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(54) Title: ALLELE-SPECIFIC AMPLIFICATION OF NUCLEIC ACIDS

(57) Abstract: The present invention includes a method of allele-specific amplification, utilizing an allele-specific oligonucleotide, at least partially complementary to more than one variant of the target sequence, having an internally-placed selective nucleotide complementary to only one variant of the target sequence wherein the allele-specific oligonucleotide is extended by a nucleic acid polymerase predominantly or exclusively when hybridized to the variant of the target sequence for which it has said complementary selective nucleotide.

ALLELE-SPECIFIC AMPLIFICATION OF NUCLEIC ACIDS

FIELD OF THE INVENTION

The invention relates to the field of nucleic acid amplification and specifically to the field of
5 allele-specific amplification.

BACKGROUND OF THE INVENTION

Allele-specific amplification of nucleic acids allows for simultaneous amplification and
analysis of the target sequence. Allele-specific amplification is commonly used when the
10 target nucleic acid has one or more variations (polymorphisms) in its sequence. Nucleic
acid polymorphisms are used in DNA profile analysis (forensics, paternity testing, tissue
typing for organ transplants), genetic mapping, distinguishing between pathogenic strains
of microorganisms as well as detection of rare mutations, such as those occurring in cancer
cells, existing in the background of cells with normal DNA.

15 In a successful allele-specific amplification, the desired variant of the target nucleic acid is
amplified, while the other variants are not, at least not to a detectable level. A typical allele-
specific amplification assay involves a polymerase chain reaction (PCR) with at least one
allele-specific primer designed such that primer extension occurs only when the primer
forms a hybrid with the desired variant of the target sequence. When the primer hybridizes
20 to an undesired variant of the target sequence, primer extension is inhibited.

Many ways of enhancing allele-specificity of primers have been proposed. However, for
many clinically-relevant nucleic acid targets lack of specificity of PCR remains a problem.
Therefore radically novel approaches to design of allele-specific primers are necessary.

25 SUMMARY OF THE INVENTION

In a first aspect, the invention relates to a method of allele-specific amplification of a variant
of a target sequence, the target existing in the form of several variant sequences, the method
comprising

(a) hybridizing a first and a second oligonucleotides to at least one variant of the target sequence; wherein the first oligonucleotide is at least partially complementary to one or more variants of the target sequence, and the second oligonucleotide is at least partially complementary to one or more variants of the target sequence, and has at least one internal selective nucleotide complementary to only one variant of the target sequence;

(b) extending the second oligonucleotide with a nucleic acid polymerase, wherein said polymerase is capable of extending said second oligonucleotide preferentially when said selective nucleotide forms a base pair with the target, and substantially less when said selective nucleotide does not form a base pair with the target.

In a second aspect, the invention relates to a method of detecting a variant of a target sequence, the target existing in the form of several variant sequences, the method comprising

(a) hybridizing a first and second oligonucleotides to at least one variant of the target sequence; wherein said first oligonucleotide is at least partially complementary to one or more variants of the target sequence and said second oligonucleotide is at least partially complementary to one or more variants of the target sequence, and has at least one internal selective nucleotide complementary to only one variant of the target sequence;

(b) extending the second oligonucleotide with a nucleic acid polymerase; wherein said polymerase is capable of extending said second oligonucleotide preferentially when said selective nucleotide forms a base pair with the target, and substantially less when said selective nucleotide does not form a base pair with the target; and

(c) detecting the products of said oligonucleotide extension, wherein the extension signifies the presence of the variant of a target sequence to which the oligonucleotide has a complementary selective nucleotide.

In a third aspect, the invention relates to a kit for allele-specific amplification of a target sequence, said target existing in the form of several variant sequences, the kit comprising

(a) a first oligonucleotide, at least partially complementary to one or more variant of the target sequence; and

(b) a second oligonucleotide, at least partially complementary to one or more variants of the target sequence having at least one internal selective nucleotide complementary to only one variant of the target sequence.

In a fourth aspect, the invention relates to an oligonucleotide for performing an allele-specific amplification of a target sequence, said target existing in the form of several variant sequences, the oligonucleotide comprising

(a) a sequence at least partially complementary to a portion of one or more variants of said target sequence;

(b) at least one internal selective nucleotide complementary to only one variant of the target sequence.

In a fifth aspect, the invention relates to a reaction mixture for allele-specific amplification of a target sequence, said target existing in the form of several variant sequences, the mixture comprising

(a) a first oligonucleotide, at least partially complementary to one or more variant of the target sequence; and

(b) a second oligonucleotide, at least partially complementary to one or more variants of the target sequence but having at least one internal selective nucleotide complementary to only one variant of the target sequence.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the results of allele-specific amplification using various nucleic acid polymerases and primers with internal selective nucleotide according to the present invention.

Figure 2 shows the results of allele-specific amplification using various nucleic acid polymerases and various primers with 3' selective nucleotide as a control.

Figure 3 shows the results of allele-specific amplification using various nucleic acid polymerases and various primers with internal selective nucleotide according to the present invention, including primers having a scorpion ARMS format.

Figure 4 shows a schematic representation of the structure of a scorpion ARMS format that can be used according to the invention.

DETAILED DESCRIPTION OF THE INVENTION

10 *Definitions*

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. In describing and claiming the present invention, the following definitions will be used.

15 The term "nucleic acid" refers to polymers of nucleotides (e.g., ribonucleotides, deoxyribonucleotides, nucleotide analogs etc.) and comprising deoxyribonucleic acids (DNA), ribonucleic acids (RNA), DNA-RNA hybrids, oligonucleotides, polynucleotides, aptamers, peptide nucleic acids (PNAs), PNA-DNA conjugates, PNA-RNA conjugates, etc., that comprise nucleotides covalently linked together, either in a linear or branched fashion.

20 A nucleic acid is typically single-stranded or double-stranded and will generally contain phosphodiester bonds, although in some cases, nucleic acid analogs are included that may have alternate backbones, including, for example, phosphoramidate (Beaucage et al. (1993) Tetrahedron 49(10):1925); phosphorothioate (Mag et al. (1991) Nucleic Acids Res. 19:1437; and U.S. Pat. No. 5,644,048), phosphorodithioate (Briu et al. (1989) J. Am. Chem. Soc.

25 111:2321), O-methylphosphoroamidite linkages (see Eckstein, Oligonucleotides and Analogues: a Practical Approach, Oxford University Press (1992)), and peptide nucleic acid backbones and linkages (see, Egholm (1992) J. Am. Chem. Soc. 114:1895). These modifications of the ribose-phosphate backbone may be done to facilitate the addition of

additional moieties such as labels, or to alter the stability and half-life of such molecules in physiological environments.

In addition to the naturally occurring heterocyclic bases that are typically found in nucleic acids (e.g., adenine, guanine, thymine, cytosine, and uracil), nucleotide analogs also may include non-naturally occurring heterocyclic bases, such as those described in, e.g., Seela et al. (1999) *Helv. Chim. Acta* 82:1640. Certain bases used in nucleotide analogs act as melting temperature (T_m) modifiers. For example, some of these include 7-deazapurines (e.g., 7-deazaguanine, 7-deazaadenine, etc.), pyrazolo[3,4-d]pyrimidines, propynyl-dN (e.g., propynyl-dU, propynyl-dC, etc.), and the like. See, e.g., U.S. Patent No. 5,990,303.

Other representative heterocyclic bases include, e.g., hypoxanthine, inosine, xanthine; 8-aza derivatives of 2-aminopurine, 2,6-diaminopurine, 2-amino-6-chloropurine, hypoxanthine, inosine and xanthine; 7-deaza-8-aza derivatives of adenine, guanine, 2-aminopurine, 2,6-diaminopurine, 2-amino-6-chloropurine, hypoxanthine, inosine and xanthine; 6-azacytidine; 5-fluorocytidine; 5-chlorocytidine; 5-iodocytidine; 5-bromocytidine; 5-methylcytidine; 5-propynylcytidine; 5-bromovinyluracil; 5-fluorouracil; 5-chlorouracil; 5-iodouracil; 5-bromouracil; 5-trifluoromethyluracil; 5-methoxymethyluracil; 5-ethynyluracil; 5-propynyluracil, and the like.

A “template nucleic acid”, “template” or “target” refers to a nucleic acid of interest to which a primer can hybridize and be extended under suitable conditions. In the context of nucleic acid amplification, “target” is preferably a region of nucleic acid, consisting of sequences at least partially complementary to at least two primer sequences, and an intervening sequence. (If the target is a single stranded nucleic acid, it consists of a sequence at least partially complementary to one primer and a sequence at least partially identical to the second primer.) Template nucleic acids can exist as isolated nucleic acid fragments or be a part of a larger nucleic acid fragment. Target nucleic acids can be derived or isolated from essentially any source, such as cultured microorganisms, uncultured microorganisms, complex biological mixtures, tissues, sera, ancient or preserved tissues or samples, environmental isolates or the like. Further, template nucleic acids optionally include or are derived from cDNA, RNA, genomic DNA, cloned genomic DNA, genomic DNA libraries, enzymatically fragmented DNA or RNA, chemically fragmented DNA or RNA, physically fragmented DNA or RNA, or the like. Template nucleic acids can also be chemically synthesized using techniques known in the art.

An “oligonucleotide” refers to a nucleic acid polymer that includes at least two, but typically 5-50 nucleotides and more typically, between 15 and 35 nucleotides. Oligonucleotides may be prepared by any suitable method known in the art, including, for example, cloning and restriction digestion of appropriate sequences, or direct chemical synthesis by a method
5 such as the phosphotriester method of Narang et al. (1979) *Meth. Enzymol.* 68:90-99; the phosphodiester method of Brown et al. (1979) *Meth. Enzymol.* 68:109-151; the diethylphosphoramidite method of Beaucage et al. (1981) *Tetrahedron Lett.* 22:1859-1862; the triester method of Matteucci et al. (1981) *J. Am. Chem. Soc.* 103:3185-3191; automated synthesis methods; the solid support method of U.S. Pat. No. 4,458,066 or any other
10 chemical method known in the art.

A “primer” is an oligonucleotide that can hybridize to a template nucleic acid and permit chain extension or elongation using a nucleotide polymerase. Although other primer lengths are sometimes utilized, primers typically range from 15 to 35 nucleotides. Short primers generally form sufficiently stable hybrids with template nucleic acids at cooler
15 temperatures. A primer need not be perfectly complementary to the template nucleic acids for the extension to occur. A primer that is at least partially complementary to the template nucleic acid is typically capable of hybridizing with the template nucleic acid for extension to occur. A primer nucleic acid can be labeled, if desired, by incorporating a label detectable by radiological, spectroscopic, photochemical, biochemical, immunochemical, or
20 chemical techniques.

An “allele-specific primer” is a primer that can hybridize to several variants of the template nucleic acid, but permit elongation by the polymerase when hybridized with only some of the variants of the template nucleic acid. With other variants of the template nucleic acid the primer-template hybrid may not be extended or is extended less efficiently by the
25 polymerase.

Nucleic acids are “extended” or “elongated” when additional nucleotides are incorporated into the nucleic acids, for example by a nucleotide incorporating biocatalyst, at the 3' end of a nucleic acid.

An amplification assay is “selective” or “allele-selective” if it yields predominance (i.e., a
30 majority but less than 100%) of one product over other possible products. An assay is described as “allele-selective” as long as amplification of the undesired (mismatched)

variant of the target sequence is detectable. The term “specific” or “allele-specific” with respect to amplification assay is used if one of the possible products is formed exclusively. An assay where amplification of the undesired target is undetectable is called “allele-specific.” However, it is understood that as the methods of detection become more
5 sensitive, some assays previously known to be allele-specific, turn out to be allele-selective, i.e. some amplification of undesired variants of the target becomes detectable. Therefore, in the context of this invention, the term “allele-specific” is meant to encompass both strictly allele-specific, as well as allele-selective amplification.

A “genotype” refers to all or part of the genetic constitution of a cell or subject, or group of
10 cells or subjects. For example, a genotype includes the particular mutations and/or alleles (e.g., polymorphisms, such as single nucleotide polymorphisms (SNPs) or the like) present at a given locus or distributed in a genome.

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

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

A “modified” enzyme refers to an enzyme comprising an amino acid polymer in which at
30 least one monomer differs from the reference sequence, such as a native or wild-type form of the enzyme. Exemplary modifications include monomer insertions, deletions, and

substitutions. Modified enzymes also include chimeric enzymes that have identifiable component sequences (e.g., structural or functional domains, etc.) derived from two or more parent enzymes. Also included within the definition of modified enzymes are those comprising chemical modifications of the reference sequence. The examples of modified polymerases include G46E E678G CS5 DNA polymerase, G46E L329A E678G CS5 DNA polymerase, G46E L329A D640G S671F CS5 DNA polymerase, G46E L329A D640G S671F E678G CS5 DNA polymerase, a G46E E678G CS6 DNA polymerase, Δ Z05 polymerase, Δ Z05-Gold polymerase, Δ Z05R polymerase, E615G Taq DNA polymerase, E678G TMA-25 polymerase, E678G TMA-30 polymerase, and the like.

