorescence emission of k-th probe is depicted as ACA_k, wherein at a particular measuring temperature (MTa) the k-th probe has percentage (dska)% in ds (double-strand) form, the fluorescence emission FEa at this measuring temperature MTa contributed by the first probe will be (ds1a)% * (ACA_1), contributed by the second probe will be (ds2a)% * (ACA_2), contributed by the k-th probe will be (dska)% * (ACA_k), contributed by the n-th probe will be (dsna)% * (ACA_n), wherein the calculation of Actual Consumed Amount (ACA) uses the following formula:

at measuring temperature MTa, the total fluorescence emission will be

FEa=(ACA1)*(ds1a)%+ (ACA2)*(ds2a)% +(ACA3)*(ds3a)% ...+(ACAn)*(dsna)%

at measuring temperature MTb, the total fluorescence emission will be

FEb=(ACA1)*(ds1b)%+ (ACA2)*(ds2b)% +(ACA3)*(ds3b)% ...+(ACAn)*(dsna)%

At measuring temperature MTc, the total fluorescence emission will be

FEc=(ACA1)*(ds1c)%+ (ACA2)*(ds2c)% +(ACA3)*(ds3c)% ...+(ACAn)*(dsna)%

and so on, wherein the ACA for each probe can be calculated from the above formulas, wherein "*" denotes "multiply by", "k" is positive integer, $1 \leqslant k \leqslant n$, and "n" is the number of probes

6. A method according to claim 5, wherein the Actual Consumed Amount of fluorescence emission of each probe is obtained through a computer program which performs the calculation at each measuring temperature at each cycle.

7. A method for assaying a sample for one or more target nucleic acids, said method comprising:

(a) contacting a sample comprising one or more target nucleic acids with a reaction mixture comprising:

two or more probes, wherein each probe comprises

a first oligonucleotide which comprises a first region which is substantially complementary to part of one target nucleic acid and a second region, and

at least one second oligonucleotide which comprises a region which is substantially complementary to the second region of the first oligonucleotide, such that the first and second oligonucleotides are capable of forming a double-stranded portion,

wherein each probe comprises a detectable label or detectable combination of labels which is/are capable of producing a changeable signal which is characteristic of the presence or absence of a double-stranded portion between the first and second oligonucleotides of that probe, and

wherein at least two of the probes comprise the same detectable label or different detectable labels with undistinguishable emission spectra and wherein the melting characteristics (melting temperature T_m) of the double-stranded portions between the first and second oligonucleotides of each of such probes are different, and are distinguishable in a melting profile analysis;

(b) performing the reaction on the sample/reaction mixture, wherein the reaction is a primer extension reaction under extension conditions, wherein, when a target nucleic acid is present, the first oligonucleotides of the corresponding probe which are extendable primers, are hybridised with target nucleic acid, therefore being consumed during the primer extension reaction, wherein the consumed oligonucleotids of probes are no longer able to participate in forming double stranded portion (the duplex) of the probe; and

(c) measuring, at least once, the melting profile of the double-stranded portions between the first and second oligonucleotides of the unconsumed probes in the reaction mixture by detecting the signal(s) from the labels in those probes as a function of temperature, wherein the melting profile provides an indication of whether or not at least one target nucleic acid is present in said sample.

8. A method for assaying a sample for one or more target nucleic acids, said method comprising:

(a) contacting a sample comprising one or more target nucleic acids with a hybridisation reaction mixture comprising:

two or more probes, wherein each probe comprises

a first oligonucleotide which comprises a first region which is substantially complementary to part of one target nucleic acid and a second region, and

at least one second oligonucleotide which comprises a region which is substantially complementary to the second region of the first oligonucleotide,

such that the first and second oligonucleotides are capable of forming a double-stranded portion,

wherein each probe comprises a detectable label or detectable combination of labels which is/are capable of producing a changeable signal which is characteristic of the presence or absence of a double-stranded portion between the first and second oligonucleotides of that probe, and

wherein at least two of the probes comprise the same detectable label or different detectable labels with undistinguishable emission spectra and wherein the melting characteristics of the double-stranded portions between the first and second oligonucleotides of each of such probes are different and are distinguishable in a melting profile analysis;

(b) performing the hybridisation reaction on the sample/reaction mixture under hybridisation conditions, wherein, when a target nucleic acid is present, the first oligonucleotides of probes which are substantially complementary to part of that target nucleic acid are hybridised with target nucleic acid, therefore being consumed during the reaction, wherein the consumed oligonucleotides of probes are no longer able to participate in forming double stranded portion (the duplex) of the probe; and

(c) measuring, at least once, the melting profile of the double-stranded portions between the first and second oligonucleotides of unconsumed probes in the reaction mixture by detecting the signal(s) from the labels in those probes as a function of temperature,
wherein the melting profile provides an indication of whether or not at least one target nucleic acid is present in said sample.

9. A method according to claim 1, wherein said amplification is an isothermal amplification or a thermal cycling amplification reaction comprising two or more denaturing, annealing, and primer extension steps.

10. A method for monitoring a PCR amplification of at least two nucleic acid targets, said method comprising:

(a) contacting a sample comprising one or more target nucleic acids with an amplification reaction mixture comprising:

(i) one or more pairs of forward/reverse oligonucleotide primers, wherein the primer pairs are capable of amplifying one or more target nucleic acids, if present in the sample,

(ii) two or more probes, wherein each probe comprises

a first oligonucleotide which comprises a first region which is substantially complementary to part of one target nucleic acid and a second region, and

at least one second oligonucleotide which comprises a region which is substantially complementary to a second region of the first oligonucleotide, such that the first and second oligonucleotides are capable of forming a double-stranded portion,

wherein each probe comprises a fluorescent label or fluorescent label/quencher pair which is capable of producing a changeable signal which is characteristic of the presence or absence of a double-stranded portion between the first and second oligonucleotides of that probe, and

wherein at least two of the probes comprise the same detectable label or different detectable labels with undistinguishable emissions spectra and wherein the melting characteristics of the double-stranded portions between the first and second oligonucleotides of each of such probes are different;

(b) performing an amplification reaction which comprises thermal cycling of the sample/amplification reaction mixture

wherein, when a target nucleic acid is present, the first oligonucleotides of probes which are substantially complementary to part of that target nucleic acid are consumed during the amplification reaction; and

wherein the step (b) further comprises the step (b1) obtaining cycle by cycle fluorescence emissions (FE) at various measuring temperatures (MT), wherein said fluorescence emissions (FE) is baseline corrected fluorescence (dR),

wherein when said amplification reaction mixture comprises "n" probes for multiplex detection of "n" nucleic acid targets, wherein first probe has a melting temperature of T_m1, second probe has a melting temperature of T_m2, third probe has a melting temperature of T_m3, n-th probe has a melting temperature of T_mn, wherein $T_m1>T_m2>T_m3...>T_mn$, wherein the percentages of the double-stranded form of each probe at a particular temperature or different temperatures are determined experimentally or are calculated in theory by a computer program, wherein a first fluorescence emission FEa is obtained at a measuring temperature MTa, at which more than 50% of first probe is in duplex form, second fluorescence emission FEb is obtained at a measuring temperature MTb, at which more than 50% of second probe is in duplex form, n-1 fluorescence emission FE(n-1) is obtained at a measuring temperature MT(n-1), at which more than 50% of (n-1)th probe is in duplex form, n-th fluorescence emission FEn is obtained at a measuring temperature MTn, at which more than 80 % of n-th probe is in duplex form, and optionally a fluorescence emission FE0 is obtained at a measuring temperature MT0, at which no more than 10 % of first probe is in duplex form, wherein n is a positive integer and $n \geqslant 2$.

wherein the step (b) further comprises the step (b2) determining cycle by cycle the Actual Consumed Amount of fluorescence emission for each probe, wherein the Actual Consumed Amount of fluorescence emission of k-th probe is depicted as ACA_k, wherein at a particular measuring temperature (MTa) said k-th probe has percentage (dska)% in ds (double-strand) form, the fluorescence emission FEa at this measuring temperature MTa contributed by the k-th probe will be (dska)% * (ACA_k), contributed by the n-th probe will be (dsna)% * (ACA_n), wherein the calculation of Actual Consumed Amount (ACA) uses the following formula:

at measuring temperature MTa, the total fluorescence emission will be

FEa=(ACA1)*(ds1a)%+ (ACA2)*(ds2a)% +(ACA3)*(ds3a)% ...+(ACAn)*(dsna)%

at measuring temperature MTb, the total fluorescence emission will be

FEb=(ACA1)*(ds1b)%+ (ACA2)*(ds2b)% +(ACA3)*(ds3b)% ...+(ACAn)*(dsna)%

At measuring temperature MTc, the total fluorescence emission will be FEc=(ACA1)*(ds1c)%+ (ACA2)*(ds2c)% +(ACA3)*(ds3c)% ...+(ACAn)*(dsna)% and so on, wherein the individual ACA can be calculated from the above formulas, wherein "*" denotes "multiply by", "k" is positive integer, $1 \leqslant k \leqslant n$, and "n" is the number of probes

wherein the Actual Consumed Amount of fluorescence emission of each probe is obtained though a computer program which performs the calculation at each measuring temperature at each cycle

and wherein the Actual Consumed Amount of fluorescence emission of each probe is related to the degree of amplification of the target nucleic acid to which the first oligonucleotide of that probe binds.

11. A method according to any one of the preceding claims, wherein said consumption of probes is achieved through hybridisation of the first oligonucleotide of the probe to the target sequence, which is followed by the incorporation of the first oligonucleotide of the probe into the amplified product, wherein when the first oligonucleotide of the probe can be incorporated into the amplified product, the first oligonucleotide is extendable primer or is one of the pair of forward/reverse oligonucleotide primers.

12. A method according to any one of the preceding claims, wherein said consumption of probes is achieved through hybridisation of the first oligonucleotide of the probe to the target sequence, which is followed by degradation of the first and/or second oligonucleotide of the probe, wherein when the first oligonucleotide of the probe is degraded during the reaction, the reaction mixture comprises double-strand dependent nuclease activity.

13. A method according to any one of the preceding claims, wherein the probe comprises a first label and a second label, wherein the first label is a fluorophore and the second label is a quencher, or vice versa.

14. A method according to claim 13, wherein the first label is attached to the first oligonucleotide and the second label is attached to the second oligonucleotide such that said first label and second label are in close proximity when the probe's internal duplex is formed.

15. A method according to claim 13, wherein labels are on one oligonucleotide of the probe, either the first oligonucleotide or the second oligonucleotide.

16. A method according to claim 15, wherein the first oligonucleotide of the probe does not comprise a label, and the second oligonucleotide of the probe comprises at least one, preferably two, labels, wherein the second oligonucleotide comprises a first label and a second label, the first label being attached at or near one end of second oligonucleotide and the second label is

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attached at or near the other end of the second oligonucleotide, whereby when the second oligonucleotide is not hybridised to the first oligonucleotide, the second oligonucleotide is in a random-coiled or a stem-loop structure which brings the first label and second label into close proximity.

17. A method according to claims 15, wherein the first oligonucleotide does not comprise a label and the second oligonucleotide comprises a label wherein, when the second oligonucleotide hybridises to the first oligonucleotide to form the double-stranded portion of the probe, the label is capable of changing the signal emission relative to the emission of the single-stranded form of the second oligonucleotide.

18. A method according to claim 15, wherein the first oligonucleotide of the probe does not comprises a label and wherein the probe comprises two second oligonucleotides which are capable of hybridising adjacently or substantially adjacently to different parts of the second region of the first oligonucleotide, wherein one of the second oligonucleotides is attached with a first label, and the other second oligonucleotide is attached with a second label, such that when the two second oligonucleotides are hybridised to the first oligonucleotide, the two labels are brought into close proximity and one label affects the signal from the other.

19. A method according to claim 13, wherein the first and second oligonucleotides of a probe are joined by a linker moiety which comprises nucleotides or a non-nucleotide chemical linker, allowing the first oligonucleotide and second oligonucleotide to form a stem-loop structure, wherein the first and second oligonucleotides are labelled with a first and second label, respectively, such that, when the probe forms an internal stem-loop structure, the labels are brought into close proximity and one label affects the signal from the other.

20. A method according to any one of the preceding claims, wherein said first region of said first oligonucleotide is not substantially overlapping with second region of said first oligonucleotide.

21. A method according to any one of the preceding claims, wherein said first region of said first oligonucleotide is substantially overlapping with the second region of said first oligonucleotide or the second region is embedded in the first region, wherein the T_m of the duplex of said first oligonucleotide hybridised with the target sequence is higher than the T_m of the duplex of said first oligonucleotide hybridised with the second oligonucleotide such that if a target is present, the first oligonucleotide forms stronger hybrids with the target and consequently melts at a higher temperature than the first/second oligonucleotide duplex.

22. A method according to any one of the preceding claims, wherein said first oligonucleotide comprises a third region which is identical or substantially identical to the sequence of a primer which is used in the amplification reaction.

23. A method according to any one of the preceding claims, wherein both the first and second oligonucleotides of probes are capable of being consumed during amplification.

24. A method according to any one of the preceding claims, wherein said first oligonucleotide is blocked at the 3' end, and wherein said second oligonucleotide is blocked at the 3' end.

25. A method for assaying a sample for one or more variant nucleotides on the target nucleic acids, said method comprising:

(a) contacting a sample comprising target nucleic acids with reaction mixture comprising:

(i) one or more pairs of forward/reverse oligonucleotide primers, wherein the primer pairs are capable of amplifying one or more target nucleic acids, if present in the sample,

(ii) at least one pair of probes, wherein first probe in the pair comprises sequence complementary to wild-type target nucleic acid sequence, second probe in the pair comprises sequence complementary to the target nucleic acid sequence containing variant nucleotides, wherein each probe in the pair comprises

a first oligonucleotide which comprises a first region which is substantially complementary to part of one target nucleic acid and a second region, and

at least one second oligonucleotide which comprises a region which is substantially complementary to the second region of the first oligonucleotide, such that the first and second oligonucleotides are capable of forming a double-stranded portion,

wherein each probe in the pair comprise the same second oligonucleotide, wherein each probe comprises a detectable label or detectable combination of labels which is/are capable of producing a changeable signal which is characteristic of the presence or absence of a double-stranded portion between the first and second oligonucleotides of that probe, and

wherein at least two of the probes comprise the same detectable label or different detectable labels with undistinguishable emission spectra and wherein the melting characteristics of the double-stranded portions between the first and second oligonucleotides of each of such probes are different;

(b) performing an amplification reaction on the sample/amplification reaction mixture wherein, when a target nucleic acid is present, the first oligonucleotides of probes which are substantially complementary to part of that target nucleic acid are consumed during the amplification reaction; and

(c) measuring, at least once, the melting profile of the double-stranded portions between the first and second oligonucleotides of unconsumed probes by detecting the signal(s) from the labels in those probes as a function of temperature,

wherein the melting profile provides an indication of whether or not at least one target nucleic acid has been amplified in said sample/amplification reaction mixture.

26. A method according to claim 25, wherein the same second oligonucleotide in the pair of the probes comprise universal base or Inosine which corresponds to the variant nucleotide in the target nucleic acid sequence, wherein said universal base is 3-nitropyrrole 2'-deoxynucleoside, 5-nitroindole, pyrimidine analog or purine analog.

27. A method according to any one of the preceding claims, wherein multiple first oligonucleotides of different probes hybridise to different sites of the same strand of a target sequence.

28. A computer software product for use with a method according to any one of the preceding claims, when run on suitable data processing means, for comparing melting profiles of probes and/or quantifying a real time PCR amplification of multiple targets which, when executed by a computer processor, performs the calculation of Actual Consumed Amount (ACA) using the following formula:

at measuring temperature MTa, the total fluorescence emission will be

FEa=(ACA1)*(ds1a)%+ (ACA2)*(ds2a)% +(ACA3)*(ds3a)% ...+(ACAn)*(dsna)%

at measuring temperature MTb, the total fluorescence emission will be

FEb=(ACA1)*(ds1b)%+ (ACA2)*(ds2b)% +(ACA3)*(ds3b)% ...+(ACAn)*(dsna)%

At measuring temperature MTc, the total fluorescence emission will be

FEc=(ACA1)*(ds1c)%+ (ACA2)*(ds2c)% +(ACA3)*(ds3c)% ...+(ACAn)*(dsna)%

and so on, wherein the individual ACA can be calculated from the above formulas, wherein "*" denotes "multiply by", "k" is positive integer, $1 \leqslant k \leqslant n$, and "n" is the number of probes

29. A computer system comprising a computer memory having a computer software program stored therein, wherein the computer software program, when executed by a processor or in a computer, performs a method according to any one of claims 1 to 27.

30. A computer system according to claim 29, wherein the computer software program performs a method comprising the step of calculating the Actual Consumed Amount of fluorescence emission t of each probe and/or determination of features of melting profiles during amplification or at the end amplification.

31. A kit for assaying for one or more nucleic acid targets, which kit comprises a probe comprising:

a first oligonucleotide of 15-150 nucleotides which comprises a first region which is substantially complementary to part of one target nucleic acid and a second region, and at least one second oligonucleotide of 4-150 nucleotides which comprises a region which is substantially complementary to the second region of the first oligonucleotide, such that the first and second oligonucleotides are capable of forming a double-stranded portion,

wherein each probe comprises a detectable label or detectable combination of labels which is/are capable of producing a changeable signal which is characteristic of the presence or absence of a double-stranded portion between the first and second oligonucleotides of that probe,

and wherein

(a) the first oligonucleotide of the probe does not comprise a label, the second oligonucleotide comprises a first label and a second label, wherein the first label is attached at or near one end of second oligonucleotide and the second label is attached at or near the other end of the second oligonucleotide, whereby when the second oligonucleotide is not hybridised with the first oligonucleotide, the second oligonucleotide is in a random-coiled or a stem-loop structure which brings the first label and second label in close proximity and wherein when the second oligonucleotide is hybridised with the first oligonucleotide, the two labels are held away from each other; or

(b) the first oligonucleotide does not comprise a label and the second oligonucleotide comprises a label, wherein when the second oligonucleotide hybridises to the first oligonucleotide to form the double-stranded portion of the probe, the label is capable of changing its detectable signal emission relative to the emission of the label when in the single-stranded form of the second oligonucleotide; or

(c) the first oligonucleotide of the probe does not comprises a label, the probe comprises two second oligonucleotides which are capable of hybridising adjacently or substantially adjacently to different parts of the second region of the first oligonucleotide, wherein one of

the second oligonucleotides is attached with a first label, and the other second oligonucleotide is attached with a second label, such that when the two second oligonucleotides are hybridised to the first oligonucleotide, the two labels are brought in close proximity and one label affects the signal from the other.

32. A kit for assaying for one or more nucleic acid targets, which kit comprises a probe mixture containing two or more probes, wherein each probes comprises:

a first oligonucleotide of 15-150 nucleotides which comprises a first region which is substantially complementary to part of one target nucleic acid and a second region, and at least one second oligonucleotide of 4-150 nucleotides which comprises a region which is substantially complementary to the second region of the first oligonucleotide, such that the first and second oligonucleotides are capable of forming a double-stranded portion,

wherein each probe comprises a detectable label or detectable combination of labels which is/are capable of producing a changeable signal which is characteristic of the presence or absence of a double-stranded portion between the first and second oligonucleotides of that probe,

and wherein

(a) the first label is attached to the second region of the first oligonucleotide and the second label is attached to the region of the second oligonucleotide which is complementary to the second region of the first oligonucleotide such that the first and second labels are brought into close proximity upon formation of the probe's internal duplex; or

(b) the first oligonucleotide of the probe does not comprise a label, the second oligonucleotide comprises a first label and a second label, wherein the first label is attached at or near one end of second oligonucleotide and the second label is attached at or near the other end of the second oligonucleotide, whereby when the second oligonucleotide is not hybridised with the first oligonucleotide, the second oligonucleotide is in a random-coiled or a stem-loop structure which brings the first label and second label in close proximity and wherein when the second oligonucleotide is hybridised with the first oligonucleotide, the two labels are held away from each other; or

(c) the first oligonucleotide does not comprise a label and the second oligonucleotide comprises a label, wherein when the second oligonucleotide hybridises to the first oligonucleotide to form the double-stranded portion of the probe, the label is capable of

changing its detectable signal emission relative to the emission of the label when in the single-stranded form of the second oligonucleotide; or

(d) the first oligonucleotide of the probe does not comprises a label, the probe comprises two second oligonucleotides which are capable of hybridising adjacently or substantially adjacently to different parts of the second region of the first oligonucleotide, wherein one of the second oligonucleotides is attached with a first label, and the other second oligonucleotide is attached with a second label, such that when the two second oligonucleotides are hybridised to the first oligonucleotide, the two labels are brought in close proximity and one label affects the signal from the other; or

(e) the first and second oligonucleotides of a probe are joined by a linker moiety which comprises nucleotides or a non-nucleotide chemical linker, allowing the first oligonucleotide and second oligonucleotide to form a stem-loop structure, wherein the first and second oligonucleotides are labelled with a first and second label, respectively, such that, when the probe forms an internal stem-loop structure, the labels are brought into close proximity and one label affects the signal from the other;

and wherein at least two of the probes comprise the same detectable label or different detectable labels with undistinguishable emission spectra and wherein the melting characteristics of the double-stranded portions between the first and second oligonucleotides of each of such probes are different.

33. A kit according to claim 31 or claim 32, wherein at least one label is a fluorescent label.

34. A kit according to claim 31, parts (a) or (c) or claim 32 parts (a), (b), (d) or (e), wherein the probe comprises a first label and a second label.

35. A kit according to claim 34, wherein the first label is a fluorophore and the second label is a quencher, or vice versa.

36. Use of a probe as defined in any one of parts (a)-(c) of claim 31, a probe mixture of claim 32, in a method as defined in any one of claims 1-27.

37. A method according to any one of claims 1 to 27, substantially as described herein and/or with reference to the Examples.

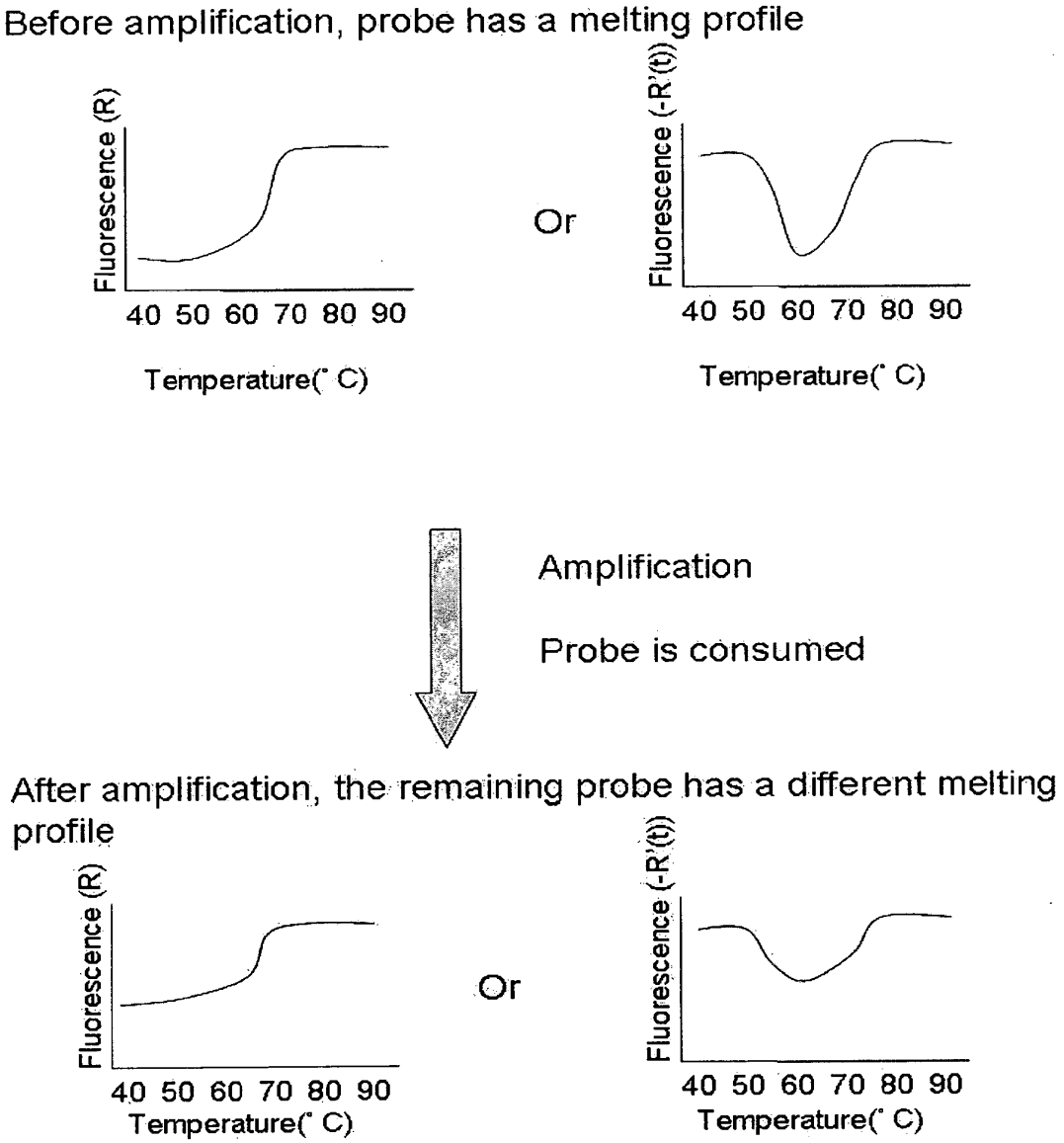

Before amplification, probe has a melting profile
Fluorescence (R)
40 50 60 70 80 90
Temperature(° C)
Or
Fluorescence (-R'(t))
40 50 60 70 80 90
Temperature(° C)
Amplification
Probe is consumed
After amplification, the remaining probe has a different melting profile
Fluorescence (R)
40 50 60 70 80 90
Temperature(° C)
Or
Fluorescence (-R'(t))
40 50 60 70 80 90
Temperature(° C)

FIG.1

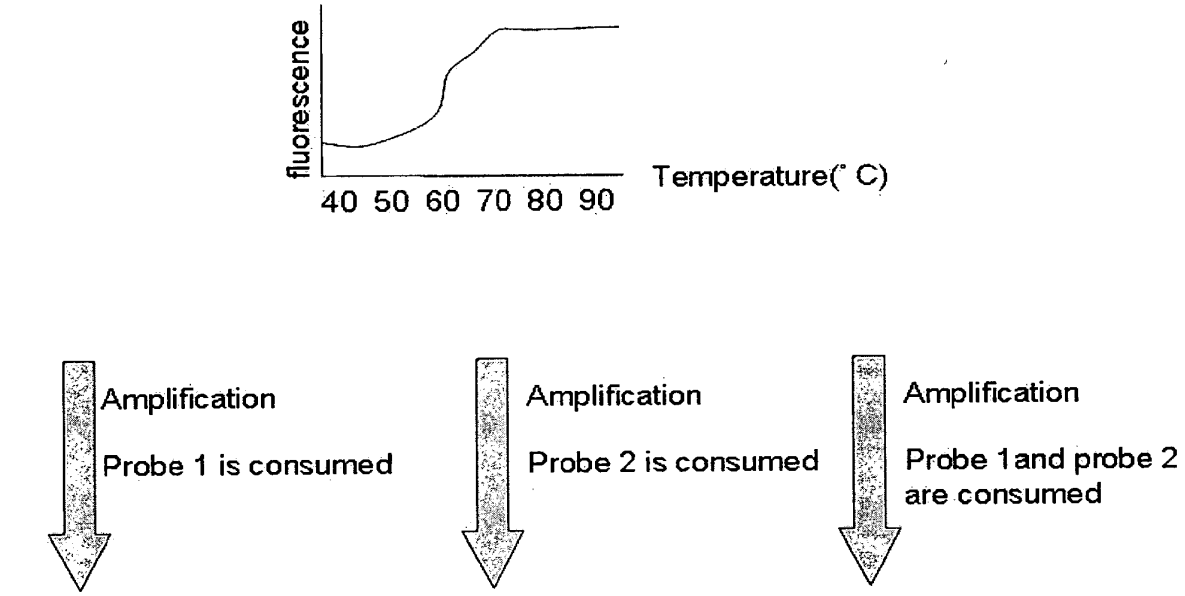

Before amplification, a mix of probe 1 and probe 2 has a melting profile
fluorescence
40 50 60 70 80 90
Temperature(° C)
Amplification
Probe 1 is consumed
Amplification
Probe 2 is consumed
Amplification
Probe 1and probe 2 are consumed
After amplification, the mix of remaining probes has a different melting profile
fluorescence
40 50 60 70 80 90
Temperature(° C)
fluorescence
40 50 60 70 80 90
Temperature(° C)
fluorescence
40 50 60 70 80 90
Temperature(° C)

FIG.2

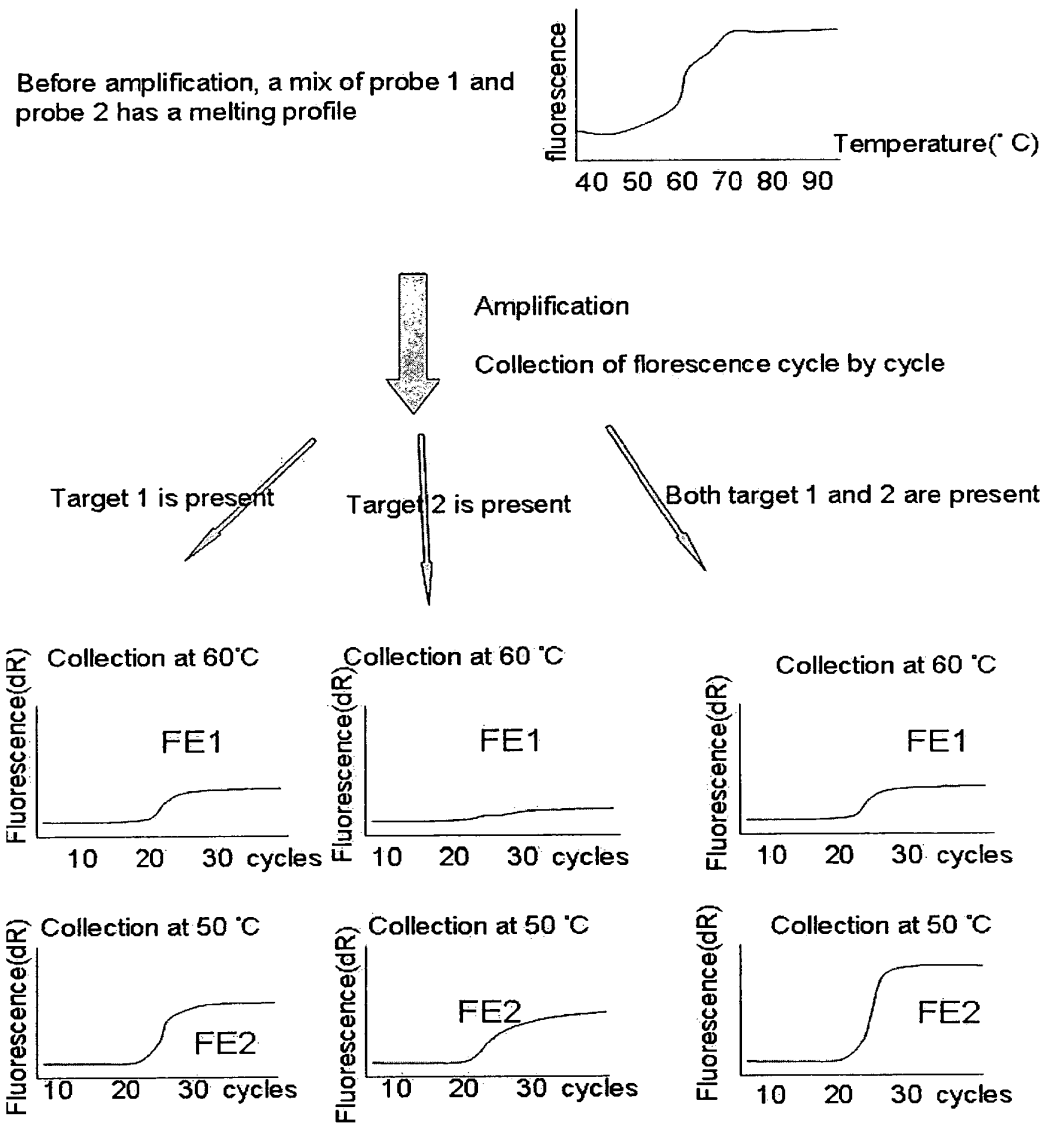

Before amplification, a mix of probe 1 and probe 2 has a melting profile
fluorescence
Temperature(° C)
40 50 60 70 80 90
Amplification
Collection of florescence cycle by cycle
Target 1 is present
Target 2 is present
Both target 1 and 2 are present
Collection at 60°C
Fluorescence(dR)
FE1
10 20 30 cycles
Collection at 60 °C
Fluorescence(dR)
FE1
10 20 30 cycles
Collection at 60 °C
Fluorescence(dR)
FE1
10 20 30 cycles
Collection at 50 °C
Fluorescence(dR)
FE2
10 20 30 cycles
Collection at 50 °C
Fluorescence(dR)
FE2
10 20 30 cycles
Collection at 50 °C
Fluorescence(dR)
FE2
10 20 30 cycles

FIG.3

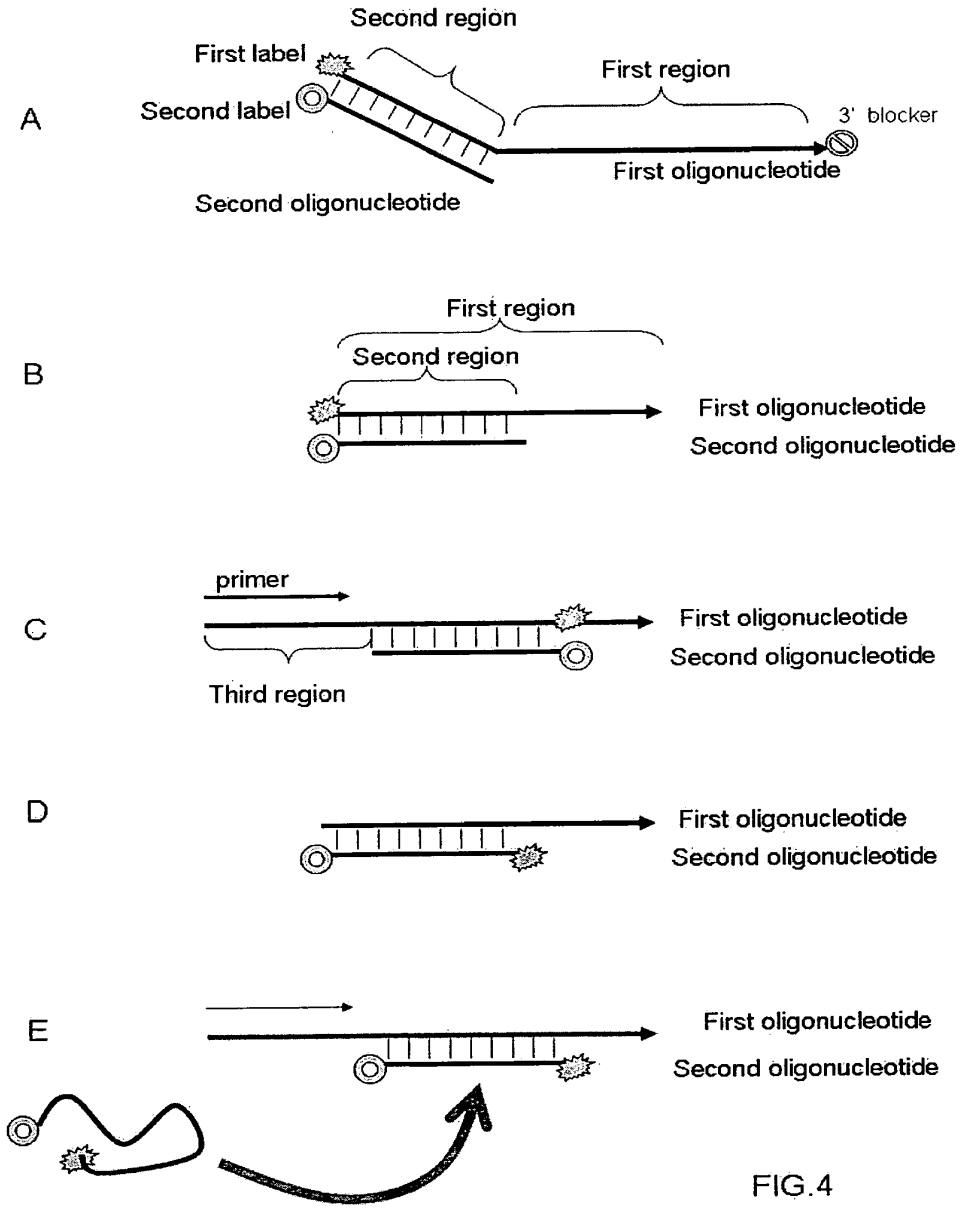

A
Second region
First label
First region
Second label
3' blocker
First oligonucleotide
Second oligonucleotide
B
First region
Second region
First oligonucleotide
Second oligonucleotide
C
primer
First oligonucleotide
Second oligonucleotide
Third region
D
First oligonucleotide
Second oligonucleotide
E
First oligonucleotide
Second oligonucleotide

