



(51) International Patent Classification:
C12Q 1/68 (2006.01)

(21) International Application Number:
PCT/EP2013/051781

(22) International Filing Date:
30 January 2013 (30.01.2013)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
1201804.0 2 February 2012 (02.02.2012) GB
1203619.0 1 March 2012 (01.03.2012) GB

(71) Applicant: **PRIMER DESIGN LTD** [GB/GB]; The Mill
Yard, Nursling street, Southampton Hampshire SO15 0AJ
(GB).

(72) Inventor: **POWELL, Robert Michael**; The Mill Yard,
Nursling Street, Southampton Hampshire SO15 0AJ (GB).

(74) Agent: **LLOYD, John Scott**; Maschio & Soames LLP, 20
Carlton Crescent, Southampton Hampshire SO15 2ET
(GB).

(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, 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, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU,
RW, 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,
ML, MR, NE, SN, TD, TG).

Published:

- *with international search report (Art. 21(3))*
- *before the expiration of the time limit for amending the
claims and to be republished in the event of receipt of
amendments (Rule 48.2(h))*

(54) Title: METHOD FOR DETECTING AND GENOTYPING TARGET NUCLEIC ACID

(57) Abstract: In a first aspect, there is provided a method for determining the presence of a target nucleic acid in a sample, the method comprising: (i) performing a nucleic acid amplification using a reaction mixture comprising: (a) a nucleic acid polymerase; (b) one or more sets of forward and reverse primers capable of hybridising to the target nucleic acid; and (c) one or more oligonucleotide probes for detecting the target nucleic acid when present in the sample and wherein the oligonucleotide probe(s) has a predicted melting temperature that is higher than the predicted melting temperature of the one or more sets of forward and reverse primers(s); and wherein the cycling conditions during the nucleic acid amplification reaction comprise at least two different sets of cycling parameters, the first set of cycling parameters including an annealing temperature in the nucleic acid amplification reaction that is less than or about the same as the temperature corresponding to the predicted melting temperature of the lowest melting temperature probe; and the second set of cycling parameters including an annealing temperature in the nucleic acid amplification reaction that is equal to or higher than the temperature corresponding to the predicted melting temperature of the highest melting temperature probe; (ii) detecting the signal produced in step (i); and (iii) determining the presence or absence of the target nucleic acid based on the signal(s) produced in step (ii).



METHOD FOR DETECTING AND GENOTYPING TARGET NUCLEIC ACID

FIELD OF THE INVENTION

The present invention relates generally to a method for determining the presence of a target nucleic acid that may be present in a sample and more specifically to an assay for detecting or determining the genotype of an amplification product at two or more allelic sites in a nucleic acid.

BACKGROUND OF THE INVENTION

Nucleic acid amplification has become an increasingly popular technique in many medical and research settings. The ability to detect and identify a specific genotype is pivotal to understanding disease and protein function. Many diseases, such as cystic fibrosis, occur as a result of a single nucleotide polymorphism (SNP) or greater genetic alteration, such as a deletion, affecting one or more base pairs within a gene. Germline mutations affecting one or both alleles of the affected gene are found throughout the cell population making the target sequences more abundant and thus technically less challenging to identify. However, even heterozygous samples have previously required complex methods to reliably elucidate a genotype. Somatic mutations are even more difficult since the mutation may be present in a small percentage of the starting material, such as a mutation within a tumour. Sensitive genotyping solutions for such mixed populations are often inadequate and unable to obtain the sensitivity required for detection of a low abundance mutated sequence in a high background of wild type sequence.

Historically there have been a number of nucleic acid amplification based methods for elucidating the genotype of a sample. One very popular method is the Polymerase Chain Reaction (PCR). For detection of the amplified nucleic acid, competing fluorescently labelled probes are often used. The method requires amplification of the DNA sequence encompassing the mutation, with the same primers also amplifying the wild type sequence. Two or more probes labelled with spectrally distinct fluorophores are designed in the same position on the resultant amplicons such that the mutation is reflected in the probe sequence of one probe and the alternative probe pairs to the wild type sequence. When bound or in solution the fluorescence of the probes is quenched by a quencher molecule bound in close proximity to the fluorescent molecule itself. Taq polymerase not only drives the nucleic acid amplification but also contains 5' nuclease activity that cleaves the bound probe as it extends the complementary

sequence. This releases the fluorophore from the quencher and thus fluorescent emission can be detected. This assay is sometimes known as the 5' nuclease assay.

Typically, a 5' nuclease assay can include the following steps. A nucleic acid amplification reaction is performed on a target sequence using a nucleic acid polymerase having 5'→3' nuclease activity. Forward and reverse primers are also used that can hybridise to a target sequence in the presence of an oligonucleotide probe which is capable of hybridising to the target sequence that is amplified by primers. The binding site of the oligonucleotide probe, which can be sited on either strand is located downstream relative to the binding site for either of the forward or reverse amplification primer. The nucleic acid polymerase extends the forward and reverse amplification primers. During extension of the primer, the polymerase encounters the probe hybridized to the target sequence and degrades the probe. Digestion of the probe can result in the formation of a detectable signal from the probe. This creates a signal that is indicative of the extension of the primer and hence the amplification of the target sequence.

One problem with the 5' nuclease approach is that a single base change within a probe is often insufficient to confer complete specificity of probe binding. The results can therefore include strong detectable signals regardless of the genotype of the sample, which is not desirable. Although the genotype can often still be deduced, this can only be achieved by comparing the ratio of signals that are produced. In practice, commercial solutions use a modified approach to this method to improve discrimination. One approach involves artificially increasing the effective melting temperature of the probe by adding a duplex stabilising moiety to the probe. This enables the design of shorter probes that still have the desired melting temperature. Shorter probes can have better discrimination since a single mismatch base in a short probe has a greater effect on overall duplex stability than the same mismatch in a longer probe. Other approaches to enhancing stability include the use of a minor groove binding moiety and a quencher that has an alternative stabilising mechanism.

A need remains in the art for a method that is able to distinguish between multiple, substantially homologous sequences in a single nucleic acid amplification reaction that can be used on commonly available instrumentation and without the use of complex chemistries to improve discriminating properties. The invention described herein seeks to satisfy this need.

SUMMARY OF THE INVENTION

Nucleic acid probes have been designed to have a predicted melting temperature that is lower than conventionally used in the art and is closer to the predicted melting temperature of the

primers used in the assay. The probe(s) can be used together with an at least two stage cycling protocol in which the second stage of the cycling protocol utilises an annealing temperature that is higher than conventionally used in the art and is optimum for probe discrimination. In use, it has been surprisingly found that the probe(s) can achieve enhanced sensitivity in the detection of nucleic acids and/or enhanced levels of discrimination between nucleic acids. This can permit highly sensitive quantification of mutations.

In a first aspect, there is provided a method for determining the presence of a target nucleic acid in a sample, the method comprising: (i) performing a nucleic acid amplification on the sample using a reaction mixture comprising: (a) a nucleic acid polymerase; (b) one or more sets of forward and reverse primers capable of hybridising to the target nucleic acid; and (c) one or more oligonucleotide probes for detecting the target nucleic acid when present in the sample and wherein the oligonucleotide probe(s) has a predicted melting temperature that is higher than the predicted melting temperature of the one or more sets of forward and reverse primers(s); and wherein the cycling conditions during the nucleic acid amplification reaction comprise at least two different sets of cycling parameters, the first set of cycling parameters including an annealing temperature in the nucleic acid amplification reaction that is less than or about the same as the temperature corresponding to the predicted melting temperature of the lowest melting temperature probe; and the second set of cycling parameters including an annealing temperature in the nucleic acid amplification reaction that is equal to or higher than the temperature corresponding to the predicted melting temperature of the highest melting temperature probe; (ii) detecting the signal produced in step (i); and (iii) determining the presence or absence of the target nucleic acid based on the signal(s) produced in step (ii).

In one embodiment, the predicted melting temperature of the nucleic acid probes is less than or equal to about 4 °C higher than the melting temperature of the nucleic acid primers.

In one embodiment, the predicted melting temperature of the nucleic acid probes is less than about 3 °C, 2 °C or 1 °C higher than the melting temperature of the nucleic acid primers.

In one embodiment, the probes have a predicted melting temperature range of between about 62 to 65 °C or 62 to 64 °C , with the proviso that the predicted melting temperature is less than or equal to about 4 °C higher than the melting temperature of the nucleic acid primers

In one embodiment, the primers have a predicted melting temperature range of between about 58 to 60 °C.

In one embodiment, the first and/or second set of cycling parameters are repeated at least 5 or more times.

In one embodiment, the probe produces a detectable signal when the target nucleic acid is present in the sample.

In one embodiment, the probe(s) comprises a fluorescence moiety and a quenching moiety.

In one embodiment, the second set of cycling parameters is repeated enough times for fluorescence data collection to occur for detection of a fluorescence spectrum.

In one embodiment, the probe is chemically modified to increase the effective melting temperature of the probe.

In one embodiment, the method includes the further step, after step (i), of treating said reaction mixture under conditions that allow for the hybridisation of the oligonucleotide probe(s) to the target nucleic acid thereby resulting in the production of a detectable signal.

In one embodiment, the nucleic acid polymerase has 5' to 3' nuclease activity.

In one embodiment, said method includes the further step, after step (i) of treating said reaction mixture under conditions that allow for the digestion of the oligonucleotide probe(s) via the nuclease activity of the polymerase when said oligonucleotide probe(s) is hybridised to the target nucleic acid.

In one embodiment, the annealing temperature in the first cycling stage is between about 58 °C to about 60 °C.

In one embodiment, the annealing temperature in the second cycling stage is between about 64 °C to about 66 °C.

In a second aspect, there is provided a method for genotyping a sample comprising, or suspected of comprising two or more nucleic acid sequences that differ from each other by at least one base position, the method comprising: (i) performing a nucleic acid amplification on the sample using a reaction mixture comprising: (a) a nucleic acid polymerase; (b) one or more sets of forward and reverse primers capable of hybridising to the nucleic acid; and (c) two or

more oligonucleotide probes for detecting each of the two or more sequences when present in the amplified nucleic acid, wherein each of the oligonucleotide probes generate a different detectable signal, and wherein the two or more oligonucleotide probes have a predicted melting temperature that is higher than the predicted melting temperature of the primers; and wherein the cycling conditions during the nucleic acid amplification reaction comprise at least two different sets of cycling parameters, the first set of cycling parameters including an annealing temperature in the nucleic acid amplification reaction that is less than the predicted melting temperature of the lowest melting temperature probe; and the second set of cycling parameters including an annealing temperature in the nucleic acid amplification reaction that is higher than the predicted melting temperature of the highest melting temperature probe; (ii) detecting the signal produced in step (i); and (iii) determining the presence or absence of the two or more sequences based on the signal(s) produced in step (iii).

In one embodiment, each of the two or more oligonucleotide probes is for distinguishing between two or more substantially homologous sequences, the two or more substantially homologous sequences differing from each other by at least one nucleotide, each probe in the set perfectly matching one of two or more substantially homologous sequences.

In one embodiment, the two or more oligonucleotide probes are allelic probes for genotyping a first allelic site and a second allelic site, suitably, wherein the probes differ from each other by at least one nucleotide.

In one embodiment, the probes each produce distinguishable fluorescence signatures for the different genotypes being detected.

In one embodiment, the nucleic acid amplification reaction is performed using the polymerase chain reaction.

In one embodiment, the method is a 5' nuclease polymerase chain reaction amplification method.

In one embodiment, the method includes the further step, after step (i), of treating said reaction mixture under conditions that allow for the hybridisation of the oligonucleotide probe(s) to the target nucleic acid thereby resulting in the production of a detectable signal.

In one embodiment, the nucleic acid polymerase has 5' to 3' nuclease activity.

In one embodiment, said method includes the further step, after step (i) of treating said reaction mixture under conditions that allow for the digestion of the oligonucleotide probe(s) via the nuclease activity of the polymerase when said oligonucleotide probe(s) is hybridised to the target nucleic acid.

In one embodiment, the predicted melting temperature of the nucleic acid probes is less than or equal to about 4 °C higher than the melting temperature of the nucleic acid primers.

In one embodiment, the predicted melting temperature of the nucleic acid probes is less than about 3 °C, 2 °C or 1 °C higher than the melting temperature of the nucleic acid primers.

In one embodiment, the probes have a predicted melting temperature range of between about 62°C to 65 °C or 62°C to 64 °C , with the proviso that the predicted melting temperature is less than or equal to about 4 °C higher than the melting temperature of the nucleic acid primers

In one embodiment, the primers have a predicted melting temperature range of between about 58 to 60 °C.

In one embodiment, the first and/or second set of cycling parameters are repeated at least 5 or more times.

In one embodiment, the probe produces a detectable signal when the target nucleic acid is present in the sample.

In one embodiment, the probe(s) comprises a fluorescence moiety and a quenching moiety.

In one embodiment, the second set of cycling parameters is repeated enough times for fluorescence data collection to occur for detection of a fluorescence spectrum.

In one embodiment, the probe is chemically modified to increase the effective melting temperature of the probe.

In one embodiment, the annealing temperature in the first cycling stage is between about 58 °C to about 60 °C.

In one embodiment, the annealing temperature in the second cycling stage is between about 64 °C to about 66 °C.

