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(54) Titre : PROCEDES, COMPOSITIONS ET NECESSAIRES DE DETECTION DE VARIANTES ALLELIQUES
(54) Title: METHODS, COMPOSITIONS, AND KITS FOR DETECTING ALLELIC VARIANTS

(57) Abrégé/Abstract:
In some embodiments, the present inventions relates generally to compositions, methods and kits for use in discriminating sequence variation between different alleles. More specifically, in some embodiments, the present invention provides for compositions, methods and kits for quantitating rare (e.g., mutant) allelic variants, such as SNPs, or nucleotide (NT) insertions or deletions, in samples comprising abundant (e.g., wild type) allelic variants with high specificity and selectivity. In particular, in some embodiments, the invention relates to a highly selective method for mutation detection referred to as competitive allele-specific TaqMan PCR ("cast-PCR").

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(54) **Title**: METHODS, COMPOSITIONS, AND KITS FOR DETECTING ALLELIC VARIANTS

(57) **Abstract**: In some embodiments, the present inventions relates generally to compositions, methods and kits for use in discriminating sequence variation between different alleles. More specifically, in some embodiments, the present invention provides for compositions, methods and kits for quantitating rare (e.g., mutant) allelic variants, such as SNPs, or nucleotide (NT) insertions or deletions, in samples comprising abundant (e.g., wild type) allelic variants with high specificity and selectivity. In particular, in some embodiments, the invention relates to a highly selective method for mutation detection referred to as competitive allele-specific TaqMan PCR ("cast-PCR").

METHODS, COMPOSITIONS, AND KITS FOR DETECTING ALLELIC VARIANTS

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] The present application claims the benefit of priority under 35 U.S.C. 119 to U.S. Provisional Application Nos. 61/138,521, filed December 17, 2009; 61/258,582, filed November 5, 2009; 61/253,501, filed October 20, 2009; 61/251,623, filed October 14, 2009; 61/186,775, filed June 12, 2009; and 61/164,230, filed March 27, 2009, all of which are incorporated herein by reference in their entireties.

BACKGROUND

[0001] Single nucleotide polymorphisms (SNPs) are the most common type of genetic diversity in the human genome, occurring at a frequency of about one SNP in 1,000 nucleotides or less in human genomic DNA (Kwok, P-Y, Ann Rev Genom Hum Genet 2001, 2: 235-258). SNPs have been implicated in genetic disorders, susceptibility to different diseases, predisposition to adverse reactions to drugs, and for use in forensic investigations. Thus, SNP (or rare mutation) detection provides great potentials in diagnosing early phase diseases, such as detecting circulating tumor cells in blood, for prenatal diagnostics, as well as for detection of disease-associated mutations in a mixed cell population.

[0002] Numerous approaches for SNP genotyping have been developed based on methods involving hybridization, ligation, or DNA polymerases (Chen, X., and Sullivan, PF, The Pharmacogeonomics Journal 2003, 3, 77-96.). For example, allele-specific polymerase chain reaction (AS-PCR) is a widely used strategy for detecting DNA sequence variation (Wu DY,

Ugozzoli L, Pal BK, Wallace RB., Proc Natl Acad Sci U S A 1989;86:2757-2760). AS-PCR, as its name implies, is a PCR-based method whereby one or both primers are designed to anneal at sites of sequence variations which allows for the ability to differentiate among different alleles of the same gene. AS-PCR exploits the fidelity of DNA polymerases, which extend primers with a mismatched 3' base at much lower efficiency, from 100 to 100,000 fold less efficient, than that with a matched 3' base (Chen, X., and Sullivan, PF, The Pharmacogeonomics Journal 2003; 3:77-96). The difficulty in extending mismatched primers results in diminished PCR amplification that can be readily detected.

[0003] The specificity and selectivity of AS-PCR, however, is largely dependent on the nature of exponential amplification of PCR which makes the decay of allele discriminating power rapid. Even though primers are designed to match a specific variant to selectively amplify only that variant, in actuality significant mismatched amplification often occurs. Moreover, the ability of AS-PCR to differentiate between allelic variants can be influenced by the type of mutation or the sequence surrounding the mutation or SNP (Ayyadevara S, Thaden JJ, Shmookler Reis RJ., Anal Biochem 2000;284:11-18), the amount of allelic variants present in the sample, as well as the ratio between alternative alleles. Collectively, these factors are often responsible for the frequent appearance of false-positive results, leading many researchers to attempt to increase the reliability of AS-PCR (Orou A, Fechner B, Utermann G, Menzel HJ., Hum Mutat 1995;6:163-169)(Imyanitov EN, Buslov KG, Suspitsin EN, Kuligina ES, Belogubova EV, Grigoriev MY, et al., Biotechniques 2002;33:484-490)(McKinzie PB, Parsons BL. Detection of rare K-ras codon 12 mutations using allele-specific competitive blocker PCR. Mutat Res 2002; 517:209-220)(Latorra D, Campbell K, Wolter A, Hurley JM., Hum Mutat 2003;22:79-85).

[0004] In some cases, the selectivity of AS-PCR has been increased anywhere from detection of 1 in 10 alleles to 1 in 100,000 alleles by using SNP-based PCR primers containing locked nucleic acids (LNAs) (Latorra, D., et al., Hum Mut 2003, 2:79-85; Nakiandwe, J. et al., Plant Method 2007, 3:2) or modified bases (Koizumi, M. et al. Anal Biochem. 2005, 340:287-294). However, these base "mimics" or modifications increase the overall cost of analysis and often require extensive optimization.

[0005] Another technology involving probe hybridization methods used for discriminating allelic variations is TaqMan® genotyping. However, like AS-PCR, selectivity using this method is limited and not suitable for detecting rare (1 in ≥1,000) alleles or mutations in a mixed sample.

SUMMARY

[0006] In some embodiments, the present inventions relates generally to compositions, methods and kits for use in discriminating sequence variation between different alleles. More specifically, in some embodiments, the present invention provides for compositions, methods and kits for quantitating rare (e.g., mutant) allelic variants, such as SNPs, or nucleotide (NT) insertions or deletions, in samples comprising abundant (e.g., wild type) allelic variants with high specificity. In particular, in some embodiments, the invention relates to a highly selective method for mutation detection referred to as competitive allele-specific TaqMan PCR ("cast-PCR").

[0007] In one aspect, the present invention provides compositions for use in identifying and/or quantitating allelic variants in nucleic acid samples. Some of these compositions can comprise: (a) an allele-specific primer; (b) an allele-specific blocker probe; (c) a detector probe; and/or (d) a locus-specific primer.

[0008] In some embodiments of the compositions, the allele-specific primer comprises a target-specific portion and an allele-specific nucleotide portion. In some embodiments, the allele-specific primer may further comprise a tail. In some exemplary embodiments, the tail is located at the 5' end of the allele-specific primer. In other embodiments, the tail of the allele-specific primer has repeated guanine and cytosine residues ("GC-rich"). In some embodiments, the melting temperature ("Tm") of the entire allele-specific primer ranges from about 50°C to 66°C. In some embodiments, the allele-specific primer concentration is between about 20-900 nM.

[0009] In some embodiments of the compositions, the allele-specific nucleotide portion of the allele-specific primer is located at the 3' terminus. In some embodiments, the selection of the allele-specific nucleotide portion of the allele-specific primer involves the use of a highly discriminating base (e.g., for detection of A/A, A/G, G/A, G/G, A/C, or C/A alleles). In some embodiments, for example when the allele to be detected involves A/G or C/T SNPs, A or G is used as the 3' allele-specific nucleotide portion of the allele-specific primer (e.g., if A or T is the target allele), or C or T is used as the 3' allele-specific nucleotide portion of the allele-specific primer (e.g., if C or G is the target allele). In other embodiments, A is used as the discriminating base at the 3' end of the allele-specific primer when detecting and/or quantifying A/T SNPs. In other embodiments, G is used as the discriminating base at the 3' end of the allele-specific primer when detecting and/or quantifying C/G SNPs.

[0010] In some embodiments of the compositions, the allele-specific blocker probe comprises a non-extendable blocker moiety at the 3' terminus. In some exemplary embodiments, the non-extendable blocker moiety is a minor groove binder (MGB). In some embodiments, the target allele position is located about 6-10, such as about 6, about 7, about 8,

about 9, or about 10 nucleotides away from the non-extendable blocker moiety of the allele-specific blocker probe. In some embodiments, the allele-specific blocker probe comprises an MGB moiety at the 5' terminus. In some exemplary embodiments, the allele-specific blocker probe is not cleaved during PCR amplification. In some embodiments, the Tm of the allele-specific blocker probe ranges from about 60°C to 66°C.

[0011] In some embodiments of the compositions, the allele-specific blocker probe and/or allele-specific primer comprise at least one modified base. In some embodiments, the modified base(s) may increase the difference in the Tm between matched and mismatched target sequences and/or decrease mismatch priming efficiency, thereby improving not only assay specificity bust also selectivity. Such modified base(s) may include, for example, 8-Aza-7-deaza-dA (ppA), 8-Aza-7-deaza-dG (ppG), locked nucleic acid (LNA) or 2′-*O*,4′-*C*-ethylene nucleic acid (ENA) bases (Fig. 4b).

[0012] In some embodiments of the compositions, the detector probe is a sequence-based or locus-specific detector probe. In other embodiments the detector probe is a 5' nuclease probe. In some exemplary embodiments, the detector probe can comprises an MGB moiety, a reporter moiety (e.g., FAM™, TET™, JOE™, VIC™, or SYBR® Green), a quencher moiety (e.g., Black Hole Quencher™ or TAMRA™;), and/or a passive reference (e.g., ROX™). In some exemplary embodiments, the detector probe is designed according to the methods and principles described in U.S. Patent No. 6,727,356 (the disclosure of which is incorporated herein by reference in its entirety). In some exemplary embodiments, the detector probe is a TaqMan® probe (Applied Biosystems, Foster City).

[0013] In some embodiments of the compositions, the composition can further comprise a polymerase; deoxyribonucleotide triphosphates (dNTPs); other reagents and/or buffers suitable

for amplification; and/or a template sequence or nucleic acid sample. In some embodiments, the polymerase can be a DNA polymerase. In some other embodiments, the polymerase can be thermostable, such as Taq DNA polymerase. In other embodiments, the template sequence or nucleic acid sample can be DNA, such as genomic DNA (gDNA) or complementary DNA (cDNA). In other embodiments the template sequence or nucleic acid sample can be RNA, such as messenger RNA (mRNA).

[0014] In another aspect, the present disclosure provides methods for amplifying an allele-specific sequence. Some of these methods can include one or more of the following: (a) hybridizing an allele-specific primer to a first nucleic acid molecule comprising a first allele (allele-1); (b) hybridizing an allele-specific blocker probe to a second nucleic acid molecule comprising a second allele (allele-2), wherein allele-2 corresponds to the same loci as allele-1; (c) hybridizing a detector probe to the first nucleic acid molecule; (d) hybridizing a locus-specific primer to the extension product of the allele-specific primer; and (e) PCR amplifying the first nucleic acid molecule comprising allele-1.

[0015] In another aspect, the present invention provides methods for detecting and/or quantitating an allelic variant in a pooled or mixed sample comprising other alleles. Some of these methods can include one or more of the following: (a) in a first reaction mixture hybridizing a first allele-specific primer to a fist nucleic acid molecule comprising a first allele (allele-1) and in a second reaction mixture hybridizing a second allele-specific primer to a first nucleic acid molecule comprising a second allele (allele-2), wherein allele-2 corresponds to the same loci as allele-1; (b) in the first reaction mixture hybridizing a first allele-specific blocker probe to a second nucleic acid molecule comprising allele-2 and in the second reaction mixture hybridizing a second allele-specific blocker probe to a second nucleic acid molecule comprising

allele-1; (c) in the first reaction mixture, hybridizing a first detector probe to the first nucleic acid molecule and in the second reaction mixture and hybridizing a second detector probe to the first nucleic acid molecule; (d) in the first reaction mixture hybridizing a first locus-specific primer to the extension product of the first allele-specific primer and in the second reaction mixture hybridizing a second locus-specific primer to the extension product of the second allele-specific primer; and (e) PCR amplifying the first nucleic acid molecule to form a first set or sample of amplicons and PCR amplifying the second nucleic acid molecule to form a second set or sample of amplicons; and (f) comparing the first set of amplicons to the second set of amplicons to quantitate allele-1 in the sample comprising allele-2 and/or allele-2 in the sample comprising allele-1.

[0016] In some embodiments of the methods, the first and/or second allele-specific primer comprises a target-specific portion and an allele-specific nucleotide portion. In some embodiments, the first and/or second allele-specific primer may further comprise a tail. In some embodiments, the Tm of the entire first and/or second allele-specific primer ranges from about 50°C to 66°C. In some embodiments the first and/or second allele-specific primer concentration is between about 20-900 nM.

[0017] In some embodiments of the methods, the target-specific portion of the first allele-specific primer and the target-specific portion of the second allele-specific primer comprise the same sequence. In other embodiments, the target-specific portion of the first allele-specific primer and the target-specific portion of the second allele-specific primer are the same sequence.

[0018] In some embodiments of the methods, the tail is located at the 5'-end of the first and/or second allele-specific primer. In some embodiments, the 5' tail of the first allele-specific primer and the 5' tail of the second allele-specific primer comprise the same sequence. In other

embodiments, the 5' tail of the first allele-specific primer and the 5' tail of the second allele-specific primer are the same sequence. In other embodiments, the tail of the first and/or second allele-specific primer is GC-rich.

[0019] In some embodiments of the methods, the allele-specific nucleotide portion of the first allele-specific primer is specific to a first allele (allele-1) of a SNP and the allele-specific nucleotide portion of the second allele-specific primer is specific to a second allele (allele-2) of the same SNP. In some embodiments of the methods, the allele-specific nucleotide portion of the first and/or second allele-specific primer is located at the 3'-terminus. In some embodiments, the selection of the allele-specific nucleotide portion of the first and/or second allele-specific primer involves the use of a highly discriminating base (e.g., for detection of A/A, A/G, G/A, G/G, A/C, or C/A alleles). In some embodiments, for example when the allele to be detected involves A/G or C/T SNPs, A or G is used as the 3' allele-specific nucleotide portion of the first and/or second allele-specific primer (e.g., if A/T is the target allele), or C or T is used as the 3' allele-specific nucleotide portion of the first and/or second allele-specific primer (e.g., if C/G is the target allele). In other embodiments, A is used as the discriminating base at the 3' end of the first and/or second allele-specific primer when detecting and/or quantifying A/T SNPs. In other embodiments, G is used as the discriminating base at the 3' end of the first and/or second allele-specific primer when detecting and/or quantifying C/G SNPs.

[0020] In some embodiments of the methods, the first and/or second allele-specific blocker probe comprises a non-extendable blocker moiety at the 3' terminus. In some exemplary embodiments, the non-extendable blocker moiety is an MGB. In some embodiments, the target allele position is located about 6-10, such as about 6, about 7, about 8, about 9, or about 10 nucleotides away from the non-extendable blocker moiety of the first and/or second allele-

specific blocker probe. In some embodiments, the first and/or second allele-specific blocker probe comprises an MGB moiety at the 5'-terminus. In other embodiments, the first and/or second allele-specific blocker probe is not cleaved during PCR amplification. In some embodiments, the Tm of the first and/or second allele-specific blocker probe ranges from about 60°C to 66°C.

[0021] In some embodiments of the methods, the first and/or second allele-specific blocker probe and/or the first and/or second allele-specific primer comprises at least one modified base. In some embodiments, the modified base(s) may increase the difference in the Tm between matched and mismatched target sequences and/or decrease mismatch priming efficiency, thereby improving not only assay specificity bust also selectivity. Such modified base(s) may include, for example, 8-Aza-7-deaza-dA (ppA), 8-Aza-7-deaza-dG (ppG), locked nucleic acid (LNA) or 2′-*O*,4′-*C*-ethylene nucleic acid (ENA) bases (Fig. 4b).

[0022] In some embodiments of the methods, the first and/or second detector probes are the same. In some embodiments, the first and/or second detector probes are different. In some embodiments, the first and/or second detector probe is a sequence-based or locus-specific detector probe. In other embodiments the first and/or second detector probe is a 5' nuclease probe. In some exemplary embodiments, the first and/or second detector probes comprises an MGB moiety, a reporter moiety (e.g., FAM™, TET™, JOE™, VIC™, or SYBR® Green), a quencher moiety (e.g., Black Hole Quencher™ or TAMRA™;), and/or a passive reference (e.g., ROX™). In some exemplary embodiments, the first and/or second detector probe is designed according to the methods and principles described in U.S. Patent No. 6,727,356 (the disclosure of which is incorporated herein by reference in its entirety). In some exemplary embodiments, the detector probe is a TaqMan® probe.

[0023] In some embodiments of the methods, the first locus-specific primer and the second locus-specific primer comprise the same sequence. In some embodiments the first locus-specific primer and the second locus-specific primer are the same sequence.

[0024] In some embodiments of the methods, the first and/or second reaction mixtures can further comprises a polymerase; dNTPs; other reagents and/or buffers suitable for PCR amplification; and/or a template sequence or nucleic acid sample. In some embodiments, the polymerase can be a DNA polymerase. In some embodiments, the polymerase can be thermostable, such as Taq DNA polymerase. In some embodiments, the template sequence or nucleic acid sample can be DNA, such as gDNA or cDNA. In other embodiments the template sequence or nucleic acid sample can be RNA, such as mRNA.

[0025] In some embodiments of the methods, the first allele-specific blocker probe binds to the same strand or sequence as the second allele-specific primer, while the second allele-specific blocker probe binds to the same strand or sequence as the first allele-specific primer. In some embodiments, the first and/or second allele-specific blocker probes are used to reduce the amount of background signal generated from either the second allele and/or the first allele, respectively. In some embodiments, first and/or second allele-specific blocker probes are non-extendable and preferentially anneal to either the second allele or the first allele, respectively, thereby blocking the annealing of, for example, the extendable first allele-specific primer to the second allele and/or the extendable second allele-specific primer to first allele.

[0026] In some exemplary embodiments, the first allele is a rare (e.g., minor) or mutant allele. In other exemplary embodiments the second allele is an abundant (e.g., major) or wild type allele.

[0027] In another aspect, the present invention provides kits for quantitating a first allelic variant in a sample comprising a second allelic variant involving: (a) a first allele-specific primer; (b) a second allele-specific primer; (c), a first locus-specific primer; (d) a second locus-specific primer; (e) a first allele-specific blocker probe; (f) a second allele-specific blocker probe; and (g) a first locus-specific detector probe and (h) a second locus-specific detector probe.

[0028] In some embodiments of the kits, the first and/or second allele-specific primer comprises a target-specific portion and an allele-specific nucleotide portion. In some embodiments, the first and/or second allele-specific primer may further comprise a tail. In some embodiments, the Tm of the entire first and/or second allele-specific primer ranges from about 50°C to 66°C. In some embodiments the first and/or second allele-specific primer concentrations are between about 20-900 nM.

[0029] In some embodiments of the kits, the target-specific portion of the first allele-specific primer and the target-specific portion of the second allele-specific primer comprise the same sequence. In other embodiments, the target-specific portion of the first allele-specific primer and the target-specific portion of the second allele-specific primer are the same sequence.

[0030] In some embodiments of the kits, the tail is located at the 5' end of the first and/or second allele-specific primer. In some embodiments, the 5' tail of the first allele-specific primer and the 5' tail of the second allele-specific primer comprise the same sequence. In other embodiments, the 5' tail of the first allele-specific primer and the 5' tail of the second allele-specific primer are the same sequence. In other embodiments, the tail of the first and/or second allele-specific primer is GC rich.

[0031] In some embodiments of the kits, the allele-specific nucleotide portion of the first allele-specific primer is specific to a first allele (allele-1) of a SNP and the allele-specific

nucleotide portion of the second allele-specific primer is specific to a second allele (allele-2) of the same SNP. In some embodiments of the disclosed methods, the allele-specific nucleotide portion of the first and/or second allele-specific primer is located at the 3' terminus. In some embodiments, the selection of the allele-specific nucleotide portion of the first and/or second allele-specific primer involves the use of a highly discriminating base (e.g., for detection of A/A, A/G, G/A, G/G, A/C, or C/A alleles) (Fig. 2). In some embodiments, for example when the allele to be detected involves A/G or C/T SNPs, A or G is used as the 3' allele-specific nucleotide portion of the first and/or second allele-specific primer (e.g., if A/T is the target allele), or C or T is used as the 3' allele-specific nucleotide portion of the first and/or second allele-specific primer (e.g., if C/G is the target allele). In other embodiments, A is used as the discriminating base at the 3' end of the first and/or second allele-specific primer when detecting and/or quantifying A/T SNPs. In other embodiments, G is used as the discriminating base at the 3' end of the first and/or second allele-specific primer when detecting and/or quantifying C/G SNPs.

[0032] In some embodiments of the kits, the first and/or second allele-specific blocker probe comprises a non-extendable blocker moiety at the 3' terminus. In some exemplary embodiments, the non-extendable blocker moiety is an MGB. In some embodiments, the target allele position is located about 6-10, such as about 6, about 7, about 8, about 9, or about 10 nucleotides away from the non-extendable blocker moiety of the first and/or second allele-specific blocker probe. In some embodiments, the first and/or second allele-specific blocker probe comprises an MGB moiety at the 5' terminus. In other embodiments, the first and/or second allele-specific blocker probe is not cleaved during PCR amplification. In some embodiments, the Tm of the first and/or second allele-specific blocker probe ranges from about

60°C to 66°C.

[0033] In some embodiments of the kits, the allele-specific blocker probe and/or the first and/or second allele-specific primer comprises at least one modified base. In some embodiments, the modified base(s) may increase the difference in the Tm between matched and mismatched target sequences and/or decrease mismatch priming efficiency, thereby improving not only assay specificity bust also selectivity. Such modified base(s) may include, for example, 8-Aza-7-deaza-dA (ppA), 8-Aza-7-deaza-dG (ppG), locked nucleic acid (LNA) or 2′-*O*,4′-*C*-ethylene nucleic acid (ENA) bases (Fig. 4b).

[0034] In some embodiments of the kits, the first and/or second detector probes are the same. In some embodiments of the disclosed kits the first and/or second detector probes are different. In some embodiments of the disclosed kits, the first and/or second detector probes are sequence-based or locus-specific detector probes. In other embodiments the first and/or second detector probe are 5' nuclease probes. In some exemplary embodiments, the first and/or second detector probes comprise an MGB moiety, a reporter moiety (e.g., FAM™, TET™, JOE™, VIC™, or SYBR® Green), a quencher moiety (e.g., Black Hole Quencher™ or TAMRA™;), and/or a passive reference (e.g., ROX™). In some exemplary embodiments, the first and/or second detector probe are designed according to the methods and principles described in U.S. Patent No. 6,727,356 (the disclosure of which is incorporated herein by reference in its entirety). In some exemplary embodiments, the detector probe is a TaqMan® probe.

[0035] In some embodiments of the kits, the first locus-specific primer and the second locus-specific primer comprise the same sequence. In some embodiments the first locus-specific primer and the second locus-specific primer are the same sequence.

[0036] In some embodiments of the kits, the first and/or second reaction mixture can further

comprise a polymerase; dNTPs; other reagents and/or buffers suitable for PCR amplification; and/or a template sequence or nucleic acid sample. In some embodiments, the polymerase can be a DNA polymerase. In some other embodiments, the polymerase can be thermostable, such as Taq DNA polymerase.

[0037] In some embodiments, the compositions, methods and kits of the present invention provide high allelic discrimination specificity and selectivity. In some embodiments, the quantitative determination of specificity and/or selectivity comprises a comparison of Ct values between a first set of amplicons and a second set of amplicons. In some embodiments, selectivity is at a level whereby a single copy of a given allele in about 1 million copies of another allele or alleles can be detected.