FIG.4

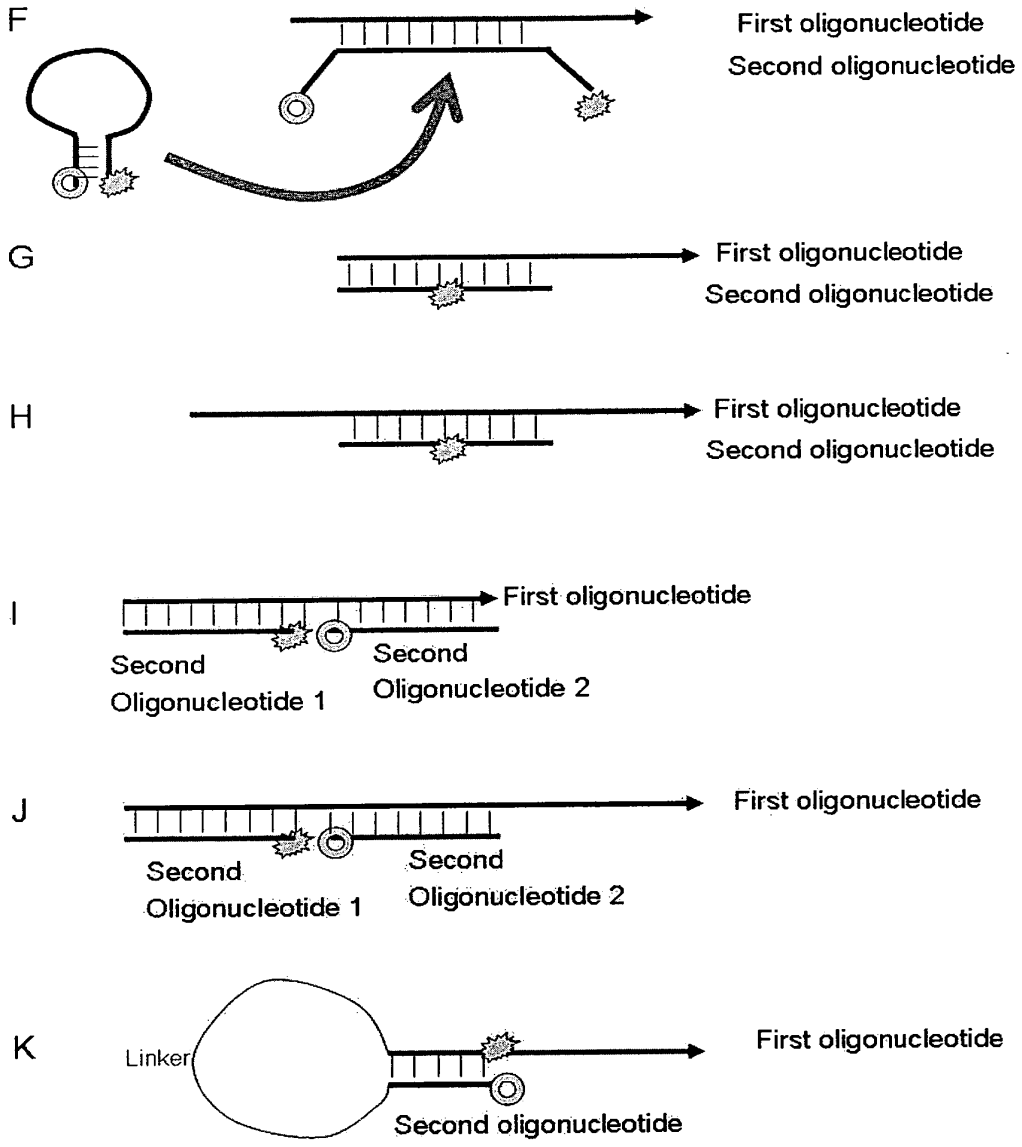


FIG.4 (continued)

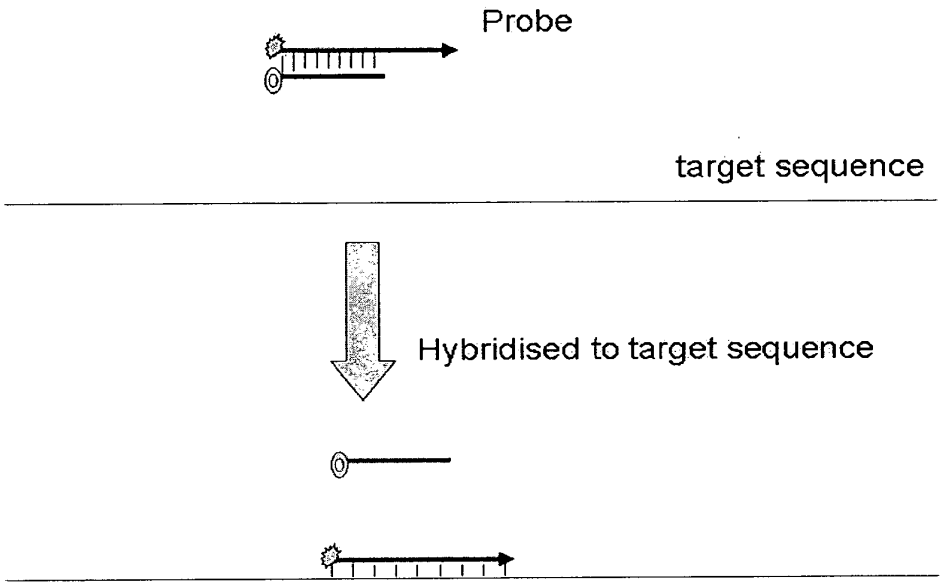


FIG.5

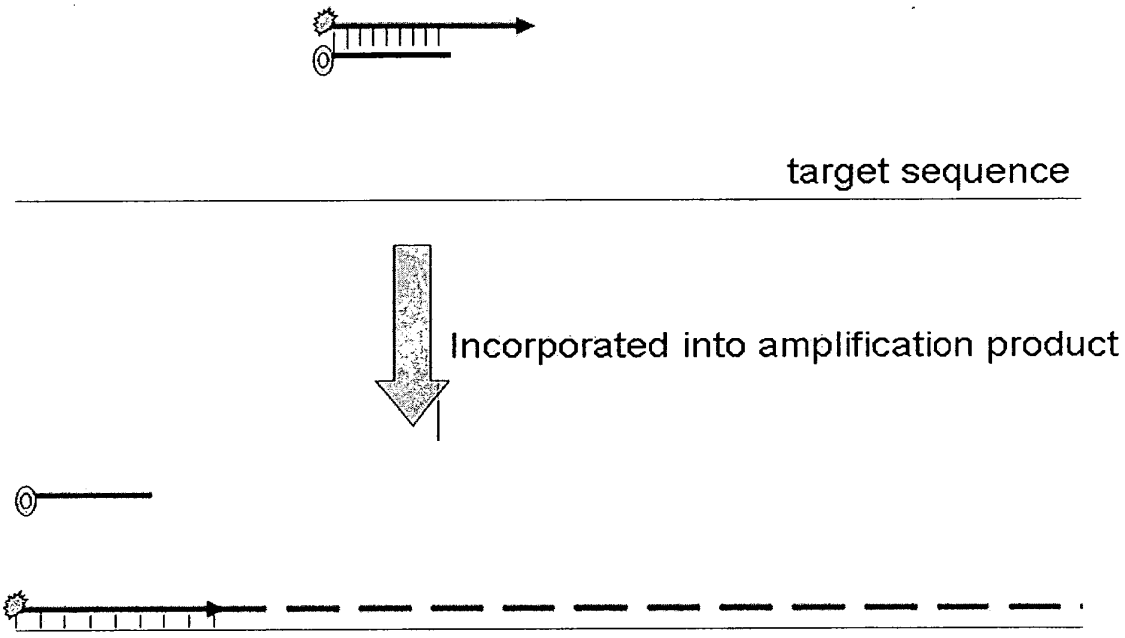

target sequence
Incorporated into amplification product

FIG.6

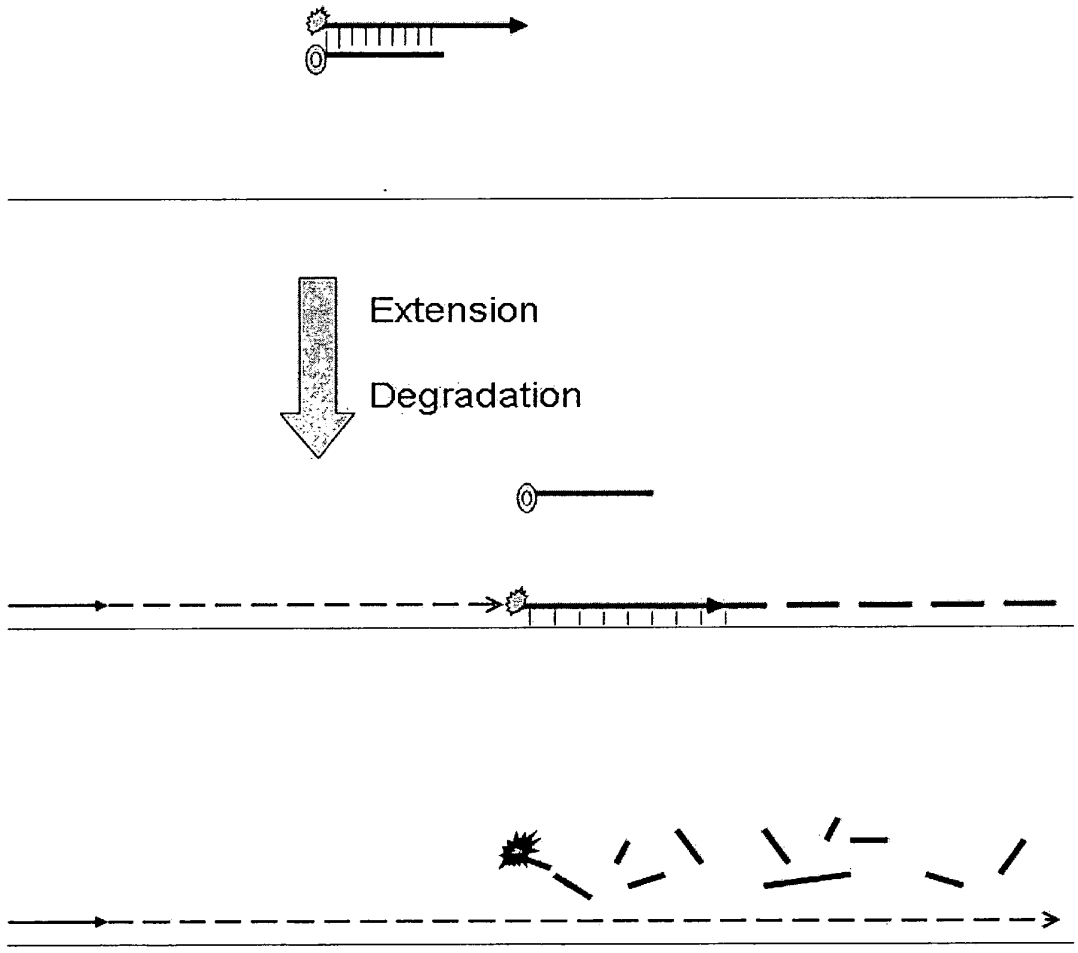


FIG.7

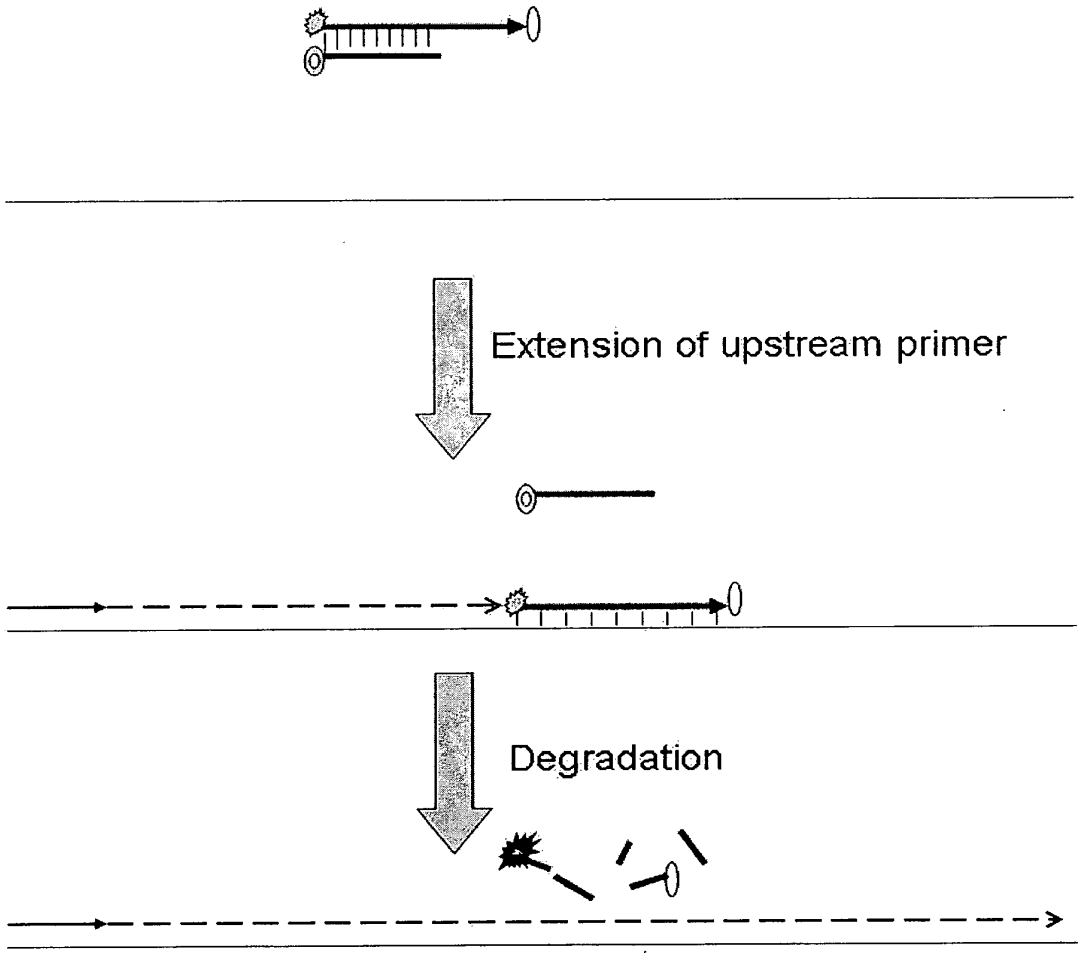
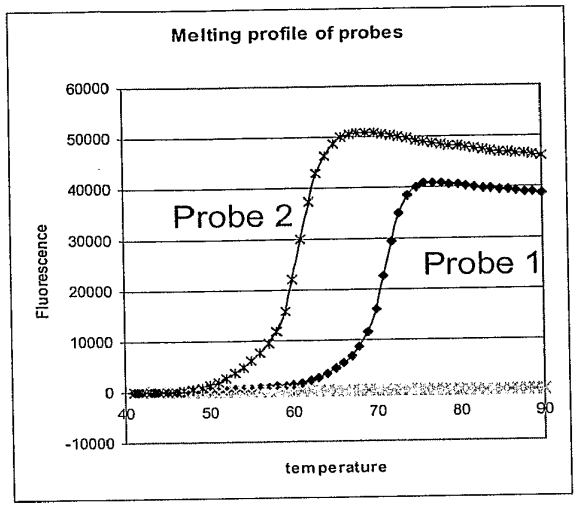


FIG.8

A



B

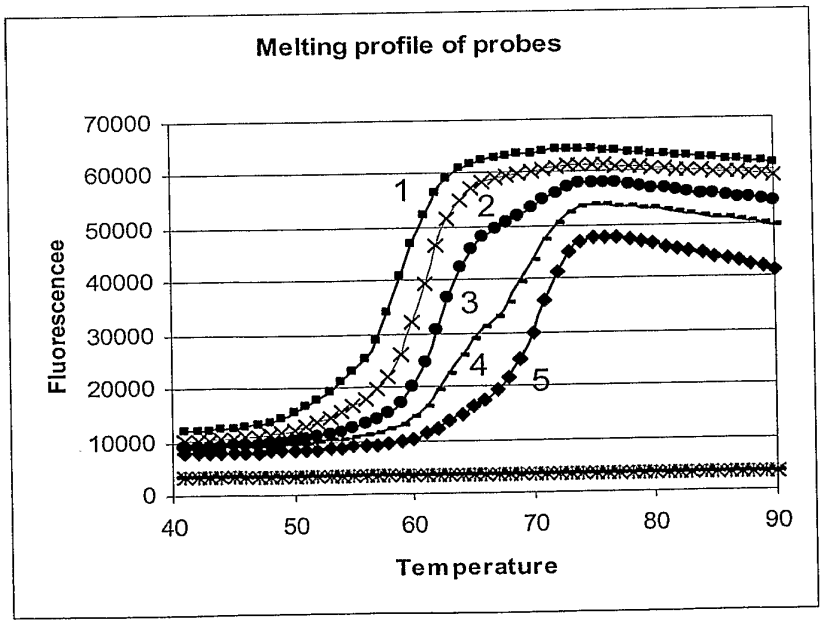


FIG. 9

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California Institute of Technology - Ex. 2003 Page 1852

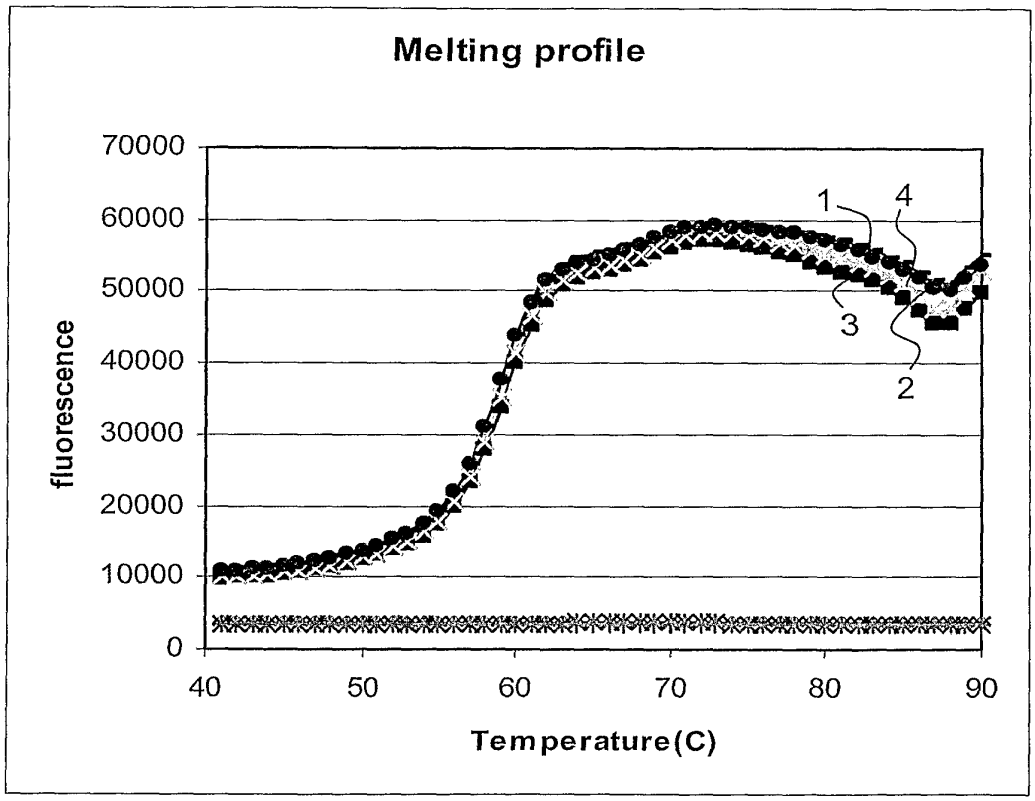


FIG. 10

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California Institute of Technology - Ex. 2003 Page 1853

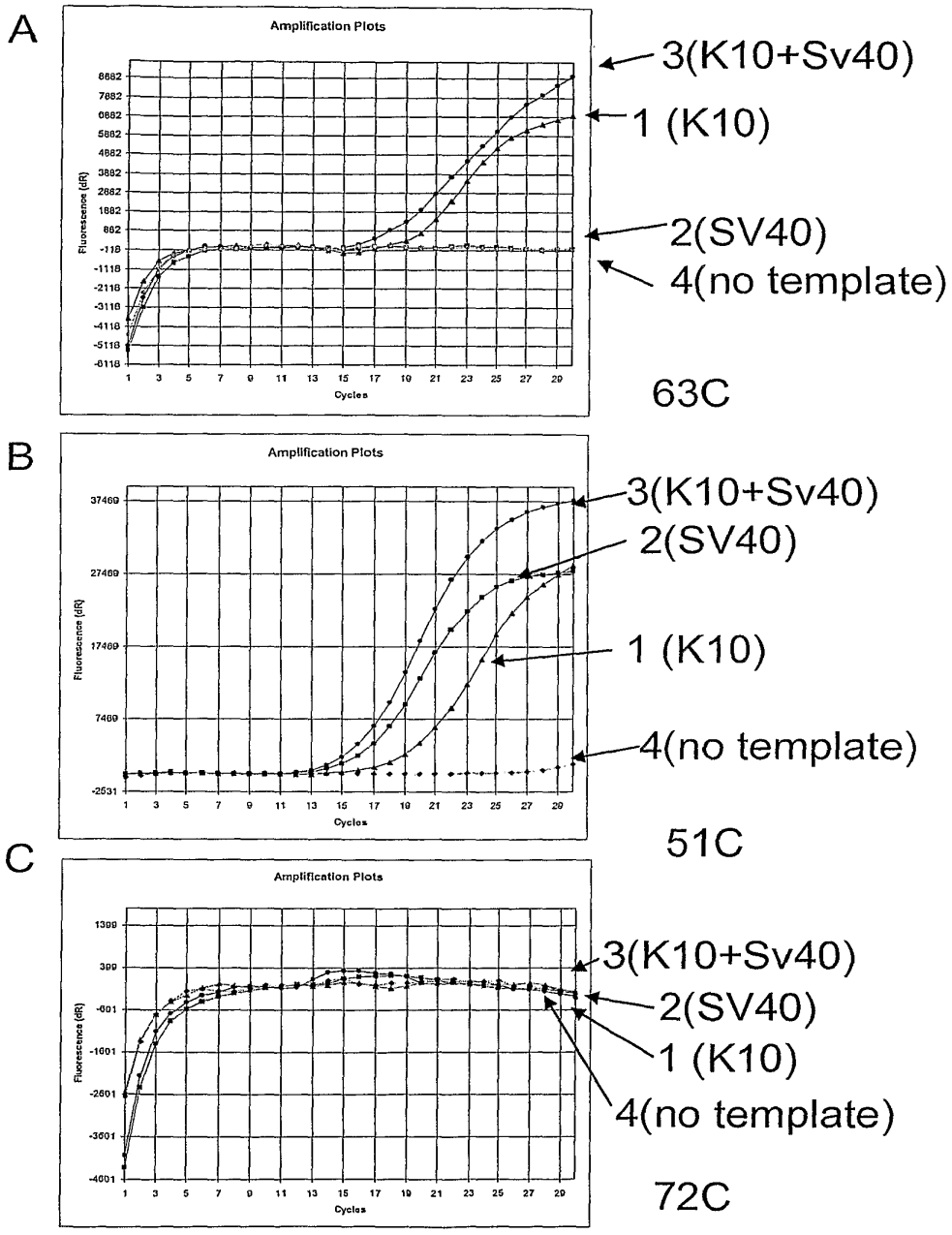


FIG. 11

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California Institute of Technology - Ex. 2003 Page 1854

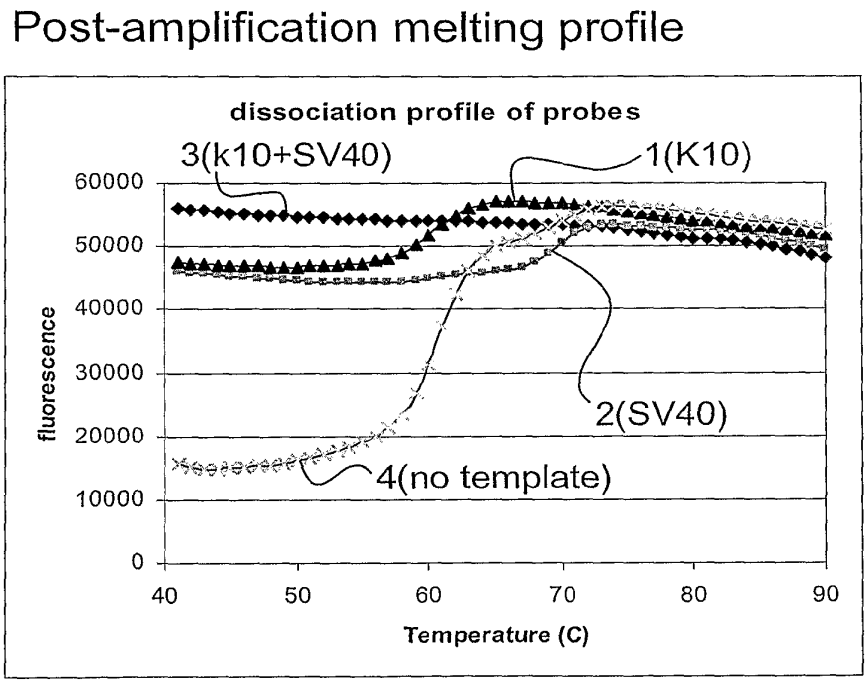


FIG. 12

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California Institute of Technology - Ex. 2003 Page 1855

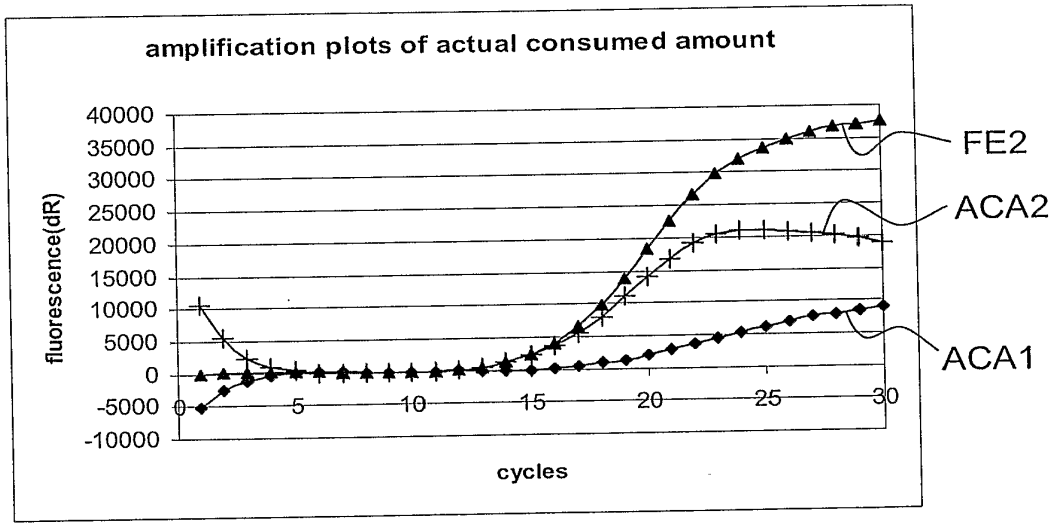


FIG. 13

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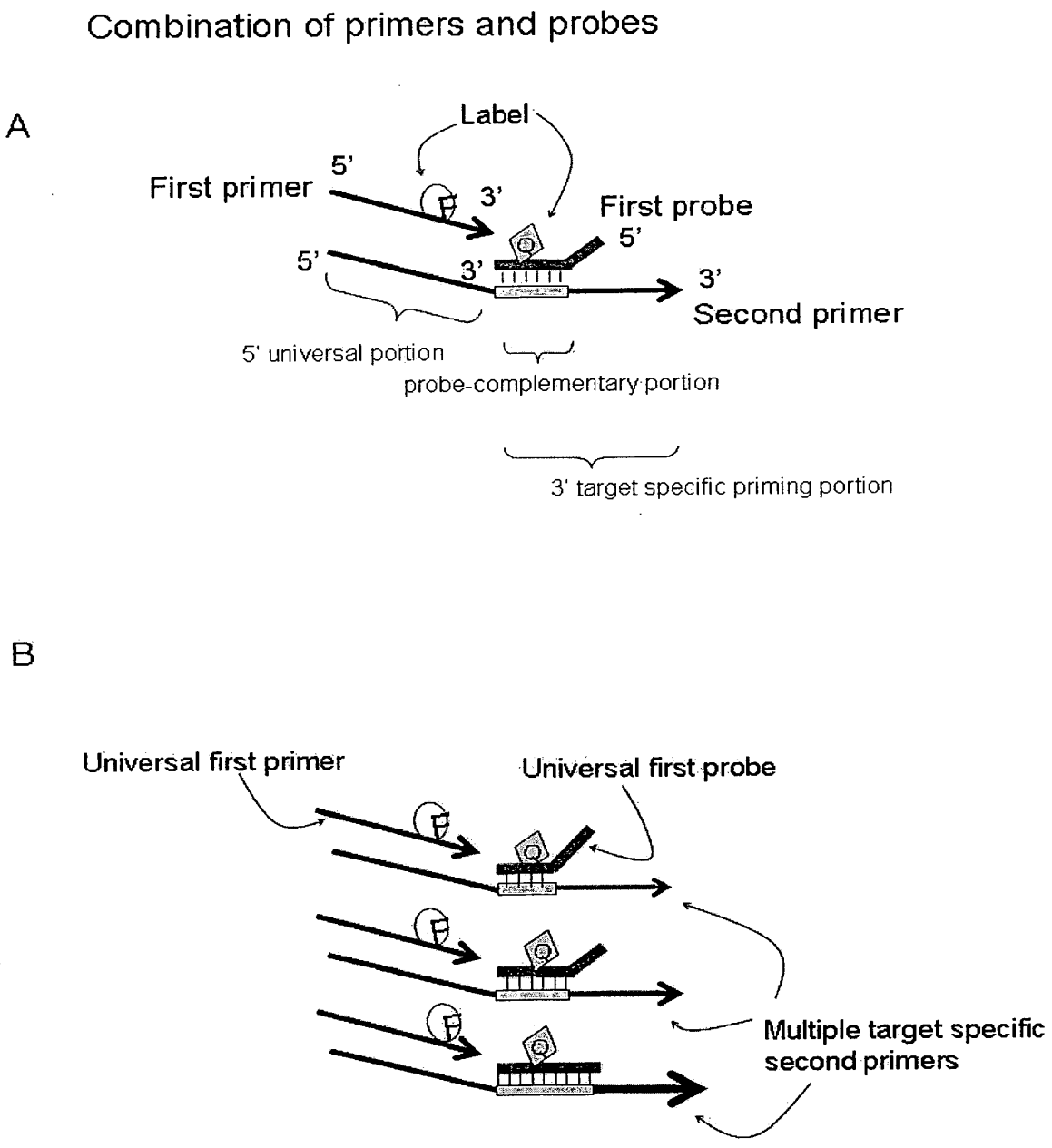


FIG.14

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California Institute of Technology - Ex. 2003 Page 1857

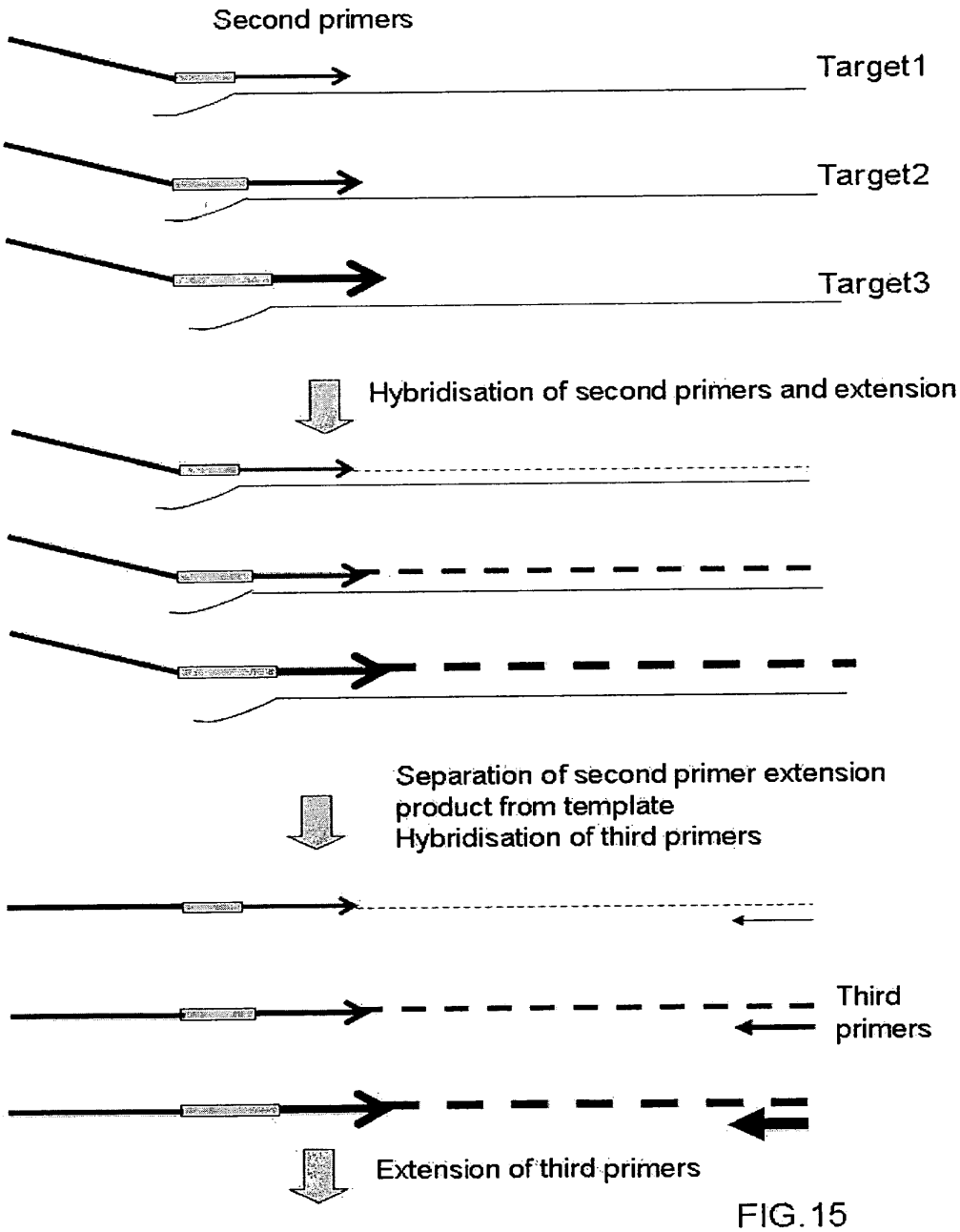

Second primers
Target1
Target2
Target3
Hybridisation of second primers and extension
Separation of second primer extension product from template
Hybridisation of third primers
Third primers
Extension of third primers