10 The term "5' to 3' nuclease activity" or "5'-3' nuclease activity" refers to an activity of a nucleic acid polymerase, typically associated with the nucleic acid strand synthesis, whereby nucleotides are removed from the 5' end of a nucleic acid strand, e.g., *E. coli* DNA polymerase I has this activity, whereas the Klenow fragment does not.

The term "3' to 5' nuclease activity" or "3'-5' nuclease activity" or "proof-reading activity" 15 refers to an activity of a nucleic acid polymerase, whereby nucleotides are removed from the 3' end of the nucleic acid strand. For example, *E. coli* DNA polymerase III has this activity, whereas the *Thermus aquaticus* (Taq) DNA polymerase does not.

A "label" refers to a moiety attached (covalently or non-covalently), to a molecule and capable of providing information about the molecule. Exemplary labels include fluorescent 20 labels, colorimetric labels, chemiluminescent labels, bioluminescent labels, radioactive labels, mass-modifying groups, antibodies, antigens, biotin, haptens, and enzymes (including peroxidase, phosphatase, etc.).

A "fidelity" or "replication fidelity" is the ability of a nucleic acid polymerase to incorporate a correct nucleotide during template-dependent polymerization. In the context of 25 replication fidelity, "correct nucleotide" on the nascent nucleotide strand is the nucleotide paired with the template nucleotide via standard Watson-Crick base pairing. Replication fidelity of a particular polymerase results from a combination of incorporating correct nucleotides and removing incorrect nucleotides from the 3'-terminus of the nascent nucleotide strand via the 3'-5' nuclease activity of the polymerase. Various methods of 30 measuring fidelity of a nucleotide polymerase are reviewed in Tindall et al. (1988) Fidelity

of DNA synthesis by the *Thermus aquaticus* DNA polymerase. *Biochemistry*, 27:6008-6013. Typically, polymerases with 3'-5' nuclease (proofreading) capability have higher fidelity than polymerases without the proof-reading activity.

5 A "hot start", in the context of a nucleic acid amplification reaction, refers to a protocol, where at least one critical reagent is withheld from the reaction mixture (or, if present in the reaction mixture, the reagent remains inactive) until the temperature is raised sufficiently to provide the necessary hybridization specificity of the primer or primers. A "hot start enzyme" is an enzyme, typically a nucleic acid polymerase, capable of acting as the "withheld" or inactive reagent in a hot start protocol.

10 A "selective nucleotide" is a nucleotide in an allele-specific primer that confers allele selectivity to the primer. The selective nucleotide is complementary to a corresponding nucleotide in the desired variant of the target nucleic acids but not complementary to the corresponding nucleotide in the undesired variants of the target nucleic acid. In a primer, more than one nucleotide may be complementary to a nucleotide in the desired variants of
15 the target nucleic acids but not complementary to the corresponding nucleotide in the undesired variants of the target nucleic acid. However, the selective nucleotide is located at a position within the primer that affects the specificity of the primer. The selective nucleotide permits efficient or inefficient amplification of the target nucleic acid, depending on whether or not it finds or does not find a complementary partner in the target nucleic
20 acid. A primer may contain more than one selective nucleotide.

A "Watson-Crick base pairing" or simply "base pairing" refers to "conventional" hydrogen bonding within a double-stranded nucleic acid molecule. Watson-Crick base pairing is hydrogen bonding between complementary bases, such as bonding between adenine and thymine, between guanine and cytosine, between adenine and uracil, and between analogs
25 of these bases.

The terms "scorpion", "scorpion-like" or "Scorpion ARMS-like" as used herein denote unimolecular primer-probe combination as described in Whitcombe et al., (1999). Detection of PCR products using self-probing amplicons and fluorescence, *Nature Biotech.* 17:804-807. Scorpion or scorpion-like primers within the meaning of the present invention
30 incorporate the typical elements of the scorpion, namely a probe portion, a stem loop

portion and a primer portion. An example of “scorpion” or “scorpion-like” unimolecular primer-probe format is illustrated in Fig. 4.

The term “internal” as used herein, for example in the expression “a second oligonucleotide, at least partially complementary to one or more variants of the target sequence, but having at least one internal selective nucleotide complementary to only one
5 variant of the target sequence” denotes any nucleotide other than the 3’ terminal, for example 1 to 5 nucleotides internally of the 3’-end.

The expression “wherein said polymerase is capable of extending said second oligonucleotide preferentially when said selective nucleotide forms a base pair with the
10 target, and substantially less when said selective nucleotide does not form a base pair with the target” means that extension of the second oligonucleotide by the polymerase is more efficient when the selective nucleotide forms a base pair with the target, than when said selective nucleotide does not form a base pair with the target. This can for example be measured or quantified with the material and methods described in example 1, the results
15 of which are shown on Figure 1.

The present invention teaches a new allele-specific amplification primer, a method of designing such primer, a method of using the primer in allele-specific amplification a reaction mixture and a kit including the primer. The method of designing the primer may be used alone or in conjunction with existing methods of designing allele-specific primers.
20 A typical allele-specific primer is designed to hybridize to a polymorphic region of the target sequence and contain at least one selective nucleotide, i.e. nucleotide complementary to the desired variants of the polymorphic nucleotide in the target and non-complementary to the undesired variants of the target. Traditionally it was considered necessary to place the selective nucleotide at the 3’-end of the primer, because the terminal mismatch was thought
25 to be a necessary prerequisite for allele-specific amplification. See Newton et al. (1989) Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). Nucl. Acids Res. 17:2503-2516.

The present inventors have discovered that internal placement of the selective nucleotide is sufficient to ensure allele-specificity of the primer. The terminal mismatch is not required
30 to confer specificity upon the primer. A sole internal mismatch is sufficient to inhibit extension of the mismatched primer by a nucleotide polymerase. According to the present

invention, the selective nucleotide is placed internally of the 3'-end of the primer, between 1 and 5 nucleotides internally of the 3'-end. When the internal nucleotide is mismatched, the undesired, mismatched template is not amplified or amplified less efficiently, while the desired, matched template is amplified efficiently.

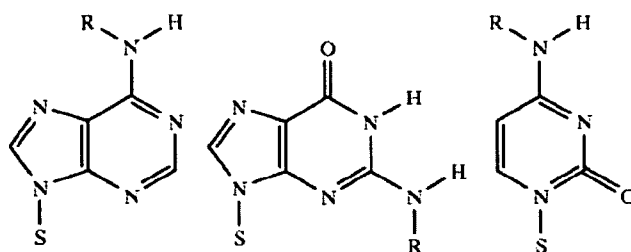
- 5 In one embodiment, the invention is an oligonucleotide (primer) for use in allele-specific PCR. The primer of the invention comprises 10-50, more preferably 15-35 nucleotides, the majority of them complementary to a sequence in more than one variant of the target sequence. The primer also contains at least one internal selective nucleotide complementary to only one variant of the target sequence.
- 10 In some embodiments, the allele-specific primer further contains one or more nucleotides with chemical modifications that further increase its specificity. For example, modifications at the exocyclic amine of a nucleobase have been described in U.S. Patent No. 6,001,611. The allele specific primer according to the present invention may have a modification at the exocyclic amine of one or more nucleobases. In some embodiments, the modified-base
- 15 nucleotide occurs between 1 and 5, but preferably 3 nucleotides upstream of the 3'-terminal nucleotide. In other embodiments, the modified-base nucleotide is the 3'-terminal nucleotide. In some embodiments, the modified-base nucleotide occurs both at the 3'-terminus as well as elsewhere within the oligonucleotide primer. In yet other embodiments, the modification may be placed on the selective nucleotide within the allele-specific primer.
- 20 According to the present invention, a suitable modification of the exocyclic amino group may be selected based on the presence of the following properties: (1) the modification interferes with, but does not prevent, Watson-Crick base pairing of the modified base with the complementary base in the double-stranded nucleic acid; (2) the modification interferes with but does not prevent the extension of the primer containing the modified
- 25 base by the nucleic acid polymerase; (3) the modification allows synthesis of the strand complementary to the strand incorporating the modified base; and (4) the modification increases selectivity of a primer incorporating the modification.

The examples of exocyclic amino groups include the amino groups in the 6-position of adenosine, 2-position of guanosine and 4-position of cytidine. Exocyclic amino groups that

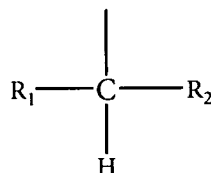
30 take part in base pairing with the complementary nucleic acid strand may also occur in various unconventional nitrogenous bases in nucleotides. Examples of nucleosides with

unconventional bases include, without limitation, 3-methyladenosine, 7-methylguanosine, 3-methylguanosine, 5-methylcytidine, and 5-hydroxymethylcytidine. Suitable modifications of exocyclic amino groups of such unconventional bases may also be selected according to the empirical method of the present invention.

- 5 The structures of the modified nucleotides containing a modified adenine, guanine, and cytosine base, respectively, are shown below,



- where S represents the sugar moiety, and R represents the modifier group. A variety of modifier groups are envisioned which possess the four properties outlined above. In certain
 10 embodiments, modifier groups have the structure:



wherein R_1 and R_2 are independently selected from the group consisting of hydrogen, alkyl, alkoxy, unsubstituted or substituted aryl and phenoxy.

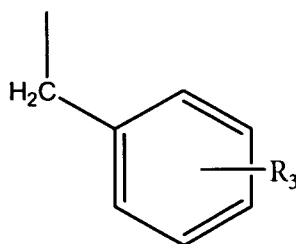
Alkyl groups may be branched or unbranched

- 15 Alkyl groups can be C_1 - C_{20} alkyls, in particular C_1 - C_{10} alkyls.

Alkoxy groups can be C_1 - C_{20} alkoxy, in particular C_1 - C_{10} alkoxy.

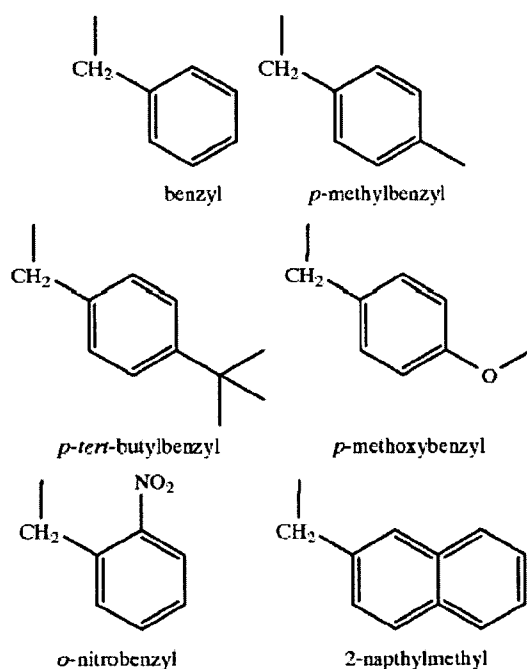
Aryl can be unsubstituted or substituted phenyl or naphthyl.

In one embodiment, R is a benzyl group or a substituted benzyl group. In certain embodiments, substituted benzyl groups can have the following structure:



wherein R_3 represents a C_1 - C_6 branched or unbranched alkyl group, more preferably a C_1 - C_4 branched or unbranched alkyl group, an alkoxy group, or a nitro group. Preferably, R_3 is attached in the para-position.

5 In some embodiments, the modifier groups are represented by structures shown below:



In general, empirical selection of a particular suitable modifier group from the class of compounds described herein can be carried out routinely by one of skill in the art, based on the presence of the four properties listed above. Preferably, suitability of a particular group is determined empirically by using the primers with modified nucleotides in an allele-specific amplification reaction. The suitability of the modification is indicated by the increased selectivity of the reaction utilizing a primer with the base modification, when compared to an identical reaction with an unmodified primer.

Additional mismatches between the primer and the template further destabilize the hybrid between the primer and the undesired variant of the target sequence. The method for optimizing the design of the allele-specific primers can be found in Newton et al. (1989) *supra*.

- 5 The allele-specific primer of the present invention may incorporate various aspects of primer design known in the art. For example, the primer may take the form of a unimolecular primer-probe combination termed "scorpion" and described in Whitcombe et al., (1999) *Detection of PCR products using self-probing amplicons and fluorescence*, *Nature Biotech.* 17:804-807. The scorpion primer designed according to the present
- 10 invention incorporates the typical elements of the scorpion, namely a probe portion, a stem loop portion and a primer portion. Further, in a scorpion designed according to the present invention, the primer portion contains the internally placed selective nucleotide. Optionally, the primer portion in a scorpion designed according to the present invention may contain one or more chemically modified nucleotides.
- 15 In summary, the allele-specific primer of the present invention possesses at a minimum the following four characteristics: 1) a 5'-portion, at least partially complementary to both desired and undesired variants of the target sequence; 2) an internal selective nucleotide, complementary only to the desired variant of the target sequence and situated within the 3'-
- 20 portion; 3) a 3'-portion, at least partially complementary to both desired and undesired variants of the target sequence; and 4) the 3'-terminal nucleotide complementary to both desired and undesired versions of the target sequence. The 5'-portion and the 3'-portion may contain additional selective nucleotides that are complementary only to the desired version of the target sequence, as long as the 3'-terminal nucleotide of the primer is complementary to both desired and undesired version of the target sequence.
- 25 Empirical selection of a suitable 5'-portion and 3'-portion of the allele-specific primer can be carried out routinely by one of skill in the art. Specifically, the length, degree of complementarity of the 5'-portion and the 3'-portion and chemical modifications of nucleotides in the 5'-portion and the 3'-portion of the primer can be varied, as long as the primer possesses the four characteristics set forth above. Preferably, suitability of a
- 30 particular allele-specific primer is determined empirically by using the primer in an allele-

specific amplification. The suitability of the primer is indicated by the selectivity of the amplification utilizing the primer.