[0038] The foregoing has described various embodiments of the invention that provide improved detection and discrimination of allelic variants using one or more of the following: (a) tailed allele-specific primers; (b) low allele-specific primer concentration; (c) allele-specific primers designed to have lower Tms; (d) allele-specific primers designed to target discriminating bases; (e) allele-specific blocker probes containing MGB, designed to prevent amplification from alternative, and potentially more abundant, allelic variants in a sample; and (f) allele-specific blocker probes and/or allele-specific primers designed to comprise modified bases in order to increase the delta Tm between matched and mismatched target sequences.

[0039] While particular embodiments employing several of the above improvements have been discussed herein, it will be apparent to the skilled artisan that depending on the nature of the sample to be examined, various combinations of the above improvements can be combined to arrive at a favorable result. Thus, for example, non-MGB blocker probes can be used with an embodiment that include methods employing allele-specific primers containing modified bases

to increase delta Tm; such primers can also be designed to target discriminating bases; and the primers can be used at low primer concentrations. Accordingly, alternative embodiments based upon the present disclosure can be used to achieve a suitable level of allelic detection.

[0040] The present disclosure provides the advantage that any of the combinations of listed improvements could be utilized by a skilled artisan in a particular situation. For example, the current invention can include a method or reaction mixture that employs improvements a, c, d and f; improvements b, c, and e; or improvements

[0041] It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention, as claimed.

[0042] The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate several exemplary embodiments of the disclosure and together with the description, serve to explain certain teachings.

BRIEF DESCRIPTION OF THE DRAWINGS

[0043] The skilled artisan will understand that the drawings described below are for illustration purposes only. The drawings are not intended to limit the scope of the present teachings in any way.

[0044] **Fig. 1** depicts a schematic of an illustrative embodiment of cast-PCR. In some embodiments, components of cast-PCR include the following: one locus-specific TaqMan probe (LST); two MGB blockers: one allele-1-specific MGB blocker (MGB1) and one allele-2-specific MGB blocker (MGB2); 3 PCR primers: one locus-specific PCR primer (LSP); one allele-1-specific primer (ASP1) and one allele-2-specific primer (ASP2).

[0045] **Fig. 2** depicts a schematic of an illustrative embodiment of cast-PCR using allele-specific blocker probes comprising highly discriminating bases for detecting rare allelic variants. Highly discriminating bases may include, for example, A/A, A/G, G/A, G/G, A/C, C/A. The least discriminating bases may include, for example, C/C, T/C, G/T, T/G, C/T. In some embodiments, for example, for detection of A-G or C-T SNPs, A & G are used as the discriminating base if A//T is allelic variant (e.g., mutant allele); or C & T are used as the discriminating base if C//G allelic variant (e.g., mutant allele).

[0046] **Fig. 3** depicts a schematic of an illustrative embodiment of cast-PCR using an allele-specific blocker probe with an MGB moiety at the 5' end. In some embodiments the blocker moiety at the 3'-end of the probe may include, for example, NH_2, biotin, MGB, PO_4, and PEG.

[0047] **Fig. 4A** depicts a schematic of an illustrative embodiment of cast-PCR using modified bases in an MGB blocker probe or allele-specific primer. (G* represents ppG.)

[0048] **Fig. 4B** depicts some examples of modified bases of an MGB blocker probe or allele-specific primer.

[0049] **Fig. 5** depicts the TaqMan-like sensitivity and dynamic range of one exemplary embodiment of cast-PCR.

[0050] **Fig. 6** depicts the sequence of KRAS mutations at codons 12 and 13 that are detectable using cast-PCR methods. KRAS mutations at codons 12 and 13 are associated with resistance to cetuxima or panitumumab in metastatic colorectal cancer (Di Nicolantonio F., *et al.*, *J Clin Oncol.* 2008; 26:5705–12.)

[0051] **Fig. 7** depicts the specificity of KRAS mutation detection using cast-PCR assays in one exemplary embodiment.

[0052] **Fig. 8** depicts one exemplary embodiment using cast-PCR methods to detect a single

copy of mutant DNA in 10^6 copies of wild-type DNA.

[0053] **Fig. 9** depicts detection of the relative copy number of mutant samples (KRAS-G12A) spiked in wild type samples using cast-PCR methods.

[0054] **Fig. 10** depicts a number of different tumor markers (SNPs) detected in tumor samples using one exemplary embodiment of cast-PCR.

[0055] **Fig. 11A-D** shows a list of exemplary allele-specific primers and probes used in cast-PCR assays.

DETAILED DESCRIPTION

[0056] I. Introduction

[0057] The selective amplification of an allele of interest is often complicated by factors including the mispriming and extension of a mismatched allele-specific primer on an alternative allele. Such mispriming and extension can be especially problematic in the detection of rare alleles present in a sample populated by an excess of another allelic variant. When in sufficient excess, the mispriming and extension of the other allelic variant may obscure the detection of the allele of interest. When using PCR-based methods, the discrimination of a particular allele in a sample containing alternative allelic variants relies on the selective amplification of an allele of interest, while minimizing or preventing amplification of other alleles present in the sample.

[0058] A number of factors have been identified, which alone or in combination, contribute to the enhanced discriminating power of allele-specific PCR. As disclosed herein, a factor which provides a greater delta C_t value between a mismatched and matched allele-specific primer is indicative of greater discriminating power between allelic variants. Such factors found to improve discrimination of allelic variants using the present methods include, for example, the use of one or more of the following: (a) tailed allele-specific primers; (b) low allele-specific

primer concentration; (c) allele-specific primers designed to have lower Tms; (d) allele-specific primers designed to target discriminating bases; (e) allele-specific blocker probes designed to prevent amplification from alternative, and potentially more abundant, allelic variants in a sample; and (f) allele-specific blocker probes and/or allele-specific primers designed to comprise modified bases in order to increase the delta Tm between matched and mismatched target sequences.

[0059] The above-mentioned factors, especially when used in combination, can influence the ability of allele-specific PCR to discriminate between different alleles present in a sample. Thus, the present disclosure relates generally to novel amplification methods referred to as cast-PCR, which utilizes a combination of factors referred to above to improve discrimination of allelic variants during PCR by increasing delta C_t values. In some embodiments, the present methods can involve high levels of selectivity, wherein one mutant molecule in a background of at least 1,000 to 1,000,000, such as about 1000-10,000, about 10,000 to 100,000, or about 100,000 to 1,000,000 wild type molecules, or any fractional ranges in between can be detected. In some embodiments, the comparison of a first set of amplicons and a second set of amplicons involving the disclosed methods provides improvements in specificity from 1,000X to 1,000,000X fold difference, such as about 1000-10,000X, about 10,000 to 100,000X, or about 100,000 to 1,000,000X fold difference, or any fractional ranges in between.

[0060] II. Definitions

[0061] For the purposes of interpreting this specification, the following definitions will apply and whenever appropriate, terms used in the singular will also include the plural and vice versa. In the event that any definition set forth below conflicts with the usage of that word in any other

document, including any document incorporated herein by reference, the definition set forth below shall always control for purposes of interpreting this specification and its associated claims unless a contrary meaning is clearly intended.

[0062] As used herein, the term "allele" refers generally to alternative DNA sequences at the same physical locus on a segment of DNA, such as, for example, on homologous chromosomes. An allele can refer to DNA sequences which differ between the same physical locus found on homologous chromosomes within a single cell or organism or which differ at the same physical locus in multiple cells or organisms ("allelelic variant"). In some instances, an allele can correspond to a single nucleotide difference at a particular physical locus. In other embodiments and allele can correspond to nucleotide (single or multiple) insertion or deletion.

[0063] As used herein, the term "allele-specific primer" refers to an oligonucleotide sequence that hybridizes to a sequence comprising an allele of interest, and which when used in PCR can be extended to effectuate first strand cDNA synthesis. Allele-specific primers are specific for a particular allele of a given target DNA or loci and can be designed to detect a difference of as little as one nucleotide in the target sequence. Allele-specific primers may comprise an allele-specific nucleotide portion, a target-specific portion, and/or a tail.

[0064] As used herein, the terms "allele-specific nucleotide portion" or "allele-specific target nucleotide" refers to a nucleotide or nucleotides in an allele-specific primer that can selectively hybridize and be extended from one allele (for example, a minor or mutant allele) at a given locus to the exclusion of the other (for example, the corresponding major or wild type allele) at the same locus.

[0065] As used herein, the term "target-specific portion" refers to the region of an allele-specific primer that hybridizes to a target polynucleotide sequence. In some embodiments, the

target-specific portion of the allele-specific primer is the priming segment that is complementary to the target sequence at a priming region 5' of the allelic variant to be detected. The target-specific portion of the allele-specific primer may comprise the allele-specific nucleotide portion. In other instances, the target-specific portion of the allele-specific primer is adjacent to the 3' allele-specific nucleotide portion.

[0066] As used herein, the terms "tail" or "5'-tail" refers to the non-3' end of a primer. This region typically will, although does not have to contain a sequence that is not complementary to the target polynucleotide sequence to be analyzed. The 5' tail can be any of about 2-30, 2-5, 4-6, 5-8, 6-12, 7-15, 10-20, 15-25 or 20-30 nucleotides, or any range in between, in length.

[0067] As used herein, the term "allele-specific blocker probe" (also referred to herein as "blocker probe," "blocker,") refers to an oligonucleotide sequence that binds to a strand of DNA comprising a particular allelic variant which is located on the same, opposite or complementary strand as that bound by an allelic-specific primer, and reduces or prevents amplification of that particular allelic variant. As discussed in greater detail herein, allele-specific blocker probes generally comprise modifications, e.g., at the 3'-OH of the ribose ring, which prevent primer extension by a polymerase. The allele-specific blocker probe can be designed to anneal to the same or opposing strand of what the allele-specific primer anneals to and can be modified with a blocking group (e.g., a "non-extendable blocker moiety") at its 3' terminal end. Thus, a blocker probe can be designed, for example, so as to tightly bind to a wild type allele (e.g., abundant allelic variant) in order to suppress amplification of the wild type allele while amplification is allowed to occur on the same or opposing strand comprising a mutant allele (e.g., rare allelic variant) by extension of an allele-specific primer. In illustrative examples, the allele-specific

blocker probes do not include a label, such as a fluorescent, radioactive, or chemiluminescent label

[0068] As used herein, the term "non-extendable blocker moiety" refers generally to a modification on an oligonucleotide sequence such as a probe and/or primer which renders it incapable of extension by a polymerase, for example, when hybridized to its complementary sequence in a PCR reaction. Common examples of blocker moieties include modifications of the ribose ring 3'-OH of the oligonucleotide, which prevents addition of further bases to the '3-end of the oligonucleotide sequence a polymerase. Such 3'-OH modifications are well known in the art. (See, e.g., Josefsen, M., et al., *Molecular and Cellular Probes*, 23 (2009):201-223; McKinzie, P. et al., *Mutagenesis.* 2006, 21(6):391-7; Parsons, B. et al., *Methods Mol Biol.* 2005, 291:235-45; Parsons, B. et al., *Nucleic Acids Res.* 1992, 25:20(10):2493-6; and Morlan, J. et al., *PLoS One* 2009, 4 (2): e4584, the disclosures of which are incorporated herein by reference in their entireties.)

[0069] As used herein, the terms "MGB," "MGB group," "MGB compound," or "MBG moiety" refers to a minor groove binder. When conjugated to the 3' end of an oligonucleotide, an MGB group can function as a non-extendable blocker moiety.

[0070] An MGB is a molecule that binds within the minor groove of double stranded DNA. Although a general chemical formula for all known MGB compounds cannot be provided because such compounds have widely varying chemical structures, compounds which are capable of binding in the minor groove of DNA, generally speaking, have a crescent shape three dimensional structure. Most MGB moieties have a strong preference for A-T (adenine and thymine) rich regions of the B form of double stranded DNA. Nevertheless, MGB compounds which would show preference to C-G (cytosine and guanine) rich regions are also theoretically

possible. Therefore, oligonucleotides comprising a radical or moiety derived from minor groove binder molecules having preference for C-G regions are also within the scope of the present invention.

[0071] Some MGBs are capable of binding within the minor groove of double stranded DNA with an association constant of 10^3M^{-1} or greater. This type of binding can be detected by well established spectrophotometric methods such as ultraviolet (UV) and nuclear magnetic resonance (NMR) spectroscopy and also by gel electrophoresis. Shifts in UV spectra upon binding of a minor groove binder molecule and NMR spectroscopy utilizing the "Nuclear Overhauser" (NOSEY) effect are particularly well known and useful techniques for this purpose. Gel electrophoresis detects binding of an MGB to double stranded DNA or fragment thereof, because upon such binding the mobility of the double stranded DNA changes.

[0072] A variety of suitable minor groove binders have been described in the literature. See, for example, Kutyavin, et al. U.S. Patent No. 5,801,155; Wemmer, D. E., and Dervan P. B., Current Opinion in Structural Biology, 7:355-361 (1997); Walker, W. L., Kopka, J. L. and Goodsell, D. S., Biopolymers, 44:323-334 (1997); Zimmer, C.& Wahnert, U. Prog. Biophys. Molec. Bio. 47:31-112 (1986) and Reddy, B. S. P., Dondhi, S. M., and Lown, J. W., Pharmacol. Therap., 84:1-111 (1999) (the disclosures of which are herein incorporated by reference in their entireties). A preferred MGB in accordance with the present disclosure is DPI_3. Synthesis methods and/or sources for such MGBs are also well known in the art. (See, e.g., U.S. Patent Nos. 5,801,155; 6,492,346; 6,084,102; and 6,727,356, the disclosures of which are incorporated herein by reference in their entireties.)

[0073] As used herein, the term "MGB blocker probe," "MBG blocker," or "MGB probe" is an oligonucleotide sequence and/or probe further attached to a minor groove binder moiety at its

3' and/or 5' end. Oligonucleotides conjugated to MGB moieties form extremely stable duplexes with single-stranded and double-stranded DNA targets, thus allowing shorter probes to be used for hybridization based assays. In comparison to unmodified DNA, MGB probes have higher melting temperatures (Tm) and increased specificity, especially when a mismatch is near the MGB region of the hybridized duplex. (See, e.g., Kutyavin, I. V., *et al.*, Nucleic Acids Research, 2000, Vol. 28, No. 2: 655-661).

[0074] As used herein, the term "modified base" refers generally to any modification of a base or the chemical linkage of a base in a nucleic acid that differs in structure from that found in a naturally occurring nucleic acid. Such modifications can include changes in the chemical structures of bases or in the chemical linkage of a base in a nucleic acid, or in the backbone structure of the nucleic acid. (See, e.g., Latorra, D. et al., *Hum Mut* 2003, 2:79-85. Nakiandwe, J. et al., p*lant Method* 2007, 3:2.)

[0075] As used herein, the term "detector probe" refers to any of a variety of signaling molecules indicative of amplification. For example, SYBR® Green and other DNA-binding dyes are detector probes. Some detector probes can be sequence-based (also referred to herein as "locus-specific detector probe"), for example 5' nuclease probes. Various detector probes are known in the art, for example (TaqMan® probes described herein (See also U.S. Patent No. 5,538,848) various stem-loop molecular beacons (See, e.g., U.S. Patent Nos. 6,103,476 and 5,925,517 and Tyagi and Kramer, 1996, Nature Biotechnology 14:303-308), stemless or linear beacons (See, e.g., WO 99/21881), PNA Molecular Beacons™ (See, e.g., U.S. Patent Nos. 6,355,421 and 6,593,091), linear PNA beacons (See, e.g., Kubista et al., 2001, SPIE 4264:53-58), non-FRET probes (See, e.g., U.S. Patent No. 6,150,097), Sunrise®/Amplifluor® probes (U.S. Patent No. 6,548,250), stem-loop and duplex Scorpion™ probes (Solinas et al., 2001,

Nucleic Acids Research 29:E96 and U.S. Patent No. 6,589,743), bulge loop probes (U.S. Patent No. 6,590,091), pseudo knot probes (U.S. Patent No. 6,589,250), cyclicons (U.S. Patent No. 6,383,752), MGB Eclipse™ probe (Epoch Biosciences), hairpin probes (U.S. Patent No. 6,596,490), peptide nucleic acid (PNA) light-up probes, self-assembled nanoparticle probes, and ferrocene-modified probes described, for example, in U.S. Patent No. 6,485,901; Mhlanga et al., 2001, Methods 25:463-471; Whitcombe et al., 1999, Nature Biotechnology. 17:804-807; Isacsson et al., 2000, Molecular Cell Probes. 14:321-328; Svanvik et al., 2000, Anal Biochem. 281:26-35; Wolffs et al., 2001, Biotechniques 766:769-771; Tsourkas et al., 2002, Nucleic Acids Research. 30:4208-4215; Riccelli et al., 2002, Nucleic Acids Research 30:4088-4093; Zhang et al., 2002 Shanghai. 34:329-332; Maxwell et al., 2002, J. Am. Chem. Soc. 124:9606-9612; Broude et al., 2002, Trends Biotechnol. 20:249-56; Huang et al., 2002, Chem Res. Toxicol. 15:118-126; and Yu et al., 2001, J. Am. Chem. Soc 14:11155-11161. Detector probes can comprise reporter dyes such as, for example, 6-carboxyfluorescein (6-FAM) or tetrachlorofluorescin (TET). Detector probes can also comprise quencher moieties such as tetramethylrhodamine (TAMRA), Black Hole Quenchers (Biosearch), Iowa Black (IDT), QSY quencher (Molecular Probes), and Dabsyl and Dabcel sulfonate/carboxylate Quenchers (Epoch). Detector probes can also comprise two probes, wherein for example a fluor is on one probe, and a quencher on the other, wherein hybridization of the two probes together on a target quenches the signal, or wherein hybridization on a target alters the signal signature via a change in fluorescence. Detector probes can also comprise sulfonate derivatives of fluorescein dyes with SO_3 instead of the carboxylate group, phosphoramidite forms of fluorescein, phosphoramidite forms of CY5 (available, for example, from Amersham Biosciences-GE Healthcare).

[0076] As used herein, the term "locus-specific primer" refers to an oligonucleotide sequence that hybridizes to products derived from the extension of a first primer (such as an allele-specific primer) in a PCR reaction, and which can effectuate second strand cDNA synthesis of said product. Accordingly, in some embodiments, the allele-specific primer serves as a forward PCR primer and the locus-specific primer serves as a reverse PCR primer, or vice versa. In some preferred embodiments, locus-specific primers are present at a higher concentration as compared to the allele-specific primers.

[0077] As used herein, the term "rare allelic variant" refers to a target polynucleotide present at a lower level in a sample as compared to an alternative allelic variant. The rare allelic variant may also be referred to as a "minor allelic variant" and/or a "mutant allelic variant." For instance, the rare allelic variant may be found at a frequency less than 1/10, 1/100, 1/1,000, 1/10,000, 1/100,000, 1/1,000,000, 1/10,000,000, 1/100,000,000 or 1/1,000,000,000 compared to another allelic variant for a given SNP or gene. Alternatively, the rare allelic variant can be, for example, less than 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 50, 75, 100, 250, 500, 750, 1,000, 2,500, 5,000, 7,500, 10,000, 25,000, 50,000, 75,000, 100,000, 250,000, 500,000, 750,000, or 1,000,000 copies per 1, 10, 100, 1,000 micro liters of a sample or a reaction volume.

[0078] As used herein, the terms "abundant allelic variant" may refer to a target polynucleotide present at a higher level in a sample as compared to an alternative allelic variant. The abundant allelic variant may also be referred to as a "major allelic variant" and/or a "wild type allelic variant." For instance, the abundant allelic variant may be found at a frequency greater than 10X, 100X, 1,000X, 10,000X, 100,000X, 1,000,000X, 10,000,000X, 100,000,000X or 1,000,000,000X compared to another allelic variant for a given SNP or gene. Alternatively, the abundant allelic variant can be, for example, greater than 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25,

50, 75, 100, 250, 500, 750, 1,000, 2,500, 5,000, 7,500, 10,000, 25,000, 50,000, 75,000, 100,000, 250,000, 500,000, 750,000, 1,000,000 copies per 1, 10, 100, 1,000 micro liters of a sample or a reaction volume.

[0079] As used herein, the terms "first" and "second" are used to distinguish the components of a first reaction (e.g., a "first" reaction; a "first" allele-specific primer) and a second reaction (e.g., a "second" reaction; a "second" allele-specific primer). By convention, as used herein the first reaction amplifies a first (for example, a rare) allelic variant and the second reaction amplifies a second (for example, an abundant) allelic variant or vice versa.

[0080] As used herein, both "first allelic variant" and "second allelic variant" can pertain to alleles of a given locus from the same organism. For example, as might be the case in human samples (e.g., cells) comprising wild type alleles, some of which have been mutated to form a minor or rare allele. The first and second allelic variants of the present teachings can also refer to alleles from different organisms. For example, the first allele can be an allele of a genetically modified organism, and the second allele can be the corresponding allele of a wild type organism. The first allelic variants and second allelic variants of the present teachings can be contained on gDNA, as well as mRNA and cDNA, and generally any target nucleic acids that exhibit sequence variability due to, for example, SNP or nucleotide(s) insertion and/or deletion mutations.

[0081] As used herein, the term "thermostable" or "thermostable polymerase" refers to an enzyme that is heat stable or heat resistant and catalyzes polymerization of deoxyribonucleotides to form primer extension products that are complementary to a nucleic acid strand. Thermostable DNA polymerases useful herein are not irreversibly inactivated when subjected to elevated temperatures for the time necessary to effect destabilization of single-stranded nucleic acids or

denaturation of double-stranded nucleic acids during PCR amplification. Irreversible denaturation of the enzyme refers to substantial loss of enzyme activity. Preferably a thermostable DNA polymerase will not irreversibly denature at about 90°-100°C under conditions such as is typically required for PCR amplification.

[0082] As used herein, the term "PCR amplifying" or "PCR amplification" refers generally to cycling polymerase-mediated exponential amplification of nucleic acids employing primers that hybridize to complementary strands, as described for example in Innis et al., PCR Protocols: A Guide to Methods and Applications, Academic Press (1990). Devices have been developed that can perform thermal cycling reactions with compositions containing fluorescent indicators which are able to emit a light beam of a specified wavelength, read the intensity of the fluorescent dye, and display the intensity of fluorescence after each cycle. Devices comprising a thermal cycler, light beam emitter, and a fluorescent signal detector, have been described, e.g., in U.S. Patent Nos. 5,928,907; 6,015,674; 6,174,670; and 6,814,934 and include, but are not limited to, the ABI Prism® 7700 Sequence Detection System (Applied Biosystems, Foster City, California), the ABI GeneAmp® 5700 Sequence Detection System (Applied Biosystems, Foster City, California), the ABI GeneAmp® 7300 Sequence Detection System (Applied Biosystems, Foster City, California), the ABI GeneAmp® 7500 Sequence Detection System (Applied Biosystems, Foster City, California), the StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, California) and the ABI GeneAmp® 7900 Sequence Detection System (Applied Biosystems, Foster City, California).

[0083] As used herein, the term "Tm"' or "melting temperature" of an oligonucleotide refers to the temperature (in degrees Celsius) at which 50% of the molecules in a population of a single-stranded oligonucleotide are hybridized to their complementary sequence and 50% of the

molecules in the population are not-hybridized to said complementary sequence. The Tm of a primer or probe can be determined empirically by means of a melting curve. In some cases it can also be calculated using formulas well know in the art (See, e.g., Maniatis, T., et al., Molecular cloning: a laboratory manual / Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.: 1982).