In another aspect, there is provided a method for determining the genotype of a sample comprising, or suspected of comprising two or more nucleic acid sequences that differ from each other by at least one base position, the method comprising: (i) performing a nucleic acid amplification on the sample using a reaction mixture comprising: (a) a nucleic acid polymerase; (b) one or more sets of forward and reverse primers capable of hybridising to the nucleic acid; and (c) two or more oligonucleotide probes for detecting each of the two or more sequences when present in the amplified nucleic acid, wherein each of the oligonucleotide probes generate a different detectable signal, and wherein the two or more oligonucleotide probes have a predicted melting temperature that is higher than the predicted melting temperature of the primers; and wherein the cycling conditions during the nucleic acid amplification reaction comprise at least two different sets of cycling parameters, the first set of cycling parameters including an annealing temperature in the nucleic acid amplification reaction that is less than the predicted melting temperature of the lowest melting temperature probe; and the second set of cycling parameters including an annealing temperature in the nucleic acid amplification reaction that is higher than the predicted melting temperature of the highest melting temperature probe; (ii) detecting the signal produced in step (i); and (iii) determining the genotype of the two or more sequences based on the signal(s) produced in step (ii).

In another aspect, there is provided a method for determining the presence of a target nucleic acid in a sample, the method comprising: (i) performing a nucleic acid amplification on the sample using a reaction mixture comprising: (a) a nucleic acid polymerase having a 5' to 3' nuclease activity; (b) one or more sets of forward and reverse primers capable of hybridising to the target nucleic acid; and (c) one or more oligonucleotide probes for detecting the target nucleic acid when present in the sample and wherein the oligonucleotide probe(s) has a predicted melting temperature that is higher than the predicted melting temperature of the one or more sets of forward and reverse primers(s); and wherein the cycling conditions during the nucleic acid amplification reaction comprise at least two different sets of cycling parameters, the first set of cycling parameters including an annealing temperature in the nucleic acid amplification reaction that is less than or about the same as the temperature corresponding to the predicted melting temperature of the lowest melting temperature primer; and the second set of cycling parameters including an annealing temperature in the nucleic acid amplification reaction that is equal to or higher than the temperature corresponding to the predicted melting temperature of the highest melting temperature probe; (ii) treating said reaction mixture under conditions that allow for the digestion of the oligonucleotide probe(s) via the nuclease activity of

the polymerase when said oligonucleotide probe(s) is hybridised to the target nucleic acid; (iii) detecting the signal produced in step (ii); and (iv) determining the presence or absence of the target nucleic acid based on the signal(s) produced in step (iii).

In another aspect, there is provided a method for genotyping a sample comprising, or suspected of comprising two or more nucleic acid sequences that differ from each other by at least one base position, the method comprising: (i) performing a nucleic acid amplification on the sample using a reaction mixture comprising: (a) a nucleic acid polymerase having a 5' to 3' nuclease activity; (b) one or more sets of forward and reverse primers capable of hybridising to the nucleic acid; and (c) two or more oligonucleotide probes for detecting each of the two or more sequences when present in the amplified nucleic acid, wherein each of the oligonucleotide probes generate a different detectable signal, and wherein the two or more oligonucleotide probes have a predicted melting temperature that is higher than the predicted melting temperature of the primers; and wherein the cycling conditions during the nucleic acid amplification reaction comprise at least two different sets of cycling parameters, the first set of cycling parameters including an annealing temperature in the nucleic acid amplification reaction that is less than the predicted melting temperature of the lowest melting temperature probe; and the second set of cycling parameters including an annealing temperature in the nucleic acid amplification reaction that is higher than the predicted melting temperature of the highest melting temperature probe; (ii) treating said reaction mixture under conditions that allow for the digestion of the oligonucleotide probe(s) via the nuclease activity of the polymerase when said oligonucleotide probe(s) is hybridised to the target nucleic acid; (iii) detecting the signal produced in step (ii); and (iv) determining the presence or absence of the two or more sequences based on the signal(s) produced in step (iii).

In another aspect, there is provided a method for genotyping a sample comprising, or suspected of comprising two or more nucleic acid sequences that differ from each other by at least one base position, the method comprising: (i) performing a nucleic acid amplification on the sample using a reaction mixture comprising: (a) a nucleic acid polymerase having a 5' to 3' nuclease activity; (b) one or more sets of forward and reverse primers capable of hybridising to the nucleic acid; and (c) two or more oligonucleotide probes for detecting each of the two or more sequences when present in the amplified nucleic acid, wherein each of the oligonucleotide probes generate a different detectable signal, and wherein the two or more oligonucleotide probes have a predicted melting temperature that is higher than the predicted melting temperature of the primers; and wherein the cycling conditions during the nucleic acid amplification reaction comprise at least two different sets of cycling parameters, the first set of cycling parameters including an annealing temperature in the nucleic acid amplification reaction

that is less than the predicted melting temperature of the lowest melting temperature probe; and the second set of cycling parameters including an annealing temperature in the nucleic acid amplification reaction that is higher than the predicted melting temperature of the highest melting temperature probe; (ii) treating said reaction mixture under conditions that allow for the digestion of the oligonucleotide probe(s) via the nuclease activity of the polymerase when said oligonucleotide probe(s) is hybridised to the target nucleic acid; (iii) detecting the signal produced in step (ii); and (iv) determining the genotype of the sample based on the signal(s) produced in step (iii).

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the fluorescence output obtained from a PCR reaction using the standard cycling conditions described in the Examples using a wild type probe with signal detected on a first channel and a SNP probe with signal detected on a second channel.

Figure 2 illustrates the fluorescence output obtained from a PCR reaction using annealing and elongation temperatures for all cycles of 60 °C and 66 °C.

Figure 3 illustrates the fluorescence output obtained from a PCR reaction using the standard cycling conditions described in the Examples (Figure 3A) and the two stage cycling conditions described in the Examples (Figure 3B). In each case a wild type probe with signal detected on a first channel and a SNP probe with signal detected on a second channel are used

Figure 4 illustrates the fluorescence output from a probe on homologous and non-homologous templates when used with a competitor probe or alone, in conjunction with standard cycling conditions (Figure 4A), and with the two stage cycling with the higher annealing and extension temperature (Figure 4B).

Figure 5 illustrates the fluorescence output obtained in a multiplex reaction where one wild type probe and multiple mutant probes have been included in the same reaction. The wild type probe only gives a fluorescent signal when the wild type template is present whilst no wild type signal is observed on mutant templates (Figure 4A). Conversely the mutant probes all give a signal when the homologous mutant template is present whilst giving no signal on the wild type template.

Figure 6 illustrates the fluorescence output obtained from a PCR reaction using the standard and two stage cycling conditions described in the Examples with a mixed nucleic acid sample at

a frequency as low as 5%. The top figure in Figure 6A relates to results obtained using standard cycling conditions described in the Examples; an enlarged view of this top figure is shown in the bottom figure; the top figure in Figure 6B relates to results obtained using the two stage cycling conditions described in the Examples; the bottom figure is an enlarged view of the top figure.

DEFINITIONS

The technical terms and expressions used within the scope of this application are generally to be given the meaning commonly applied to them in the pertinent art of plant and molecular biology. All of the following term definitions apply to the complete content of this application. The word "comprising" does not exclude other elements or steps, and the indefinite article "a" or "an" does not exclude a plurality. A single step may fulfill the functions of several features recited in the claims. The terms "essentially", "about", "approximately" and the like in connection with an attribute or a value particularly also define exactly the attribute or exactly the value, respectively. The term "about" in the context of a given numerate value or range refers to a value or range that is within 20 %, within 10 %, or within 5 % of the given value or range.

Melting temperature. Duplex stability between complementary nucleic acid molecules is expressed by the duplexes "melting temperature", which is often abbreviated as T_m . The melting temperature indicates the temperature at which a duplex nucleic acid dissociates into single-stranded nucleic acids. For multiple copies of DNA molecules, the melting temperature is defined as the temperature at which half of the DNA strands are in the double-helical state and half are in the random coil states. The melting temperature can depend on variables including the length of the molecule, the specific nucleotide sequence composition of that molecule and the molarity of the salt and the nucleic acid in the solution. Duplex stability and melting temperature are important in nucleic acid amplification where thermocycling may be involved. During such thermocycling steps, the temperature is raised high enough above the melting temperature so that duplexes of the target nucleic acid and its complement are dissociated. In subsequent steps of reannealing the temperature is brought to below the melting temperature such that duplexes of the target nucleic acid and primer(s) and/or probe(s) are able to form, while still remaining high enough to avoid non-specific hybridization. Melting temperature and how to measure it is thoroughly described in the art. See for example, *Nucleic Acids Research* (1990) 18, 6409-6412 and *Bioinformatics*, V. 12(12), pp. 1226-1227. Theoretical or empirical models that relate duplex stability to nucleotide sequence can be used to predict melting temperatures for nucleic acids. For example, *Proc. Natl. Acad. Sci. U.S.A.* 1986, 83:3746-3750, *Biochemistry* 1996, 35:3555-3562; and *Proc. Natl. Acad. Sci. U.S.A.* 1998, 95:1460-1465 describe a model for predicting melting temperature that is widely used in the art and is known

as the "nearest neighbour model". This model takes account of both the length of the oligonucleotide and the sequence of the bases within it.

Predicted melting temperature. The predicted melting temperature refers to the theoretically calculated melting temperature of an oligonucleotide that has not been conjugated with any moieties. It is usually an estimate of the actual melting temperature of an oligonucleotide and is based on the sequence of the oligonucleotide typically without considering the effect of any labels and any associated linkers and the like. Given the exact sequence of an oligonucleotide, the melting temperature of the oligonucleotide can be predicted as discussed above. The predicted melting temperature according to the present invention is calculated, in one embodiment, using the nearest neighbour model. In a further embodiment, the predicted melting temperature according to the present invention is calculated using the nearest neighbour thermodynamic theory using SantaLucia values (SantaLucia, J., Jr. (1998) *Proc. Natl. Acad. Sci. U.S.A.* (1998) 95, 1460-1465). The SantaLucia values are contained in various computer programs that can be used to predict melting temperatures. In one embodiment, the "Beacon Designer" software package is used. In one embodiment, the SantaLucia values are used with the following reaction conditions; nucleic acid concentration of 0.25 nM; monovalent ion concentration of 50 mM; free magnesium ion concentration of 5mM; total Na⁺ equivalent of 332.84mM; and temperature for free energy calculations of 25 °C. Regardless of the choice of the particular software programs or methodologies used, the same software program or methodology is desirably used, suitably, with its default settings, to ascertain the predicted melting temperatures of the primer(s) and the probe(s) used in the present invention.

Amplification. This refers to any method that results in the formation of one or more copies of a nucleic acid molecule (for example, exponential amplification) or in the formation of one or more copies of only the complement of a nucleic acid or polynucleotide molecule (for example, linear amplification). One of the most widely used techniques is the polymerase chain reaction (PCR). PCR is a primer extension reaction that provides a method for amplifying specific nucleic acids *in vitro*. PCR can produce million fold copies of a DNA template in a single enzymatic reaction mixture within a matter of hours, enabling researchers to determine the size and sequence of target DNA. General procedures for PCR are taught in U.S. Patent Nos. 4,683,195 and 4,683,202. In one embodiment, the amplification method is an amplification method that incorporates the use of a polymerase which has 5' nuclease activity, suitably 5' exonuclease activity. In one embodiment, the amplification method is the polymerase chain reaction. In one embodiment, the amplification method is or is based on a 5' nuclease polymerase chain reaction.

Nucleic acid. This term refers to a compound or composition that is a polymeric nucleotide or nucleic acid polymer. The nucleic acid may be a natural compound or a synthetic compound. The nucleic acid can have from about 10 to 5,000,000 or more nucleotides. The larger nucleic acids are generally found in the natural state. In an isolated state the nucleic acid can have about 30 to 50,000 or more nucleotides – such as about 100 to 20,000 nucleotides or about 500 to 10,000 nucleotides. The nucleic acid can include nucleic acids, and fragments thereof, from any source in purified or unpurified form including DNA (dsDNA and ssDNA) and RNA, including tRNA, mRNA, rRNA, mitochondrial DNA and RNA, chloroplast DNA and RNA, DNA/RNA hybrids, or mixtures thereof, genes, chromosomes, plasmids, the genomes of biological material – such as bacteria, yeasts, viruses, viroids, moulds, fungi, plants, animals, humans, and the like. Also included are genes, oncogenes, cDNA, and the like. The nucleic acid, where appropriate, may be cleaved to obtain a fragment that contains a target nucleotide sequence, for example, by shearing or by treatment with a restriction endonuclease or other site specific chemical cleavage method. The nucleic acid may be at least partially denatured or single stranded or treated to render it denatured or single stranded. Such treatments are well known in the art and include, for instance, heat or alkali treatment, or enzymatic digestion of one strand. For example, dsDNA can be heated to a high temperature for a period of about 1 to 15 minutes to produce denatured material.

Oligonucleotide. This refers to a nucleic acid that is usually single stranded and is usually a synthetic nucleic acid but may be a naturally occurring nucleic acid. Oligonucleotide(s) are generally comprised of at least about 5 contiguous nucleotides, suitably, about 10 to 100 contiguous nucleotides, more suitably, about 20 to 50 nucleotides, more suitably 10 to 30 nucleotides, more suitably, 20 to 30 nucleotides, more suitably, 22 to 26 nucleotides, more suitably, 23 to 25 nucleotides in length. In some embodiment, the oligonucleotides are about 23 or about 25 nucleotides in length. The precise length will depend to some extent on the required melting temperature of the oligonucleotide. Various techniques can be employed for preparing an oligonucleotide that include biological or chemical synthesis. The oligonucleotide can be synthesised using standard methods such as those used in commercial automated nucleic acid synthesisers. Chemical synthesis of DNA on a suitably modified glass or resin can result in DNA covalently attached to the surface. This may offer advantages in washing and sample handling. For longer sequences standard replication methods employed in molecular biology can be used.