In another aspect, the present invention is a method of allele-specific amplification of a target nucleic acid. The amplification involves the use of an allele-specific primer having a selective nucleotide, placed internally of the 3'-end of the primer, for example between 1 and 5 nucleotides internally of the 3'-end.

In one embodiment, the present invention is a method of allele-specific amplification of a variant of a target sequence, which exists in the form of several variant sequences, the method comprising: providing a sample, possibly containing at least one variant of a target sequence; providing a first oligonucleotide, at least partially complementary to one or more variants of the target sequence; providing a second oligonucleotide, at least partially complementary to one or more variants of the target sequence, but having at least one internal selective nucleotide complementary to only one variant of the target sequence; providing conditions suitable for the hybridization of said first and second oligonucleotides to at least one variant of the target sequence; providing conditions suitable for the oligonucleotide extension by a nucleic acid polymerase; wherein said polymerase is capable of extending said second oligonucleotide when it is hybridized to the variant of the target sequence for which it has said complementary internal selective nucleotide, and substantially less when said second oligonucleotide is hybridized to the variant of the target sequence for which it has a non-complementary internal selective nucleotide; and repeating the sequence of hybridization and extension steps multiple times.

In some embodiments of the invention, the amplification involves the polymerase chain reaction, i.e. repeated cycles of template denaturation, annealing (hybridization) of the oligonucleotide primer to the template, and extension of the primer by the nucleic acid polymerase. In some embodiments, annealing and extension occur at the same temperature step.

In some embodiments, the amplification reaction involves a hot start protocol. In the context of allele-specific amplification, the selectivity of the allele-specific primers with respect to the mismatched target may be enhanced by the use of a hot start protocol. Many hot start protocols are known in the art, for example, the use of wax, separating the critical reagents from the rest of the reaction mixture (U.S. Patent No. 5,411,876), the use of a

nucleic acid polymerase, reversibly inactivated by an antibody (U.S. Patent No. 5,338,671), a nucleic acid polymerase reversibly inactivated by an oligonucleotide that is designed to specifically bind its active site (U.S. Patent No. 5,840,867) or the use of a nucleic acid polymerase with reversible chemical modifications, as described e.g. in U.S. Patent Nos. 5,677,152 and 5,773,528.

In some embodiments of the invention, the allele-specific amplification assay is real-time PCR assay. In a real-time PCR assay, the measure of amplification is the “threshold cycle” or Ct value. In the context of the allele-specific real-time PCR assay, the difference in Ct values between the matched and the mismatched templates is a measure of discrimination between the alleles or the selectivity of the assay. A greater difference indicates a greater delay in amplification of the mismatched template and thus a greater discrimination between alleles. Often the mismatched template is present in much greater amounts than the matched template. For example, in tissue samples, only a small fraction of cells may be malignant and carry the mutation targeted by the allele-specific amplification assay (“matched template”). The mismatched template present in normal cells may be amplified less efficiently, but the overwhelming numbers of normal cells will overcome any delay in amplification and erase any advantage of the mutant template. To detect rare mutations in the presence of the wild-type template, the specificity of the allele-specific amplification assay is critical.

The allele-specific amplification assay of the present invention may employ any suitable nucleic acid polymerase known in the art. For an allele-specific PCR assay of the present invention, any thermostable nucleic acid polymerase may be used. A modified, engineered or chimeric polymerase may also be used. It is sometimes desirable to use an enzyme without the proofreading (3'-5'-exonuclease) activity, such as for example, Taq DNA polymerase. It may also be desirable to use enzymes, substantially or entirely lacking the 5'-3' nuclease activity, such as described in U.S. Patent No. 5,795,762. One example of such an enzyme is $\Delta Z05$ polymerase. It may sometimes be desirable to have an enzyme with a “hot start” capability, such as the reversibly modified enzymes described in U.S. Patent Nos. 5,677,152 and 5,773,528. One example of a hot-start enzyme is $\Delta Z05$ -Gold polymerase. It is generally known that the specificity of an allele-specific primer may vary somewhat among different enzymes. See Newton et al. (1989) Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). Nucl. Acids Res. 17:2503-2516.

Based on the protocols described in Newton (*supra*) a person of ordinary skill would be able to optimize the reaction parameters, for example, by changing the salt concentration and temperature profile in order to achieve maximum specificity with each enzyme and select the best enzyme for a particular assay.

- 5 A special advantage of the allele-specific PCR of the present invention is the ability to use polymerases with proofreading 3'-5'-nuclease activity. Examples of such enzymes can be found in US 7,148,049. Such enzymes comprise for example *Thermatoga Maritima*. These enzymes typically have higher fidelity (i.e. fewer misincorporated nucleotides in the final product) than the enzymes without the proofreading activity. For example, error rate for
- 10 Taq DNA polymerase (which does not have a proofreading function) is about 10^{-4} . Tindall et al. (1988) Fidelity of DNA synthesis by the *Thermus aquaticus* DNA polymerase. *Biochemistry*, 27:6008-6013. By comparison, the error rate of a proofreading thermostable Pfu DNA polymerase is about 10^{-6} . Andre et al. (1997) Fidelity and mutational spectrum of Pfu DNA polymerase on a human mitochondrial DNA sequence, *Genome Res.* 7:843-852.
- 15 Prior to the present invention, a high-fidelity proofreading polymerase could not be used with allele-specific PCR. See U.S. Patent No. 5,639,611. The nuclease activity of the enzyme would remove the mismatched selective nucleotide from the 3'-terminus of the primer thus eliminating allele-specificity of the primer. In the present invention, the allele-specific primer has a selective nucleotide not at the 3'-end but internally. The internal
- 20 mismatch is an inefficient substrate for the exonuclease activity of a proofreading enzyme. It has been observed that the ability of the exonuclease to remove mismatched nucleotides drops dramatically when the mismatch is located away from the 3'-end. Fidalgo-Da Silva et al. (2007) DNA polymerase proofreading: active site switching catalyzed by the bacteriophage T4 DNA polymerase, *Nucl. Acids Res.* 35:5452-5463. The rate of removal of
- 25 three nucleotides is much lower than two nucleotides. Reddy et al. (1992) Processive proofreading is intrinsic to T4 DNA polymerase. *J. Biol. Chem.* 267:14157-14166. Therefore the primer with an internal selective nucleotide of the present invention may be used with a proofreading nucleotide polymerase. A person of skill in the art would recognize how to optimize reaction conditions, for example by changing the composition of
- 30 the reaction buffer and concentration of nucleic acid precursors in order to minimize exonuclease activity of the enzyme without compromising allele-specific amplification. See e.g. Goodman et al. (1993) Biochemical basis of DNA replication fidelity, *Crit. Rev.*

Biochem. Mol. Biol. 28:83-126 for conditions favoring polymerization and conditions favoring nuclease digestion activities of various nucleic acid polymerases.

In some embodiments of the method, the amplification products may be detected by any technique known in the art, including but not limited to the use of labeled primers and probes as well as various nucleic acid-binding dyes. The means of detection may be specific to one variant of the target sequence, or may be generic to all variants of the target sequence or even to all double-stranded DNA.

The amplification products may be detected after the amplification has been completed, for example, by gel electrophoresis of unlabeled products and staining of the gel with a nucleic acid-binding dye. Alternatively, the amplification products may carry a radioactive or a chemical label, either by virtue of incorporation during synthesis or by virtue of being the extension products of a labeled primer. After, or during electrophoresis, the labeled amplification products may be detected with suitable radiological or chemical tools known in the art. After electrophoresis, the products may also be detected with a target-specific probe labeled by any one of the methods known in the art. The labeled probe may also be applied to the target without electrophoresis, i.e. in a "dot blot" assay or the like.

In other embodiments, the presence of the amplification product may be detected in a homogeneous assay, i.e. an assay where the nascent product is detected during the cycles of amplification, or at least in the same unopened tube, and no post-amplification handling is required. A homogeneous amplification assay has been described for example, in U.S. Patent No. 5,210,015. Homogeneous amplification assay using nucleic acid-intercalating dyes has been described for example, in U.S. Patent Nos. 5,871,908 and 6,569,627. The homogeneous assay may also employ fluorescent probes labeled with two interacting fluorophores, such as "molecular beacon" probes (Tyagi et al., (1996) Nat. Biotechnol., 14:303-308) or fluorescently labeled nuclease probes (Livak et al., (1995) PCR Meth. Appl., 4:357-362). The amplification products may also be detected using a unimolecular primer-probe combination termed "scorpion." Whitcombe et al., (1999) Detection of PCR products using self-probing amplicons and fluorescence, Nature Biotech. 17:804-807. The primer portion of the scorpion oligonucleotide may be an allele-specific primer designed according to the present invention.

In certain variations of the method of the present invention, the amplification product may also be identified by virtue of its distinctive melting temperature, see U.S. Patent Nos. 5,871,908 and 6,569,627.

In another embodiment, the invention provides a reaction mixture for selectively
5 amplifying the desired variant of the target sequence, the target sequence existing in the form of several variant sequences, the mixture comprising a first oligonucleotide, at least partially complementary to one or more variants of the target sequence; and a second oligonucleotide, at least partially complementary to one or more variants of the target sequence, but having at least one internal selective nucleotide complementary to only one
10 variant of the target sequence. The reaction mixture may also contain a nucleic acid polymerase which is capable of extending said second oligonucleotide when it is hybridized to the variant of the target sequence for which it has said complementary internal selective nucleotide, and substantially less when said second oligonucleotide is hybridized to the variant of the target sequence for which it has a non-complementary internal selective
15 nucleotide. In some embodiments, the reaction mixture further comprises the reagents and solutions generally necessary for the amplification of nucleic acids, including nucleic acid precursors, i.e. nucleoside triphosphates, and organic and inorganic ions, suitable for the support of the activity of the nucleotide polymerase.

In another embodiment, the invention provides kits for conducting allele-specific
20 amplification according to the invention. The kit generally includes assay-specific components as well as components generally required for performing nucleic acid amplification. As the assay-specific components, the allele-specific amplification kit of the present invention contains a first oligonucleotide, at least partially complementary to one or more variants of the target sequence; a second oligonucleotide, at least partially
25 complementary to one or more variants of the target sequence, but having at least one internal selective nucleotide complementary to only one variant of the target sequence; and optionally, a control nucleic acid sequence comprising an amount of at least one variant of the target sequence, at least partially complementary to the oligonucleotides enclosed in the kit. In some embodiments, more than one variant of the control nucleic acid sequence may
30 be enclosed. As the components generally required for nucleic acid amplification, the kit of the present invention may include one or more of a nucleic acid polymerase, nucleic acid precursors, such as nucleoside triphosphates deoxy-ribonucleoside triphosphates or

ribonucleoside triphosphates, a pyrophosphatase, for minimizing pyrophosphorolysis of nucleic acids, a uracil N-glycosylase (UNG) for protection against carry-over contamination of amplification reactions, pre-made reagents and buffers necessary for the amplification reaction and detection, and a set of instructions for conducting allele-specific amplification according to the present invention.

The following examples and figures are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

10

EXAMPLES

In the examples below, two variants of the template sequence were used: a matched variant, with a sequence complementary to the selective nucleotide in the allele-specific primer and a mismatched variant, with a sequence non-complementary to the selective nucleotide in the allele-specific primer.

As a matched target, the examples utilized the V600E mutation of the human BRAF gene (GeneBank reference). The matched variant was a plasmid DNA with the insert incorporating the BRAF V600E mutant sequence (SEQ ID NO: 19), while the mismatched variant was the same plasmid with the BRAF wild-type sequence (SEQ ID NO: 20).

20 SEQ ID NO: 19 (BRAF V600E mutant sequence fragment)

5'-AGTAAAAATAGGTGATTTTGGTCTAGCTACAGAGAAATCTCGATGGAGTGGG
TCCCATCAGTTTGAACAGTTGTCTGGATCCATTTTGTGGATGGTAAGAATTGAGG
CTA-3'

SEQ ID NO: 20 (BRAF wild-type sequence fragment)

25 5'-AGTAAAAATAGGTGATTTTGGTCTAGCTACAGTCAAATCTCGATGGAGTGGG
TCCCATCAGTTTGAACAGTTGTCTGGATCCATTTTGTGGATGGTAAGAATTGA
GGCTA-3'

This mutation is a valine-to-glutamate change of amino acid 600, that results from a thymine (T) to adenine (A) transition at nucleotide 1799 of the BRAF gene. The mutation is found in many cancers and is thought to contribute to cancer progression, as it results in constitutive activation of the MAPK pathway. Detection of this single nucleotide change in a population of tumor cells has utility in the diagnosis and treatment of human cancers.