[0084] As used herein, the term "sensitivity" refers to the minimum amount (number of copies or mass) of a template that can be detected by a given assay. As used herein, the term "specificity" refers to the ability of an assay to distinguish between amplification from a matched template versus a mismatched template. Frequently, specificity is expressed as $\Delta Ct = Ct_{mismatch} - Ct_{match}$. An improvement in specificity or "specificity improvement" or "fold difference" is expressed herein as $2^{(\Delta Ct_condition1 - (\Delta Ct_condition2)}$. The term "selectivity" refers to the extent to which an AS-PCR assay can be used to determine minor (often mutant) alleles in mixtures without interferences from major (often wild type) alleles. Selectivity is often expressed as a ratio or percentage. For example, an assay that can detect 1 mutant template in the presence of 100 wild type templates is said to have a selectivity of 1:100 or 1%. As used herein, assay selectivity can also be calculated as $1/2^{\Delta Ct}$ or as a percentage using $(1/2^{\Delta Ct} \times 100)$.

[0085] As used herein, the term "C_t" or "C_t value" refers to threshold cycle and signifies the cycle of a PCR amplification assay in which signal from a reporter that is indicative of amplicon generation (e.g., fluorescence) first becomes detectable above a background level. In some embodiments, the threshold cycle or "C_t" is the cycle number at which PCR amplification becomes exponential.

[0086] As used herein, the term "delta C_t" or "ΔC_t" refers to the difference in the numerical cycle number at which the signal passes the fixed threshold between two different samples or

reactions. In some embodiments delta C_t is the difference in numerical cycle number at which exponential amplification is reached between two different samples or reactions. The delta C_t can be used to identify the specificity between a matched primer to the corresponding target nucleic acid sequence and a mismatched primer to the same corresponding target nucleic acid sequence.

[0087] In some embodiments, the calculation of the delta C_t value between a mismatched primer and a matched primer is used as one measure of the discriminating power of allele-specific PCR. In general, any factor which increases the difference between the C_t value for an amplification reaction using a primer that is matched to a target sequence (e.g., a sequence comprising an allelic variant of interest) and that of a mismatched primer will result in greater allele discrimination power.

[0088] According to various embodiments, a Ct value may be determined using a derivative of a PCR curve. For example, a first, second, or nth order derivative method may be performed on a PCR curve in order to determine a Ct value. In various embodiments, a characteristic of a derivative may be used in the determination of a Ct value. Such characteristics may include, but are not limited by, a positive inflection of a second derivative, a negative inflection of a second derivative, a zero crossing of the second derivative, or a positive inflection of a first derivative. In various embodiments, a Ct value may be determined using a thresholding and baselining method. For example, an upper bound to an exponential phase of a PCR curve may be established using a derivative method, while a baseline for a PCR curve may be determined to establish a lower bound to an exponential phase of a PCR curve. From the upper and lower bound of a PCR curve, a threshold value may be established from which a Ct value is determined. Other methods for the determination of a Ct value known in the art, for example, but

not limited by, various embodiments of a fit point method, and various embodiments of a sigmoidal method (See, e.g., U.S. Patent Nos. 6,303,305; 6,503,720; 6,783,934, 7,228,237 and U.S. Application No. 2004/0096819; the disclosures of which are herein incorporated by reference in their entireties).

[0089] III. Compositions, methods and kits

[0090] In one aspect, the present invention provides compositions for use in identifying and/or quantitating an allelic variant in a nucleic acid sample. Some of these compositions can comprise: (a) an allele-specific primer; (b) an allele-specific blocker probe; (c) a detector probe; and/or (d) a locus-specific primer. In some embodiments of the compositions, the compositions may further comprise a polymerase, dNTPs, reagents and/or buffers suitable for PCR amplification, and/or a template sequence or nucleic acid sample. In some embodiments, the polymerase can be thermostable.

[0091] In another aspect, the invention provides compositions comprising: (i) a first allele-specific primer, wherein an allele-specific nucleotide portion of the first allele-specific primer is complementary to the first allelic variant of a target sequence; and (ii) a first allele-specific blocker probe that is complementary to a region of the target sequence comprising the second allelic variant, wherein said region encompasses a position corresponding to the binding position of the allele-specific nucleotide portion of the first allele-specific primer, and wherein the first allele-specific blocker probe comprises a minor groove binder.

[0092] In some illustrative embodiments, the compositions can further include a locus-specific primer that is complementary to a region of the target sequence that is 3' from the first allelic variant and on the opposite strand.

[0093] In further embodiments, the compositions can further include a detector probe.

[0094] In another aspect, the present invention provides methods for amplifying an allele-specific sequence. Some of these methods can include: (a) hybridizing an allele-specific primer to a first nucleic acid molecule comprising a target allele; (b) hybridizing an allele-specific blocker probe to a second nucleic acid molecule comprising an alternative allele wherein the alternative allele corresponds to the same loci as the target allele; (c) hybridizing a locus-specific detector probe to the first nucleic acid molecule; (d) hybridizing a locus-specific primer to the extension product of the allele-specific primer and (e) PCR amplifying the target allele.

[0095] In another aspect, the present invention provides methods for detecting and/or quantitating an allelic variant in a mixed sample. Some of these methods can involve: (a) in a first reaction mixture hybridizing a first allele-specific primer to a fist nucleic acid molecule comprising a first allele (allele-1) and in a second reaction mixture hybridizing a second allele-specific primer to a first nucleic acid molecule comprising a second allele (allele-2), wherein the allele-2 corresponds to the same loci as allele-1; (b) in the first reaction mixture hybridizing a first allele-specific blocker probe to a second nucleic acid molecule comprising allele-2 and in the second reaction mixture hybridizing a second allele-specific blocker probe to a second nucleic acid molecule comprising allele-1; (c) in the first reaction mixture, hybridizing a first detector probe to the first nucleic acid molecule and in the second reaction mixture, hybridizing a second detector probe to the first nucleic acid molecule; (d) in the first reaction mixture hybridizing a first locus-specific primer to the extension product of the first allele-specific primer and in the second reaction mixture hybridizing a second locus-specific primer to the extension product of the second allele-specific primer; and (e) PCR amplifying the first nucleic acid molecule to form a first set or sample of amplicons and PCR amplifying the second nucleic acid

molecule to form a second set or sample of amplicons; and (f) comparing the first set of amplicons to the second set of amplicons to quantitate allele-1 in the sample comprising allele-2 and/or allele-2 in the sample comprising allele-1.

[0096] In yet another aspect, the present invention provides methods for detecting and/or quantitating allelic variants. Some of these methods can comprise: (a) PCR amplifying a first allelic variant in a first reaction comprising (i) a low-concentration first allele-specific primer, (ii) a first locus-specific primer, and (iii) a first blocker probe to form first amplicons; (b) PCR amplifying a second allelic variant in a second reaction comprising (i) a low-concentration second allele-specific primer, (ii) a second locus-specific primer, and (iii) a second blocker probe to form second amplicons; and (d) comparing the first amplicons to the second amplicons to quantitate the first allelic variant in the sample comprising second allelic variants.

[0097] In yet another aspect, the present invention provides methods for detecting a first allelic variant of a target sequence in a nucleic acid sample suspected of comprising at least a second allelic variant of the target sequence. Methods of this aspect include forming a first reaction mixture by combining the following: (i) a nucleic acid sample; (ii) a first allele-specific primer, wherein an allele-specific nucleotide portion of the first allele-specific primer is complementary to the first allelic variant of the target sequence; (iii) a first allele-specific blocker probe that is complementary to a region of the target sequence comprising the second allelic variant, wherein said region encompasses a position corresponding to the binding position of the allele-specific nucleotide portion of the first allele-specific primer, and wherein the first allele-specific blocker probe comprises a minor groove binder; (iv) a first locus-specific primer that is complementary to a region of the target sequence that is 3' from the first allelic variant and on the opposite strand; and (v) a first detector probe.

[0098] Next an amplification reaction, typically a PCR amplification reaction, is carried out on the first reaction mixture using the first locus-specific primer and the first allele-specific primer to form a first amplicon. Then, the first amplicon is detected by a change in a detectable property of the first detector probe upon binding to the amplicon, thereby detecting the first allelic variant of the target gene in the nucleic acid sample. The detector probe in some illustrative embodiments is a 5' nuclease probe. The detectable property in certain illustrative embodiments is fluorescence.

[0099] In some embodiments, the 3' nucleotide position of the 5' target region of the first allele-specific primer is an allele-specific nucleotide position. In certain other illustrative embodiments, including those embodiments where the 3' nucleotide position of the 5' target region of the first allele-specific primer is an allele-specific nucleotide position, the blocking region of the allele-specific primer encompasses the allele-specific nucleotide position. Furthermore, in illustrative embodiments, the first allele-specific blocker probe includes a minor groove binder. Furthermore, the allele-specific blocker probe in certain illustrative embodiments does not have a label, for example a fluorescent label, or a quencher.

[00100] In certain illustrative embodiments, the quantity of the first allelic variant is determined by evaluating the change in a detectable property of the first detector probe.

[00101] In certain illustrative embodiments, the method further includes forming a second reaction mixture by combining (i) the nucleic acid sample; (ii) a second allele-specific primer, wherein an allele-specific nucleotide portion of the second allele-specific primer is complementary to the second allelic variant of the target sequence; (iii) a second allele-specific blocker probe that is complementary to a region of the target sequence comprising the first allelic variant, wherein said region encompasses a position corresponding to the binding position

of the allele-specific nucleotide portion of the second allele-specific primer, and wherein the second allele-specific blocker probe comprises a minor groove binder; (iv) a second locus-specific primer that is complementary to a region of the target sequence that is 3' from the second allelic variant and on the opposite strand; and (v) a second detector probe. Next, an amplification reaction is carried out on the second reaction mixture using the second allele-specific primer and the locus-specific primer, to form a second amplicon. Then the second amplicon is detected by a change in a detectable property of the detector probe.

[00102] In certain embodiments, the method further includes comparing the change in a detectable property of the first detector probe in the first reaction mixture to the change in a detectable property of the second detector probe in the second reaction mixture.

[00103] In yet another aspect, the present invention provides a reaction mixture that includes the following (i) nucleic acid molecule; (ii) an allele-specific primer, wherein an allele-specific nucleotide portion of the allele-specific primer is complementary to a first allelic variant of a target sequence; (iii) an allele-specific blocker probe that is complementary to a region of the target sequence comprising a second allelic variant, wherein said region encompasses a position corresponding to the binding position of the allele-specific nucleotide portion of the allele-specific primer, and wherein the allele-specific blocker probe comprises a minor groove binder; (iv) a locus-specific primer that is complementary to a region of the target sequence that is 3' from the first allelic variant and on the opposite strand; and (v) a detector probe.

[00104] In certain embodiments, the methods of the invention are used to detect a first allelic variant that is present at a frequency less than 1/10, 1/100, 1/1,000, 1/10,000, 1/100,000, 1/1,000,000, 1/10,000,000, 1/100,000,000 or 1/1,000,000,000, and any fractional ranges in between, of a second allelic variant for a given SNP or gene. In other embodiments, the methods

are used to detect a first allelic variant that is present in less than 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 50, 75, 100, 250, 500, 750, 1,000, 2,500, 5,000, 7,500, 10,000, 25,000, 50,000, 75,000, 100,000, 250,000, 500,000, 750,000, 1,000,000 copies per 1, 10, 100, 1,000 micro liters, and any fractional ranges in between, of a sample or a reaction volume.

[00105] In some embodiments the first allelic variant is a mutant. In some embodiments the second allelic variant is wild type. In some embodiments, the present methods can involve detecting one mutant molecule in a background of at least 1,000 to 1,000,000, such as about 1000 to 10,000, about 10,000 to 100,000, or about 100,000 to 1,000,000 wild type molecules, or any fractional ranges in between. In some embodiments, the methods can provide high sensitivity and the efficiency at least comparable to that of TaqMan®-based assays.

[00106] In some embodiments, the comparison of the first amplicons and the second amplicons involving the disclosed methods provides improvements in specificity from 1,000X to 1,000,000X fold difference, such as about 1000 to 10,000X, about 10,000 to 100,000X, or about 100,000 to 1,000,000X fold difference, or any fractional ranges in between. In some embodiments, the size of the amplicons range from about 60-120 nucleotides long.

[00107] In another aspect, the present invention provides kits for quantitating a first allelic variant in a sample comprising an alternative second allelic variants that include: (a) a first allele-specific primer; (b) a second allele-specific primer; (c), a first locus-specific primer; (d) a second locus-specific primer; (e) a first allele-specific blocker probe; (f) a second allele-specific blocker probe; and (g) a polymerase. In some embodiments of the disclosed kits, the kit further comprises a first locus-specific detector probe and a second locus-specific detector probe.

[00108] In another aspect, the present invention provides kits that include two or more containers comprising the following components independently distributed in one of the two or

more containers: (i) a first allele-specific primer, wherein an allele-specific nucleotide portion of the first allele-specific primer is complementary to the first allelic variant of a target sequence; and (ii) a first allele-specific blocker probe that is complementary to a region of the target sequence comprising the second allelic variant, wherein said region encompasses a position corresponding to the binding position of the allele-specific nucleotide portion of the first allele-specific primer, and wherein the first allele-specific blocker probe comprises a minor groove binder.

[00109] In some illustrative embodiments, the kits can further include a locus-specific primer that is complementary to a region of the target sequence that is 3' from the first allelic variant and on the opposite strand.

[00110] In other embodiments, the kits can further include a detector probe.

[00111] In some embodiments, the compositions, methods, and/or kits can be used in detecting circulating cells in diagnosis. In one embodiment, the compositions, methods, and/or kits can be used to detect tumor cells in blood for early cancer diagnosis. In some embodiments, the compositions, methods, and/or kits can be used for cancer or disease-associated genetic variation or somatic mutation detection and validation. In some embodiments, the compositions, methods, and/or kits can be used for genotyping tera-, tri-and di-allelic SNPs. In some embodiments, the compositions, methods, and/or kits can be used for DNA typing from mixed DNA samples for QC and human identification assays, cell line QC for cell contaminations, allelic gene expression analysis, virus typing/rare pathogen detection, mutation detection from pooled samples, detection of circulating tumor cells in blood, and/or prenatal diagnostics.

[00112] In some embodiments, the compositions, methods, and/or kits are compatible with various instruments such as, for example, SDS instruments from Applied Biosystems (Foster City, CA).

[00113] Allele-specific primers

[00114] Allele-specific primers (ASPs) designed with low Tms exhibit increased discrimination of allelic variants. In some embodiments, the allele-specific primers are short oligomers ranging from about 15-30, such as about 16-28, about 17-26, about 18-24, or about 20-22, or any range in between, nucleotides in length. In some embodiments, the Tm of the allele-specific primers range from about 50°C to 70°C, such as about 52°C to 68°C (e.g., 53°C), about 54°C to 66°C, about 56°C to 64°C, about 58°C to 62°C, or any range in between. In other embodiments, the Tm of the allele-specific primers is about 3°C to 6°C higher than the anneal/extend temperature of the PCR cycling conditions employed during amplification.

[00115] Low allele-specific primer concentration can also improve selectivity. Reduction in concentration of allele-specific primers below 900 nM can increase the delta Ct between matched and mismatched sequences. In some embodiments of the disclosed compositions, the concentration of allele-specific primers ranges from about 20 nM to 900 nM, such as about 50 nM to 700 nM, about 100 nM to 500 nM, about 200 nM to 300 nM, about 400 nM to 500 nM, or any range in between. In some exemplary embodiments, the concentration of the allele-specific primers is between about 200 nM to 400 nM.

[00116] In some embodiments, allele-specific primers can comprise an allele-specific nucleotide portion that is specific to the target allele of interest. The allele-specific nucleotide portion of an allele-specific primer is complementary to one allele of a gene, but not another allele of the gene. In other words, the allele-specific nucleotide portion binds to one or more

variable nucleotide positions of a gene that is nucleotide positions that are known to include different nucleotides for different allelic variants of a gene. The allele-specific nucleotide portion is at least one nucleotide in length. In exemplary embodiments, the allele-specific nucleotide portion is one nucleotide in length. In some embodiments, the allele-specific nucleotide portion of an allele-specific primer is located at the 3' terminus of the allele-specific primer. In other embodiments, the allele-specific nucleotide portion is located about 1-2, 3-4, 5-6, 7-8, 9-11, 12-15, or 16-20 nucleotides in from the 3'most-end of the allele-specific primer.

[00117] Allele-specific primers designed to target discriminating bases can also improve discrimination of allelic variants. In some embodiments, the nucleotide of the allele-specific nucleotide portion targets a highly discriminating base (e.g., for detection of A/A, A/G, G/A, G/G, A/C, or C/A alleles). Less discriminating bases, for example, may involve detection of C/C, T/C, G/T, T/G, C/T alleles. In some embodiments, for example when the allele to be detected involves A/G or C/T SNPs, A or G may be used as the 3' allele-specific nucleotide portion of the allele-specific primer (e.g., if A/T is the target allele), or C or T may be used as the 3' allele-specific nucleotide portion of the allele-specific primer (e.g., if C/G is the target allele). In other embodiments, A may be used as the nucleotide-specific portion at the 3' end of the allele specific primer (e.g., the allele-specific nucleotide portion) when detecting and/or quantifying A/T SNPs. In other embodiments, G may be used as the nucleotide-specific portion at the 3' end of the allele specific primer when detecting and/or quantifying C/G SNPs.

[00118] In some embodiments, the allele-specific primer can comprise a target-specific portion that is specific to the polynucleotide sequence (or locus) of interest. In some embodiments the target-specific portion is about 75-85%, 85-95%, 95-99% or 100% complementary to the target polynucleotide sequence of interest. In some embodiments, the

target-specific portion of the allele-specific primer can comprise the allele-specific nucleotide portion. In other embodiments, the target-specific portion is located 5' to the allele-specific nucleotide portion. The target-specific portion can be about 4-30, about 5-25, about 6-20, about 7-15, or about 8-10 nucleotides in length. In some embodiments, the Tm of the target specific portion is about 5°C below the anneal/extend temperature used for PCR cycling. In some embodiments, the Tm of the target specific portion of the allele-specific primer ranges from about 51°C to 60°C, about 52°C to 59°C, about 53°C to 58°C, about 54°C to 57°C, about 55°C to 56°C, or about 50°C to about 60°C.

[00119] In some embodiments of the disclosed methods and kits, the target-specific portion of the first allele-specific primer and the target-specific portion of the second allele-specific primer comprise the same sequence. In other embodiments, the target-specific portion of the first allele-specific primer and the target-specific portion of the second allele-specific primer are the same sequence.

[00120] In some embodiments, the allele-specific primer comprises a tail. Allele-specific primers comprising tails, enable the overall length of the primer to be reduced, thereby lowering the Tm without significant impact on assay sensitivity.

[00121] In some exemplary embodiments, the tail is on the 5' terminus of the allele-specific primer. In some embodiments, the tail is located 5' of the target-specific portion and/or allele-specific nucleotide portion of the allele-specific primer. In some embodiments, the tail is about 65-75%, about 75-85%, about 85-95%, about 95-99% or about 100% non-complementary to the target polynucleotide sequence of interest. In some embodiments the tail can be about 2-40, such as about 4-30, about 5-25, about 6-20, about 7-15, or about 8-10 nucleotides in length. In some embodiments the tail is GC-rich. For example, in some embodiments the tail sequence is

comprised of about 50-100%, about 60-100%, about 70-100%, about 80-100%, about 90-100% or about 95-100% G and/or C nucleotides.

[00122] The tail of the allele-specific primer may be configured in a number of different ways, including, but not limited to a configuration whereby the tail region is available after primer extension to hybridize to a complementary sequence (if present) in a primer extension product. Thus, for example, the tail of the allele-specific primer can hybridize to the complementary sequence in an extension product resulting from extension of a locus-specific primer.

[00123] In some embodiments of the disclosed methods and kits, the tail of the first allele-specific primer and the tail of the second allele-specific primer comprise the same sequence. In other embodiments, the 5' tail of the first allele-specific primer and the 5' tail of the second allele-specific primer are the same sequence.

[00124] Allele-specific blocker probes

[00125] Allele-specific blocker probes (or ASBs) (herein sometimes referred to as "blocker probes") may be designed as short oligomers that are single-stranded and have a length of 100 nucleotides or less, more preferably 50 nucleotides or less, still more preferably 30 nucleotides or less and most preferably 20 nucleotides or less with a lower limit being approximately 5 nucleotides.

[00126] In some embodiments, the Tm of the blocker probes range from 60°C to 70°C, 61°C to 69°C, 62°C to 68°C, 63°C to 67°C, 64°C to 66°C, or about 60°C to about 63°C, or any range in between. In yet other embodiments, the Tm of the allele-specific blocker probes is about 3°C to 6°C higher than the anneal/extend temperature in the PCR cycling conditions employed during amplification.

[00127] In some embodiments, the blocker probes are not cleaved during PCR amplification. In some embodiments, the blocker probes comprise a non-extendable blocker moiety at their 3'-ends. In some embodiments, the blocker probes can further comprise other moieties (including, but not limited to additional non-extendable blocker moieties, quencher moieties, fluorescent moieties, etc) at their 3'-end, 5'-end, and/or any internal position in between. In some embodiments, the allele position is located about 5-15, such as about 5-11, about 6-10, about 7-9, about 7-12, or about 9-11, such as about 6, about 7, about 8, about 9, about 10, or about 11 nucleotides away from the non-extendable blocker moiety of the allele-specific blocker probes when hybridized to their target sequences. In some embodiments, the non-extendable blocker moiety can be, but is not limited to, an amine (NH_2), biotin, PEG, DPI_3, or PO_4. In some preferred embodiments, the blocker moiety is a minor groove binder (MGB) moiety. (The oligonucleotide-MGB conjugates of the present invention are hereinafter sometimes referred to as "MGB blocker probes" or "MGB blockers.")

[00128] As disclosed herein, the use of MGB moieties in allele-specific blocker probes can increase the specificity of allele-specific PCR. One possibility for this effect is that, due to their strong affinity to hybridize and strongly bind to complementary sequences of single or double stranded nucleic acids, MGBs can lower the Tm of linked oligonucleotides (See, for example, Kutyavin, I., et al., Nucleic Acids Res., 2000, Vol. 28, No. 2: 655-661). Oligonucleotides comprising MGB moieties have strict geometric requirements since the linker between the oligonucleotide and the MGB moiety must be flexible enough to allow positioning of the MGB in the minor groove after DNA duplex formation. Thus, MGB blocker probes can provide larger Tm differences between matched versus mismatched alleles as compared to conventional DNA blocker probes.

[00129] In general, MGB moieties are molecules that binds within the minor groove of double stranded DNA. Although a generic chemical formula for all known MGB compounds cannot be provided because such compounds have widely varying chemical structures, compounds which are capable of binding in the minor groove of DNA, generally speaking, have a crescent shape three dimensional structure. Most MGB moieties have a strong preference for A-T (adenine and thymine) rich regions of the B form of double stranded DNA. Nevertheless, MGB compounds which would show preference to C-G (cytosine and guanine) rich regions are also theoretically possible. Therefore, oligonucleotides comprising a radical or moiety derived from minor groove binder molecules having preference for C-G regions are also within the scope of the present invention.

[00130] Some MGBs are capable of binding within the minor groove of double stranded DNA with an association constant of 10^3M^{-1} or greater. This type of binding can be detected by well established spectrophotometric methods such as ultraviolet (UV) and nuclear magnetic resonance (NMR) spectroscopy and also by gel electrophoresis. Shifts in UV spectra upon binding of a minor groove binder molecule and NMR spectroscopy utilizing the "Nuclear Overhauser" (NOSEY) effect are particularly well known and useful techniques for this purpose. Gel electrophoresis detects binding of an MGB to double stranded DNA or fragment thereof, because upon such binding the mobility of the double stranded DNA changes.

[00131] A variety of suitable minor groove binders have been described in the literature. See, for example, Kutyavin, et al. U.S. Patent No. 5,801,155; Wemmer, D. E., and Dervan P. B., Current Opinion in Structural Biology, 7:355-361 (1997); Walker, W. L., Kopka, J. L. and Goodsell, D. S., Biopolymers, 44:323-334 (1997); Zimmer, C.& Wahnert, U. Prog. Biophys. Molec. Bio. 47:31-112 (1986) and Reddy, B. S. P., Dondhi, S. M., and Lown, J. W., Pharmacol.

