(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
23 May 2013 (23.05.2013)

WIPO | PCT

(10) International Publication Number
WO 2013/074977 A1

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

(21) International Application Number:
PCT/US2012/065606

(22) International Filing Date:
16 November 2012 (16.11.2012)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/561,703 18 November 2011 (18.11.2011) US
61/673,933 20 July 2012 (20.07.2012) US

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

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

— *with sequence listing part of description (Rule 5.2(a))*

WO 2013/074977 A1

(54) **Title**: SELECTIVE AMPLIFICATION AND DETECTION OF MUTANT GENE ALLELES

(57) **Abstract**: The present invention provides methods for selectively amplifying a mutant allele of a gene. These methods include (a) contacting a sample containing a mutant and a normal allele of a gene with at least one primer that selectively modifies the normal allele of the gene and causes further amplification of the normal allele to fail or to be substantially reduced; and (b) selectively amplifying the mutant allele of the gene or a portion thereof. Other methods for detecting a mutant allele of a gene in a sample are also provided.

SELECTIVE AMPLIFICATION AND DETECTION OF MUTANT GENE ALLELES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims benefit to U.S. Provisional Patent Application Serial Nos. 61/561,703 filed November 18, 2011 and 61/673,933, filed July 20, 2012. The contents of the above application are incorporated by reference as if recited in full.

FIELD OF INVENTION

[0002] The present invention provides, *inter alia,* methods for selectively amplifying a mutant allele and enhancing the sensitivity of detection of gene mutations.

INCORPORATION BY REFERENCE OF SEQUENCE LISTING

[0003] This application contains references to amino acids and/or nucleic acid sequences that have been filed concurrently herewith as sequence listing text file "0344404.txt", file size of 20 KB, created on November 15, 2012. The aforementioned sequence listing is hereby incorporated by reference in its entirety pursuant to 37 C.F.R. § 1.52(e)(5).

BACKGROUND OF THE INVENTION

[0004] A large number of solid tumor cancers, including breast cancer, are caused by single point mutations or small base pair insertions/deletions in susceptible genes. The detection of these mutations typically relies on the

polymerase chain reaction (PCR) followed by a variety of post-amplification analyses. However, a major limitation of all of these methods is their low sensitivities since amplification of fewer copies of the abnormal gene is competitively inhibited by preferential amplification of the more abundant normal gene. In fact, detection of the abnormal gene is often not possible until it represents greater than 5-10% of the total alleles present meaning that early detection of a low number of cancer cells is severely limited.

[0005] Accordingly, there is a need for selective amplification of and early detection of mutant gene alleles present in low abundance in, *inter alia*, breast and other solid organ cancers. The present invention is directed to meeting this and other needs.

SUMMARY OF THE INVENTION

[0006] Disclosed herein are methods that modify standard nucleic acid amplification methods such as, *e.g.*, PCR reactions to specifically and significantly block the amplification of a normal gene and thus enhance the detection of limited copies of a mutant allele. One of the methods involves the selective degradation of a normal allele using thermostable restriction endonucleases before and/or during the PCR reaction. Another method involves the design of primers that, when extended, create amplicons of the normal allele that cannot be used for further amplification as well as mutant amplicons that can be further amplified.

[0007] Accordingly, one embodiment of the present invention is a method for selectively amplifying a mutant allele. This method comprises:

(a) contacting a sample comprising a mutant and a normal allele of a gene with at least one primer that selectively modifies the normal allele of the gene and

causes further amplification of the normal allele to fail or to be substantially reduced; and

(b) selectively amplifying the mutant allele of the gene or a portion thereof.

[0008] Another embodiment of the present invention is a method for detecting a mutant allele of a gene, if present, in a sample. This method comprises:

(a) contacting the sample with a thermostable restriction endonuclease that recognizes a sequence in a normal allele of the gene but not in a mutant allele of the gene;

(b) amplifying the mutant allele of the gene or a portion thereof, if present in the sample; and

(c) before or during step (b), selectively degrading the normal allele of the gene.

[0009] Yet another embodiment of the present invention is a method for detecting a mutant allele of a gene, if present, in a sample using a PCR. This method comprises:

(a) contacting the sample with a first primer that produces a first and a second amplicon after two rounds of amplification, the first amplicon being an amplicon of the normal allele and cannot serve as a template for subsequent amplification, and the second amplicon being an amplicon of the mutant allele; and

(b) contacting the sample with a second primer that anneals to the second amplicon.

[0010] A further embodiment of the present invention is a template tool for use in selecting optimal PCR conditions for a method of detecting a mutant allele of a gene in a sample, which method selectively degrades a normal allele of the gene in

the sample by a thermostable restriction endonuclease. This template tool comprises:

a first polynucleotide sequence comprising recognition sites for a plurality of thermostable endonucleases, and

a second polynucleotide sequence homologous to the first polynucleotide except that it does not contain a single recognition site for any one of the plurality of thermostable endonucleases.

[0011] Another embodiment of the present invention is a template tool for use in selecting optimal PCR conditions for a method of selectively amplifying mutant alleles present in low abundance in solid tumors, which method selectively degrades a normal allele or an amplicon of the normal allele in the sample by a thermostable restriction endonuclease. This template tool comprises:

a first polynucleotide sequence comprising recognition sites for a plurality of thermostable endonucleases, and

a second polynucleotide sequence homologous to the first polynucleotide except that it does not contain a single recognition site for any one of the plurality of thermostable endonucleases.

[0012] An additional embodiment of the present invention is a kit. This kit comprises any template tool disclosed herein.

BRIEF DESCRIPTION OF THE DRAWINGS

[0013] Figure 1 is a schematic showing that the normal allele and its amplicons are degraded before and during lower-denaturation-temperature PCR by

a thermostable restriction endonuclease that recognizes a sequence natively present or introduced by primer design.

[0014] Figure 2 shows a DNA gel demonstrating that a 466 basepair (bp) fragment from mouse adenylate cyclase subtype 5 and a 178 bp fragment from mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) can be effectively amplified under denaturing temperatures of 90°C and 87.5°C, respectively. Annealing and extension were performed at 72°C for both reactions.

[0015] Figure 3 shows a DNA gel demonstrating that a 185 bp fragment from human β-actin can be effectively amplified under a denaturing temperature of 85°C. Annealing and extension were performed at 55°C and 70°C, respectively.

[0016] Figure 4 shows a DNA gel demonstrating that a 179 bp fragment from human β-actin can be effectively amplified under a denaturing temperature of 81°C. Annealing and extension were performed at 55°C and 70°C, respectively.

[0017] Figure 5 shows a DNA gel demonstrating that a 131 bp fragment from human chromosome 6 (AL731777 / GI29801752) can be effectively amplified under denaturing temperatures of 72.5°C-75.0°C. Annealing and extension were both performed at 60°C. The forward primer used for this reaction was CATTTACATCTTTCAATTATGTATATTCTT (SEQ. ID NO: 49), and the reverse primer was ATGCAAATACTTCATTAGTAAAATTTACTT (SEQ. ID NO: 50)

[0018] Figure 6 shows a DNA gel demonstrating that PhoI, but not MwoI or BstNI, can retain activity under a 92°C denaturing step and effectively block the amplification of a 388 bp fragment from human GAPDH. The fragment contained PhoI (GGCC), MwoI (GCNNNNNNNGC), and BstNI (CCWGG) restriction sites. The amplification was performed using brain cDNA with an Advantage 2 PCR kit

(Clontech Laboratories Inc., Mountain View, CA). Denaturing, annealing, and extension temperatures were 92°C, 60°C, and 75°C, respectively.

[0019] Figure 7 shows a DNA gel demonstrating that MwoI can retain activity under a 83°C-88°C denaturing step and effectively block the amplification of a 86 bp fragment from human guanylate cyclase 2F containing a MwoI (GCNNNNNNNGC) restriction site. The amplification was performed using brain cDNA with an Advantage 2 PCR kit. Annealing and extension temperatures were 60°C and 70°C, respectively.

[0020] Figure 8 shows a DNA gel demonstrating that a 131 bp fragment from human chromosome 6 can be effectively amplified under a denaturing temperature of 66.4°C with 20% glycerol in the reaction mixture. The same fragment can be effectively amplified alternatively under a denaturing temperature of 69.0°C with 10% glycerol in the reaction mixture. Annealing and extension temperatures were 35°C and 60°C, respectively.

[0021] Figure 9 is a schematic showing the extension of a primer carrying a sequence that enhances hairpin formation during the PCR amplification of a normal allele to allow for the selective amplification of a mutant allele.

[0022] Figure 10 is a PCR product analysis of rat (as mutant) and mouse (as wild) cDNA mixed at the given ratio. The PCR product was digested with either XbaI or Tru9I.

[0023] Figure 11 is a table showing primer design and thermostable restriction endonuclease selection for enhanced detection of specific breast cancer mutations.

[0024] Figure 12 is a table showing primer design to induce amplicon hairpin formation from normal alleles to allow enhanced detection of breast cancer mutations.

[0025] Figure 13 is a bar graph showing the distribution of PCR primer lengths (cited from Yuryev, Anton. *PCR Primer Design*, Humana Press, 2007.).

[0026] Figure 14 is a bar graph showing the distribution of PCR product sizes for all primer pairs in the VirOligo database (Oklahoma State University, Stillwater, OK) (Yuryev, Anton. *PCR Primer Design*, Humana Press, 2007.).

[0027] Figure 15 is a bar graph showing the distribution of melting temperatures (T_m) for approximately 100 randomly selected amplification products from ten mRNA sequences (NM_000901-NM_000910). Products were selected with automatic searching using Oligo software, version 7 (Molecular Biology Insights, Inc., Cascade, CO).

[0028] Figure 16 is a scatter plot and regression line showing the relationship between T_m and PCR product length using data from Figure 15.

[0029] Figure 17 is a line graph showing that T_m is lower in PCR products with low guanine-cytosine (G-C) content.

[0030] Figure 18 is a line graph showing the relationship between T_m and PCR product length.

[0031] Figure 19 shows the amplification plots of synthetic B-raf templates representing wild type or mutant sequences of the B-raf gene in the absence or presence of the thermostable enzyme TspRI.

[0032] Figure 20 shows the DNA sequencing results of PCR products following amplification of a wild type and a mutant template of the B-raf gene present in a 1,000 : 1 ratio.

[0033] Figures 21A-21G show the amplification plots of wild type genomic DNA in the absence or presence of thermostable restriction endonucleases known to

digest wild type regions of several genes that commonly encode mutations in solid tumors.

[0034] Figure 22 shows the DNA sequencing results following enriched PCR amplification of a synthetic mutant site in a set of custom synthesized universal templates, which allows for the identification of optimal conditions for the activities of thermostable enzymes. Examples of two experiments are presented. A mixture of a wild type universal template and a mutant universal template were mixed in a 100:1 ratio and subjected to PCR amplification at a reduced denaturation temperature (85°C) in the presence of BseLI (*top tracing*) and Bse JI (*bottom tracing*). These enzymes successfully degraded the wild type amplification products allowing for the equal detection of the mutant product (*top tracing*) or the detection of only the mutant product (*bottom tracing*).

DETAILED DESCRIPTION OF THE INVENTION

[0035] One embodiment of the present invention is a method for selectively amplifying a mutant allele. This method comprises:

(a) contacting a sample comprising a mutant and a normal allele of a gene with at least one primer that selectively modifies the normal allele of the gene and causes further amplification of the normal allele to fail or to be substantially reduced; and

(b) selectively amplifying the mutant allele of the gene or a portion thereof.

[0036] As used herein, a "normal allele" is a variation of a gene that is the most prevalent in a population or a subpopulation of interest, such as *e.g.*, the wild type. A "mutant allele" is a variation of the gene other than the normal allele.

[0037] In this invention, a sample, which comprises nucleic acids, may be obtained from an organism, such as a mammal, for example, a human, a farm animal, a laboratory animal, or a pet. Some examples of farm animals include cows, pigs, horses, goats, etc. Some examples of pets include dogs, cats, etc. Some examples of laboratory animals include rats, mice, rabbits, guinea pigs, etc.

[0038] As used herein, a "primer" is a short strand of nucleic acid, which (or a portion of which) anneals to a gene of interest or a portion thereof. The primer serves as a starting point for the amplification of the gene or a portion thereof. Exemplary sequences of primers suitable for use in the methods of the present invention include those disclosed in Figures 11 and 12 (such as, *e.g.*, SEQ ID NOs: 1-2, 5-6, 9-10, 13-14, 17-18, 21, 23, 25, 27, 29-31, 34-36, and 39-41). Primers include forward and reverse primers. A forward primer initiates elongation of a nucleic acid strand from the 5' end to the 3' end. Reverse primers initiate the elongation of a nucleic acid strand from the 3' end to the 5' end.

[0039] In the present method, amplification step(s) may be carried out using any suitable technique known in the art. Non-limiting examples of methods for amplification of nucleic acids include PCR, ligation amplification (or ligase chain reaction (LCR)), amplification methods based on the use of Q-beta replicase or template-dependent polymerase (see, *e.g.*, US Patent Publication Number US20050287592); helicase-dependent isothermal amplification (Vincent *et al.*, "Helicase-dependent isothermal DNA amplification". EMBO reports 5 (8): 795-800 (2004)); strand displacement amplification (SDA); thermophilic SDA nucleic acid sequence based amplification (3SR or NASBA) and transcription-associated amplification (TAA). Preferably, the amplification step(s) of the present invention are carried out with PCR.

[0040] In the present invention, selective modification of the normal allele of a gene by a primer includes, for example, introducing a thermostable restriction endonuclease site into the normal allele after the first two rounds of PCR, or causing an amplicon of the normal allele to form a hairpin, for example, a hairpin in the 3' end of the amplicon, after the first two rounds of PCR. As used herein, an "amplicon" means a nucleic acid product formed as a result of an amplification process, such as, *e.g.*, PCR. As used herein, each "round" of PCR is a cycle of denaturation of double-stranded nucleic acids, annealing of the primers to the single-stranded nucleic acids, and extension of the primers. As used herein, a "thermostable" restriction endonuclease means a restriction enzyme that retains its activity or a portion thereof after exposure to elevated temperature (*e.g.*, greater than about 70°C). Preferably, the thermostable restriction endonuclease retains its activity or a portion thereof after exposure to temperatures of greater than about 80°C. Non-limiting examples of restriction endonucleases for use in accordance with the methods of the present invention include Ack1, Apa LI, Ape KI, Bam HI, Bam HI-HF, Bcl I, Bgl II, Blp I, Bsa AI, Bsa XI, BseJI, BseLI, BseSI, Bsi HKAI, BsmI, Bso BI, BsrI, Bsr FI, BsrSI, Bst BI, Bst EII, BstHHI, BstSF1, Bst NI, Bst UI, Bst Z17I, Bts CI, CspCI, Cvi QI, EsaBC3I, EsaBC4I, Hpa I, Hyp188I, Kpn I, MjaI, MjaII, MjaIII, MjaIV, MjaV, MspNI, Mwo I, Nci I, PabI, Pae R7I, Pho I, Ppu MI, PspGI, Pvu II, Sfi I, Sfo I, SmlI, SuiI, TaaI, TaiI, TaqI, Tti I, Taq52, TasI, TatI, TauI, TceI, TfiI, TfiL, TliI, TmaI, TneI, Tru1I, TscAI, TseI, Tsp1I, Tsp32I, Tsp32II, Tsp4C, Tsp8E, Tsp49I, TspMI, Tsp45C, Tsp45I, Tsp504I, Tsp505I, Tsp507I, Tsp509I, Tsp510I, Tsp514I, Tsp560I, TspAI, TspDTI, TspGWI, Tsp MI, TspRI, TteI, Tth111I, Tth111II, Tth24I, TthHB27I, TthHB8I, TthRQI, TtmI, TtmII, TtrI and Zra I. Combinations of any of the foregoing endonucleases are also contemplated herein.

[0041] In one aspect of this embodiment, amplification is carried out with a PCR, which reaction includes denaturing, extension, and annealing steps.

[0042] Preferably, the primer introduces a thermostable restriction endonuclease site into an amplicon of the normal allele but not an amplicon of the mutant allele after two rounds of PCR, and selectively amplifying the mutant allele of the gene or a portion thereof is accomplished by contacting the sample with a thermostable restriction endonuclease that recognizes the site introduced into the amplicon of the normal allele.

[0043] In this embodiment, the thermostable restriction endonuclease may be active in a PCR reaction mix. As used herein, "active", with respect to restriction endonucleases, means having the ability to digest DNA at the recognition site. A PCR reaction mix is well known in the art. PCR reaction mixes can be purchased from numerous vendors or mixed in the laboratory. Typically, a PCR reaction mix comprises several components, including, a DNA polymerase, deoxynucleotides, a buffer solution, the PCR template, forward and reverse primers, and water.

[0044] The denaturing step of the PCR may be carried out at a temperature that does not substantially irreversibly inactivate the thermostable restriction endonuclease. For example, the denaturing step of the PCR may be carried out at a temperature between about 70°C and about 90°C, such as about 70°C, 71°C, 72°C, 73°C, 74°C, 75°C, 76°C, 77°C, 78°C, 79°C, 80°C, 81°C, 82°C, 83°C, 84°C, 85°C, 86°C, 87°C, 88°C, 89°C, and 90°C. Ranges within any of these points are also contemplated.