The mutant target is “matched”, i.e. forms an A-T Watson-Crick pair with the selective nucleotide of each of the allele-specific primers (Table A). The mismatched target is the wild-type BRAF sequence. The mismatched target forms a mismatch with the selective nucleotide of each of the allele-specific primers.

10 *Table A*

Primers

Seq ID	Function	Sequence
1	Forward primer	5'-TAAAAATAGGTGATTTTGGTCTAGCTACAG <u>A</u> GA-3'*
2	Forward primer	5'- TAAAAATAGGTGATTTTGGTCTAGCTACYG <u>A</u> GA-3'
3	Forward primer	5'- TAAAAATAGGTGATTTTGGTCTAGCTACAG <u>Y</u> GA-3'
4	Forward primer	5'- TAAAAATAGGTGATTTTGGTCTAGCTACAG <u>A</u> GX-3'
5	Forward primer	5'-TAAAAATAGGTGATTTTGGTCTAGCTACYG <u>A</u> GY-3'
6	Forward primer	5'-GTAAAAATAGGTGATTTTGGTCTAGCTACAG <u>A</u> G-3'
7	Forward primer	5'-GTAAAAATAGGTGATTTTGGTCTAGCTACAG <u>Y</u> G-3'
8	Forward primer	5'-GTAAAAATAGGTGATTTTGGTCTAGCTACYG <u>A</u> G-3'

9	Forward primer	5'-GTAAAAATAGGTGATTTTGGTCTAGCTACYG <u>Y</u> G-3'
10	Forward primer	5'-GTAAAAATAGGTGATTTTGGTCTAGCTAZAG <u>Y</u> G-3'
11	Reverse primer	5'-TAGCCTCAATTCTTACCATCCACAX-3'

* selective nucleotide is underlined

X – N⁶-benzyl-dA

Y – N⁶-para-tert-butyl-benzyl-dA

5 Z – N⁶-para-tert-butyl-benzyl-dC

Example 1

Allele-specific amplification using a primer with an internal selective nucleotide

In this example, two variants of the template sequence were used: a matched variant, with a sequence complementary to the selective nucleotide in the allele-specific primer and a mismatched variant, with a sequence non-complementary to the selective nucleotide in the allele-specific primer. The matched variant was a plasmid DNA with the insert representing the BRAF sequence with a V600E mutation. The mismatched variant was the same plasmid with the BRAF wild-type sequence. The forward primers (SEQ ID NO: 1-4) and reverse primer (SEQ ID NO: 11) are shown in Table A. The forward allele-specific primers were designed with the selective nucleotide internal of the 3' terminus, at the N-2 position. Some primers contained chemical modifications where indicated.

Each 50 µL reaction contained 10⁵ copies of either target, 8% glycerol, 50 mM tricine (pH 7.7), 45 mM potassium acetate (pH 7.5), 200 µM each dATP, dCTP and dGTP, 400 µM dUTP, 0.1 µM forward primer, 0.7 µM reverse primer, 2 µM Syto-13 intercalating dye, 1% DMSO, 2 units of uracil-N-glycosylase (UNG), 50 units of ΔZ05-Gold DNA polymerase, and 3 mM magnesium acetate. Amplification and analysis were done using the Roche LightCycler 480 instrument. The reactions were subjected to the following temperature profile: 50°C for 5 minutes (UNG step), 95°C for 10 minutes, followed by 60-70 cycles of

95°C for 15 seconds and 59°C for 40 seconds. Fluorescence data was collected at 495-525nm at the end of each 59°C step.

The results are shown on Figure 1 and Table 1. The selectivity of the amplification is measured by the difference in the C_t value (ΔC_t) between the matched and the mismatched targets. ΔC_t for each experiment is indicated on each diagram. The data shows that the matched (mutant) variant of the target was amplified selectively over the mismatched (wild-type) variant. The selectivity was enhanced by chemical modification of the nucleotides in the primers. In this table, position N denotes the nucleotide position relative to the 3' end.

Table 1

Forward primer	Position of the selective nucleotide	Chemical modification	Average C_t wild-type target	Average C_t mutant target	ΔC_t
SEQ ID NO: 1	N-2	none	33.7	29.4	4.3
SEQ ID NO. 2	N-2	Y*at N-4	44.0	31.9	12.1
SEQ ID NO. 3	N-2	Y at N-2	42.1	32.1	10.0
SEQ ID NO. 4	N-2	X at 3' end	49.0	33.3	15.7

10

*Y – N⁶-para-tert-butyl-benzyl-dA

X – N⁶-benzyl-dA

Example 2:

15 *Allele-specific amplification using primers with an internal selective nucleotide and different nucleic acid polymerases*

In this example, the same matched (mutant) and mismatched (wild-type) targets as in Example 1 were amplified using primers shown in Table A. Amplification was carried out in the presence of Z05, Δ Z05, or Δ Z05-Gold polymerase.

All reactions were done in triplicate, in 15 μ L volumes containing 10^5 copies of either target, 200 μ M each dATP, dCTP and dGTP, 400 μ M dUTP, 0.1 μ M forward primer, 0.7 μ M reverse primer, 2 μ M Syto-13 intercalating dye, 1% DMSO, 0.04U/ μ l uracil-N-glycosylase (UNG), and 3mM magnesium acetate. Z05 reactions contained 3U of polymerase, 130 mM potassium acetate (pH 7.5), 5% glycerol, and 50 mM Tricine (pH 8.3). Δ Z05 reactions contained 3U of polymerase, 25 mM potassium acetate (pH 7.5), 5% glycerol, and 50 mM Tricine (pH 8.3). Δ Z05-Gold reactions contained 15U of polymerase, 45 mM potassium acetate (pH 7.5), 8% glycerol, and 50 mM Tricine (pH 7.7).

Amplification and analysis were done using the Roche LightCycler 480 instrument. The reactions were subjected to the following temperature profile: 50°C for 5 minutes (UNG step), 95°C for 10 minutes, followed by 99 cycles of 95°C for 15 seconds and 59°C for 40 seconds. Fluorescence data was collected at 465-510nm at the end of each 59°C step.

The results are shown in Table 2. The selectivity of amplification is measured by the difference in the C_t value (ΔC_t) between the matched and the mismatched targets. ΔC_t for each experiment is indicated in Table 2. The data shows that the matched (mutant) variant of the target was amplified selectively over the mismatched (wild-type) variant. The selectivity was enhanced by the alkyl modification of the nucleotides in the primers. In this table, position N denotes the nucleotide position relative to the 3' end.

20 TABLE 2

SEQ ID	SELECTIVE NUCLEOTIDE POSITION	Z05			DZ05			Δ Z05-GOLD		
		Mut Ct avg	Wt Ct avg	ΔC_t (wt - mut)	Mut Ct avg	Wt Ct avg	ΔC_t (wt - mut)	Mut Ct avg	Wt Ct avg	ΔC_t (wt - mut)
1	N-2	24.1	24.9	0.8	25.9	27.3	1.4	29.8	34.0	4.2
3	N-2	25.1	29.6	4.5	27.2	38.4	11.2	35.1	51.5	16.4
5	N-2	29.0	47.0	18.0	N/A	N/A	N/A	94.0	100.0	6.0

6	N-1	25.0	27.0	2.0	25.2	32.1	6.9	28.9	42.0	13.2
7	N-1	24.2	30.5	6.3	26.3	38.8	12.5	31.7	60.0	28.2
8	N-1	24.3	31.0	6.8	26.4	39.0	12.6	31.3	55.7	24.4
9	N-1	25.3	39.9	14.5	48.4	100.0	51.6	56.2	92.6	36.4
10	N-1	24.3	36.8	12.6	38.5	77.3	38.7	56.7	89.3	32.6

N/A – no data

Example 3:

5 *Allele-specific amplification using Scorpion ARMS-like primers with internal base modifications and/or internal selective nucleotide*

TABLE 3

SEQ ID	FUNCTION	PRIMER SEQUENCE
12	FORWARD PRIMER	AGTAAAAATAGGTGATTTGGTCTAGCTACAG <u>A</u>
13	FORWARD PRIMER, PROBE	FCCCGCGCGGACCCACTCCATCGAGAGCGGGGQJAGTAAAAATAGGTGATTTGGTCTAGCTACAG <u>A</u>
14	FORWARD PRIMER	AGTAAAAATAGGTGATTTGGTCTAGCTACYG <u>A</u>
15	FORWARD PRIMER, PROBE	FCCCGCGCGGACCCACTCCATCGAGAGCGGGGQJAGTAAAAATAGGTGATTTGGTCTAGCTACYG <u>A</u>
6	FORWARD PRIMER	GTAAAAATAGGTGATTTGGTCTAGCTACAG <u>G</u>
16	FORWARD PRIMER, PROBE	FCCCGCGCGGACCCACTCCATCGAGAGCGGGGQJGTAAAAATAGGTGATTTGGTCTAGCTACAG <u>G</u>
9	FORWARD PRIMER	GTAAAAATAGGTGATTTGGTCTAGCTACYG <u>G</u>

17	FORWARD PRIMER, PROBE	FCCCGCGCGGACCCACTCCATCGAGAGCGCGGGQJGTAAAAATAGGTGATTTTGGTCTAGCTACYGYG
11	REVERSE PRIMER	TAGCCTCAATTCTTACCATCCACAX
18	PROBE	FTCTCGATGGAGTGGGTCCQp

X – N⁶-benzyl-dA

Y – N⁶-para-tert-butyl-benzyl-dA

F – cx-FAM donor fluorophore

5 Q – BHQ-2 “Black Hole” quencher

J-HEG

p – 3'-phosphate

* The allele selective nucleotide is underlined (N or N-1 position from 3' terminus)

- 10 In this example, two variants of the template sequence were present in equal amounts, a matched variant, complementary to the primer sequence and a mismatched variant. The matched variant was a plasmid DNA with the insert representing the BRAF V600E mutant sequence (SEQ ID NO: 1), while the mismatched variant was the same plasmid with the BRAF wild-type sequence (SEQ ID NO: 2). The forward primers (SEQ ID NO: 6, 9, 12- 17)
- 15 and reverse primer (SEQ ID NO: 11) are as described in Table 3. The forward, ASPCR primers, were designed with the SNP at, or near the 3' terminal position, either with or without N⁶-tert-butyl-benzyl-dA modification(s). The ASPCR primer is paired with a downstream detection probe (SEQ ID: 18) or linked to the probe complement in a closed Scorpion ARMS-like format.
- 20 Each 50uL reaction contained 10⁵ copies of either target, 5% glycerol, 50mM tricine (pH 8.3), 150mM potassium acetate (pH 7.5), 200μM each of dATP, dCTP and dGTP, 400μM dUTP, 0.4μM forward primer, 0.4μM reverse primer, 1% DMSO, 2 units uracil-N-glycosylase (UNG), 10 units Z05 polymerase, and 3mM magnesium acetate. 0.2uM of detection probe was added to reactions containing Primers 6, 9, 12 and 14 where the probe
- 25 complement is not linked to the forward primer.

Amplification and analysis were done using the Roche LightCycler 480 instrument. The reactions were subjected to the following temperature profile: 50°C for 5 minutes (UNG

step) followed by 95 cycles of 95°C for 15 seconds and 59°C for 40 seconds. Fluorescence data was collected at the 495-525nm range at the end of each 59°C anneal/extend step.

The results are shown on Figure 2 and Table 4. The selectivity of the amplification is measured by the difference in the Ct value (ΔC_t) between the matched and the mismatched targets. ΔC_t for each experiment is indicated on each diagram and summarized in Table 4. The data shows that the matched (mutant) variant of the target was amplified selectively over the mismatched (wild-type) variant using either the individual primer and probe or the primer and probe linked in a closed Scorpion ARMS-like format. Discrimination was achieved whether the selective nucleotide was at the 3' terminus or internal. Additionally, the selectivity of amplification was enhanced by addition of one or more alkyl modifications.