Therap., 84:1-111 (1999). In one group of embodiments, the MGB is selected from the group consisting of CC1065 analogs, lexitropsins, distamycin, netropsin, berenil, duocarmycin, pentamidine, 4, 6-diamino-2-phenylindole and pyrrolo[2,1-c][1,4]benzodiazepines. A preferred MGB in accordance with the present disclosure is DPI_3 (see U.S. Patent No. 6,727,356, the disclosure of which is incorporated herein by reference in its entirety).

[00132] Suitable methods for attaching MGBs through linkers to oligonucleotides or probes and have been described in, for example, U.S. Patent Nos. 5,512,677; 5,419,966; 5,696,251; 5,585,481; 5,942,610; 5,736,626; 5,801,155 and 6,727,356. (The disclosures of each of which are incorporated herein by reference in their entireties.) For example, MGB-oligonucleotide conjugates can be synthesized using automated oligonucleotide synthesis methods from solid supports having cleavable linkers. In other examples, MGB probes can be prepared from an MGB modified solid support substantially in accordance with the procedure of Lukhtanov et al. Bioconjugate Chern., 7: 564-567 (1996). (The disclosure of which is also incorporated herein by reference in its entirety.) According to these methods, one or more MGB moieties can be attached at the 5'-end, the 3'-end and/or at any internal portion of the oligonucleotide.

[00133] The location of an MGB moiety within an MGB-oligonucleotide conjugate can affect the discriminatory properties of such a conjugate. An unpaired region within a duplex will likely result in changes in the shape of the minor groove in the vicinity of the mismatched base(s). Since MGBs fit best within the minor groove of a perfectly-matched DNA duplex, mismatches resulting in shape changes in the minor groove would reduce binding strength of an MGB to a region containing a mismatch. Hence, the ability of an MGB to stabilize such a hybrid would be decreased, thereby increasing the ability of an MGB-oligonucleotide conjugate to discriminate a mismatch from a perfectly-matched duplex. On the other hand, if a mismatch lies outside of the

region complementary to an MGB-oligonucleotide conjugate, discriminatory ability for unconjugated and MGB-conjugated oligonucleotides of equal length is expected to be approximately the same. Since the ability of an oligonucleotide probe to discriminate single base pair mismatches depends on its length, shorter oligonucleotides are more effective in discriminating mismatches. The first advantage of the use of MGB-oligonucleotides conjugates in this context lies in the fact that much shorter oligonucleotides compared to those used in the art (i.e., 20-mers or shorter), having greater discriminatory powers, can be used, due to the pronounced stabilizing effect of MGB conjugation. Consequently, larger delta Tms of allele-specific blocker probes can improve AS-PCR assay specificity and selectivity.

[00134] Blocker probes having MGB at the 5' termini may have additional advantages over other blocker probes having a blocker moiety (e.g., MGB, PO_4, NH_2, PEG, or biotin) only at the 3' terminus. This is at least because blocker probes having MGB at the 5' terminus (in addition to a blocking moiety at the 3'-end that prevents extension) will not be cleaved during PCR amplification. Thus, the probe concentration can be maintained at a constant level throughout PCR, which may help maintain the effectiveness of blocking non-specific priming, thereby increasing cast-PCR assay specificity and selectivity (Fig. 3).

[00135] In some embodiments, the allele-specific blocker probe can comprise one or more modified bases in addition to the naturally occurring bases adenine, cytosine, guanine, thymine and uracil. In some embodiments, the modified base(s) may increase the difference in the Tm between matched and mismatched target sequences and/or decrease mismatch priming efficiency, thereby improving not only assay specificity, bust also selectivity (Fig. 4A).

[00136] Modified bases are considered to be those that differ from the naturally-occurring bases by addition or deletion of one or more functional groups, differences in the heterocyclic

ring structure (i.e., substitution of carbon for a heteroatom, or vice versa), and/or attachment of one or more linker arm structures to the base. Such modified base(s) may include, for example, 8-Aza-7-deaza-dA (ppA), 8-Aza-7-deaza-dG (ppG), locked nucleic acid (LNA) or 2′-*O*,4′-*C*-ethylene nucleic acid (ENA) bases (Fig. 4B). Other examples of modified bases include, but are not limited to, the general class of base analogues 7-deazapurines and their derivatives and pyrazolopyrimidines and their derivatives (described in PCT WO 90/14353; and U.S. Application No. 09/054,630, the disclosures of each of which are incorporated herein by reference in their entireties). These base analogues, when present in an oligonucleotide, strengthen hybridization and improve mismatch discrimination. All tautomeric forms of naturally occurring bases, modified bases and base analogues may be included in the oligonucleotide primer and probes of the invention.

[00137] Similarly, modified sugars or sugar analogues can be present in one or more of the nucleotide subunits of an oligonucleotide conjugate in accordance with the invention. Sugar modifications include, but are not limited to, attachment of substituents to the 2', 3' and/or 4' carbon atom of the sugar, different epimeric forms of the sugar, differences in the α- or β-configuration of the glycosidic bond, and other anomeric changes. Sugar moieties include, but are not limited to, pentose, deoxypentose, hexose, deoxyhexose, ribose, deoxyribose, glucose, arabinose, pentofuranose, xylose, lyxose, and cyclopentyl.

[00138] Modified internucleotide linkages can also be present in oligonucleotide conjugates of the invention. Such modified linkages include, but are not limited to, peptide, phosphate, phosphodiester, phosphotriester, alkylphosphate, alkanephosphonate, thiophosphate, phosphorothioate, phosphorodithioate, methylphosphonate, phosphoramidate, substituted phosphoramidate and the like. Several further modifications of bases, sugars and/or

internucleotide linkages, that are compatible with their use in oligonucleotides serving as probes and/or primers, will be apparent to those of skill in the art.

[00139] In addition, in some embodiments, the nucleotide units which are incorporated into the oligonucleotides of the MGB blocker probes of the present invention may have a cross-linking function (an alkylating agent) covalently bound to one or more of the bases, through a linking arm.

[00140] The "sugar" or glycoside portion of some embodiments of the MGB blocker probes of the present invention may comprise deoxyribose, ribose, 2-fiuororibose, 2-0 alkyl or alkenylribose where the alkyl group may have 1 to 6 carbons and the alkenyl group 2 to 6 carbons. In the naturally occurring nucleotides and in the herein described modifications and analogs the deoxyribose or ribose moiety forms a furanose ring. the glycosydic linkage is of the ~ configuration and the purine bases are attached to the sugar moiety via the 9-position. the pyrimidines via the I-position and the pyrazolopyrimidines via the I-position. The nucleotide units of the oligonucleotide are interconnected by a "phosphate" backbone, as is well known in the art. The oligonucleotide of the oligonucleotide-MGB conjugates (MGB blocker probes) of the present invention may include, in addition to the "natural" phosphodiester linkages, phosphorothiotes and methylphosphonates.

[00141] In some embodiments of the methods and kits, the first allele-specific blocker probe binds to the same strand or sequence as the first allele-specific primer, while the second allele-specific blocker probe binds to the opposite strand and/or complementary sequence as the first allele-specific primer.

[00142] Detector probes

[00143] In some embodiments, detector probe is designed as short oligomers ranging from about 15-30 nucleotides, such as about 16, about 18, about 22, about 24, about 30, or any number in between. In some embodiments, the Tm of the detector probe ranges from about 60°C to 70°C, about 61°C to 69°C, about 62°C to 68°C, about 63°C to 67°C, or about 64°C to 66°C, or any range in between.

[00144] In some embodiments, the detector probe is a locus-specific detector probes (LST). In other embodiments the detector probe is a 5' nuclease probe. In some exemplary embodiments, the detector probe can comprises an MGB moiety, a reporter moiety (e.g., FAM™, TET™, JOE™, VIC™, or SYBR® Green), a quencher moiety (e.g., Black Hole Quencher™ or TAMRA™;), and/or a passive reference (e.g., ROX™). In some exemplary embodiments, the detector probe is designed according to the methods and principles described in U.S. Patent No. 6,727,356 (the disclosure of which is incorporated herein by reference in its entirety). In some exemplary embodiments, the detector probe is a TaqMan® probe (Applied Biosystems, Foster City). In exemplary embodiments, the locus-specific detector probe can be designed according to the principles and methods described in U.S. Patent No. 6,727,356 (the disclosure of which is incorporated herein by reference in its entirety). For example, fluorogenic probes can be prepared with a quencher at the 3' terminus of a single DNA strand and a fluorophore at the 5' terminus. In such an example, the 5'-nuclease activity of a Taq DNA polymerase can cleave the DNA strand, thereby separating the fluorophore from the quencher and releasing the fluorescent signal. In some embodiments, the detector probes are hybridized to the template strands during primer extension step of PCR amplification (e.g., at 60-65°C.). In yet other embodiments, an MGB is covalently attached to the quencher moiety of the locus-specific detector probes (e.g., through a linker).

[00145] In some embodiments of the disclosed methods and kits, the first and second detector probes are the same and/or comprise the same sequence or are the same sequence.

[00146] Locus-specific primers

[00147] In some embodiments, locus-specific primer (LSP) is designed as a short oligomer ranging from about 15-30 nucleotides, such as about 16, about 18, about 22, about 24, about 30, or any number in between. In some embodiments, the Tm of the locus-specific primer ranges from about 60°C to 70°C, about 61°C to 69°C, about 62°C to 68°C, about 63°C to 67°C, or about 64°C to 66°C, or any range in between.

[00148] In some other embodiments of the disclosed methods and kits, the first locus-specific detector probe and/or second locus-specific detector probes comprise the same sequence or are the same sequence.

[00149] Additional components

[00150] Polymerase enzymes suitable for the practice of the present invention are well known in the art and can be derived from a number of sources. Thermostable polymerases may be obtained, for example, from a variety of thermophilic bacteria that are commercially available (for example, from American Type Culture Collection, Rockville, Md.) using methods that are well-known to one of ordinary skill in the art (See, e.g., U.S. Patent No. 6,245,533). Bacterial cells may be grown according to standard microbiological techniques, using culture media and incubation conditions suitable for growing active cultures of the particular species that are well-known to one of ordinary skill in the art (See, e.g., Brock, T. D., and Freeze, H., J. Bacteriol. 98(1):289-297 (1969); Oshima, T., and Imahori, K, Int. J. Syst. Bacteriol. 24(1):102-112 (1974)). Suitable for use as sources of thermostable polymerases are the thermophilic bacteria Thermus aquaticus, Thermus thermophilus, Thermococcus litoralis, Pyrococcus furiosus, Pyrococcus

woosii and other species of the Pyrococcus genus, Bacillus stearothermophilus, Sulfolobus acidocaldarius, Thermoplasma acidophilum, Thermus flavus, Thermus ruber, Thermus brockianus, Thermotoga neapolitana, Thermotoga maritima and other species of the Thermotoga genus, and Methanobacterium thermoautotrophicum, and mutants of each of these species. Preferable thermostable polymerases can include, but are not limited to, Taq DNA polymerase, Tne DNA polymerase, Tma DNA polymerase, or mutants, derivatives or fragments thereof.

[00151] Various sources and/or preparation methods of nucleic acids

[00152] Sources of nucleic acid samples in the disclosed compositions, methods and/or kits include, but are not limited to, human cells such as circulating blood, buccal epithelial cells, cultured cells and tumor cells. Also other mammalian tissue, blood and cultured cells are suitable sources of template nucleic acids. In addition, viruses, bacteriophage, bacteria, fungi and other micro-organisms can be the source of nucleic acid for analysis. The DNA may be genomic or it may be cloned in plasmids, bacteriophage, bacterial artificial chromosomes (BACs), yeast artificial chromosomes (YACs) or other vectors. RNA may be isolated directly from the relevant cells or it may be produced by in vitro priming from a suitable RNA promoter or by in vitro transcription. The present invention may be used for the detection of variation in genomic DNA whether human, animal or other. It finds particular use in the analysis of inherited or acquired diseases or disorders. A particular use is in the detection of inherited diseases.

[00153] In some embodiments, template sequence or nucleic acid sample can be gDNA. In other embodiments, the template sequence or nucleic acid sample can be cDNA. In yet other embodiments, as in the case of simultaneous analysis of gene expression by RT-PCR, the template sequence or nucleic acid sample can be RNA. The DNA or RNA template sequence or

nucleic acid sample can be extracted from any type of tissue including, for example, formalin-fixed paraffin-embedded tumor specimens.

[00154] The following examples are intended to illustrate but not limit the invention.

EXAMPLES

[00155] I. General Assay Design:

[00156] The general schema for the cast-PCR assays used in the following examples is illustrated in Fig. 1. For each SNP that was analyzed, allele-specific primers were designed to target a first allele (i.e. allele-1) and a second allele (i.e. allele-2). The cast-PCR assay reaction mixture for allele-1 analysis included a tailed allele-1-specific primer (ASP1), one MGB allele-2 blocker probe (MGB2), one common locus-specific TaqMan probe (LST) and one common locus-specific primer (LSP). The cast-PCR assay reaction mixture for analysis of allele-2 included a tailed allele-2-specific primer (ASP2), one MGB allele-1 blocker probe (MGB1), one common locus-specific TaqMan probe (LST) and one common locus-specific primer (LSP).

[00157] II. Reaction conditions:

[00158] Each assay reaction mixture (10 µl total) contained 1X TaqMan Genotyping Master Mixture (Applied Biosystems, Foster City, CA; P/N 437135), 0.5 ng/µL genomic DNA or 1 million copies of plasmid DNA (as indicated), 300 nM (unless specified otherwise) tailed-, or in some cases untailed-, allele-specific primer (ASP1 for detection of allele-1 or ASP2 for detection of allele-2), 200 nM TaqMan probe (LST), 900 nM locus-specific primer (LSP), 150 nM allele-specific MGB blocker probe (MGB1 for detection of allele-2 or MGB2 for detection of allele-1). The reactions were incubated in a 384-well plate at 95°C for 10 minutes, then for 5 cycles at 95°C for 15 seconds each, then 58°C for 1 minute, then by 45 cycles at 95°C for 15 seconds each

and then 60°C for 1 minute. All reactions were run in duplicate or higher replication in an ABI PRISM 7900HT® Sequence Detection System, according to the manufacturer's instructions.

[00159] III. Nucleic Acid Samples:

[00160] Plasmids containing specific SNP sequences were designed and ordered from BlueHeron (Bothell, WA). (See Table 1 for a list of plasmids comprising SNPs used in the following examples.) The plasmids were quantified using TaqMan RNase P Assay (Applied Biosystems, Foster City, CA; P/N 4316838) according to the manufacturer's instructions and were used as templates (See Table 1, RNase P Control) to validate sensitivity, linear dynamic range, specificity, and selectivity of the given assays.

[00161] Genomic DNAs were purchased from Coriell Institute for Medical Research (Camden, NJ; NA17203, NA17129, NA17201). The genotypes of target SNPs were validated with TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, CA; P/N 4332856) according to the manufacturer's instructions.

[00162] Table 1: Plasmid SNP Sequences (target alleles are indicated in brackets).

CV11201742	GCTCTGCTTCATTCCTGTCTGAAGAAGGGCAGATAGTTTGGCTGCTCCTGTG**[C/T]**TGTCACCTGCAATTCT CCCTTATCAGGGCCATTGGCCTCTCCCTTCTCTCTGTGAGGGATATTTTCTCTGACTTGTCAATCCACATCTT CC
CV11349123	GGCTTGCAATGGCTCCAACCGGAAGGGCGGTGCTCGAGCTGTGGTGCGTGC**[C/T]**GCTAAGTTGTGCGTTC CAGGGTGCACTCGC
CV1207700	GCAACTATACCCTTGATGGATGGAGATTTA**[C/T]**GCAATGTGTTTTACTGGGTAGAGTGACAGACCTT
CV25594064	CCTGAACTTATTTGGCAAGAGCGATGAGTACTCTTAAAATTACTATCTGGAAATTATATTATTTAGAATCTG CCAATTACCTAGATCCCCCCT**[C/G]**AACAATTGTTTCACCAAGGAACTTCCTGAA
CV25639181	GAATTGGTTGTCTCCTTATGGGAACTGGAAGTATTTTGACA**[G/T]**CTTTACCACATTTCTTCATGGGATAGT AAGTGTTAAACAGCTCTGAGCCATTTATTATCAGCTACTTGTAAATTAGCAGTAGAATTTTATTTTTATACT TGTAAGTGGGCAGTTACCTTTTGAGAGGAATACCTATAG
RNaseP Control	GCGGAGGGAAGCTCATCAGTGGGGCCACGAGCTGAGTGCGTCCTGTCACTCCACTCCCATGTCCCTTGGGA AGGTCTGAGACTAGGG
BRAF-1799TA	TACTACACCTCAGATATATTTCTTCATGAAGACCTCACAGTAAAAATAGGTGATTTTGGTCTAGCTACAG **[T/A]**GAAATCTCGATGGAGTGGGTCCCATCAGTTTGAACAGTTGTCTGGATCCATTTTGTGGATGGTAAGA ATTGAGGCTATTTTTCCACTGATTAAATTTTTGGCCCTGAGATGCTGCTGAGTT

CTNNB1-121AG	TGCTAATACTGTTTCGTATTTATAGCTGATTTGATGGAGTTGGACATGGCCATGGAACCAGACAGAAAAGC GGCTGTTAGTCACTGGCAGCAACAGTCTTACCTGGACTCTGGAATCCATTCTGGTGCCACT**[A/G]**CCACAG CTCCTTCTCTGAGTGGTAAAGGCAATCCTGAGGAAGAGGATGTGGATACCTCCCAAGTC
CTNNB1-134CT	TTTGATGGAGTTGGACATGGCCATGGAACCAGACAGAAAAGCGGCTGTTAGTCACTGGCAGCAACAGTCTT ACCTGGACTCTGGAATCCATTCTGGTGCCACTACCACAGCTCCTT**[C/T]**TCTGAGTGGTAAAGGCAATCCT GAGGAAGAGGATGTGGATACCTCCCAAGTCCTGTATGAGTGGGAA
EGFR-2369CT	GTGGACAACCCCCACGTGTGCCGCCTGCTGGGCATCTGCCTCACCTCCACCGTGCAGCTCATCA**[C/T]**GCA GCTCATGCCCTTCGGCTGCCTCCTGGACTATGTCCGGGAACACAAAGACAATATTGGCTCCCAGTACCTGCT CAACTGGTGTGTGCAGATCGCAAAGGTAATCAGGGAAGGGA
EGFR-2573TG	GCATGAACTACTTGGAGGACCGTCGCTTGGTGCACCGCGACCTGGCAGCCAGGAACGTACTGGTGAAAAC ACCGCAGCATGTCAAGATCACAGATTTTGGGC**[T/G]**GGCCAAACTGCTGGGTGCGGAAGAGAAAGAATAC CATGCAGAAGGAGGCAAAGTAAGGAGGTG
KRAS-176CG	CAGGATTCCTACAGGAAGCAAGTAGTAATTGATGGAGAAACCTGTCTCTTGGATATTCTCGACACAG**[C/G]**AGGTCAAGAGGAGTACAGTGCAATGAGGGACCAGTACATGAGGACTGGGGAGGGCTTTCTTTGTGTATTT GCCATAAATAATACTAAATCATTTGAAGATATTC
KRAS-183AC	ACAGGAAGCAAGTAGTAATTGATGGAGAAACCTGTCTCTTGGATATTCTCGACACAGCAGGTCA**[A/C]**GA GGAGTACAGTGCAATGAGGGACCAGTACATGAGGACTGGGGAGGGCTTTCTTTGTGTATTTGCCATAAATA ATACTAAATCATTTGAAGATATTCACCATTATAGGTGGGTTTAAATTGAATATAATAAGCTGACATTAA
KRAS-34GA	TATTAACCTTATGTGTGACATGTTCTAATATAGTCACATTTTCATTATTTTTATTATAAGGCCTGCTGAAAAT GACTGAATATAAACTTGTGGTAGTTGGAGCT**[G/A]**GTGGCGTAGGCAAGAGTGCCTTGACGATACAGCTA ATTCAGAATCATTTTGTGGACGAATATGA
KRAS-35GA	TATTAACCTTATGTGTGACATGTTCTAATATAGTCACATTTTCATTATTTTTATTATAAGGCCTGCTGAAAAT GACTGAATATAAACTTGTGGTAGTTGGAGCTG**[G/A]**TGGCGTAGGCAAGAGTGCCTTGACGATACAGCTA ATTCAGAATCATTTTGTGGACGAATATGATC
KRAS-38GA	CATTATTTTTATTATAAGGCCTGCTGAAAATGACTGAATATAAACTTGTGGTAGTTGGAGCTGGTG**[G/A]**C GTAGGCAAGAGTGCCTTGACGATACAGCTAATTCAGAATCATTTTGTGGACGAATATGATCCAACAATAGA GGTAAATCTTGTTTTAATATGCATATTACTGGTGCAGGACCATTCTTTGATACAGATAAAGGTTTCTCTGAC CATTTTCATGAGTACTTAT
NRAS-181CA	ATTCTTACAGAAAACAAGTGGTTATAGATGGTGAAACCTGTTTGTTGGACATACTGGATACAGCTGGA**[C/ A]**AAGAAGAGTACAGTGCCATGAGAGACCAATACATGAGGACAGGCGAAGGCTTCCTCTGTGTATTTGCCA TCAATAATAGCAAGTCATTTGCGGATATTAACCTCTACAGGTACTAGGAGCATTATTTTCTCTGAAAGGATG
NRAS-183AT	TTACAGAAAACAAGTGGTTATAGATGGTGAAACCTGTTTGTTGGACATACTGGATACAGCTGGACA**[A/T]**G AAGAGTACAGTGCCATGAGAGACCAATACATGAGGACAGGCGAAGGCTTCCTCTGTGTATTTGCCATCAAT AATAGCAAGTCATTTGCGGATATTAACCTCTACAGGTACTAGGAGCATTATTTTCTCTGAAAGGATG
NRAS-35GA	TGGTTTCCAACAGGTTCTTGCTGGTGTGAAATGACTGAGTACAAACTGGTGGTGGTTGGAGCAG**[G/A]**TGG TGTTGGGAAAAGCGCACTGACAATCCAGCTAATCCAGAACCACTTTGTAGATGAATATGATCCCACCATAG AGGTGAGGCCCAGTGGTAGCCCG
NRAS-38GA	TTTCCAACAGGTTCTTGCTGGTGTGAAATGACTGAGTACAAACTGGTGGTGGTTGGAGCAGGTG**[G/A]**TGT TGGGAAAAGCGCACTGACAATCCAGCTAATCCAGAACCACTTTGTAGATGAATATGATCCCACCATAGAGG TGAGGCCCAGTGGTAGCCC
TP53-524GA	GGCACCCGCGTCCGCGCCATGGCCATCTACAAGCAGTCACAGCACATGACGGAGGTTGTGAGGC**[G/A]**CT GCCCCCACCATGAGCGCTGCTCAGATAGCGATGGTGAGCAGCTGGGGCTGGAGAGACGACAGGGCTGGTT GCCCAGGGTCCCCAGGCCTCTGATTCCTCACTGATTGCTCTTAGGTCTGGCC
TP53-637CT	CCTCCTCAGCATCTTATCCGAGTGGAAGGAAATTTGCGTGTGGAGTATTTGGATGACAGAAACACTTTT**[C/ T]**GACATAGTGTGGTGGTGCCCTATGAGCCGCCTGAGGTCTGGTTTGCAACTGGGGTCTCTGGGAGGAGGG GTTAAGGGTGGTTGTCAGTGGCCCTC
TP53-721TG	CTTGGGCCTGTGTTATCTCCTAGGTTGGCTCTGACTGTACCACCATCCACTACAACTACATGTGTAACAGT**[T/G]**CCTGCATGGGCGGCATGAACCGGAGGCCCATCCTCACCATCATCACACTGGAAGACTCCAGGTCAGG AGCCACTTGCCACCCTGCACACTGGCCTGCTGTGCCCCAGCCTC
TP53-733GA	TAGGTTGGCTCTGACTGTACCACCATCCACTACAACTACATGTGTAACAGTTCCTGCATGGGC**[G/A]**GCAT GAACCGGAGGCCCATCCTCACCATCATCACACTGGAAGACTCCAGGTCAGGAGCCACTTGCCACCCTGCAC ACTGGCCTGCTGTGCCCCAGCCTC
TP53-742CT	CTGACTGTACCACCATCCACTACAACTACATGTGTAACAGTTCCTGCATGGGCGGCATGAAC**[C/T]**GGAGG CCCATCCTCACCATCATCACACTGGAAGACTCCAGGTCAGGAGCCACTTGCCACCCTGCACACTGGCCTGC TGTGCCCCAGCCTCTGCTTGCCTC
TP53-743GA	TGACTGTACCACCATCCACTACAACTACATGTGTAACAGTTCCTGCATGGGCGGCATGAACC**[G/A]**GAGGC CCATCCTCACCATCATCACACTGGAAGACTCCAGGTCAGGAGCCACTTGCCACCCTGCACACTGGCCTGCT GTGCCCCAGCCTCTGCTTGCCTC

TP53-817CT	CCTCTTGCTTCTCTTTTCCTATCCTGAGTAGTGGTAATCTACTGGGACGGAACAGCTTTGAGGTG**[C/T]**GTG TTTGTGCCTGTCCTGGGAGAGACCGGCGCACAGAGGAAGAGAATCTCCGCAAGAAAGGGGAGCCTCACCA CGAGCTGCCCCCAGGGAGCACTAAGCGAGGTAAGCAA

[00163] Data Analysis:

[00164] An automatic baseline and manual threshold of 0.2 were used to calculate the threshold cycle (C_t) which is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. The ΔCt between amplification reactions for matched vs. mismatched sequences is defined as the specificity of cast-PCR ($\Delta Ct = Ct_{mismatch} - Ct_{match}$). The larger the ΔCt between mismatched and matched targets, the better assay specificity. The $2^{\Delta Ct}$ value was used to estimate the power of discrimination (or selectivity) which is equal to $1/2^{\Delta Ct}$ or, in some cases, calculated as % ($1/2^{\Delta Ct}$ x 100).