Oligonucleotide primer. Oligonucleotide primers are employed in a chain extension on a nucleic acid template such as in, for example, the amplification of nucleic acid. The oligonucleotide primer is usually a synthetic nucleotide that is single stranded, containing a

sequence at its 3'-end that is capable of hybridising with the target nucleic acid. Normally, an oligonucleotide primer has at least 80%, preferably 90%, more preferably 95%, most preferably 100% complementarity to a target nucleic acid sequence or primer binding site. The number of nucleotides in the oligonucleotide primer should be such that stringency conditions used to hybridize the oligonucleotide primer will prevent excessive random non-specific hybridisation. Usually, the number of nucleotides in the oligonucleotide primer will be at least about 5 contiguous nucleotides, suitably, about 10 to 100 contiguous nucleotides, more suitably, about 20 to 50 nucleotides, more suitably 10 to 30 nucleotides, more suitably, 20 to 30 nucleotides, more suitably, 22 to 26 nucleotides, more suitably, 23 to 25 nucleotides in length. In some embodiment, the oligonucleotides are about 23 or about 25 nucleotides in length. The precise length will depend to some extent on the required melting temperature of the primer. In general, in primer extension, amplification primers hybridise to, and are extended along (chain extended), at least the target nucleotide sequence within the target polynucleotide and, thus, the target sequence acts as a template. The target sequence usually lies between two defined sequences. In general, the primers hybridise with the defined sequences or with at least a portion of such target polynucleotide, usually at least a ten-nucleotide segment at the 3'-end thereof and preferably at least 15, frequently a 20 to 50 nucleotide segment thereof.

Oligonucleotide probe. This refers to an oligonucleotide employed to bind to a portion of a polynucleotide such as another oligonucleotide or a target nucleotide sequence. Suitably, the oligonucleotide probe is able to produce a detectable signal that is indicative that the nucleic acid that it is designed to detect is present in the sample. Usually, the number of nucleotides in the oligonucleotide probe will be at least about 5 contiguous nucleotides, suitably, about 10 to 100 contiguous nucleotides, more suitably, about 20 to 50 nucleotides, more suitably 10 to 30 nucleotides, more suitably, 20 to 30 nucleotides, more suitably, 22 to 26 nucleotides, more suitably, 23 to 25 nucleotides in length. In some embodiment, the oligonucleotides are about 23, 24 or 25 nucleotides in length. Suitably, each of the oligonucleotide probes is able to produce a different or a distinct detectable signal – such as spectrally distinct signal. Suitably, the oligonucleotide probe includes at least one fluorescer and at least one quencher which can be digested by the 5' nuclease activity of a polymerase in order to detect any amplified target oligonucleotide sequences. In general, the oligonucleotide probes will have a sufficient number of phosphodiester linkages adjacent to its 5' end so that the 5'→3' nuclease activity employed can efficiently degrade the bound probe to separate the fluorescer(s) and quencher(s), for example. The fluorescer can be any molecule capable of generating a fluorescence signal. The quencher molecule can be any molecule capable of absorbing the fluorescence energy of the excited fluorescer, thereby quenching the fluorescence signal that would otherwise be released from the excited fluorescer. In order for a quencher molecule to quench an excited

fluorescer, the quencher must generally be within a minimum quenching distance of the excited fluorescer at some time prior to the fluorescer releasing the stored fluorescence energy. Suitably, each of the oligonucleotide probes is able to produce a spectrally distinct fluorescent signal.

Target nucleotide sequence. This refers to the nucleotide sequence that is to be amplified and/or detected and can contain one or more mutations or polymorphisms to be detected. A nucleotide sequence to which primers and/or probes can anneal is considered to be a target nucleotide sequence. In general, the nucleotide sequence of a nucleic acid is described from the 5'-end to the 3'-end of the sense strand. Further, the target nucleotide sequence can include not only the sense strand but also the nucleotide sequence of the complementary strand thereof, *i.e.* the antisense strand.

Sample. A sample can include biological or clinical samples that may contain or may be suspected of containing nucleic acids or fragments of nucleic acids to be detected or genotyped. The sample may be a complex mixture, a partially purified sample or a purified sample. For example, the sample may be a partially purified sample or a purified sample of nucleic acid. A sample may be or may be derived from a biological sample that can include any tissue or material derived or derivable from a living or dead mammal or organism, including, without limitation, blood cells, saliva, blood, plasma, serum, mucous and biopsies. Samples may comprise *in vitro* cell culture constituents. The sample may be treated to physically or mechanically disrupt tissue or cells to release intracellular nucleic acids into a solution which may contain enzymes, buffers, salts, detergents and the like, to prepare the sample for analysis. Suitably, the methods exclude the physical step of obtaining a sample from a subject.

Mutation. This refers to a difference (*eg.* one or more differences or two or more differences) in the nucleotide sequence of a nucleic acid and is typically observed among individual organisms of the same origin. With respect to multicellular organisms, differences observed among organs, cells, and so on within an individual are also included as mutations. Mutations can arise from point mutations, deletions, insertions, duplications, inversions, and translocations. Further, mutations may occur within gene regions – such as protein coding regions, intron sequences or expression regulatory regions, which can include promoters or enhancers and the like. Differences in nucleotide sequences found in other genomic sequences are also considered as mutations.

Polymorphism. Among the above mutations, those that are present at a frequency of 1% or more within a certain population are particularly referred to as "polymorphisms". Among

polymorphisms, polymorphisms consisting of displacement, insertion, or deletion of a single nucleotide are particularly referred to as single nucleic polymorphisms (or "SNPs").

Homology, identity and similarity. These terms refer to the degree of sequence similarity between two polypeptides or between two polynucleotide molecules compared by sequence alignment. The degree of homology between two discrete polynucleotide sequences being compared is a function of the number of identical, or matching, nucleotides at comparable positions. The degree of similarity expressed in terms of percent identity may be determined by visual inspection and mathematical calculation. Alternatively, the percent identity of two polynucleotide sequences may be determined by comparing sequence information using the GAP computer program, version 6.0 described by Devereux *et al.* (*Nucl. Acids Res.* 12:387, 1984) and available from the University of Wisconsin Genetics Computer Group (UWGCG), ClustalW, BLAST, FASTA or Smith-Waterman. Typical default parameters for the GAP program include: (1) a unary comparison matrix (containing a value of 1 for identities and 0 for non-identities) for nucleotides, and the weighted comparison matrix of Gribskov and Burgess, *Nucl. Acids Res.* 14:6745, 1986, as described by Schwartz and Dayhoff, eds., *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation, pp. 353-358, 1979; (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps. Various programs known to persons skilled in the art of sequence comparison can be alternatively utilized.

DETAILED DESCRIPTION

One of the improvements in the methods described herein relates to the predicted melting temperature of the nucleic acid probe that is used in the assay as compared to the predicted melting temperature of the nucleic acid primers. In the context of the nucleic acid amplification methods that are described in the art, the probe is allowed to anneal to all of the nucleic acid targets present in order to saturate the sample, without the primers annealing at the same time. This permits sequential binding of the probe followed by the primers when ramping down to the annealing temperature used in the amplification reaction. This step is therefore conventionally performed at a temperature that is too high for the primers to anneal, but low enough for the probes to anneal. When the temperature is at the annealing temperature, which will be below the melting temperature of the primers, the primers will extend and in certain embodiments can cleave off the probe. It is not desirable for the probe to come off the target nucleic acid simply because of temperature. Instead, it is desired in some embodiments that the probe comes off the target because it was removed by the action of the 5' nuclease. Conventionally therefore, the probe will have a predicted melting temperature that is greater than the predicted melting

temperature of the primers by about 5-10°C. The predicted melting temperature of each of the primers will be very similar to each other, if not about the same.

Contrary to this convention in the art, the present invention utilises one or more probes that have a melting temperature that is less than about 5 °C higher than the melting temperature of the nucleic acid primer(s). Thus, according to this scenario, it is believed that the thermodynamic effects of a mismatch in the probe will be of greater discriminating effect when the melting temperature of the probe has been lowered. Without wishing to be bound by any particular theory, it is understood that this modification increases specificity since a mismatched probe has less time to bind to the wrong sequence before primer extension removes the probe binding site from this cycle of the nucleic acid amplification reaction.

In one embodiment, the probe(s) has a predicted melting temperature that is only about 4 °C higher than the melting temperature of the nucleic acid primer(s). In one embodiment, the probe(s) has a predicted melting temperature that is only about 3 °C higher than the melting temperature of the nucleic acid primer(s). In one embodiment, the probe(s) has a predicted melting temperature that is only about 2 °C higher than the melting temperature of the nucleic acid primer(s). In one embodiment, the probe(s) has a predicted melting temperature that is only about 1 °C higher than the melting temperature of the nucleic acid primer(s). In one embodiment, the probe(s) has a predicted melting temperature that is only about 0.5 °C higher than the melting temperature of the nucleic acid primer(s). In one embodiment, the probe(s) has a predicted melting temperature that is only about 0.1 °C higher than the melting temperature of the nucleic acid primer(s).

In one embodiment, the probe(s) has a predicted melting temperature that is between about 0.1 and about 4 °C higher than the melting temperature of the nucleic acid primer(s). In one embodiment, the probe(s) has a predicted melting temperature that is between about 0.5 and about 4 °C higher than the melting temperature of the nucleic acid primer(s). In one embodiment, the probe(s) has a predicted melting temperature that is between about 1 and about 4 °C higher than the melting temperature of the nucleic acid primer(s). In one embodiment, the probe(s) has a predicted melting temperature that is between about 2 and about 4 °C higher than the melting temperature of the nucleic acid primer(s). In one embodiment, the probe(s) has a predicted melting temperature that is between about 3 and about 4 °C higher than the melting temperature of the nucleic acid primer(s).

Surprisingly, the full benefit of the use of probes that have a melting temperature that is less than about 5 °C higher than the melting temperature of the nucleic acid primer(s) is fully realised when probe discrimination is allowed to occur, for at least a period of time, at a higher than normal annealing temperature in the nucleic acid amplification reaction than would otherwise have been used. Surprisingly, the present inventors have found that the enhanced specificity of the probe for its cognate template is intrinsic to the design specification of that particular probe and to the cycling parameters used in the method. The methods of the prior art require the use of multiple competing probes to synergise stringent signalling since they will compete against each other for the range of possible binding sites. Each one will preferentially bind to its cognate template having a higher melting temperature for that target than any other probes in the mix which will all differ by at least one base. Sequestration of alternative binding sites greatly reduces the potential for mis-binding on non cognate targets. In the current method, very high stringency can be achieved with a single probe. Mechanistically there are various possible models to explain this finding. It is possible that the lower melting temperature probe does not bind to the target sequence at the higher annealing temperature when a mismatch is present. Alternatively, when a mismatch is present, the probe may bind, but with such a weak interaction that it is preferentially strand displaced by the polymerase, rather than degraded to produce a fluorescent signal. Without wishing to be bound by any particular theory, it is believed that the method of the present invention adds a new level of probe specificity to the nucleic acid amplification reaction since a mismatched probe, even when used alone, can allow substantially complete exclusion of mis-hybridisation to non-complimentary target sequences. Thus, a single probe can advantageously be used to achieve highly stringent probe binding between sequences that differ by just one nucleotide base.

In one embodiment, the probes have a predicted melting temperature range of between about 62°C to 65 °C or about 62°C to 64 °C with the proviso that the predicted melting temperature is less than or equal to about 4 °C, about 3 °C, about 2 °C, about 1 °C, about 0.5 °C or about 0.1 °C higher than the melting temperature of the nucleic acid primers.

In one embodiment, the probes have a predicted melting temperature range of between about 62°C to 65 °C or about 62°C to 64 °C with the proviso that the predicted melting temperature is less than or equal to about 0.1 and about 4 °C higher than the melting temperature of the nucleic acid primer(s), about 0.5 and about 4 °C higher than the melting temperature of the nucleic acid primer(s), about 1 and about 4 °C higher than the melting temperature of the nucleic acid primer(s), about 2 and about 4 °C higher than the melting temperature of the

nucleic acid primer(s), about 3 and about 4 °C higher than the melting temperature of the nucleic acid primer(s).

In one embodiment of the invention, the cycling protocol is separated into two different cycling stages.

The first cycling stage allows for efficient amplification of the target sequence since the annealing temperature is optimal for efficient nucleic acid priming and amplification. These early cycles can be used to initiate efficient nucleic acid amplification in a complex biological mixture to increase the abundance of the target nucleic acid sequence. Efficient nucleic acid amplification typically requires that the annealing and extension temperature are less than or similar to the melting temperature of the primers and so the early cycles are expected to take place at about or around the optimal predicted melting temperature for primer annealing to occur. During the early cycles of the nucleic acid amplification, the amount of probe cleaved in each cycle will normally be insufficient to raise the signal produced by probe cleavage to above the background level. Probe discrimination and signal detection are not therefore relevant to these early amplification cycles and data need not normally be collected during this first cycling stage.

Suitably, the annealing temperature used in the first cycling stage of the nucleic acid amplification reaction is equal to or less than about 2 °C of the predicted melting temperature of the lowest melting temperature primer. Suitably, the annealing temperature used in the first cycling stage of the nucleic acid amplification reaction is equal to or less than about 1 °C of the predicted melting temperature of the lowest melting temperature primer. Suitably, the annealing temperature used in the first cycling stage of the nucleic acid amplification reaction is equal to or less than about 0.5 °C of the predicted melting temperature of the lowest melting temperature primer. Suitably, the annealing temperature used in the first cycling stage of the nucleic acid amplification reaction is equal to or less than about 0.1 °C of the predicted melting temperature of the lowest melting temperature primer.