TABLE 4

SEQ ID	PRIMER SEQUENCE	POSITION OF SELECTIVE NUCLEOTIDE	PRIMER FORMAT	MODIFICATION OF PRIMING SEGMENT	WT $C_{T_{AVG}}$	MUT $C_{T_{AVG}}$	ΔC_t
12	AGTAAAAATAGGTGATTTTGGTCTAGCTACAGA	3' terminus	Traditional	none	31.6	30.2	1.4
13	FCCCGCGGGGACCCACTCCATCGA GAGCGCGGGQJAGTAAAAATAGGT GATTTTGGTCTAGCTACAGA	3' terminus	Scorpion ARMS	none	34.7	29.2	5.5
14	AGTAAAAATAGGTGATTTTGGTCTAGCTACYGA	3' terminus	Traditional	Y at N-2	38.1	29.5	8.6
15	FCCCGCGGGGACCCACTCCATCGA GAGCGCGGGQJAGTAAAAATAGGT GATTTTGGTCTAGCTACYGA	3' terminus	Scorpion ARMS	Y at N-2	51.4	32.6	18.8
6	GTAAAAATAGGTGATTTTGGTCTAGCTACAGAG	3' penultimate	Traditional	none	30.8	29.9	0.9
16	FCCCGCGGGGACCCACTCCATCGA GAGCGCGGGQJGTAAAAATAGGTG ATTTTGGTCTAGCTACAGAG	3' penultimate	Scorpion ARMS	none	31.4	29.3	2.1
9	GTAAAAATAGGTGATTTTGGTCTAGCTACYGYG	3' penultimate	Traditional	Y at N-1 and N-3	50.1	31.7	18.4
17	FCCCGCGGGGACCCACTCCATCGA GAGCGCGGGQJGTAAAAATAGGTG ATTTTGGTCTAGCTACYGYG	3' penultimate	Scorpion ARMS	Y at N-1 and N-3	Not Amplified	34.7	> 60.3

While the invention has been described in detail with reference to specific examples, it will be apparent to one skilled in the art that various modifications can be made within the scope of this invention. Thus the scope of the invention should not be limited by any of the examples described herein, but by the claims presented below.

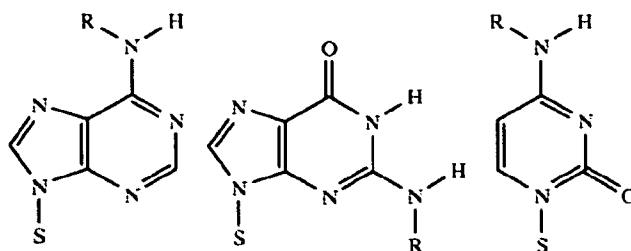
CLAIMS

1. A method of allele-specific amplification of a variant of a target sequence, the target existing in the form of several variant sequences, the method comprising
 - 5 (a) hybridizing a first and a second oligonucleotides to at least one variant of the target sequence; wherein the first oligonucleotide is at least partially complementary to one or more variants of the target sequence, and the second oligonucleotide is at least partially complementary to one or more variants of the target sequence, and has at least one internal selective nucleotide complementary to only one variant of the target sequence;
10
 - (b) extending the second oligonucleotide with a nucleic acid polymerase, wherein said polymerase is capable of extending said second oligonucleotide preferentially when said selective nucleotide forms a base pair with the target, and substantially less when said selective nucleotide does not form a base pair with the target.
15
2. The method of claim 1, wherein said nucleic acid polymerase in step (b) is capable of extending said second oligonucleotide exclusively when said selective nucleotide forms a base pair with the target.
3. The method of claim 1, wherein said selective nucleotide is at the position between 1
20 and 5 nucleotides near the 3'-terminus of the oligonucleotide.
4. The method of claim 1, further comprising a step (c) of detecting the product of primer extension in step (b).
5. The method of claim 1 or 2, wherein said nucleic acid polymerase is selected from a
25 group consisting of Taq DNA polymerase, Z05 DNA polymerase, Δ Z05 DNA polymerase and Δ Z05-Gold DNA polymerase.

6. The method of claim 1 wherein said nucleic acid polymerase possesses 3'-5' nuclease activity.
7. The method of claim 6, wherein said nucleic acid polymerase is selected from a group consisting of Pfu DNA polymerase and *Thermatoga Maritima*.
- 5 8. The method of claim 1, wherein said variant of the sequence in step (a) is a V600E mutation of the human BRAF, EGFR, PIK3CA or KRAS gene.
9. The method of claim 1, wherein said first oligonucleotide is SEQ ID NO: 11.
10. The method of claim 1, wherein said second oligonucleotide is selected from a group consisting of SEQ ID NOs: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 13, 14, 15, 16 and 17.
- 10 11. A method of detecting a variant of a target sequence, the target existing in the form of several variant sequences, the method comprising:
 - (a) hybridizing a first and second oligonucleotides to at least one variant of the target sequence; wherein said first oligonucleotide is at least partially complementary to one or more variants of the target sequence and said second
15 oligonucleotide is at least partially complementary to one or more variants of the target sequence, and has at least one internal selective nucleotide complementary to only one variant of the target sequence;
 - (b) extending the second oligonucleotide with a nucleic acid polymerase; wherein said polymerase is capable of extending said second oligonucleotide
20 preferentially when said selective nucleotide forms a base pair with the target, and substantially less when said selective nucleotide does not form a base pair with the target; and
 - (c) detecting the products of said oligonucleotide extension, wherein the extension signifies the presence of the variant of a target sequence to which the
25 oligonucleotide has a complementary selective nucleotide.

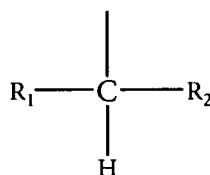
12. A kit for allele-specific amplification of a target sequence, said target existing in the form of several variant sequences, the kit comprising:
 - (a) a first oligonucleotide, at least partially complementary to one or more variant of the target sequence; and
 - 5 (b) a second oligonucleotide, at least partially complementary to one or more variants of the target sequence having at least one internal selective nucleotide complementary to only one variant of the target sequence.
13. The kit of claim 12, further comprising a nucleic acid polymerase, nucleoside triphosphates, buffer suitable for the extension of nucleic acids by the nucleic acid
10 polymerase and a set of instructions for performing allele-specific amplification.
14. An oligonucleotide for performing an allele-specific amplification of a target sequence, said target existing in the form of several variant sequences, the oligonucleotide comprising
 - (a) a sequence at least partially complementary to a portion of one or more variants
15 of said target sequence;
 - (b) at least one internal selective nucleotide complementary to only one variant of the target sequence.
15. The oligonucleotide of claim 14, further comprising at least one nucleotide with a base covalently modified at the exocyclic amino group.
- 20 16. The oligonucleotide of claim 15, wherein the structures of one nucleotide with a base covalently modified at the exocyclic amino group is selected from the group consisting of:

31



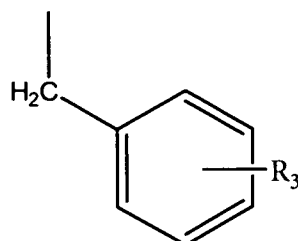
wherein S represents a sugar moiety, and R represents a modifier group.

17. The oligonucleotide of claim 16, wherein said modified nucleotide occurs at positions -5, -4, -3, -2 or -1 relative to the 3'-terminus.
- 5 18. The oligonucleotide of claim 17, wherein said base covalently modified at the exocyclic amino group comprises a modifier of the following formula:



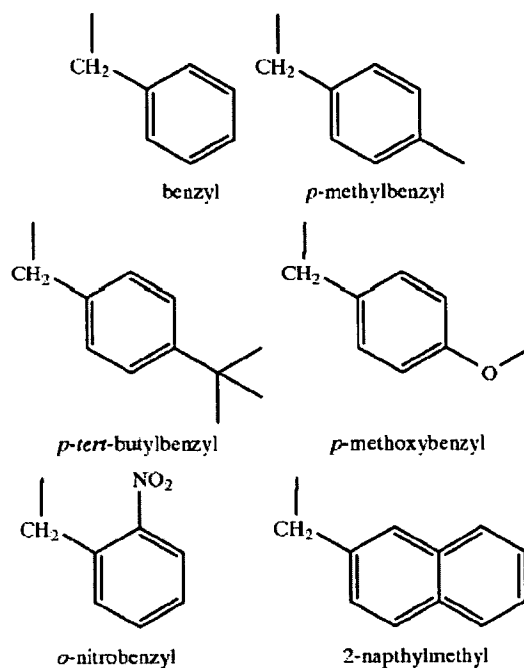
wherein R_1 and R_2 are independently selected from the group consisting of hydrogen, alkyl, alkoxy, unsubstituted or substituted aryl and phenoxy.

- 10 19. The oligonucleotide of claim 18, wherein the modifier has the following formula:



wherein R_3 is selected from the group consisting of C_1 - C_6 alkyl, alkoxy and nitro.

20. The oligonucleotide of claim 19, wherein the modifier is selected from the group consisting of:



21. The oligonucleotide of claim 16, wherein said base, covalently modified at the
5 exocyclic amino group is selected from a group consisting of N⁶-benzyl-adenine, N⁶-para-tert-butyl-benzyl adenine, N²-alkyl-guanine and N⁴-benzyl-cytosine.
22. The oligonucleotide of claim 14, with a sequence selected from a group consisting of SEQ ID NOs: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 13, 14, 15, 16 and 17.
23. The oligonucleotide of claim 15, wherein it has a scorpion format.
- 10 24. A reaction mixture for allele-specific amplification of a target sequence, said target existing in the form of several variant sequences, the mixture comprising:
- (a) a first oligonucleotide, at least partially complementary to one or more variant of the target sequence; and

- (b) a second oligonucleotide, at least partially complementary to one or more variants of the target sequence but having at least one internal selective nucleotide complementary to only one variant of the target sequence.
25. The reaction mixture of claim 24, further comprising a nucleic acid polymerase,
5 nucleoside triphosphates and a buffer suitable for the extension of nucleic acids by the nucleic acid polymerase.

AMENDED CLAIMS
received by the International Bureau on 15 April 2011 (15.04.2011)

CLAIMS

1. A method of allele-specific amplification of a variant of a target sequence, the target existing in the form of several variant sequences, the method comprising
 - (a) hybridizing a first and a second oligonucleotides to at least one variant of the target sequence; wherein the first oligonucleotide is at least partially complementary to one or more variants of the target sequence, and the second oligonucleotide is at least partially complementary to one or more variants of the target sequence, has at least one internal selective nucleotide complementary to only one variant of the target sequence, and has a 3'-terminal nucleotide complementary to any variant of the target sequence; wherein said second oligonucleotide incorporates at least one nucleotide with a base covalently modified at the exocyclic amino group;
 - (b) extending the second oligonucleotide with a nucleic acid polymerase, wherein said polymerase is capable of extending said second oligonucleotide preferentially when said second oligonucleotide is hybridized to a variant of the target sequence which is complementary to the at least one internal selective nucleotide, and substantially less when said second oligonucleotide is hybridized to a variant of the target sequence which is not complementary to the at least one internal selective nucleotide.
2. The method of claim 1, wherein said nucleic acid polymerase in step (b) is capable of extending said second oligonucleotide exclusively when said selective nucleotide forms a base pair with the target.
3. The method of claim 1, wherein said selective nucleotide is at the position between 1 and 5 nucleotides near the 3'-terminal nucleotide of the oligonucleotide.
4. The method of claim 1, further comprising a step (c) of detecting the product of primer extension in step (b).
5. The method of claim 1, wherein said nucleic acid polymerase is selected from a group consisting of Taq DNA polymerase, Z05 DNA polymerase, ΔZ05 DNA polymerase and ΔZ05-Gold DNA polymerase.

6. The method of claim 1, wherein said nucleic acid polymerase possesses 3'-5' nuclease activity.
7. The method of claim 6, wherein said nucleic acid polymerase is selected from a group consisting of Pfu DNA polymerase and *Thermatoga Maritima*.
8. The method of claim 1, wherein said variant of the sequence in step (a) is a V600E mutation of the human BRAF, EGFR, PIK3CA or KRAS gene.
9. The method of claim 1, wherein said first oligonucleotide is SEQ ID NO: 11.
10. The method of claim 1, wherein said second oligonucleotide is selected from a group consisting of SEQ ID NOs: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 13, 14, 15, 16 and 17.
11. A method of detecting a variant of a target sequence, the target existing in the form of several variant sequences, the method comprising:
 - (a) hybridizing a first and second oligonucleotides to at least one variant of the target sequence; wherein said first oligonucleotide is at least partially complementary to one or more variants of the target sequence and said second oligonucleotide is at least partially complementary to one or more variants of the target sequence, has at least one internal selective nucleotide complementary to only one variant of the target sequence, and has a 3'-terminal nucleotide complementary to any variant of the target sequence; wherein said second oligonucleotide incorporates at least one nucleotide with a base covalently modified at the exocyclic amino group;
 - (b) extending the second oligonucleotide with a nucleic acid polymerase; wherein said polymerase is capable of extending said second oligonucleotide preferentially when said second oligonucleotide is hybridized to a variant of the target sequence which is complementary to the at least one internal selective nucleotide, and substantially less when said second oligonucleotide is hybridized to a variant of the target sequence which is not complementary to the at least one internal selective nucleotide; and

(c) detecting the products of said oligonucleotide extension, wherein the extension signifies the presence of the variant of a target sequence to which the oligonucleotide has a complementary selective nucleotide.

12. A kit for allele-specific amplification of a target sequence, said target existing in the form of several variant sequences, the kit comprising:

(a) a first oligonucleotide, at least partially complementary to one or more variant of the target sequence; and

(b) a second oligonucleotide, at least partially complementary to one or more variants of the target sequence having at least one internal selective nucleotide complementary to only one variant of the target sequence, and has a 3'-terminal nucleotide complementary to any variant of the target sequence; wherein said second oligonucleotide incorporates at least one nucleotide with a base covalently modified at the exocyclic amino group.