[0045] Methods of lowering the temperature of the denaturing step in a PCR reaction are known in the art, such as, *e.g.*, those disclosed below in the Examples section. For instance, co-solvents such as, *e.g.*, glycerol, DMSO, NMP, formamide,

and combinations thereof, may be used to lower the temperature of the denaturing step. Furthermore, shortening the length of the PCR product, shortening the length of the primers, and/or lowering the guanine-cytosine (G-C) content of the PCR product may also be used effectively to lower the denaturing temperature.

[0046] The annealing step and/or the extension step of the PCR may be carried out at a temperature at which the thermostable restriction endonuclease is active.

[0047] For example, the annealing step of the PCR may be carried out at a temperature between about 55°C and about 72°C, such as about 55°C, 56°C, 57°C, 58°C, 59°C, 60°C, 61°C, 62°C, 63°C, 64°C, 65°C, 66°C, 67°C, 68°C, 69°C, 70°C, 71°C, and 72°C. Ranges within any of these points are also contemplated.

[0048] Additionally, the extension step of the PCR may be carried out at a temperature between 60°C and 72°C, such as 60°C, 61°C, 62°C, 63°C, 64°C, 65°C, 66°C, 67°C, 68°C, 69°C, 70°C, 71°C, and 72°C. Ranges within any of these points are also contemplated.

[0049] In a further aspect of this embodiment, the thermostable restriction endonuclease is selected from the group consisting of ApeKI, BclI, BgLII, BlpI, BsaXI, BseJI, BseLI, BseSI, BsiHkAI, BsmI, BsoBI, BsR FI, BsrI, BstBI, BstEII, BstHHI, BstNI, BstSF1, BstUI, BstZ17I, BtsCI, CspCI, EsaBC3I, EsaBC4I, Hyp188I, MjaI, MjaII, MjaIII, MjaIV, MjaV, MspNI, MwoI, PabI, PhoI, PspGI, SfiI, SmlI, SmlI, SuiI, TaaI, TaiI, TaqI, TasI, TatI, TauI, TceI, TfiI, TfiL, TliI, TmaI, TneI, Tru1I, TscAI, TseI, Tsp1I, Tsp32I, Tsp32II, Tsp45C, Tsp45I, Tsp504I, Tsp505I, Tsp507I, Tsp509I, Tsp510I, Tsp514I, Tsp560I, TspAI, TspDTI, TspGWI, TspRI, TteI, Tth111I, Tth111II, Tth24I, TthHB27I, TthHB8I, TthRQI, TtmI, TtmII, and TtrI. Preferably, the thermostable restriction endonuclease is selected from ApeKI, BsaXI, BseJI, BseLI,

BsmI, BsrI, BstBI, BstHHI, Hyp188I, MwoI, PhoI, PspGI, TaqI, TasI, TfiI, TseI, Tsp45C, Tsp45I, Tsp509I, and TspRI.

[0050] In another aspect of this embodiment, the amount of the mutant allele is at least about 0.02% of the normal allele in the sample such as, *e.g.*, at least about 0.03%, 0.04%, 0.05%, 0.06%, 0.07%, 0.08%, 0.09%, 0.1%, 0.2%, 0.3%,0.4%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, 1%, 1.5%, 2%, 2.5%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, or 10% of the normal allele in the sample. Ranges within any of these points are also contemplated.

[0051] In yet another aspect of this embodiment, the mutant allele is a marker for a cancer or other disease state. Non-limiting examples of cancers for which the mutant allele is a marker include, *e.g.*, adrenocortical carcinoma, anal cancer, bladder cancer, bone cancer, brain tumor, breast cancer, carcinoid tumor, carcinoma, cervical cancer, colon cancer, endometrial cancer, esophageal cancer, extrahepatic bile duct cancer, Ewing family of tumors, extracranial germ cell tumor, eye cancer, gallbladder cancer, gastric cancer, germ cell tumor, gestational trophoblastic tumor, head and neck cancer, hypopharyngeal cancer, islet cell carcinoma, kidney cancer, laryngeal cancer, leukemia, lip and oral cavity cancer, liver cancer, lung cancer, lymphoma, malignant mesothelioma, Merkel cell carcinoma, mycosis fungoides, myelodysplastic syndrome, myeloproliferative disorders, nasopharyngeal cancer, neuroblastoma, oral cancer, oropharyngeal cancer, osteosarcoma, ovarian epithelial cancer, ovarian germ cell tumor, pancreatic cancer, paranasal sinus and nasal cavity cancer, parathyroid cancer, penile cancer, pituitary cancer, plasma cell neoplasm, prostate cancer, rhabdomyosarcoma, rectal cancer, renal cell cancer, transitional cell cancer of the renal pelvis and ureter, salivary gland cancer, Sezary syndrome, skin cancer (such as cutaneous t-cell

lymphoma, Kaposi's sarcoma, and melanoma), small intestine cancer, soft tissue sarcoma, stomach cancer, testicular cancer, thymoma, thyroid cancer, urethral cancer, uterine cancer, vaginal cancer, vulvar cancer, Wilms' tumor. Preferably, the cancer is a breast cancer or other solid tumor cancer, such as lung cancer and colon cancer.

[0052] In yet another aspect of this embodiment, the mutant allele is selected from the group consisting of 185 deletion AG in BRCA1, 5382 insertion C in BRCA1, 6174 deletion T (6174 del T) in BRCA2, G12D 35G>A/T/C in K-ras, 38G>A/T/C in K-ras, V600E 1798 T>A in B-raf, E545K 1633 G>A in PIK3CA, 1624G>A in PIK3CA, 3140A>G in PIK3C1, and L858R 2573 T>G in EGFR.

[0053] In yet another aspect of this embodiment, a first forward primer produces a first and a second amplicon after two rounds of amplification, the first amplicon being an amplicon of the normal allele, which cannot serve as a template for subsequent amplification, and the second amplicon being an amplicon of the mutant allele.

[0054] In one preferred embodiment, the sample is contacted with a second forward primer that anneals to the second amplicon. Preferably, the ratio of the first forward primer to the second forward primer is less than 1:10, such as 1:20, 1:30, 1:40, 1:50, 1:60, 1:70, 1:80, 1:90, 1:100, 1:200, 1:300, 1:400, 1:500, 1:600, 1:700, 1:800, 1:900, 1:1000, 1:1500, 1:2000.

[0055] In another aspect of the present embodiment, the first amplicon forms a hairpin.

[0056] In another aspect of the present embodiment, the first forward primer is selected from the group consisting of TCTGTCCTGGGATTCTCTTGAGATGTGGTCAATGGAAGAAACCACCAAG (SEQ

ID NO: 29), GGGACACTCTAAGATTTTCTTCGCGTTGAAGAAGTACAAAATGTCATTA (SEQ ID NO: 34), and GACAGATTTTCCACTTGCTGTGCGGAAGCTTCATAAGTCAGTCTCATCTGCAAA TAC (SEQ ID NO: 39); and the second forward primer is selected from the group consisting of CCTGGGATTCTCTTGAGATGTGGTCAAT(SEQ ID NO: 30), ACACTCTAAGATTTTCTTCGCGTT (SEQ ID NO: 35), and ATTTTCCACTTGCTGTGCGGAAGCTTCATA (SEQ ID NO: 40), respectively.

[0057] In a further aspect of the present embodiment, the mutant allele is selected from the group consisting of 5382 insertion C in BRCA1, 185 deletion AG in BRCA1, and 6174 del T in BRCA2.

[0058] Another embodiment of the present invention is a method for detecting a mutant allele of a gene, if present, in a sample. The method comprises:

(a) contacting the sample with a thermostable restriction endonuclease that recognizes a sequence in a normal allele of the gene but not in a mutant allele of the gene;

(b) amplifying the mutant allele of the gene or a portion thereof, if present in the sample; and

(c) before or during step (b), selectively degrading the normal allele of the gene.

[0059] In this embodiment, the mutant allele is present in the sample, as a percentage of the normal allele, as set forth above. Preferably, the mutant allele is present in the sample in an amount that is at least about 0.02% of the normal allele. In addition, suitable thermostable restriction endonucleases for use in this embodiment are as disclosed above.

[0060] In one aspect of this embodiment, the amplification step comprises using a PCR, which reaction includes denaturing, extension, and annealing steps. Preferably, the denaturing step of the PCR is carried out at a temperature that does not substantially irreversibly inactivate the thermostable restriction endonuclease. Furthermore, the annealing step and/or the extension step of the PCR is preferably carried out at a temperature at which the thermostable restriction endonuclease is active. Suitable temperatures for each step are as disclosed above.

[0061] In another aspect of this embodiment, the sequence in the normal allele recognized by the thermostable restriction endonuclease is natively present in the normal allele. Alternatively, the sequence recognized by the restriction endonuclease may be introduced by a primer used to amplify a gene or a portion thereof. In the latter case, the sequence recognized by the restriction endonuclease will be introduced after the first two rounds of amplification. Non-limiting examples of the primers used in these reactions are disclosed in Figure 11.

[0062] In yet another aspect of this embodiment, the mutant allele of the gene is a marker for a cancer or other disease state. Various types of cancer that may be detected using the methods of the invention are as disclosed previously.

[0063] In yet another aspect of this embodiment, the mutant allele is selected from the group consisting of 185 deletion AG in BRCA1, 5382 insertion C in BRCA1, 6174 del T in BRCA2, G12D 35G>A/T/C in K-ras, 38G>A/T/C in K-ras, V600E 1798 T>A in B-raf, E545K 1633 G>A in PIK3CA, 1624G>A in PIK3CA, 3140A>G in PIK3C1, and L858R 2573 T>G in EGFR.

[0064] As set forth above, the source of the sample may be obtained from any organism, such as a mammal, *e.g.*, a human, a farm animal, a laboratory animal, or a pet.

[0065] Yet another embodiment of the present invention is a method for detecting a mutant allele of a gene, if present, in a sample using a PCR. The method comprises:

(a) contacting the sample with a first primer that produces a first and a second amplicon after two rounds of amplification, the first amplicon being an amplicon of the normal allele and cannot serve as a template for subsequent amplification, and the second amplicon being an amplicon of the mutant allele; and

(b) contacting the sample with a second primer that anneals to the second amplicon.

[0066] In one aspect of this embodiment, the first and the second primers are forward primers. The ratios of the first forward primer to the second forward primer are as disclosed above. Preferably, the ratio of the first forward primer to the second forward primer is less than 1:10.

[0067] In another aspect of this embodiment, the first amplicon forms a hairpin.

[0068] In an additional aspect of this embodiment, the first primer is selected from the group consisting of TCTGTCCTGGGATTCTCTTGAGATGTGGTCAATGGAAGAAACCACCAAG (SEQ ID NO: 29), GGGACACTCTAAGATTTTCTTCGCGTTGAAGAAGTACAAAATGTCATTA (SEQ ID NO: 34), and GACAGATTTTCCACTTGCTGTGCGGAAGCTTCATAAGTCAGTCTCATCTGCAAA TAC (SEQ ID NO: 39); and the second primer is selected from the group consisting of CCTGGGATTCTCTTGAGATGTGGTCAAT(SEQ ID NO: 30),

ACACTCTAAGATTTTCTTCGCGTT (SEQ ID NO: 35), and ATTTTCCACTTGCTGTGCGGAAGCTTCATA (SEQ ID NO: 40), respectively.

[0069] In a further aspect of this embodiment, the mutant allele is selected from the group consisting of 5382 insertion C in BRCA1, 185 deletion AG in BRCA1, and 6174 del T in BRCA2.

[0070] Another embodiment of the present invention is a template tool for use in selecting optimal PCR conditions for a method of detecting a mutant allele of a gene in a sample, which method selectively degrades a normal allele of the gene in the sample by a thermostable restriction endonuclease. This template tool comprises:

a first polynucleotide sequence comprising recognition sites for a plurality of thermostable endonucleases, and

a second polynucleotide sequence homologous to the first polynucleotide except that it does not contain a single recognition site for any one of the plurality of thermostable endonucleases.

[0071] As used herein, PCR conditions include the temperature and the duration of the various steps of the PCR, such as the denaturing step, the annealing step, and the extension step, as well as buffer selection for the PCR.

[0072] As used herein, "homologous" means two or more sequences that have at least 50%, 60% identity, preferably 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher identity when compared and aligned for maximum correspondence. Methods of aligning sequences for comparison are known in the art. (*See, e.g.*, Smith & Waterman, Adv. Appl. Math. 2:482 (1981); Needleman & Wunsch, J. Mol. Biol. 48:443 (1970), Pearson & Lipman, Proc. Nat'l. Acad. Sci. USA 85:2444 (1988)). In this aspect of the present invention,

in the second polynucleotide sequence, such recognition site may contain 1 or more mutations, or differences, in nucleotide sequence, such that each recognition site is non-functional or substantially non-functional. The first and second polynucleotides of this aspect of the invention are exemplified by the so-called "wildtype" and mutant sequences or templates, respectively, disclosed in Example 3 herein.

[0073] In one aspect of this embodiment, the first polynucleotide sequence comprises recognition sites for 10-50 different thermostable endonucleases, such as 15-45 different thermostable endonucleases, 20-40 different thermostable endonucleases, 25-35 different thermostable endonucleases, including 26, 27,28,29, 30, 31, 32, 33, 34, or 35 different thermostable endonucleases. Preferably, the first polynucleotide sequence comprises recognition sites for 30 different thermostable endonucleases.

[0074] In another aspect of this embodiment, the plurality of thermostable endonucleases are selected from the group consisting of any thermostable endonucleases disclosed herein or that are subsequently selected according to at least the following criteria: (1) the enzyme can survive in environments that are greater than 80°C for at least 20 minutes; and (2) the organism from which the enzyme originates grows in environments having a temperature between 50-70°C. Preferably, the plurality of thermostable endonucleases are selected from the group consisting: TasI, Tru1I, TspDTI, BstNI, PspGI, TscAI, TspRI, PhoI, TaaI, BseLI, MwoI, TseI, ApeKI, BtsCI, TfiI, Tsp45I, BstSF1, SmlI, TatI, TaiI, BsiHkAI, BseSI, BstHHI, TaqI, TspGWI, TauI, BclI, BstUI, Tth111I, BseJI, and combinations thereof.

[0075] In a further aspect of this embodiment, the recognition site for each of the plurality of thermostable endonucleases is selected from the group consisting of: AATT, TTAA, ATGAA, CCWGG, CCWGG, CASTG, CASTG, GGCC, ACNGT,

CCNNNNNNNGG, GCNNNNNNNGC, GCWGC, GCWGC, GGATG, GAWTC, GTSAC, CTRYAG, CTYRAG, WGTACW, ACGT, GWGCWC, GKGCMC, GCGC, TCGA, ACGGA, GCSGC, TGATCA, CGCG, GACNNNGTC, GATNNNNATC, and combinations thereof.

[0076] In an additional aspect of this embodiment, the first and the second polynucleotide sequences are from about 100 base pairs to about 600 base pairs in length, such as about 150 to about 550 base pairs, about 200 to about 500 base pairs, about 250 to about 450 base pairs, or about 300 to about 400 base pairs in length. Preferably, the first and the second polynucleotide sequences are about 300 base pairs in length. Also preferably, the first and the second polynucleotide sequences are the same length.

[0077] In another aspect of this embodiment, the melting temperature of the first and the second polynucleotide sequences are from about 75°C to about 90°C, including from about 76°C to about 88°C, from about 78°C to about 85°C, or from about 80°C to about 83°C. Preferably, the melting temperature of the first and the second polynucleotide sequences are about 80°C. Also preferably, the melting temperature of the first and the second polynucleotide sequences are the same. As used herein, the "melting temperature" of a polynucleotide means the temperature at which 50% of the oligonucleotide and its perfect complement are in duplex. Methods of determining melting temperature are known in the art. The most accurate method is to carry out the determination empirically, but various studies have derived accurate equations for melting temperature using thermodynamic basis sets for nearest neighbor interactions. (See, *e.g.*, Breslauer, K.J.; Frank, R.; Blšcker, H.; Marky, L.A. Proc. Natl. Acad. Sci. USA 83, 3746-3750(1986)).

[0078] In an additional aspect of this embodiment, the first polynucleotide sequence comprises a sequence as depicted in SEQ ID NO:69 and the second polynucleotide sequence comprises a sequence as depicted in SEQ ID NO:70.

[0079] Another embodiment of the present invention is a kit. This kit comprises any template tool disclosed herein. The kit may optionally include directions for its use, buffers and other reagents for facilitating use of the template tool. Furthermore, each of the first and second polynucleotide sequences may be packaged together or separately. Each of the polynucleotide sequences may be packaged in any convenient form, *e.g.*, they may be lyophilized or in solution.

[0080] This template tool may be used in conjunction with other methods disclosed herein, including for example, a method for detecting a mutant allele of a gene, if present, in a sample. The method comprises:

(a) contacting the sample with a thermostable restriction endonuclease that recognizes a sequence in a normal allele of the gene but not in a mutant allele of the gene;

(b) amplifying the mutant allele of the gene or a portion thereof, if present in the sample; and

(c) before or during step (b), selectively degrading the normal allele of the gene.