Suitably, the annealing temperature used in the first cycling stage of the nucleic acid amplification reaction is equal to or less than about 2 °C lower than the predicted melting temperature of the lowest melting temperature primer. Suitably, the annealing temperature used in the first cycling stage of the nucleic acid amplification reaction is equal to or less than about 1 °C lower than the predicted melting temperature of the lowest melting temperature primer. Suitably, the annealing temperature used in the first cycling stage of the nucleic acid

amplification reaction is equal to or less than about 0.5 °C lower than the predicted melting temperature of the lowest melting temperature primer. Suitably, the annealing temperature used in the first cycling stage of the nucleic acid amplification reaction is equal to or less than about 0.1 °C lower than the predicted melting temperature of the lowest melting temperature primer.

Suitably, the annealing temperature in the nucleic acid amplification reaction is substantially the same as the predicted melting temperature of the lowest melting temperature primer.

Suitably, the annealing temperature in the nucleic acid amplification reaction is the same as the predicted melting temperature of the lowest melting temperature primer.

In one embodiment, the first cycling stage comprises activating the enzyme used for amplification – such as a DNA polymerase – for a period of time followed by repeat cycles of melting and annealing/extension. In one embodiment, the activation step is carried out at about 95 °C for between about 5 to 10 minutes, suitably about 10 minutes. In one embodiment, the melting step is carried out at a temperature of about 95 °C for about 10 seconds. In one embodiment, the annealing/extension is carried out at a temperature of between about 58 °C and about 60 °C – such as at about 58 °C, 59 °C or 60 °C.

The number of repeat cycles used in the first cycling stage can be at least about 5 cycles or at least about 10 cycles, or at least about 15 cycles, or at least about 20 cycles, or at least about 25 cycles, or at least about 30 cycles.

One exemplary first cycling stage comprises an activation step at about 95 °C for about 10 minutes, followed by about 15 or more repeat cycles of about 95 °C for about 10 seconds and about 60 °C for about 60 seconds.

In the second cycling stage, it is intended that efficient probe discrimination occurs to thereby give maximum discrimination between the probes which can ensure binding only to nucleic acid sequences with which they share 100% identity. Without wishing to be bound by any particular theory, it is believed that probe discrimination is improved at higher predicted melting temperatures due to the fundamental kinetics of binding. At temperatures close to the effective melting temperature of the probe, bound probes are destabilised and a mismatch has a greater perturbing effect on binding and hence mis-probing on the wrong template is reduced. The second cycling conditions are predicated on the first stage cycles since it is understood that if a

high annealing temperature was used from the outset, the nucleic acid amplification would probably fail to amplify efficiently leading to a failed amplification. The method is therefore understood to depend on the shifting thermodynamic properties of primer binding as the target copy number increases throughout the nucleic acid amplification reaction.

During the first cycling stage, the target copy number in the reaction is exponentially increased from the starting copy number. The target copy number present in the reaction following the first cycling stage will typically be between about 1 to 20 million copies (depending on the number of first stage cycles and the input copy number). Without wishing to be bound by any particular theory, at high target copy number, the effective melting temperature of the primers will be greatly increased allowing efficient nucleic acid amplification at annealing temperatures far higher than the predicted melting temperature of the primers and far higher than would be possible at the start of the nucleic acid amplification reaction. In the second cycling stage, the annealing temperature is higher than the predicted melting temperature of either the primers or the probe(s) which is understood to result in maximum probe discrimination without compromising the efficiency of the amplification.

Suitably, the annealing temperature used in the second cycling stage of the nucleic acid amplification reaction is equal to or greater than about 0.1 °C higher than the predicted melting temperature of the highest melting temperature probe. Suitably, the annealing temperature used in the second cycling stage of the nucleic acid amplification reaction is equal to or greater than about 0.5 °C higher than the predicted melting temperature of the highest melting temperature probe. Suitably, the annealing temperature used in the second cycling stage of the nucleic acid amplification reaction is equal to or greater than about 1 °C higher than the predicted melting temperature of the highest melting temperature probe. Suitably, the annealing temperature used in the second cycling stage of the nucleic acid amplification reaction is equal to or greater than about 2 °C higher than the predicted melting temperature of the highest melting temperature probe. Suitably, the annealing temperature used in the second cycling stage of the nucleic acid amplification reaction is equal to or greater than about 3 °C higher than the predicted melting temperature of the highest melting temperature probe. Suitably, the annealing temperature used in the second cycling stage of the nucleic acid amplification reaction is equal to or greater than about 4 °C higher than the predicted melting temperature of the highest melting temperature probe. Suitably, the annealing temperature used in the second cycling stage of the nucleic acid amplification reaction is equal to or greater than about 5 °C higher than the predicted melting temperature of the highest melting temperature probe.

Without wishing to be bound by any particular theory, the higher annealing temperature in the second cycling stage is believed to be important for discrimination since this will lead to very stringent probe hybridising conditions wherein the probe can only hybridise to its complementary template and subsequently be cleaved to produce a detectable signal.

In one embodiment, the second stage comprises repeat cycles of activation and annealing/extension at a temperature that is higher than in the first cycling stage. In one embodiment, the melting step is carried out at a temperature of about 95 °C for about 10 seconds. In one embodiment, the annealing/extension is carried out at a temperature of greater than or equal to about 64 °C. In one embodiment, the annealing/extension is carried out at a temperature of between about 64 °C and about 66 °C – such as about 64 °C, 65 °C or 66 °C. In one embodiment, the melting temperature is about 95 °C and the annealing/extension is carried out at a temperature of between about 64 °C to about 66 °C. In one embodiment, the melting temperature is about 95 °C and the annealing/extension is carried out at a temperature of about 64 °C. In one embodiment, the melting temperature is about 95 °C and the annealing/extension is carried out at a temperature of about 65 °C. In one particularly suitable embodiment, the melting temperature is about 95 °C and the annealing/extension is carried out at a temperature of about 66 °C.

The number of repeat cycles used in the second cycling stage can be at least about 5 cycles, or at least about 10 cycles, or at least about 15 cycles, or at least about 20 cycles, or at least about 25 cycles, or at least about 30 cycles, or at least about 35 cycles, or at least about 40 cycles, or at least about 45 cycles or at least about 50 cycles. One exemplary second cycling stage comprises 30 or more repeat cycles of about 95 °C for about 10 seconds and between about 64 °C and 66 °C for about 60 seconds. One exemplary second cycling stage comprises 30 or more repeat cycles of about 95 °C for about 10 seconds and about 64 °C for about 60 seconds. One exemplary second cycling stage comprises 30 or more repeat cycles of about 95 °C for about 10 seconds and about 65 °C for about 60 seconds. One exemplary second cycling stage comprises 30 or more repeat cycles of about 95 °C for about 10 seconds and about 66 °C for about 60 seconds.

The first and second cycling stages may be carried out sequentially or substantially sequentially.

Many different amplification methods are known in the art including the polymerase chain reaction (PCR). Briefly, amplification of nucleic acids by PCR involves repeated cycles of heat-denaturing the DNA, annealing two primers to sequences that flank the target nucleic acid

segment to be amplified, and extending the annealed primers with a polymerase. The primers hybridize to opposite strands of the target nucleic acid and are oriented so that the synthesis by the polymerase proceeds across the segment between the primers, effectively doubling the amount of the target segment. Moreover, because the extension products are also complementary to and capable of binding primers, each successive cycle essentially doubles the amount of target nucleic acids synthesized in the previous cycle. This results in exponential accumulation of the specific target nucleic acids at approximately a rate of 2^n , where n is the number of cycles. A typical conventional PCR thermal cycling protocol comprises 30 cycles of (a) denaturation at a range of 90°C to 95°C for 0.5 to 1 minute, (b) annealing at a temperature ranging from 50°C to 65°C for 1 to 2 minutes, and (c) extension at 68°C to 75°C for at least 1 minute. Other protocols including but not limited to universal protocol as well as fast cycling protocol can be performed. A variant of the conventional PCR is a reaction termed "Hot Start PCR". Hot Start PCR techniques focus on the inhibition of polymerase activity during reaction preparation. By limiting polymerase activity prior to PCR cycling, nonspecific amplification is reduced and the target yield is increased. Common methods for Hot Start PCR include chemical modifications to the polymerase, inhibition of the polymerase by a polymerase-specific antibody, and introduction of physical barriers in the reaction site to sequester the polymerase before the thermal cycling takes place. The reagents necessary for performing Hot Start PCR are conveniently packaged in kits that are commercially available. Another variation of the conventional PCR that can be performed with the subject probes is "nested PCR" using nested primers. The method is preferred when the amount of target nucleic acid in a sample is extremely limited for example, where archival, forensic samples are used. In performing nested PCR, the nucleic acid is first amplified with an outer set of primers capable of hybridizing to the sequences flanking a larger segment of the target nucleic acid. This amplification reaction is followed by a second round of amplification cycles using an inner set of primers that hybridizes to target sequences within the large segment. Another variant is ligase chain polymerase chain reaction (LCR-PCR). The method involves ligating the target nucleic acids to a set of primer pairs, each having a target-specific portion and a short anchor sequence unrelated to the target sequences. A second set of primers containing the anchor sequence is then used to amplify the target sequences linked with the first set of primers. Procedures for conducting LCR-PCR are well known in the art.

In certain embodiments, the methods can exploit the 5' to 3' nuclease activity of a polymerase – such as when the polymerase is used in conjunction with PCR. During extension of the primer, the polymerase encounters the probe hybridised to the target nucleic acid first displaces the 5' end of the probe and then degrades it. The probe(s) can be added concomitantly with the primer(s) at the start of PCR, and the signal generated from hydrolysis of the labelled

nucleotide(s) of the probe provides a means for detection of the target nucleic acid sequence during its amplification. Thus, in one aspect, there is provided a method for determining the presence of a target nucleic acid in a sample, the method comprising: (i) performing a nucleic acid amplification using a reaction mixture comprising: (a) a nucleic acid polymerase having a 5' to 3' nuclease activity; (b) one or more sets of forward and reverse primers capable of hybridising to the target nucleic acid; and (c) one or more oligonucleotide probes for detecting the target nucleic acid when present in the sample and wherein the oligonucleotide probe(s) has a predicted melting temperature that is higher than the predicted melting temperature of the one or more sets of forward and reverse primers(s); and wherein the cycling conditions during the nucleic acid amplification reaction comprise at least two different sets of cycling parameters, the first set of cycling parameters including an annealing temperature in the nucleic acid amplification reaction that is less than or about the same as the temperature corresponding to the predicted melting temperature of the lowest melting temperature primer; and the second set of cycling parameters including an annealing temperature in the nucleic acid amplification reaction that is equal to or higher than the temperature corresponding to the predicted melting temperature of the highest melting temperature probe; (ii) treating said reaction mixture under conditions that allow for the digestion of the oligonucleotide probe(s) via the nuclease activity of the polymerase when said oligonucleotide probe(s) is hybridised to the target nucleic acid; (iii) detecting the signal produced in step (ii); and (iv) determining the presence or absence of the target nucleic acid based on the signal(s) produced in step (iii). In another aspect, there is provided a method for genotyping a sample comprising, or suspected of comprising two or more nucleic acid sequences that differ from each other by at least one base position, the method comprising: (i) performing a nucleic acid amplification using a reaction mixture comprising: (a) a nucleic acid polymerase having a 5' to 3' nuclease activity; (b) one or more sets of forward and reverse primers capable of hybridising to the nucleic acid; and (c) two or more oligonucleotide probes for detecting each of the two or more sequences when present in the amplified nucleic acid, wherein each of the oligonucleotide probes generate a different detectable signal, and wherein the two or more oligonucleotide probes have a predicted melting temperature that is higher than the predicted melting temperature of the primers; and wherein the cycling conditions during the nucleic acid amplification reaction comprise at least two different sets of cycling parameters, the first set of cycling parameters including an annealing temperature in the nucleic acid amplification reaction that is less than the predicted melting temperature of the lowest melting temperature probe; and the second set of cycling parameters including an annealing temperature in the nucleic acid amplification reaction that is higher than the predicted melting temperature of the highest melting temperature probe; (ii) treating said reaction mixture under conditions that allow for the digestion of the oligonucleotide probe(s) via the nuclease activity of the polymerase when said oligonucleotide probe(s) is hybridised to the

target nucleic acid; (iii) detecting the signal produced in step (ii); and (iv) determining the presence or absence of the two or more sequences based on the signal(s) produced in step (iii).

Numerous polymerases are suited to catalyse primer and template-dependent nucleic acid synthesis and possess the 5' to 3' nuclease activity. Non-limiting examples include DNA polymerases - such as *E. coli* DNA polymerase I, *Thermus thermophilus* (Tth) DNA polymerase, *Bacillus stearothermophilus* DNA polymerase, *Thermococcus littoralis* DNA polymerase, and *Thermus aquaticus* (Taq) DNA polymerase and polymerases extracted from the thermostable bacteria *Thermus flavus*, *Thermus ruber*, *Thermus thermophilus*, *Bacillus stearothermophilus*, *Thermus lacteus*, *Thermus rubens*, *Thermotoga maritima*, *Thermococcus littoralis*, and *Methanothermus fervidus*.

Nucleic acid amplification can be performed with polymerases that exhibit strand-displacement activity (also known as rolling circle polymerization). Strand displacement can result in the synthesis of tandem copies of a circular DNA template, and is particularly useful in isothermal PCR reaction. Non-limiting examples of rolling circle polymerases include but are not limited to T5 DNA polymerase, T4 DNA polymerase holoenzyme, phage M2 DNA polymerase, phage PRDI DNA polymerase and Klenow fragment of DNA polymerase I.