13. The kit of claim 12, further comprising a nucleic acid polymerase, nucleoside triphosphates, buffer suitable for the extension of nucleic acids by the nucleic acid polymerase and a set of instructions for performing allele-specific amplification.

14. An oligonucleotide for performing an allele-specific amplification of a target sequence, said target existing in the form of several variant sequences, the oligonucleotide comprising

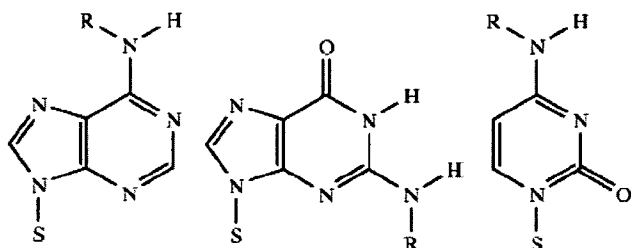
(a) a sequence at least partially complementary to a portion of one or more variants of said target sequence;

(b) at least one internal selective nucleotide complementary to only one variant of the target sequence;

(c) a 3'-terminal nucleotide complementary to any variant of the target sequence;

(d) at least one nucleotide with a base covalently modified at the exocyclic amino group.

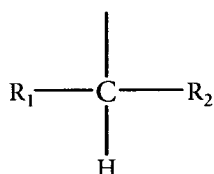
15. The oligonucleotide of claim 14, wherein the structures of one nucleotide with a base covalently modified at the exocyclic amino group is selected from the group consisting of:



wherein S represents a sugar moiety, and R represents a modifier group.

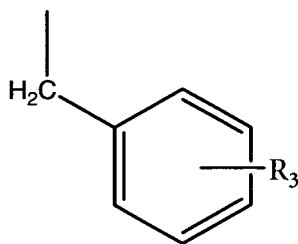
16. The oligonucleotide of claim 15, wherein said modified nucleotide occurs at positions -5, -4, -3, -2 or -1 relative to the 3'-terminal nucleotide.

17. The oligonucleotide of claim 16, wherein said base covalently modified at the exocyclic amino group comprises a modifier of the following formula:



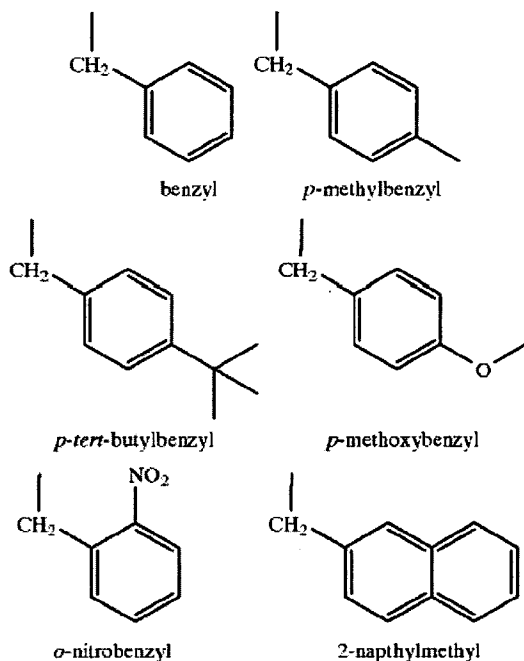
wherein R₁ and R₂ are independently selected from the group consisting of hydrogen, alkyl, alkoxy, unsubstituted or substituted aryl and phenoxy.

18. The oligonucleotide of claim 17, wherein the modifier has the following formula:



wherein R₃ is selected from the group consisting of C₁-C₆ alkyl, alkoxy and nitro.

19. The oligonucleotide of claim 18, wherein the modifier is selected from the group consisting of:



20. The oligonucleotide of claim 15, wherein said base, covalently modified at the exocyclic amino group is selected from a group consisting of N⁶-benzyl-adenine, N⁶-para-tert-butyl-benzyl adenine, N²-benzyl-guanine and N⁴-benzyl-cytosine.

21. The oligonucleotide of claim 14, comprising a sequence selected from a group consisting of SEQ ID NOs: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 13, 14, 15, 16 and 17.

22. The oligonucleotide of claim 14, wherein it has a scorpion format.

23. A reaction mixture for allele-specific amplification of a target sequence, said target existing in the form of several variant sequences, the mixture comprising:

- (a) a first oligonucleotide, at least partially complementary to one or more variant of the target sequence; and
- (b) a second oligonucleotide, at least partially complementary to one or more variants of the target sequence but having at least one internal selective nucleotide complementary to only one variant of the target sequence, and

has a 3'-terminal nucleotide complementary to any variant of the target sequence; wherein said second oligonucleotide incorporates at least one nucleotide with a base covalently modified at the exocyclic amino group.

24. The reaction mixture of claim 23, further comprising a nucleic acid polymerase, nucleoside triphosphates and a buffer suitable for the extension of nucleic acids by the nucleic acid polymerase.

FIGURE 1-1

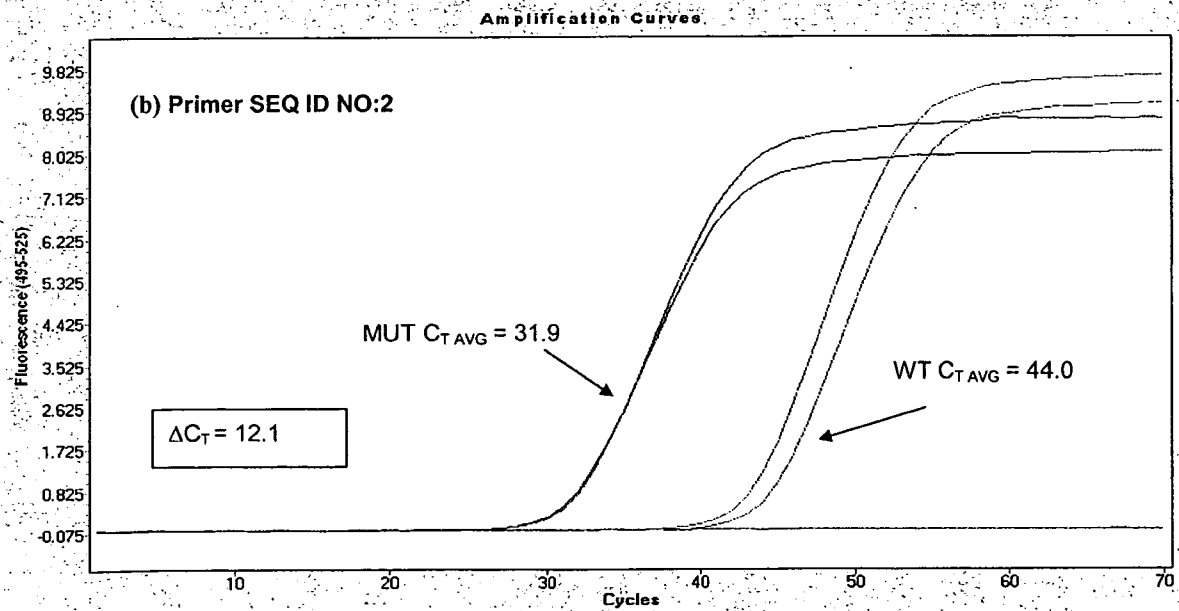
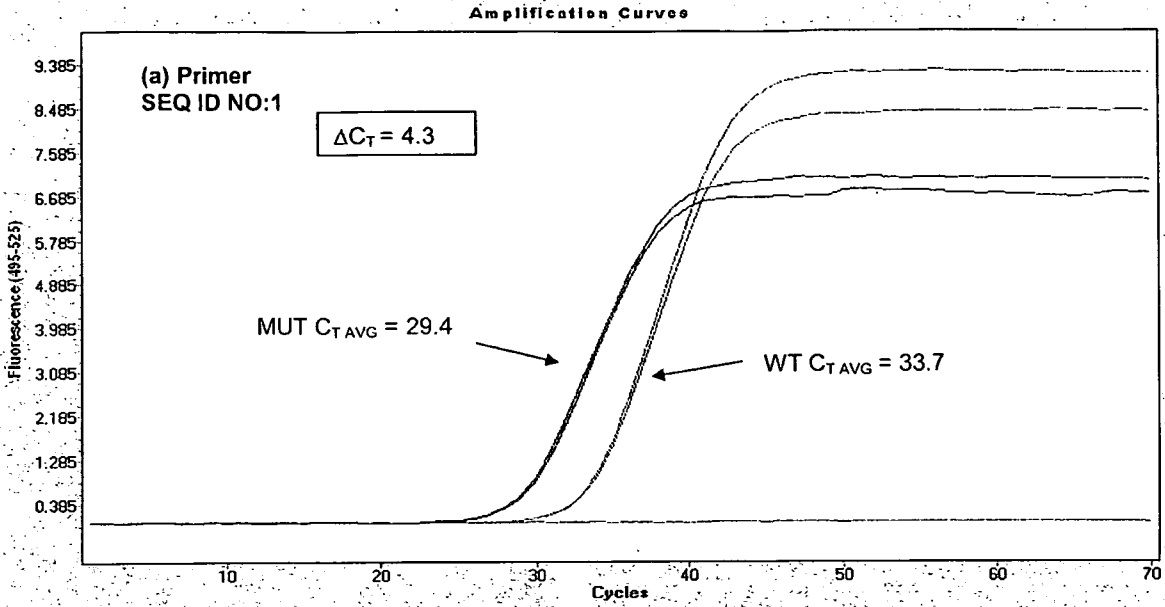


FIGURE 1-2

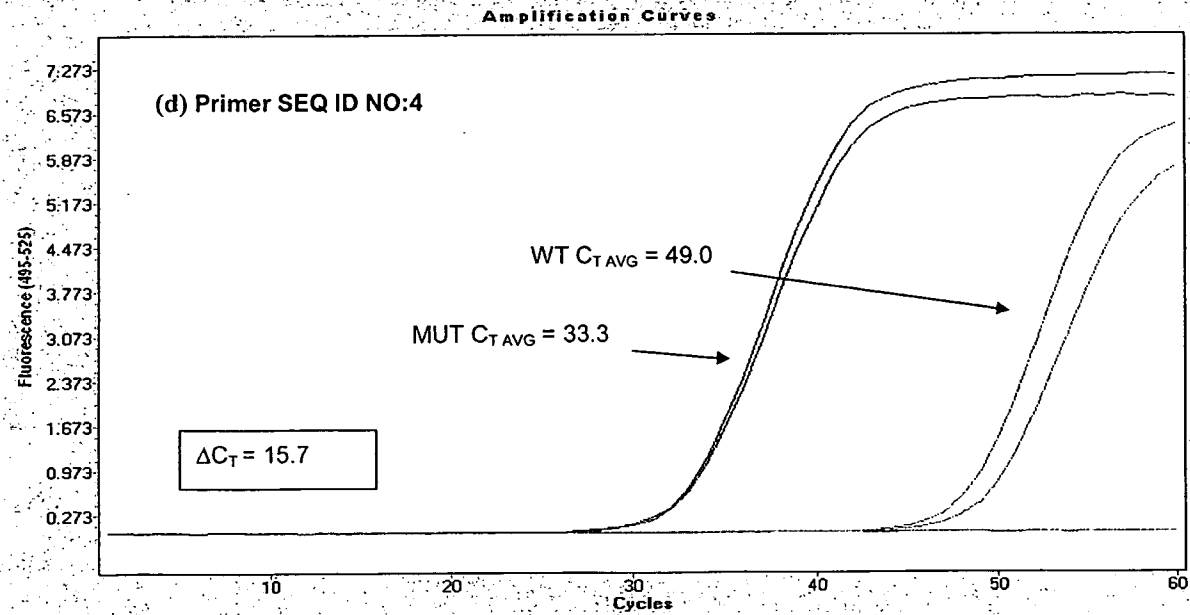
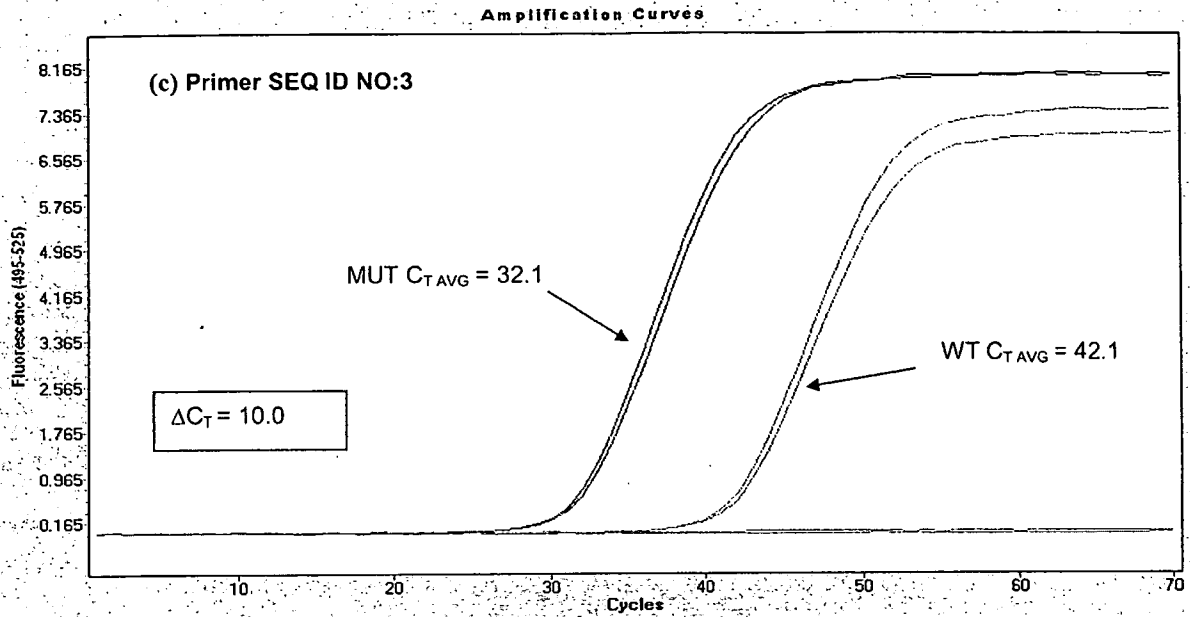