FIG.15

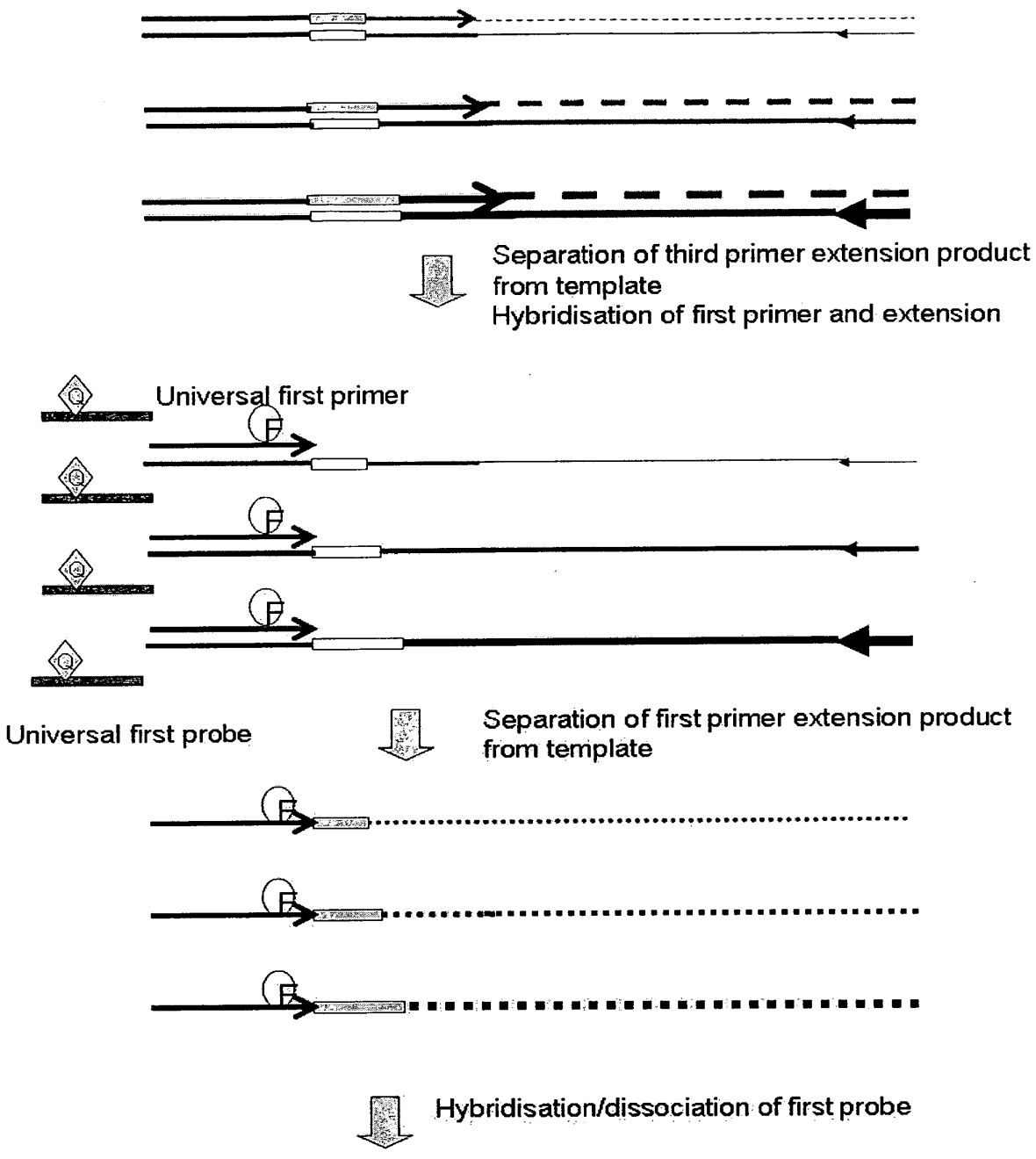


FIG. 15 continued

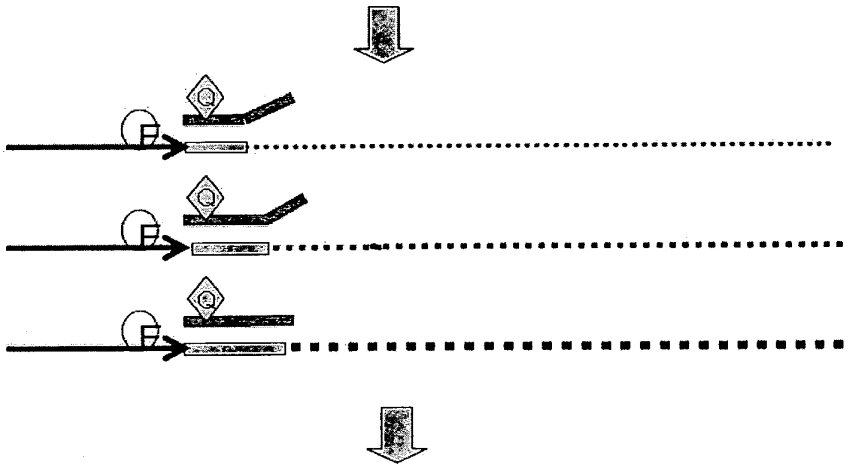


FIG. 15 continued

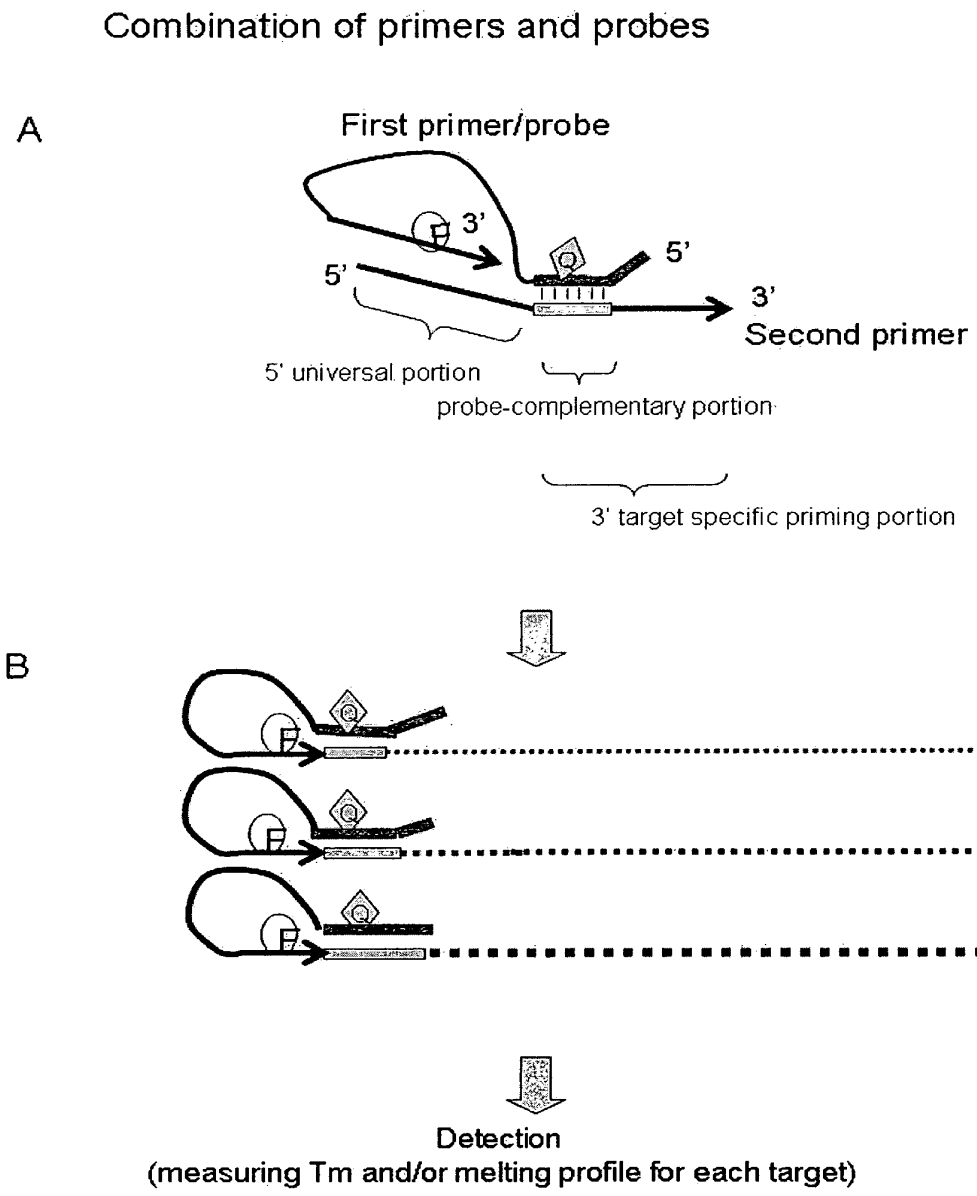

Combination of primers and probes
A
First primer/probe
3'
5'
5'
3'
Second primer
5' universal portion
probe-complementary portion
3' target specific priming portion
B
Detection
(measuring Tm and/or melting profile for each target)

FIG. 16

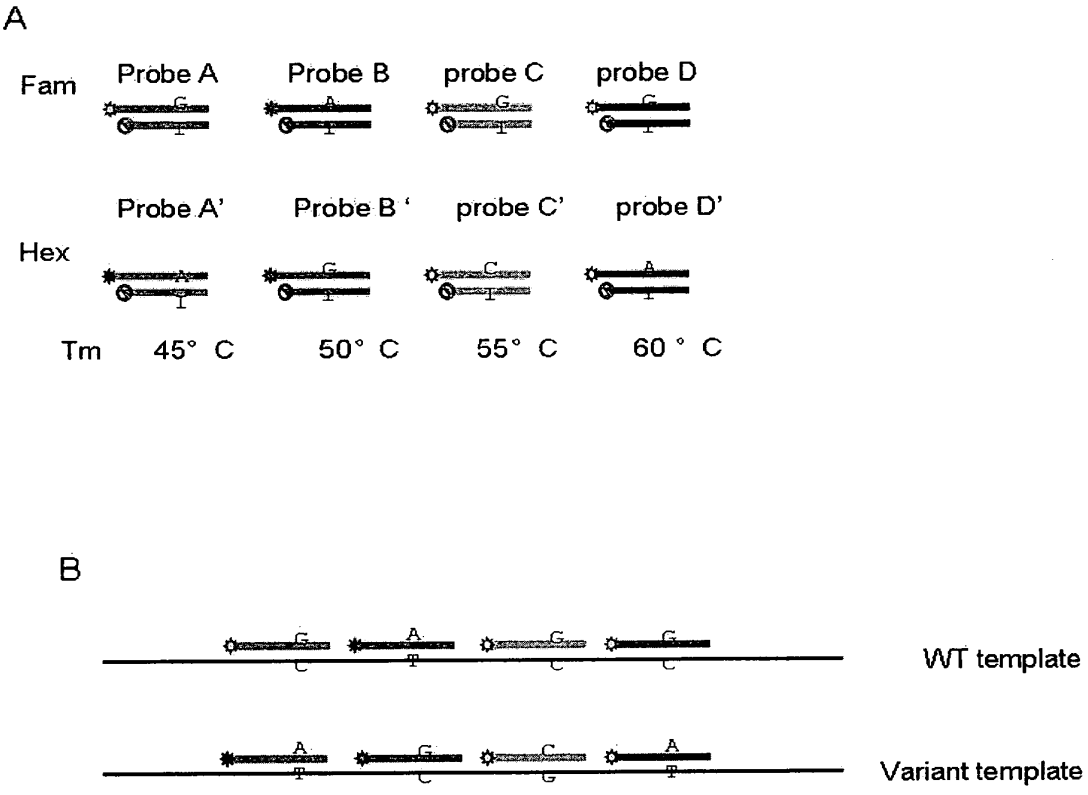

A
Fam
Probe A
Probe B
probe C
probe D
Probe A'
Probe B '
probe C'
probe D'
Hex
Tm
45° C
50° C
55° C
60 ° C
B
WT template
Variant template

FIG. 17

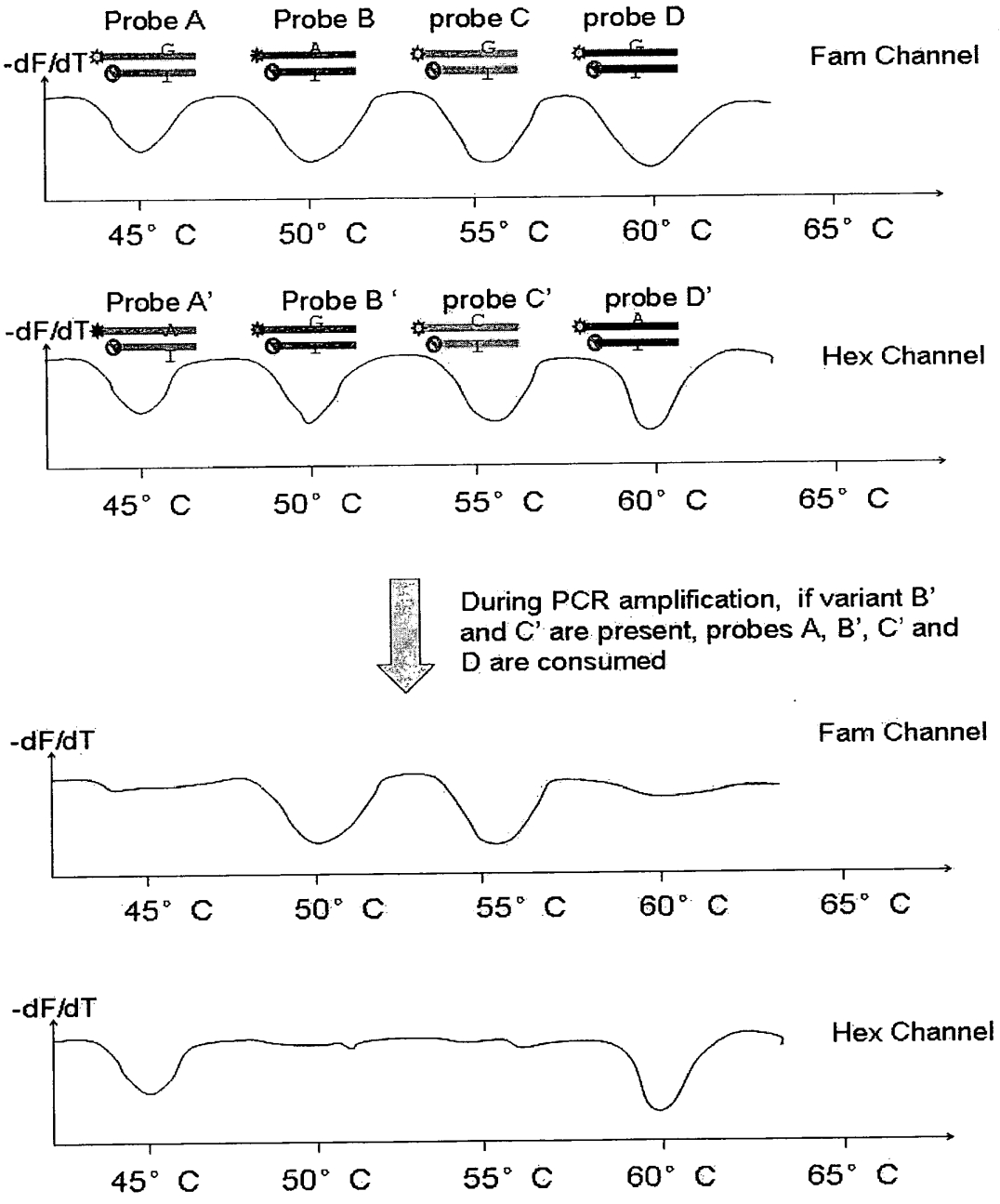

Probe A
Probe B
probe C
probe D
-dF/dT
Fam Channel
45° C
50° C
55° C
60° C
65° C
Probe A'
Probe B '
probe C'
probe D'
-dF/dT
Hex Channel
45° C
50° C
55° C
60° C
65° C
During PCR amplification, if variant B' and C' are present, probes A, B', C' and D are consumed
-dF/dT
Fam Channel
45° C
50° C
55° C
60° C
65° C
-dF/dT
Hex Channel
45° C
50° C
55° C
60° C
65° C

FIG. 18

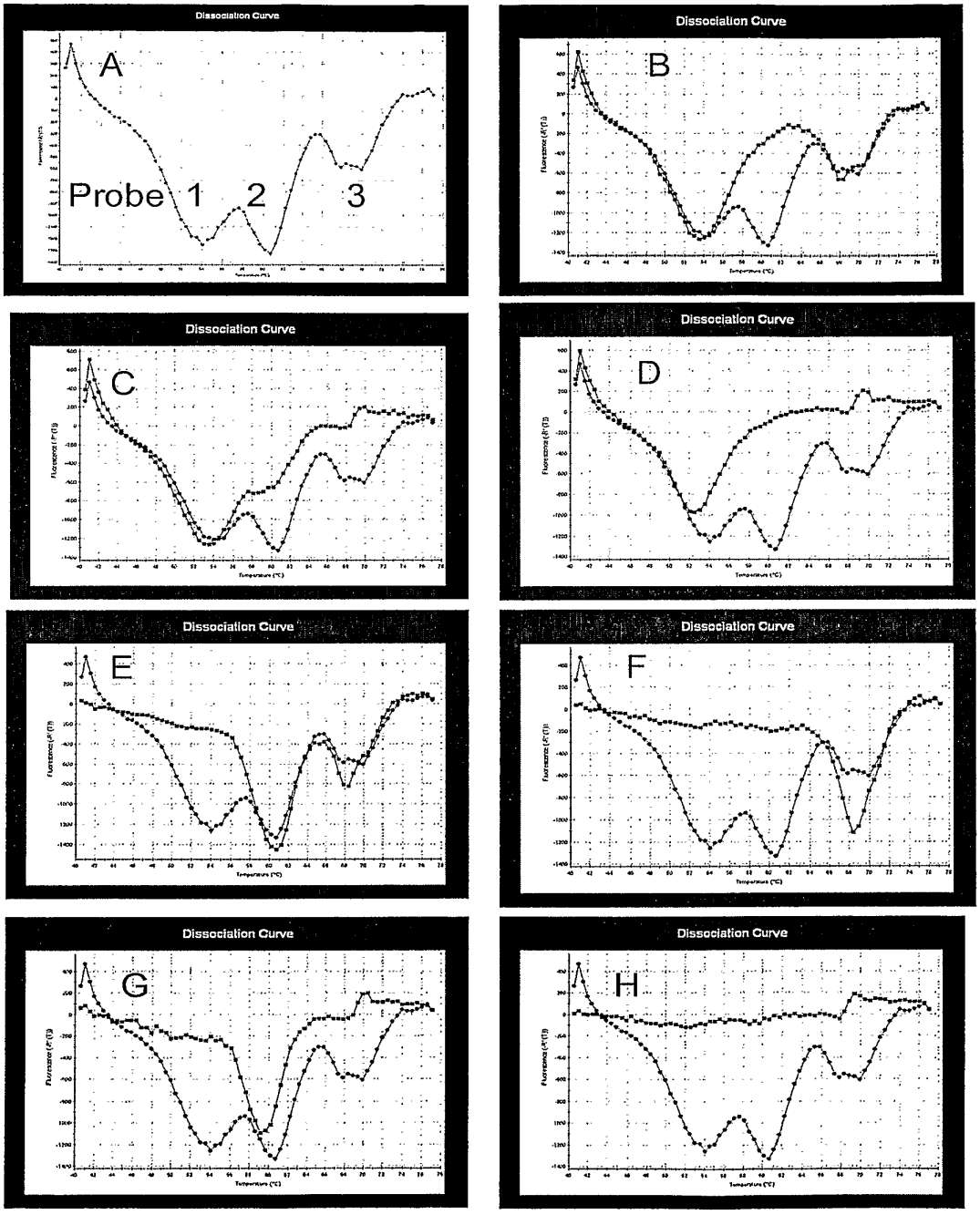


FIG. 19

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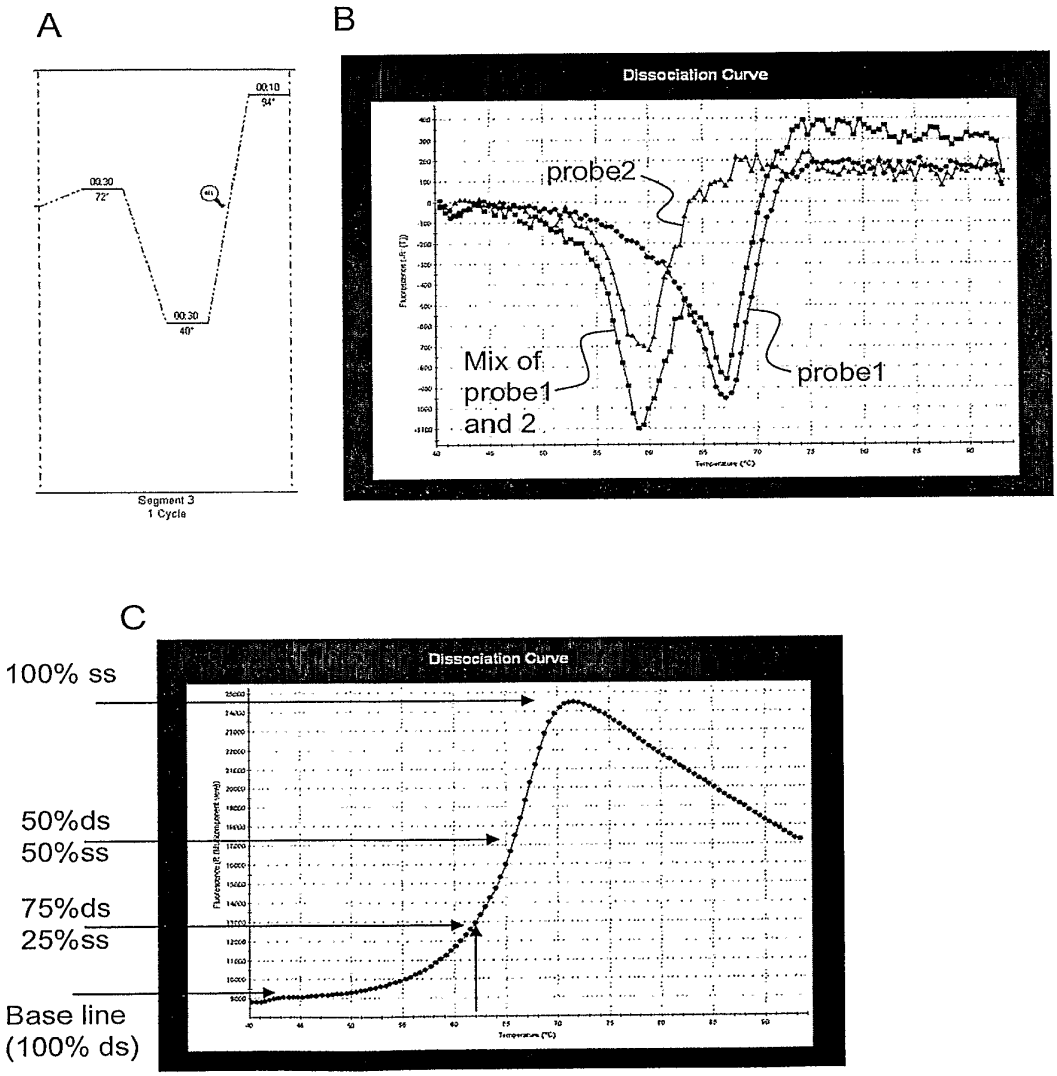


FIG. 20

IPR2024-01178
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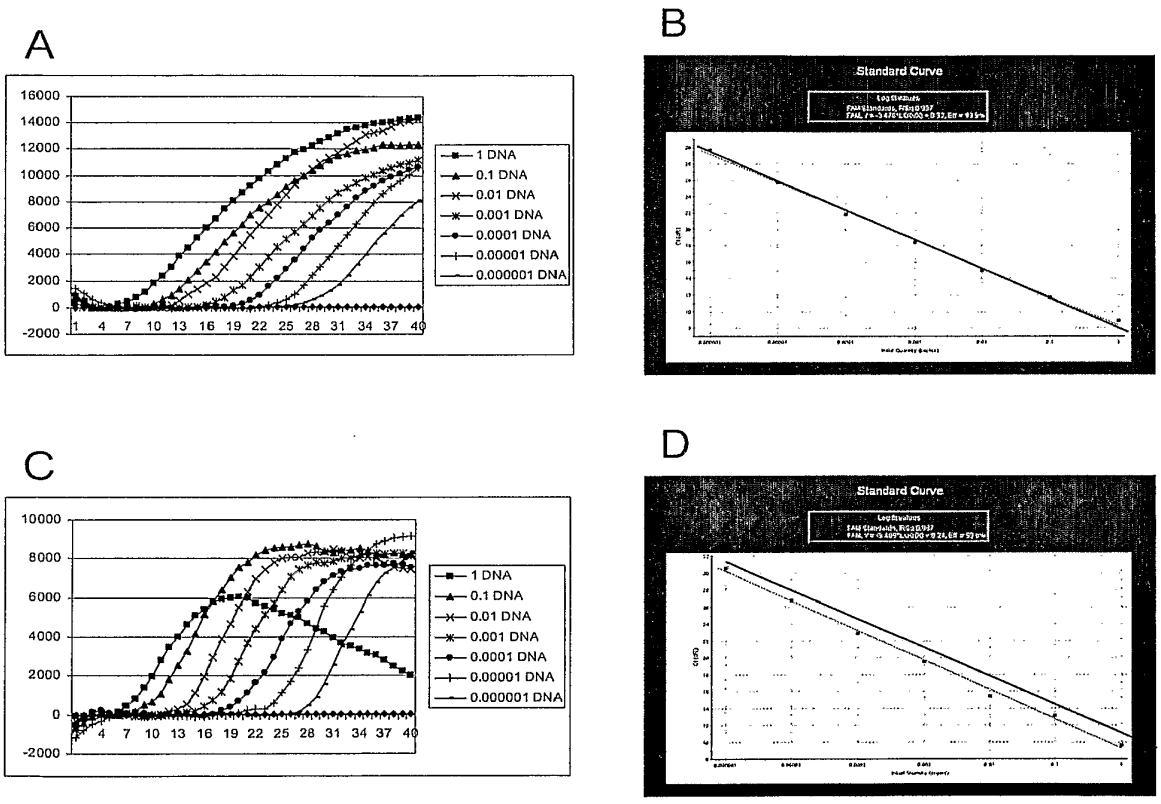
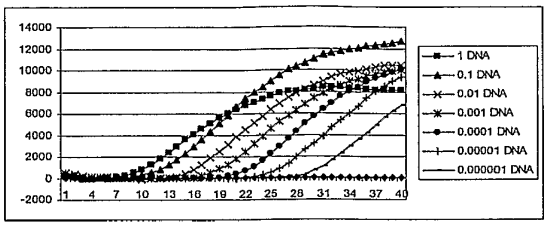


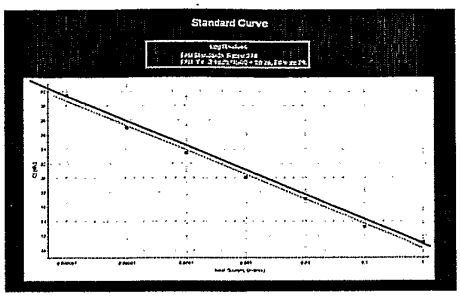
FIG. 21

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California Institute of Technology - Ex. 2003 Page 1866

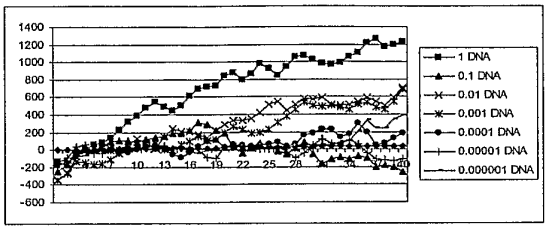
E



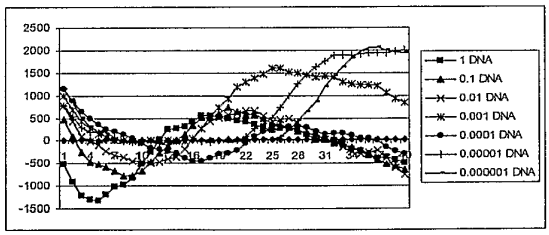
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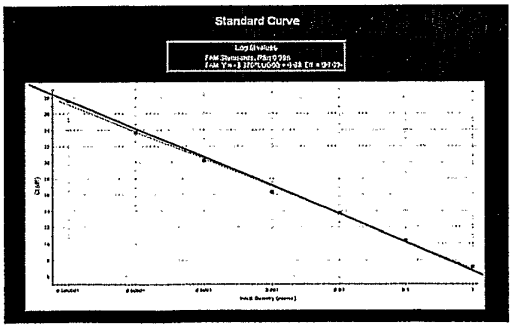
G



H



J



I

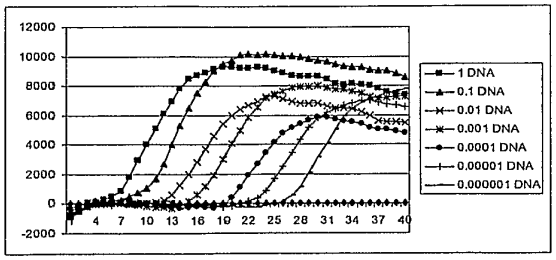


FIG. 21CONT'D

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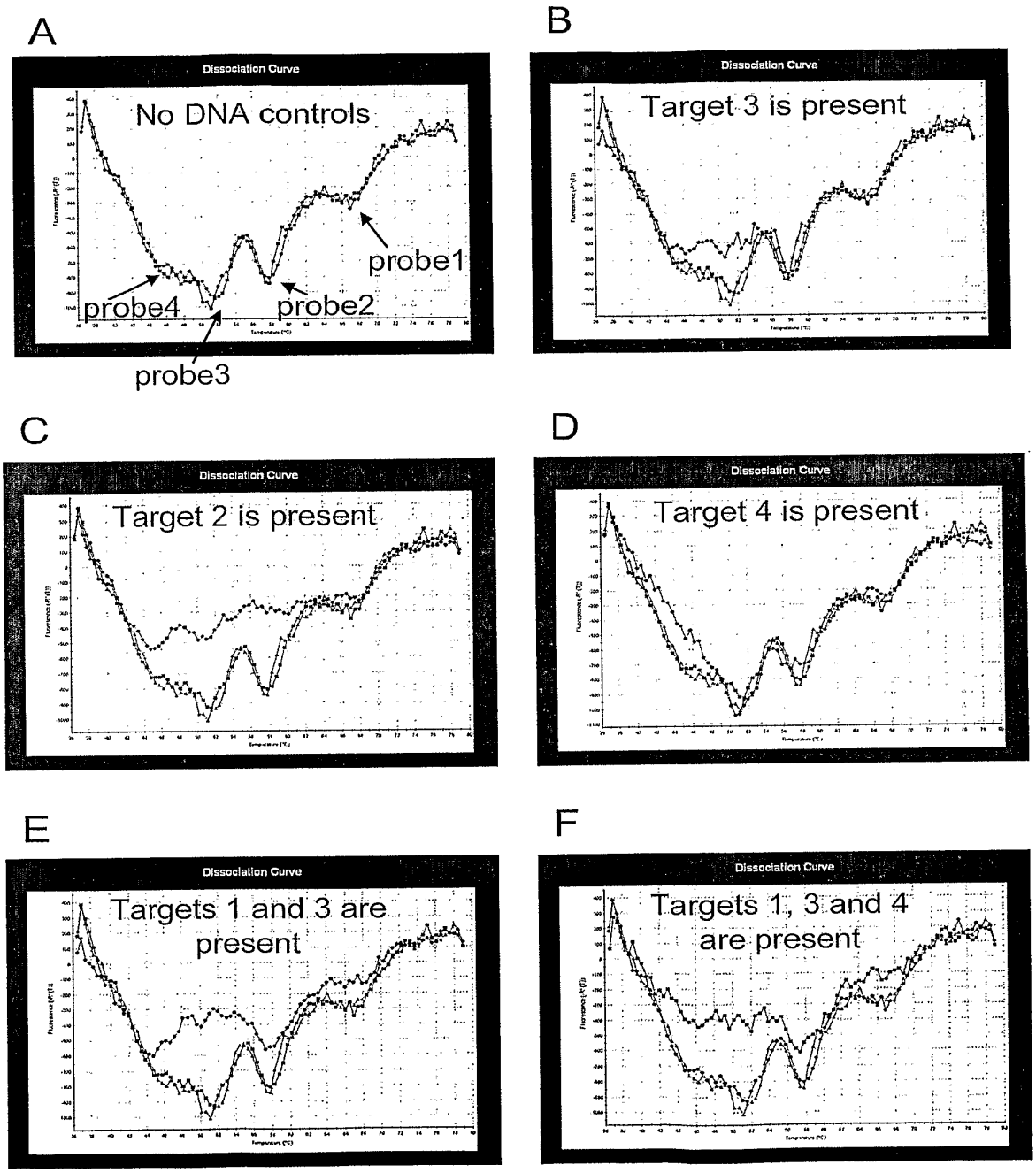

A
Dissociation Curve
No DNA controls
probe1
probe4
probe2
probe3
B
Dissociation Curve
Target 3 is present
C
Dissociation Curve
Target 2 is present
D
Dissociation Curve
Target 4 is present
E
Dissociation Curve
Targets 1 and 3 are present
F
Dissociation Curve
Targets 1, 3 and 4 are present

FIG. 22

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2009/001897

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LI QINGGE ET AL: "A new class of homogeneous nucleic acid probes based on specific displacement hybridization." NUCLEIC ACIDS RESEARCH 15 JAN 2002, vol. 30, no. 2, 15 January 2002 (2002-01-15), page E5, XP002545143 ISSN: 1362-4962 cited in the application	
A	HUANG SHIHAI ET AL: "Thermodynamically modulated partially double-stranded linear DNA probe design for homogeneous real-time PCR" NUCLEIC ACIDS RESEARCH, vol. 35, no. 16, August 2007 (2007-08), page Article No.: e101, XP002545144 ISSN: 0305-1048	

-/--

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 September 2009	28/09/2009

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Fax: (+31–70) 340–3016	Aslund, Fredrik

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB2009/001897

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2008/044129 A (BIO RAD PASTEUR [FR]; DAGLAND TYPHAINE [FR]; RIEUNIER FRANCOIS [FR]) 17 April 2008 (2008-04-17) claim 1; figure 2	
A	LUK ET AL: "Partially double-stranded linear DNA probes: Novel design for sensitive detection of genetically polymorphic targets" JOURNAL OF VIROLOGICAL METHODS, ELSEVIER BV, NL, vol. 144, no. 1-2, 27 July 2007 (2007-07-27), pages 1-11, XP022170232 ISSN: 0166-0934	

Form PCT/ISA/210 (continuation of second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/GB2009/001897

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2008044129 A	17-04-2008	AT 437965 T	15-08-2009
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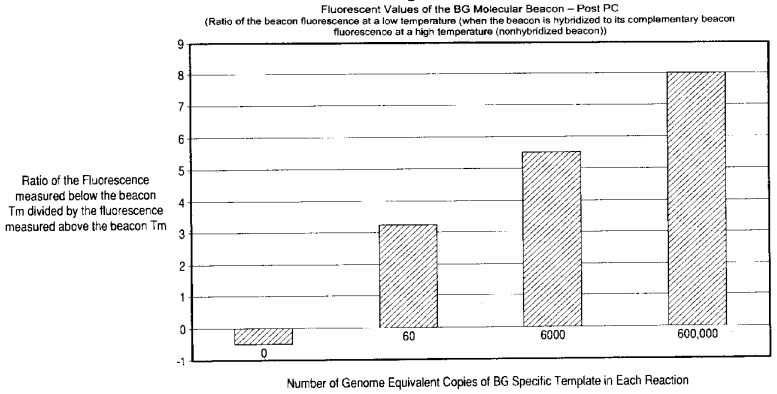
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(54) **Title**: DETECTION ALGORITHM FOR PCR ASSAY

Fig. 1



(57) **Abstract**: The application provides methods for improving the detection accuracy of the binding of labeled nucleic acid probes, such as those used in PCR reactions. One such method comprises measuring the label intensity, e.g. fluorescence, at two different temperatures, a higher temperature and a lower temperature, and then calculating the ratio of the label intensity at the lower temperature over the label intensity at the higher temperature. Another method comprises measuring the label intensity at least two points in time post-PCR and calculating the slope of the label intensity as a function of time. Measuring the hybridization kinetics of the probe binding to the target nucleic acid allows an on-rate slope to be calculated which gives this method good specificity of detection.