Nucleic acid amplification is generally performed with the use of amplification reagents. Amplification reagents typically include enzymes, aqueous buffers, salts, primer(s), probe(s), target nucleic acid, and nucleoside triphosphates. Depending upon the context, amplification reagents can be either a complete or incomplete amplification reaction mixture.

The assay may be a homogeneous assay. In such an assay, a target nucleic acid is detected and/or quantified without the requirement of post-assay processing to record the result of the assay. For example, a homogeneous PCR reaction can be carried out in a closed sample holder and no further addition or removal of reagents is necessary to record the result once the assay is started. Homogeneous assays allow recordation of the result of the assay in real time. Where desired, in practicing the subject methods, the result of the assay can be continuously recorded as the assay progresses in time or recorded intermittently at one or more point during the assay or upon completion of the assay. Where desired, homogeneous assays can be multiplexed, such that more than one target nucleic acid can be detected in one assay. In a multiplex assay, two or more specific nucleic acid probes, which differ in the nature of their covalently attached dyes, are added to the mixture to be assayed. The dyes are chosen to produce distinguishable signals from each specific nucleic acid probe. The signals of the different dye combinations of the nucleic acid probes can be recorded simultaneously to detect

and/or quantify the corresponding target nucleic acids. Multiplexing greatly reduces the cost of analysis and can tremendously increase throughput in high volume settings.. Thus, in a further embodiment, the method is a multiplexed amplification method.

The method described herein can be used for the detection of low abundance target sequences in the presence of a high background of alternative substantially homologous sequence(s). Thus, for example, a target sequence that is present at as little as about 5% of a mixed population of nucleic acid sequences can be accurately identified.

The method described herein can be used to detect or determine mutations, including SNPs. Methods are provided that can detect a single mismatch or more between the probe sequence and a target nucleic acid sequence. Another application of the method described herein is for determining the genotype of a sample of nucleic acid at two or more different allelic sites. The two or more different allelic sites may be on a single strand of nucleic acid or may be on different strands of nucleic acid. The two or more different allelic sites may be amplified by a single amplification primer, for example when the allelic sites are on the same strand of nucleic acid and adjacent each other, or by multiple different amplification primers. In some embodiments, this assay can be performed under competitive conditions, since multiple probes to the same allelic site can be present in the same reaction vessel. Part of the discrimination against a mismatch is that the probe that is perfectly matched functions to prevent the mismatched probe from binding because of the perfectly matched probe's stable hybridisation to the sequence being amplified.

The choice of primers for use in nucleic acid amplification will depend on the target nucleic acid sequence. In general, the following factors are considered in primer design: a) each individual primer of a pair preferably does not self-hybridize in an amplification reaction; b) the individual pairs preferably do not cross-hybridize in an amplification reaction; c) the selected pair preferably have the appropriate length, and sequence homology in order to anneal to two distinct regions flanking the nucleic acid segment to be amplified; and d) the selected primers should have the predicted melting temperature. The primer sequence need not reflect the exact sequence of the target nucleic acid. For example, 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 target. Alternatively, non-complementary bases can be interspersed into the primer, provided that the primer sequence has sufficient complementarity with the target for annealing to occur and allow synthesis of a complementary nucleic acid strand.

The oligonucleotide primers and/or probes can comprise DNA, RNA, single-stranded or double-stranded and any chemical modifications thereof, such as PNA and LNA. Modifications include, but are not limited to, those that provide other chemical groups that incorporate additional charge, polarisability, hydrogen bonding, electrostatic interaction, and functionality to the individual nucleic acid bases or to the nucleic acid as a whole. Such modifications include, but are not limited to, modified bases such as 2'-position sugar modifications, 5-position pyrimidine modifications, 8-position purine modifications, modifications at cytosine exocyclic amines, substitution of 5-bromo-uracil; backbone modifications, methylations, unusual base-pairing combinations such as the isobases isocytidine and isoguanidine and the like. Modifications can also include 3' and 5' modifications such as capping. The presence of phosphorothioates and methylphosphonates is also contemplated.

In one embodiment, the primers have a predicted melting temperature range of between about 58 to 60 °C. In one embodiment, the primers have a predicted melting temperature of about 58, about 59 °C or about 60 °C.

In one embodiment, the assay utilises one or more labelled probes to identify a target nucleic acid. The label may be detectable by spectroscopic, photochemical, biochemical, immunochemical or chemical means. When two or more probes are used then each of the probes may be separately or distinctly detectable. In one embodiment, the assay utilises one or more fluorescer-quencher probes to identify a target nucleic acid. In another embodiment, the assay utilises one or more fluorescer-quencher probes with spectrally distinct fluorophores to identify a target nucleic acid. In another embodiment, the assay utilises combinations of fluorescer-quencher probes to identify members of a first set of two or more substantially homologous sequences that may be present, or suspected of being present, in a sample of nucleic acid and a second set of fluorescer-quencher probes in the same reaction to identify which members of a second set of two or more substantially homologous sequences are also present in the sample, or suspected of being present. Each of the fluorescer-quencher probes can have spectrally distinct fluorophores to separately identify the sets of sequences. The assay can be performed in a single reaction containing both the first and second sets of probes. The assay enables one to determine which members of the first set of substantially homologous sequences are present in the sample while simultaneously enabling one to determine which members of the second set of substantially homologous sequences are present in the sample.

Suitably, each of the two or more oligonucleotide probes is for distinguishing between two or more substantially homologous sequences, the two or more substantially homologous

sequences differing from each other by at least one nucleotide, each probe in the set perfectly matching one of two or more substantially homologous sequences.

Suitably, the two or more oligonucleotide probes are allelic probes for genotyping a first allelic site and a second allelic site, suitably, wherein the probes differ from each other by at least one nucleotide.

Suitably, the probes each produce distinguishable signals or signatures – such as fluorescent signals or signatures for each of the different genotypes being detected.

The number of probes that are used in the assay can vary depending upon the application of the method. Thus, 1 or more, 2 or more, 3 or more, 4 or more, 5 or more, 6 or more, 7 or more, 8 or more, 9 or more, 10 or more, 15 or more, or 20 or more probes can be used in each assay. One or more of the probes may differ by one or more nucleotides – such as two or more or three or more nucleotides. Each of the probes may differ by one or more nucleotides – such as two or more or three or more nucleotides. In certain embodiments, it may be desirable to determine only that a mutation is present rather than identifying the specific type of mutation. Thus the method can be used in a multiplex assay format using multiple probes, each of which differs by at least one nucleotide. Suitably, the probes produce the same or a different detectable signal. Suitably, each of the probes produces the same or a different detectable signal.

Detailed descriptions of nucleic acid amplification reactions employing fluorescer-quencher probes can be found in, for example, *PNAS* (USA) 88:7276–7280 (1992); *Clinical Chemistry*, 38:462–463 (1992); *Nucleic Acid Research*, 21: 3761–3766 (1993) and *PCR Methods and Applications*, 4:357–362 (1995). A variety of different fluorescer-quencher probes are known in the art. The fluorescer and quencher need not be positioned at a specific distance within a probe in order to achieve effective quenching. This facilitates the design and synthesis of the probes wherein the probe hybridizes to itself to form a hair-pin loop such that the quencher molecule is brought into proximity with the fluorescer in the absence of a complementary nucleic acid sequence. Exemplary fluorescer-quencher probes are molecular beacons. Molecular beacons are hairpin shaped molecules with an internally quenched fluorophore whose fluorescence is restored once bound to a target nucleic acid sequence. The loop portion is a probe sequence complementary to a target nucleic acid sequence. The stem is formed by the annealing of complementary arm sequences on the ends of the probe sequence. A fluorescent moiety is attached to the end of one arm and a quenching moiety is attached to the end of the other arm. The stem keeps these two moieties in close proximity to each other, causing the

fluorescence of the fluorophore to be quenched by energy transfer so that it is unable to fluoresce. When the probe encounters a target nucleic acid sequence, it forms a hybrid that is longer and more stable than the stem and its rigidity and length preclude the simultaneous existence of the stem hybrid. Thus, the molecular beacon undergoes a spontaneous conformational reorganization that forces the stem apart, and causes the fluorophore and the quencher to move away from each other, leading to the restoration of fluorescence. Thus, in one embodiment, the probe is molecular beacon. Upon binding of the probe to the nucleic acid target sequence the probe dye and quencher are separated without the requirement for a polymerase enzyme with 5'-3' nuclease activity. Molecular beacons are described in WO90/03446.

Thus, in one embodiment, the method includes the step of treating said reaction mixture under conditions that allow for the hybridisation of the oligonucleotide probe(s) to the target nucleic acid thereby resulting in the production of a detectable signal.

In another embodiment, the assay involves the digestion of an oligonucleotide probe containing a fluorescer and quencher during a nucleic acid amplification reaction to evidence nucleic acid amplification. Thus, the oligonucleotide probe can include at least one fluorescer and at least one quencher. During extension of the primer, the polymerase encounters the probe hybridised to the target nucleic acid and degrades the probe via the nuclease activity of the polymerase. Alternatively, digestion of the probe results in release of the fluorescer (or quencher) from the probe. This causes the fluorescer and quencher on the probe to become spatially separated from each other, thereby creating a change in fluorescence in the sample to indicate the extension of the primer and hence the amplification of the target nucleic acid.

Suitably, the fluorescers are fluorescent organic dyes derivatised for attachment to the terminal 3' carbon or terminal 5' carbon of the probe via a linking moiety. Methods also exist for linking both fluorescers and quenchers to internal moieties within the oligonucleotide. Suitably, quencher molecules can include organic dyes, which may or may not be fluorescent. Whether the quencher molecule is fluorescent or simply releases the transferred energy from the fluorescer by non-radioactive decay, the absorption band of the quencher should substantially overlap the fluorescent emission band of the fluorescer.

There is a great deal of practical guidance available in the literature for selecting appropriate fluorescer-quencher pairs. The literature also includes references providing exhaustive lists of fluorescent and chromogenic molecules and their relevant optical properties for choosing reporter-quencher pairs. There is also extensive guidance in the literature for derivatising

reporter and quencher molecules for covalent attachment via common reactive groups that can be added to an oligonucleotide. There are many linking moieties and methodologies for attaching fluorescer or quencher molecules to the 5' or 3' termini of oligonucleotides known in the art. Commercially available linking moieties can be employed that can be attached to an oligonucleotide during synthesis.

When desired, the amplification reaction can be carried out as an automated process.

The present invention also provides a reaction mixture useful for amplification of a target nucleic acid target comprising: (i) a nucleic acid polymerase, suitably, having a 5' to 3' nuclease activity; (ii) one or more sets of forward and reverse primers capable of hybridising to the target nucleic acid; and (iii) one or more or more oligonucleotide probe(s) for detecting the target nucleic acid when present in the amplified nucleic acid, wherein the oligonucleotide probes generate a detectable signal, and wherein the oligonucleotide probes have a predicted melting temperature that is higher than the predicted melting temperature of the primers; and optionally (iv) reagents and buffers required to carry out the amplification reaction.

The present invention also provides a kit useful for amplification of a target nucleic acid comprising: (i) a nucleic acid polymerase, suitably, having a 5' to 3' nuclease activity; (ii) one or more sets of forward and reverse primers capable of hybridising to the target nucleic acid; and (iii) one or more or more oligonucleotide probes for detecting the target nucleic acid when present in the amplified nucleic acid, wherein the oligonucleotide probes generate a detectable signal, and wherein the oligonucleotide probes have a predicted melting temperature that is higher than the predicted melting temperature of the primers; and optionally (iv) reagents and buffers required to carry out the amplification reaction.

According to another embodiment, the reaction mixture or the kit includes at least two sets of probes where each set of probes is for distinguishing between two or more substantially homologous sequences, the two or more substantially homologous sequences differing from each other by at least one nucleotide, and each probe in the set perfectly matching one of two or more substantially homologous sequences, and wherein the oligonucleotide probes have a predicted melting temperature that is higher than the predicted melting temperature of the primers.

In another embodiment, the reaction mixture or the kit includes at least a first set of allelic probes for genotyping a first allelic site and second set of allelic probes for genotyping a second allelic site. Each set of probes includes at least two probes which are capable of hybridizing to

the allelic site but differ from each other by at least one nucleotide. The at least two sets of probes are selected so as to produce distinguishable fluorescence signatures for the different genotypes being detected.

The reaction mixture or kit may also include sample nucleic acid which can serve as a control in the assay. The reaction mixture or kit may also include a fluorescent material for use as a passive internal standard.

Optionally, the reaction mixture or kit may also include buffer or other reagents for performing the method. Reagents can be supplied in a solid form or dissolved/suspended in a liquid buffer suitable for inventory storage, and later for exchange or addition into the reaction mixture when the hybridization assay is performed. Suitable individual packaging is normally provided and instructions for users are generally supplied as well. Diagnostic or prognostic procedures using the kits of this invention can be performed by clinical laboratories, experimental laboratories, practitioners, or private individuals.

The invention will be further described in the following examples, which are not intended to limit the scope of the invention described in the claims.

EXAMPLES

Materials and methods

All stated melting temperatures are derived from the "Beacon Designer version 6.02" software package (Premier Biosoft, Palo Alto, CA). The melting temperature calculations within this software are based on the nearest neighbour thermodynamic theory using SantaLucia values (SantaLucia, J., Jr. (1998) "A Unified View of Polymer, Dumbbell, and Oligonucleotide DNA Nearest-neighbour Thermodynamics", *Proc. Natl. Acad. Sci. U.S.A.* 95, 1460-1465.)