Example 1

Tailed Primers Improve Discrimination of Allelic Variants

[00165] The following example demonstrates that the application of allele-specific primers comprising tails significantly improves the discrimination of allelic variants.

[00166] In conventional AS-PCR, the discrimination of 3' nucleotide mismatches is largely dependent on the sequence surrounding the SNP and the nature of the allele. The ΔCT between the amplification reactions for matched and mismatched primers varies. To improve the discrimination between the amplification of matched and mismatched sequences, allele-specific primers were designed to comprise tails at their 5' termini and then tested for their suitability in AS-PCR assays.

[00167] Assays were performed using the general experimental design and reaction conditions indicated above (with the exception that no blocker probes were included), using 0.5 ng/uL

genomic DNA containing the hsv11711720 SNP comprising one of three alleles (A, C, or T) as the nucleic acid template (see Table 2). The three genotypes are indicated in Table 2a. Primers and probes were designed according to the sequences shown in Table 3.

[00168] Table 2: Genomic DNA Sequence for hsv11711720 SNP (target alleles are indicated in brackets).

AGAAAATAACTAAGGGAAGGAGGAAAGTGGGGAGGAAGGAAGAACAGTGTGAAG
ACAATGGCCTGAAAACTGAAAAAGTCTGTTAAAGTTAATTATCAGTTTTTGAGTCCA
AGAACTGGCTTTGCTACTTTCTGTAAGTTTCTAATTTACTGAATAAGCATGAAAAAG
ATTGCTTTGAGGAATGGTTATAAACACATTCTTAGAGCATAGTAAGCAGTAGGGAGT
AACAAAATAACACTGATTAGAATACTTTACTCTACTTAATTAATCAATCATATTTAG
TTTGACTCACCTTCCCAG**[A/C/T]**ACCTTCTAGTTCTTTCTTATCTTTCAGTGCTTGTC
CAGACAACATTTTCATTTCAACAACTCCTGCTATTGCAATGATGGGTACAATTGCTA
AGAGTAACAGTGTTAGTTGCCAACCATAGATGAAGGATATAATTATTCCTGTCCCAA
GATTTGCTATATTCTGGGTAATTACAGCAAGCCTGGAACCTATAGCCTGCAAAACAA
AACAAATTAGAGAAATTTTAAAAATATTATCTTCACAACTCATGCTTCTATTTTCTGA
AAACTCACCTTCATGAGACTATATTCATTATTTTAT

Table 2a: Genotypes of Genomic DNA Sequence for hsv11711720 SNP

Genomic DNA ID	**Genotype**
NA17203	AA
NA17129	CC
NA17201	TT

[00169] Table 3: List and Sequences of Primers and Probes: conventional allele-specific primers ("ASP-tail"); tailed allele-specific primers ("ASP"); locus-specific TaqMan probe (LST); locus-specific primer (LSP). The nucleotides shown in lower case are the tailed portion of the primers. The nucleotide-specific portion of each allele-specific primer is at the 3'-most terminus of each primer (indicated in bold).

Primer/Probe ID	Sequence (5' to 3')	Tm (°C)
17129-ASP	ATATTTAGTTTGACTCACCTTCCCAG**C**	63.2
17129-tailASP	**acc**ACTCACCTTTCCCAG**C**	63.0
17203-ASP	ATATTTAGTTTGACTCACCTTCCCAG**A**	62.0
17203-tailASP	**acc**ACTCACCTTTCCCAG**A**	63.7
17201-ASP	ATATTTAGTTTGACTCACCTTCCCAG**T**	62.2
17201-tailASP	**acc**ACTCACCTTTCCCAG**T**	64.0
LST	(6-FAM)-TGGACAAGCACTGAAAGA-(MGB)	67.4
LSP	GCAGGAGTTGTTGAAATGAAAATGTTG	62.5

[00170] As shown in Table 4, when using non-tailed ASPs ("ASP-tail"), the discrimination of 3' nucleotide mismatch is largely dependent on the nature of the allele, as a considerable range of ΔCt values is observed depending on the identity of the 3' terminal base. The range of ΔCt values between matched and mismatched nucleotides ("NT") were from -0.1 to 10. However, with tailed ASPs, the discrimination of 3' nucleotide mismatch was significantly improved. In

fact, as Table 4 shows, the ΔCt value between matched and mismatched nucleotides was consistently equal to or greater than 10 when tailed ASPs were used. The Ct values for amplification of matched sequences using tailed ASPs were comparable to those using conventional or non-tailed ASPs. These results indicate that tailed ASP, can improve the specificity of AS-PCR, but may not improve the sensitivity of detection.

[00171] Table 4: Tailed allele-specific primers ("ASP") significantly improve discrimination of allelic variants. The specificity ("fold difference") was calculated based on the difference between Ct values using tailed vs. untailed primers ($2^{(\Delta Ct(ASP) - (\Delta Ct(ASP\text{-}tail)}$). The mismatched nucleotides of the 3' allele-specific nucleotide portion of the ASPs (+/- tail) and the target allele are also indicated ("NT mismatch").

CA	0.9	11.5	1552.1
CT	1.2	11.5	1278.3
AC	10.0	11.9	3.7
AG	9.8	11.9	4.3
TG	2.3	11.5	588.1
TC	-0.1	11.5	3104.2
Average	4.0	11.6	1088.5

Example 2

Low Primer Concentrations Improve Discrimination of Allelic Variants

[00172] Assays were performed using the general experimental design and reaction conditions indicated above, in the presence of 1 million copies of plasmid DNA containing various SNP target sequences (see Table 1) and 200 nM or 800 nM tailed ASP (as indicated). Assay primers and probes were designed according to the sequences shown in Fig. 11A-D.

[00173] The effect of tailed ASP concentration on discrimination of allelic variants is summarized in Table 5. The ΔCt between the amplification reactions for matched and mismatched primers demonstrate that lower tailed ASP concentrations improve discrimination of allelic variants.

[00174] Table 5: Assay Results Using Different Concentrations of Tailed Allele-specific Primers

CV11201742	14.1	15.2	2.14
CV11349123	8.2	10	3.48
CV1207700	5.2	6.6	2.64
CV25594064	20.1	19.1	0.5
CV25639181	11.9	12.9	2
Average	12.6	13.44	2.14

Example 3

Primers Designed with Reduced Tms Improves Discrimination of Allelic Variants

[00175] Assays were performed using the general experimental design and reaction conditions indicated above, in the presence of 1 million copies of plasmid DNA containing various SNP target sequences (see Table 1) using tailed ASP with a higher Tm (~57 °C) or tailed ASP with a lower Tm (~53°C). Assay primers and probes were designed according to the sequences shown in Fig. 11A-D.

[00176] The effect of allele-specific primer Tm on discrimination of allelic variants is summarized in Table 6. The ΔCt of allele-specific primers with a lower Tm are significantly higher than those of allele-specific primers with a higher Tm. Allele-specific primers designed with reduced Tms improved discrimination of allelic variants by as much as 118-fold in some cases or an average of about 13-fold difference.

[00177] Table 6: ΔCt Values Using Tailed ASPs with Lower Tm (~53°C) or with Higher Tm (~57°C)

BRAF-1799TA	12.2	19.1	118.9
CTNNB1-121AG	11.6	14.9	10.0
KRAS-176CG	18.8	22.5	13.1
NRAS-35GA	13.0	14.0	1
TP53-721TG	14.7	19.1	20.6
CTNNB1-134CT	8.6	14.1	44.8

EGFR-2369CT	9.7	10.7	2
KRAS-183AC	22.2	23.1	1.8
NRAS-38GA	14.0	14.3	1.2
TP53-733GA	13.6	13.5	1.0
EGFR-2573TG	16.7	20.2	10.9
KRAS-34GA	14	14.8	1.8
KRAS-38GA	11.2	14.4	8.9
NRAS-181CA	24.0	27.1	8.6
TP53-742CT	9.1	8.0	0.5
KRAS-35GA	11.5	15.1	12.3
NRAS-183AT	23.6	22.7	0.5
TP53-524GA	11.4	13.5	4.6
TP53-637CT	11.4	14.4	7.8
TP53-743GA	10.1	13.2	8.4
TP53-817CT	13.6	13.9	1.2
Average	14.1	16.3	13.3

Example 4

Use of Blocker Probes Improves Discrimination of Allelic Variants

[00178] The following example illustrates that the use of MGB blocker probes improves the discrimination between 3' nucleotide mismatched and matched primers to target sequences in PCR reactions.

[00179] Assays were performed using the general cast-PCR schema and reaction conditions indicated above, using 1 million copies of plasmid DNA containing various SNP target sequences (see Table 1) in the presence of MGB blocker probes or in the absence of MGB blocker probes. Assay primers and probes were designed according to the sequences shown in Fig. 11A-D.

[00180] To improve the selectivity of AS-PCR, blocker probes were synthesized to comprise an MGB group at their 3' terminus. (See, for example, Kutyavin, I. V., *et al.*, Nucleic Acids Research, 2000, Vol. 28, No. 2: 655-661, U.S. Pat. Nos. 5,512,677; 5,419,966; 5,696,251; 5,585,481; 5,942,610 and 5,736,626.)

[00181] The results of cast-PCR using MGB blocker probes are summarized in Table 7. The ΔCt between cast-PCR with MGB blocker probes is larger than that without MGB blocker probes. As shown, MGB blocker probes improve the discrimination of allelic variants.

[00182] Table 7: MGB Blocker Probes Improve Discrimination of Allelic Variants

BRAF-1799TA	11.4	14.9	11.5
CTNNB1-121AG	11.6	14.1	5.4
KRAS-176CG	17.8	20.9	9
NRAS-35GA	13.9	14.3	1.4
TP53-721TG	12.5	14.7	4.4
CTNNB1-134CT	6.7	10.2	11.6
EGFR-2369CT	7.7	10.1	5.3

KRAS-183AC	22.4	23	1.5
NRAS-38GA	14.5	14.6	1.1
TP53-733GA	13.2	14.4	2.3
EGFR-2573TG	18.2	21.8	11.6
KRAS-34GA	14.4	15.1	1.7
KRAS-38GA	11.9	15.1	1.7
NRAS-181CA	19.3	24.2	30.2
TP53-742CT	12.7	13.6	1.9
KRAS-35GA	11.0	13.7	6.5
NRAS-183AT	20.2	21.7	2.9
TP53-524GA	13.5	13.5	1
TP53-637CT	9.3	12.1	7.0
TP53-743GA	9.9	11.5	3.1
TP53-817CT	12.6	13.2	1.5
Average	13.6	15.5	6.0

Example 5

Primers Designed to Target Discriminating Bases Improves Discrimination of Allelic Variants

[00183] Assays were performed using the general cast-PCR schema and reaction conditions indicated above, in the presence of 1 million copies of plasmid DNA containing SNP target sequences (see Table 1). Assay primers and probes were designed according to the sequences

shown in Fig. 11A-D.

[00184] According to the data summarized in Table 8, the discrimination of cast-PCR was dependent on the nature of the allele being analyzed. As Table 8 indicates, the ΔCt between mismatched and matched sequences for allele-1 were different from ΔCt between mismatched and matched sequences for allele-2. However, both A and G bases, as compared to a T base, were highly discriminating for allele-1 and allele-2 in all four SNPs examined.

[00185] Table 8: Primers Designed to Target Discriminating Bases Improve Discrimination of Allelic Variants

	ASP design		SNP allele-1			SNP allele-2		
SNP ID	3' NT of ASP1	3' NT of ASP2	Allele NT	ΔCt (Ct_mismatch - Ct_match)	Specificity (fold difference)	Allele NT	ΔCt (Ct_mismatch - Ct_match)	Specificity (fold difference)
KRAS-38GA	G	A	C	13.4	10809	T	8.2	294
NRAS-181CA	C	A	G	27.5	189812531	T	9.8	891
NRAS-183AT	A	T	T	17.9	244589	A	23.4	11068835
TP53-742CT	C	T	G	12.3	5043	A	8.3	315

Example 6

Determination of the sensitivity and dynamic range for cast-PCR

[00186] In this example, the sensitivity and dynamic range of cast-PCR was determined by performing cast-PCR using various copy numbers of a target plasmid.

[00187] Assays were performed using the general cast-PCR schema and reaction conditions indicated above, using 1 X 10^0 (1 copy) to 1 X 10^7 copies of plasmid DNA containing the

NRAS-181CA SNP target sequence (see Table 1). Assay primers and probes were designed according to the sequences shown in Fig. 11A-D.

[00188] As shown in Figure 5, the use of tailed primers and MGB-blocker probes does not adversely affect the sensitivity of cast-PCR, as the sensitivity of cast-PCR is comparable to TaqMan assays which do not utilize tailed primers or blocker probes. Furthermore, Figure 5 shows that the cast-PCR assay shows a linear dynamic range over at least 7 logs.

Example 7

Determination of the specificity of cast-PCR

[00189] In this example, the specificity of cast-PCR was determined by comparing the amplification of particular alleles of KRAS using either matched or mismatched ASPs to a given allele in the presence of their corresponding blocker probes.

[00190] Assays were performed using the general cast-PCR schema and reaction conditions indicated above, using 1 X 10^6 copies of plasmid DNA containing either one of two alleles of the NRAS-181CA SNP target sequence (see Table 1). Assay primers and probes were designed according to the sequences shown in Fig. 11A-D.

[00191] The left panel of Figure 7 shows the an amplification plot of cast-PCR on allele-1 DNA using matched (A1) primers in the presence of A2 blocker probes or mismatched (A2) primers in the presence of A1 blocker probes. The right hand panel shows a similar experiment in which cast-PCR was performed on allele-2 DNA. As indicated in the data summary in Fig. 7, a robust ΔCt values of over 20 were observed for cast-PCR on both alleles of KRAS-183AC tested. This corresponds to a specificity as determined by a calculation of $2^{\Delta Ct}$ of 9 X 10^6, and 2 X 10^6, respectively, for alleles A1 and A2. Furthermore, a calculation of selectivity ($1/2^{\Delta Ct}$)

indicates that values of $1/1.1 \times 10^7$ and $1/5.0 \times 10^7$ are observed for alleles A1 and A2, respectively.

Example 8

cast-PCR is able to detect a single copy mutant DNA in one million copies of wild type DNA

[00192] In this example, the selectivity of cast-PCR, i.e., the ability of cast-PCR to detect a rare mutant DNA in an excess of wild type DNA, was determined.

[00193] Assays were performed using the general cast-PCR schema and reaction conditions indicated above, using various copy numbers of mutant KRAS-183AC plasmid DNA (1 copy to 1×10^6 copies) mixed with 1×10^6 copies of wild type KRAS-183AC plasmid DNA (see Table 1). Assay primers and probes were designed according to the sequences shown in Fig. 11A-D, and cast-PCR reactions were performed using wild type or mutant ASP and corresponding MGB blocker probes.

[00194] Fig. 8 shows that cast-PCR is able to detect as little as one copy of a mutant DNA sequence, even when surrounded by a million-fold excess of a wild type sequence.

Example 9

Selectivity of cast-PCR in discriminating tumor cell DNA from normal cell DNA

[00195] In this example, the selectivity of cast-PCR was determined by performing assays on samples in which various amounts of tumor cell genomic DNA were mixed with or "spiked" into genomic DNA from normal cells. DNA samples were extracted using QIAmp DNA Mini Prep Kits (Qiagen). Wild type DNA was extracted from the SW48 cell line and mutant DNA was extracted the H1573 cell line.

[00196] The mutant DNA contained the KRAS-G12A mutation. The percentage of tumor cell DNA in the spiked samples varied from 0.5 to 100%. cast-PCR was used to detect the presence of tumor cell DNA when present in these percentages.

[00197] Assays were performed using the general cast-PCR schema and reaction conditions indicated above, using 30 ng of gDNA per reaction. Assay primers and probes were designed according to the sequences corresponding to KRAS-G12A SNP ID, as shown in Fig. 11A-D.

[00198] As shown in Figure 9, tumor cell DNA, even when present only at a level of 0.5% as compared to normal cell DNA, is easily detected using cast-PCR.

Example 10

Use of cast-PCR to detect tumor cells in tumor samples

[00199] In this example, cast-PCR was used to detect and determine the percentage of tumor cells in tumor samples. Various normal and tumor samples were obtained and assayed by cast-PCR for the presence of a number of SNPs associated with cancer as shown in Figure 10.

[00200] Assays were performed using the general cast-PCR schema and reaction conditions indicated above, using 5 ng of gDNA or 1.5 ng cDNA derived from either normal or tumor samples. Assay primers and probes corresponding to the SNPs shown in Figure 10 were designed according to the sequences as shown in Fig. 11A-D.

[00201] The results shown in Figure 10 indicate that cast-PCR has a low false positive rate as indicated by the failure of cast-PCR to detect the presence of mutant cells in normal samples. In contrast, cast-PCR was able to provide a determination of the percentage of tumor cells in various tumor samples that ranged from just under 2% for a tumor sample containing the NRAS-183AT SNP to 80% for a sample containing the EGFR-2573TG SNP.

[00202] It is noted that, as used in this specification and the appended claims, the singular forms "a," "an," and "the," include plural referents unless expressly and unequivocally limited to one referent. The use of "or" means "and/or" unless stated otherwise. The use of "comprise," "comprises," "comprising," "include," "includes," and "including" are interchangeable and not intended to be limiting. Furthermore, where the description of one or more embodiments uses the term "comprising," those skilled in the art would understand that, in some specific instances, the embodiment or embodiments can be alternatively described using the language "consisting essentially of" and/or "consisting of."

[00203] The section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described. All documents, or portions of documents, cited in this application, including but not limited to patents, patent applications, articles, books, and treatises are hereby expressly incorporated by reference in their entirety for any purpose. In the event that one or more of the incorporated documents defines a term that contradicts that term's definition in this application, this application controls.

[00204] All references cited herein, including patents, patent applications, papers, text books, and the like, and the references cited therein, to the extent that they are not already, are hereby incorporated by reference in their entirety. In the event that one or more of the incorporated literature and similar materials differs.

We claim:

1. A method for detecting a first allelic variant of a target sequence in a nucleic acid sample suspected of comprising at least a second allelic variant of the target sequence, comprising:

a) forming a first reaction mixture by combining:

i.the nucleic acid sample;

ii.a first allele-specific primer, wherein an allele-specific nucleotide portion of the first allele-specific primer is complementary to the first allelic variant of the target sequence;

iii.a first allele-specific blocker probe that is complementary to a region of the target sequence comprising the second allelic variant, wherein said region encompasses a position corresponding to the binding position of the allele-specific nucleotide portion of the first allele-specific primer, and wherein the first allele-specific blocker probe comprises a minor groove binder;

iv.a first locus-specific primer that is complementary to a region of the target sequence that is 3' from the first allelic variant and on the opposite strand; and

v.a first detector probe;

b) carrying out an amplification reaction on the first reaction mixture using the first locus-specific primer and the first allele-specific primer to form a first amplicon; and

c) detecting the first amplicon by detecting a change in a detectable property of the first detector probe, thereby detecting the first allelic variant of the target gene in the nucleic acid sample.

2. The method of claim 1, further comprising using the change in a detectable property of the first detector probe to quantitate the first allelic variant.

3. The method of claim 1, further comprising:

d) forming a second reaction mixture by combining:

i) the nucleic acid sample;

ii) a second allele-specific primer, wherein an allele-specific nucleotide portion of the second allele-specific primer is complementary to the second allelic variant of the target sequence;

iii) a second allele-specific blocker probe that is complementary to a region of the target sequence comprising the first allelic variant, wherein said region encompasses a position corresponding to the binding position of the allele-specific nucleotide portion of the second allele-specific primer, and wherein the second allele-specific blocker probe comprises a minor groove binder;

iv) a second locus-specific primer that is complementary to a region of the target sequence that is 3' from the second allelic variant and on the opposite strand; and

v) a second detector probe;

e) carrying out an amplification reaction on the second reaction mixture using the second allele-specific primer and the locus-specific primer, to form a second amplicon; and

f) detecting the second amplicon by detecting a change in a detectable property of the detector probe, thereby detecting the second allelic variant of the target gene in the nucleic acid sample.

4. The method of claim 3, further comprising comparing the change in a detectable property of the first detector probe in the first reaction mixture to the change in a detectable property of the second detector probe in the second reaction mixture.

5. The method of claim 1 or 3, wherein said first and/or second allele-specific primer comprises a tail.

6. The method of claim 5, wherein said tail is GC-rich.

7. The method of claim 5, wherein said tail is between 2-30 nucleotides long.

8. The method of claim 5, wherein said tail is at the 5' end of said first and/or second allele-specific primer.

9. The method of claim 1 or 3, wherein the Tm of said first and/or second allele-specific primer is between 50°C to 55°C.

10. The method of claim 1 or 3, wherein said concentration of said first and/or second allele-specific primer is between 20-900 nM.

11. The method of claim 1 or 3, wherein said first and/or second allele-specific primer is designed to comprise a highly discriminating base at the 3' terminus.

12. The method of claim 1 or 3, wherein said allele-specific nucleotide portion of said first and/or second allele-specific primer is located at the 3' terminus of said first and/or second allele-specific primer.

13. The method of claim 12, wherein A or G is used as the 3' allele-specific nucleotide portion of said first and/or second allele-specific primer if A/T is the allelic variant; or C or T is used as the 3' allele-specific nucleotide portion of said first and/or second allele-specific primer if C/G is the allelic variant.

14. The method of claim 1 or 3, wherein said first and/or second allele-specific primer and/or first and/or second allele-specific blocker probe comprises at least one modified base.

15. The method of claim 14, wherein said modified base is a 8-aza-7-deaza-dN (ppN) base analog, where N is adenine (A), cytosine (C), guanine (G), or thymine (T).