[0081] As set forth above, the amplification step (step (b)) may comprise using a PCR, which reaction includes denaturing extension, and annealing steps. Thus, conditions for these PCR steps may be selected by using a template tool according to the present invention, which comprises:

a first polynucleotide sequence comprising recognition sites for a plurality of thermostable endonucleases, and

a second polynucleotide sequence homologous to the first polynucleotide except that it does not contain a single recognition site for any one of the plurality of thermostable endonucleases.

[0082] Another embodiment of the present invention is a template tool for use in selecting optimal PCR conditions for a method of selectively amplifying mutant alleles present in low abundance in solid tumors, which method selectively degrades a normal allele or an amplicon of the normal allele in the sample by a thermostable restriction endonuclease, the template tool comprising:

a first polynucleotide sequence comprising recognition sites for a plurality of thermostable endonucleases, and

a second polynucleotide sequence homologous to the first polynucleotide except that it does not contain a single recognition site for any one of the plurality of thermostable endonucleases.

[0083] As used herein, "low abundance" means that the mutant allele is less than 50% of the normal allele, including less than 20%, less than 10%, less than 5%, less than 2%, less than 1%, less than 0.5%, less than 0.1%, less than 0.05%, and less than 0.02%.

[0084] In one aspect of this embodiment, the first polynucleotide sequence comprises recognition sites for any number of different thermostable endonucleases, including, *e.g.*, for 10-50 different thermostable endonucleases, such as 15-45 different thermostable endonucleases, 20-40 different thermostable endonucleases, 25-35 different thermostable endonucleases, including 26, 27, 28, 29, 30, 31, 32, 33, 34, or 35 different thermostable endonucleases. Preferably, the first polynucleotide sequence comprises recognition sites for 30 different thermostable endonucleases

[0085] In another aspect of this embodiment, the plurality of thermostable endonucleases are selected from the group consisting of any thermostable endonucleases disclosed herein or as subsequently selected using as least the criteria set forth above. Preferably, the plurality of thermostable endonucleases are selected from the group consisting: TasI, Tru1I, TspDTI, BstNI, PspGI, TscAI, TspRI, PhoI, TaaI, BseLI, MwoI, TseI, ApeKI, BtsCI, TfiI, Tsp45I, BstSF1, SmlI, TatI, TaiI, BsiHkAI, BseSI, BstHHI, TaqI, TspGWI, TauI, BclI, BstUI, Tth111I, BseJI, and combinations thereof.

[0086] In a further aspect of this embodiment, the recognition site for each of the plurality of thermostable endonucleases is selected from the group consisting of: AATT, TTAA, ATGAA, CCWGG, CCWGG, CASTG, CASTG, GGCC, ACNGT, CCNNNNNNNGG, GCNNNNNNNGC, GCWGC, GCWGC, GGATG, GAWTC, GTSAC, CTRYAG, CTYRAG, WGTACW, ACGT, GWGCWC, GKGCMC, GCGC, TCGA, ACGGA, GCSGC, TGATCA, CGCG, GACNNNGTC, GATNNNNATC, and combinations thereof.

[0087] In an additional aspect of this embodiment, the first and the second polynucleotide sequences are from about 100 base pairs to about 600 base pairs in length, such as about 150 to about 550 base pairs, about 200 to about 500 base pairs, about 250 to about 450 base pairs, or about 300 to about 400 base pairs in length. Preferably, the first and the second polynucleotide sequences are about 300 base pairs in length. Also preferably, the first and the second polynucleotide sequences are the same length.

[0088] In another aspect of this embodiment, the melting temperature of the first and the second polynucleotide sequences are from about 75°C to about 90°C, including from about 76°C to about 88°C, from about 78°C to about 85°C, or from

about 80°C to about 83°C. Preferably, the melting temperature of the first and the second polynucleotide sequences are about 80°C. Also preferably, the melting temperature of the first and the second polynucleotide sequences are the same.

[0089] In an additional aspect of this embodiment, the first polynucleotide sequence comprises a sequence as depicted in SEQ ID NO:69 and the second polynucleotide sequence comprises a sequence as depicted in SEQ ID NO:70.

[0090] As used herein, the terms “nucleic acid," "oligonucleotide," and "polynucleotide" are used interchangeably. In the present invention, these terms mean at least two nucleotides covalently linked together, or any artificially constructed molecules that mimic such a chain. Nucleic acids include without limitation, deoxyribonucleic acid (DNA), ribonucleic acid (RNA), peptide nucleic acid (PNA), morpholino and locked nucleic acid (LNA), glycol nucleic acid (GNA) and threose nucleic acid (TNA).

[0091] Nucleic acids may be single stranded or double stranded, or may contain portions of both double stranded and single stranded sequences. As noted above, the nucleic acid may be DNA, both genomic and cDNA, RNA, or a hybrid, where the nucleic acid may contain combinations of deoxyribo- and ribo-nucleotides, and combinations of bases including uracil, adenine, thymine, cytosine, guanine, inosine, xanthine, hypoxanthine, isocytosine and isoguanine. Nucleic acids may be synthesized as a single stranded molecule or expressed in a cell (*in vitro* or *in vivo*) using a synthetic gene. Nucleic acids may be obtained by chemical synthesis methods or by recombinant methods.

[0092] As noted above, the nucleic acid may also be a RNA such as a mRNA, tRNA, shRNA, siRNA, Piwi-interacting RNA, pri-miRNA, pre-miRNA, miRNA, or anti-miRNA, as described, *e.g.*, in U.S. Patent Application Nos. 11/429,720, 11/384,049,

11/418,870, and 11/429,720 and Published International Application Nos. WO 2005/116250 and WO 2006/126040.

[0093] A nucleic acid will generally contain phosphodiester bonds, although nucleic acid analogs for use, *e.g.*, in the primers of the present invention, may be included that may have at least one different linkage, *e.g.*, phosphoramidate, phosphorothioate, phosphorodithioate, or O-methylphosphoroamidite linkages and peptide nucleic acid backbones and linkages. Other analog nucleic acids include those with positive backbones; non-ionic backbones, and non-ribose backbones, including those disclosed in U.S. Pat. Nos. 5,235,033 and 5,034,506. Nucleic acids containing one or more non-naturally occurring or modified nucleotides are also included within the definition of nucleic acid.

[0094] In this invention, any suitable method for detecting amplified nucleic acids may be used. For example, the amplified nucleic acid species may be sequenced directly using any suitable nucleic acid sequencing method, such as conventional dideoxy sequencing methods, pyrosequencing, nanopore based sequencing methods (*e.g.*, sequencing by synthesis), sequencing by ligation, sequencing by hybridization, and microsequencing (primer extension based polymorphism detection).

[0095] The following examples are provided to further illustrate the methods of the present invention. These examples are illustrative only and are not intended to limit the scope of the invention in any way.

EXAMPLES

Example 1

Selective Amplification of Low-Quantity Mutant Alleles by Inclusion of Restriction Endonucleases in PCR Reaction

[0096] As illustrated in Figure 1, to selectively amplify the mutant gene allele, one strategy includes (1) primer design that allows amplified products to denature at lower temperatures and creates a thermostable restriction endonuclease site in normal alleles lacking endogenous restriction sites; (2) inclusion of a thermostable endonuclease chosen to destroy the amplicon of the normal gene during each PCR cycle; (3) a change in the typical PCR denaturation step (95°C / 30 seconds) to an optimized lower temperature and shorter duration that does not destroy endonuclease activity but does allow denaturation of amplified dsDNA products; and (4) inclusion of co-solvents that reduce dsDNA denaturation temperatures from the typical 94°C or 95°C and enhance enzyme thermo-stability.

[0097] Primer design for selective amplification of low quantity mutant alleles should account for primer length, product length, and melting temperature. As seen in Figure 13, a large number of designed PCR primers are 20 bp in length. Additionally, as seen in Figure 14, PCR products commonly range in length from about 100-300 bp. Using these criteria, over one hundred 60-300 bp high stringency amplification products were selected through an automatic search using Oligo software, version 7 (Molecular Biology Insights, Inc., Cascade, CO). Figure 15 shows that 68% of these products had a melting temperature (T_m) that was less than 85°C while 90% of products had T_m less than 90°C. Using the same data, Figure 16 exhibits a correlation between T_m and PCR product length and Figure 18 shows the same phenomenon with a different data set. Therefore, T_m can be reduced by

designing smaller PCR products. Another method of reducing T_m is to design PCR products with low guanine-cytosine (G-C) content (Figure 17).

[0098] As seen in Figure 2, a 466 bp fragment from mouse adenylate cyclase subtype 5 and a 178 bp fragment from glyceraldehyde-3-phosphate dehydrogenase (GAPDH) can be effectively amplified under denaturing temperatures of 90°C and 87.5°C, respectively. Annealing and extension were performed at 72°C for both reactions. Additionally, as seen in Figure 3, a 185 bp fragment from human β-actin can be effectively amplified under a denaturing temperature of 85°C with annealing and extension performed at 55°C and 70°C, respectively. Furthermore, a 179 bp fragment from human β-actin can also be effectively amplified under a denaturing temperature of 81°C, with annealing and extension temperatures of 55°C and 70°C, respectively. (Figure 4). Finally, a 131 bp fragment from human chromosome 6 (AL731777/GI29801752) can be effectively amplified under a denaturing temperature of 72.5°C, with annealing and extension both performed at 60°C (Figure 5).

[0099] The denaturing temperatures may also be reduced using optimum co-solvents in the PCR reaction mix. For example, Tables 1 and 2 below (cited from Innis *et al*. ed. PCR Strategies, Academic Press, 1995) show the influence of some common co-solvents on the melting temperature (T_m) and strand dissociation temperature (T_{ss}).

TABLE 1

		PCR Buffer		PCR Buffer: 5% formamide, 10% glycerol			
DNA	% G+C	T_m	T_{ss}	T_m	T_{ss}	ΔT_m	ΔT_{ss}
Lambda	50	90.2	95	84	90-91	-6.2	-4-5
Herring sperm	42	86.5	(98)	82	86-92	-4.5	(-6)
Clostridium	32	80.6	85	76	78-79	-4.6	-6
Micrococcus luteus	72	99.5	(102.5)	93	94-97	-6.5	(-7)
Human – HL60	42	86	96-98	80.6	87-92	-5.4	-6

( ): estimated value.

TABLE 2

DNA	T_m (°C)	ΔT_m (°C)
PCR Buffer Alone	90.2	
+ 10% Glycerol	87.2	-3.0
+ 10% DMSO	84.4	-5.8
+ 10% NMP	79.6	-10.6
+ 10% Formamide	83.6	-6.6
+ 5% Formamide and 10% Glycerol	84.0	-6.2

[0100] As seen in Figure 8, a 131 bp fragment from human chromosome 6 can be effectively amplified under a denaturing temperature of 66.4°C with 20% glycerol as a co-solvent. Annealing and extension temperatures were 35°C and 60°C, respectively. Likewise, the same fragment can be effectively amplified under a denaturing temperature of 69°C with 10% glycerol as a co-solvent. Annealing and extension temperatures were 35°C and 60°C, respectively. These experiments show that PCR may be carried out at denaturing temperatures that are lower than the typical 94°C or 95°C.

[0101] Lowering the T_m allows for the inclusion of restriction endonucleases in the PCR reaction mix without substantially irreversibly inactivate them. Several restriction endonucleases come from source organisms that grow at high temperatures (Table 3). Thus, they can withstand and even retain their endonuclease activity at high temperatures. Other available thermostable restriction

endonucleases are shown in Tables 4 and 5. Furthermore, as little as eight thermostable restriction endonucleases can recognize nearly 60% of SNPs randomly generated from genes NM_000015 to NM_000034 (Table 6).

TABLE 3

Growth Temperatures for Microorganisms that Express Given Thermostable Restriction Endonucleases

Restriction endonuclease	Growth Temperature of the source microorganisms(°C)
ApeKI	95
PspGI	95
PhoI	98
PabI	103
TmaI	80
EsaBC3I	85
EsaBC4I	85
MjaI	85
MjaII	85
MjaIII	85
MjaIV	85
MjaV	85
TceI	85
TliI	85
TneI	85
Tsp1I	70
Tsp32I	70
Tsp32II	70
Tsp45I	70
Tsp504I	70
Tsp505I	70
Tsp507I	70
Tsp509I	70
Tsp510I	70
Tsp514I	70
TspAI	70
MspNI	75
SuiI	75
TteI	75
Tth24I	75
Tth111I	75
Tth111II	75
TthHB8I	75
TthHB27I	75
TthRQI	75

Restriction endonuclease	Growth Temperature of the source microorganisms(°C)
TtmI	75
TtmII	75
TtrI	75

TABLE 4

Thermostable Restriction Endonucleases (from New England Biolabs, Ipswich, MA) and Their Compatibility with PCR Mixtures

Enzyme	Recognition Sequence	Optimum Temperature (°C)	Inactivated at 80°C / 20 min	PCR buffer 1	PCR buffer 2	PCR buffer 3	PCR buffer 4
PspGI	CCWGG	75	n	+++	+++	+++	+++
PhoI	GGCC	75	n	<++	++	+++	+++
MwoI	GC7NGC	60	Y	+++	+++	+++	+++
ApeKI	GCWGC	75	n	<++	<++	+++	++
BsmI	CGAATCGCN	65	y	+++	+++	<+	+
BsrI	ACTGGN	65	y	+++	+++	+++	+
BstBI	TTCGAA	65	y	+++	+++	+++	+++
taqI	TCGA	65	y	+++	+++	+++	+++
TfiI	CAWTG	65	y	<++	<++	+	+++
TspRI	NNCASTGNN	65	n	+	+	<+	++
TseI	GCWGC	65	n	+++	+++	+++	+++
Tsp509I	AATT	65	n	+++	+++	+++	+++
Tsp45I	GTSAC	65	n	<+	+++	-	++

TABLE 5

Restriction Enzymes That Do Not Inactivate When Exposed To 80°C for 20 Minutes (from New England Biolabs, Ipswich, MA)

Enzyme	Optimum Temperature (°C)
BclI	50
BgLII	37
BlpI	37
BsaXI	37
BsoBI	37
BsR FI	37
BstBI	65
BstEII	60
BstNI	60
BstUI	60
BstZ17I	37
CspCI	37

Enzyme	Optimum Temperature (°C)
MwoI	60
PhoI	75
PspGI	75
SfiI	50
SmlI	55
TfiL	75
TliI	75
TseI	65
Tsp45I	65
Tsp509I	65
TspRI	65
Tth111I	65

TABLE 6

Percentages of SNP Sequences Recognized by Eight Selected Thermo-Tolerant Restriction Endonucleases (100 Randomly Picked SNPs from Genes NM_000015 - NM_000034)

Enzyme	Recognition Sequence	%
BseLI	CCNNNNNNGG	31
MwoI	GCNNNNNNNGC	18
BsaXI	ACNNNNNCTTC	12
TaqI	TCGA	4
TasI	AATT	4
BstHHI	GCGC	6
TspRI	CASTG	4
Tsp45C	GTSAC	4

[0102] As seen in Figure 6, the restriction endonuclease PhoI retains activity under a 92°C denaturing temperature and blocks the amplification of a 388 bp fragment from human GAPDH containing PhoI, MwoI, and BstNI restriction sites. The fragment was amplified using brain cDNA by Advantage 2 PCR kit (Clontech, Mountain View, CA). Denaturing, annealing, and extension temperatures were 92°C, 60°C, and 75°C, respectively. In Figure 7, MwoI retains activity under an 83°C-88°C denaturing temperature and effectively blocks the amplification of an 86 bp fragment from human guanylate cyclase 2F containing an MwoI restriction site. Annealing and extension temperatures were 60°C and 70°C, respectively.

[0103] Because the denaturing temperature may be lowered, thermostable restriction endonucleases that are active during PCR are widely available, and because the large number of SNP wild type gene sequences that are recognized by just a small number of thermostable restriction endonucleases, this strategy of selective amplification of low-quantity mutant alleles by inclusion of restriction endonucleases in a one-pot PCR reaction should be widely applicable in most circumstances.

Example 2

Selective Amplification of Low-Quantity Mutant Alleles by Inclusion of Primers Designed to Induce Hairpin Formation in Wild-type Amplicons

[0104] As illustrated in Figure 9, to selectively amplify a mutant gene allele, another strategy includes (1) inclusion of a low concentration of a specific forward primer that produces a hairpin-forming amplicon of the normal allele after the first round of PCR that cannot serve as a template for subsequent amplification; and (2) inclusion of a high concentration of a second forward primer with an appropriate T_m that anneals only to amplification products resulting from extension of the initial forward primer and not to the starting DNA, as well as a reverse primer, allowing for selective PCR amplification of the mutant allele.