Real time PCR reactions are performed on an ICycler IQ5 or an Illumina ECO thermocycler. Each assay contains 300nM (final concentration) of forward and reverse primer as well as 300nM (final concentration) of both the wild type (WT) specific probe and the SNP specific probe. The PCR reaction is driven by the use of 0.5 Units of thermostable Taq DNA polymerase in an appropriate buffer containing 5mM MgCl₂. dNTPs are included at a final concentration of 0.15mM each. Target DNA is added as either synthetic vector template (present at 10⁴ copies per reaction) or as gDNA at 10ng per reaction. The nucleic acid primers and probes used are shown in Table 1.

Cycling protocols are as follows:

Standard cycling conditions:

95 °C for 10 minutes followed by 50 cycles at: 95 °C for 10 seconds and 60 °C for 60 seconds.

Two stage cycling conditions:

95°C for 10 minutes followed by 15 cycles at 95 °C for 10 seconds and 60°C for 60 seconds, followed by 35 cycles at 95°C for 10 seconds and 66°C for 60 seconds.

Data collection during the for the wild type probe occurs through the VIC channel and data collection for the SNP specific probe occurs through the ROX channel. The sequences of the primers and probes used in this experiment are set forth in Table 1.

Results

The use of 'standard cycling conditions' for all cycles leads to a lack of discrimination between genotypes (Figure 1). Two low melting temperature probes were included in the assay, each complimentary to a sequence where that sequence differed by one nucleotide and each with a unique fluorophore that was read through different channels. 'Standard cycling conditions' as set forth above with an annealing/elongation temperature of 60°C were used for 50 cycles. Both probes gave a fluorescent signal on both WT and SNP samples as shown in Figure 1. Unbroken lines: WT target sequence ; Dotted lines: SNP target sequence.

The use of an elevated annealing and elongation temperature for all cycles leads to a loss of sensitivity and end point fluorescence (Figure 2). Low melting temperature probes were used in the assay which is carried out with an annealing/elongation stage at 60°C using the 'standard cycling conditions' or increasing the annealing/elongation temperature to 66°C. Comparison of the amplification plots reveals a later C_q value for the higher temperature 'standard cycling conditions' as compared with the normal 'standard cycling conditions' at 60°C. This indicates a loss of sensitivity, and a loss of approximately 70% of end point fluorescence is also observed. Both factors are important for the functionality of the assay (Figure 2). See Figure 2. Unbroken lines: Fluorescent signal from probe binding the complimentary template; Dotted lines: Fluorescent signal from probe binding the non-complimentary template.

Two stage cycling utilising both standard and high temperature annealing/elongation stages significantly improves probe binding discrimination without loss of assay sensitivity or the end

point fluorescence achieved (Figure 3). Low melting temperature probes were used in the assay. When the annealing/elongation stage is held at 60°C for 50 cycles under the 'standard cycling conditions' both probes gave a fluorescent signal on both WT and SNP target sequences (Figure 3A). In contrast, by increasing the annealing/elongation temperature after 15 cycles from 60°C to 66°C ('two stage cycling conditions'), a considerable loss of non-complimentary probe binding was observed and subsequently each probe only gave a fluorescent signal on its homologous target sequence (Figure 3B). Unbroken lines: WT target sequence; Dotted lines: SNP target sequence.

The specificity of the probe interaction was tested in a reduced assay format using a single probe without a competitor probe (Figure 4). Using a single probe without a competitor probe, in conjunction with standard cycling, leads to a very strong fluorescent signal on both a homologous sample as well as a sample including a single mismatch under the probe target sequence (Figure 4A). In contrast, a single probe used in conjunction with the two stage cycling method demonstrated complete specificity (Figure 4B), *i.e.* a fluorescent signal was only obtained when the target sequence under the probe was 100% homologous. Unbroken lines: fluorescent signal from probe binding to non homologous template. Broken lines: fluorescent signal from probe binding to 100% homologous template.

Having demonstrated that a single probe has complete specificity for its cognate template, where the specificity is not contingent on the presence of another competitor template, it was decided to test the method in a multiplex system containing multiple probes, each of which differed by just one base (see Table 2). Multiple PCR reactions were performed using the two stage method and a range of wild-type and mutant templates. The system was configured such that a single wild type probe would produce fluorescence through one channel and all six mutant probes would report through the second channel (Figure 5). The results show that the wild type homologous probe only produced a strong fluorescent trace on the wild type template. Conversely, a mutant probe signal was produced through channel 2 when any of the mutant templates were present in the PCR reaction.

Improved discrimination of probe binding allows detection and quantification of genotypes when present in a mixed sample at a frequency as low as 5%. Figure 6A depicts that genotype quantification can be achieved through analysis of end point fluorescence since the fluorescent signal output is directly related to the number of probe binding and cleavage events. The false probe signal on its non-cognate channel is observed at 60°C (see Figure 6A and B) but not when using the two stage cycling protocol (see Figures 6B and 4D). This allows considerably improved detection limits down to 5% of the starting sample. Figure 6B is an enlarged section of

Figure 6A where at 60°C no discrimination between false signal and low abundance signal can be made whereas the use of low melting temperature probes with two stage cycling conditions gives very clear genotype identification when the template is present at a frequency of 5% of the starting sample – see Figure 6B. unbroken line: 100% probe target template; dotted line: 100% non cognate sequence; dashed and dotted lines: fluorescent signal from probe target template present at 5% of starting DNA sample.

Any publication cited or described herein provides relevant information disclosed prior to the filing date of the present application. Statements herein are not to be construed as an admission that the inventors are not entitled to antedate such disclosures. All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in cellular, molecular and plant biology or related fields are intended to be within the scope of the following claims.

TABLE 1

Exemplary primer and probe sequences and their associated predicted melting temperatures

	Sequence	Predicted melting temperature
Forward primer	TTACAACGTGTCTCTGCTTCTCC	59.3
Reverse primer	GATAGACATGGGTATGGCCTCTAAA	58.9
WT probe	CAGTCCCTTTCTCGTCGATGGTCAG	63.9
SNP probe	CAGTCCCTTTCTTGTCGATGGTCAG	62.4

TABLE 2

Exemplary primer and probe sequences for multiplex genotyping and their associated predicted melting temperatures

	Sequence	Predicted melting temperature
Forward primer	GCCTGCTGAAAATGACTGAATATAAAC	59
Reverse primer	CGTCCACAAAATGATTCTGAATTAGC	58.9
WT probe	TGCCTACGCCACCAGCTCCAA	64.7
G12D probe	TGCCTACGCCATCAGCTCCAA	62.4
G12V probe	TGCCTACGCCAACAGCTCCAA	62.9
G12S probe	TGCCTACGCCACTAGCTCCAA	61.8
G12C probe	TGCCTACGCCACAAGCTCCAA	62.9
G12A probe	TGCCTACGCCAGCAGCTCCAA	65
G13D probe	TGCCTACGTCACCAGCTCCAA	62

CLAIMS

1. A method for determining the presence of a target nucleic acid in a sample, the method comprising:

(i) performing a nucleic acid amplification on the sample using a reaction mixture comprising:

(a) a nucleic acid polymerase;

(b) one or more sets of forward and reverse primers capable of hybridising to the target nucleic acid; and

(c) one or more oligonucleotide probes for detecting the target nucleic acid when present in the sample and wherein the oligonucleotide probe(s) has a predicted melting temperature that is higher than the predicted melting temperature of the one or more sets of forward and reverse primers(s); and wherein

the cycling conditions during the nucleic acid amplification reaction comprise at least two different sets of cycling parameters,

the first set of cycling parameters including an annealing temperature in the nucleic acid amplification reaction that is less than or about the same as the temperature corresponding to the predicted melting temperature of the lowest melting temperature probe; and

the second set of cycling parameters including an annealing temperature in the nucleic acid amplification reaction that is equal to or higher than the temperature corresponding to the predicted melting temperature of the highest melting temperature probe;

(ii) detecting the signal produced in step (i); and

(iii) determining the presence or absence of the target nucleic acid based on the signal(s) produced in step (ii).

2. The method according to claim 1, wherein the predicted melting temperature of the nucleic acid probes is less than or equal to about 4 °C higher than the melting temperature of the nucleic acid primers, preferably, wherein the predicted melting temperature of the nucleic acid probes is less than about 3 °C, about 2 °C or about 1 °C higher than the melting temperature of the nucleic acid primers.
3. The method according to claim 1 or claim 2, wherein the probes have a predicted melting temperature range of between about 62 to about 65 °C or about 62 to about 64 °C, with the proviso that the predicted melting temperature is less than or equal to about 4 °C higher than the melting temperature of the nucleic acid primers
4. The method according to any of the preceding claims, wherein the primers have a predicted melting temperature range of between about 58°C to about 60 °C.
5. The method according to any of the preceding claims, wherein the first and/or second set of cycling parameters are repeated at least 5 or more times.
6. The method according to any of the preceding claims, wherein the probe produces a detectable signal when the target nucleic acid is present in the sample.
7. The method according to any of the preceding claims, wherein the probe(s) comprises a fluorescence moiety and a quenching moiety.
8. The method according to claim 7, wherein the second set of cycling parameters is repeated enough times for fluorescence data collection to occur for detection of a fluorescence spectrum.
9. The method according to any of the preceding claims, wherein the nucleic acid polymerase has 5' to 3' nuclease activity.
10. The method according to claim 9, where said method includes the further step, after step (i) of treating said reaction mixture under conditions that allow for the digestion of the oligonucleotide probe(s) via the nuclease activity of the polymerase when said oligonucleotide probe(s) is hybridised to the target nucleic acid;

11. A method for genotyping a sample comprising, or suspected of comprising two or more nucleic acid sequences that differ from each other by at least one base position, the method comprising:

(i) performing a nucleic acid amplification on the sample using a reaction mixture comprising:

(a) a nucleic acid polymerase;

(b) one or more sets of forward and reverse primers capable of hybridising to the nucleic acid; and

(c) two or more oligonucleotide probes for detecting each of the two or more sequences when present in the amplified nucleic acid, wherein each of the oligonucleotide probes generate a different detectable signal, and wherein the two or more oligonucleotide probes have a predicted melting temperature that is higher than the predicted melting temperature of the primers; and wherein

the cycling conditions during the nucleic acid amplification reaction comprise at least two different sets of cycling parameters,

the first set of cycling parameters including an annealing temperature in the nucleic acid amplification reaction that is less than the predicted melting temperature of the lowest melting temperature probe; and

the second set of cycling parameters including an annealing temperature in the nucleic acid amplification reaction that is higher than the predicted melting temperature of the highest melting temperature probe;

(ii) detecting the signal produced in step (i); and

(iii) determining the presence or absence of the two or more sequences based on the signal(s) produced in step (ii).

12. The method according to claim 11, wherein each of the two or more oligonucleotide probes is for distinguishing between two or more substantially homologous sequences, the two or more substantially homologous sequences differing from each other by at least one

nucleotide, each probe in the set perfectly matching one of two or more substantially homologous sequences; or wherein the two or more oligonucleotide probes are allelic probes for genotyping a first allelic site and a second allelic site, suitably, wherein the probes differ from each other by at least one nucleotide.

13. The method according to claim 11 or claim 12, wherein the probes each produce distinguishable fluorescence signatures for the different genotypes being detected.

14. The method according to any of the preceding claims, wherein the nucleic acid amplification reaction is performed using the polymerase chain reaction.

15. The method according to any of claims 11 to 14, wherein the nucleic acid polymerase has 5' to 3' nuclease activity, preferably, where said method includes the further step, after step (i) of treating said reaction mixture under conditions that allow for the digestion of the oligonucleotide probe(s) via the nuclease activity of the polymerase when said oligonucleotide probe(s) is hybridised to the target nucleic acid.