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DETECTION ALGORITHM FOR PCR ASSAY

[0001] This application claims priority under 35 U.S.C. § 119(e) to U.S. provisional application no. 61/136,040, filed 8 August 2008, which is hereby incorporated by reference in its entirety.

BACKGROUND

[0002] The present application relates generally to the field of detection of nucleic acids using polymerase chain reaction (PCR). The application provides methods for increasing specificity, and therefore sensitivity, of the detection of target nucleic acids using PCR by calculating the effect of temperature on the hybridization of a labeled probe to the target, and by calculating the hybridization rate of the probe to the target as a function of label intensity as a function of time.

[0003] The amplification of nucleic acids has been an invaluable tool for the detection of specific nucleic acids in a sample. PCR is used to amplify the nucleic acid using thermocycling of a heat stable DNA polymerase, such as Taq polymerase, in a reaction comprising the target nucleic acid, primers for DNA polymerization that are complementary to the target nucleic acid, as well as the necessary nucleosides and buffer reagents, as described, for instance, in U.S. Pat. No. 4,683,202. Many variations of PCR have been developed for specific needs, such as can be found in *Current Protocols in Molecular Biology* (April 2008, Print ISSN: 1934-3639; Online ISSN: 1934-3647). It is a widely used technique for detecting the presence of DNA and RNA targets for a variety of purposes, including, for example, pathogen detection ex vivo and from environmental samples, in vitro diagnostics, genetic analyses, forensics, food and agricultural testing, and parentage testing.

[0004] PCR has evolved from technique performed only under controlled laboratory conditions to a technique useful for field testing. This evolution has been made possible by the advent of handheld PCR devices. Such devices may be particularly suited for the detection of pathogens, forensic sampling, or even rapid diagnosis of medical conditions without the need for expensive, time consuming and laborious laboratory processing. The use of PCR in the field is particularly important to counter bioterrorism, because rapid and accurate identification of bioweapons is crucial.

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[0005] Field applications still require a robust and accurate assay, despite the lack of a controlled laboratory environment. While field assays are about on par with laboratory assays in detection of target DNA, comparison of PCR assays versus traditional culture techniques for the diagnosis of bacterial infection from tissue samples indicated the PCR methods were less sensitive than a 72 hour laboratory culture (Emanuel et al. *J. Clin. Microbiol.* (2002) 41:689-693). Thus, improved PCR sensitivity is a goal for developing rapid detection methods.

[0006] Signal specificity remains a major limitation of the sensitivity of any PCR assay, particularly when using fluorescent reporter molecules for measuring the reaction kinetics and amount of amplified target in the reaction. While subtraction of the background fluorescence in a negative control reaction is generally used to account for non-specific hybridization, this method remains crude, at best, limiting the lower threshold amount at which a target nucleic must be present in order to be detected.

SUMMARY OF THE INVENTION

[0007] Provided herein is a method for detecting hybridization of a labeled nucleic acid probe to its complementry nucleic acid target comprising (a) contacting a sample suspected of containing a target nucleic acid with a labeled nucleic acid probe that hybridizes with the target nucleic acid; (b) measuring the label intensity at a first temperature and at a second temperature, wherein the first temperature is lower than the second temperature; (c) calculating the ratio of (i) the label intensity at the first temperature to (i) the label intensity at the second temperature, wherein a ratio of at least 0.8 indicates the presence of the target nucleic acid. In a further embodiment, steps (b) and (c) are repeated at least twice. In a further embodiment, steps (b) and (c) are repeated at the same first and second temperatures. The measurement of the first temperature can occur before the measurement of the second temperature or vice versa. In a further embodiment, the method is used as a post PCR detection technique. In a further embodiment, the method further comprises measuring the label intensity at single temperature at 3 or more points in time, for instance 15, 30, and 45 seconds, after the sample is brought to the said temperature. In a further embodiment, the method further comprises measuring the label intensity at different temperatures at three or more points in time, such as at 15, 30, and 45 seconds.

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[0008] In one embodiment, a ratio of at least 0.9 indicates the presence of the target nucleic acid.

[0009] In another embodiment, the first temperature is below the Tm of the labeled nucleic acid probe and the second temperature is above the Tm of the labeled nucleic acid probe. In a further embodiment, the first temperature is about 40° C, about 41° C, about 42° C, about 43° C, about 44° C, about 45° C, about 46° C, about 47° C, about 48° C, about 49° C, about 50 ° C, about 51° C, about 52° C, about 53° C, about 54° C, about 55° C, about 56° C, about 57° C, about 58° C, about 59° C, about 60° C, about 61° C, about 62° C, about 63° C, about 64° C, about 65° C, about 66° C, about 67° C, about 68° C, about 69° C, about 70° C , about 71° C or about 72 °C.

[0010] In another embodiment, the second temperature is about 85° C, about 86° C, about 87° C, about 88° C, about 89° C, about 90° C, about 91° C, about 92° C, about 93° C, about 94° C, or about 95 °C.

[0011] In another embodiment, the second temperature is about 85° C, about 86° C, about 87° C, about 88° C, about 89° C, about 90° C, about 91° C, about 92° C, about 93° C, about 94° C, or about 95 °C.

[0012] In another embodiment, the first temperature is about 50° C and the second temperature is about 95° C.

[0013] In another embodiment, the labeled nucleic acid probe comprises a fluorescent label. In a further embodiment, the nucleic acid probe further comprises a quencher molecule that absorbs the emission of the fluorescent label such that when the quencher molecule and fluorescent label are in relatively close proximity, the fluorescent emission of the fluorescent label is undetectable or at least less detectable than when the quencher molecule and fluorescent label are not in close proximity. In a further embodiment, the nucleic acid probe is a molecular beacon or a linear probe.

[0014] In one embodiment, the target nucleic acid is DNA or RNA.

[0015] In one embodiment, an average ratio is calculated based on repeated measurements.

[0016] In a further embodiment, the measuring is done following a PCR reaction.

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[0017] In another embodiment, the PCR reaction comprises i) contacting a sample suspected of containing the target nucleic acid with the labeled nucleic acid probe in a solution comprising suitable primers, enzymes and substrates to form a reaction mixture; and ii) cycling said reaction mixture at denaturing, annealing and extension temperatures suitable for amplification of the target nucleic acid.

[0018] Further provided herein is a method for detecting hybridization of a labeled nucleic acid probe to its target nucleic acid comprising (a) contacting a sample suspected of containing a target nucleic acid with a labeled nucleic acid probe that hybridizes with the target nucleic acid; (b) measuring the label intensity at least two different points in time; and (c) calculating the slope of the label intensity as a function of time. The measuring at different points in time, as done in step (b), can be done under isothermic conditions or at different temperatures.

[0019] In a further embodiment, a positive slope indicates the presence of the target nucleic acid when the intensity of the signal generated by the labeled nucleic acid probe increases over time when bound to the target nucleic acid as compared to the intensity of the signal generated by the labeled nucleic acid probe when it is not bound to the target nucleic acid. In a further embodiment, a negative or zero slope indicates the absence of the target nucleic acid

[0020] In a further embodiment, the nucleic acid probe is a molecular beacon or a linear probe.

[0021] In one embodiment, the target nucleic acid is DNA or RNA.

[0022] In one embodiment, the measuring is done during isothermal conditions. In a further embodiment, the measuring is done following a PCR reaction. In a further embodiment, the measuring is completed within the time period of about 1 to about 10 minutes following the completion of the PCR reaction. In a further embodiment, the measuring is done at least five different points in time. In a further embodiment, the measuring is done at 15, 30, and 45 seconds following the completion of PCR.

[0023] In another embodiment, the PCR reaction comprises (i) contacting a sample suspected of containing the target nucleic acid with the labeled nucleic acid probe in a solution comprising suitable primers, enzymes, substrates and buffer to form a reaction mixture; and (ii) cycling said reaction mixture at denaturing, annealing and extension

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temperatures suitable for amplification of the target nucleic acid. In another embodiment, the annealing and extension temperatures are the same.

[0024] In one embodiment, the slope is calculated by taking the first derivative of the label intensity as a function of time. In a further embodiment, the label intensity as a function of time is calculated by least squares fitting the label intensity measurements as a function of time. In a further embodiment, the slope is calculated by fitting the label intensity data as a function of time to the following equation: $y = mx + b$, wherein y is label intensity, x is time, and m is the slope.

[0025] In a further embodiment, the hybridization kinetics of the labeled nucleic acid probe and target nucleic acid based on the slope of label intensity is calculated as a function of time.

BRIEF DESCRIPTION OF THE DRAWINGS

[0026] FIG. 1 is a graph showing the ratio of the fluorescent values below the beacon Tm divided by the fluorescence values above the beacon Tm. Positive ratios (+) were found only for samples containing *bacillus globigii* (BG) template, which is a surrogate organism for studying biological weapons, and negative ratios (-) were found for control samples that lacked BG template. Thus, FIG. 1 shows that the use of ratio values can be used to positively detect even small quantities of target sample while minimizing false positives.

[0027] FIG. 2 shows the results of measuring the slope of the fluorescence values over 3-10 minutes of six samples. Only samples containing BG templates, the target sequence, showed positive slopes.

[0028] FIG. 3 shows the results of PCR reactions performed to determine whether label intensity at two temperatures at two different times can be used to accurately detect the presence of a target nucleic acid, a nucleic acid specific for anthrax, while minimizing false positives. The results show that anthrax was reliably detected.

[0029] FIG. 4 shows the results of PCR reactions performed to determine whether label intensity at two temperatures at two different times can be used to accurately detect the presence of a target nucleic acid, a nucleic acid specific for tularemia, while minimizing false positives. The results show that tularemia was reliably detected.

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DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0030] By "nucleic acid probe," it is meant an oligonucleotide which is RNA or DNA that is complementary to the target sequence and thus hybridizes specifically to the target sequence. The probe can be any suitable length, and in some embodiments, the probe is from 20 to 1000 bases long. Optionally, the probe is labeled, for example linked to at least one detectable reporter molecule. A fluorescent reporter molecule can be used as the reporter molecule. The fluorescent reporters may be attached to one end of the oligonucleotide and a fluorescent quencher molecule to the opposite end of the oligonucleotide such that when the reporter is in close proximity to the quencher, the fluorescent emission from the reporter is at least partially absorbed by the quencher, thus decreasing the detectable signal of the reporter. These molecules could be also be attached to the internal portion of the oligonucleotide. The mechanism of this quenching is known as fluorescent resonance energy transfer (FRET) and results in the probe having a higher label intensity when bound to the target versus when the probe is unbound. Other methods for labeling the probe include linking radioisotopes, single fluorophores, DNA intercalating dyes (such as SYBR Green), chemiluminescent molecules, affinity tags, and the like. Hybridization of the probe to the target can be calculated using methods and equations known in the art, such at those described in Tsourkas et al. *Nuc. Acids Res.* (2003) 31:1319-1330, which is hereby incorporated by reference in its entirety.

[0031] By "molecular beacon," it is meant a probe that has sequence complementary to the target in the middle of the probe, with a heterologous sequence at the 5' and 3' ends, which forms a stem-loop structure when the probe is not bound to the target and is at a temperature below the effective T_M of the stem structure. The complementary sequence of the molecular probe may be at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, or at least 99% complementary to the target. The stem-loop brings the 5' and 3' ends in close proximity, allowing the reporter and quencher molecules to interact, resulting in a reduced label intensity. Upon binding the target, the reporter and quencher molecules become more distant, allowing for an increased label intensity. Thus, free molecular beacons generate little or no signal, while molecular beacons that are bound to the target sequence have a much greater label intensity at temperatures near or below the effective T_M of the beacon-target hybrid. Molecular beacon probes are well-known in the art and are described in Maras et al., *Clinica Chemica Acta* (2006) 363:48-60, for example.

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[0032] By "linear probe," it is meant a probe that has no particular secondary structure. The probe may be 100% complementary to the target or only partially complementary to the target. For example, the probe may be at least 85%, at least 90%, at least 95%, at least 97%, at least 98%, or at least 99% complementary to the target.

[0033] Unless otherwise specified, "probe" refers generally to a nucleic acid probe, a molecular beacon, and a linear probe. In other words, "probe," as used herein, encompasses a nucleic acid probe, a molecular beacon, and a linear probe unless otherwise specified.

[0034] By "target nucleic acid," it is meant the nucleic acid in a sample to be detected using the complementary PCR primers and probes. The target may be DNA, such as genomic DNA, bacterial DNA, viral DNA, episomal DNA, or synthetic DNA. The target may be RNA, such as mRNA, rRNA, tRNA, viral RNA, bacterial RNA, or synthetic RNA. Samples containing the target nucleic acid may be from any source. Such samples include biological samples, environmental samples, clinical samples, *in vitro* samples, and tissue samples, for example. For example, the samples can come from a material suspected of being contaminated with a biowarfare agent. Specific examples of target nucleic acid include, but are not limited to, nucleic acids encoding at least a portion of the anthrax, tularemia, plague, and pan orthopox genomes. Methods for extracting the nucleic acid from such samples for use in PCR reactions are well known in the art and may be used.

[0035] Probes and primers may be multiplexed to detect more than one target in a single reaction. In general, different sets of primers will amplify different target nucleic acids, and the probes that detect the targets may have different labels, such as different fluorophores, such that the two targets may be distinguishable in the same reaction. Such methods are well known in the art, such as those described in Belanger et al. *J. Clin. Microbiol.* (2002) 40:1436-1440. In another embodiment, different sets of primers will amplify different target nucleic acids, and the probes that detect the targets can have the same labels, such as identical fluorophores. The probes can hybridize at different temperatures, which allows each probe to be distinguishable in the same reaction despite the same label. This can be thought of as multiplexing in temperature space.

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[0036] Additionally, multiple reactions can be used to screen for multiple targets, such as a screen for pathogenic organisms in a sample or disease genes. Such reactions may be performed in a multiwell format, for example a 96-well plate.

[0037] By "label intensity," it is meant the amplitude of the signal detected from the probe. The particular signal detected will depend on the probe. For example, fluorescence will be detected for a probe labeled with a fluorescent probe. When using FRET-based probe labeling, label intensity is proportional to the amount of bound probe to the target and, therefore, proportional to the amount of target in the sample. Likewise, when a DNA intercalating reporter molecule is used, such as SYBR Green, fluorescence increases as more reporter is incorporated in the newly-synthesized double stranded DNA. Therefore, the label intensity can be used as a means to determine whether the target nucleic acid is present in the sample, and optionally, the amount of target present in the sample. For instance, the label intensity can be compared to a standard curve generated using known amounts of the target, interpolating the results, and determining the amount of target in the sample. Such methods are well known in the art.

[0038] In some embodiments, a proxy unit will be used to record or represent label intensity. For example, a device may detect or record the fluorescence from a fluorescent probe as a particular voltage. This proxy unit, voltage in this specific example, can be considered the label intensity.

[0039] By "hybridize," it is meant when two single stranded polynucleotides combine to form one strand of double stranded polynucleotide. The nucleic acids may be DNA or RNA, and may form DNA-DNA, RNA-RNA, or DNA-RNA double stranded polynucleotides or three standed hybrids. Hybridization is sequence specific, and the kinetics of hybridization can be calculated using a second order equation, as described by Tsourkas *et al. Nuc. Acids Res.* (2003) 31:1319-1330, for example, which is hereby incorporated by reference. The kinetics of hybridization can also be calculated by measuring the fluorescent increase of a molecular beacon over time during its hybridization to a homologous sequence. A molecular beacon is labeled on one end with a fluorescent molecule and on the other with a quencher molecule. As the molecular beacon hybridizes to its complementary sequence, the reporter fluor is physically separated from the quencher molecule and the fluorescence increases. The

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kinetics of hybridization can then be measured as the rate of fluorescent increase using the slope of the label intensity.

[0040] By "Tm" or "melting temperature," it is meant the temperature at which 50% of the probe molecules are hybridized to target nucleic acids, while 50% of the probe molecules remain free in solution. The Tm can be calculated, for example, using the formula Tm = 2[A+T] + 4[G+C], or determined by software programs developed specifically for this purpose. Calculation of Tm is well known within the art.

[0041] By "slope of the label intensity as a function of time" it is meant the slope of a line that is a plot of the label intensity or change in label intensity as a function of time. As the probe hybridizes to its specific complementary template, the label intensity increases. Over a given time period more probe hybridizes to the template, which results in an increase of fluorescence (label intensity) over time. Thus, the slope may be measured as intensity over time or change in intensity over time. Such slopes can be calculated, for example by taking the first derivative of the label intensity as a function of time, by calculating the least squares fitting the label intensity measurements as a function of time, or by fitting the label intensity data as a function of time to the following equation: $y = mx + b$, where y is label intensity, x is time, and m is the slope. Any suitable method known in the art can be used to determine the slope. Further the hybridization kinetics of the labeled nucleic acid probe and target nucleic acid may be calculated based on the slope of label intensity as a function of time in this manner.

[0042] The slope of a line defined by points (x_1, y_1) and (x_2, y_2) can be determined using the following formula:

$$m = \frac{y_2 - y_1}{x_2 - x_1}$$

[0043] where m is the slope of the line and $x_1 \neq x_2$. This equation can be employed any number of ways by one of skill in the field. For example, a line can be fit to data and then the equation can be applied to determine the slope m. Alternatively, the slope m can be calculated for a number of different points and the values averaged to determine a slope. In some embodiments, the slope will be calculated repeatedly using data points sequential in time.

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[0044] By "about," it is meant a value that is the indicated value, plus or minus five percent of that value.

[0045] By "PCR" it is meant a repetitive target nucleic acid amplification reaction based on serial cycling of the temperature of a reaction comprising the target, which may be from a sample; a DNA polymerase such as Taq polymerase or other heat stable polymerase; at least one primer complementary to the target; a probe complementary to the amplified portion of the target; deoxynucleoside triphosphates; and a buffer solution comprising divalent cations. Suitable concentrations for the components are well known in the art and may be adjusted according to known optimization parameters. Each cycle typically includes three steps: denaturation at 85-100 °C, annealing at 37-60 °C, and elongation at 40-75 °C. Each step may be 10-300 seconds long, preferably 30-120 seconds. The exact temperature of the steps may be varied according to the sequence of the target according to well-known parameters. For instance, the annealing temperature may be a temperature approximately 5 °C lower than the Tm of the primers, which can be calculated as described above. There are many software programs available for calculating the optimal temperatures of the PCR steps. Each cycle can comprise two steps: the first step being denaturation and the second step combining the annealing and elongation steps together. An optional initial denaturation step may be included for "hot start" polymerases. Further, a final elongation step may also be included to ensure any remaining single stranded DNA molecule is fully extended. Specific protocols for performing PCR are well known in the art and may be found, for example, in *Current Protocols in Molecular Biology* (April 2008, Print ISSN: 1934-3639; Online ISSN: 1934-3647).

[0046] The PCR reaction cycles can be characterized as being early, late and final stages. In the early stage, exponential amplification occurs as near 100% efficiency of doubling the target sequence occurs. As reagents are exhausted, the reaction enters the late stage and efficiency drops off. This stage is sometimes called the linear stage, though the reaction actually has a high degree of variability at this stage, depending on the availability of the reagents and polymerase performance. The final stage is a plateau at which no further target sequence accumulates due to exhaustion of the reagents and enzyme.

[0047] "Real time PCR," also referred to as quantitative or Q-PCR and originally described as the 5' nuclease PCR assay, refers to measuring signals generated by the enzymatic

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cleavage of a dual labeled probe (label intensity) during the PCR reaction. The signal generated by the cleavage of the probe which is bound to the target sequence is proportional to the amount of target sequence in the reaction. By comparing the signal to known standards, the amount of target present in the sample may be determined.

[0048] "Endpoint PCR" typically refers to measuring the label intensity in the late or final stage of the reaction. Complete exhaustion of the reagents is not required. While the target in the sample may be less precisely quantified using this method as compared to real time PCR, the target has been maximally amplified, allowing for robust detection of its present due to its greater abundance in the final stages of the PCR reaction.

[0049] "Assymetric PCR" refers to a PCR technique used to preferentially amplify one strand of the target nucleic acid more than the other. Generally, preferential amplification of the target nucleic acid is accomplished by using a large excess of the primer for the preferred nucleic acid. Specifically asymmetric PCT protocols are well known in the art.

[0050] Specific detection of the amplified target remains a limiting characteristic for all PCR reactions regardless of type or time of measuring the label intensity.

[0051] PCR can be performed in any suitable thermocycler and format, such as 96 or 384 well plates. Alternatively, the PCR reaction may be performed in a field-suitable device, such as a BIOSEEQ™ PLUS device available from Smiths Detection.

[0052] The slope of the label intensity as a function of time can be calculated automatically using computer software or hardware, for example. In some embodiments, label intensities are detected, and the output of this detection is transmitted to a processor for data manipulation. The processor performs the necessary calculations and returns the data in numerical, graphical or other interpretable output to the user. In other embodiments, the user obtains the signal intensity data and manipulates the data manually. The data can be manually manipulated using standard mathematical methods or computer programs, such as Microsoft's EXCEL program, including the 2007 version.

[0053] In one embodiment, the hybridization of a labeled nucleic acid probe to its target nucleic acid is detected by measuring the label intensity at at least two different temperatures. In one embodiment, the method comprises contacting a sample suspected of containing a target nucleic acid with a labeled nucleic acid probe that hybridizes with the target nucleic

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acid and measuring the label intensity at a first temperature and at a second temperature, wherein the first temperature is less than the second temperature or reversed.

[0054] The ratio is calculated as the label intensity at the lower temperature divided by the label intensity at the higher temperature. Thus, the higher the resulting number, the higher the concentration of target contained in the reaction. The specific value of the ratio number depends on a number of factors including the label intensity scale of the instrument being used. A ratio of greater than one can be indicative of the presence of the target. In some embodiments, a ratio of about 0.9, 1.0, 1.1, 1.2, 1.5, 2, 5, 10, 20, or 50 indicates the presence of a target nucleic acid. The ratio can also be used to calculate the amount of target present. Such a determination can be made by comparing the ratios to standards, for example. Another aspect in determining the ratio indicating the presence of a target nucleic acid is to determine the baseline ratio of reactions which contain no target nucleic acids which could be called the noise of the assay. A ratio determining the presence of target nucleic acids could be 5 or more standard deviations above the noise, for example. In some embodiments, a ratio indicating the presence of target nucleic acids could be 2, 4, 6, 8, 10, or 15 or more standard deviations above the noise. Depending on the probe used, the amount may also be calculated directly from the ratio without need for a comparison to standards.

[0055] The temperatures at which the label intensity are measured can vary depending on the particular application, and suitable temperatures can be readily calculated by one of skill in the art. The selection of temperatures will depend on the Tm and sequence of the probe. Specifically, the higher temperature will be greater than Tm of the probe, and the lower temperature will be less than the Tm of the probe. In some embodiments, the higher temperature will be at least about 90°C and the lower temperature will be about 50°C or less. For example, the higher temperature can be at least 95°C, 100°C, 105°C, or 110°C, and the lower temperature can be 5 °C, 10 °C, 15 °C, 20 °C, 25 °C, 30° C, 35 °C, 40 °C, or 45°C.

[0056] In some embodiments, intensities will be measured at more than two temperatures. For example, intensity measurements can be taken at a first, second, and third temperatures, wherein each of the first, second, and third temperatures are different. Similar values at all temperatures are indicative of specific binding. Similar values can mean values that differ by no more than 20%, no more than 15%, no more than 10%, no more than 5%, no more than

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3%, or no more than 1%. By increasing the number of temperatures used, the confidence in the results can be increased.

[0057] The ratio can be calculated at one or several points in time. For example, label intensity can be measured following a PCR reaction to determine the presence of a target nucleic acid that is the subject of the PCR reaction. The label intensity can also be measured before a PCR reaction is performed. A ratio indicative of the presence of the target nucleic acid may therefore allow unnecessary PCR to be avoided.

[0058] The ratio can be calculated based on a single reading at each of the higher and lower temperatures. In the alternative, multiple readings can be taken at the lower or higher temperature and the values averaged. Such averaging can be used to prevent errors caused by incorrect readings.

[0059] In one embodiment, the hybridization of a labeled nucleic acid probe to its target nucleic acid can be detected by measuring the slope of label intensity as a function of time. Because the amount of target in samples containing target increases over time due to PCR, more probe hybridizes to the target over time. Thus, label intensity as a function of time can be used to determine the presence of target in a sample. Moreover, the label intensity as a function of time can be used to determine the hybridization kinetics of the probe/target interaction.

[0060] In one embodiment, the method comprises contacting a sample suspected of containing a target nucleic acid with a labeled nucleic acid probe that hybridizes with the target nucleic acid and measuring the label intensity at different points in time. The slope of the label intensity of a function of time is indicative of both the presence of the target nucleic acid and the kinetics of the hybridization of the probe to the target nucleic acid. Specifically, a positive slope indicates the presence of the target nucleic acid when the label intensity of the probe increases when hybridized to the target, and a negative or zero slope indicates the absence of target nucleic acid when the label intensity of the probe decreases when there is no target for the probe to hybridize with. Similarly, the slope also can be used to determine the kinetics of hybridization using well-known kinetic equations.

[0061] The slope of the label intensity as a function of time can be calculated in any number of ways readily known to one of skill in the art. For example, the label intensity data

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can be fitted with a line using known numerical methods. These methods include least squares fitting (both linear and non-linear), linear regression ($y = mx + b$), best fit exponential curve, quadratic regression, cubic regression, and polynomial regression. The slope of the label intensity can be calculated, either analytically or numerically, by finding the first derivative of the line fit to the data. Such mathematical computations are well-known to those of skill in the art.

[0062] The label intensity can be measured under isothermal conditions or at different temperatures. In one embodiment, all label intensity measurements are taken at a single temperature. In another embodiment, the label intensity measurements can be taken at different temperatures. This situation may occur during the cooling or heating of a PCR reaction mixture. In yet another example, the label intensity can be measures at different temperatures. Based on this data, different slopes can be calculated. For example, multiple data points can be taken at the first temperature, T1, followed by taking multiple data points at the second temperature, T2. Based on this data, two different slopes can be calculated, one corresponding to the T1 data and one corresponding to the T2 data. By varying the temperature at which label intensity is measured, the kinetics of hybridization can be studied.

[0063] The intensity measurements can be taken at any time. The measurements can be taken following PCR or taken during PCR. In some embodiments, the measurements are taken in the ten minutes following PCR. The measurements can also be taken in five, four, three, two, or one minutes following PCR. In one embodiment, measurements are taken at about 15 sec., 30 sec., and 45 sec. The time between measurements can vary. For example, the measurements can be separated by at least about 10 sec., about 15 sec., or about 20 sec. The measurements can also be taken in rapid succession over some period of time.

[0064] The number of intensity measurements can also vary. Generally, increasing the number of data points can increase the confidence in the slope of the line. But as few as two data points can be used to determine the slope. In some embodiments, two, three, or four data points are used to determine the slope.

EXAMPLES

Example 1: Reducing false positives by measuring label intensity at two temperatures.

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[0065] PCR reactions were performed to determine whether label intensity at two temperatures can be used to accurately detect the presence of a target nucleic acid while minimizing false positives. The PCR reactions, which were 25μL each, are described below in Table 1, and the primer sequences used are described in Table 2.

Table 1 - PCR Reactions

Component	Volume	Final Concentration
5X Platinum® *Tfi* Reaction Buffer	5 μl	1X
10 mM dNTP mix, PCR grade	0.5 μl	200 μM each
50 mM MgCl2	1.5 μl	3 mM
Primer mix (10 μM each)	1 μl	0.2 μM each
Template DNA	≥1 μl as required	
Platinum® *Tfi* Exo(-) DNA Polymerase	0.2–0.4 μl	2-4 units
Autoclaved distilled water	to 25 μl	

Table 2 (Sequences are 5' to 3') - Primers

TGCGTTCTGACTGAACAGCTGATCGAG	BG_Limiting Primer
TCCTCTTGAAATTCCCGAATGG	BG_Excess Primer
Fam-CTCGAGAAAGGTTGTCGTAAAACGCCTCGAG-Dabcyl	BG_beacon

[0066] Four sets of duplicate reactions were set up containing the following amounts of synthetic *bacillus globigii* (BG) template: 0, 60, 6000, or 600,000 genome copy equivalents. The reactions were cycled 55 times from between 85°C and 95°C to between 50°C and 70°C. The reactions were then reduced to below the Tm of the beacons, between 40°C and 60°C. Fluorescent reads were taken every 15 seconds for about 5 minutes. The reactions were then heated to between 80°C and 95°C and five 15 second reads were taken. The ratios of the fluorescence at 40°C to 60°C divided by the fluorescence at 80°C to 95°C are shown below in Table 3.

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Table 3		
BG Template	Fluorescent	% Increase in ratios
Copies	Ratios	over 0 copies
0	0.35	0%
60	1.36	285%
6000	2.36	569%
600,000	2.75	680%

[0067] A fluorescent ratio below 1 indicates no target nucleic acid was included in the reaction, while a fluorescent ratio above 1 indicates target nucleic acid was included, as described above. Thus, the results demonstrate that at least as few as 60 copies of plague can be detected reliably using the ratios described herein.

Example 2: Reducing false positives by measuring label intensity over time.

[0068] PCR reactions were performed to determine whether the label intensity as a function of time could be used to determine the presence of a target nucleic acid. PCR reactions were performed, as described above in Example 1.

[0069] The ratio of the fluorescent values below the beacon Tm divided by the fluorescence values above the beacon Tm were calculated. Figure 1 and Table 4 below show the calculated ratios for the PCR reactions. Positive ratios were found only in the samples containing the template, while a negative ratio was found for the negative control.

Table 4	
BG Template Copies	Fluorescent Ratios
0	-0.5
60	3.25
6000	5.5
600,000	8

[0070] Label intensity was also measured using powder samples that did or did not contain BG template. Specifically, six white powder samples were run on the Bioseeq PLUS instrument, which is available from Smiths Detection, using Training Assay consumables. One sample received negative powder (sample labeled – in Fig. 2), and the remaining five samples received positive BG powder (samples labeled + in Fig. 2). The samples were

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cycled 55 times from between 85°C and 95°C to between 50°C and 70°C. The reaction temperatures were then reduced to below the Tm of the beacons, between 40°C and 60°C, and fluorescent reads were taken every 15 seconds for 10 minutes. The fluorescent readings taken from minutes 3 to 10 were plotted on a scatter plot and the slope was calculated by fitting the data using linear regression. This slope value is shown in Figure 2.

[0071] This example demonstrates that measuring label intensity over time can effectively be used to determine whether a target sequence is present. No false positives were found.

Example 3: Reducing false positives by measuring label intensity over time and at different temperatures with the Anthrax pX01 assay.

[0072] PCR reactions were performed to determine whether label intensity at two temperatures at two different times can be used to accurately detect the presence of a target nucleic acid while minimizing false positives. The PCR reactions, which were 25μL each, are described above in Table 1, and the primer sequences used are described below in Table 5.

Table 5 (Sequences are 5' to 3') - Primers

TGGCTAATCAGCTTAAGGAACATCCCACAGAC	Anthrax pX01_Limiting Primer
TGCATAAAGCTGTAAAACATCACGA	Anthrax pX01_Excess Primer
CAL 610-CAACGTGGAACAAAATAGCAATGAGGTAACGTTG-Dabcyl	Anthrax pX01_beacon

[0073] Figure 2 shows the results of the measurements. Specifically, the values in the target rate column illustrate the on-rate slope and are examples of the differential seen between a positive (POS) and a negative (NEG) call. The three samples that contained no Anthrax template (modules 1,2 and 3) gave NEG calls. The three samples that contained ~60,000 anthrax genome copy equivalents (modules 4, 5, and 6) gave POS calls. The NEG calls show a negative slope while the POS calls show a positive slope. The slope was calculated using the 2nd and 4th data point shown in the above plots using the slope function

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in EXCEL, which is b = Sum((x - Avg(x)) * (y - Avg(y))) / Sum((x - Avg(x))^2) where x is the field in slope that contains the x coordinates and y is the field in slope that contains the y coordinates. The results demonstrate that label intensity at two temperatures at two different times can be used to accurately detect the presence of a target nucleic acid while minimizing false positives.

Example 4: Reducing false positives by measuring label intensity over time and at different temperatures with the tularemia assay.

[0074] PCR reactions were performed to determine whether label intensity at two temperatures at two different times can be used to accurately detect the presence of a target nucleic acid while minimizing false positives. The PCR reactions, which were 25μL each, are described below in Table 1, and the primer sequences used are described in Table 6.

Table 6 (Sequences are 5' to 3') - Primers	
AGCGTAAGATTACAATGGCAGGCTCCAGA	Tularemia_Limiting Primer
GCCCAAGTTTTATCGTTCTTCTCA	Tularemia _Excess Primer
CAL 610- CCTCGTAAGTGCCATGATACAAGCCGAGG-Dabcyl	Tularemia _beacon

[0075] Figure 3 shows the results of the measurements. Specifically, the values in the target rate column illustrate the on-rate slope and are examples of the differential seen between a positive(POS) and a negative(NEG) call. The three samples that contained no Tularemia template (modules 1, 2, and 3) gave NEG calls. The three samples that contained ~60,000 Tularemia genome copy equivalents (modules 4, 5, and 6) gave POS calls. Two of the 3 NEG calls show a negative slope while the third NEG call shows a weak positive slope that is below the threshold used for a POS call, which in this case was 0.002. This is an illustration of the background noise of the assay. POS calls show a positive slope. The slope was calculated using the 2nd and 4th data point shown in the above plots using the slope function in EXCEL, which is b = Sum((x - Avg(x)) * (y - Avg(y))) / Sum((x - Avg(x))^2) where x is the field in slope which contains the x coordinates and y is the field in slope which contains the y coordinates. The results demonstrate that label intensity at two temperatures at

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two different times can be used to accurately detect the presence of a target nucleic acid while minimizing false positives.

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WHAT IS CLAIMED IS:

1. A method for detecting hybridization of a labeled nucleic acid probe to its target nucleic acid comprising:

(a) contacting a sample suspected of containing a target nucleic acid with a labeled nucleic acid probe that hybridizes with the target nucleic acid;

(b) measuring the label intensity at a first temperature and at a second temperature, wherein the first temperature is less than the second temperature;

(c) calculating the ratio of (i) the label intensity at the first temperature to (ii) the label intensity at the second temperature, wherein a ratio of at least 0.8 indicates the presence of the target nucleic acid.

2. The method of claim 1, wherein a ratio of at least 0.9 indicates the presence of the target nucleic acid.

3. The method of claim 1, wherein the first temperature is below the Tm of the labeled nucleic acid probe and the second temperature is above the Tm of the labeled nucleic acid probe.

4. The method of claim 3, wherein said second temperature is about 85° C, about 86° C, about 87° C, about 88° C, about 89° C, about 90° C, about 91° C, about 92° C, about 93° C, about 94° C, or about 95 °C.

5. The method of claim 3, wherein said first temperature is about 40° C, about 41° C, about 42° C, about 43° C, about 44° C, about 45° C, about 46° C, about 47° C, about 48° C, about 49° C, about 50 ° C, about 51° C, about 52° C, about 53° C, about 54° C, about 55° C, about 56° C, about 57° C, about 58° C, about 59° C, about 60° C, about 61° C, about 62° C, about 63° C, about 64° C, about 65° C, about 66° C, about 67° C, about 68° C, about 69° C, about 70° C , about 71° C, or about 72 °C.

6. The method of claim 3, wherein said first temperature is about 50° C and said second temperature is about 95° C.

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7. The method of claim 1, wherein said labeled nucleic acid probe comprises a fluorescent label.

8. The method of claim 7, wherein said nucleic acid probe further comprises a quencher molecule that absorbs the emission of the fluorescent label such that when the quencher molecule and fluorescent label are in close proximity, the fluorescent emission of the fluorescent label is undetectable or at least less than when the quencher molecule and fluorescent label are not in close proximity.

9. The method of claim 1, wherein said nucleic acid probe is a molecular beacon or a linear probe.

10. The method of claim 1, wherein the target nucleic acid is DNA.

11. The method of claim 1, wherein the target nucleic acid is RNA.

12. The method of claim 1, wherein (b) and (c) are repeated at least twice.

13. The method of claim 12, wherein an average ratio is calculated based on the repeated measurements.

14. The method of claim 1, wherein (b) and (c) are repeated at the same first and second temperatures.

15. The method of claim 13, wherein the measuring is done following a PCR reaction.

16. The method of claims 13 or 14, wherein the PCR reaction comprises:

i) contacting a sample suspected of containing the target nucleic acid with the labeled nucleic acid probe in a solution comprising suitable primers, enzymes and substrates to form a reaction mixture; and

ii) cycling said reaction mixture at denaturing, annealing and extension temperatures suitable for amplification of the target nucleic acid.