16. The method of claim 14, wherein said modified base is a locked nucleic acid (LNA) base.

17. The method of claim 14, wherein said modified base is any modified base that increases the Tm between matched and mismatched target sequences and/or nucleotides.

18. The method of claim 1 or 3, wherein said first and/or second allele-specific blocker probe comprises an MGB moiety at the 3'-end, the 5'-end and/or at an internal position within said first and/or second allele-specific blocker probe.

19. The method of claim 18, wherein said first and/or second allele-specific blocker probe is non-extendable at the 3' end.

20. The method of claim 18, wherein said MGB moiety or moieties is/are not cleaved from said first and/or second allele-specific blocker probe during said amplification reaction.

21. The method of claim 1 or 3, wherein said first and/or second detector probe is a locus-specific detector probe

22. The method of claim 21, wherein said locus-specific detector probe is a 5' nuclease probe.

23. The method of claim 1, wherein said nucleic acid sample is genomic DNA (gDNA).

24. The method of claim 1 or 3, wherein said first and/or second reaction mixture further comprises a polymerase, dNTPs, and/or other reagents or buffers suitable for PCR amplification.

25. The method of claim 24, wherein said polymerase is thermostable.

26. The method of claim 1 or 3, wherein said amplification reaction is a real time polymerase chain reaction (PCR).

27. The method of claim 1, wherein said nucleic acid sample is derived from a tumor sample.

28. The method of claim 1, wherein said nucleic acid sample is derived from a blood sample comprising circulating tumor cells.

29. The method of claim 27, wherein said tumor sample is a breast or a lung cancer tumor sample.

30. The method of claim 27, wherein said tumor comprises mutations in Ras, EGFR, Kit, pTEN, and/or p53.

31. The method of claim 30, wherein said Ras mutation is a KRAS or and NRAS mutation.

32. The method of claim 31, wherein said KRAS mutation is in codon 12 and/or codon 13 as depicted in Fig 6.

33. A reaction mixture comprising:

a) a nucleic acid molecule;

b) an allele-specific primer, wherein an allele-specific nucleotide portion of the allele-specific primer is complementary to a first allelic variant of a target sequence;

c) an allele-specific blocker probe that is complementary to a region of the target sequence comprising a second allelic variant, wherein said region encompasses a position corresponding to the binding position of the allele-specific nucleotide portion of the allele-specific primer, and wherein the allele-specific blocker probe comprises a minor groove binder;

d) a locus-specific primer that is complementary to a region of the target sequence that is 3' from the first allelic variant and on the opposite strand; and

e) a detector probe.

34. The reaction mixture of claim 33, wherein said allele-specific primer comprises a tail.

35. The reaction mixture of claim 34, wherein said tail is GC-rich.

36. The reaction mixture of claim 34, wherein said tail is between 2-30 nucleotides long.

37. The reaction mixture of claim 34, wherein said tail is at the 5' end of said allele-specific primer.

38. The reaction mixture of claim 33, wherein the Tm of said allele-specific primer is between 50°C to 55°C.

39. The reaction mixture of claim 33, wherein said concentration of said allele-specific primer is between 20-900 nM.

40. The reaction mixture of claim 33, wherein said allele-specific primer is designed to comprise a highly discriminating base at the 3' terminus.

41. The reaction mixture of claim 33, wherein said allele-specific nucleotide portion of said allele-specific primer is located at the 3' terminus of said allele-specific primer.

42. The reaction mixture of claim 41, wherein A or G is used as the 3' allele-specific nucleotide portion of said allele-specific primer if A/T is the allelic variant; or C or T is used as the 3' allele-specific nucleotide portion of said allele-specific primer if C/G is the allelic variant.

43. The reaction mixture of claim 33, wherein said allele-specific primer and/or first and/or allele-specific blocker probe comprises at least one modified base.

44. The reaction mixture of claim 43, wherein said modified base is a 8-aza-7-deaza-dN (ppN) base analog, where N is adenine (A), cytosine (C), guanine (G), or thymine (T).

45. The reaction mixture of claim 43, wherein said modified base is a locked nucleic acid (LNA) base.

46. The reaction mixture of claim 43, wherein said modified base is any modified base that increases the Tm between matched and mismatched target sequences and/or nucleotides.

47. The reaction mixture of claim 33, wherein said allele-specific blocker probe comprises an MGB moiety at the 3'-end, the 5'-end and/or at an internal position within said allele-specific

blocker probe.

48. The reaction mixture of claim 47, wherein said allele-specific blocker probe is non-extendable at the 3' end.

49. The reaction mixture of claim 33, wherein said detector probe is a locus-specific detector probe.

50. The reaction mixture of claim 49, wherein said locus-specific detector probe comprises a label.

51. The reaction mixture of claim 49 wherein said locus-specific detector probe is a 5' nuclease probe.

52. The reaction mixture of claim 33, wherein said nucleic acid molecule is genomic DNA (gDNA).

53. The reaction mixture of claim 33, wherein said reaction mixture comprises a polymerase, dNTPs, and/or other reagents or buffers.

54. The reaction mixture of claim 53, wherein said polymerase is thermostable.

55. The reaction mixture of 33, wherein reaction mixture is used for a real time polymerase chain reaction (PCR).

56. The reaction mixture of claim 33, wherein said nucleic acid molecule is derived from a tumor sample.

57. The reaction mixture of claim 33, wherein said nucleic acid molecule is derived from a blood sample comprising circulating tumor cells.

58. The reaction mixture of claim 56, wherein said tumor sample is a breast or a lung cancer tumor sample.

59. The reaction mixture of claim 56, wherein said tumor comprises mutations in Ras, EGFR, Kit, pTEN, and/or p53.

60. The reaction mixture of claim 59, wherein said Ras mutation is a KRAS or and NRAS mutation.

61. The reaction mixture of claim 60, wherein said KRAS mutation is in codon 12 and/or codon 13 as depicted in Fig 6.

62. A composition comprising:

a) a first allele-specific primer, wherein an allele-specific nucleotide portion of the first allele-specific primer is complementary to the first allelic variant of a target sequence;

b) a first allele-specific blocker probe that is complementary to a region of the target sequence comprising the second allelic variant, wherein said region encompasses a position corresponding to the binding position of the allele-specific nucleotide portion of the first allele-specific primer, and wherein the first allele-specific blocker probe comprises a minor groove binder;

63. The composition of claim 62, further comprising a locus-specific primer that is complementary to a region of the target sequence that is 3' from the first allelic variant and on the opposite strand.

64. The composition of claim 62, further comprising a detector probe.

65. The composition of claim 62, wherein said allele-specific primer comprises a tail.

66. The composition of claim 65, wherein said tail is GC-rich.

67. The composition of claim 65, wherein said tail is between 2-30 nucleotides long.

68. The composition of claim 65, wherein said tail is at the 5' end of said allele-specific primer.

69. The composition of claim 62, wherein the Tm of said allele-specific primer is between 50°C to 55°C.

70. The composition of claim 62, wherein said concentration of said allele-specific primer is between 20-900 nM.

71. The composition of claim 62, wherein said allele-specific primer is designed to comprise a highly discriminating base at the 3' terminus.

72. The composition of claim 62, wherein said allele-specific nucleotide portion of said allele-specific primer is located at the 3' terminus of said allele-specific primer.

73. The composition of claim 72, wherein A or G is used as the 3' allele-specific nucleotide portion of said allele-specific primer if A/T is the allelic variant; or C or T is used as the 3' allele-specific nucleotide portion of said allele-specific primer if C/G is the allelic variant.

74. The composition of claim 62, wherein said allele-specific primer and/or first and/or allele-specific blocker probe comprises at least one modified base.

75. The composition of claim 74, wherein said modified base is a 8-aza-7-deaza-dN (ppN) base analog, where N is adenine (A), cytosine (C), guanine (G), or thymine (T).

76. The composition of claim 74, wherein said modified base is a locked nucleic acid (LNA) base.

77. The composition of claim 74, wherein said modified base is any modified base that increases the Tm between matched and mismatched target sequences and/or nucleotides.

78. The composition of claim 62, wherein said allele-specific blocker probe comprises an MGB moiety at the 3'-end, the 5'-end and/or at an internal position within said allele-specific blocker probe.

79. The composition of claim 78, wherein said allele-specific blocker probe is non-extendable at the 3' end.

80. The composition of claim 64, wherein said detector probe is a locus-specific detector probe.

81. The composition of claim 80, wherein said locus-specific detector probe comprises a label.

82. The composition of claim 64 wherein said locus-specific detector probe is a 5' nuclease probe.

83. The composition of claim 62, wherein said composition further comprises a nucleic acid molecule, a polymerase, dNTPs, and/or other reagents or buffers.

84. The composition of claim 83, wherein said nucleic acid molecule is genomic DNA (gDNA).

85. The composition of claim 84, wherein said polymerase is thermostable.

86. The composition of claim 83, wherein said nucleic acid molecule is derived from a tumor sample.

87. The composition of claim 83, wherein said nucleic acid molecule is derived from a blood sample comprising circulating tumor cells.

88. The composition of claim 86, wherein said tumor sample is a breast or a lung cancer tumor sample.

89. The composition of claim 86, wherein said tumor comprises mutations in Ras, EGFR, Kit, pTEN, and/or p53.

90. The composition of claim 89, wherein said Ras mutation is a KRAS or and NRAS mutation.

91. The composition of claim 90, wherein said KRAS mutation is in codon 12 and/or codon 13 as depicted in Fig 6.

92. A kit comprising, two or more containers comprising the following components independently distributed in one of the two or more containers:

a) a first allele-specific primer, wherein an allele-specific nucleotide portion of the first allele-specific primer is complementary to the first allelic variant of a target sequence; and

b) a first allele-specific blocker probe that is complementary to a region of the target sequence comprising the second allelic variant, wherein said region encompasses a position corresponding to the binding position of the allele-specific nucleotide portion of the first allele-specific primer, and wherein the first allele-specific blocker probe comprises a minor groove binder.

93. The kit of claim 92, further comprising a locus-specific primer that is complementary to a region of the target sequence that is 3' from the first allelic variant and on the opposite strand.

94. The kit of claim 92, further comprising a detector probe.

95. The kit of claim 92, wherein the first allele-specific blocker probe does not comprise a label.

96. The kit of claim 92, wherein said allele-specific primer comprises a tail.

97. The kit of claim 96, wherein said tail is GC-rich.

98. The kit of claim 96, wherein said tail is between 2-30 nucleotides long.

99. The kit of claim 96, wherein said tail is at the 5' end of said allele-specific primer.

100. The kit of claim 92, wherein the Tm of said allele-specific primer is between 50°C to 55°C.

101. The kit of claim 92, wherein said concentration of said allele-specific primer is between 20-900 nM.

102. The kit of claim 92, wherein said allele-specific primer is designed to comprise a highly discriminating base at the 3' terminus.

103. The kit of claim 92, wherein said allele-specific nucleotide portion of said allele-specific primer is located at the 3' terminus of said allele-specific primer.

104. The kit of claim 103, wherein A or G is used as the 3' allele-specific nucleotide portion of said allele-specific primer if A/T is the allelic variant; or C or T is used as the 3' allele-specific nucleotide portion of said allele-specific primer if C/G is the allelic variant.

105. The kit of claim 92, wherein said first allele-specific primer and/or first allele-specific blocker probe comprises at least one modified base.

106. The kit of claim 105, wherein said modified base is a 8-aza-7-deaza-dN (ppN) base analog, where N is adenine (A), cytosine (C), guanine (G), or thymine (T).

107. The kit of claim 105, wherein said modified base is a locked nucleic acid (LNA) base.

108. The kit of claim 105, wherein said modified base is any modified base that increases the Tm between matched and mismatched target sequences and/or nucleotides.

109. The kit of claim 92, wherein said allele-specific blocker probe comprises an MGB moiety at the 3'-end, the 5'-end and/or at an internal position within said allele-specific blocker probe.

110. The kit of claim 109, wherein said allele-specific blocker probe is non-extendable at the 3' end.

111. The kit of claim 94, wherein said detector probe is a locus-specific detector probe.

112. The kit of claim 111, wherein said locus-specific detector probe comprises a label.

113. The kit of claim 111 wherein said locus-specific detector probe is a 5' nuclease probe.

114. The kit of claim 92, wherein said kit further comprises a polymerase, dNTPs, and/or other reagents or buffers.

115. The kit of claim 114, wherein said polymerase is thermostable.

116. The kit of one of claims 92-94, wherein said kit is used to detect mutations in Ras, EGFR, Kit, pTEN, and/or p53.

117. The kit of claim 117, wherein said Ras mutation is a KRAS or and NRAS mutation.

118. The kit of claim 118, wherein said KRAS mutation is in codon 12 and/or codon 13 as depicted in Fig 6.

119. The method of claim 1 or 3, wherein said method has a selectivity of detection of at least 1:1000, 1:10,000, 1:100,000 or 1:1,000,000.

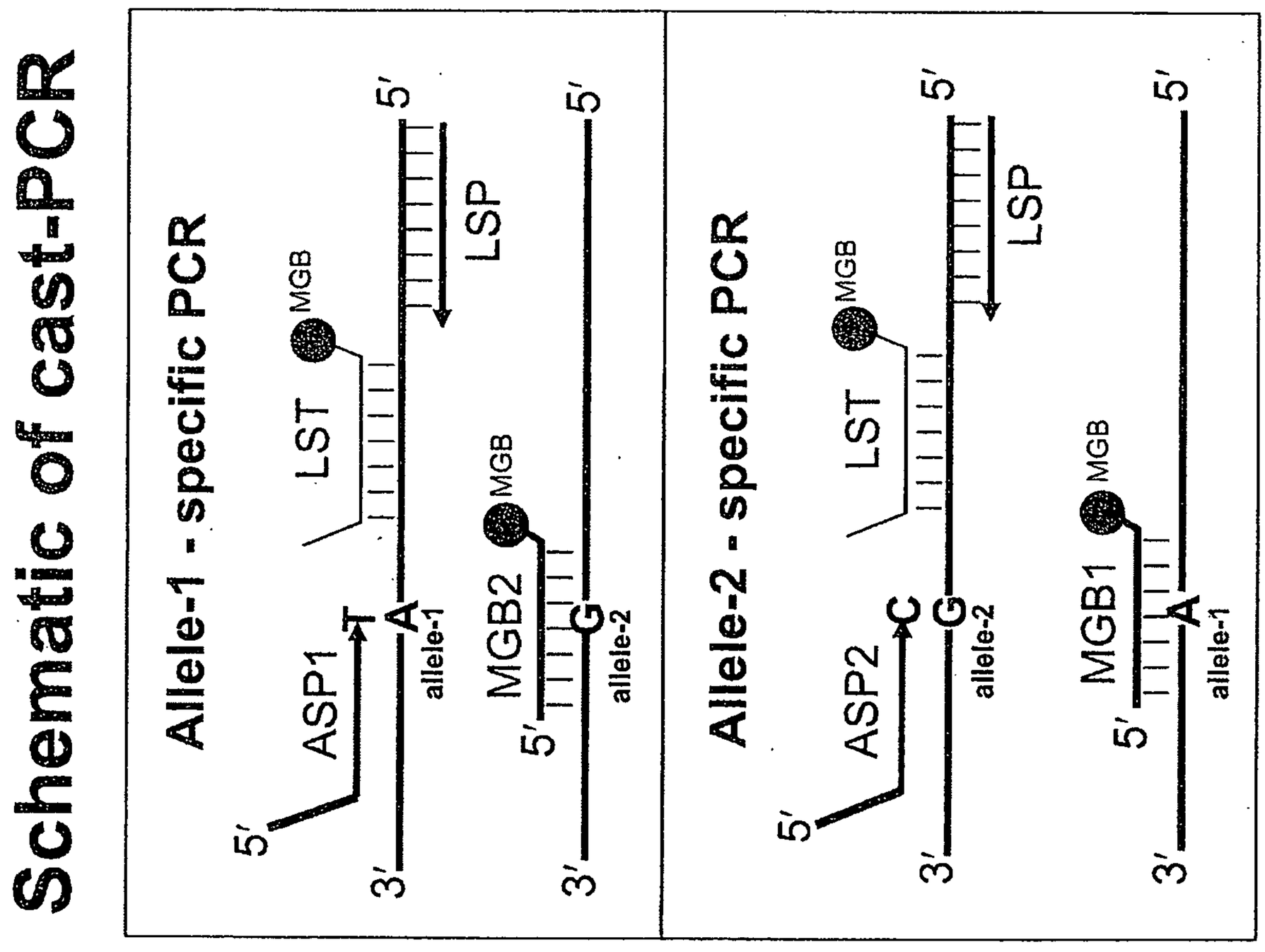

Schematic of cast-PCR
Allele-1 - specific PCR
5′
ASP1
T
A
allele-1
3′
LST
LSP
MGB
MGB2
G
allele-2
Allele-2 - specific PCR
ASP2
C
MGB1
A

Fig. 1

Use of highly discriminating bases for allele detection (e.g., mutant allele)

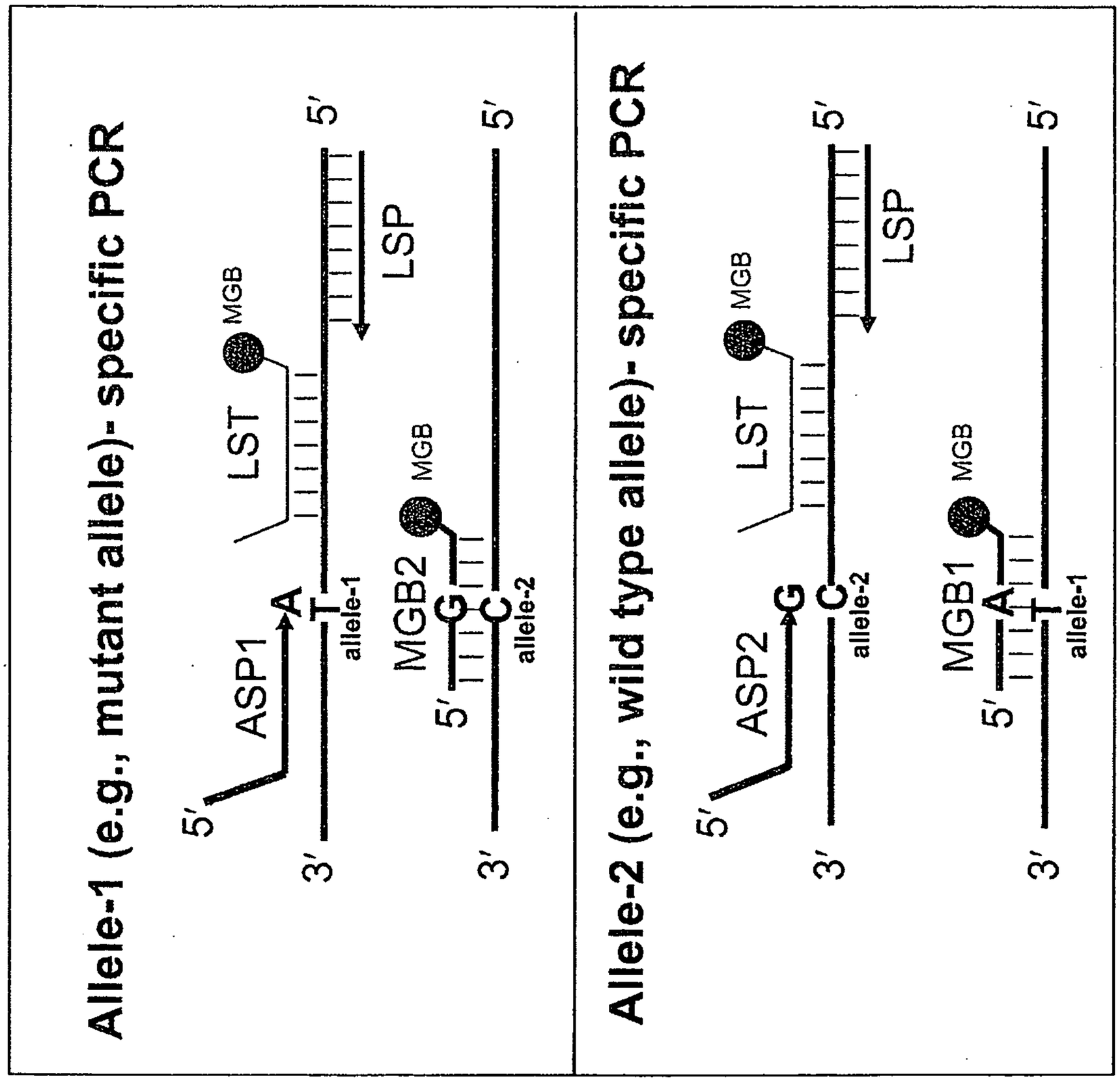


Fig. 2

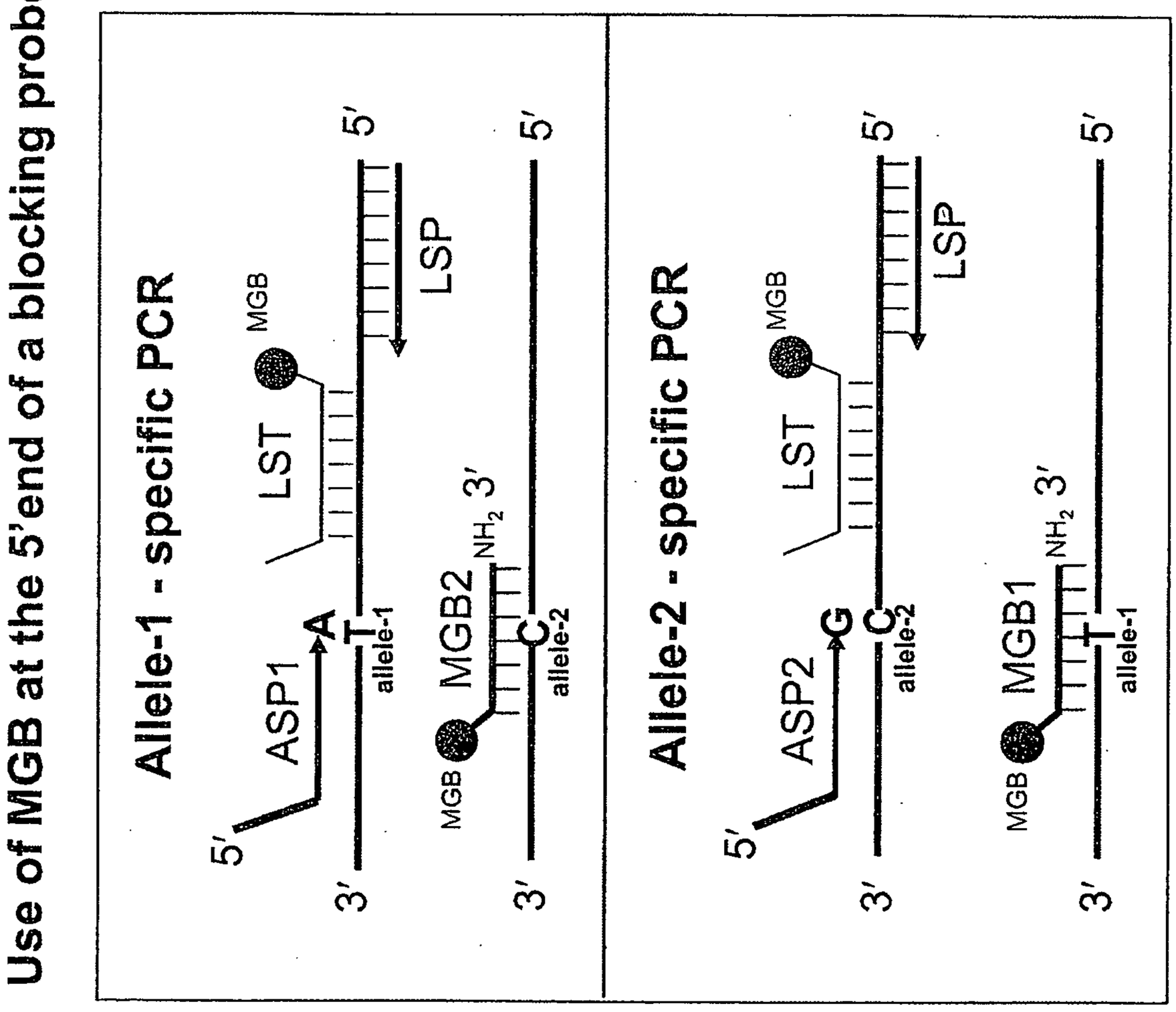

Use of MGB at the 5'end of a blocking probe
Allele-1 - specific PCR
LST
MGB
5'
LSP
ASP1
A
T
allele-1
3'
MGB
MGB2
NH2
C
allele-2
Allele-2 - specific PCR
ASP2
G
C
MGB1