FIGURE 1

1/9

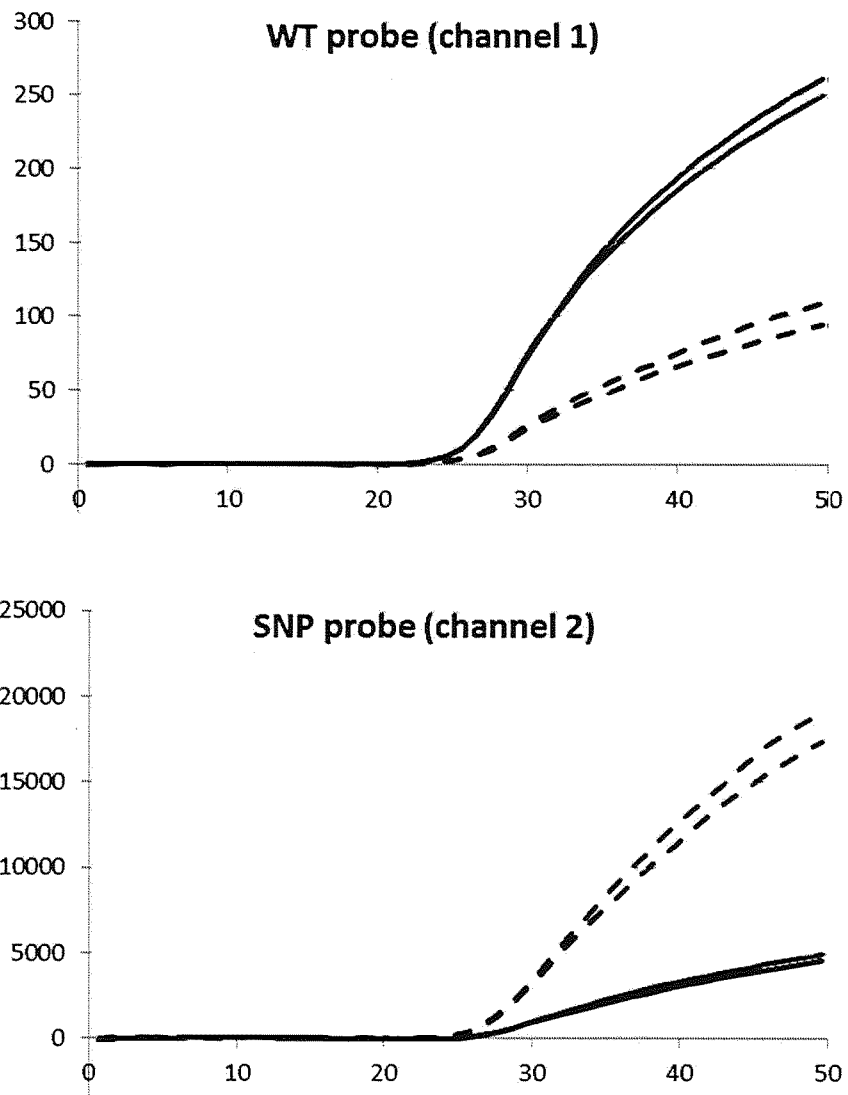


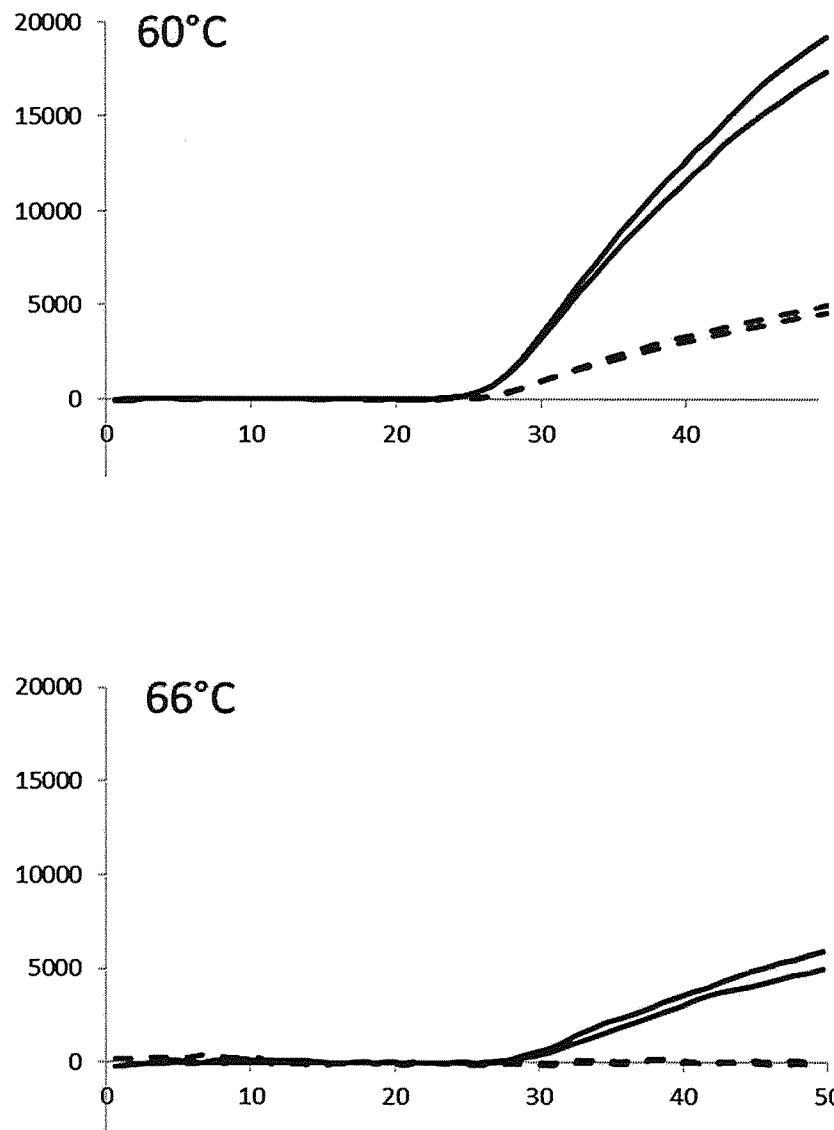
FIGURE 2
2/9

FIGURE 3A
3/9

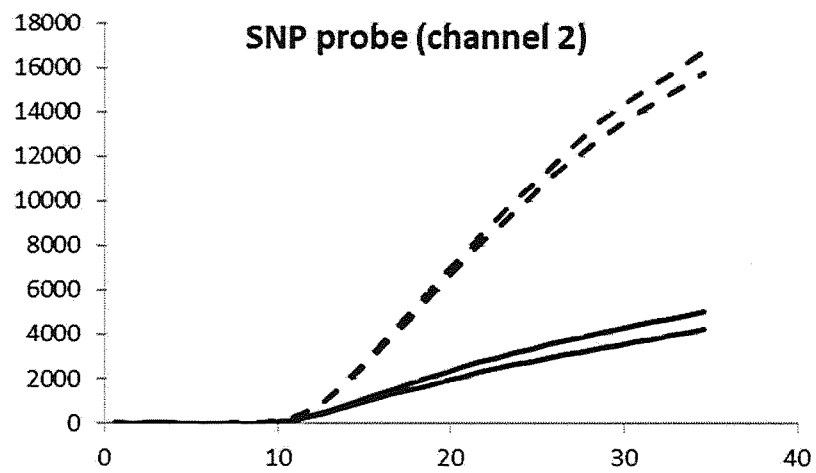
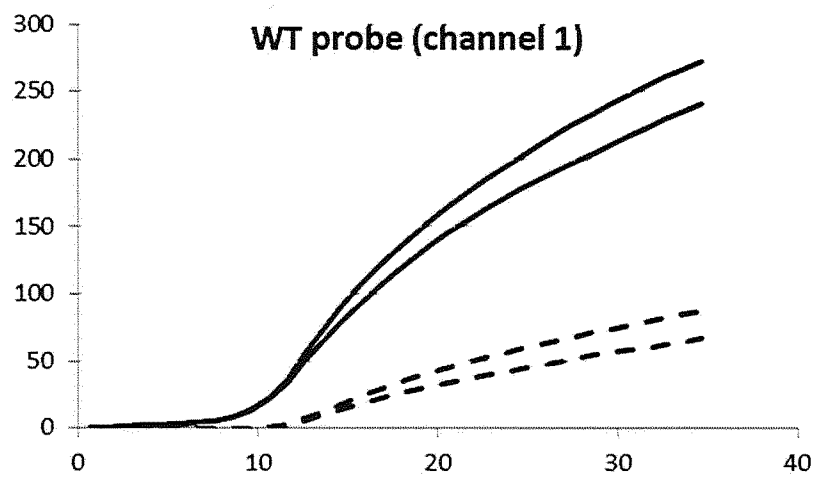


FIGURE 3B
4/9

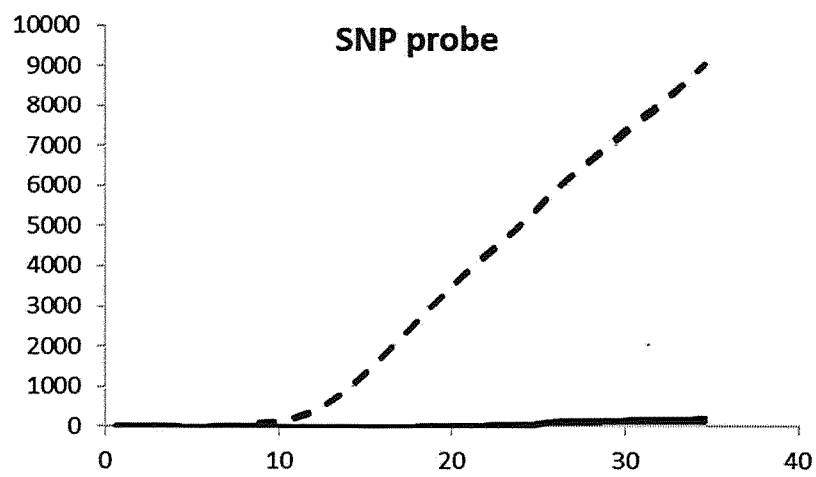
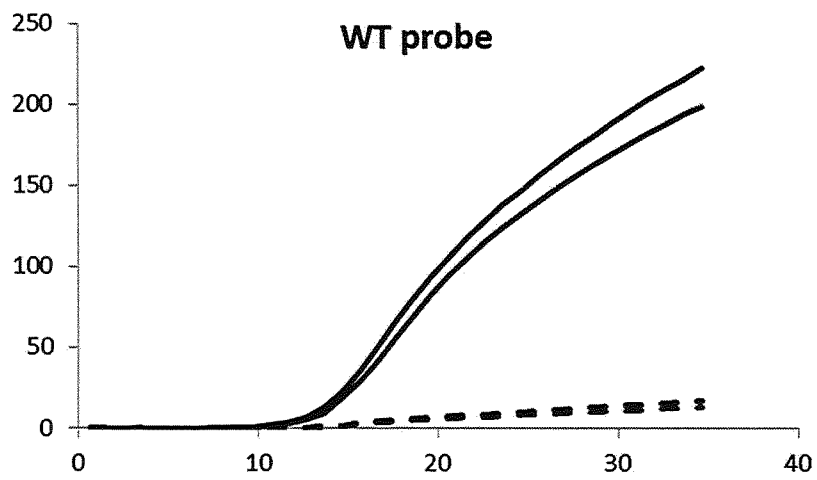


FIGURE 4A
5/9

WT probe Channel 1 60°C

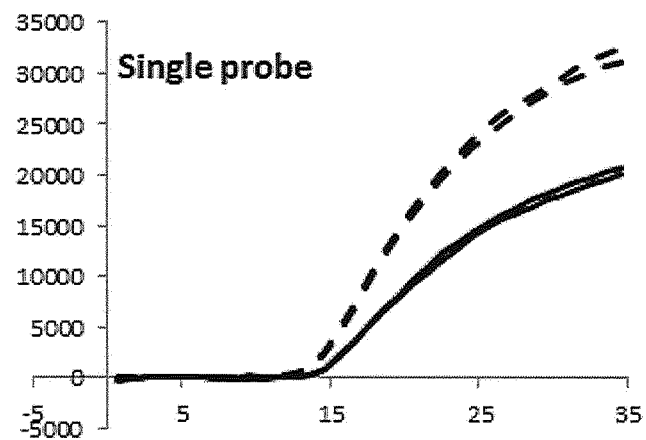
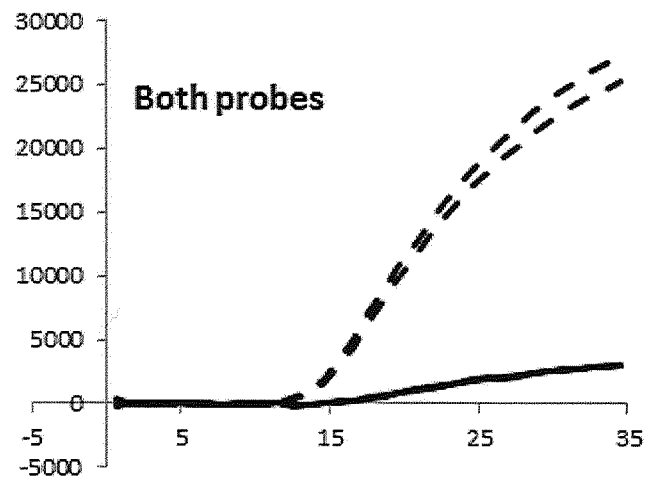


FIGURE 4B
6/9

WT probe Channel 1 66°C (2 stage cycling)

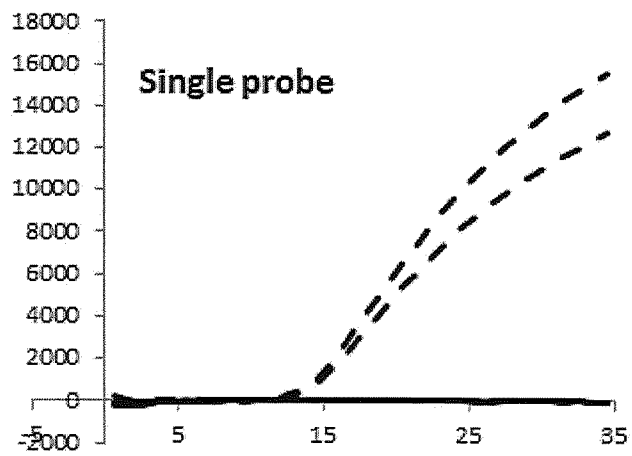
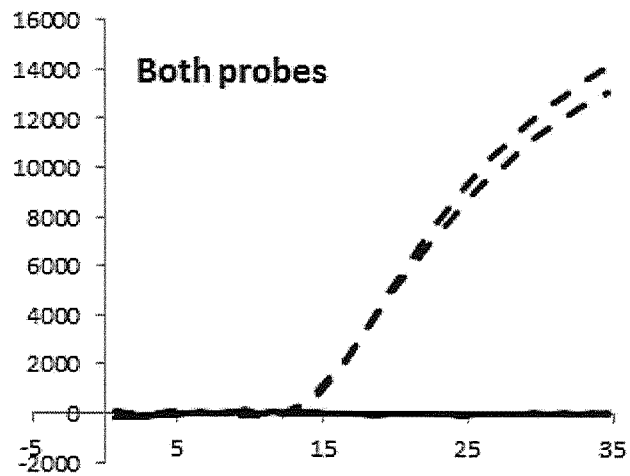