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17. A method for detecting hybridization of a labeled nucleic acid probe to its target nucleic acid comprising:

(a) contacting a sample suspected of containing a target nucleic acid with a labeled nucleic acid probe that hybridizes with the target nucleic acid;

(b) measuring the label intensity at least two different points in time; and

(c) calculating the slope of the label intensity as a function of time.

18. The method of claim 17, wherein a positive slope indicates the presence of the target nucleic acid when the intensity of the signal generated by the labeled nucleic acid probe is greater when bound to the target nucleic acid as compared to the intensity of the signal generated by the labeled nucleic acid probe when it is not bound to the target nucleic acid.

19. The method of claim 17, wherein a negative or zero slope indicates the absence of the target nucleic acid

20. The method of claim 17, wherein the target nucleic acid is DNA.

21. The method of claim 17, wherein the target nucleic acid is RNA.

22. The method of claim 17, wherein the labeled nucleic acid probe is a fluorescently labeled nucleic acid probe.

23. The method of claim 22, wherein said nucleic acid probe is a molecular beacon or a linear probe.

24. The method of claim 17, wherein the measuring is done during isothermal conditions.

25. The method of claim 17, wherein the measuring is done following a PCR reaction.

26. The method of claim 25, wherein the measuring is completed within the time period of about 1 to about 10 minutes following the completion of the PCR reaction.

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27. The method of claim 25, wherein the measuring is done at least five different points in time.

28. The method of claim 25, wherein the PCR reaction comprises:

(i) contacting a sample suspected of containing the target nucleic acid with the labeled nucleic acid probe in a solution comprising suitable primers, enzymes, substrates and buffer to form a reaction mixture; and

(ii) cycling said reaction mixture at denaturing, annealing and extension temperatures suitable for amplification of the target nucleic acid.

29. The method of claim 17, wherein the slope is calculated by taking the first derivative of the label intensity as a function of time.

30. The method of claim 29, wherein the label intensity as a function of time is calculated by least squares fitting the label intensity measurements as a function of time.

31. The method of claim 17, wherein the slope is calculated by fitting the label intensity data as a function of time to the following equation:

$y = mx + b$,

wherein y is label intensity, x is time, and m is the slope.

32. The method of claim 17, wherein the slope of a line is calculated using the formula:

$$m = \frac{y_2 - y_1}{x_2 - x_1}$$

where m is the slope of the line, (x_1, y_1) and (x_2, y_2) are the at least two different points in time, and $x_1 \neq x_2$.

33. The method of claim 17, further comprising calculating the hybridization kinetics of the labeled nucleic acid probe and target nucleic acid based on the slope of label intensity as a function of time.

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34. The method of claim 17, wherein there are at least two labeled nucleic acid probes that each hybridize with a different target nucleic acid, and wherein each probe hybridizes at a different temperature.

35. The method of claim 34, wherein the labeled nucleic acid probes each have the same label.

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Fig. 1

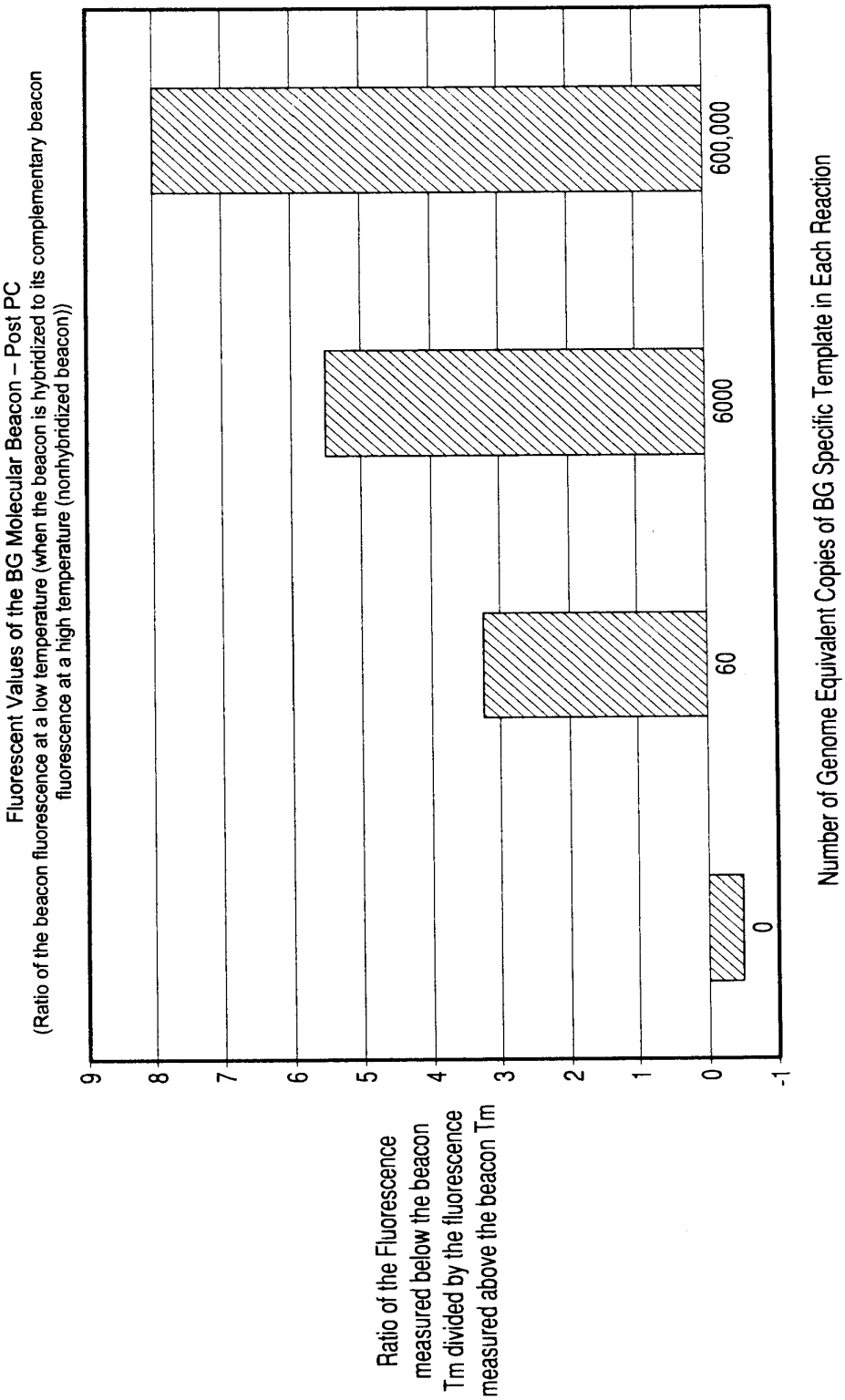

Fluorescent Values of the BG Molecular Beacon – Post PC
(Ratio of the beacon fluorescence at a low temperature (when the beacon is hybridized to its complementary beacon fluorescence at a high temperature (nonhybridized beacon))
Ratio of the Fluorescence measured below the beacon Tm divided by the fluorescence measured above the beacon Tm
9
8
7
6
5
4
3
2
1
0
-1
0
60
6000
600,000
Number of Genome Equivalent Copies of BG Specific Template in Each Reaction

Fig. 2

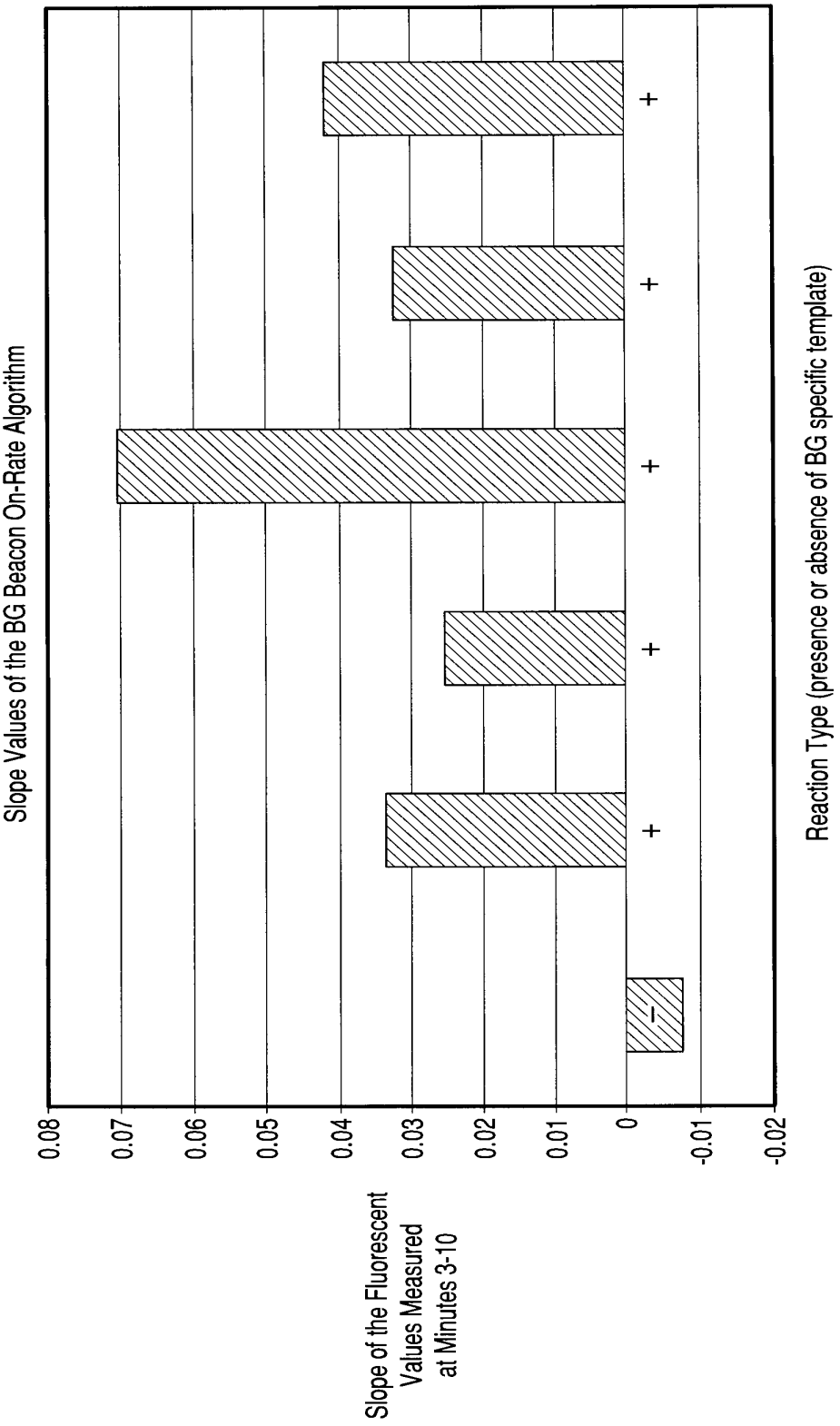

Slope Values of the BG Beacon On-Rate Algorithm
Slope of the Fluorescent Values Measured at Minutes 3-10
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
-0.01
-0.02
+
+
+
+
–
Reaction Type (presence or absence of BG specific template)

Fig. 3

Anthrax pX01 Assay

Module	Sample ID	Target Rate	Control Rate	Error	Serial Number
1	NEG [4]	-0.0086	0.0101	0	2202
2	NEG [4]	-0.0102	0.0223	0	2221
3	NEG [4]	-0.0121	0.0251	0	2222
4	POS [5]	0.0125	0.007	0	2161
5	POS [1]	0.014	-0.0008	0	2228
6	POS [5]	0.0189	0.0053	0	2166

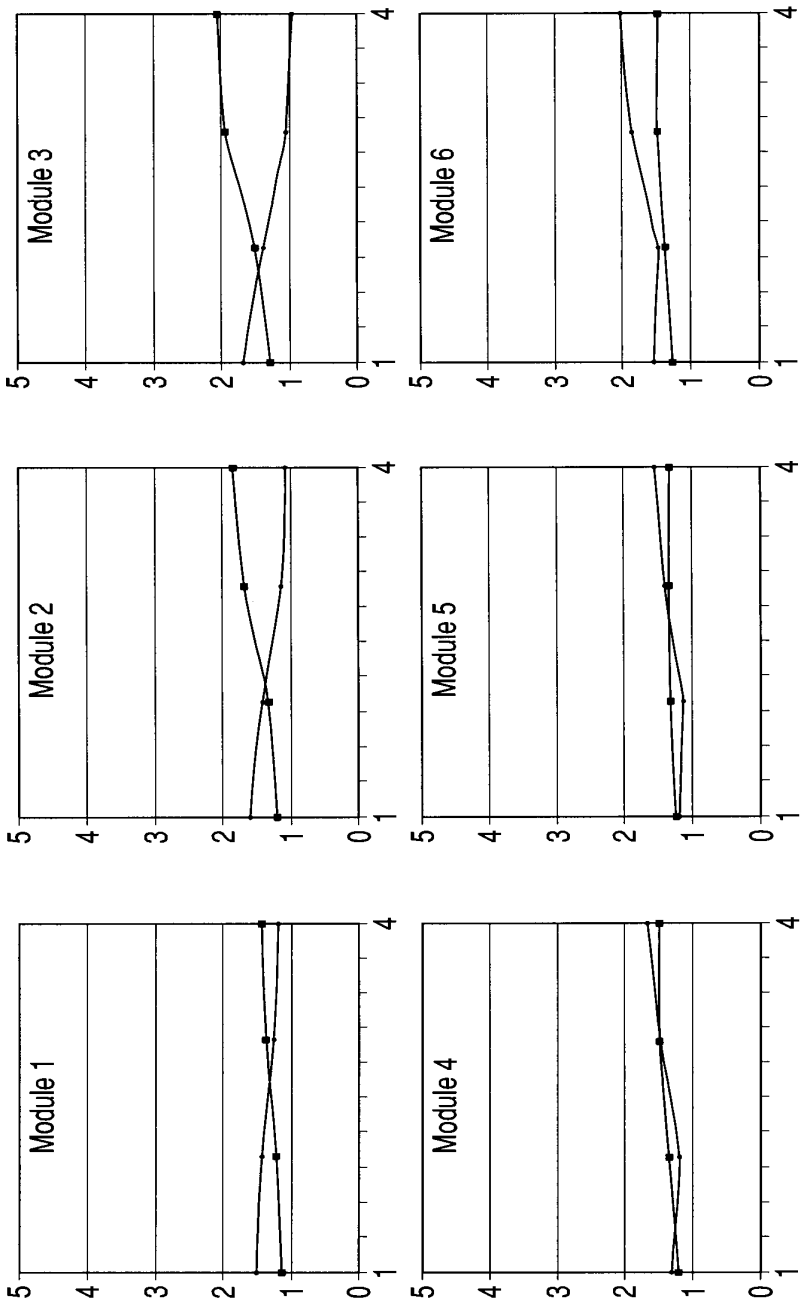
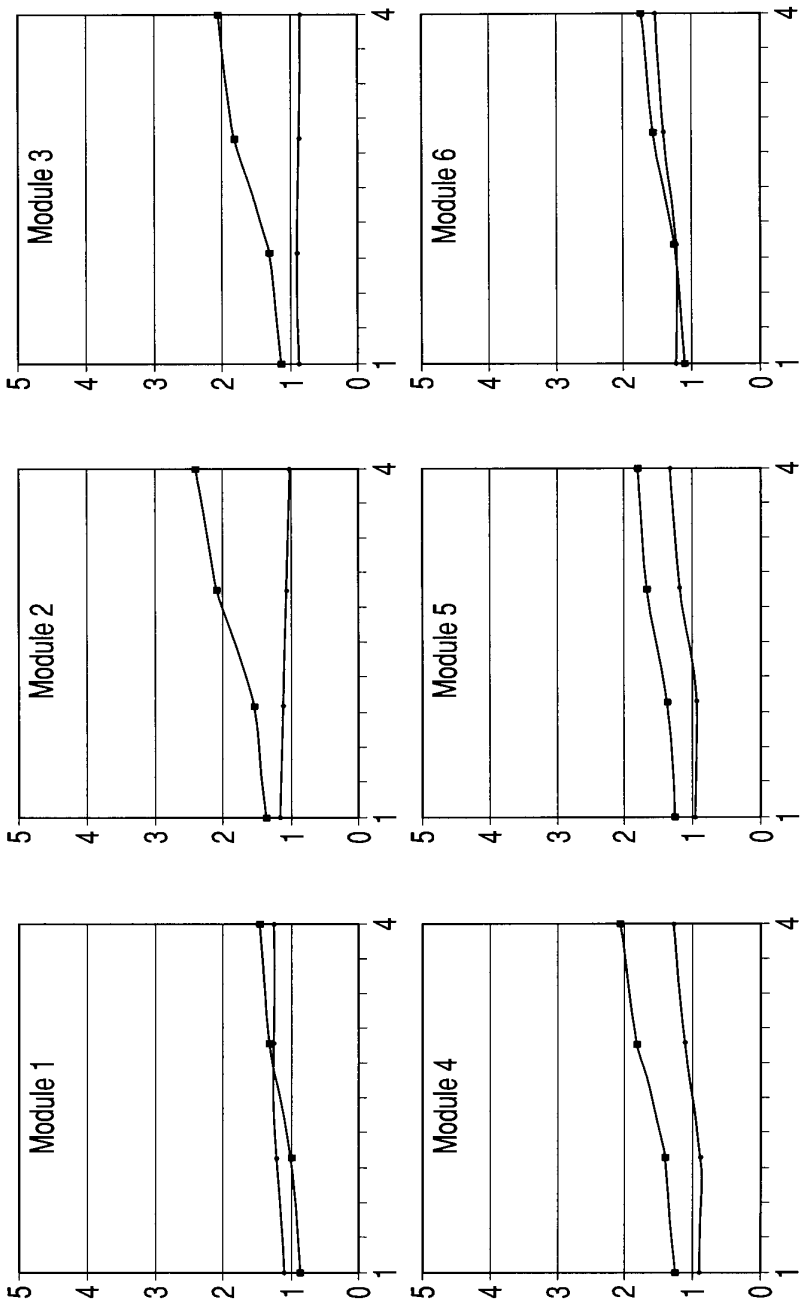


Fig. 4



Tularemia Assay

Module	Sample ID	Target Rate	Control Rate	Error	Serial Number
1	NEG [4]	0.0012	0.0214	0	2202
2	NEG [4]	-0.002	0.0398	0	2221
3	NEG [4]	-0.0007	0.0336	0	2222
4	POS [5]	0.0111	0.028	0	2161
5	POS [5]	0.0134	0.0233	0	2228
6	POS [5]	0.0109	0.0249	0	2166

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2009/053253

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, EMBASE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2007/026421 A1 (SUNDBERG S.A. ET AL.) 1 February 2007 (2007-02-01) abstract; figure 6	1-16
X	US 2005/202470 A1 (SUNDBERG S.A. ET AL.) 15 September 2005 (2005-09-15) abstract; figure 6	1-16
X	US 2004/005720 A1 (CREMER P.S. ET AL.) 8 January 2004 (2004-01-08) abstract; figures 8-12 paragraphs [0046] - [0048]	1-16

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 November 2009	07/12/2009

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Barz, Wolfgang

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2009/053253

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BULLOCK G.C. ET AL.: "Hepatitis C genotype determination by melting curve analysis with a single set of fluorescence resonance energy transfer probes" CLINICAL CHEMISTRY, AMERICAN ASSOCIATION FOR CLINICAL CHEMISTRY, WASHINGTON, DC, vol. 48, no. 12, 1 December 2002 (2002-12-01), pages 2147-2154, XP002414623 ISSN: 0009-9147 abstract; figures 1,2; table 3 * discussion *	1-16
X	WITTWER C.T. ET AL.: "CONTINUOUS FLUORESCENCE MONITORING OF RAPID CYCLE DNA AMPLIFICATION" BIOTECHNIQUES, INFORMA LIFE SCIENCES PUBLISHING, WESTBOROUGH, MA, US, vol. 22, no. 1, 1 January 1997 (1997-01-01), pages 130/131,134-138, XP000683698 ISSN: 0736-6205 the whole document	17-35
A	TSOURKAS A. ET AL.: "Hybridization kinetics and thermodynamics of molecular beacons" NUCLEIC ACIDS RESEARCH, OXFORD UNIVERSITY PRESS, SURREY, GB, vol. 31, no. 4, 15 February 2003 (2003-02-15), pages 1319-1330, XP002455592 ISSN: 0305-1048 abstract; figures 2,3	1-35

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2009/053253

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2007026421 A1	01-02-2007	CN 101426933 A	06-05-2009
		EP 1987163 A2	05-11-2008
		JP 2009525759 T	16-07-2009
		WO 2007092643 A2	16-08-2007
US 2005202470 A1	15-09-2005	NONE	
US 2004005720 A1	08-01-2004	NONE	

Form PCT/ISA/210 (patent family annex) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2009/053253

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

   a. type of material

   [X] a sequence listing

   [ ] table(s) related to the sequence listing

   b. format of material

   [X] on paper

   [X] in electronic form

   c. time of filing/furnishing

   [ ] contained in the international application as filed

   [ ] filed together with the international application in electronic form

   [X] furnished subsequently to this Authority for the purpose of search

2. [X] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

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[Continued on next page]

(54) Title: MULTIPLEXING AND QUANTIFICATION IN PCR WITH REDUCED HARDWARE AND REQUIREMENTS

(57) Abstract: Methods and algorithms for a multiplexed single detection channel amplification process and quantification of generated amplicons is presented. Various mathematical approaches for quantifying and verifying the amplicons in a reaction are presented. Usage of such methods and approaches allow upgrading of existing single and multiple channel instruments for further multiplexing capabilities.

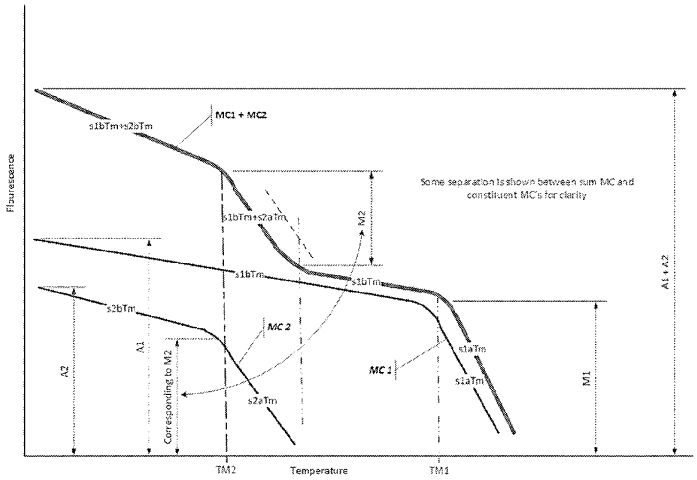


FIG. 3

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Multiplexing and Quantification in PCR with Reduced Hardware and Requirements

CROSS REFERENCE TO RELATED APPLICATIONS

[001] The present application claims priority to US provisional application No. 61/679,547 for "Multiplexing and Quantification in PCR with Reduced Hardware and Requirements", filed on Aug. 3rd, 2012, which is herein incorporated by reference in its entirety.

TECHNICAL FIELD

[002] The present disclosure relates to the field of biochemical technology, and more specifically to methods and algorithms used in a multiplexed real-time quantitative polymerase chain reaction (qPCR) technology.

BACKGROUND

[003] Multiplexing in real-time quantitative polymerase chain reaction (qPCR) allows detection and quantification of different amplified targets (e.g. amplicons) within a single qPCR assay, thus conserving sample material (e.g. enzyme, nucleotides, etc.) and avoiding well to well variation which can occur if multiplexing is done by splitting the sample into multiple separate chambers, well or tubes. Typical implementation of multiplexed qPCR requires reporters with different spectra (e.g. emission wavelengths) for each different amplicon and different detection channels to detect each of the different spectra. In one particular case, special reporters (e.g. target specific probes) are used to allow multiplexed qPCR using a same emission spectrum (e.g. wavelength) thus reducing the hardware required for the detection of the emission (e.g. reduced number of detection channels).Teachings according to the present disclosure allow for multiplexed qPCR and quantification in a single channel detection, by using, for example, simple non-specific dyes (e.g. intercalating dyes) and a same emission spectrum, thus providing a simple and cost effective multiplexing and quantification solution.

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SUMMARY

[004] According to a first aspect of the present disclosure, a method is provided, the method comprising: detecting a plurality of different amplicons generated during a multiplexed amplification reaction, using a single channel detector generating a sum amplitude signal in correspondence of the plurality of different amplicons; quantifying the plurality of detected different amplicons, and verifying the plurality of detected different amplicons, wherein the quantifying and the verifying comprise using mathematical analysis of the generated sum amplitude signal.

[005] According to a second aspect of the present disclosure, a processor-based hardware analyzer for analyzing a multiplexed amplification reaction is provided, wherein the processor-based hardware analyzer is configured to quantify and verify the multiplexed amplification reaction based on a provided digital representation of a sum amplitude signal in correspondence of a plurality of different amplicons of the multiplexed amplification reaction.

BRIEF DESCRIPTION OF THE DRAWINGS

[006] The accompanying drawings, which are incorporated into and constitute a part of this specification, illustrate one or more embodiments of the present disclosure and, together with the description of example embodiments, serve to explain the principles and implementations of the disclosure.

FIGs. 1A-1D show the various possible relationships between the amplitude curves of a multiplexed reaction with two amplicons.

FIG. 2 shows a flowchart of an algorithm according to the present disclosure for a single color detection multiplexed reaction.

FIG. 3 shows an exemplary sum melt curve of two amplicons and its two constituent melt curves associated to each of the amplicons.

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FIG. 4 shows exemplary amplification curves of two amplicons with threshold cycle (C_T) points close to each other.

FIGs. 5A and 5B show a typical three step and two step respectively temperature cycle of an amplification process and melting temperatures associated to amplified amplicons.

FIG. 6B shows an embodiment according to the present disclosure of a modified temperature cycle of the three step cycle depicted in FIG. 6A, wherein segments of a transition region around various melting temperatures of amplicons are modified to have less steep slopes, the transition region being a ramping down of the temperature of the cycle.

FIGs. 7A and 7B are equivalent to FIGs. 6A/6B for a case of a two step cycle and wherein the transition region is a ramping up of the temperature of the cycle.

FIG. 8 shows a excitation and emission spectra of SYBR Green and Alexa Fluor dies, together with a wide detection filter used to detect portion of the emission spectra of both dies.

FIG. 9 shows an estimation model which according to an embodiment of the present disclosure can be used to represent a multiplexed amplification process.

FIG. 10 shows error in minimum cumulative point wise distance between two normalized and two non-normalized curves.

FIG. 11 is an exemplary embodiment of a processor-controlled hardware used in a single color detection multiplexed amplification reaction.

DETAILED DESCRIPTION

[007] According to several embodiments of the present disclosure, methods and algorithms for multiplexing and quantification of assays are disclosed. Such methods and algorithms can be used in conjunction with any technique which can measure total or targeted amplicon

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concentration during amplification, including but not limited to real-time quantitative polymerase chain reaction (qPCR) with fluorescence detection, electrochemical detection or any other real-time detection technique which can quantify the amount of DNA, whether total or specific.

[008] For Point of Care (POC) applications, a single color multiplexing instrument (e.g. detecting single emission wavelength, single channel detection) has the benefits of being simpler, more compact and cheaper to manufacture as compared to a multi-color instrument. Such single color multiplexing instrument can also be faster since it can be more efficient in collecting emitted light and hence reducing the time needed to observe a corresponding signal. The skilled person will know that current multi-color instruments have the extra complexity of using dedicated detection channels per color, where each detection channel can contain a separate excitation light source, reflector, excitation filter, dedicated optical elements (e.g. prisms, gratings, dispersive elements, etc.) and narrow band (e.g. band pass) emission filter, to detect a different emitted spectrum while rejecting other spectra used in the instrument. Due to overlapping emission spectra of dyes used in a multiplex real time qPCR, band pass filters are typically used to differentiate between the various emitted spectral bands, but a portion of the emission from the dye is not used to reduce spectral overlap, thus a less efficient detection can be obtained which can require a longer integration time.

[009] According to some embodiments of the present disclosure, a single color (e.g. single detection channel) multiplexing instrument can be made by using absorbance, interference or combination of filters at the emission side with a wider bandwidth, such as a greater portion of an emission spectrum (e.g. wider range of wavelengths) can be used to detect the amplification process, which can in turn increase a detected signal intensity and thus reduce integration time while conducting fluorescence detection. According to further embodiments of the present disclosure, such single color multiplexing instrument can be used to detect, quantify and verify amplicons emitting at different wavelengths peaks associated to the different dyes and/or probes/primers used for each amplicon. In some cases, only portion of the emitted spectra associated to an amplicon is detected which can be taken into consideration during the quantification phase of the amplification process.

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[0010] Testing for a disease is very useful in various applications. However, to ensure quality of a reaction, a positive and negative control is useful to determine whether contamination has occurred and whether the amplification is occurring properly. By conducting qPCR in one well (e.g. cartridge, reaction vessel), one can ease the design of fluidics, save reagents cost, as well as reduce error and contamination. Reducing of errors and contamination can in turn eliminate the requirement for addition of positive and negative controls in separate wells. In case of lyophilized reagents only the eluted DNA has to be added and the reaction can be run in one chamber.

[0011] Embodiments according to the present disclosure can be used in conjunction with various quantitative methods for detecting amplicon concentration using, for example, intercalating dyes which bind to any double string DNA in the assay, or methods using fluorophores attached to specific primers (e.g. Promega Plexor primers) which bind only to a targeted DNA string. Various other detection methods, using for example hybridization-probes such as hydrolysis, molecular beacons, fluorescence resonance energy transfer (FRET), hybridization, etc…, as well as electrochemical methods and electrophoresis methods are compatible with the various embodiments according to the present disclosure.

[0012] The various embodiments according to the present disclosure allow for multiplex detection and quantification of more targets (e.g. different targets) in the following cases (e.g. allows greater multiplexing)

a. Single fluorophore (e.g. single emission wavelength) for different targets using a single excitation (e.g. single wavelength). In such embodiments a same fluorophore, and thus a single detector, can be used with different sequence specific primers and yet detect different amplicons.
b. Different fluorophores (e.g. different emission wavelengths) for different targets using a single excitation (e.g. FRET probes which can be all excited using single excitation wavelength).
c. Different fluorophores for different targets using multiple excitation wavelengths with a single detector and a single emission filter. The single emission filter can be a

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wider bandwidth filter to allow the inclusion of larger portions of the emission signal from the various fluorophores (e.g. dyes) such as to obtain an equivalent sum of the emitted intensities.

d. Different fluorophores for different targets using multiple excitation wavelengths with multiple detectors and emission filters.

[0013] The various embodiments according to the present disclosure further allow for use of DNA binding dyes (e.g. fluorescent) like SYBR Green and FAM, or probes which bind to specific targets, primer attached fluorophores or other DNA binding probes. In further embodiments according to the present disclosure, multiplexing and quantification based on analysis of fluorescence versus temperature curves at various points during an amplification process (e.g. qPCR) other than just at the end of the annealing or extension phases are also provided.

[0014] The various embodiments according to the present disclosure further describe methods for obtaining multiple (e.g. different) quantifications (e.g. of amplicons) by using, for example, a single emission wavelength. It should be noted that the amplicons are obtained from a same assay containing different targets (e.g. DNA sequences or RNA sequences). It should also be noted that for the sake of clarity, the exemplary cases of two different amplicons obtained by amplification of two different targets is presented. Such exemplary cases of two amplicons should not be considered as a limitation of the presented embodiments but rather an exemplary case of the inventive concept as disclosed herein.

[0015] Amplification techniques of target genes or sequences are used to determine (e.g. quantify) an initial concentration of the target genes or sequences. As known by the skilled person, such initial concentration is referred to as, or identified by, the threshold cycle (C_T) which is defined by a detectable initial knee in the amplification curve corresponding to a detectable concentration of amplicons at an amplification cycle (e.g. a qPCR temperature cycle). A detected C_T value can then be correlated to known prior concentrations to derive a concentration of the target genes thus completing the quantification process. Typically derivation of the C_T value is performed after determining a successful (e.g. proper) amplification reaction

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which can be verified (e.g. verification process) by conducting, for example, an end point melting curve analysis on the amplicons or using positive and negative controls. In the exemplary case where two different amplicons are amplified (e.g. two different target genes), the end point melting curve analysis can determine whether a single specific amplicon is amplified, whether both amplicons are amplified or whether neither of the amplicons is amplified. In a successful amplification, also referred to as a proper reaction, both amplicons are amplified if the targets for both are present, and therefore one can proceed to find the C_T value for each of the amplicons. In some cases one of the amplification reactions (e.g. creating amplicons) is a control reaction for which the C_T value (e.g. concentration) is known. In other cases concentration of both targets are unknown. According to the various embodiments of the present disclosure, the unknown initial concentrations (e.g. C_T values) can be found in both cases by analyzing the amplitude curve of the multiplexed reaction, also referred to as the sum amplitude curve, which relates to the total number of amplicons per detection cycle. In some cases, the analysis of the sum amplitude curve uses information obtained by analyzing of the melt curve (e.g. melting curve analysis). According to several embodiments of the present application, detailed analysis of the sum amplitude curve and the melt curve can be performed using powerful known mathematical functions and models which the person skilled in the art of signal processing, estimation theory and the like is well aware of.

[0016] When using intercalating dyes, the fluorescence intensity is about proportional to the length of the amplicon (e.g. the longer the amplicon the stronger the fluorescence intensity). According to some embodiments of the present disclosure, methods of analyzing the sum amplitude curve (e.g. corresponding to a total detected intensity) of the multiplexed amplicons is provided which enable to derive the C_T and a corresponding amplitude curve for each of the multiplexed reactions. Such methods are also compatible for the case where primers with attached fluorophores are used. In other embodiment according to the present disclosure more dye molecules can be attached to one primer set vs. the other such as to obtain a higher fluorescence (e.g. amplitude) for one amplicons versus the other as a distinguishing feature of an associated amplification curve. As previously noted, methods according to the various embodiments of the present disclosure can be extended to multiple (e.g. more than two) amplicons. In some embodiments according to the present disclosure, the efficiency of the

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reactions can be different or designed to be different such as to limit a corresponding slope and shape of the amplification curve (e.g. of a given reaction) in order to facilitate detection of the various individual curves and corresponding C_T values. In an exemplary embodiment, efficiency in amplification can be changed by using different primer concentrations for the different targets used in the multiplexed reaction such as to obtain individual target amplification curves with different slopes and shapes. Algorithms according to the various embodiments of the present disclosure can be used to separate the different amplification curves, which are superimposed onto each other (e.g. as per a single detection channel). According to further embodiments of the present disclosure, methods to obtain the total fluorescence of each reaction using the melt curve are provided.

[0017] FIGs. 1A-1D show the various possible relationships between the amplitude curves for the case of a multiplexed reaction using two target genes, each identified by a corresponding amplicon (amplicon1, amplicon2) respectively. It is assumed that the reaction is proper, such as the correct genes are amplified at an expected efficiency (e.g. amplitude slope), and verified, for example, via an end point melting curve analysis. For each of the four different cases represented by FIGs. 1A-1D, the total detected signal (e.g. total emitted fluorescence intensity detected via a single detection channel) is represented by the sum amplitude curve (sum), which corresponds to the sum of the individual amplitude curves (amplicon1) and (amplicon2). Furthermore, the derivative of the sum amplitude curve (sum), as well as its double derivative and its normalized double derivative are displayed for each of the four cases.

[0018] In the cases represented by FIGs. 1C and 1D, the amplifications of the two targets occur at differing cycle numbers making it relatively easy to determine the C_T value for each of the reactions by using simple mathematical analysis, such as for example by using the double derivative of the sum amplitude curve, since there is very little overlap (e.g. of the amplification slope) of the two amplification curves as further displayed by the two distinct peaks in the derivative curves. In the cases represented by FIGs. 1C and 1D the slopes associated to the two amplicons are far enough apart such as to allow finding the C_T value for each of the reactions by analyzing the flat portions and/or the portions with slope of the (sum) curve. For example, looking at the graph represented by FIG. 1D, at around cycle 8 a steep slope starts, which can

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indicate the start of a detectable amplification, and again at around cycle 19 a second steep slope starts, which can indicate the start of another amplification. By further examining, for example, the initial amplitude of each slope, one can determine that a single amplicon is being amplified during each slope (e.g. by verifying a slope amplitude representing an exponential amplification rate). Melting curve analysis can verify that both target amplicons are amplified. Although C_T points are established, one still needs to associate these points to a specific amplicon of the multiplexed reaction. According to various embodiments of the present disclosure, a combination of sum curve analysis and melting curve analysis can provide said association.