Fig. 3

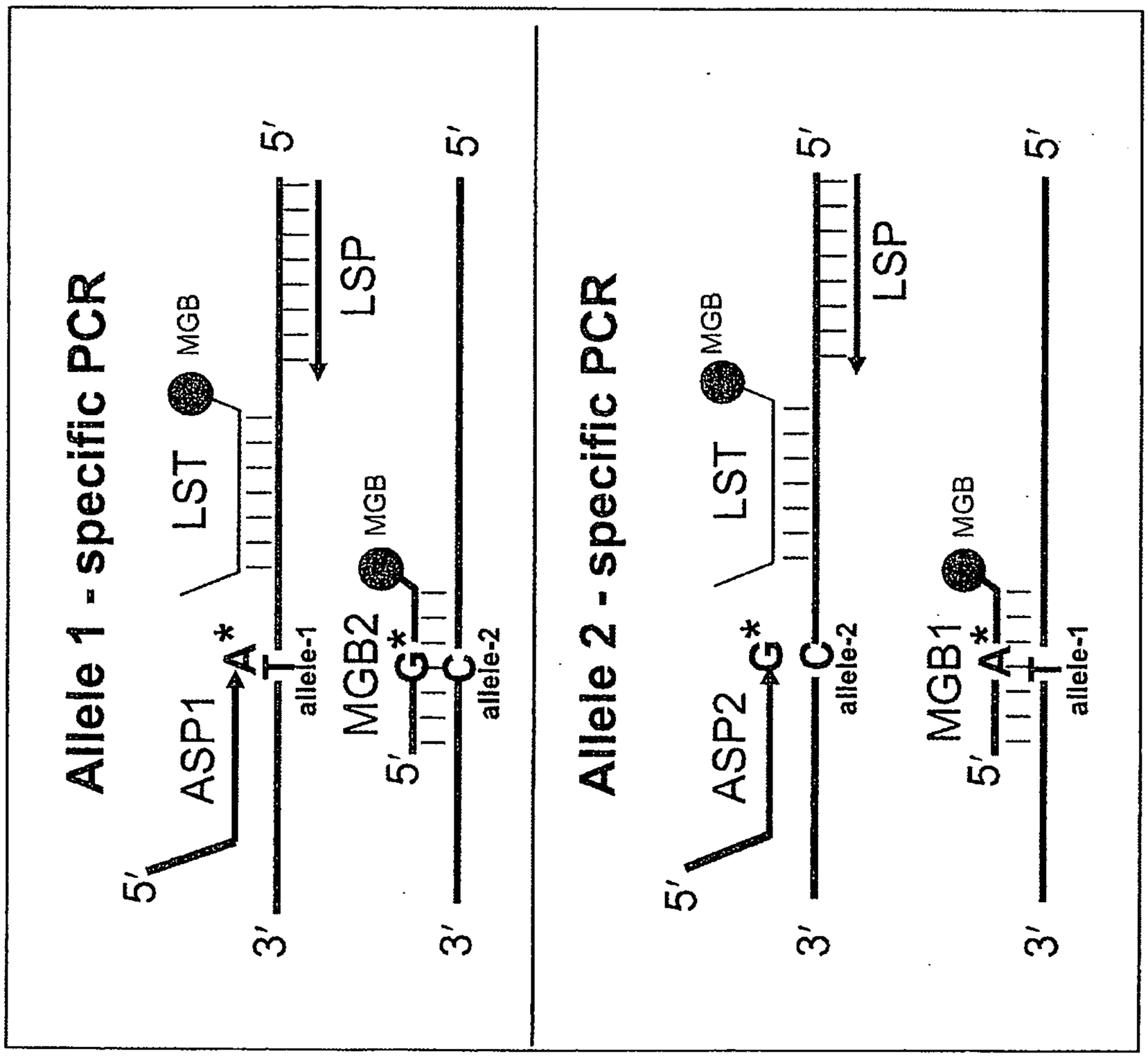

Use of modified bases in MGB blockers and allele-specific PCR primers (ASP1 and ASP2)
Allele 1 - specific PCR
LST
MGB
LSP
ASP1
A*
T
allele-1
MGB2
G*
C
allele-2
5′
3′
Allele 2 - specific PCR
ASP2
G*
C
MGB1
A*
T

Fig. 4A

Examples of Modified Nucleosides

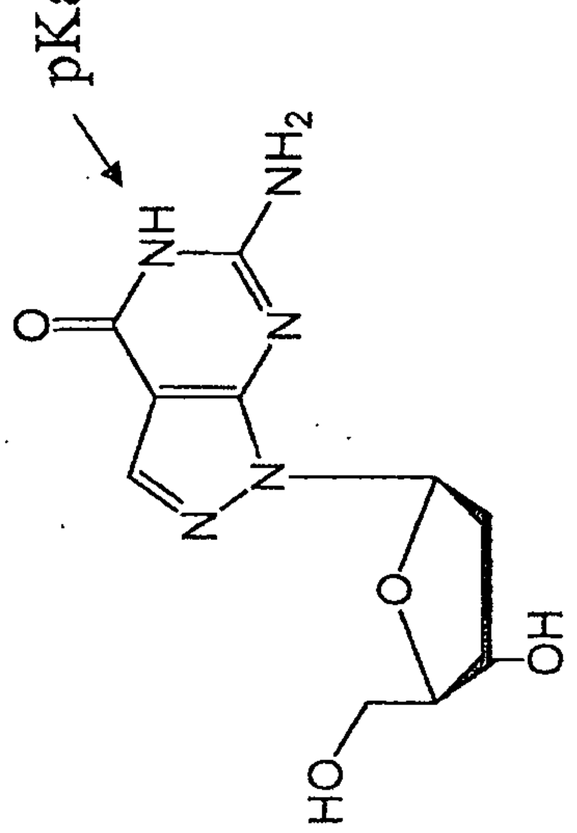
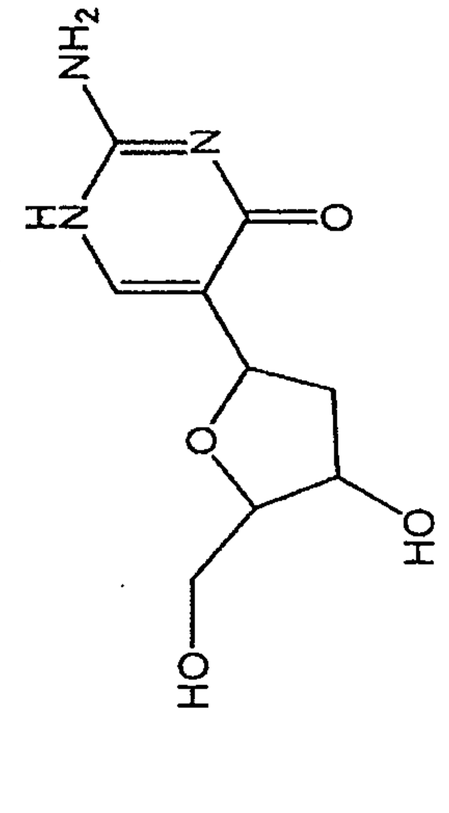
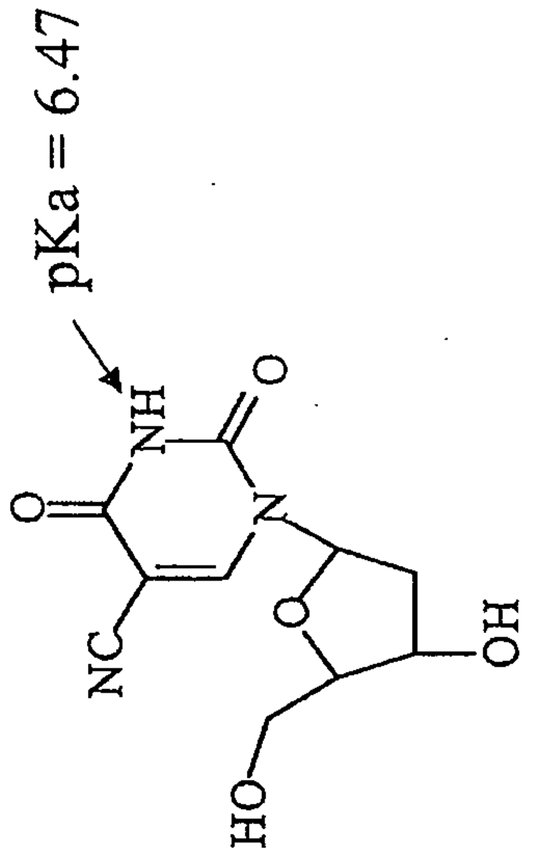
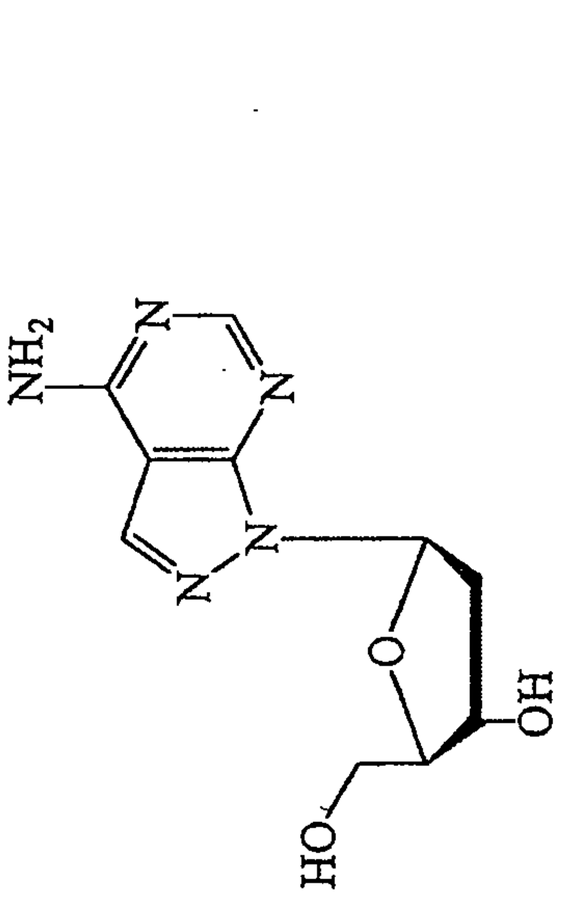
	
8-Aza-7-deaza-dG (ppG)	2'-Deoxy-pseudoisocytidine
	
5-Cyano-2'-deoxyuracil	8-Aza-7-deaza-dA (ppA)

Fig. 4B

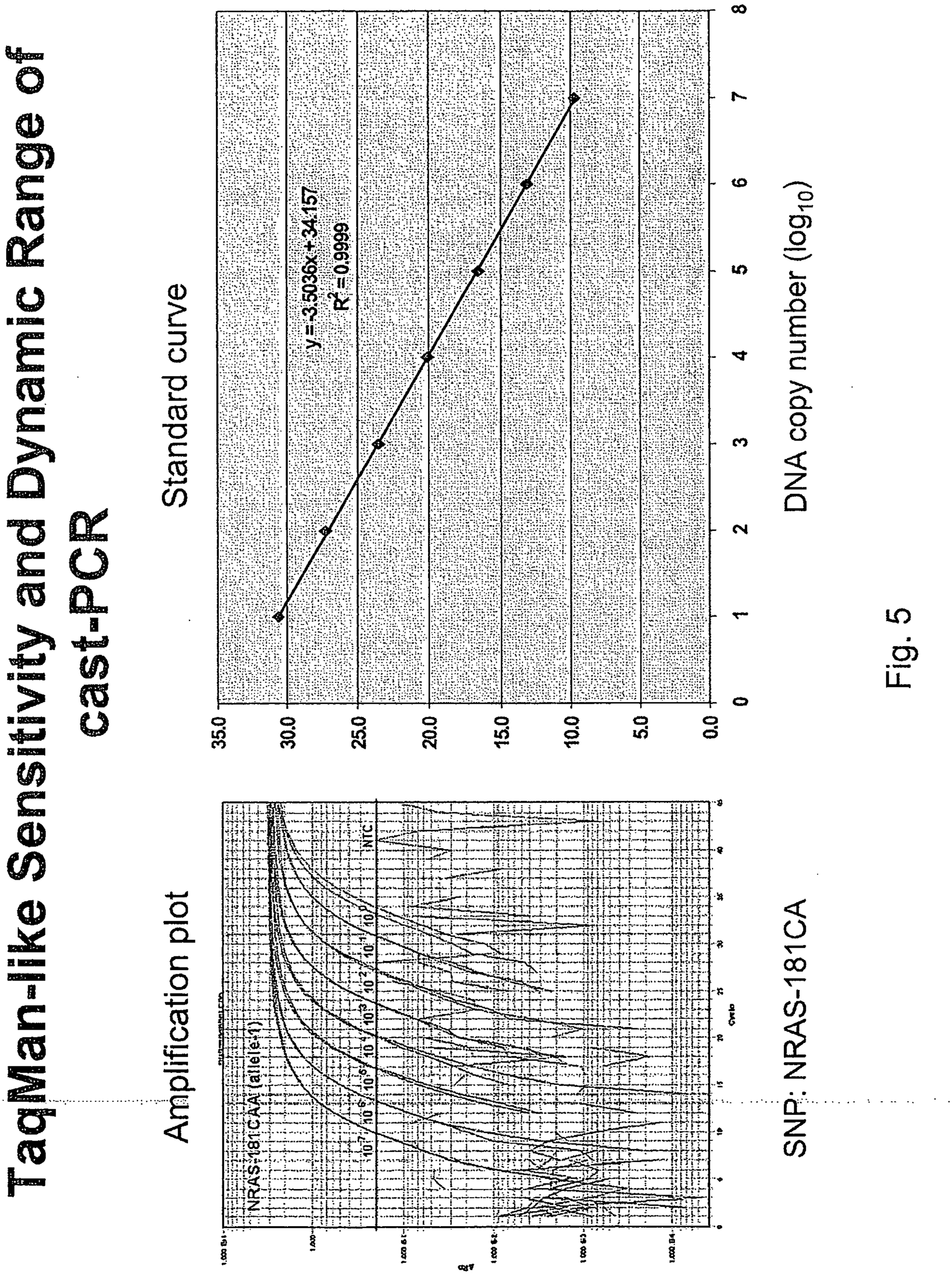
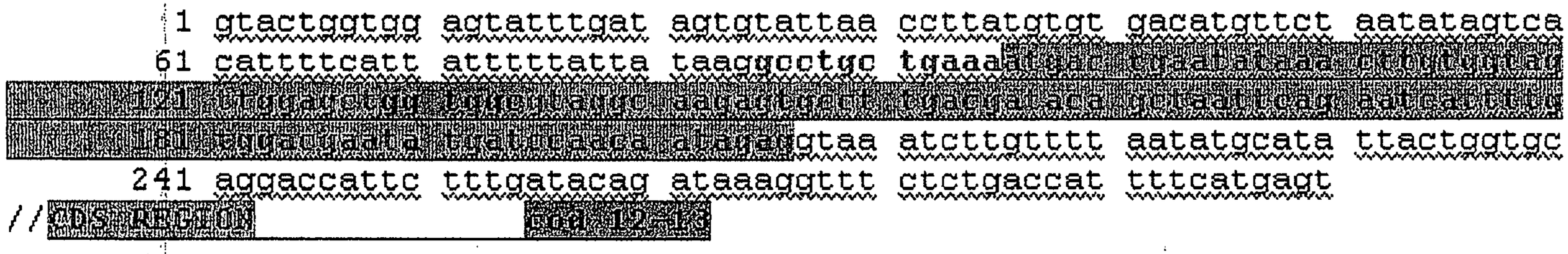

TaqMan-like Sensitivity and Dynamic Range of Cascast-PCR
Amplification plot
SNP: NRAS-181CA
NRAS181CAA (allele-1)
NTC
Standard curve
y = -3.5036x + 34.157
R² = 0.9999
DNA copy number (log10)
Ct
Cycle

Fig. 5

KRAS mutations



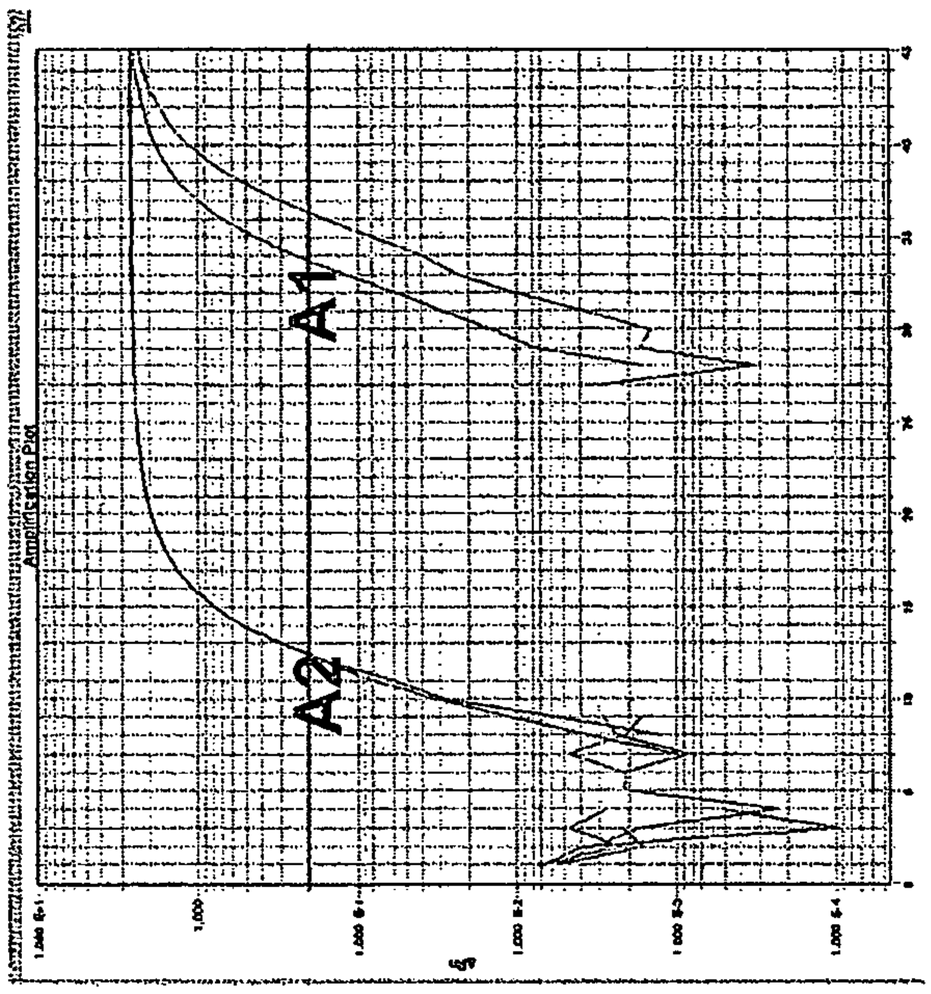
KRAS mutations

- 12 Asp (GGT>GAT)
- 12 Val (GGT>GTT)
- 12 Cys (GGT>TGT)
- 12 Ser (GGT>AGT)
- 12 Ala (GGT>GCT)
- 12 Arg (GGT>CGT)
- 13 Asp (GGC>GAC)

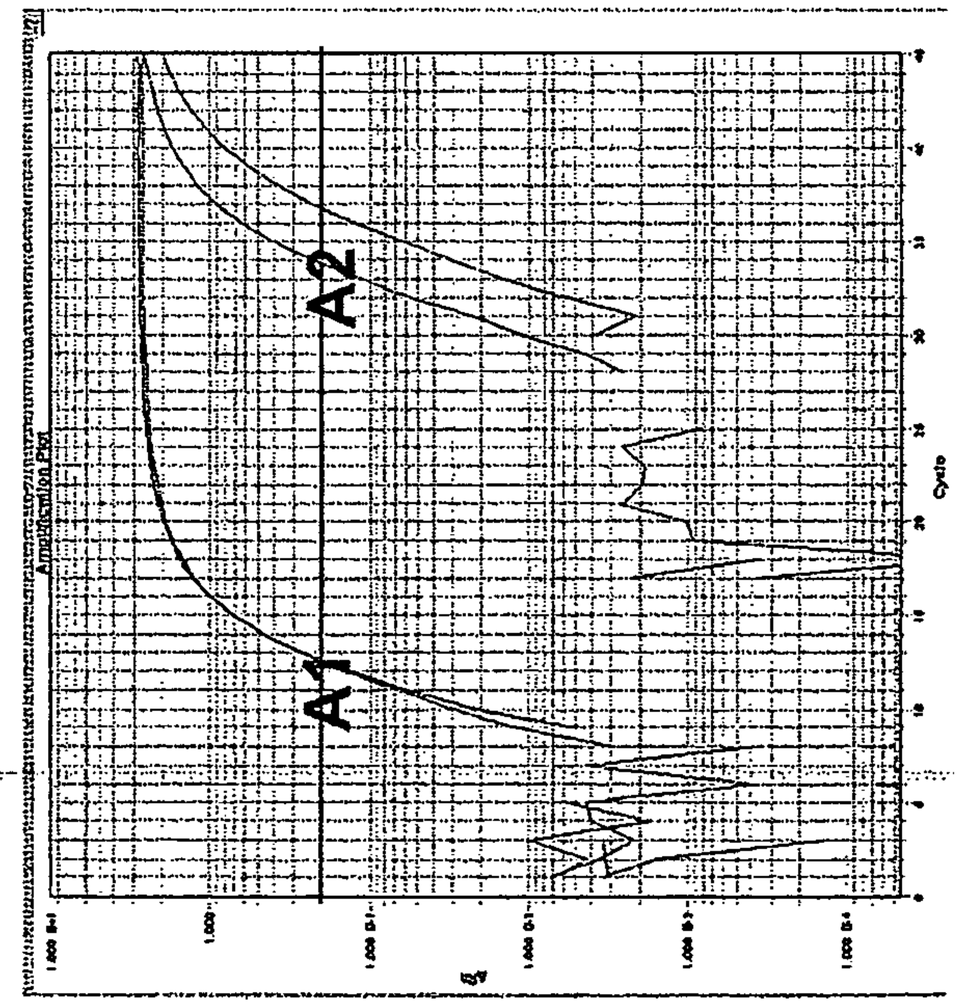
Fig. 6

Specificity of KRAS-183AC cast-PCR Assays

10^6 copies of allele-2 DNA



10^6 copies of allele-1 DNA



	Ct_A2	Δ Ct	Specificity Improvement (fold difference)
KRAS-183AC (alelle-1)	35.7	23.1	$9x10^6$
KRAS-183AC (allele-2)	13.5	20.9	$2x10^6$