FIGURE 2-1

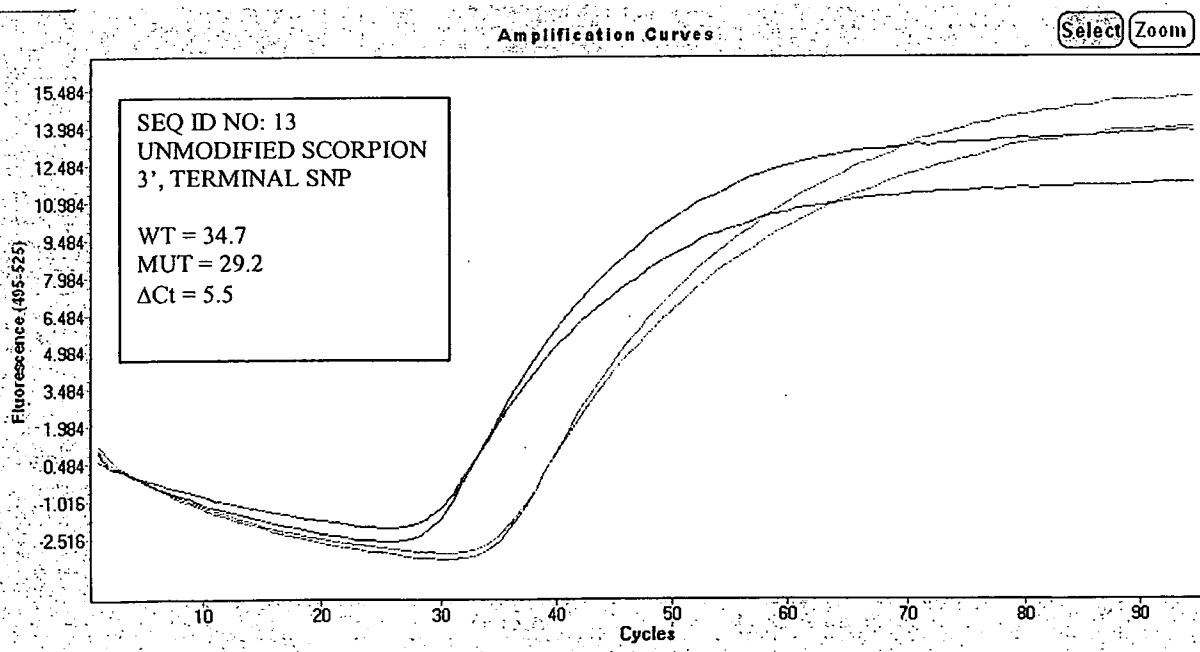
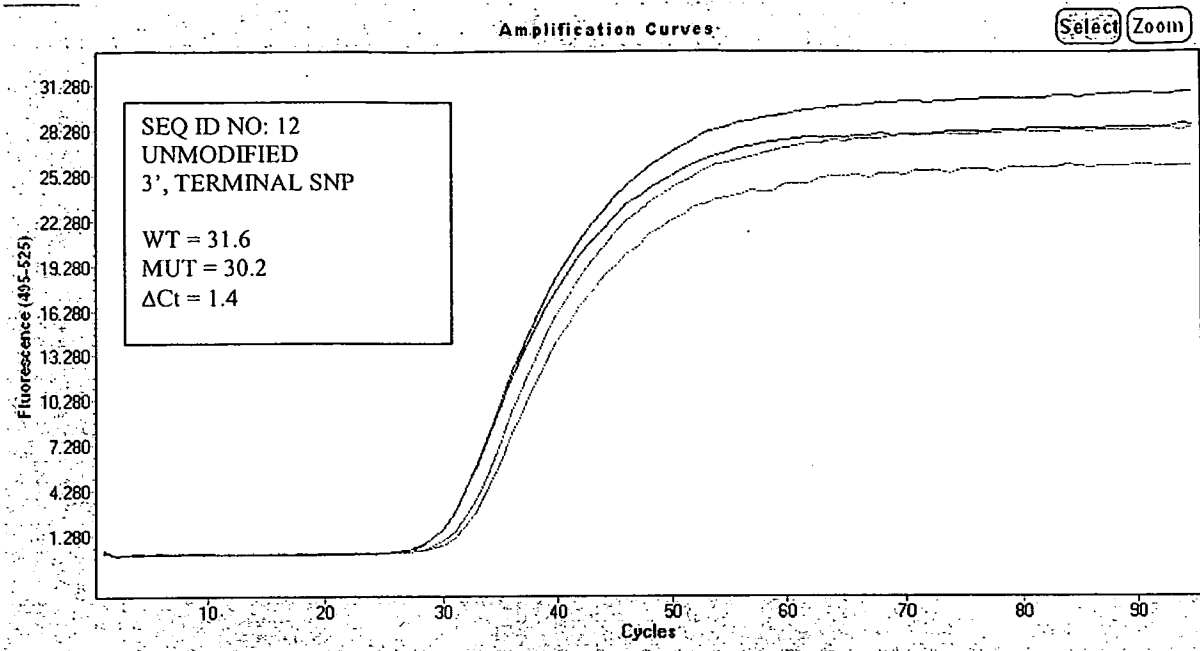


FIGURE 2-2

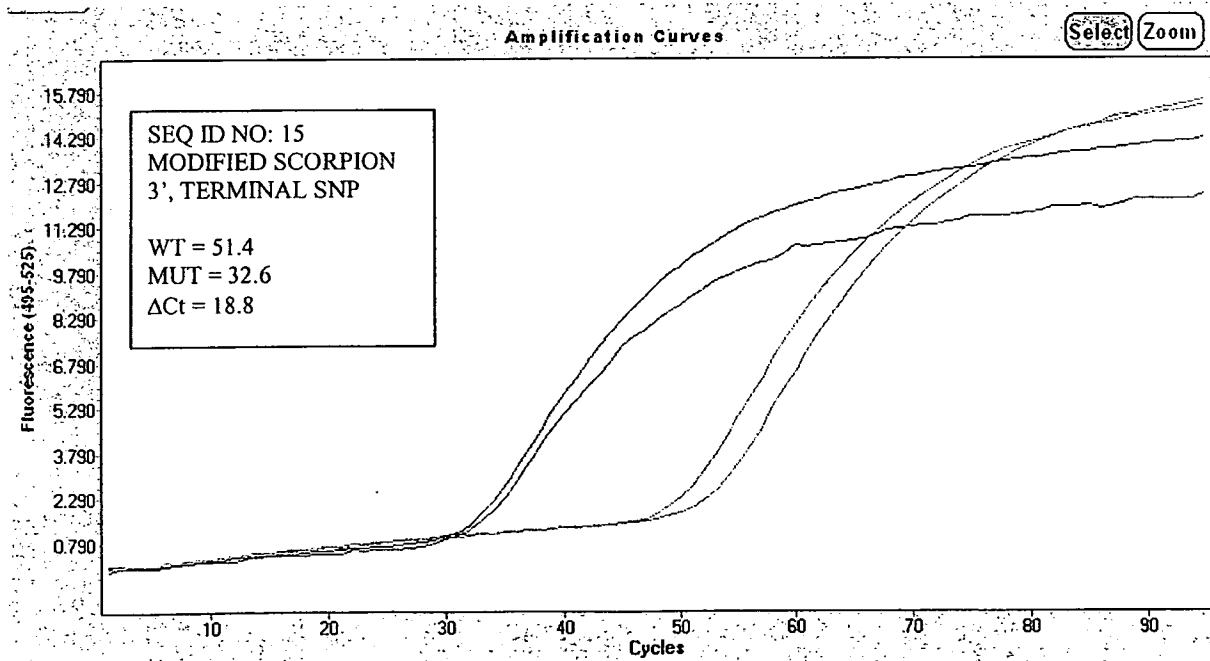
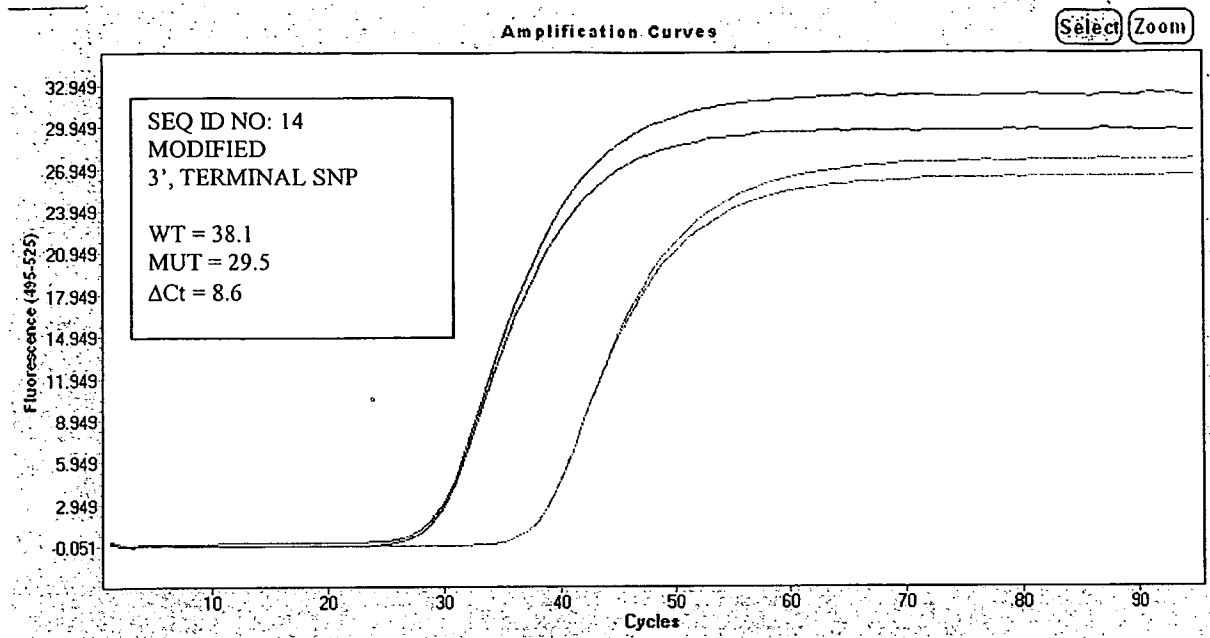