[0019] In the case represented by FIG. 1A, the C_T values are close, and therefore there are no additional peaks in the derivative and double derivative of the (sum) curve as compared to a single amplification curve of a single amplicon, thus rendering determination of the C_T values difficult.

[0020] In the case represented by FIG. 1B, the CT values are close and there is a single peak in the derivative of the sum curve. However, a closer attention to the derivative curve shows an asymmetrical (bell) curve shape due to a skew of the two CT points. According to some embodiments of the present disclosure, the amount of asymmetry in the derivative curve can be used to determine the relative position of the two CT points. Also in the case represented by FIG. 1B, the double derivative has more peaks than the amplification curve of a single amplicon which further indicates a skew of the two CT points.

[0021] According to the various embodiments of the present disclosure, methods are provided such as to enable derivation of the CT values and association to specific amplicons of a single detection channel multiplexed reaction for all of the possible cases (e.g. as represented by FIGs. 1A-1D), by mathematically analyzing the sum amplitude curve and/or the melt curve.

[0022] According to an embodiment of the present disclosure, FIG. 2 shows a flowchart of an algorithm for a single color detection of one target with positive and negative controls. Such as in a case of a qPCR reaction, it is here assumed that the reaction is run in a sequence of repeated cycles, wherein further amplification may be obtained with each run of the cycle. The proposed

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algorithm provides a method to derive the concentration of the multiplexed target and positive control via detection of the corresponding C_T values irrelevant of their relative distance. The algorithm of FIG. 2 performs the following steps:

1. *At the end of the reaction (e.g. all the cycles) run a melting curve analysis which yields the following:*
    a. *Which amplicons did amplify, as each amplicons has a different melting temperature (T_M) per design.*
    b. *What was the amplitude of the fluorescence for each amplified amplicon assuming it reached a plateau level. It also gives the amplitude, which is at the end of the cycles, even if it did not plateau.*
    c. *The background fluorescence, for example by observing the detected level of fluorescence at the end of the melting curve analysis.*
    d. *Quality of amplification to some extent, for example by contrasting the observed shape of the melt curve and an expected shape based on known parameters.*
2. *If negative control did amplify then reaction has failed.*
3. *If positive control did not amplify then the reaction has failed.*
4. *If target did not amplify then target is absent/not detectable. Report this and go to End.*
5. *Otherwise do analysis of the sum amplification curve:*
    a. *Analyze the sum amplitude curve to look for peaks:*
        i. *Using derivatives and double derivatives of the sum amplitude curve.*
        ii. *Using various peak detection algorithms known in the art, such as for example CFAR type detection algorithms (e.g. as used in radar theory).*
    b. *If enough peaks are detected with confidence, then the C_T values are sufficiently distant:*
        i. *Derive the C_T values of both the amplicon and the positive control. To be noted that the C_T value of the positive control may be known in advance. The C_T value of positive control can be made to be at later cycles, for example, it can be put after the maximum C_T which can be accepted for clinical relevance or acceptance of the reaction. This simplifies finding the C_T value of a valid amplification as the positive control will not affect the determination of the C_T*

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value of the target. The amplitude of positive control at or after the plateau phase can also be limited by limiting, for example, the amount of primers.

ii. *Optionally verify the derived C_T values using the distance-based method as described below. (step c.vi.)*

c. *If not enough peaks are detected, then the C_T values are near (assuming amplifications with efficiency as expected/specified):*

iii. *Optionally normalize amplitudes of target and positive control using the amplitudes obtained from the melting curve analysis.*

(Amplitude may be normalized such as to assume same amplitude for each of the individual amplitude curves as per reference data used to correlate C_T points to known concentrations, otherwise accuracy can be degraded as shown in FIG. 10). Use the total fluorescence intensity (e.g. amplitude) and the points where the melting occurs for each amplicon to estimate the maximum of each individual fluorescence curve.

iv. *Do a skew analysis on peaks to estimate a C_T difference of the two amplicons. Amplitude information of both amplicons (e.g. target and positive control) as obtained via the melting curve analysis can be used for this step.*

v. *Assume different values of C_T for target amplification and for positive control amplification and based on the assumed different values generate the corresponding sum amplitude curves. Running various experiments and storing the corresponding data or using mathematical models or a combination of both can obtain these C_T values. If the C_T value of the positive control is known, then it can be fixed and the sum amplitude curves can only be different by the assumed C_T values of the target amplification.*

vi. *Take the difference of absolute values between the sum amplitude curves (e.g. absolute or squared) based on the assumed C_T values and the actual measured sum amplitude curve. Analyze the result and select the C_T value (or values in the case where positive control value is not known), which is estimated to be the closest to the actual value. Optionally the CT value corresponding to a minimum distance can be selected as the actual value.*

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Optionally the shape of the distance curve/surface/function may be taken into account.

6. *End.*

[0023] Using the algorithm represented by FIG. 2 to implement a single color detection method to amplify a target with both positive and negative controls is attractive as it allows a reliable detection in POC instruments and in commercial machines.

[0024] In multicolor machines (e.g. having multiple detectors), this multiplexing technique (e.g. FIG. 2) can be used for each color thus enabling the following:

- Have positive and negative control on one color and use other colors separately for detection of other targets
- Have positive and negative control for each color and target
- Increase the number of amplicons, which can be detected by two or three fold. (later we show how to increase this even further)

[0025] According to an embodiment of the present disclosure a method for finding amplitude of amplification curves via sum melt curve analysis and finding constituent melt curves from the observed sum melt curve via a single channel detection is provided. FIG. 3 shows an exemplary sum melt curve (MC1+MC2) corresponding to two amplicons (a1, a2) generated through an amplification process. Based on this sum melt curve it is desired to extract its constituent curves (MC1) and (MC2) which are further shown in FIG. 3. The sum melt curve changes slope appreciably near (TM1) and (TM2) which are the melting temperatures of amplicons (a1) and (a2) respectively. According to the observed sum melt curve (MC1+MC2), the slopes at various regions of the curve are known and can be associated to the slopes of the various regions of the individual curves (MC1) and (MC2) as seen in FIG. 3. By defining:

s1bTm: slope of (MC1) before its melt temperature (TM1)
s1aTm: slope of (MC1) after its melt temperature (TM1)
s2bTm: slope of (MC1) before its melt temperature (TM2)

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s2aTm: slope of (MC1) after its melt temperature (TM2)

based on the observed sum melt curve (MC1+MC2) and as depicted in FIG. 3, the following expressions are known:

(a) s1bTm + s2bTm
(b) s1bTm + s2aTm
(c) s1bTm
(d) s1aTm

These four independent expressions therefore allow to determination of each of the two slopes associated to the constituent melt curves (MC1) and (MC2).
Assuming the amplitudes of the constituent melt curves (MC1) and (MC2) are (A1) and (A2) respectively, then based on the observed single channel sum melt curve, the following amplitudes are known:

(e) A1+A2
(f) M1
(g) M2

Using well known mathematical techniques, the skilled person will know how to estimate (A1) and (A2) by using the relationship of (M1, M2) with (TM1, TM2).

[0026] Finding amplitudes of the various constituent melt curves (e.g. of the sum melt curve) as per the method described above and depicted in FIG. 3, allows to correlate amplitude curves (e.g. C_T values) to specific amplicons, by using for example the relative amplitudes obtained via the sum melt curve analysis provided above (e.g. FIG. 3) and the amplitude curves obtained from the sum amplitude curve as per any one of the various embodiments of the present disclosure. The melt curves can associate an amplicon to a relative amplitude (e.g. via known melt temperature), which can then be correlated to an amplitude of an amplitude curve. For example, and in relation to FIG. 3, amplitude (A2) which corresponds to amplicon (a2) is relatively larger than amplitude

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(A1) of amplicon (a1). Therefore, if analysis of the sum amplitude curve corresponding to generation of amplicons (a1, a2), yields in two separate C_T values, the larger value will correspond to amplicons (a2) and the smaller value to amplicon (a1).

[0027] In some cases the slope of the amplitude curves in the plateau phase (e.g. minimal amplification region subsequent to the exponential phase) may be difficult to determine. Therefore, according to an embodiment of the present disclosure the exponential phase only of the assumed amplitude curves (e.g. FIG. 4 later described) may be used for the analysis (e.g. step 5 of the algorithm). Similarly, a phase of an amplitude curve where no amplification is detectable (e.g. relatively flat region of the curve) according to an assumed and/or the actual amplitude curve, can be excluded from the analysis. Such technique of eliminating regions (e.g. phases) of the curve containing minimal information from the analysis step can give a better and more robust analysis result. It should be noted that the region of interest for the analysis is typically the area of exponential amplification of an amplitude curve which is at the vicinity of a corresponding C_T point. Assuming that the amplification is proper (e.g. maximum efficiency and expected amplicons generated), then it is expected that the amplitude change (e.g. amplification) at the vicinity of a C_T point follows an exponential curve, as at the vicinity of a C_T point amplification occurs at a rate of 2^N where N is a number associated to an amplification cycle. The skilled person will not need further explanation on this and will appreciate the benefits of using/analyzing the relevant regions (e.g. slopes) of the amplitude curves in order to detect the various C_T points.

[0028] FIG. 4 shows exemplary amplification curves of two amplicons with C_T points close to each other. As per prior paragraphs, by observing and analyzing a slope (e.g. represented by an efficiency *e* of the reaction) in FIG. 4) of the sum amplification curve it is possible to directly determine the C_T point of each amplicon. By tracking the exponential phase only of the sum amplitude curve and assuming that each reaction worked properly (e.g. amplified at the proper rate), each of the constituent amplitude curves can be represented as shown in FIG. 4. When the first amplicon starts to be detected above a corresponding C_T value while the second amplicon is not being detected, the efficiency *e* is close to 1 (e.g. one amplicon amplified properly) as shown in the first segment of FIG. 4. When the second amplicon crosses its corresponding C_T value,

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two proper amplification reactions are detectable with each being in a corresponding exponential phase. Thus in the second segment of FIG. 4 the corresponding efficiency (e.g. slope) value is close to 2, showing that both amplifications are in exponential phase. Finding these two initial segments are enough to find the C_T value of each of the two amplicons, based on the fact that an exponential phase of either amplicon has an efficiency (e.g. slope) contribution to the observed sum amplitude curve of approximately 1. In the case where the two C_T values are the same, then the two segments are merged and a single segment with an efficiency value of approximately 2 is observed. In the third segment the efficiency of amplification of one or both amplicons starts decreasing, thus the total observed efficiency also starts decreasing from the value of approximately 2 as an amplification starts approaching a plateau phase. In the third segment the efficiency value (e.g. slope) changes from a value of less than 2 to 0 in the case where both amplifications enter the plateau phase.

[0029] The technique disclosed above to detect C_T values based on the slope of the sum amplitude curve is easily adaptable to cases where C_T values are far apart by easy extension of this technique. This technique is robust since it seeks to find the exponential phase of amplification which occurs during the parts of reaction (e.g. of one of the constituent amplicons) where efficiency is close to 1 and is thus not dependent on the amplification curve taking other shapes due to, for example, limited reagents availability during the later stages of the reaction.

[0030] According to a further embodiment of the present disclosure, the disclosed algorithm for a single color detection of one target with positive and negative controls can be further extended to include more than two targets while keeping both the positive and the negative controls. As for the single target case, the peaks in derivative and double derivative curves and the amplitude (e.g. as detected via the melting curve analysis) can be used to separate the amplitude curves for each of the multiple amplicons from the sum amplitude curve (e.g. steps 5.a. and 5.b. of single amplicons algorithm).

[0031] For the case of multiple targets with one positive control and one negative control, same algorithm as used for the case of single target can be sued, with the exception of steps 5.c.v. and 5.c.vi. which may be replaced by the following steps:

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For each amplified amplicon assume some C_T values based on prior analysis results.

For all assumed C_T values

Generate all or part of the sum amplitude curve

Find the distance over all or portion of the generated sum amplitude curve to the measured sum amplitude curve (e.g. may calculate distance over a portion such as the exponential phase of the curves)

End for

Select the most probable C_T values (e.g. which minimize the distance)

[0032] According to some embodiments of the present disclosure, the proposed multiplexing algorithm can be used to increase the multiplexing capability of instruments used for amplification of target genes which have multiple optical detection channels. For example by implementing such algorithm in a 4 channel instrument, the instrument can be upgraded to detect 12 targets with 3 targets multiplexed per color using the disclosed multiplexing algorithm. In the case where 4 targets are multiplexed per color using the disclosed algorithm, then the same 4 channel instrument can be upgraded to detect 16 targets. The skilled person will know that such instruments perform all or part of their data analysis of the observed/detected emitted intensity (e.g. fluorescence) based on algorithms implemented in a combination of software and/or firmware code embedded within a processor-controlled hardware of the instrument, such as, for example, the processor-controlled hardware depicted in FIG. 11. Such algorithms perform upon a digital representation of the observed/detected emitted intensity read into a memory module accessible by (or part of) the processor-controlled hardware. Such digital representation can be obtained, for example, via an analog to digital converter module which converts an analog signal representing the observed/detected emitted signal to an equivalent digital representation. The skilled person will realize the possible upgrade to all or portion of such software and/or firmware to include the proposed single channel multiplexed algorithm. In alternative embodiments according to the present disclosure, data observed/detected by an instrument in its standard configuration may be analyzed off-line using the proposed multiplexing algorithm such as to not disturb an existing instrument. In this case the proposed multiplexing algorithm may be entirely implemented in software and/or firmware running on a computer and/or processor-controlled hardware.

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[0033] FIG. 11 is an exemplary embodiment of a processor-controlled hardware (10) (e.g. a computer system) as discussed in the previous paragraphs. This processor-controlled hardware comprises a processor (15), a memory bank (20), a local interface bus (35) and one or more Input/Output devices (40). The processor may execute one or more instructions related to the implementation of the discussed mathematical analyses and related algorithms for quantification and verification of the amplification process and as provided by the Operating System (25) based on some executable program stored in the memory (20). These instructions are carried to the processors (20) via the local interface (35) and as dictated by some data interface protocol specific to the local interface and the processor (15). It should be noted that the local interface (35) is a symbolic representation of several elements such as controllers, buffers (caches), drivers, repeaters and receivers that are generally directed at providing address, control, and/or data connections between multiple elements of a processor based system. In some embodiments the processor (15) may be fitted with some local memory (cache) where it can store some of the instructions to be performed for some added execution speed. Execution of the instructions by the processor may require usage of some input/output device (40), such as inputting data from a file stored on a hard disk (e.g. data representing a sum amplitude and/or a sum melting curves), inputting commands from a keyboard, outputting data to a display, or outputting data to a USB flash drive. In some embodiments, the operating system (25) facilitates these tasks by being the central element to gathering the various data and instructions required for the execution of the program and provide these to the microprocessor. In some embodiments the operating system may not exist, and all the tasks are under direct control of the processor (15), although the basic architecture of the processor-controlled hardware device (10) will remain the same as depicted in FIG. 11. In some embodiments a plurality of processors may be used in a parallel configuration for added execution speed. In such a case, the executable program may be specifically tailored to a parallel execution. Also, in some embodiments the processor (15) may execute part of the quantification and/or verification, and some other part may be implemented using dedicated hardware/firmware placed at an Input/Output location accessible by the target hardware (10) via local interface (35) (e.g. dedicated PCB boards for digitizing a detected amplification signal). The processor-controlled hardware (10) may include a plurality of executable program (30), wherein each may run independently or in combination with one another.

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[0034] As known by the skilled person, when intercalating dyes are used for detection of an amplification reaction, the amplitude of the fluorescence (e.g. associated with the intercalating dyes) will change with the amplicon length. In the case where sequence specific probes (e.g. bind only to specific targets) are used to monitor an amplification reaction, intercalating dyes can be added to the reaction (e.g. the assay comprising the various reagents) without affecting the monitoring efficiency of the amplification reaction and provide the advantage of allowing a melting curve analysis. The skilled person will know that some sequence specific probes are not compatible with a melting curve analysis thus addition of an intercalating dye can be beneficial in, for example, performing the disclosed single color multiplexed reaction detection algorithm.

[0035] For a probe-based reaction, the number of fluorophores on the probes can be different for different amplicons. The reporter molecules can be different but emit in the same wavelength or in two wavelengths that are substantially the same, such as a same detector can detect both wavelengths with a sufficiently high efficiency. For example FAM, Eva Green and SYBR Green dyes have similar, or substantially same, emission spectra. Such dyes can be used for target specific probes and still allow multiplexed detection of several targets using the disclosed single color multiplexed reaction detection algorithm. This, for example, can allow probe-based detection in a same detection channel. Thus it is possible to get specificity of probes and quantification of different targets using a single detection channel.

[0036] The single color detection algorithm disclosed is also compatible for a multiplexed reaction where different spectra dyes are used. In this case the relatively wide emission spectrum of the various dyes is used to detect all or a portion of the emission spectrum through, for example, a same absorbance filter. For example Texas Red will have a greater portion of its spectrum going through an absorbance filter with a cut-off wavelength of 520 nm (e.g. passes wavelengths above 520nm) than, for example, SYBR Green.

[0037] In the case where FRET probes are used, which have FAM as acceptor dye (e.g. single excitation wavelength) and different donor dyes (e.g. different emission spectra), the amplitude of the different amplicons generated through a multiplexed reaction can be different while being able to use a single excitation wavelength. Distinction in amplitude of the different amplicons

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can facilitate correlation of the amplicon specific amplitudes detected via a melting curve analysis, and the C_T points detected from the sum amplitude curves as per the analysis disclosed in the single color multiplexed reaction detection algorithm of this disclosure.

[0038] In some cases different excitation wavelengths are used with different probes designed to emit at different wavelengths. Using different emission wavelengths will yield different amplicon amplitudes for various probes which can be used to further differentiate the various amplicons based on amplitude. For example Alexa Fluor 610 is minimally excited by wavelength of 475 nm (475/25 blue filter) so a blue LED (or a blue filter) can be used to excite SYBR Green only, which yields in detection of amplicons specific to probes with SYBR Green. On the other hand, a wavelength of 410nm can excite both the SYBR Green and the Alexa Fluor dies, and thus yielding in detection of amplicons specific to probes with both SYBR Green and Alexa Fluor. As depicted in FIG. 8, which was taken from the webpage http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Cell-Analysis/Labeling-Chemistry/Fluorescence-SpectraViewer.html, retrieved on August 2, 2013, a detection filter (e.g. 600/200 Em Filter) with a wide enough bandwidth covering a wavelength ranges of [500nm, 700nm] can be chosen to detect emission spectra (e.g. portion of) corresponding to both the SYBR Green and the Alexa Fluor dies. Detecting emission of both amplicons with one excitation source and then emission of only one amplicon with a second excitation source can allow determining of the emission of each amplicon. As depicted by FIG. 8, one can multiplex using a single detector but different excitation sources and still capture a large portion of the spectrum of dyes in contrast to using bandpass filters specific to each of the emission wavelengths. One can get the amplitude information by getting melting curves with multiple excitation sources in series to get the amplitudes of both. This means select an excitation source, run a multiplexed reaction and analyze reaction using provided algorithm, then select a different excitation source, run a multiplexed reaction and analyze reaction using same algorithm. Multiplexing of the excitation source thus allows increasing detection capability of an instrument for each detection channel.

[0039] To control the amplitude of an amplicon the primers can be limited. For example the positive control can have limited primers so that it plateaus close to a designed value. As

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previously mentioned, controlling the amplitude of an amplicon in a multiplexed reaction can facilitate detection of a corresponding CT value as per the disclosed single color multiplexed reaction detection algorithm.

[0040] The initial period of a reaction, where none of the amplicons in the amplification has entered an exponential phase (e.g. corresponding to amplitude curve region with steepest slope), can be treated as background fluorescence and can be subtracted from the total fluorescence waveform (e.g. sum amplitude curve).

[0041] According to one embodiment of the present disclosure, partial melting curve analysis is performed during each cycle of an amplification reaction, such as for example a multiplexed qPCR reaction. Such cycle by cycle partial melting curve analysis can determine the quantification of various amplicons during the course of the amplification reaction. FIGs. 5A and 5B show a typical cycle (e.g. temperature cycle) of an amplification process, whereby a temperature of an assay containing genes to be amplified is subjected to various levels corresponding to a denaturation phase, an annealing phase and an extension phase of a DNA sequence associated to the gene. As represented in FIG. 5A, the temperature levels for the annealing and extension phases are different whereas in the case of FIG. 5B these levels are equal. The skilled person will need no additional information on the inner working of an amplification process and the relevance of the various temperature levels as related to the three main phases of the amplification process. In a typical amplification process, the transition regions from one temperature level of a cycle corresponding to a phase of the cycle to another temperature level corresponding to a different phase of the cycle is not critical to the amplification process and is typically a function of characteristics associated to the heating/cooling elements and a chamber of an associated amplification process instrument. FIGs. 5A and 5B show melting temperatures (TM1, TM2) associated to two different amplicons being amplified. As known by the skilled person these melting temperatures are known a priori and set per design of the target specific primers used in the reaction. According to various embodiments of the present disclosure, melt analysis of the various amplicons of the amplification process, for example as associated to the melt temperatures (TM1, TM2) can be performed at the vicinity of these temperatures, either during a region of ramping up of the temperature or a region of

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ramping down of the temperature in the cycle. In some embodiments according to the present disclosure, the cycles can be slowed down such as to allow better detection/resolution during such partial melting curve analysis. In some embodiments in order to maintain a relatively fast cycle, the temperature within a cycle can be controlled to quickly go to the expected temperatures of the melt curve (e.g. partial melt curve) where various amplicons show a signature (e.g. melting temperature as per design of each of the different amplicons) and pass through other temperature points rapidly. Such controlling of the temperature within the cycle can be performed during either a ramping up or a ramping down of the temperature, with better results obtained dependent on the heating method used. FIG. 6B shows a modified cycle of the cycle represented in FIG. 6A whereby the temperature of the cycle corresponding to the transition between the denaturation and the annealing phases is controlled such as to allow for the partial melt curve analysis at the vicinity of the two melting temperature points (TM1) and (TM2). In the embodiment depicted by FIG. 6B, the transition region between the two temperature levels associated to the denaturation and annealing phases is segmented into regions at the vicinity of and including each distinct melting temperature point and segments devoid of a melting point, such as the sum of the segments contain the entire transition region. As depicted by FIG. 6B, segments containing a melting point provide a lower slope corresponding to a lower temperature change of rate such as to allow for better melt curve analysis, whereas segments devoid of a melting point have a larger slope such as to quickly pass through a temperature transition and minimize duration of the cycle. Although in the exemplary case depicted by FIG. 6B the partial melting curve analysis is performed during a ramping down of the temperature profile of the cycle, according to other embodiments of the present disclosure the partial melting curve analysis can be performed during a ramping up of the temperature profile of the cycle as depicted for example in FIGs. 7A and 7B. In some cases, the partial melting curve can be performed only during some (e.g. not all) of the cycles of the amplification process. In a case where the thermal characteristics of the system (e.g. including qPCR fluid) are such that the ramp rate need not be reduced but still a signature is obtained, the cycle can proceed at normal ramp without slowing down near (TM1) or (TM2).

[0042] In some embodiments partial melting curve analysis in some of the cycles can be skipped such as to reduce total reaction time (e.g. sum of all cycles times) and then quantify based on

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threshold only. Normally the Ct value is desired to be quantified. In some embodiments looking for a melt signature (e.g. a specific change in shape/slope of the detected fluorescence versus temperature at a melting temperature point) for an amplicon can be stopped as soon as its signature becomes detectable. In some embodiments it may be desirable to run a final melting curve analysis in order to verify the reaction and cross check against the observed amplicons (e.g. no unexpected amplicons generated).

A partial melting curve analysis algorithm is as follows:

Start cycling
For each cycle
- If fluorescence is above a threshold (signals start of an amplification)
- Run melting curve for undetected targets (or look for expected signature instead of going slowly through all temperatures)
- If a target is detected, mark it and optionally exclude analyzing it in next cycles (no need to ramp slowly across this melting temperature for next cycles)
- If all targets are detected, break
End for
Optionally verify using melting curve at end

[0043] In some cases it can be desirable to run full melting curve analysis in between cycles of an amplification reaction, at the expense of the extra time required to acquire the melting curve. However, since the melting temperature of each amplicon is known in advance, one can measure fluorescence (e.g. emitted intensity) at particular temperatures (e.g. at the vicinity of a known melting temperature of an amplicon) and skip temperature ranges which do not contain a melting temperature of an amplicon, instead of recording (e.g. measuring fluorescence) at more temperatures as is typically done in a melting curve analysis. By tracking the peaks in observed fluorescence for each of the known melting temperatures (e.g. associated to an amplicon) for each run melting curve, one can detect the presence of an amplicon (e.g. at a concentration). Such a method of accelerating the melting curve analysis can save overall time (e.g. for detection/quantification of amplicons) while quantification can be obtained for many amplicons

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with different melting temperatures. Also the amplitude information, obtained via the sum amplitude curve, can be used to detect two amplicons with the same or very close melting temperatures. For example, fluorescence increasing at double the rate (e.g. compared to an amplification rate of a single amplicon during an exponential amplification phase) or similar, is indicative of two amplicons being amplified instead of one.

[0044] According to a further embodiment of the present disclosure, an algorithm for an accelerated melting curve analysis is presented below. Such algorithm can be used in cases where it is desired to find out the concentration of an amplicon or amplicons (e.g. corresponding C_Ts) within, for example, a multiplexed reaction. It is assumed that each reaction will go through exponential phase, or in other words that the reactions are proper:

Start cycling
For each cycle
- *Measure fluorescence at temperatures related to each amplicon*
- *If a target is detected, mark it and optionally exclude analyzing it in next cycles (do not ramp slowly across the melting temperature of a detected target for next cycles)*
- *If all targets are detected, break*

End for
Optionally verify using melting curve at end

Same algorithm presented above can be used in the case of multiple amplicons with similar melting temperatures.

[0045] Non-specific probes, such as for example intercalating dyes, can be used to qualitatively find out which amplicon has amplified by using, for example, a melting curve analysis. Non-specific probes can be used to make an assay with targets which need quantification and targets which do not need quantification (e.g. only need to find out their presence and not concentration). Sequence specific probes can be used to measure quantification (e.g. C_T values). In a two channel (e.g. detection) instrument, one channel can be used to perform qualitative

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analysis of a multiplexed assay using non-specific probes, whereas a second channel can be used to perform a quantification of specific targets of the assay using sequence specific probes. Such a configuration thus provides two degrees of freedom (e.g. color and melting temperature) in multiplex assays.

[0046] In the exemplary case of three amplicons, corresponding to a positive control, a negative control and a target, one can use both non-specific dyes and sequence specific hybridization probes (e.g. for all three corresponding amplicons) in a same reaction. According to an embodiment of the present disclosure, such multiplexed reaction can be analyzed, both quantitatively and qualitatively, using two excitation sources and only one detector. For example and as depicted by FIG. 8, if the non-specific dye is SYBR Green and the dye used for sequence specific detection is Alexa Fluor 610 then one can use a blue LED (e.g. 475nm/25 blue filter) to excite the green dye while the Alexa Fluor is not excited appreciably. This will give melting curve and qualitative answer to the amplification reaction.

[0047] Quantification can be determined by measuring the fluorescence during cycles, while a UV LED (e.g. 410nm/25 UV filter) provides the excitation wavelength, which does not excite the green dye appreciably. The advantage is that only one detector and emission filter is used thus possibly allowing more light capture. In this case the amplification of controls does not interfere with the florescence signal of the target amplicon.

[0048] According to further embodiments of the present disclosure, various mathematical transforms and functions used in signal processing (e.g. FFT, DFT, z-transform, etc...) can be used to compare, match or further analyze signals/amplitudes detected during a multiplexed reaction. Such transforms/functions can detect a phase difference between two shifted waveforms, such as slopes detected and/or assumed, and/or various derivatives of sum amplitude and/or melting curves. They can also help in normalizing the amplitudes and providing more insight into the behavior of the multiplexed reaction than by using only slopes of the observed signals. These transforms/functions can be applied to the entire and/or different parts of the observed/detected signals.

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[0049] According to a further embodiment of the present disclosure, teachings from the signal processing theory as well as the estimation theory can be used to further analyze the observed/detected signals during a multiplexed amplification reaction. In an exemplary embodiment according to the present disclosure, the estimation model given in FIG. 9 can be used to represent the amplification process.

In the estimation model depicted by FIG. 9:

$C_Tk = C_T$ of a corresponding amplification

Ak = amplitude of a corresponding amplification

Sk = slope/efficiency of a corresponding amplification

where k = (1,2,…) depending on the number of multiplexed amplicons.

By feeding an observed signal $\overline{y}$, from the multiplexed amplification reaction, to an estimator corresponding to the modeled process represented by a matrix $\overline{h}$, one can derive an estimation of the process parameter $\overline{x}$, which includes the main information of the reaction (e.g. C_T, amplitude, slope/efficiency).

[0050] One can use various estimators like ML (maximum likelihood), MAP (Maximum A Priori) and various others to estimate the values of parameters. The process ($\overline{h}$) can be modeled in various ways. It can be modeled recursively and a Kalman filter can be used to smooth out and or estimate the parameters. Various other control algorithms known to a person skilled in the art of signal processing and estimation theory can be used to analyze the observed/detected signals during the multiplexed reaction. As previously mentioned, such algorithms can be implemented in a combination of software/firmware to be used to upgrade an existing instrument and/or used in a new instrument configured to perform the multiplexed reaction (e.g. single channel detector) and/or configured to simply analyze off line data/signals detected/observed.

[0051] According to a further embodiment of the present disclosure, quality and concentration of a multiplexed reaction can be obtained by limiting the primers used in the reaction process such as to control the plateau levels of the various amplicons of the multiplexed reaction. Using such

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method therefore allows to find the C_T value of each amplicon and whether various amplicons amplified using only the amplification curves and without melting curve analysis at the end. By limiting the primers and control the number of cycles it takes to reach plateau phase for various amplicons then an estimate of the slope of fluorescence for each amplicon can be made. In some cases the peaks of derivative and double derivatives would be apart such as to allow easy quantification and detection. In overlapping cases of several C_T values , the prior slope knowledge obtained by limiting the amplicon specific primers can be used to resolve the amplicon amplified. For example, if a slope of the amplification is higher than an expected slope of any of the amplicon (e.g., in case of a same C_T value) then it can be estimated that two amplicons are amplified. Also the fluorescence value at the end of all amplifications can give a sum which can be used to find out how many amplicons did amplify, since plateau level of each amplicon is known per design. Mathematical analysis as described in prior paragraphs can further help to analyze the observed/detected amplitudes/slopes.

[0052] Therefore, in accordance with the present disclosure, systems and methods for multiplexing and quantification in PCR with reduced hardware and requirements are provided, which allow detection of different amplicons via a single detector and quantification of said amplicons using known mathematical analysis methods.

[0053] The person skilled in the art will be able to extend the teachings of the present disclosure to any type of process which can measure total or targeted amplicon concentration during an amplification, including but not limited to real-time quantitative polymerase chain reaction (qPCR) with fluorescence detection, electrochemical detection or any other real-time detection technique which can quantify the amount of DNA, whether total or specific, in a manner similar to what has already been discussed in the present disclosure. While the devices and methods have been described by means of specific embodiments and applications thereof, it is understood that numerous modifications and variations could be made thereto by those skilled in the art without departing from the spirit and scope of the disclosure. It is therefore to be understood that within the scope of the claims, the disclosure may be practiced otherwise than as specifically described herein

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[0054] A number of embodiments of the present inventive concept have been described. Nevertheless, it will be understood that various modifications may be made without departing from the scope of the inventive teachings.

[0055] Accordingly, it is to be understood that the inventive concept is not to be limited by the specific illustrated embodiments, but only by the scope of the appended claims. The description may provide examples of similar features as are recited in the claims, but it should not be assumed that such similar features are identical to those in the claims unless such identity is essential to comprehend the scope of the claim. In some instances the intended distinction between claim features and description features is underscored by using slightly different terminology.

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CLAIMS

1. A method comprising:
   detecting a plurality of different amplicons generated during a multiplexed amplification reaction, using a single channel detector generating a sum amplitude signal in correspondence of the plurality of different amplicons;
   quantifying the plurality of detected different amplicons, and
   verifying the plurality of detected different amplicons,
   wherein the quantifying and the verifying comprise using mathematical analysis of the generated sum amplitude signal.

2. The method of claim 1, wherein the sum amplitude signal is in correspondence of a sum of amplitude signals of each different amplicon of the plurality of different amplicons.

3. The method of claim 1, wherein the detecting is based on emitted wavelengths from sequence specific probes and/or sequence specific primers.

4. The method of claim 1, wherein the detecting is based on emitted wavelengths from fluorescent DNA binding dyes.

5. The method according to any one of claims 1-4, wherein the mathematical analysis is performed on a portion of the sum amplitude signal.

6. The method of claim 5, wherein the portion comprises slopes regions of the sum amplitude signal.

7. The method of claim 6, wherein the mathematical analysis of the slopes regions comprises analysis of the slopes variations.

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8. The method of claim 7, wherein the analysis of the slopes variations is used to detect a threshold cycle of an amplicon of the plurality of different amplicons.

9. The method of claim 8, wherein the slopes are dependent on different primer concentrations used for different amplicons of the plurality of different amplicons.

10. The method of claim 5, wherein the portion of the sum amplitude signal comprises flat regions of the sum amplitude signal.

11. The method of claim 10, wherein the mathematical analysis of the flat regions is used to identify an amplicon of the plurality of different amplicons.

12. The method of claim 11, wherein the flat regions are dependent on different primer concentrations used for different amplicons of the plurality of different amplicons.

13. The method according to any one of claims 1-12, wherein the mathematical analysis further comprises:

using signal processing techniques to separate an amplitude curve of an amplicon of the plurality of different amplicons from a sum amplitude curve in correspondence of the sum amplitude signal, and

deriving a value of a threshold cycle of the amplicon based on the amplitude curve.

14. The method of claim 13, wherein the signal processing techniques comprise one or more of: a) fast Fourier transform (FFT), b) discrete Fourier transform, and b) z-transform.

15. The method of claim 13 further comprising:

for each amplicon of the plurality of different amplicons, assuming a threshold cycle value based on prior mathematical analysis of the sum amplitude curve, and

based on the assuming, generating an assumed amplitude curve in correspondence of the each amplicon of the plurality of different amplicons,

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based on the generating, generating an assumed sum amplitude curve,

based on the generating, measuring a distance between the sum amplitude curve and the assumed sun amplitude curve,

repeating the assuming, generating, generating and measuring, and

based on the repeating, deriving for each amplicon of the plurality of different amplicons the value of the threshold cycle from the assumed value.

16. The method of claim 15, wherein the measuring of the distance is performed over a portion of the sum amplitude curve and the assumed sum amplitude curve.

17. The method of claim 16, wherein the portion comprises an exponential phase of the curves.

18. The method according to any one of claims 1-4, wherein the mathematical analysis further comprises using a model for modeling the multiplexed amplification reaction, the model being defined by a plurality of process parameters, and estimating the values of the process parameters based on an estimator associated to the model.

19. The method of claim 18, wherein a plurality of process parameters defining the model comprise: a) a threshold cycle, b) an amplitude, and c) a slope, of a corresponding amplification of the multiplexed amplification reaction.

20. The method of claim 18 or claim 19, wherein the estimator comprises one of: a) a maximum likelihood estimator, and b) a maximum a priori estimator.

21. The method according to any one of claims 18-20, wherein the model is modeled recursively using a Kalman filter.

22. The method according to any one of claims 1-5, 10, 13, 15 and 18, wherein the verifying further comprises:

running a melting curve analysis;

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based on the running, verifying a number of different amplicons of the plurality of different amplicons, and

based on the running, detecting presence of an amplicon of the plurality of different amplicons.