[0105] Experiments have been performed for this strategy using a single nucleotide base difference between the rat (as mutant) (SEQ ID NO: 47) and mouse (as wild) β-actin gene (SEQ ID NO: 48). Mouse and rat brain total RNA were isolated using TRIzol reagent (Ambion / Life technologies, Grand Island, NY). 1 μg total RNA was used for cDNA synthesis with an Advantage RT-for PCR kit (Clontech, Mountain View, CA) using an oligo(dT) primer according to the manufacturer's protocol and diluted to 100 μl. Rat cDNA was diluted 10, 50, 100,

200, 500, 1000, 2000 and 5000 fold, and mixed 1:1 with undiluted mouse cDNA. PCR#was performed using an Advantage 2 PCR kit (Clontech, Mountain View, CA). Each 20 μl reaction contained a 2 μl mouse and rat cDNA mixture. The final concentration of initial forward primer (SEQ ID NO: 44), successive forward primer (SEQ ID NO: 45), and reverse primer (SEQ. ID NO: 46) were 0.008 μM, 8 μM, and 1 μM, respectively. The first cycle of the PCR was carried out at 94°C for 1 minute, 89°C for 15 seconds, and 70°C for 10 minutes. Then, PCR was carried out at 94°C for 1 minute, 89°C for 15 seconds, and 70°C for 1 minute for 36 cycles.

[0106] Next, a 15 μl digestion reaction containing 7 μl of the above PCR product and 0.75μl XbaI (20U / μl, New England Biolabs, Ipswich, MA) was incubated at 37°C for one hour. Alternatively, the digestion reaction containing 7 μl of the above PCR product and 0.50 μl Tru9I (12U / μl, Promega, Madison, WA) was incubated at 65°C for one hour.

[0107] The results of this experiment (Figure 10) showed that this method allowed the detection of the rat allele even when its starting DNA copies represented only 0.02% of the mouse allele.

Example 3

Selective Amplification of Mutant Alleles by Addition of Thermostable Restriction Endonucleases

[0108] Quantitative polymerase chain reaction (qPCR) was performed on an Applied Biosystems 7500 PCR system using version 2.06 software (Applied Biosystems/Life technologies, Carlsbad, CA). Two different qPCR kits were used: SYBR Advantage qPCR premix (Clontech Laboratories, Inc., Mountain View, CA) and Power SYBR Green PCR Master Mix (Applied Biosystems). qPCR conditions followed the manufacturers' recommendations. Reactions were carried out in 20 μl reaction volumes and included 0 (control) or 2-3 units of the restriction endonuclease

as indicated in the Figures 19-22. In studies in which the wildtype template was degraded before qPCR, Applied Biosystems' kit was used. PCR was initiated at the temperature that was optimum for the restriction enzyme in use for 10 to 15 minutes. The temperature was then increased to 95°C for 10 minutes and was followed by 40 cycles of denaturation temperatures of 84°C-87°C and annealing/extension temperatures of 65°C for 1 to 2 minutes. In studies in which both the wildtype template and the amplification product were degraded during qPCR, Clontech's qPCR kit was used. PCR was initiated at 85°C for 2 minutes to denature the template. This was followed by 40 cycles of PCR that included a denaturation step at 85°C for 15 seconds and annealing/extension and degrading wild type product at 65°C for 1 to 2 minutes or 65°C for 1 minute and increasing to 75°C for another 1 minute. PCR products were purified using the QAquick PCR purification kit and sequenced by the Sanger sequence method (Genewiz, NJ). The sequencing results were analyzed by Chromas Lite software.

[0109] Figure 19 shows the amplification plots of synthetic B-raf templates representing wild type (SEQ ID NO:67) or mutant (SEQ ID NO:68) sequences of the B-raf gene in the absence or presence of the thermostable enzyme TspRI. Quantitative PCR was performed on a synthetic wild type B-raf sequence (containing a TspRI endonuclease recognition site) or a synthetic mutant B-raf sequence (lacking a site, *i.e.*, the nucleotide sequence of the recognition site has been changed by at least one nucleotide compared to the wild type sequence). PCR was performed at a reduced denaturation temperature of 85°C for 40 cycles. The primer used for the reaction is listed in Table 7 below. Note that the TspRI enzyme destroys products of the wild type template during the PCR reaction (top tracing) resulting in a rightward shift in the amplification plot and Ct value indicative of a

>99% enzymatic destruction in the PCR product of the wild type allele. This would allow for an enrichment of the amplification of a less abundant mutant allele.

[0110] Figure 20 shows the DNA sequencing results of PCR products following amplification of a wild type and a mutant template of the B-raf gene present in a 1,000 : 1 ratio. PCR was performed with a 1,000-fold excess of wild type to mutant template co-incubated in the presence of the thermostable endonuclease TspRI which selectively destroys products of the wild type template. Note that despite a 1,000-fold greater amount of wild type template, the product of the mutant allele (bottom arrow) was still detectable following PCR.

[0111] Figures 21A-21G show the amplification plots of wild type genomic DNA in the absence or presence of thermostable restriction endonucleases known to digest wild type regions of several genes that commonly encode mutations in solid tumors. This feasibility study demonstrates that these enzymes degrade PCR products of wild type alleles which would allow for enhanced PCR amplification of mutant alleles which lack recognition sites for these enzymes.

[0112] Figure 22 shows the DNA sequencing results following enriched PCR amplification of a synthetic mutant site in a set of custom synthesized universal templates (SEQ ID NO:69-70), which allows for the identification of optimal conditions for the activities of thermostable enzymes. These enzymes would be used to degrade products of wild type amplicons allowing for the selective amplification of mutant templates present in low abundance in solid tumors. A custom synthetic DNA template containing enzyme recognition sites for 30 thermostable endonucleases was designed ("wild type template", SEQ ID NO:69) using the following selection criteria: (1) the enzyme can survive in environments that are greater than 80°C for at least 20 minutes; and (2) the organism from which

the enzyme originates grows in environments having a temperature between 50-70°C. These enzyme sites would be present in wild type genes but absent in mutant alleles. A second synthetic universal DNA template containing the complimentary mutations was synthesized ("mutant template", SEQ ID NO:70). Known ratios of this wild type and mutant template were combined in the presence of selected thermostable enzymes. Examples of two experiments are presented; a mixture of a wild type universal template and a mutant universal template were mixed in a 100:1 ratio and subjected to PCR amplification at a reduced denaturation temperature (85°C) in the presence of BseLI (*top tracing*) and Bse JI (*bottom tracing*). These enzymes successfully degraded the wild type amplification products allowing for the equal detection of the mutant product (*top tracing*) or the detection of only the mutant product (*bottom tracing*).

[0113] The primers used in the experimental results shown in Figures 19-22 are as follows:

Table 7

List of Primers

Primer for	Accession number	Primer sequence（5' to 3')
Figure 19, B-raf synthetic (wild/mutant) template		CTTATTGACTCTAAGAGGAAAGATGAAGTACTATG (SEQ ID NO: 51)
		CAATTCTTACCATCCACAAAATGGATCCAG (SEQ ID NO: 52)
Figure 21A B-raf	NM_004333	CTTATTGACTCTAAGAGGAAAGATGAAGTACTATG (SEQ ID NO: 53)
		CAATTCTTACCATCCACAAAATGGATCCAG (SEQ ID NO: 54)
Figure 21B BRCA2	NM_000059	CTGCAAATACTTGTGGGATTTTTAGCACAGCCAG (SEQ ID NO: 55)*

Primer for	Accession number	Primer sequence（5' to 3')
		GACTTGCTTGGTACTATCTTCTATTTCAGAAAACA (SEQ ID NO: 56)
Figures 21C and 21F PIK3CA	NM_006218	GAGACAATGAATTAAGGGAAAATGACAA (SEQ ID NO: 57)
		CTGTGACTCCATAGAAAATCTTTCTC (SEQ ID NO: 58)
Figure 21D K-ras	NM_033360	GCTGAAAATGACTGAATATAAACTTGTGGTAGTTGGAACTG (SEQ ID NO: 59)*
		ATATTCGTCCACAAAATGATTCTGAATTAGCTGTA (SEQ ID NO: 60)
Figure 21E K-ras	NM_033360	GACTGAATATAAACTTGTGGTAGTTGGCGCTGGTG(SEQ ID NO: 61)*
		AGAGAAACCTTTATCTGTATCAAAGAATGGTCCTG(SEQ ID NO: 62)
Figure 21G PIK3CA	NM_006218	AGACCCTAGCCTTAGATAAAACTGAGCAAG (SEQ ID NO: 63)
		TCAATGCATGCTGTTTAATTGTGTGGAAGA (SEQ ID NO: 64)
Figure 22 For universal template		TTGACTCTATGATGAAAGATGTTGTT (SEQ ID NO: 65)
		ATTACGATCCACAAAATGTATGCAG (SEQ ID NO: 66)

*Mismatch to construct thermo-stable restriction endonuclease recognition site

[0114] The universal wildtype template contains 30 thermostable restriction sites, which sites are not in the universal mutant template. These sites are listed in Table 8 below.

Table 8

Thermostable Restriction Sites in the Universal Wildtype Template

recognition sequence	Name	Optimum temperature	Growth Temperature	average fragment length for enzyme over entire human genome	Commercial Availability
AATT	TasI	65	72	133	Fermentas
TTAA	Tru1I	65	60	150	Fermentas
ATGAA	TspDTI	70	70	276	Eurx
CCWGG	BstNI	60	55	292	NEB
CCWGG	PspGI	75	95	292	NEB
CASTG	TscAI	65	70	330	Fermentas
CASTG	TspRI	65	70	330	NEB
GGCC	PhoI	75	98	343	NEB
ACNGT	TaaI	65	72	380	Fermentas
CCNNNNNNNGG	BseLI	55	55	382	Fermentas
GCNNNNNNNGC	MwoI	60	60	469	NEB
GCWGC	TseI	65	70	556	NEB
GCWGC	ApeKI	75	95	556	NEB
GGATG	BtsCI	50	55	558	NEB
GAWTC	TfiI	65	70	610	NEB
GTSAC	Tsp45I	65	70	726	NEB
CTRYAG	BstSF1	60	55	827	SibEnzymes
CTYRAG	SmlI	55	30	1059	NEB
WGTACW	TatI	65	65	1063	Fermentas
ACGT	TaiI	65	65	1330	Fermentas
GWGCWC	BsiHkAI	65	37	1406	NEB
GKGCMC	BseSI	55	55	1444	Fermentas
GCGC	BstHHI	50	55	1728	SibEnzymes

recognition sequence	Name	Optimum temperature	Growth Temperature	average fragment length for enzyme over entire human genome	Commercial Availability
TCGA	TaqI	65	70	1894	Fermentas
ACGGA	TspGWI	70	70	2878	Eurx
GCSGC	TauI	55	70	3188	Fermentas
TGATCA	BclI	50	70	3911	NEB
CGCG	BstUI	60	55	3941	NEB
GACNNNGTC	Tth111I	65	75	4068	NEB
GATNNNNATC	BseJI	65	65	6436	Fermentas

Example 4

Cancer Detection By Selective Amplification of Mutant Alleles

[0115] Additional PCR conditions (primer design, denaturation temperatures, choice of thermostable restriction endonucleases) for both of the strategies set forth in the above Examples have been determined herein for the most common single nucleotide point or small insertion/deletion mutations in breast cancer (*e.g.* BRCA1, BRCA2, K-ras, B-raf, PIK3CA and EGFR) and other solid tumor cancers. The PCR conditions are shown in Figures 11 and 12.

[0116] The methods disclosed herein enhance the sensitivity of detecting cancer in tissue samples. The methods modify existing PCR methods to detect, with greater sensitivity, the existence of, *e.g.*, a single nucleotide point mutation known to cause a variety of solid tumors (breast, colon, lung cancer).

[0117] The methods disclosed herein offer much greater sensitivity for detection of cancer when only a small fraction of the cells have the mutation. Current

PCR methods can only amplify a mutant allele from a mixture of mutant and normal cells when the mutant allele represents at least about 10% of the total cells. The methods of the present invention enhance this sensitivity as much as 500-fold such that mutant cells may be detected when they represent only 0.02% of the total cells.

[0118] All documents cited in this application are hereby incorporated by reference as if recited in full herein.

[0119] Although illustrative embodiments of the present invention have been described herein, it should be understood that the invention is not limited to those described, and that various other changes or modifications may be made by one skilled in the art without departing from the scope or spirit of the invention.

WHAT IS CLAIMED IS:

1. A method for selectively amplifying a mutant allele comprising:

(a) contacting a sample comprising a mutant and a normal allele of a gene with at least one primer that selectively modifies the normal allele of the gene and causes further amplification of the normal allele to fail or to be substantially reduced; and

(b) selectively amplifying the mutant allele of the gene or a portion thereof.

2. The method according to claim 1, which is carried out with a polymerase chain reaction (PCR), which reaction includes denaturing, extension, and annealing steps.

3. The method according to claim 2, wherein the primer introduces a thermostable restriction endonuclease site into an amplicon of the normal allele but not an amplicon of the mutant allele after two rounds of PCR, and wherein step (b) comprises contacting the sample with a thermostable restriction endonuclease that recognizes the site introduced into the amplicon of the normal allele.

4. The method according to claim 3, wherein the thermostable restriction endonuclease is active in a PCR reaction mix.

5. The method according to claim 3, wherein the denaturing step of the PCR is carried out at a temperature that does not substantially irreversibly inactivate the thermostable restriction endonuclease.

6. The method according to claim 5, wherein the denaturing step of the PCR is carried out at a temperature between about 70°C and about 90°C.

7. The method according to claim 3, wherein the annealing step and/or the extension step of the PCR are carried out at a temperature at which the thermostable restriction endonuclease is active.

8. The method according to claim 7, wherein the annealing step of the PCR is carried out at a temperature between about 55°C and about 72°C.

9. The method according to claim 7, wherein the extension step of the PCR is carried out at a temperature between about 60°C and about 72°C.

10. The method according to claim 3, wherein the thermostable restriction endonuclease is selected from the group consisting of ApeKI, BclI, BgLII, BlpI, BsaXI, BseJI, BseLI, BseSI, BsiHkAI, BsmI, BsoBI, BsR FI, BsrI, BstBI, BstEII, BstHHI, BstNI, BstSF1, BstUI, BstZ17I, BtsCI, CspCI, EsaBC3I, EsaBC4I, Hyp188I, MjaI, MjaII, MjaIII, MjaIV, MjaV, MspNI, MwoI, PabI, PhoI, PspGI, SfiI, SmlI, SmlI, SuiI, TaaI, TaiI, TaqI, TasI, TatI, TauI, TceI, TfiI, TfiL, TliI, TmaI, TneI, Tru1I, TseI, Tsp1I, Tsp32I, Tsp32II, Tsp45C, Tsp45I, Tsp504I, Tsp505I, Tsp507I, Tsp509I, Tsp510I, Tsp514I, Tsp560I, TspAI, TspDTI, TspGWI, TspRI, TtiI, Tth111I, Tth111II, Tth24I, TthHB27I, TthHB8I, TthRQI, TtmI, TtmII, and TtrI.

11. The method according to claim 10, wherein the thermostable restriction endonuclease is selected from the group consisting of ApeKI, BsaXI, BseJI, BseLI, BsmI, BsrI, BstBI, BstHHI, Hyp188I, MwoI, PhoI, PspGI, TaqI, TasI, TfiI, TseI, Tsp45C, Tsp45I, Tsp509I, and TspRI.

12. The method according to claim 1, wherein the amount of the mutant allele is at least about 0.02% of the normal allele in the sample.

13. The method according to claim 1, wherein the mutant allele is a marker for a cancer.

14. The method according to claim 1, wherein the mutant allele is selected from the group consisting of 185 deletion AG in BRCA1, 5382 insertion C in BRCA1, 6174 deletion T (6174 del T) in BRCA2, G12D 35G>A/T/C in K-ras, 38G>A/T/C in K-ras, V600E 1798 T>A in B-raf, E545K 1633 G>A in PIK3CA, 1624G>A in PIK3CA, 3140A>G in PIK3C1, and L858R 2573 T>G in EGFR.

15. The method according to claim 1, wherein a first forward primer produces a first and a second amplicon after two rounds of amplification, the first amplicon being an amplicon of the normal allele, which cannot serve as a template for subsequent amplification, and the second amplicon being an amplicon of the mutant allele.

16. The method according to claim 15, further comprising contacting the sample with a second forward primer that anneals to the second amplicon.

17. The method according to claim 16, wherein the ratio of the first forward primer to the second forward primer is less than 1:10.

18. The method according to claim 15, wherein the first amplicon forms a hairpin.

19. The method according to claim 15, wherein the first forward primer is selected from the group consisting of TCTGTCCTGGGATTCTCTTGAGATGTGGTCAATGGAAGAAACCACCAAG (SEQ ID NO: 29), GGGACACTCTAAGATTTTCTTCGCGTTGAAGAAGTACAAAATGTCATTA (SEQ ID NO: 34), and GACAGATTTTCCACTTGCTGTGCGGAAGCTTCATAAGTCAGTCTCATCTGCAAA TAC (SEQ ID NO: 39); and the second forward primer is selected from the group consisting of CCTGGGATTCTCTTGAGATGTGGTCAAT(SEQ ID NO: 30), ACACTCTAAGATTTTCTTCGCGTT (SEQ ID NO: 35), and ATTTTCCACTTGCTGTGCGGAAGCTTCATA (SEQ ID NO: 40), respectively.