FIGURE 5
7/9

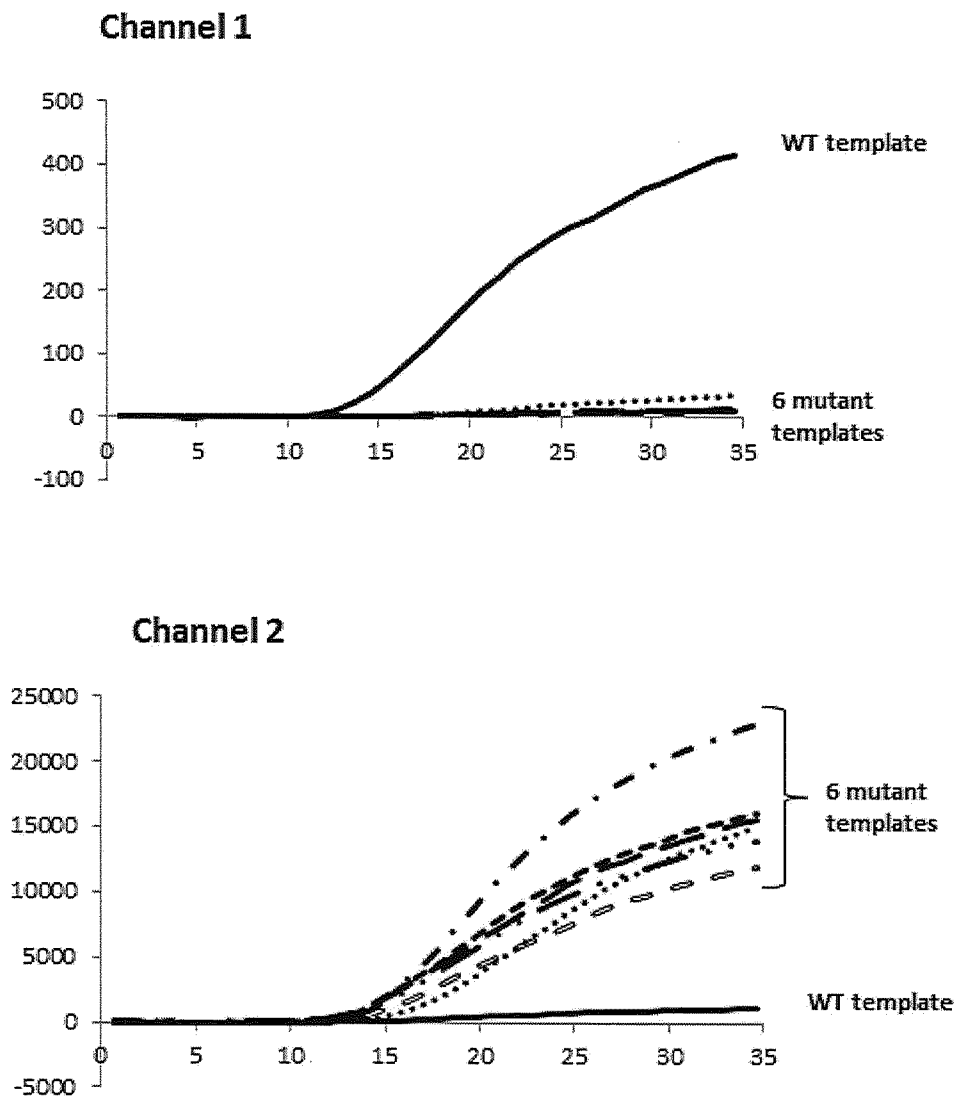


FIGURE 6A
8/9

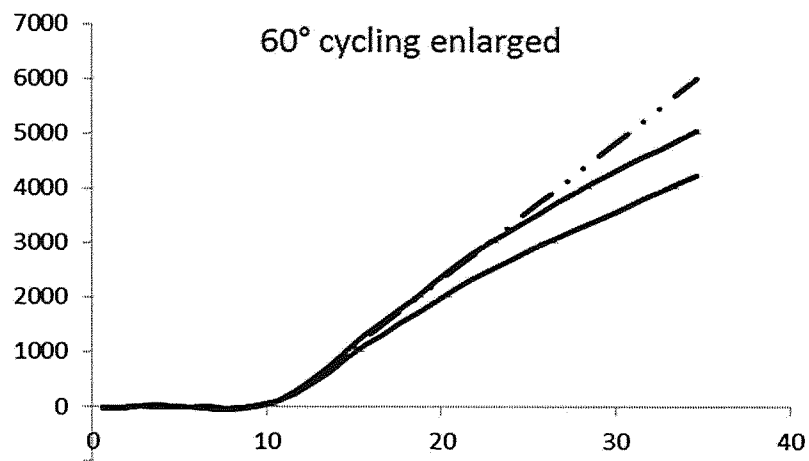
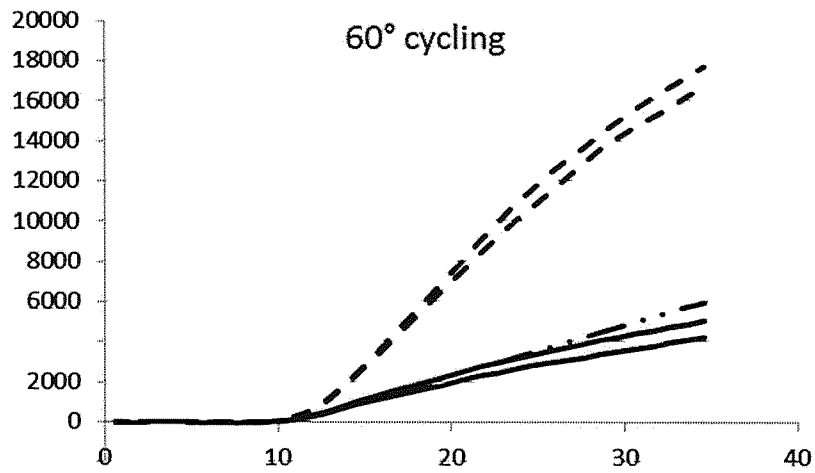
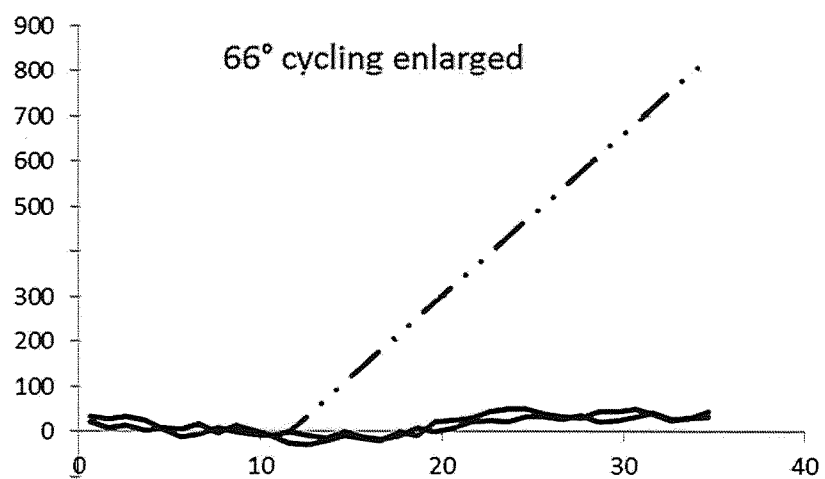
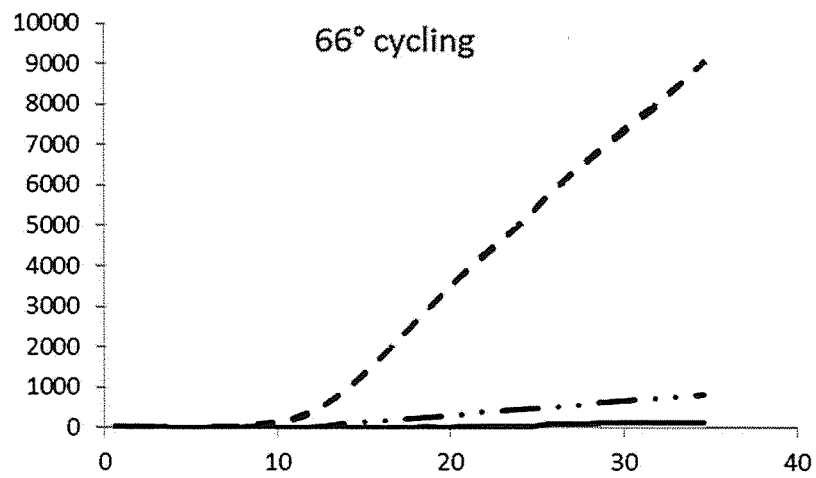


FIGURE 6B
9/9



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2013/051781

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/68
ADD.

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 practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 2 228 454 A1 (ROCHE DIAGNOSTICS GMBH [DE]; HOFFMANN LA ROCHE [CH]) 15 September 2010 (2010-09-15)	1-10
Y	abstract page 3, paragraph 4 - paragraph 5 page 6, paragraph 8 - page 9, paragraph 2 page 10, paragraph 1 - paragraph 5; claims 1-7; figure 1	11-15
Y	----- US 6 174 670 B1 (WITTEWER CARL T [US] ET AL) 16 January 2001 (2001-01-16) examples 21,23	11-15
A	----- WO 2012/009813 A1 (SOCRA SCIENCES SANTE ET HUMAINES S E C [CA]; FROST ERIC [CA]; DESLAND) 26 January 2012 (2012-01-26) the whole document ----- -/-	1-15



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 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

1 July 2013

Date of mailing of the international search report

12/07/2013

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

Tilkorn, A

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2013/051781

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 2007/045890 A1 (PRIMERDESIGN LTD [GB]; POWELL ROBERT [GB]) 26 April 2007 (2007-04-26) the whole document -----	1-15
A	WO 2010/139937 A1 (OXITEC LTD [GB]; FU GUOLIANG [GB]) 9 December 2010 (2010-12-09) the whole document -----	1-15
A	ZHU LING-XIANG ET AL: "Multiplex asymmetric PCR-based oligonucleotide microarray for detection of drug resistance genes containing single mutations in Enterobacteriaceae", ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, AMERICAN SOCIETY FOR MICROBIOLOGY, WASHINGTON, DC, US, vol. 51, no. 10, 1 October 2007 (2007-10-01), pages 3707-3713, XP002532643, ISSN: 0066-4804, DOI: 10.1128/AAC.01461-06 [retrieved on 2007-07-23] abstract page 3708, column 2, paragraph 2 - paragraph 3 -----	1-15
X,P	WO 2012/013733 A1 (HOFFMANN LA ROCHE [CH]; ROCHE DIAGNOSTICS GMBH [DE]; EICKHOFF MEIKE [C] 2 February 2012 (2012-02-02) abstract page 27, line 24 - page 28, line 6 page 34, line 7 - page 35, line 16; example 1 -----	1-10

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2013/051781

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 2228454	A1	15-09-2010	CA 2696652 A1 10-09-2010
		CN 101831496 A 15-09-2010	
		EP 2228454 A1 15-09-2010	
		JP 2010207220 A 24-09-2010	
		US 2010233686 A1 16-09-2010	

US 6174670	B1	16-01-2001	AT 295427 T 15-05-2005
		AT 318327 T 15-03-2006	
		AT 428801 T 15-05-2009	
		AU 726501 B2 09-11-2000	
		CA 2257109 A1 11-12-1997	
		CA 2591550 A1 11-12-1997	
		CA 2658290 A1 11-12-1997	
		DE 69733282 D1 16-06-2005	
		DE 69733282 T2 19-01-2006	
		DE 69735313 T2 02-11-2006	
		DK 0912766 T3 10-08-2009	
		DK 1179600 T3 05-09-2005	
		EP 0912766 A1 06-05-1999	
		EP 1033411 A2 06-09-2000	
		EP 1179600 A1 13-02-2002	
		ES 2243393 T3 01-12-2005	
		ES 2326050 T3 29-09-2009	
		JP 4118932 B2 16-07-2008	
		JP 4401416 B2 20-01-2010	
		JP 4540754 B2 08-09-2010	
		JP 2000509608 A 02-08-2000	
		JP 2007185188 A 26-07-2007	
		JP 2008173127 A 31-07-2008	
		JP 2010046063 A 04-03-2010	
		NZ 333136 A 27-03-2000	
		NZ 502323 A 28-09-2001	
		PT 912766 E 16-07-2009	
		PT 1179600 E 31-08-2005	
		US 6174670 B1 16-01-2001	
		US 6232079 B1 15-05-2001	
		US 6245514 B1 12-06-2001	
		US 2002058258 A1 16-05-2002	
		US 2004002098 A1 01-01-2004	
		US 2006029965 A1 09-02-2006	
		US 2009258414 A1 15-10-2009	
		US 2009311673 A1 17-12-2009	
		US 2012258524 A1 11-10-2012	
		WO 9746714 A1 11-12-1997	

WO 2012009813	A1	26-01-2012	NONE

WO 2007045890	A1	26-04-2007	NONE

WO 2010139937	A1	09-12-2010	AU 2010255523 A1 19-01-2012
		EP 2438189 A1 11-04-2012	
		US 2012164645 A1 28-06-2012	
		WO 2010139937 A1 09-12-2010	

WO 2012013733	A1	02-02-2012	CA 2802563 A1 02-02-2012
		CN 103025891 A 03-04-2013	
		DE 112011102524 T5 11-07-2013	
		EP 2598654 A1 05-06-2013	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2013/051781

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
W0 2012013733 A1 02-02-2012			