23. The method of claim 22, wherein the melting curve analysis further comprises:

defining a temperature range comprising a plurality of melt temperatures of the plurality of different amplicons;

segmenting the temperature range in temperature regions containing a melt temperature of the plurality of different amplicons and temperature regions devoid of a melt temperature of the plurality of different amplicons;

defining a slow temperature ramp rate and a fast temperature ramp rate;

associating the slow temperature ramp rate to the temperature regions containing a melt temperature and associating the fast temperature ramp rate to temperature regions devoid of a melt temperature;

based on the associating, ramping a melting curve analysis temperature over the temperature range, and

based on the ramping, analyzing during the slow ramp rate a melt curve in correspondence of the melting of the plurality of different amplicons.

24. The method of claim 23, wherein the quantifying further comprises:

waiting for a completion of a cycle of the multiplexed amplification reaction;

running a melting curve analysis;

based on the running, obtaining a quantity of an amplicon of the plurality of different amplicons, and

repeating the waiting, the running and the obtaining.

25. The method of claim 23, wherein the quantifying further comprises running one or more melting curve analyses during one or more corresponding cycles of the multiplexed amplification reaction.

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26. The method of claim 25, wherein the running of the one or more melting curve analyses is performed during a region of temperature transition between one of: a) a denaturation phase and an annealing phase of the one or more corresponding cycle, and b) an extension phase and a denaturation phase of the one or more corresponding cycles.

27. The method according to any one of claims 1-5, 10, 13, 15, 18 and 22, wherein the mathematical analysis further comprises taking into account a difference in amplitude between a first amplicon of the plurality of different amplicons and a second different amplicon of the plurality of amplicons, the difference in amplitude being based on a length difference between the first amplicon and the second amplicon.

28. The method according to any one of claims 1-5, 10, 13, 15, 18 and 22, wherein the mathematical analysis further comprises taking into account a difference between a first amplitude signal associated to a first amplicon of the plurality of different amplicons and a second amplitude signal associated to a second amplicon of the plurality of different amplicons, the difference being based on a difference in primer concentration used for the first and the second amplicons.

29. The method according to any one of claims 1-5, 10, 13, 15, 18, 22, 27 and 28, wherein the detecting further comprises:

using a filter configured to detect a wider range of wavelengths;

based on the using, detecting different wavelengths associated to different amplicons of the plurality of different amplicons, and

based on the detecting, generating the sum amplitude signal.

30. The method according to any one of claims 1-5, 10, 13, 15, 18, 27 and 28, wherein the quantifying further comprises:

running a melting curve analysis;

based on the running, deriving a relative amplitude of an amplicon of the plurality of different amplicons;

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associating the relative amplitude to a threshold point derived from a sum melt curve associated to the sum amplitude signal, and

based on the associating, determining the threshold point of the amplicon, wherein the threshold point quantifies the amplicon.

31. A method for further multiplexing a single channel detector multiplexed amplification reaction, the method comprising:

providing a plurality of excitation sources;

activating one of the plurality of excitation sources;

based on the activating, exciting one or more fluorophores in correspondence of one or more different amplicons from a plurality of different amplicons;

based on the exciting, performing the detecting, the quantifying and the verifying of the one or more different amplicons in accordance to the method according to any one of claims 1-5, 10, 13, 15, 18, 22, 25, 27 and 28;

deactivating the one excitation source; and

repeating the activating, the exciting, the performing, and the deactivating for each remaining excitation source of the plurality of excitation sources, wherein the repeating detects, quantifies and verifies the plurality of different amplicons.

32. The method according to any one of claims 1-5, 10, 13, 15, 18, 22, 25, 27, 28 and 31, wherein the detecting further comprises:

using a filter configured to detect a wider range of wavelengths;

based on the using, detecting different wavelengths associated to different amplicons of the one or more different amplicons, and

based on the detecting, generating the sum amplitude signal.

33. A processor-based hardware analyzer for analyzing a multiplexed amplification reaction, wherein the processor-based hardware analyzer is configured to quantify and verify the multiplexed amplification reaction based on a provided digital representation of a sum amplitude signal in correspondence of a plurality of different amplicons of the multiplexed amplification reaction.

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34. The processor-based analyzer of claim 33, wherein the quantification and verification is performed using mathematical analysis of the digital representation of the sum amplitude signal, the mathematical analysis being implemented in software and/or firmware code and executed on the processor-based analyzer.

35. The processor-based analyzer of claim 34, wherein the mathematical analysis further comprises signal processing techniques adapted to separate an amplitude curve of an amplicon of the plurality of different amplicons from the digital representation of the sum amplitude signal, and adapted to derive a value of a threshold cycle of the amplicon based on the amplitude curve.

36. The processor-based analyzer according to any one of claims 33-35, wherein the processor-based analyzer is further configured to derive a relative amplitude of an amplicon of the plurality of different amplicons based on a provided sum melt curve.

37. An apparatus for performing a multiplexed amplification reaction, the apparatus comprising:

a single channel detector configured to detect a sum amplitude signal in correspondence of a plurality of different amplicons of the multiplexed amplification reaction and generate a digital representation of said signal; and

the processor-based analyzer of any one of claims 33-35, configured to receive the digital representation of the sum amplitude signal.

38. The apparatus of claim 37 further comprising a plurality of excitation sources.

39. The apparatus of claim 37 or claim 38, further comprising a wider bandwidth filter adapted to detect a wider range of wavelengths.

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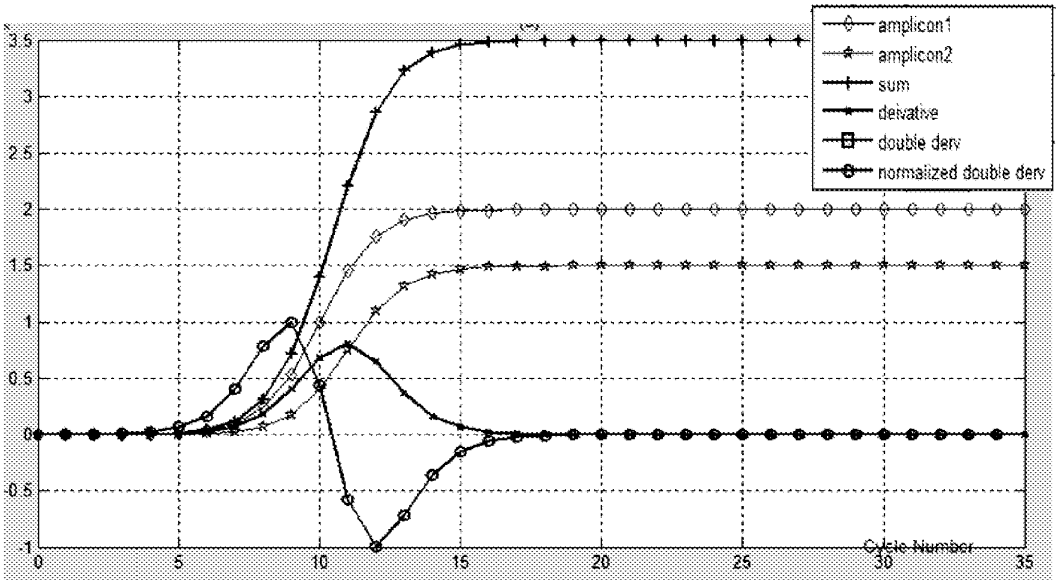

amplicon1
amplicon2
sum
deivative
double derv
normalized double derv
Cycle Number

FIG. 1A

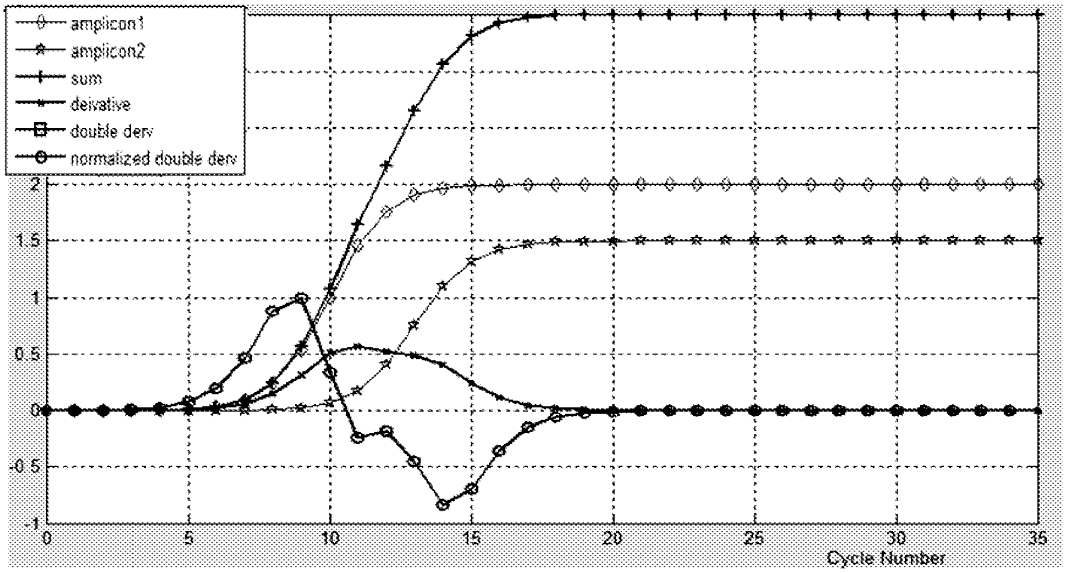

amplicon1
amplicon2
sum
deivative
double derv
normalized double derv
Cycle Number

FIG. 1B

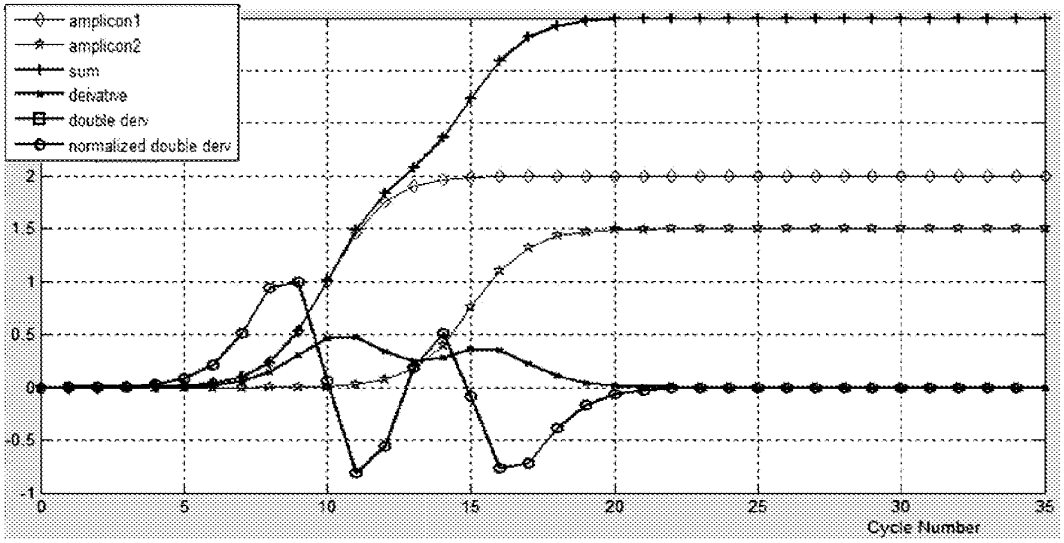

amplicon1
amplicon2
sum
derivative
double derv
normalized double derv
2
1.5
1
0.5
0
-0.5
-1
0
5
10
15
20
25
30
35
Cycle Number

FIG. 1C

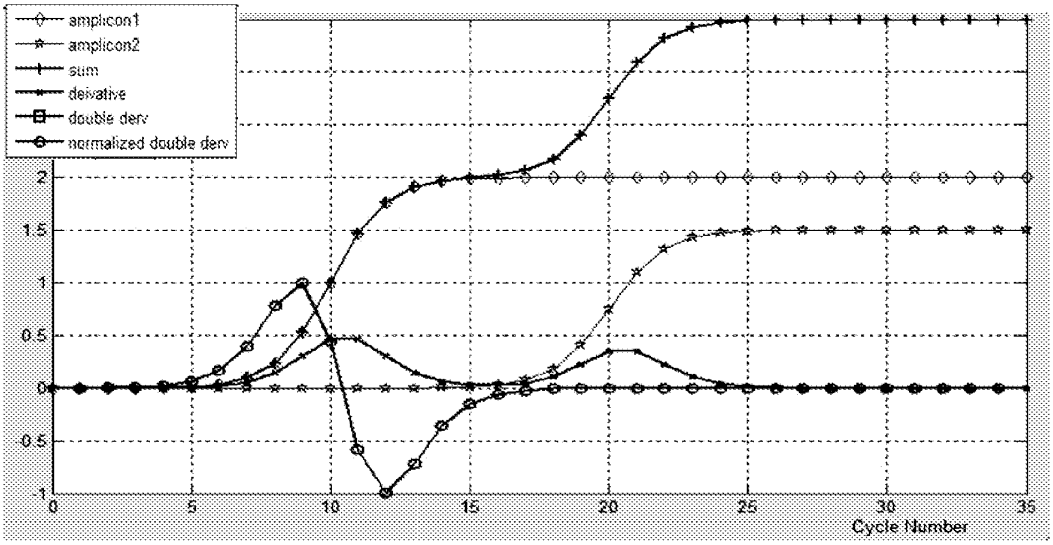

amplicon1
amplicon2
sum
derivative
double derv
normalized double derv
2
1.5
1
0.5
0
-0.5
-1
0
5
10
15
20
25
30
35
Cycle Number

FIG. 1D

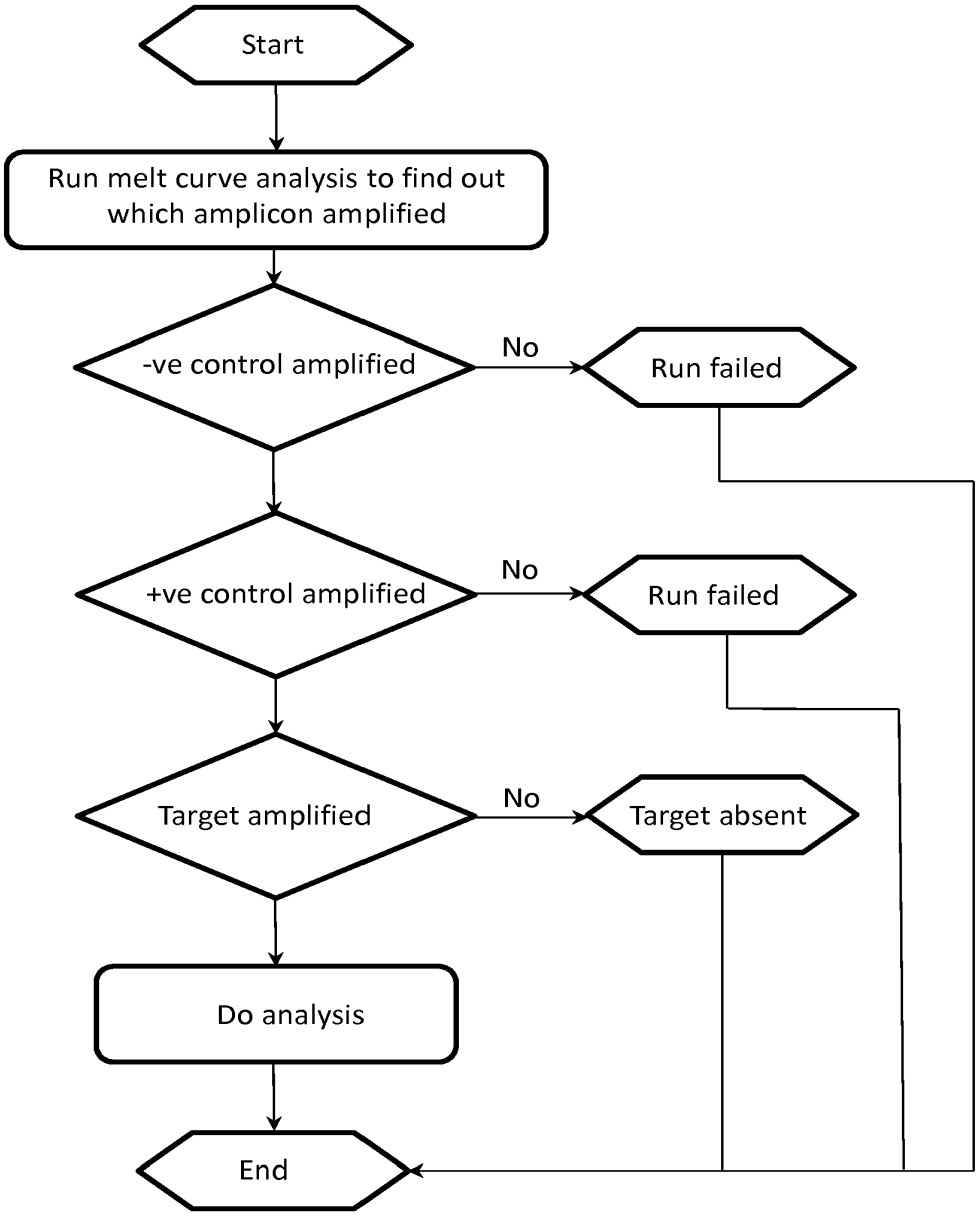

Start
Run melt curve analysis to find out which amplicon amplified
-ve control amplified
No
Run failed
+ve control amplified
No
Run failed
Target amplified
No
Target absent
Do analysis
End

FIG. 2

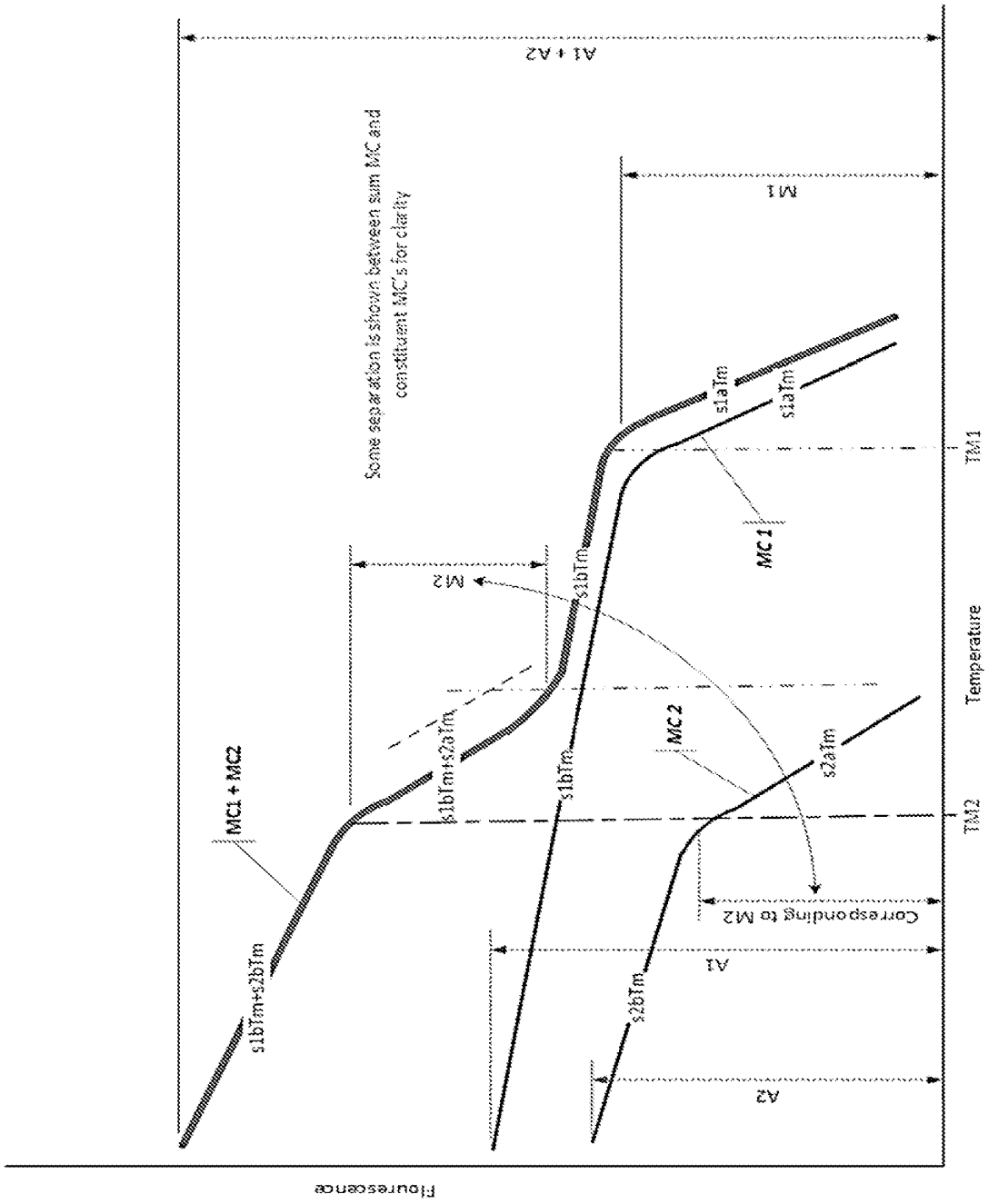

A1 + A2
Some separation is shown between sum MC and constituent MCs for clarity
M1
M2
s1aTm
s1aTm
s1bTm
s1bTm
MC1
MC2
MC1 + MC2
s1bTm+s2aTm
s1bTm+s2bTm
s2aTm
s2bTm
TM1
TM2
Temperature
Corresponding to M2
A1
A2
Fluorescence

FIG 3

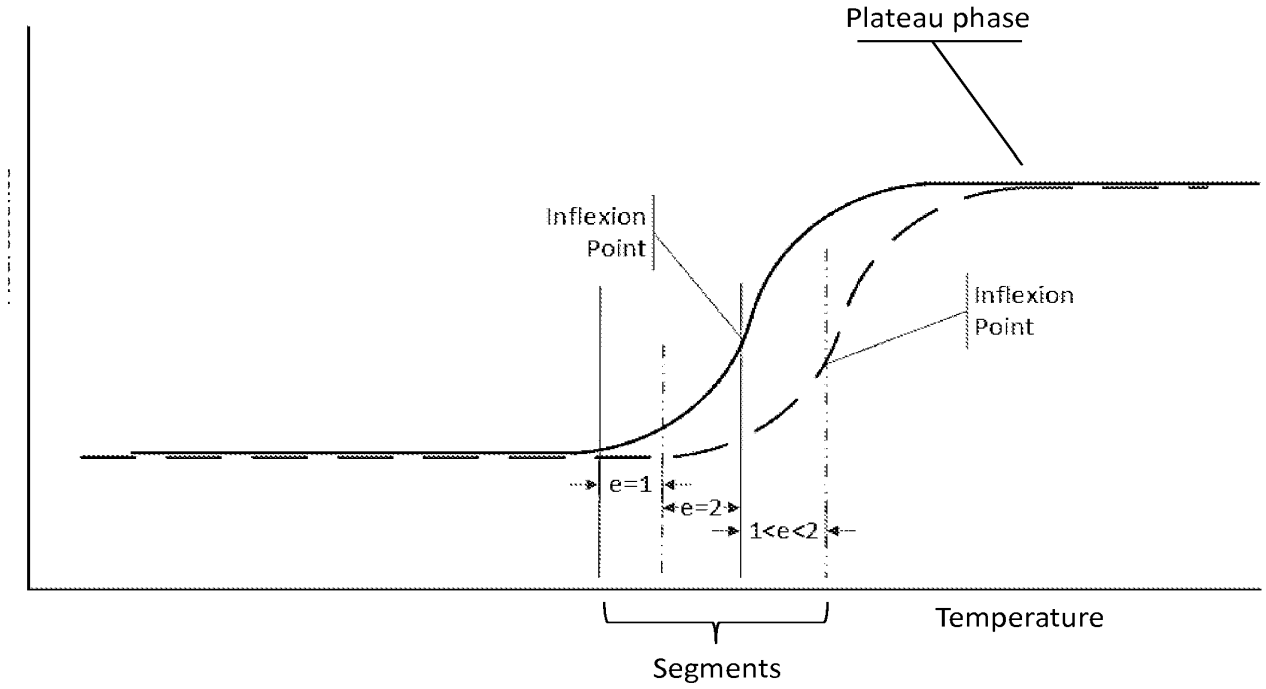

Plateau phase
Inflexion Point
Inflexion Point
e=1
e=2
1<e<2
Temperature
Segments

FIG. 4

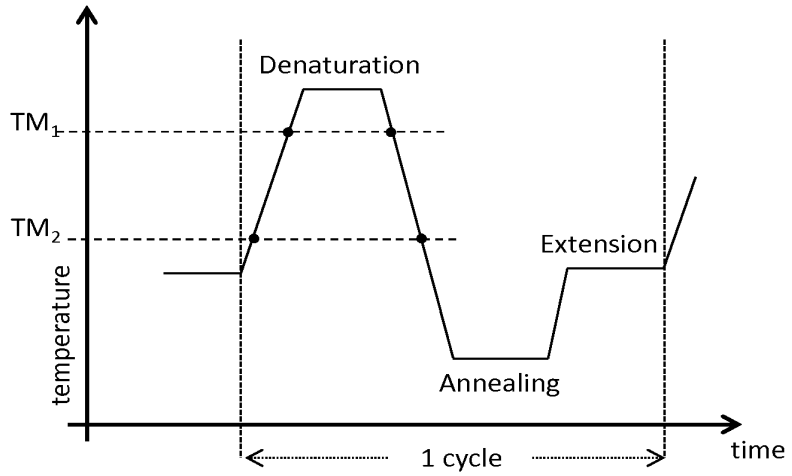

Denaturation
TM1
TM2
Extension
temperature
Annealing
1 cycle
time

FIG. 5A

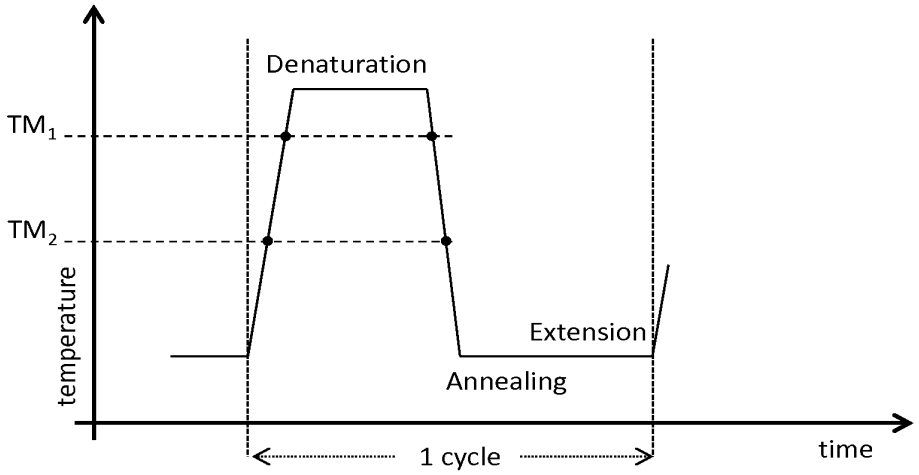

Denaturation
TM1
TM2
temperature
Extension
Annealing
1 cycle
time

FIG. 5B

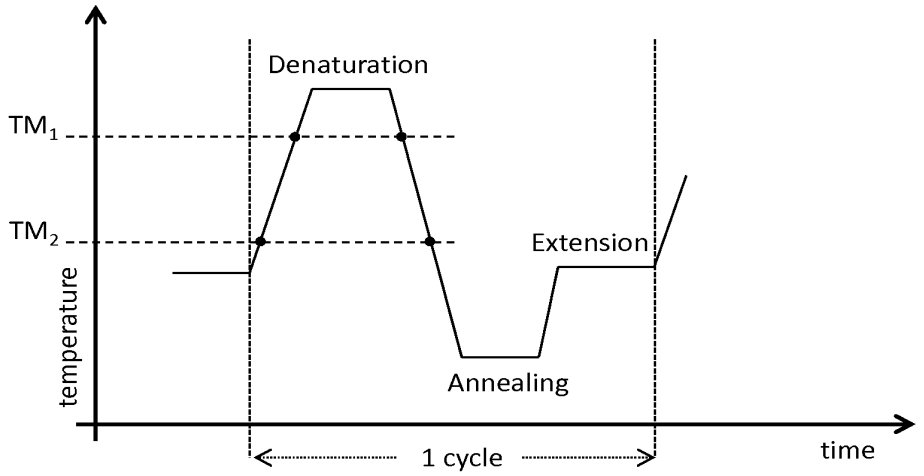

Denaturation
TM1
TM2
Extension
temperature
Annealing
1 cycle
time

FIG. 6A

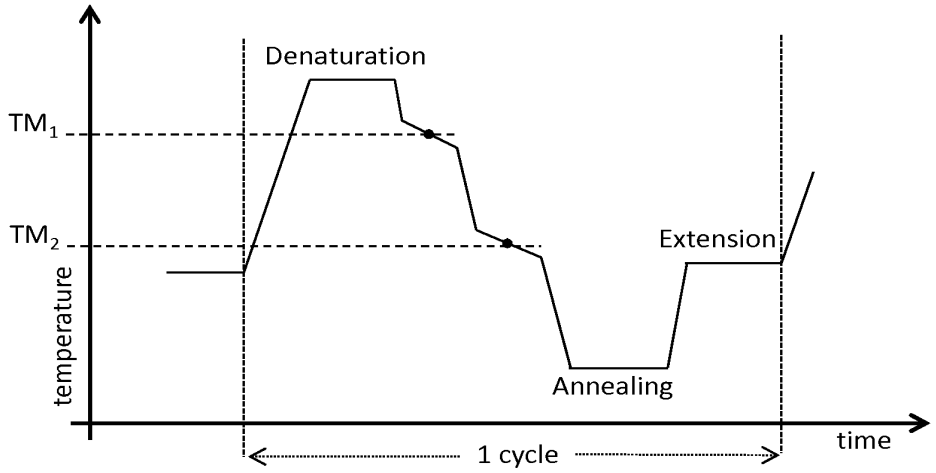

Denaturation
TM1
TM2
Extension
temperature
Annealing
1 cycle
time

FIG. 6B

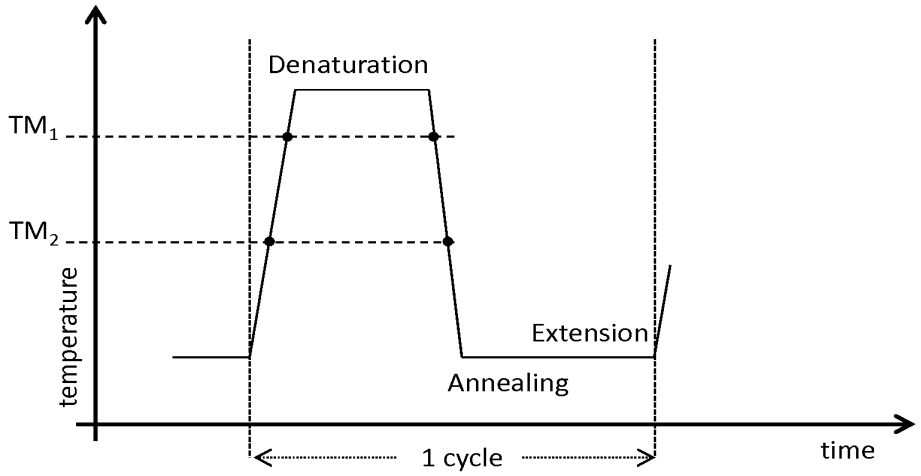

Denaturation
TM1
TM2
temperature
Extension
Annealing
1 cycle
time

FIG. 7A

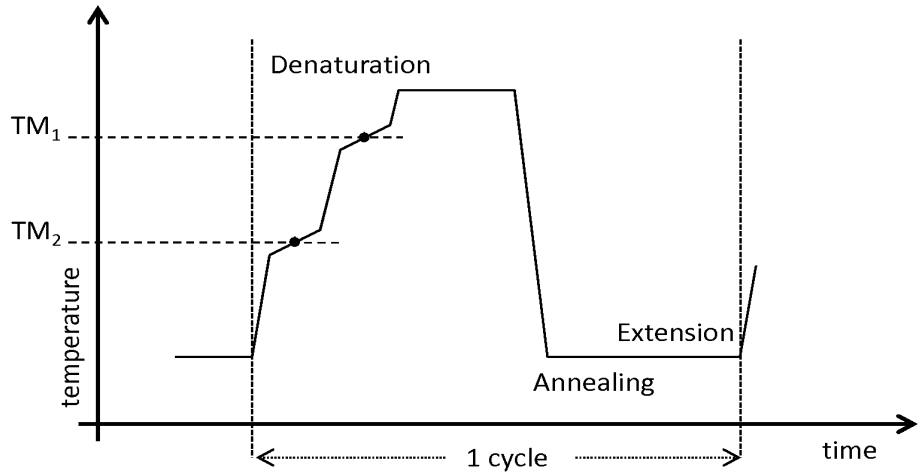

Denaturation
TM1
TM2
temperature
Extension
Annealing
1 cycle
time

FIG. 7B

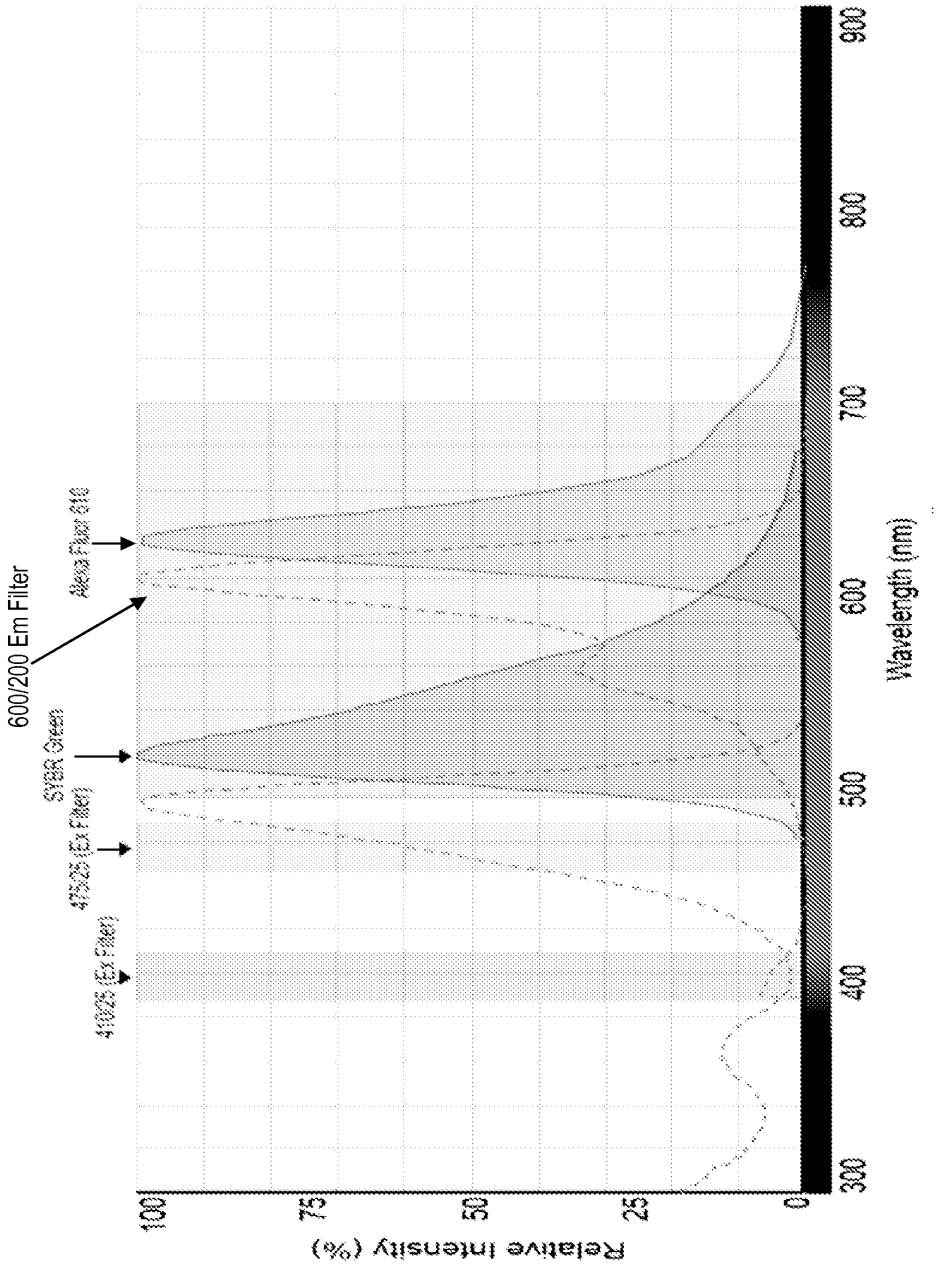

Relative Intensity (%)
100
75
50
25
0
300
400
500
600
700
800
900
Wavelength (nm)
410/25 (Ex Filter)
475/25 (Ex Filter)
SYBR Green
600/200 Em Filter
Alexa Fluor 610

FIG. 8

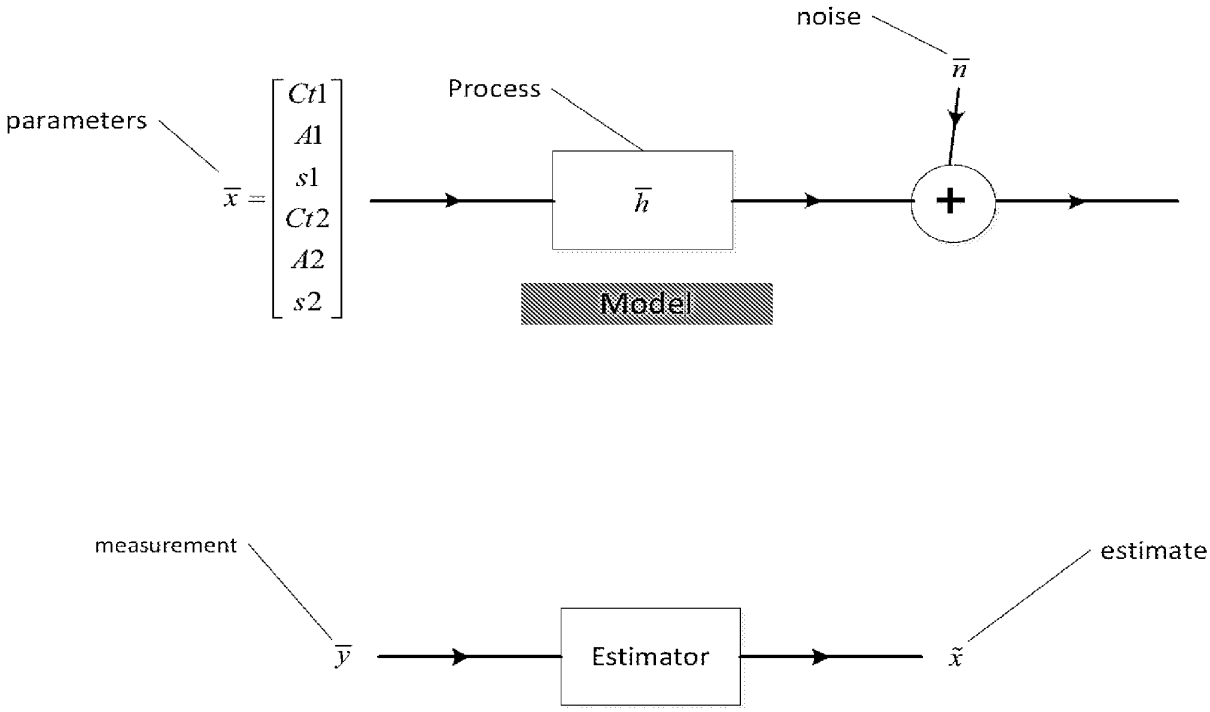
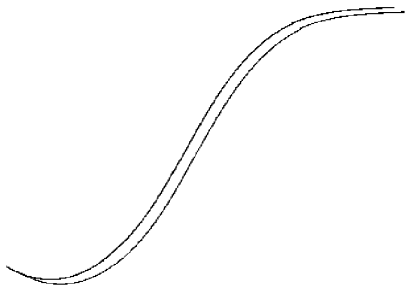
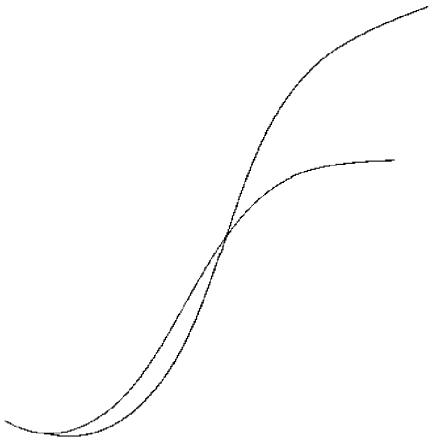

noise
$\overline{n}$
parameters
Process
$\overline{x} = \begin{bmatrix} Ct1 \\ A1 \\ s1 \\ Ct2 \\ A2 \\ s2 \end{bmatrix}$
$\overline{h}$
+
Model
measurement
estimate
$\overline{y}$
Estimator
$\tilde{x}$

FIG. 9



Amplitude normalized:
These two amplification curves with same Ct should overlap for minimum cumulative point wise distance.