20. The method according to claim 15, wherein the mutant allele is selected from the group consisting of 5382 insertion C in BRCA1, 185 deletion AG in BRCA1, and 6174 del T in BRCA2.

21. A method for detecting a mutant allele of a gene, if present, in a sample comprising:

(a) contacting the sample with a thermostable restriction endonuclease that recognizes a sequence in a normal allele of the gene but not in a mutant allele of the gene;

(b) amplifying the mutant allele of the gene or a portion thereof, if present in the sample; and

(c) before or during step (b), selectively degrading the normal allele of the gene.

22. The method according to claim 21, wherein step (b) comprises using a polymerase chain reaction (PCR), which reaction includes denaturing, extension, and annealing steps.

23. The method according to claim 21, wherein the amount of the mutant allele is at least about 0.02% of the normal allele in the sample.

24. The method according to claim 21, wherein the thermostable restriction endonuclease is selected from the group consisting of ApeKI, BclI, BgLII, BlpI, BsaXI, BseJI, BseLI, BseSI, BsiHkAI, BsmI, BsoBI, BsR FI, BsrI, BstBI, BstEII, BstHHI, BstNI, BstSF1, BstUI, BstZ17I, BtsCI, CspCI, EsaBC3I, EsaBC4I, Hyp188I, MjaI, MjaII, MjaIII, MjaIV, MjaV, MspNI, MwoI, PabI, PhoI, PspGI, SfiI, SmlI, SmlI, SuiI, TaaI, TaiI, TaqI, TasI, TatI, TauI, TceI, TfiI, TfiL, TliI, TmaI, TneI, Tru1I, TscAI, TseI, Tsp1I, Tsp32I, Tsp32II, Tsp45C, Tsp45I, Tsp504I, Tsp505I, Tsp507I, Tsp509I, Tsp510I, Tsp514I, Tsp560I, TspAI, TspDTI, TspGWI, TspRI, TteI, Tth111I, Tth111II, Tth24I, TthHB27I, TthHB8I, TthRQI, TtmI, TtmII, and TtrI.

25. The method according to claim 24, wherein the thermostable restriction endonuclease is selected from the group consisting of ApeKI, BsaXI, BseJI, BseLI, BsmI, BsrI, BstBI, BstHHI, Hyp188I, MwoI, PhoI, PspGI, TaqI, TasI, TfiI, TseI, Tsp45C, Tsp45I, Tsp509I, and TspRI.

26. The method according to claim 21, wherein the sequence in the normal allele recognized by the thermostable restriction endonuclease is natively present in the normal allele.

27. The method according to claim 21, wherein the sequence in the normal allele recognized by the thermostable restriction endonuclease is introduced by a primer used to amplify the gene or a portion thereof.

28. The method according to claim 21, wherein the mutant allele of the gene is a marker for a cancer.

29. The method according to claim 21, wherein the mutant allele is selected from the group consisting of 185 deletion AG in BRCA1, 5382 insertion C in BRCA1, 6174 del T in BRCA2, G12D 35G>A/T/C in K-ras, 38G>A/T/C in K-ras, V600E 1798 T>A in B-raf, E545K 1633 G>A in PIK3CA, 1624G>A in PIK3CA, 3140A>G in PIK3C1, and L858R 2573 T>G in EGFR.

30. The method according to claim 21, wherein the sample is obtained from a human.

31. The method according to claim 22, wherein the denaturing step of the PCR is carried out at a temperature that does not substantially irreversibly inactivate the thermostable restriction endonuclease.

32. The method according to claim 22, wherein the annealing step and/or the extension step of the PCR is carried out at a temperature at which the thermostable restriction endonuclease is active.

33. A method for detecting a mutant allele of a gene, if present, in a sample using a polymerase chain reaction (PCR) comprising:

(a) contacting the sample with a first primer that produces a first and a second amplicon after two rounds of amplification, the first amplicon being an amplicon of the normal allele and cannot serve as a template for subsequent amplification, and the second amplicon being an amplicon of the mutant allele; and

(b) contacting the sample with a second primer that anneals to the second amplicon.

34. The method according to claim 33, wherein the first and the second primers are forward primers.

35. The method according to claim 34, wherein the ratio of the first forward primer to the second forward primer is less than 1:10.

36. The method according to claim 33, wherein the first amplicon forms a hairpin.

37. The method according to claim 33, wherein the first primer is selected from the group consisting of TCTGTCCTGGGATTCTCTTGAGATGTGGTCAATGGAAGAAACCACCAAG (SEQ ID NO: 29), GGGACACTCTAAGATTTTCTTCGCGTTGAAGAAGTACAAAATGTCATTA (SEQ ID NO: 34), and GACAGATTTTCCACTTGCTGTGCGGAAGCTTCATAAGTCAGTCTCATCTGCAAA TAC (SEQ ID NO: 39); and the second primer is selected from the group consisting of CCTGGGATTCTCTTGAGATGTGGTCAAT(SEQ ID NO: 30), ACACTCTAAGATTTTCTTCGCGTT (SEQ ID NO: 35), and ATTTTCCACTTGCTGTGCGGAAGCTTCATA (SEQ ID NO: 40), respectively.

38. The method according claim 33, wherein the mutant allele is selected from the group consisting of 5382 insertion C in BRCA1, 185 deletion AG in BRCA1, and 6174 del T in BRCA2.

39. A template tool for use in selecting optimal PCR conditions for a method of detecting a mutant allele of a gene in a sample, which method selectively degrades a normal allele of the gene in the sample by a thermostable restriction endonuclease, the template tool comprising:

a first polynucleotide sequence comprising recognition sites for a plurality of thermostable endonucleases, and

a second polynucleotide sequence homologous to the first polynucleotide except that it does not contain a single recognition site for any one of the plurality of thermostable endonucleases.

40. The template tool according to claim 39, wherein the first polynucleotide sequence comprises recognition sites for 10-50 different thermostable endonucleases.

41. The template tool according to claim 39, wherein the first polynucleotide sequence comprises recognition sites for 30 different thermostable endonucleases.

42. The template tool according to claim 39, wherein the plurality of thermostable endonucleases are selected from the group consisting of: TasI, Tru1I, TspDTI, BstNI, PspGI, TscAI, TspRI, PhoI, TaaI, BseLI, MwoI, TseI, ApeKI, BtsCI, TfiI, Tsp45I, BstSF1, SmlI, TatI, TaiI, BsiHkAI, BseSI, BstHHI, TaqI, TspGWI, TauI, BclI, BstUI, Tth111I, BseJI, and combinations thereof.

43. The template tool according to claim 39, wherein the recognition site for each of the plurality of thermostable endonucleases is selected from the group consisting of: AATT, TTAA, ATGAA, CCWGG, CCWGG, CASTG, CASTG, GGCC, ACNGT, CCNNNNNNNGG, GCNNNNNNNGC, GCWGC, GCWGC, GGATG, GAWTC, GTSAC, CTRYAG, CTYRAG, WGTACW, ACGT, GWGCWC, GKGCMC, GCGC, TCGA, ACGGA, GCSGC, TGATCA, CGCG, GACNNNGTC, GATNNNNATC, and combinations thereof.

44. The template tool according to claim 39, wherein the first and the second polynucleotide sequences are from about 100 base pairs to about 600 base pairs in length.

45. The template tool according to claim 39, wherein the melting temperature of the first and the second polynucleotide sequences are from about 75°C to about 90°C.

46. The template tool according to claim 39, wherein the first polynucleotide sequence comprises a sequence as depicted in SEQ ID NO:69 and the second polynucleotide sequence comprises a sequence as depicted in SEQ ID NO:70.

47. A kit comprising the template tool according to claim 39.

48. A kit comprising the template tool according to claim 46.

49. The method according to claim 22, wherein conditions for the PCR are selected by using a template tool comprising:

a first polynucleotide sequence comprising recognition sites for a plurality of thermostable endonucleases, and

a second polynucleotide sequence homologous to the first polynucleotide except that it does not contain a single recognition site for any one of the plurality of thermostable endonucleases.

50. A template tool for use in selecting optimal PCR conditions for a method of selectively amplifying mutant alleles present in low abundance in solid tumors, which method selectively degrades a normal allele or an amplicon of the normal allele in the sample by a thermostable restriction endonuclease, the template tool comprising:

a first polynucleotide sequence comprising recognition sites for a plurality of thermostable endonucleases, and

a second polynucleotide sequence homologous to the first polynucleotide except that it does not contain a single recognition site for any one of the plurality of thermostable endonucleases.

51. The template tool according to claim 50, wherein the first polynucleotide sequence comprises recognition sites for 30 different thermostable endonucleases.

52. The template tool according to claim 50, wherein the plurality of thermostable endonucleases are selected from the group consisting of: TasI, Tru1I, TspDTI, BstNI, PspGI, TscAI, TspRI, PhoI, TaaI, BseLI, MwoI, TseI, ApeKI, BtsCI, TfiI, Tsp45I, BstSF1, SmlI, TatI, TaiI, BsiHkAI, BseSI, BstHHI, TaqI, TspGWI, TauI, BclI, BstUI, Tth111I, BseJI, and combinations thereof.

53. The template tool according to claim 50, wherein the recognition site for each of the plurality of thermostable endonucleases is selected from the group consisting of: AATT, TTAA, ATGAA, CCWGG, CCWGG, CASTG, CASTG, GGCC, ACNGT, CCNNNNNNNGG, GCNNNNNNNGC, GCWGC, GCWGC, GGATG, GAWTC, GTSAC, CTRYAG, CTYRAG, WGTACW, ACGT, GWGCWC, GKGCMC, GCGC, TCGA, ACGGA, GCSGC, TGATCA, CGCG, GACNNNGTC, GATNNNNATC, and combinations thereof.

54. The template tool according to claim 50, wherein the first polynucleotide sequence comprises a sequence as depicted in SEQ ID NO:69 and the second polynucleotide sequence comprises a sequence as depicted in SEQ ID NO:70.

55. A kit comprising the template tool according to claim 50.

56. A kit comprising the template tool according to claim 54.

Figure 1

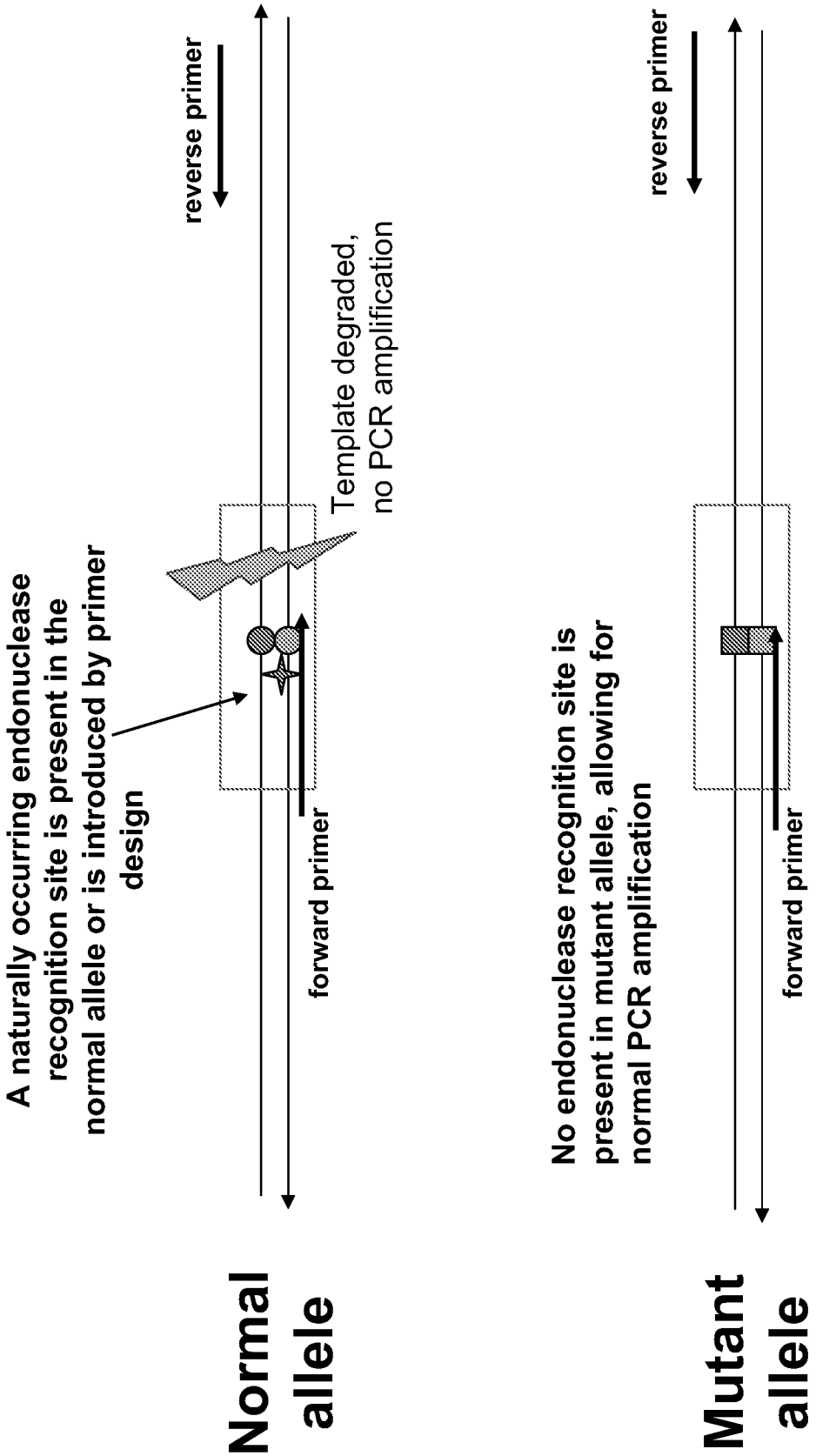

Normal allele
A naturally occurring endonuclease recognition site is present in the normal allele or is introduced by primer design
reverse primer
forward primer
Template degraded, no PCR amplification
Mutant allele
No endonuclease recognition site is present in mutant allele, allowing for normal PCR amplification
reverse primer
forward primer

Figure 2

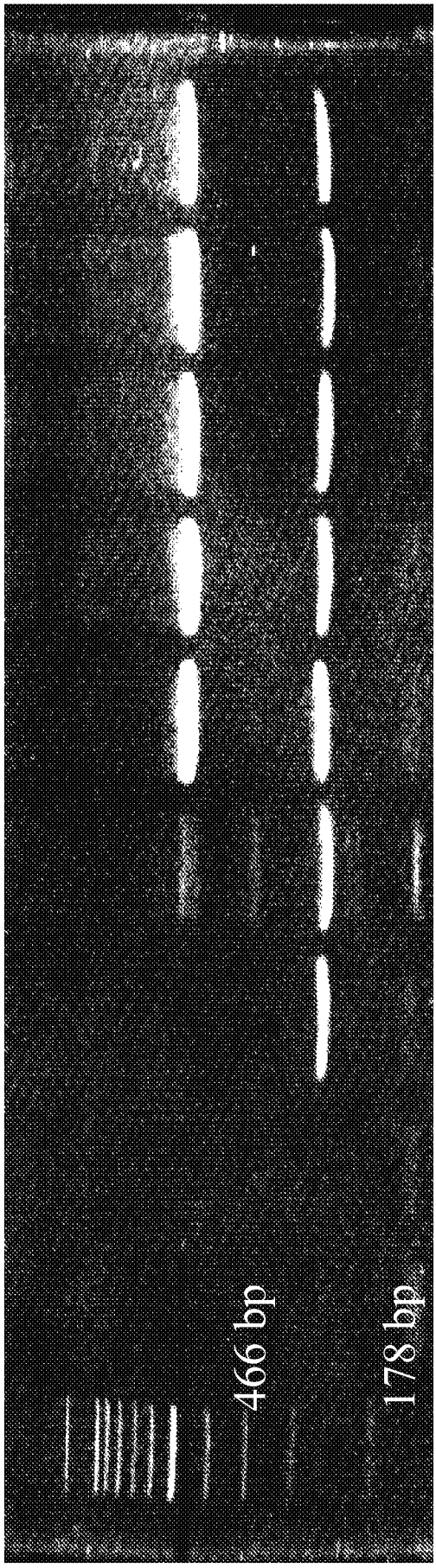

Denaturing temperature:
85.3
86.3
87.5
88.9
90.0
90.9
91.5
91.9
92.0
466 bp
178 bp
178 bp