FIGURE 3-1

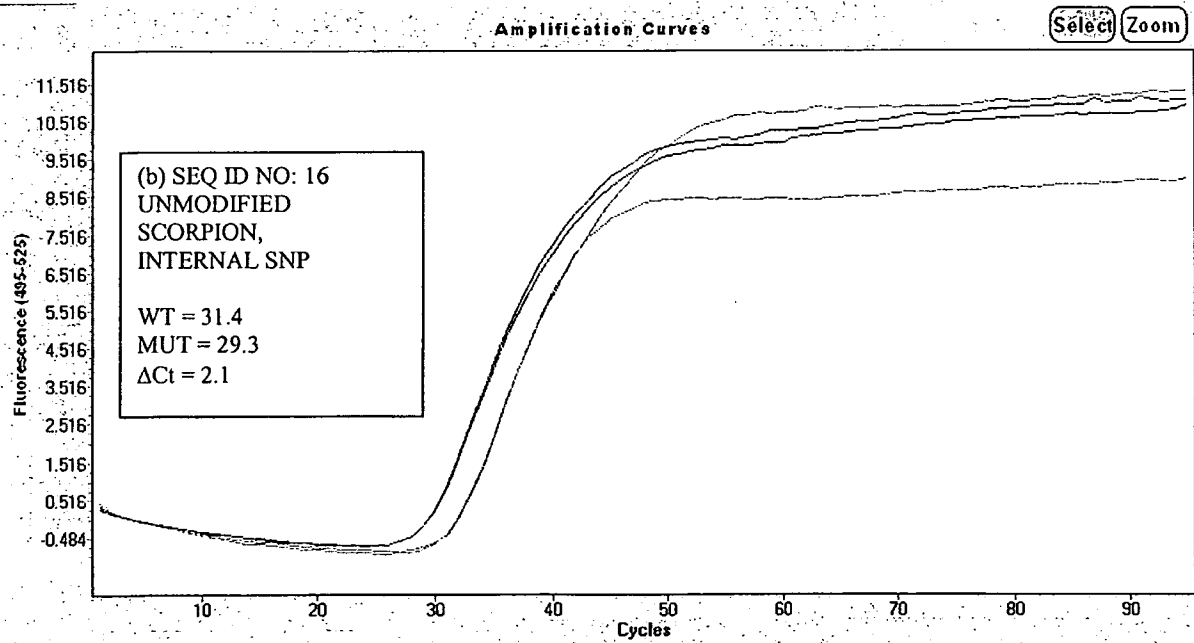
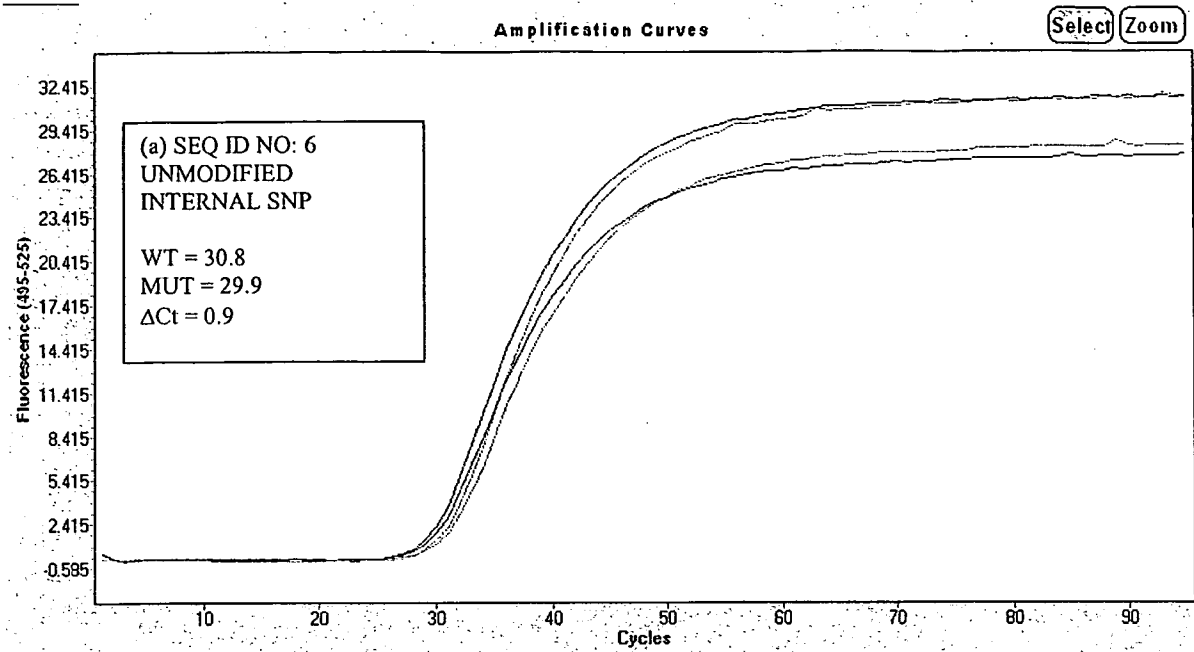


FIGURE 3-2

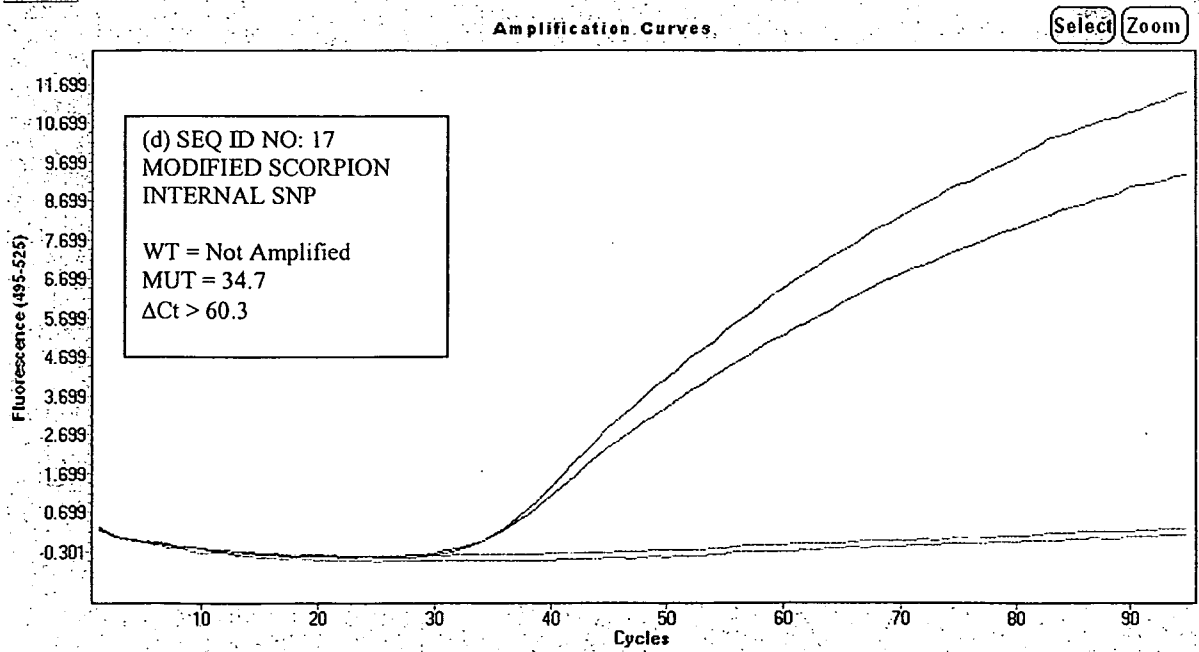
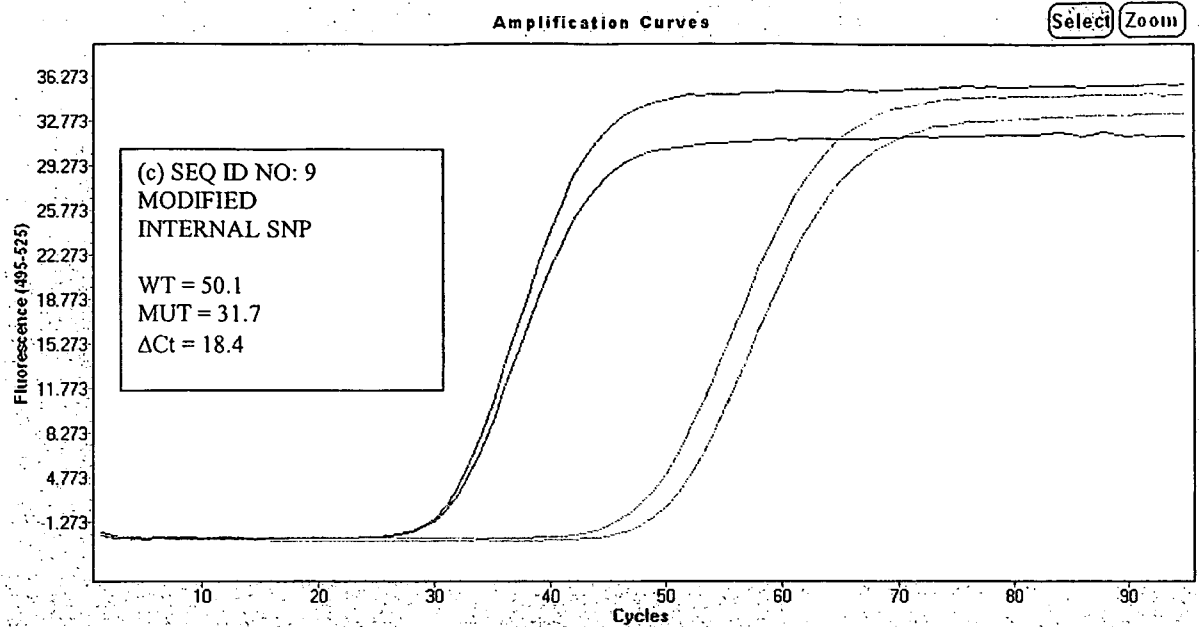
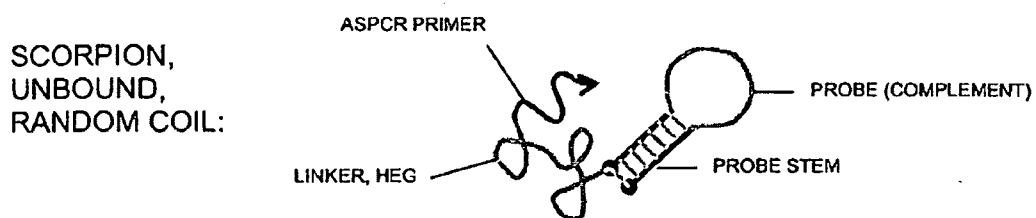
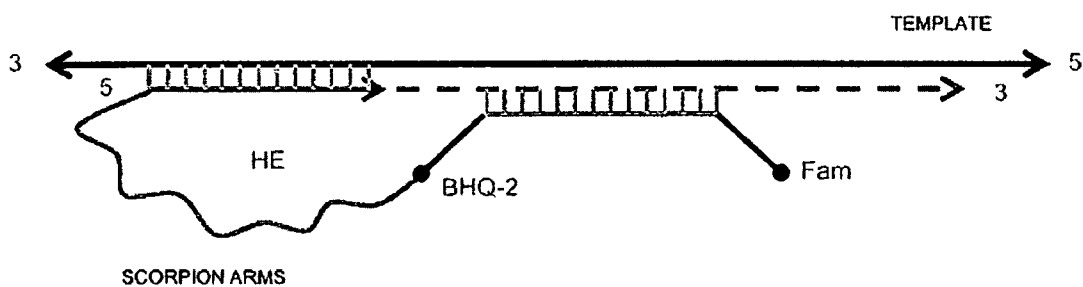


FIGURE 4

SCORPION ARMS (UNI-MOLECULAR, CLOSED FORMAT)



SCORPION, BOUND:



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2010/007560

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 practical, search terms used)
EPO-Internal, BIOSIS, Sequence Search, 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	US 2007/117118 A1 (YAKU HIDENOBU [JP] ET AL) 24 May 2007 (2007-05-24) see whole doc. esp. claims -----	1-25
X	WO 03/072814 A2 (ROCHE DIAGNOSTICS GMBH [DE]; HOFFMANN LA ROCHE [CH]; PINGOUD ALFRED [D]) 4 September 2003 (2003-09-04) the whole document -----	1-25
A	US 6 001 611 A (WILL STEPHEN GORDON [US]) 14 December 1999 (1999-12-14) cited in the application -----	1-25
	-/--	

Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents :

<p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"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)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p>	<p>"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</p> <p>"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</p> <p>"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.</p> <p>"&" document member of the same patent family</p>
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Date of the actual completion of the international search 8 February 2011	Date of mailing of the international search report 17/02/2011
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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 Mueller, Frank
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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2010/007560

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>WHITCOMBE D ET AL: "Detection of PCR products using self-probing amplicons and fluorescence", NATURE BIOTECHNOLOGY, NATURE PUBLISHING GROUP, NEW YORK, NY, US, vol. 17, no. 8, 1 August 1999 (1999-08-01) , pages 804-807, XP002226672, ISSN: 1087-0156, DOI: DOI:10.1038/11751 cited in the application the whole document</p> <p style="text-align: center;">-----</p>	1-25
A	<p>US 2006/246476 A1 (POLSKY DAVID [US] ET AL) 2 November 2006 (2006-11-02) the whole document</p> <p style="text-align: center;">-----</p>	1-25
X,P	<p>WO 2010/046067 A1 (ROCHE DIAGNOSTICS GMBH [DE]; HOFFMANN LA ROCHE [CH]) 29 April 2010 (2010-04-29) the whole document</p> <p style="text-align: center;">-----</p>	1-25

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2010/007560

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:
 - a. (means)
 - on paper
 - in electronic form
 - b. (time)
 - in the international application as filed
 - together with the international application in electronic form
 - subsequently to this Authority for the purpose of search
2. In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2010/007560

Patent document cited in search report	Publication date	Patent family member(s)	Publication date	
US 2007117118	A1	24-05-2007	JP 3859687 B1	20-12-2006
			JP 2007135521 A	07-06-2007

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			JP 2966389 B2	25-10-1999
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			PL 325439 A1	28-09-1998
			RU 2159248 C2	20-11-2000
			TW 567188 B	21-12-2003

US 2006246476	A1	02-11-2006	NONE	

WO 2010046067	A1	29-04-2010	US 2010099110 A1	22-04-2010