Amplitude not normalized:
These two amplification curves with same Ct are not at correct location for minimum cumulative point wise distance.

FIG. 10

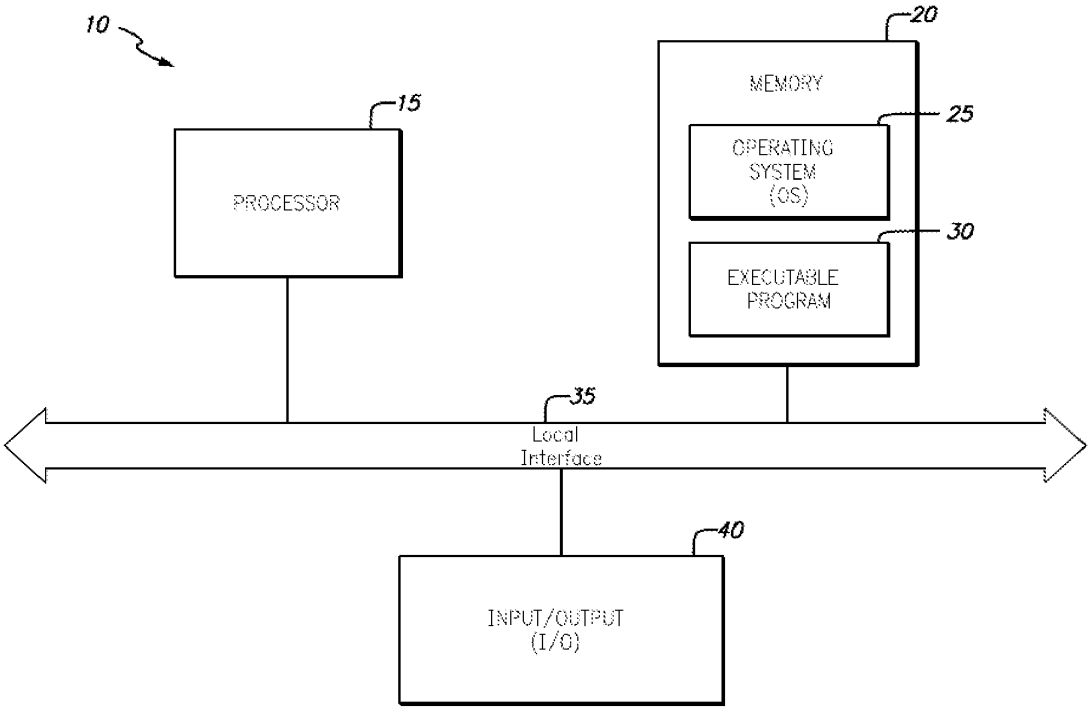

10
15
PROCESSOR
20
MEMORY
25
OPERATING SYSTEM (OS)
30
EXECUTABLE PROGRAM
35
Local Interface
40
INPUT/OUTPUT (I/O)

FIG. 11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/053512

A. CLASSIFICATION OF SUBJECT MATTER

C12Q 1/68(2006.01)i, C12M 1/38(2006.01)i, C12N 15/11(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q 1/68; C12M 1/00; G06F 19/00; G01N 21/64; C12P 19/34; G06F 19/10; C12M 1/34; C12M 1/38; C12N 15/11

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords: amplicons, multiplexed amplification, single channel detector, sum amplitude signal, processor-based hardware analyzer

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	FU et al., 'Multiplex detection and SNP genotyping in a single fluorescence channel' PLoS One, Vol.7, Issue 1, Article No.e30340, pp.1-8 (January 2012) See the whole document.	1-12,18-19,33-39
A	US 2011-0223602 A1 (WHITMAN et al.) 15 September 2011 See the whole document.	1-12,18-19,33-39
A	US 2010-0324834 A1 (TREPTOW et al.) 23 December 2010 See the whole document.	1-12,18-19,33-39
A	US 2012-0040352 A1 (WANGH et al.) 16 February 2012 See the whole document.	1-12,18-19,33-39
A	US 7507575 B2 (BEDINGHAM et al.) 24 March 2009 See the whole document.	1-12,18-19,33-39
A	US 2012-0101740 A1 (ORPANA et al.) 26 April 2012 See the whole document.	1-12,18-19,33-39

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 October 2013 (16.10.2013)	**16 October 2013 (16.10.2013)**

Name and mailing address of the ISA/KR
Korean Intellectual Property Office
189 Cheongsa-ro, Seo-gu, Daejeon Metropolitan City, 302-701, Republic of Korea
Facsimile No. +82-42-472-7140

Authorized officer
KIM Seung Beom
Telephone No. +82-42-481-3371

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/053512

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 14-17,23-26
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
Claims 14-17 and 23-26 are unclear since they are referring to the multiple dependent claims which do not comply with PCT Rule 6.4(a).

3. ☒ Claims Nos.: 13,20-22,27-32
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required addtional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
☐ No protest accompanied the payment of additional search fees.

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/US2013/053512

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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California Institute of Technology - Ex. 2003 Page 1955

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/US2013/053512

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		WO 2010-128206 A1	11/11/2010

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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

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31 July 2014 (31.07.2014)

WIPO | PCT

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23 January 2014 (23.01.2014)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/756,343 24 January 2013 (24.01.2013) US

(71) Applicant: **CALIFORNIA INSTITUTE OF TECHNOLOGY** [US/US]; 1200 E. California Blvd., Pasadena, CA 91125 (US).

(72) Inventors: **RAJAGOPAL, Aditya**; 27 Lee, Irvine, CA 92620 (US). **GOLDBERG, Mark, D.**; 7027 Margo Street, Alta Loma, CA 91701 (US). **GARCIA, Erika, F.**; 1615 Yosemite Dr. #102, Los Angeles, CA 90041 (US). **MADERO, Xiomara, L.**; 401 Roads End Rd., Glendale, CA 91205 (US). **TOMBRELLO, Thomas, A.**; 2938 Santa Rosa Avenue, Altadena, CA 91001 (US). **SCHERER, Axel**; 1661 North Road, Barnard, VT 05031 (US).

(74) Agents: **STOPAK, Kimberly** et al.; WILSON SONSINI GOODRICH & ROSATI, 650 Page Mill Road, Palo Alto, CA 94304-1050 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

WO 2014/116884 A1

(54) Title: CHROMOPHORE-BASED CHARACTERIZATION AND DETECTION METHODS

(57) Abstract: This disclosure provides methods, compositions and kits for the detection of a plurality of analytes in a sample. In some examples, this disclosure provides methods, compositions, and kits for detecting analytes, genetic variations, monitoring reaction process, and monitoring analyte-analyte interactions by measuring signals. In some examples, the presence of signals or changes in signals may be used to construct signal profiles which can be used to determine the presence or absence of analytes.

CHROMOPHORE-BASED CHARACTERIZATION AND DETECTION METHODS

CROSS-REFERENCE

[0001] This application claims the benefit of U.S. provisional application 61/756,343, filed January 24, 2013, which is incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

[0002] Nucleic acid analyte identification is a critical procedure in a variety of biomedical applications, such as in research and clinical diagnostic environments. Identification of an analyte is primarily done by sequencing or by amplification-based detection. For example, in the latter scheme, the polymerase chain reaction is often used to increase the quantity of the nucleic acid analyte present. Then, the nucleic acid analytes are discriminated using one of several additional techniques including fluorescence intensity measurement (e.g., fluorescent probes or intercalating dyes), length discrimination (e.g., using gel electrophoresis or melt curve analysis), or chromatography (e.g., haptin-based nucleic acid capture). Thus, current amplification-based detection technology indirectly detects analytes and requires a secondary technique (such as gel electrophoresis or mass spectroscopy) for analyte detection. Amplification or polymerization-based techniques that directly detect analytes would improve efficiency, time and cost.

SUMMARY OF THE INVENTION

[0003] Disclosed herein are methods, compositions, and kits for detecting analytes, particularly polynucleotides and/or polypeptides. The methods generally involve using oligonucleotides (e.g., primers, probes) attached to chromophores (e.g., fluorophores, quenchers, etc.) in amplification or polymerization reactions in order to detect a polynucleotide analyte. In some embodiments, provided herein are methods of detecting at least one polynucleotide analyte in a sample, comprising: (a) combining the sample with a first primer and a first oligonucleotide, wherein a first chromophore is attached to the first primer, a second chromophore is attached to the first oligonucleotide, the first primer and the first oligonucleotide are specific for a first polynucleotide analyte and the first chromophore is different from the second chromophore; (b) measuring a first signal generated by the first and second chromophores; (c) performing at least one polymerization reaction with the first primer using the first polynucleotide analyte as a template; and (d) measuring a second signal

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generated by the first and second chromophores; wherein the first and second signals are used to detect the first polynucleotide analyte.

[0004] In some cases, the first oligonucleotide is a second primer. In some cases, the first chromophore is attached to the 5' end of the first primer. In some cases, the first chromophore is an inorganic or organic dye, a fluorophore or a quencher. In some cases, the first chromophore is a fluorophore. In some cases, the fluorophore is 6-FAM (Fluorescein), 6-FAM (NHS Ester), Fluorescein dT, HEX, JOE (NHS Ester), MAX, TET, ROX, TAMRA, TARMA (NHS Ester), TEX 615, ATTO 488, ATTO 532, ATTO 550, ATTO 565, ATTO Rho101, ATTO 590, ATTO 633, ATTO 647N, TYE 563, TYE 665 or TYE 705. In some cases, the second chromophore is attached to the 5' end of the second primer. In some cases, the second chromophore is an inorganic or organic dye, a fluorophore or a quencher. In some cases, the second chromophore is a quencher. In some cases, the quencher is Iowa Black FG, Iowa Black RG, BHQ1, BHQ2 or BHQ3. In some cases, the first chromophore is a fluorophore and the second chromophore is a quencher.

[0005] In some cases, the methods described herein further comprise comparing the first and second signals, wherein a change in the first and second signals indicates the presence of the first polynucleotide analyte. In some cases, the change in the first and second signals is a decrease in fluorescent intensity. In some cases, the change is an increase in intensity. In some cases, the decrease in fluorescent intensity is at least about a 30% decrease in signal (or at least about 10%, 20%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, or 75%). In some cases, the second signal is measured after a second polymerization reaction. In some cases, the polymerization reaction is a polymerase chain reaction process or an isothermal process. In some cases, the polymerase chain reaction process is an end-point polymerase chain reaction process, a real-time polymerase chain reaction process, a digital polymerase chain reaction process, a droplet digital polymerase chain reaction process, or a quantitative polymerase chain reaction process. In some cases, the first primer is a forward primer and the second primer is a reverse primer. In some cases, the first primer is a reverse primer and the second primer is a forward primer. In some cases, the first and second chromophores interact through an electron-transfer process. In some cases, the first polynucleotide analyte is from about 10 to about 500 nucleotides in length. In some cases, the concentration of the first polynucleotide analyte is from about 10μM to about 10aM. In some cases, the first polynucleotide analyte is a DNA polynucleotide analyte. In some cases, the first polynucleotide analyte is an RNA polynucleotide analyte.

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[0006] In some cases, the first polynucleotide analyte comprises a genetic variation. In some cases, the genetic variation comprises a substitution, an addition, a deletion or a translocation. In some cases, the genetic variation comprises a single-nucleotide polymorphism (SNP). In some cases, the at least one polynucleotide analyte is from a source selected from a human, a non-human mammal, a plant, a bacteria, a fungus, an archaea, a parasite, or a virus. In some cases, the virus is a human immunodeficiency virus, an influenza type A virus, an influenza type B virus, a respiratory syncytial virus type A (RsvA), a respiratory syncytial virus type B virus (RsvB), a human rhinovirus (Hrv), a human metapneumovirus (Hmpv) or a human parainfluenza virus type 3 (PIV-3). In some cases, the sample is a forensic sample, a clinical sample, a food sample, an environmental sample, a pharmaceutical sample, or a sample from a consumer product. In some cases, the methods disclosed herein further comprising detecting at least one additional polynucleotide analyte within the sample with an additional fluorophore attached to a primer, wherein the first chromophore is a fluorophore with the same color as the additional fluorophore. In some cases, the first primer is not attached to two or more chromophores. In some cases, the first oligonucleotide is not attached to two or more chromophores. In some cases, step b) of the method further comprises measuring the first signal at a first denaturing step and a third signal at a first annealing step; step d) further comprises measuring the second signal at a second denaturing step and a fourth signal at a second annealing step; and the method further comprises comparing the first signal with the second signal to obtain a first ratio and comparing the third signal with the fourth signal to obtain a second ratio; wherein when the first ratio is about 1 and the second ratio is greater than or less than 1 indicate the presence of the first polynucleotide analyte.

[0007] In some cases, step a) of the method described herein further comprises combining the sample with a third primer and a second oligonucleotide, wherein a third chromophore is attached to the third primer and a fourth chromophore is attached to the second oligonucleotide , and the third primer and the second oligonucleotide are specific for a second polynucleotide analyte; step b) further comprises measuring a fifth signal generated by the third and fourth chromophores; step d) further comprises measuring a sixth signal generated by the third and fourth chromophores; wherein the fifth and sixth signals are used to detect the second polynucleotide analyte. In some cases, the methods disclosed herein further comprising comparing the fifth and sixth signals, wherein a change in the fifth and

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sixth signals indicates the presence of the second polynucleotide analyte. In some cases, the second oligonucleotide is a fourth primer.

[0008] Also disclosed herein are methods of detecting at least one genetic variation in an analyte comprising: (a) combining a first analyte with a first primer and a second primer, wherein a first chromophore is attached to the first primer, a second chromophore is attached to the second primer, at least one of the first and the second primers are specific for a first genetic variation in the first analyte and the first chromophore is different from the second chromophore; (b) measuring a first signal generated by the first and second chromophores; (c) performing at least one polymerization reaction with the first primer and the second primer using the first analyte as a template; and (d) measuring a second signal generated by the first and second chromophores; wherein the first and second signals are used to detect the first genetic variation in the first analyte. In some cases, the methods described herein further comprise comparing the first and second signals, wherein a change in the first and second signals indicates the presence of the genetic variation in the first analyte.

[0009] In some cases, the analyte is a polynucleotide analyte. In some cases, the genetic variation comprises a substitution, an addition, a deletion or a translocation. In some cases, the genetic variation comprises a single-nucleotide polymorphism (SNP). In some cases, the first primer comprises a sequence encoding the SNP or the first primer binds to a region of the analyte encoding the SNP. In some cases, the second primer comprises a sequence not encoding the SNP or the second primer comprises a sequence complementary to a region of the analyte not encoding the SNP. In some cases, the first primer encodes a region of the analyte less than 500 base pairs apart from a region of the analyte encoded by the second primer. In some cases, the change in signal is distinct for at least two of the mismatched base pairs selected from the group consisting of UU, UT, UG, UC, UA, AA, TT, GG, CC, AG, AC, TG and TC. In some cases, the change in signal from a mismatched base pair is distinct from a change in signal from a complementary base pair.

[0010] In some cases, step a) of the methods described herein further comprises combining the first analyte with a third primer and a fourth primer, wherein a third chromophore is attached to the third primer, a fourth chromophore is attached to the fourth primer, the third and the fourth primers are specific for a second genetic variation in the first analyte and the third chromophore is different from the fourth chromophore; step b) further comprises measuring a third signal; step d) further comprises measuring a fourth signal; and the method further comprises comparing the third and fourth signals; wherein a change in the

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third and fourth signals indicates the presence of the second single genetic variation in the first analyte. In some cases, step a) of the method further comprises combining a second analyte with a third primer and a fourth primer, wherein a third chromophore is attached to the third primer, a fourth chromophore is attached to the fourth primer comprises, the third and the fourth primers are specific for a second genetic variation in the second analyte and the third chromophore is different from the fourth chromophore; step b) further comprises measuring a third signal; step d) further comprises measuring a fourth signal; and the method further comprises comparing the third and fourth signals; wherein a change in the third and fourth signals indicates the presence of the second single genetic variation in the second analyte. In some cases, step a) further comprises combining a second analyte with a third primer and a fourth primer, wherein the first chromophore is attached to the third primer, the second chromophore is attached to the fourth primer, and the third and the fourth primers are specific for a second genetic variation in the second analyte. In some cases, the polymerization reaction is a PCR process or an isothermal reaction. In some cases, the PCR process is an end-point PCR process, a digital PCR process, a real-time PCR process, a droplet digital PCR process, or a quantitative PCR process. In some cases, the polymerization reaction is a quantitative PCR process. In some cases, the detecting comprises a quantitative PCR method. In some cases, the detecting comprises a quantitative PCR method and a second method. In some cases, the second method is a digital PCR process. In some cases, at least one SNP is detected in a gene. In some cases, at least one SNP is associated with a disease. In some cases, the disease is a genetic disorder, an autoimmune disease, a neurological disease, a cardiovascular disease, or a cancer.

[0011] Also disclosed herein are methods of detecting a plurality of analytes, comprising: a) combining the plurality of analytes with a plurality of primer pairs, wherein each primer pair is specific to a single analyte and each primer of the primer pair is attached to at least one chromophore; b) measuring a first set of signals generated by the chromophores; c) performing at least one polymerization reaction with the plurality of primer pairs using the plurality of analytes as templates; and d) measuring a second set of signals generated by the chromophores; wherein the first and second set of signals are used to detect each analyte of the plurality of analytes. In some cases, the method described herein further comprises: e) repeating step c and d at least once; and f) generating a set of signature profiles; wherein the presence of each analyte of the plurality of analytes is detected by comparing the set of signature profiles to a control set of signature profiles.

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[0012] In some cases, the signature profile is an end-point signature profile or a signature curve. In some cases, the plurality of analytes has different lengths. In some cases, each of the plurality of primer pairs comprises a forward primer and a reverse primer. In some cases, one, two or more chromophores are attached to the forward primer. In some cases, one chromophore is attached to the 5' end of the forward primer. In some cases, at least one chromophore is attached to the reverse primer. In some cases, one chromophore is attached to the 5' end of the reverse primer. In some cases, the chromophore is an inorganic or organic dye, a fluorophore or a quencher. In some cases, the chromophore is a fluorophore. In some cases, the chromophore is a quencher.

[0013] Also disclosed herein are methods of generating a signature curve profile for a polynucleotide analyte, comprising: (a) contacting the polynucleotide analyte with a first primer and a second primer, wherein a first chromophore is attached to the first primer and a second chromophore is attached to the second primer, the first primer and the second primer are specific for the polynucleotide analyte and the first chromophore is different from the second chromophore; (b) measuring a first signal at a first temperature; (c) performing at least one polymerization reaction with the first primer and the second primer using the polynucleotide analyte as a template; (d) measuring a second signal at the first temperature; and (e) repeating step c and d at least once; wherein the signals create the signature curve profile of the polynucleotide analyte. In some cases, the method disclosed herein further comprises: f) changing the temperature; g) measuring a third signal at a second temperature; and h) repeating steps f and g at least once; wherein the signals create the signature curve profile of the polynucleotide analyte.

[0014] In some cases, the signature curve is a length curve, a morphology curve, a melt curve, or a SNP curve. In some cases, the polymerization reaction is a polymerase chain reaction process. In some cases, the polymerase chain reaction process is an end-point polymerase chain reaction process, a real-time polymerase chain reaction process, a digital polymerase chain reaction process or a quantitative polymerase chain reaction process. In some cases, the first chromophore is an inorganic or organic dye, a fluorophore or a quencher. In some cases, the first chromophore is a fluorophore. In some cases, the fluorophore is 6-FAM (Fluorescein), 6-FAM (NHS Ester), Fluorescein dT, HEX, JOE (NHS Ester), MAX, TET, ROX, TAMRA, TARMA (NHS Ester), TEX 615, ATTO 488, ATTO 532, ATTO 550, ATTO 565, ATTO Rho101, ATTO 590, ATTO 633, ATTO 647N, TYE 563, TYE 665 or TYE 705. In some cases, the second chromophore is an inorganic or organic

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dye, a fluorophore or a quencher. In some cases, the second chromophore is a quencher. In some cases, the quencher is Iowa Black FG, Iowa Black RG, BHQ1, BHQ2 or BHQ3. In some cases, the polynucleotide analyte is a DNA polynucleotide analyte. In some cases, the polynucleotide analyte is an RNA polynucleotide analyte. In some cases, the polynucleotide analyte is from a source selected from the group consisting of a human, a non-human mammal, a plant, a bacteria, an archaea, a fungus, a parasite, and a virus. In some cases, the virus is a human immunodeficiency virus, an influenza type A virus, an influenza type B virus, a respiratory syncytial virus type A (RsvA), a respiratory syncytial virus type B virus (RsvB), a human rhinovirus (Hrv), a human metapneumovirus (Hmpv) or a human parainfluenza virus type 3 (PIV-3).

[0015] Also disclosed herein is a method of monitoring an amplification reaction comprising: (a) contacting a first polynucleotide analyte with: i) a first primer and a second primer, wherein the first primer and the second primer are specific for the first polynucleotide analyte; and ii) at least two chromophores capable of specific incorporation into a product amplified from the first polynucleotide analyte; (b) subjecting the combination in step (a) to a temperature capable of denaturing double-stranded DNA; (c) measuring a first signal in step (b) generated by the at least two chromophores; (d) subjecting the combination in step b) to a temperature capable of annealing polynucleotides; (e) measuring a second signal in step (d) generated by the at least two chromophores; and (f) repeating steps b) – e) to obtain a third signal and a fourth signal; and (g) comparing the first and the third signals to obtain a first ratio and the second and the fourth signals to obtain a second ratio; wherein an amplification reaction occurs when the first ratio is about 1 and the second ratio is greater than or less than 1. In some cases, at least one chromophore is attached to the first primer, for example exactly one chromophore may be attached to the first primer. In some cases, one chromophore (or exactly one chromophore) is attached to the 5' end of the first primer. In some cases, one, two or more chromophores are attached to the second primer. In some cases, one chromophore is attached to the 5' end of the second primer. In some cases, the chromophore is an inorganic or organic dye, a fluorophore or a quencher.

[0016] Also disclosed herein are methods of detecting a morphology of an analyte, comprising: (a) providing a sample comprising the analyte, wherein a first chromophore, a second chromophore and a third chromophore are attached to the analyte, wherein the first, second and third chromophores are different; (b) measuring a signal from the first chromophore, second chromophore and the third chromophore at a first and second

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temperature, wherein the first and second temperatures are different; and (c) using the measured signals to detect the morphology of the analyte. In some cases, the analyte is a protein, a polypeptide, a lipid or a polynucleotide. In some cases, the analyte is a polynucleotide. In some cases, the polynucleotide is a DNA polynucleotide. In some cases, the polynucleotide is an RNA polynucleotide. In some cases, the first chromophore is an inorganic or organic dye, a fluorophore or a quencher. In some cases, the first chromophore is a first fluorophore. In some cases, the first fluorophore is 6-FAM (Fluorescein), 6-FAM (NHS Ester), Fluorescein dT, HEX, JOE (NHS Ester), MAX, TET, ROX, TAMRA, TARMA (NHS Ester), TEX 615, ATTO 488, ATTO 532, ATTO 550, ATTO 565, ATTO Rho101, ATTO 590, ATTO 633, ATTO 647N, TYE 563, TYE 665 or TYE 705. In some cases, the second chromophore is an inorganic or organic dye, a fluorophore or a quencher. In some cases, the second chromophore is a second fluorophore. In some cases, the second fluorophore is 6-FAM (Fluorescein), 6-FAM (NHS Ester), Fluorescein dT, HEX, JOE (NHS Ester), MAX, TET, ROX, TAMRA, TARMA (NHS Ester), TEX 615, ATTO 488, ATTO 532, ATTO 550, ATTO 565, ATTO Rho101, ATTO 590, ATTO 633, ATTO 647N, TYE 563, TYE 665 or TYE 705. In some cases, the third chromophore is an inorganic or organic dye, a fluorophore or a quencher. In some cases, the third chromophore is a quencher. In some cases, the quencher is Iowa Black FG, Iowa Black RG, BHQ1, BHQ2 or BHQ3. In some cases, the analyte comprises more than one fluorophore. In some cases, the analyte comprises more than one quencher. In some cases, the polynucleotide comprises a quencher at its 3' end.

[0017] Also disclosed herein are methods of detecting a polynucleotide analyte comprising: (a) combining the polynucleotide analyte with at least two chromophores, wherein the at least two chromophores are each attached to a separate polynucleotide that is complementary to a region within the polynucleotide analyte; (b) performing at least one polymerization reaction to incorporate the at least two chromophores into products of the polymerization reaction; and (c) detecting a fluorescent intensity from the at least two chromophores at a first timepoint and a second timepoint, wherein the second timepoint is later than the first timepoint and wherein a change (particularly a decrease) in fluorescent intensity at the second timepoint relative to the first timepoint is indicative of the presence of the polynucleotide analyte. In some cases, the polymerization reaction is a polymerase chain reaction. In some cases, the first timepoint is after step a) and the second timepoint is after step b). In some cases, the products of the at least one polymerization reaction each comprise

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a first polynucleotide strand and a second polynucleotide strand, wherein the first polynucleotide strand and the second polynucleotide strand are complementary. In some cases, the at least two chromophores are different. In some cases, the at least two chromophores comprise a fluorophore. In some cases, the at least two chromophores comprise a fluorophore and a quencher. In some cases, the fluorophore is incorporated into the first polynucleotide strand and the quencher is incorporated into the second polynucleotide strand. In some cases, the fluorophore is incorporated at the 5' end of first polynucleotide strand and the quencher is incorporated at the 5' end of the second polynucleotide strand.

[0018] Also disclosed herein are compositions and kits, particularly for detecting polynucleotide or polypeptide analytes. In some cases, the kits comprise oligonucleotides (e.g., primers, probes, etc.) attached to a chromophore (e.g., fluorophore, quencher). In some cases, the kit comprises: (a) a first primer or probe attached to a fluorophore; and (b) a second primer or probe attached to a quencher. In some cases, the kit comprises: (a) a first primer or probe attached to exactly one first chromophore, wherein the first chromophore is a fluorophore; and (b) a second primer or probe attached to exactly one second chromophore, wherein the first chromophore is different from the second chromophore. In some cases, the second chromophore is a quencher. In some cases, the second chromophore is a fluorophore. In some cases, the first primer or probe is a primer. In some cases, the second primer or probe is a primer. In some cases, the first primer or probe comprises an oligonucleotide sequence that is complementary to the sequence of a target analyte. In some cases, the second primer or probe comprises an oligonucleotide sequence complementary to the sequence of a target analyte. In some cases, the fluorophore is 6-FAM (Fluorescein), 6-FAM (NHS Ester), Fluorescein dT, HEX, JOE (NHS Ester), MAX, TET, ROX, TAMRA, TARMA (NHS Ester), TEX 615, ATTO 488, ATTO 532, ATTO 550, ATTO 565, ATTO Rho101, ATTO 590, ATTO 633, ATTO 647N, TYE 563, TYE 665 or TYE 705. In some cases, the quencher is Iowa Black FG, Iowa Black RG, BHQ1, BHQ2 or BHQ3. In some cases, the kit comprises at least three primers, wherein each primer is attached to a different chromophore.

[0019] In some cases, the kit comprises: (a) a first primer or probe attached to a first chromophore, wherein the first chromophore is a fluorophore; and (b) a second primer or probe attached to a second chromophore, wherein the first chromophore is different from the second chromophore. In some cases, the kit comprises: (a) a first primer or probe attached to exactly one first chromophore; and (b) a second primer or probe attached to exactly one

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second chromophore, wherein the first chromophore is different from the second chromophore. In some cases, the kit comprises: (a) a first primer or probe attached to exactly one first chromophore, wherein the first chromophore is a fluorophore; and (b) a second primer or probe attached to exactly one second chromophore, wherein the first chromophore is different from the second chromophore. In some cases, the second chromophore is a quencher. In some cases, the second chromophore is a fluorophore. In some cases, the first primer or probe is a primer. In some cases, the second primer or probe is a primer. In some cases, the first primer or probe comprises an oligonucleotide sequence that is complementary to the sequence of a target analyte. In some cases, the second primer or probe comprises an oligonucleotide sequence complementary to the sequence of a target analyte. In some cases, the fluorophore is 6-FAM (Fluorescein), 6-FAM (NHS Ester), Fluorescein dT, HEX, JOE (NHS Ester), MAX, TET, ROX, TAMRA, TARMA (NHS Ester), TEX 615, ATTO 488, ATTO 532, ATTO 550, ATTO 565, ATTO Rho101, ATTO 590, ATTO 633, ATTO 647N, TYE 563, TYE 665 or TYE 705. In some cases, the quencher is Iowa Black FG, Iowa Black RG, BHQ1, BHQ2 or BHQ3. In some cases, the kit comprises at least three primers, wherein each primer is attached to a different chromophore.

INCORPORATION BY REFERENCE

[0020] All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

[0021] Various aspects of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

[0022] **Fig. 1** exemplifies a mechanism for detection of a nucleic acid analyte using chromophore-attached primers (a fluorophore-attached forward (FWD) primer and a quencher-attached reverse (or rewind (RWD)) primer) during a polymerase chain reaction (PCR). Figs. 1A-1B illustrate the denaturation of a double-stranded polynucleotide analyte. Figs. 1C-1D illustrate the annealing of the chromophore-attached primers to opposite strands

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of the analyte and for a polymerase to extend the primers during a PCR reaction. Figs. 1E-1F illustrate the formation of a double-stranded PCR products containing both chromophores (fluorophore and a quencher) which leads to the generation of a signal (e.g., quenching of fluorescence).

[0023] **Fig. 2** exemplifies an electron transfer state. Fig. 2A illustrates an energy transfer formula. Fig. 2B illustrates a Jablonski diagram showing Förster resonance energy transfer with typical timescales indicated.

[0024] **Fig. 3** illustrates a diagnostic protocol and treatment method for use with a detection method described herein.

[0025] **Fig. 4** illustrates a conceptual schematic of an exemplary computer server to be used for processing a method described herein.

[0026] **Fig. 5** exemplifies the detection of HIV TPP analytes at varying concentrations using quantitative PCR (qPCR). Fig. 5A illustrates the qPCR detection of 80bp HIV TPP analytes at 10nM concentration. Fig. 5B illustrates the qPCR detection of 80bp HIV TPP analytes at 1nM concentration. Fig. 5C illustrates the qPCR detection of 80bp HIV TPP analytes at 100pM concentration. Fig. 5D illustrates the qPCR detection of 80bp HIV TPP analytes at 10pM concentration. All successful detection curves exhibit the inverse-sigmoidal characteristic.

[0027] **Fig. 6** illustrates the sensitivity of detection of HIV TPP using an exemplary detection method. Fig. 6A illustrates the qPCR detection of 80bp HIV TPP analytes at 1pM concentration. Fig. 6B illustrates the qPCR detection of 80bp HIV TPP analytes at 100fM concentration. Fig. 6C illustrates the qPCR detection of 80bp HIV TPP analytes at 10fM concentration. Fig. 6D illustrates the qPCR detection of 80bp HIV TPP analytes at 1fM concentration.

[0028] **Fig. 7** shows that the extent of signal generation (quenching) is strongly correlated with the distance between a quencher and a fluorophore. Analytes were varied in length from 40bp to 120bp for a single set of primers. Fig. 7A illustrates the extent of quenching using a 40mer analyte. Fig. 7B illustrates the extent of quenching using a 60mer analyte. Fig. 7C illustrates the extent of quenching using a 80mer analyte.

[0029] **Fig. 8** illustrates the effect of a single nucleotide polymorphism on signal generation. Signal level changes significantly when a mis-priming event occurs (Fig. 8A and Fig. 8B).

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