Figure 3

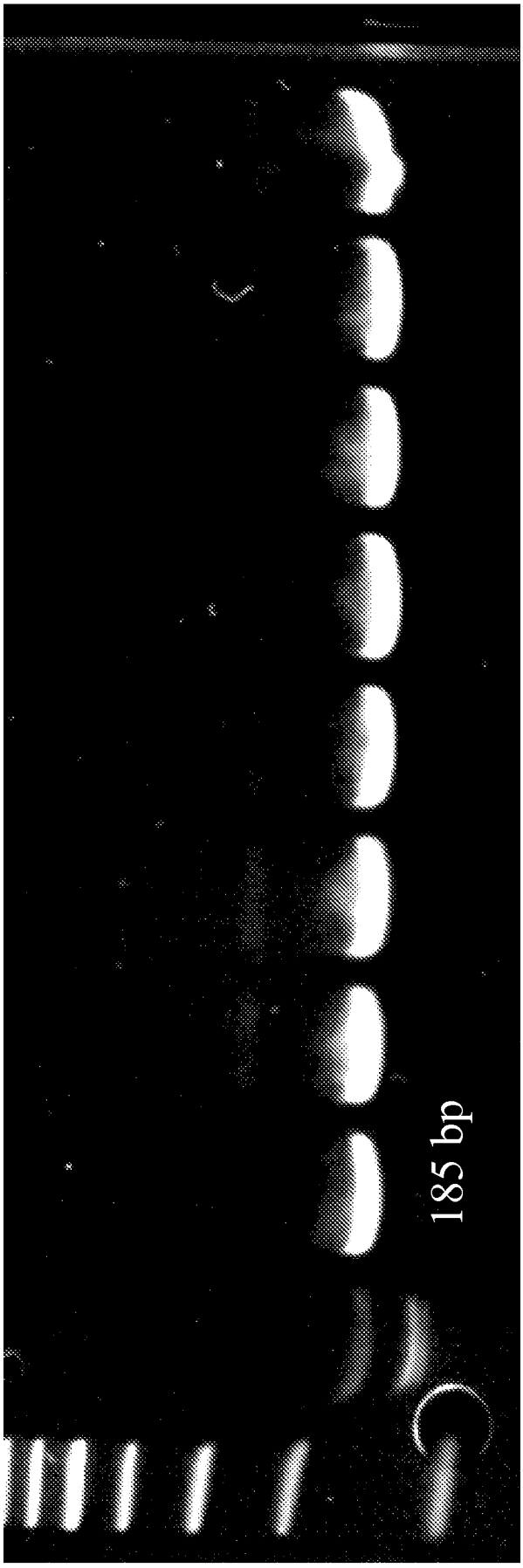

Denaturing temperature:
84.0
84.9
86.0
87.5
89.3
91.3
93.0
94.2
96.0
185 bp

Figure 4

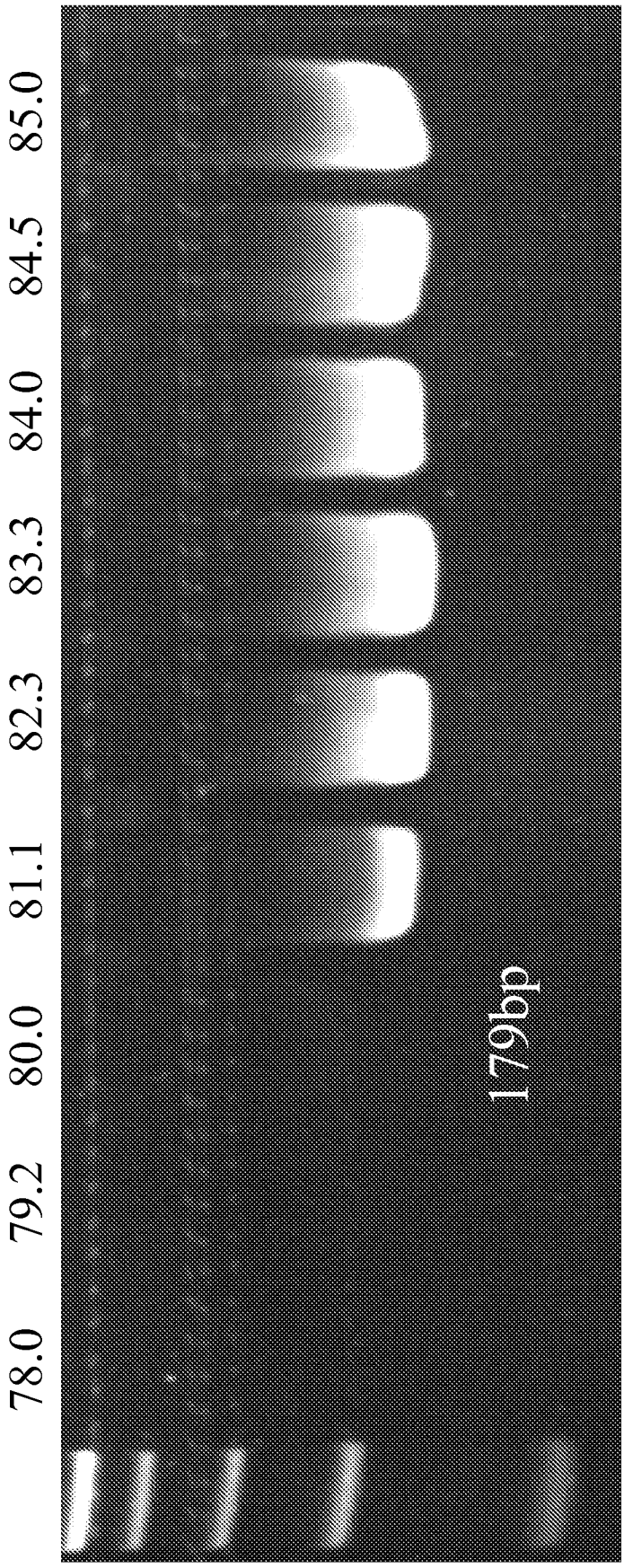

Denaturing temperature:
78.0
79.2
80.0
81.1
82.3
83.3
84.0
84.5
85.0
179bp

Figure 5

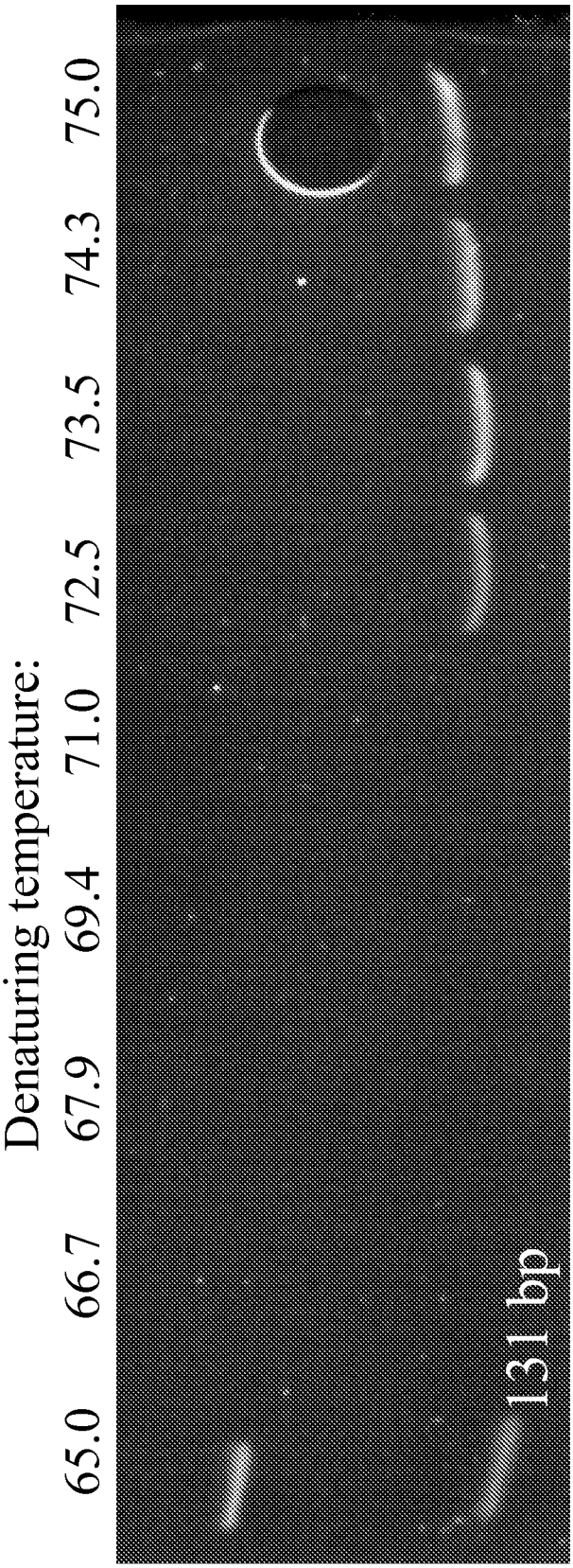

Denaturing temperature:
65.0
66.7
67.9
69.4
71.0
72.5
73.5
74.3
75.0
131 bp

Figure 6

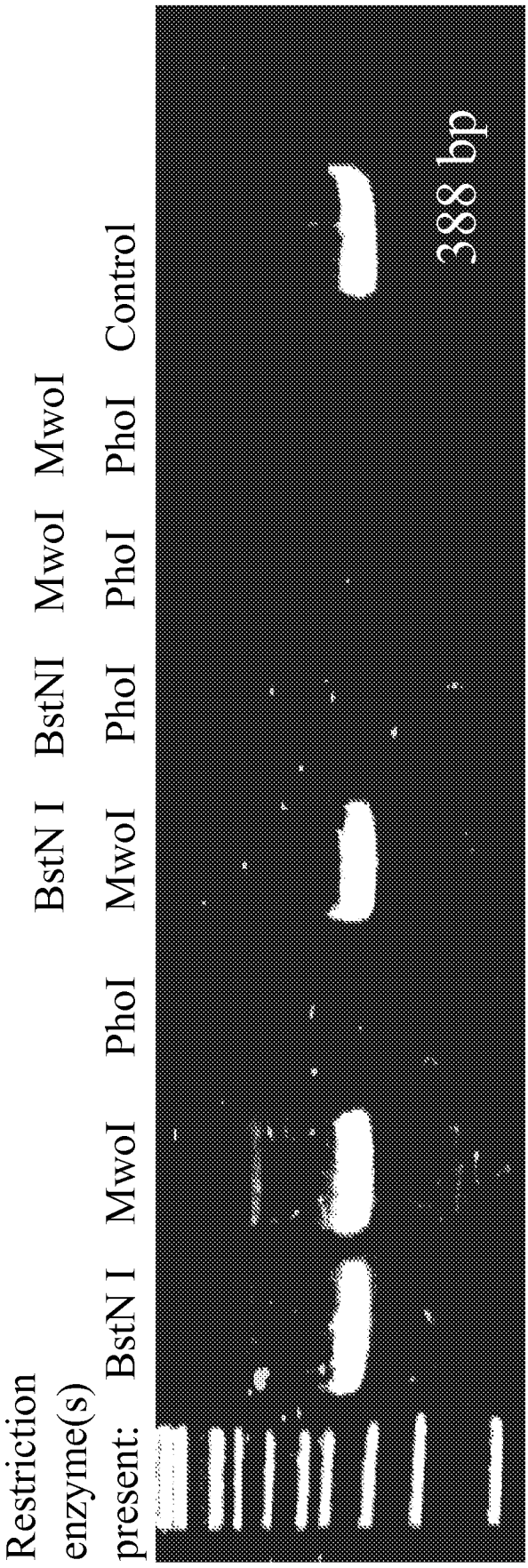

BstN I
Restriction enzyme(s) present:
BstN I
MwoI
PhoI
BstN I
BstNI
PhoI
MwoI
PhoI
MwoI
PhoI
Control
388 bp

Figure 7

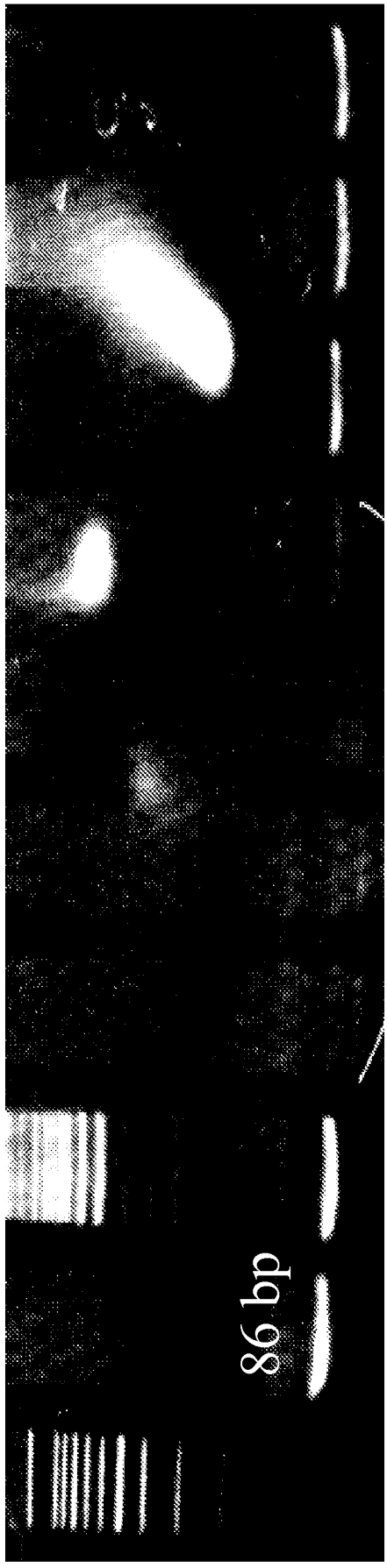

Denaturing temperature:
83.7 92.0 83.7 84.9 86.4 88.1 89.5 90.6 92.0
86 bp
— No MwoI —
1ul MwoI / 25 ul PCR reaction

Figure 8

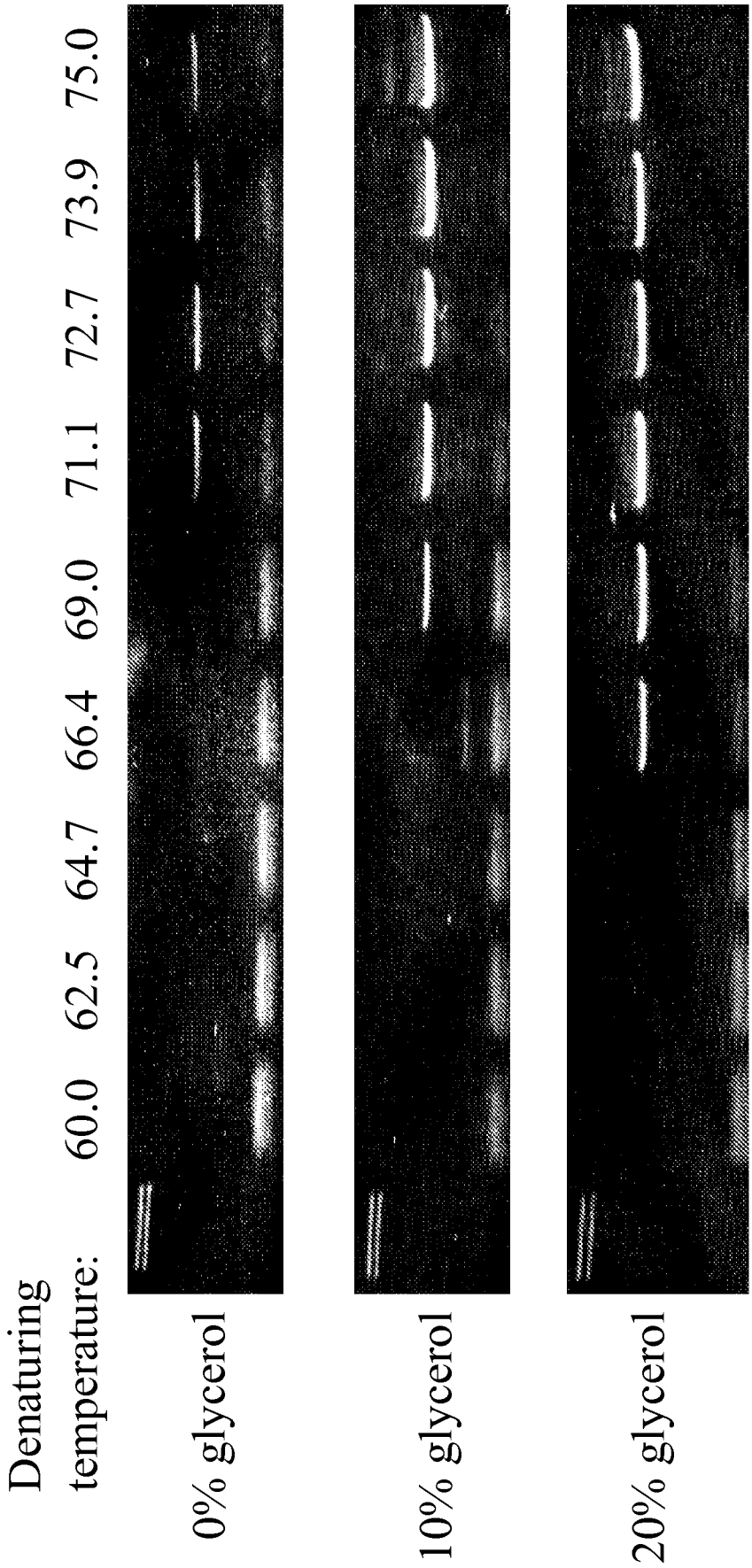

Denaturing temperature:
60.0
62.5
64.7
66.4
69.0
71.1
72.7
73.9
75.0
0% glycerol
10% glycerol
20% glycerol