	Ct_A1
KRAS-183AC (alelle-1)	12.6
KRAS-183AC (allele-2)	34.4

Fig. 7

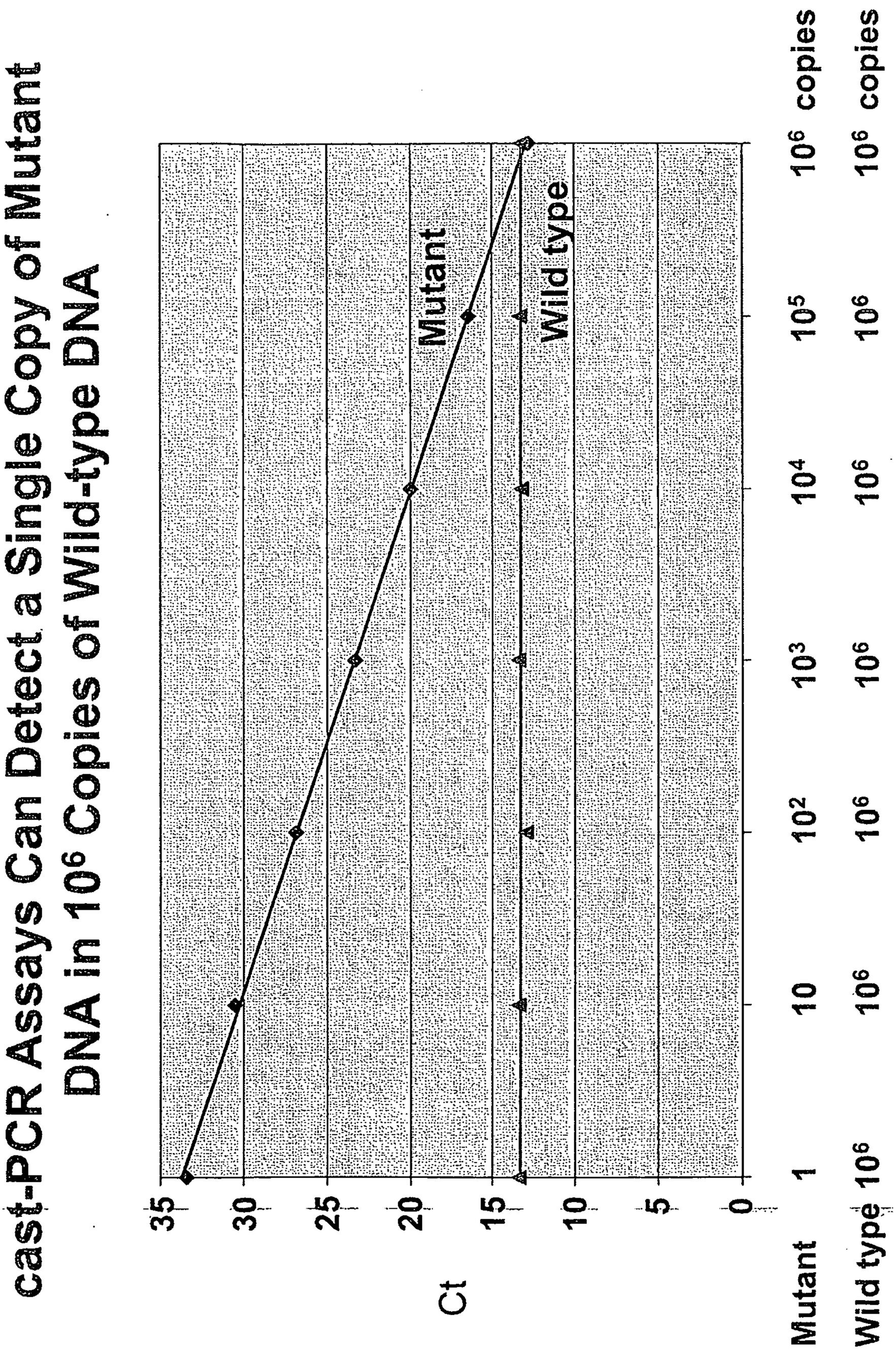
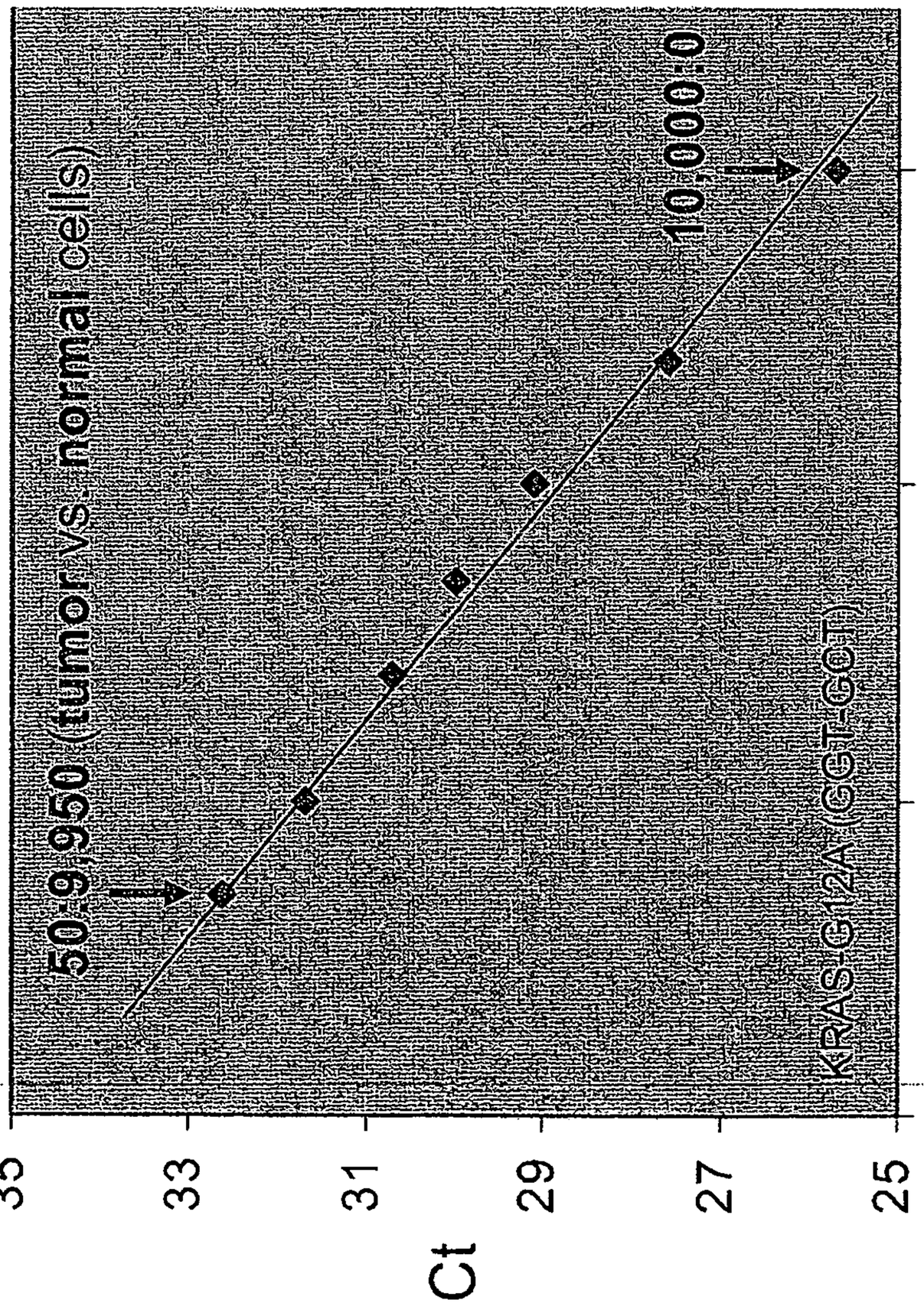

cast-PCR Assays Can Detect a Single Copy of Mutant DNA in 10^6 Copies of Wild-type DNA
Mutant
Wild type
Ct
35
30
25
20
15
10
5
0
1
10
10^2
10^3
10^4
10^5
10^6
10^6
10^6
10^6
10^6
10^6
10^6
Mutant
Wild type

Fig. 8

Specificity of cast-PCR Assays – Tumor Cell DNA Spiking



% tumor cell DNA	Ct
0.5	32.6
1	31.7
2.5	30.7
5	30.0
10	29.1
25	27.6
100	25.7

Fig. 10

Detection of Mutant DNA from Tumor Samples by cast-PCR

SNP ID	Sample ID	Ct_WT	Ct_Mutant	Normalized ΔCt	Mutant cell DNA (%)
KRA-S38GA	Normal-1	23.2	40.0	16.8	0.00
	Tumor-01	27.8	30.3	3.6	7.62
EGFR-2573TG	Normal-1	22.9	40.0	17.1	0.00
	Tumor-02	27.7	25.4	-2	80.00
CTNNB1-134CT	Normal-1	22.8	40	17.2	0.00
	Tumor-03	27.6	31.3	-2.7	86.66
KRAS-35GA	Normal-1	23.8	40	16.2	0.00
	Tumor-04	30.9	31.7	0	50.00
BRAF-1799TA	Normal-1	24.5	40	15.5	0.00
	Tumor-05	24.5	25	1.4	27.48
NRAS-183AT	Normal-1	24.2	40	15.8	0.00
	Tumor-06	29.8	40.0	5.9	1.65
TP53-733GA	Normal-1	21.8	40	18.2	0.00
	Tumor-07	26.2	26.1	1.2	30.33

Fig. 10

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List of Primers and Probes used in cast-PCR Assays

SNP ID	Tailed Allele-specific Primer 1 (ASP1)	Sequence of ASP1	Tailed Allele-specific Primer 2 (ASP2)	Sequence of ASP2
CV11201742	ASP1	cccGCTGCTCCTGTGC	ASP2	cccGGCTGCTCCTGTGT
CV11349123	ASP1	acccAACGCACAACTTAGCG	ASP2	gcccGAACGCACAACTTAGCA
CV1207700	ASP1	cccgCCTTGATGGATGGAGATTTAC	ASP2	cccgCCTTGATGGATGGAGATTTAT
CV25594064	ASP1	gcacCCTTGGTGAAACAATTGTTG	ASP2	tgcacCCTTGGTGAAACAATTGTTC
CV25639181	ASP1	cgccGAACTGGAAGTATTTTGACAG	ASP2	cgccGAACTGGAAGTATTTTGACAT
SNP ID	Locus-specific Primer (LSP)	Sequence of LSP	Locus-specific TaqMan Probe (LST)	Sequence of LST
CV11201742	LSP	TATCCCTCACAGAGAGAAGGGAG	LST	(6-FAM)ATGGCCCTGATAAGGGAGA(MGB)
CV11349123	LSP	GCTTGCAATGGCTCCAACC	LST	(6-FAM)AGGGCGGTGCTCGAG(MGB)
CV1207700	LSP	GGAGCCATTGCAAGCCAAG	LST	(6-FAM)TGTCACTCTACCCAGTAAA(MGB)
CV25594064	LSP	TGGCAAGAGCGATGAGTACTC	LST	(6-FAM)AGAATCTGCCAATTACCTAGA(MGB)
CV25639181	LSP	CTCTCAAAAGGTAACTGCCCACTTA	LST	(6-FAM)ACAAGTAGCTGATAATAAATG(MGB)
SNP ID	MGB Blocker 1 (MGB1)	Sequence of MGB1	MGB Blocker 2 (MGB2)	Sequence of MGB2
CV11201742	MGB1	CTCCTGTGCTGTCACC(MGB)	MGB2	GCTCCTGTGTTGTCACC(MGB)
CV11349123	MGB1	CTTAGCGGCACGCAC(MGB)	MGB2	CTTAGCAGCACGCACC(MGB)
CV1207700	MGB1	GGAGATTTACGCAATGTG(MGB)	MGB2	TGGAGATTTATGCAATGTG(MGB)
CV25594064	MGB1	ACAATTGTTGAGGGGGG(MGB)	MGB2	ACAATTGTTCAGGGGGG(MGB)
CV25639181	MGB1	AAGTATTTTGACAGCTTTAC(MGB)	MGB2	AAGTATTTTGACATCTTTAC(MGB)

Fig. 11A

SNP ID	Tailed Allele-specific Primer 1 (ASP1)	Sequence of ASP1	Tailed Allele-specific Primer 2 (ASP2)	Sequence of ASP2
BRAF-1799TA	ASP1	GCCTGATTTTGGTCTAGCTACAGA	ASP2	CCGGATTTTGGTCTAGCTACAGT
CTNNB1-121AG	ASP1	CGCGAGAAGGAGCTGTGGT	ASP2	GCCAGAAGGAGCTGTGGC
CTNNB1-134CT	ASP1	CCGTGCCTTTACCACTCAGAG	ASP2	CCGTGCCTTTACCACTCAGAA
EGFR-2369CT	ASP1	GCGCGTGCAGCTCATCAC	ASP2	GCGCGTGCAGCTCATCAT
EGFR-2573TG	ASP1	CGGCAGCAGTTTGGCCC	ASP2	CGGCAGCAGTTTGGCCA
KRAS-176CG	ASP1	GCCGGATATTCTCGACACAGC	ASP2	CGCTGGATATTCTCGACACAGG
KRAS-183AC	ASP1	GCGGACACAGCAGGTCAA	ASP2	GCGGACACAGCAGGTCAC
KRAS-183AT	ASP1	GCGGACACAGCAGGTCAA	ASP2	GCGGACACAGCAGGTCAT
KRAS-34GA	ASP1	CGCCTTGCCTACGCCACT	ASP2	GCGTTGCCTACGCCACC
KRAS-34GC	ASP1	CGCTGCCTACGCCACG	ASP2	GCGTTGCCTACGCCACC
KRAS-34GT	ASP1	GCGTTGCCTACGCCACC	ASP2	GCGTTGCCTACGCCACA
KRAS-35GA	ASP1	CGCCTCTTGCCTACGCCAT	ASP2	GCGTCTTGCCTACGCCAC
KRAS-35GC	ASP1	GCGTCTTGCCTACGCCAG	ASP2	GCGTCTTGCCTACGCCAC
KRAS-35GT	ASP1	GCGTCTTGCCTACGCCAC	ASP2	GCGTCTTGCCTACGCCAA
KRAS-3GA	ASP1	CGCGTAGTTGGAGCTGGTGA	ASP2	GCCTAGTTGGAGCTGGTGG
NRAS-181CA	ASP1	CCGATACTGGATACAGCTGGAA	ASP2	CCGATACTGGATACAGCTGGAC
NRAS-183AT	ASP1	CCGTGGATACAGCTGGACAA	ASP2	CGCCTGGATACAGCTGGACAT
NRAS-35GA	ASP1	CCGGGTGGTTGGAGCAGA	ASP2	CGCGTGGTTGGAGCAGG
NRAS-38GA	ASP1	CCGGGTTGGAGCAGGTGA	ASP2	CGCGTTGGAGCAGGTGG
TP53-524GA	ASP1	CCGGGAGGTTGTGAGGCA	ASP2	GGCGAGGTTGTGAGGCG
TP53-637CT	ASP1	GCCGGATGACAGAAACACTTTTC	ASP2	GCCTGGATGACAGAAACACTTTTT
TP53-721TG	ASP1	GCGTACAACTACATGTGTAACAGTG	ASP2	GGCCTACAACTACATGTGTAACAGTT
TP53-733GA	ASP1	GCCTTCCTGCATGGGCA	ASP2	GCCTCCTGCATGGGCG
TP53-742CT	ASP1	GCCGGCGGCATGAACC	ASP2	GCCGGCGGCATGAACT
TP53-743GA	ASP1	GCCGCGGCATGAACCA	ASP2	GCCGCGGCATGAACCG
TP53-817CT	ASP1	GCCAACAGCTTTGAGGTGC	ASP2	CGCGAACAGCTTTGAGGTGT

Fig. 11B

SNP ID	Locus-specific Primer (LSP)	Sequence of LSP	Locus-specific TaqMan Probe (LST)	Sequence of LST
BRAF-1799TA	LSP	AGCCTCAATTCTTACCATCCACAAAA	LST	(6-FAM)CAGACAACTGTTCAAACTG(MGB)
CTNNB1-121AG	LSP	CATGGAACCAGACAGAAAAGCG	LST	(6-FAM)CTGGCAGCAACAGTCT(MGB)
CTNNB1-134CT	LSP	GTCACTGGCAGCAACAGTCTTA	LST	(6-FAM)TAGTGGCACCAGAATGG(MGB)
EGFR-2369CT	LSP	TGGGAGCCAATATTGTCTTTGTGTT	LST	(6-FAM)CCCTTCGGCTGCCTCC(MGB)
EGFR-2573TG	LSP	AGGAACGTACTGGTGAAAACACC	LST	(6-FAM)CAGCATGTCAAGATCAC(MGB)
KRAS-176CG	LSP	CCCTCCCCAGTCCTCATGTA	LST	(6-FAM)CTGGTCCCTCATTGCAC(MGB)
KRAS-183AC	LSP	TCTTCAAATGATTTAGTATTATTTATGGCAAATACACAAAG	LST	(6-FAM)CCCTCCCCAGTCCTCA(MGB)
KRAS-183AT	LSP	TCTTCAAATGATTTAGTATTATTTATGGCAAATACACAAAG	LST	(6-FAM)CCCTCCCCAGTCCTCA(MGB)
KRAS-34GA	LSP	TGTGTGACATGTTCTAATATAGTCACAT	LST	(6-FAM)CCTGCTGAAAATGAC(MGB)
KRAS-34GC	LSP	TGTGTGACATGTTCTAATATAGTCACAT	LST	(6-FAM)CCTGCTGAAAATGAC(MGB)
KRAS-34GT	LSP	TGTGTGACATGTTCTAATATAGTCACAT	LST	(6-FAM)CCTGCTGAAAATGAC(MGB)
KRAS-35GA	LSP	TGTGTGACATGTTCTAATATAGTCACAT	LST	(6-FAM)CCTGCTGAAAATGAC(MGB)
KRAS-35GC	LSP	TGTGTGACATGTTCTAATATAGTCACAT	LST	(6-FAM)CCTGCTGAAAATGAC(MGB)
KRAS-35GT	LSP	TGTGTGACATGTTCTAATATAGTCACAT	LST	(6-FAM)CCTGCTGAAAATGAC(MGB)
KRAS-3GA	LSP	GGTCCTGCACCAGTAATATGCA	LST	(6-FAM)TCGTCCACAAAATGATTC(MGB)
NRAS-181CA	LSP	GCAAATGACTTGCTATTATTGATGGCAAA	LST	(6-FAM)CCTTCGCCTGTCCTCATG(MGB)
NRAS-183AT	LSP	GCAAATGACTTGCTATTATTGATGGCAAA	LST	(6-FAM)CCTTCGCCTGTCCTCATG(MGB)
NRAS-35GA	LSP	AGTGGTTCTGGATTAGCTGGATTG	LST	(6-FAM)CAGTGCGCTTTTCC(MGB)
NRAS-38GA	LSP	GTGGTTCTGGATTAGCTGGATTGT	LST	(6-FAM)CAGTGCGCTTTTCC(MGB)
TP53-524GA	LSP	GCAACCAGCCCTGTCGTC	LST	(6-FAM)CTGCTCACCATCGCTATC(MGB)
TP53-637CT	LSP	AGACCCCAGTTGCAAACCAG	LST	(6-FAM)CCTCAGGCGGCTCATAG(MGB)
TP53-721TG	LSP	CTGGAGTCTTCCAGTGTGATGATG	LST	(6-FAM)ATGGGCCTCCGGTTCAT(MGB)
TP53-733GA	LSP	CTGGAGTCTTCCAGTGTGATGAT	LST	(6-FAM)ACCGGAGGCCCATCCT(MGB)
TP53-742CT	LSP	TGTGCAGGGTGGCAAGT	LST	(6-FAM)CACACTGGAAGACTCC(MGB)
TP53-743GA	LSP	TGTGCAGGGTGGCAAGT	LST	(6-FAM)CACACTGGAAGACTCC(MGB)
TP53-817CT	LSP	CTTTCTTGCGGAGATTCTCTTCCT	LST	(6-FAM)CTGTGCGCCGGTCTC(MGB)

Fig. 11C

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SNP ID	MGB Blocker 1 (MGB1)	Sequence of MGB1	MGB Blocker 2 (MGB2)	Sequence of MGB2
BRAF-1799TA	MGB1	GCTACAGAGAAATCTC(MGB)	MGB2	GCTACAGTGAAATCTC(MGB)
CTNNB1-121AG	MGB1	GAGCTGTGGTAGTGGCA(MGB)	MGB2	AGCTGTGGCAGTGGCA(MGB)
CTNNB1-134CT	MGB1	CACTCAGAGAAGGAGC(MGB)	MGB2	CACTCAGAAAAGGAGC(MGB)
EGFR-2369CT	MGB1	CATCACGCAGCTCATG(MGB)	MGB2	TCATCATGCAGCTCATG(MGB)
EGFR-2573TG	MGB1	AGTTTGGCCCGCCCAA(MGB)	MGB2	AGTTTGGCCAGCCCAA(MGB)
KRAS-176CG	MGB1	CTCGACACAGCAGGTCA(MGB)	MGB2	CTCGACACAGGAGGTCA(MGB)
KRAS-183AC	MGB1	GCAGGTCAAGAGGAGTAC(MGB)	MGB2	CAGGTCACGAGGAGTAC(MGB)
KRAS-183AT	MGB1	GCAGGTCAAGAGGAGTAC(MGB)	MGB2	GCAGGTCATGAGGAGTAC(MGB)
KRAS-34GA	MGB1	CGCCACTAGCTCCA(MGB)	MGB2	CGCCACCAGCTCC(MGB)
KRAS-34GC	MGB1	CCACGAGCTCCAACTA(MGB)	MGB2	CCACCAGCTCCAACTA(MGB)
KRAS-34GT	MGB1	ACGCCACCAGCTCC(MGB)	MGB2	ACGCCACAAGCTCCAA(MGB)
KRAS-35GA	MGB1	CGCCATCAGCTCC(MGB)	MGB2	CGCCACCAGCTCC(MGB)
KRAS-35GC	MGB1	CGCCAGCAGCTCC(MGB)	MGB2	CGCCACCAGCTCC(MGB)
KRAS-35GT	MGB1	CGCCACCAGCTCCA(MGB)	MGB2	CGCCAACAGCTCCAA(MGB)
KRAS-3GA	MGB1	GCTGGTGACGTAGGC(MGB)	MGB2	GCTGGTGGCGTAGGC(MGB)
NRAS-181CA	MGB1	GCTGGAAAAGAAGAGTAC(MGB)	MGB2	GCTGGACAAGAAGAGTAC(MGB)
NRAS-183AT	MGB1	CAGCTGGACAAGAAGAG(MGB)	MGB2	CAGCTGGACATGAAGAG(MGB)
NRAS-35GA	MGB1	TTGGAGCAGATGGTGTT(MGB)	MGB2	TGGAGCAGGTGGTGTT(MGB)
NRAS-38GA	MGB1	TGGAGCAGGTGATGTT(MGB)	MGB2	TGGAGCAGGTGGTGTT(MGB)
TP53-524GA	MGB1	TGAGGCACTGCCC(MGB)	MGB2	TGAGGCGCTGCCC(MGB)
TP53-637CT	MGB1	GAAACACTTTTCGACATAGTGTGGTG(MGB)	MGB2	AGAAACACTTTTTGACATAGTGTGGTG(MGB)
TP53-721TG	MGB1	TGTGTAACAGTGCCTGCATG(MGB)	MGB2	ATGTGTAACAGTTCCTGCATG(MGB)
TP53-733GA	MGB1	GCATGGGCAGCATG(MGB)	MGB2	GCATGGGCGGCATG(MGB)
TP53-742CT	MGB1	GCATGAACCGGAGGC(MGB)	MGB2	GCATGAACTGGAGGCC(MGB)
TP53-743GA	MGB1	CATGAACCAGAGGCC(MGB)	MGB2	CATGAACCGGAGGCC(MGB)
TP53-817CT	MGB1	TTGAGGTGCGTGTTTGTG(MGB)	MGB2	TTTGAGGTGTGTGTTTGTGC(MGB)

Fig. 11D