Figure 9

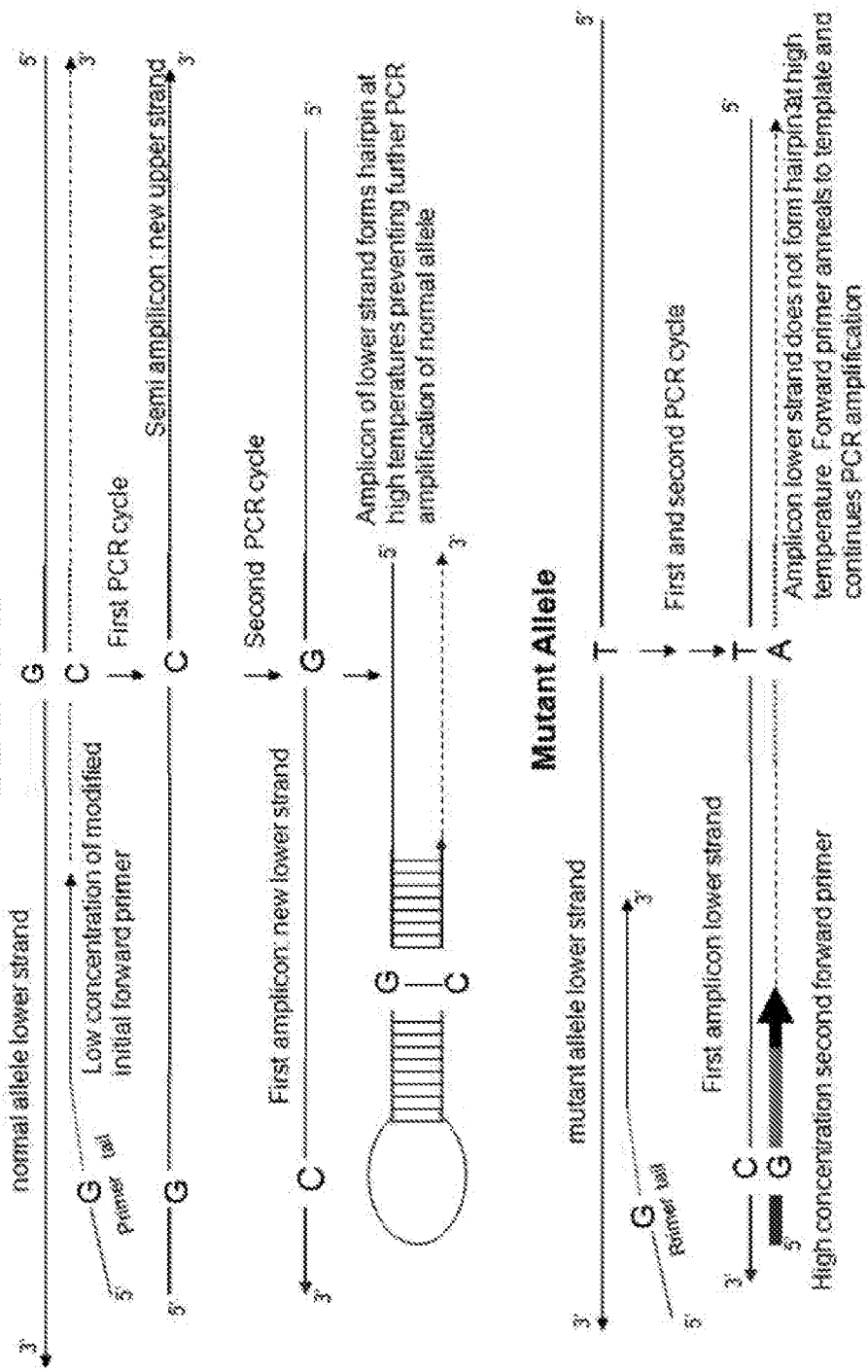
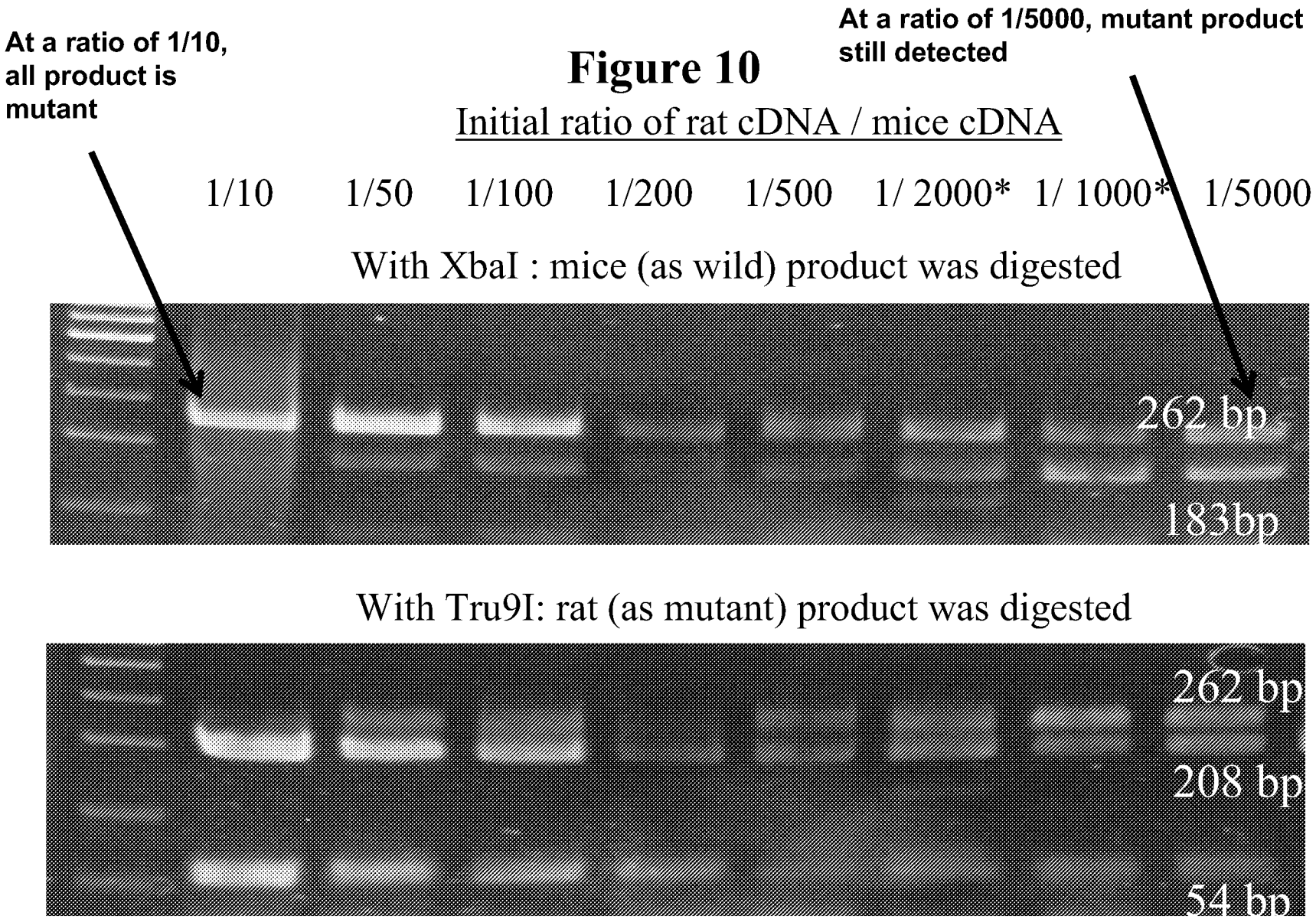

Normal Allele
normal allele lower strand
Low concentration of modified initial forward primer
First PCR cycle
Semi amplicon: new upper strand
Second PCR cycle
First amplicon: new lower strand
Amplicon of lower strand forms hairpin at high temperatures preventing further PCR amplification of normal allele
Mutant Allele
mutant allele lower strand
First and second PCR cycle
First amplicon lower strand
Amplicon lower strand does not form hairpin at high temperature. Forward primer anneals to template and continues PCR amplification
High concentration second forward primer

Figure 10


At a ratio of 1/10, all product is mutant
At a ratio of 1/5000, mutant product still detected
Initial ratio of rat cDNA / mice cDNA
1/10
1/50
1/100
1/200
1/500
1/ 2000*
1/ 1000*
1/5000
With XbaI : mice (as wild) product was digested
262 bp
183bp
With Tru9I: rat (as mutant) product was digested
262 bp
208 bp
54 bp

Gene / mutation /accession number	Primer Location	Primer sequence (5' to 3') *	allele site** wild / mutant	Amplification product Tm	Enzyme Sequence/ optimum temp / 80°C 20min inactivation
BRCA1 185 del eltion AG gDNA	1408-1503	CGTTGAAGAAGTACAAAATGTCATTAATGCTATGCAGAAA AGCTTA (SEQ ID NO: 1)	GCTTAGAGTGC / (SEQ ID NO: 3)	77.6°C	MwoI GCN/GC / 60°C / No
	1455-1482 CTTG	TGCTGACTTACCAGATGGGGCACT (SEQ ID NO: 2)	GCTTAGTGC (SEQ ID NO: 4)		
BRCA1 5382 Insertion C gDNA	68379-68418 AT (SEQ ID NO: 5)	AATGGAAGAAACCACCAAGGTCCAAAGCGAGCAAGAGGC	GCATCCCAGGC / (SEQ ID NO: 7)	82°C	MwoI GCN/GC / 60°C / No
	68421-68453 ACTTGAG	GGAGGGAGCTTTACCTTTCTGGCCTG (SEQ ID NO: 6)	GCATCCCCAGGC (SEQ ID NO: 8)		
BRCA2 6174 del T (NM_000059) in same exon	6139-6372 CTG	CAAATACTTGTGGGATTTTTAGCACAGCCAG (SEQ ID NO: 9)	CAGTG / (SEQ ID NO: 11)	79.3°C	TspRI NNCASTGNN / 65°C / No
	6223-6257 GACTT	GCTTGGTACTATCTTCTATTTCAGAAAACA (SEQ ID NO: 10)	CAGGG (SEQ ID NO: 12)		
K-ras G12D 35G>A (NM_033360 216G>A) In same exon	175-215 G (SEQ ID NO: 13)	GCTGAAAATGACTGAATATAAACTTGTGGTAGTTGGAACT	ACTGGG / (SEQ ID NO: 15)	79.2°C	BsrSI ACTGGN / 65°C / not available
	243-277 A	TATTCGTCCACAAAATGATTCTGAATTAGCTGTA (SEQ ID NO: 14)	ACTGAG (SEQ ID NO: 16)		
B-raf V600E 1798 T>A (NM_004333) In same exon	1809-1843 TTCTT	CATGAAGACCTCACAGTAAAAATAGGTGAT (SEQ ID NO: 17)	CAGTG / (SEQ ID NO: 19)	79.4°C	TspRI NNCASTGNN / 65°C / No
	1884-1916 ACAAAA	TGGATCCAGACAACTGTTCAAACTGAT (SEQ ID NO: 18)	CAGAG (SEQ ID NO: 20)		
PIK3CA E545K	1713-1740 GAGACAA	TGAATTAAGGGAAAATGACAA (SEQ ID NO: 21)	CACTG / (SEQ ID NO: 22)	78.9°C	TspRI NNCASTGNN

Figure 11

primer design and thermostable restriction endonuclease selection for enhanced detection of specific breast cancer mutations

1633 G>A (NM_006218 1789 G>A) In same exon	1796-1821 CTGT	GACTCCATAGAAAATCTTTCTC (SEQ ID NO: 23)	CACTA (SEQ ID NO: 24)		/ 65°C / No
EGFR L858R 2573 T>G (NM_005228 2819 T>G) In same exon	2772-2818	CGTACTGGTGAAAACACCGCAGCATGTCAAGATCACAGA TTTTGGGC (SEQ ID NO: 25)	CCTGG / (SEQ ID NO: 26) CCGGG	83.2°C	PspGI CCWGG / 75°C / No
	2877-2910 GTGT	AAAATTGATTCCAATGCCATCCACTTGATA (SEQ ID NO: 27)	(SEQ ID NO: 28)		

* bold / italic: mismatched base
underline: recognition site for thermo stable restriction endonuclease

Figure 11, con't

primer design to induce amplicon hairpin formation in normal alleles to allow enhanced detection of breast cancer mutations

Gene / mutation Accession number	Primer	Location	Primer sequence (5' to 3') *	Designed Hairpin Formation in normal allele amplicon and Tm normal / mutant
BRCA1 /5382 Insertion C /gDNA	Initial forward primer*	68369-68397	TCTGTCCTGGGATTCTCTTGAGATGTGGTCAATGGAAGAAACCACCAAG (SEQ ID NO: 29)	TCTGTCCTGGGATTCTCTTGAGATGTGGTCAATGGAAGAA |||||||||||||||||||||||| : : A AGACAGGACCCTAAGAACAGCAGCCAACCTGGAACCACC 79.6°C (SEQ ID NO: 32) / TCTGTCCTGGGATTCTCTTGAGATGTGGTCAATGGAAGAA : ||||||||||||| : : A AGACAGGACCCTAAGAACAGCAGCCAACCTGGAACCACC 62.3°C (SEQ ID NO: 33)
	successive forward positive		CCTGGGATTCTCTTGAGATGTGGTCAAT (SEQ ID NO: 30)	
	Reverse primer	68483-68507	CCCATCTCTGCAAAGGGGAGTGGAA (SEQ ID NO: 31)	
BRCA1 /185 deletion AG /gDNA	Initial forward primer*	1402-1433	GGGACACTCTAAGATTTTCTTCGCGTTGAAGAAGTACAAAATGTCATTA (SEQ ID NO: 34)	GGGACACTCTAAGATTTTCTTCGCGTTGAAGAAGTACAA |||||||||||||||||||| : :) CCCTGTGAGATTCTAAAAGACGTATCGTAATTACTGTAA 78.1°C (SEQ ID NO: 37) / GGGACACTCTAAGATTTTCTTCGCGTTGAAGAAGTACAA : || ||||||||||| : :) CCCTGTGAGATTCTAAAAGACGTATCGTAATTACTGTAA 64.1°C (SEQ ID NO: 38)
	successive forward positive		ACACTCTAAGATTTTCTTCGCGTT (SEQ ID NO: 35)	
	Reverse primer	1510-1540	AGGTCAATTCTGTTCATTTGCATAGGAGATA (SEQ ID NO: 36)	
BRCA2 /6174 del T /(NM_000059) in same exon	Initial forward primer*	6115-6148	GACAGATTTTGCACTTGCTGTGCGGAAGCTTCATAAGTCAGTCTCATCTGCAAATAC (SEQ ID NO: 39)	GACAGATTTTGCACTTGCTGTGCGGAAGCTTCATAAGTCAGTCTCA |||||||||||||||||||||||| || : : ||| : T CTGTCTAAAACGTGAACGACACGATTTTACGCTGTCATAAACGTC °C (SEQ ID NO: 42) / GACAGATTTTGCACTTGCTGTGCGGAAGCTTCATAAGTCAGTCTCA ||| : |||||||||| || : : ||| : T CTGTCTAAAACGTGAACGACACGATTTTACGCTGTCATAAACGTC 63.9°C (SEQ ID NO: 43)
	successive forward primer		ATTTTGCACTTGCTGTGCGGAAGCTTCATA (SEQ ID NO: 40)	
	Reverse primer	6211-6247	GTACTATCTTCTATTTCAGAAAACACTTGTCTTGCGT (SEQ ID NO: 41)	

*Initial forward primer designed to create hairpin forming amplicon from normal allele

underline: primer tail extension

Figure 12

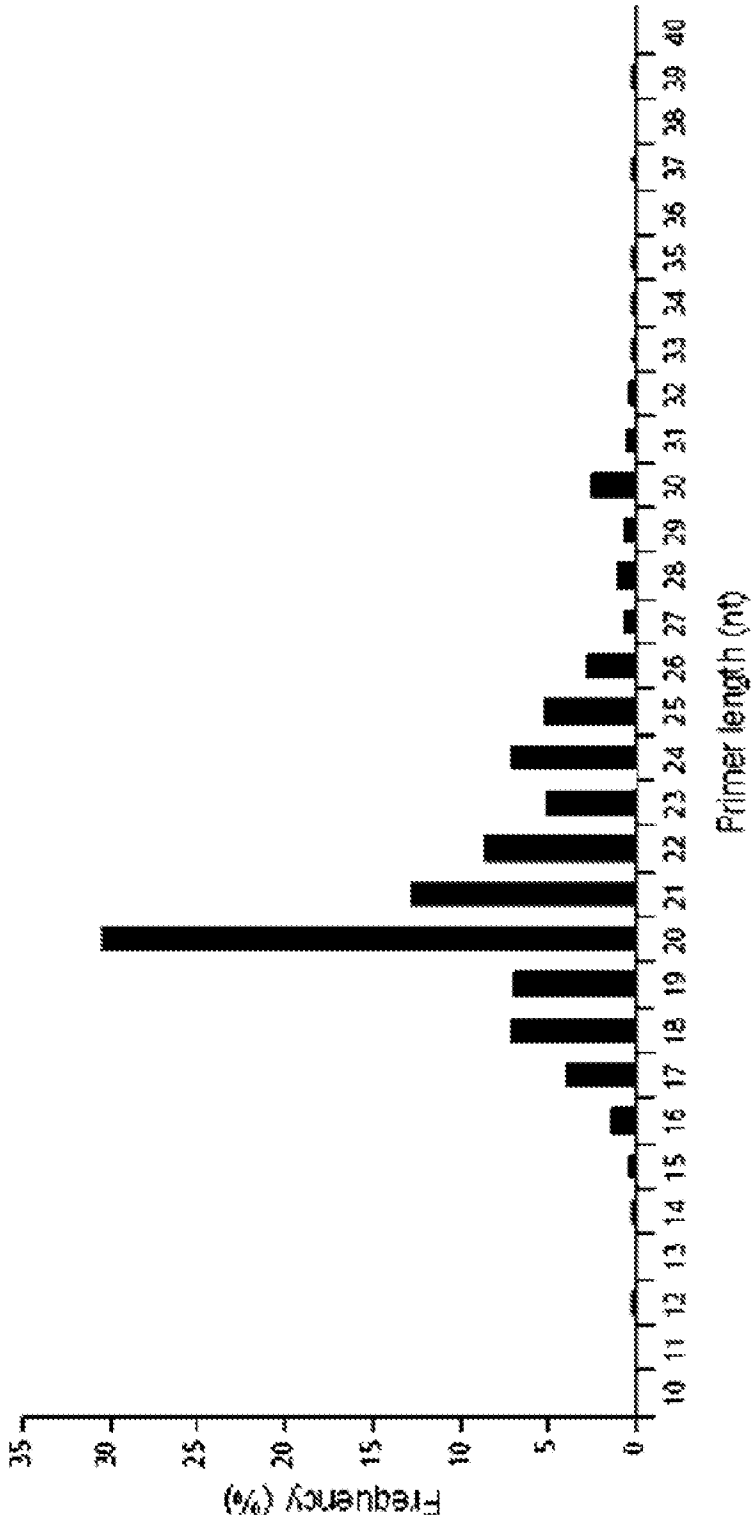

Frequency (%)
0
5
10
15
20
25
30
35
Primer length (nt)
10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40

Figure 13

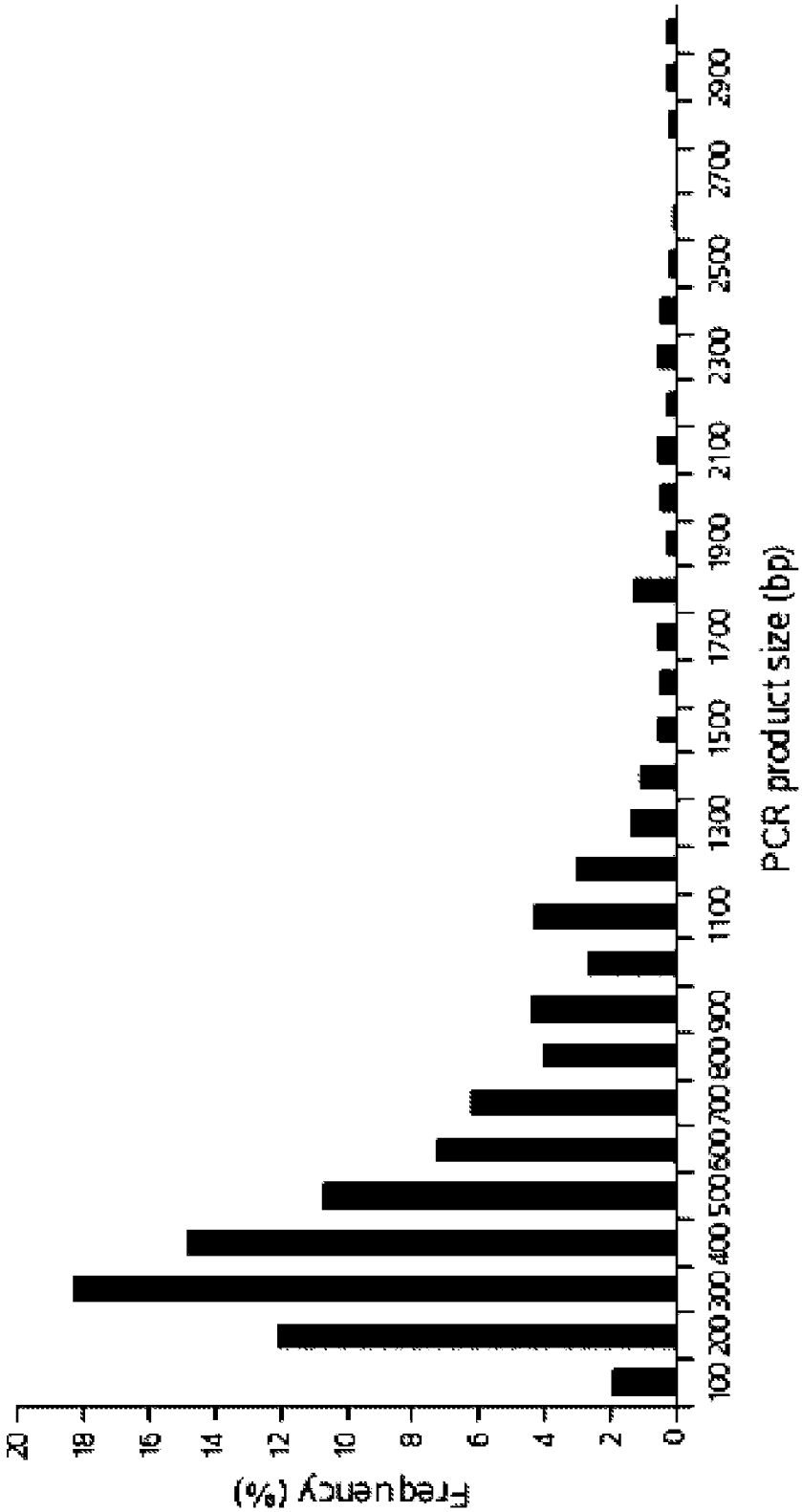

Frequency (%)
0
2
4
6
8
10
12
14
16
18
20
100 200 300 400 500 600 700 800 900 1100 1300 1500 1700 1900 2100 2300 2500 2700 2900
PCR product size (bp)

Figure 14

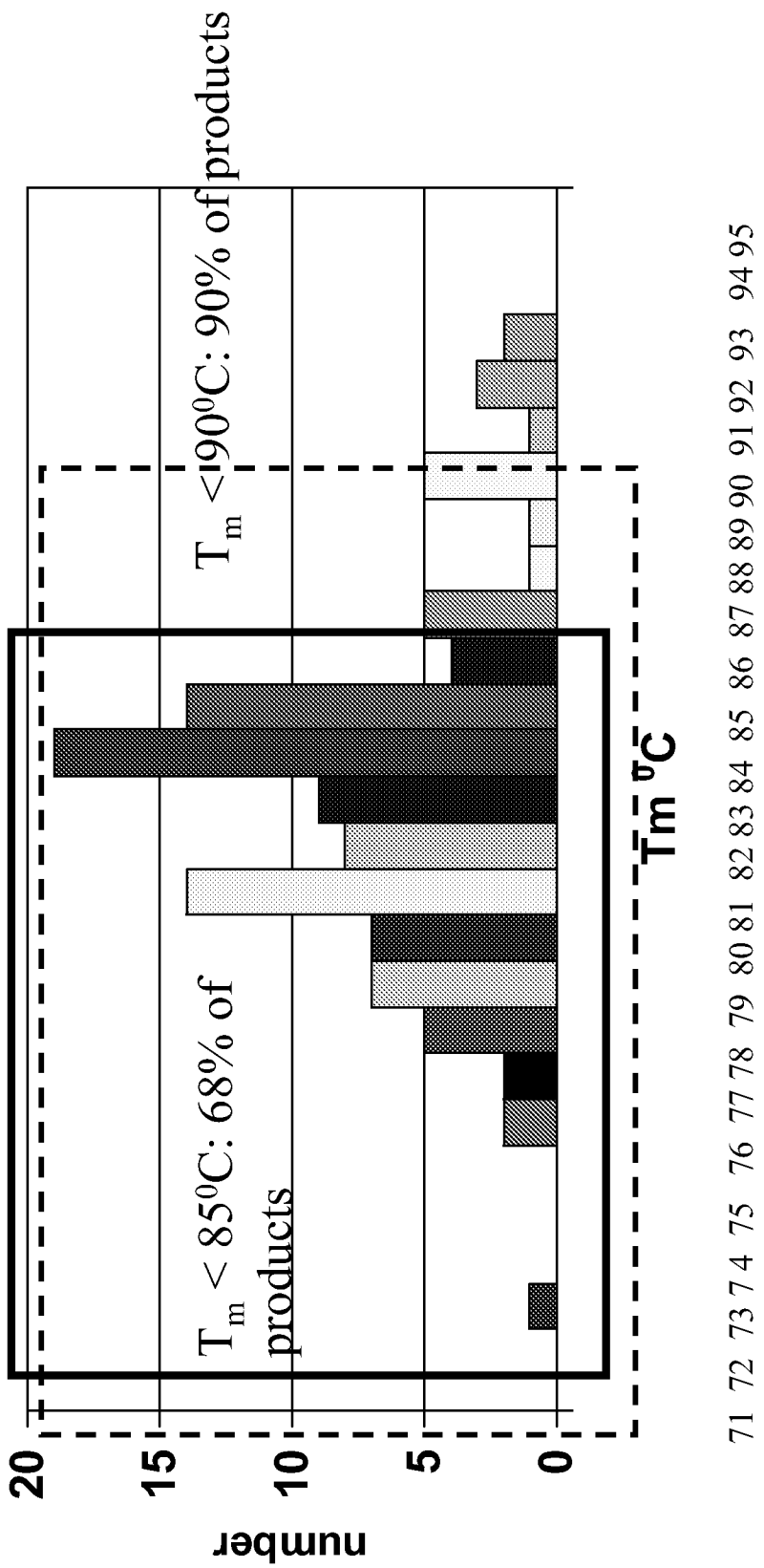

number
20
15
10
5
0
T_m <90^{0}C: 90% of products
T_m < 85^{0}C: 68% of products
71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95
Tm ^{0}C

Figure 15

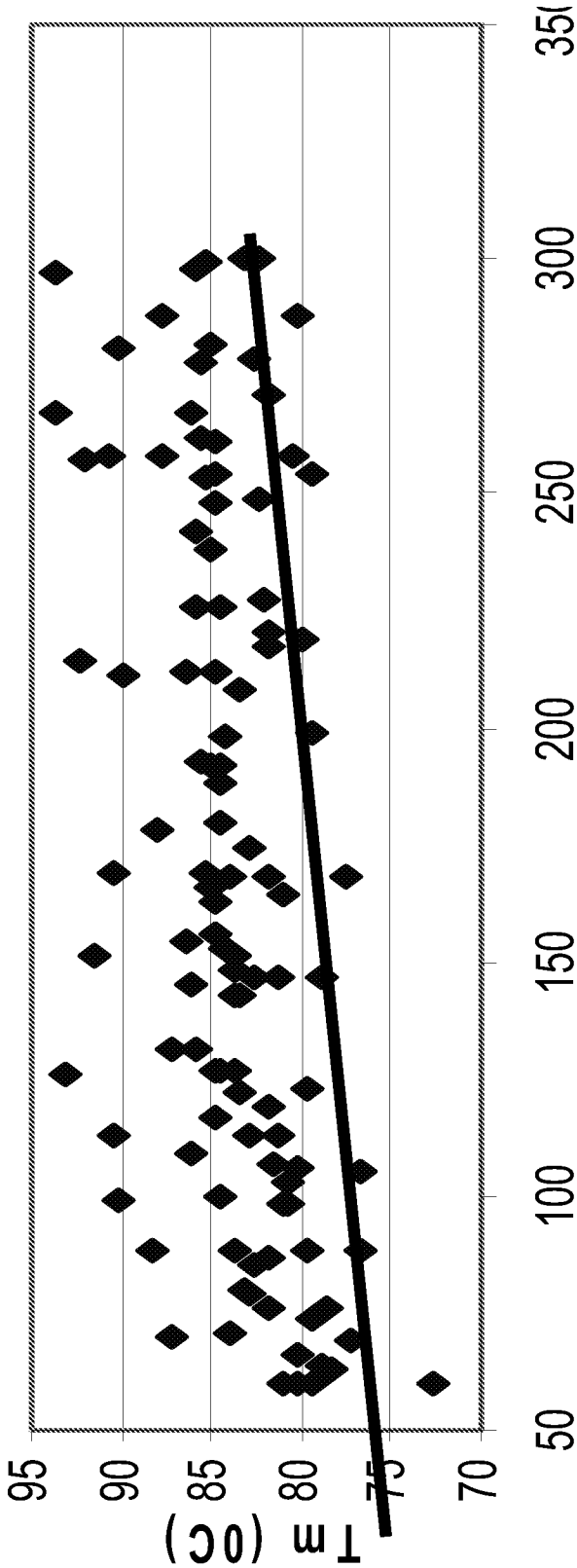


Figure 16

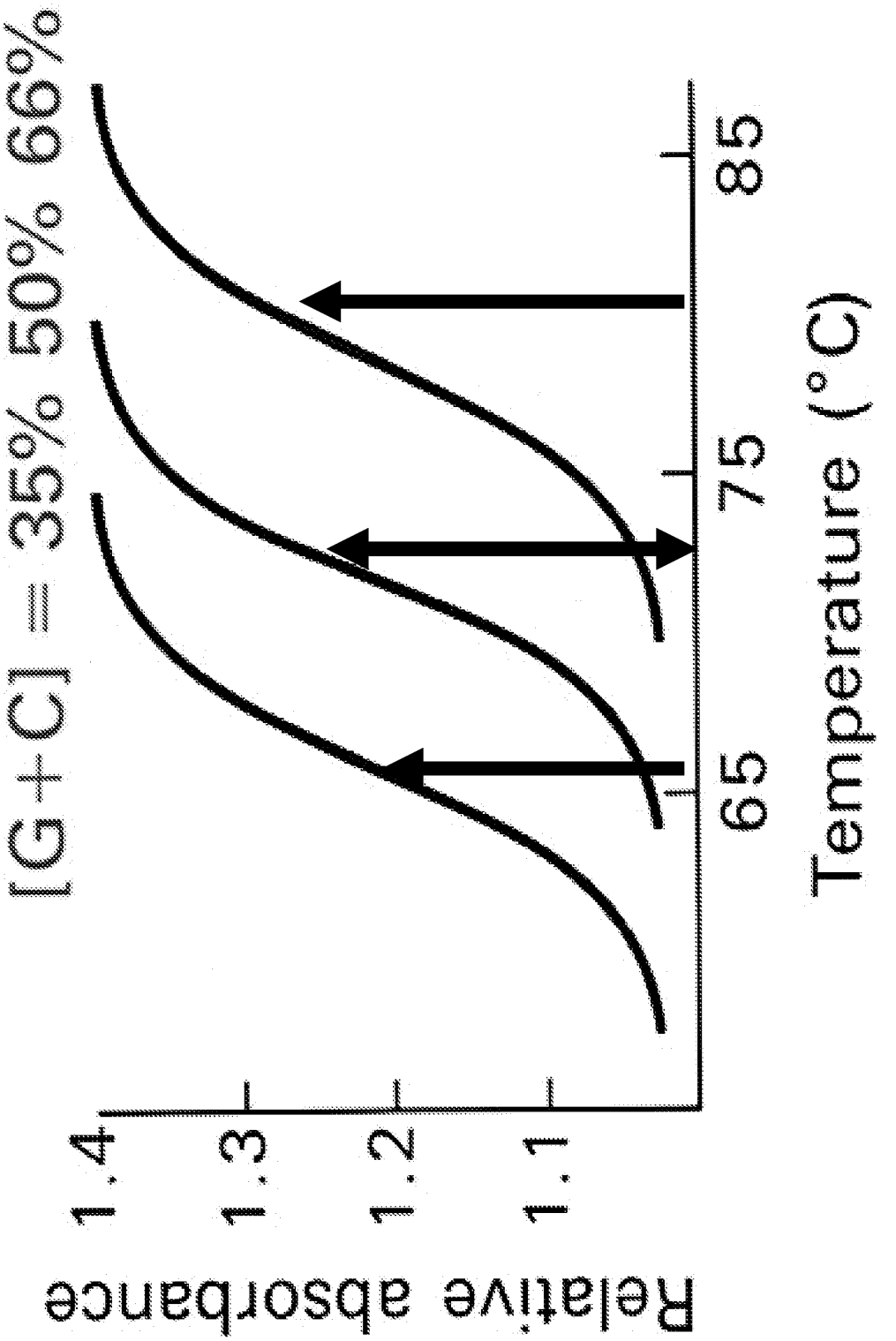

[G+C] = 35% 50% 66%
Relative absorbance
1.4
1.3
1.2
1.1
65
75
85
Temperature (°C)

Figure 17

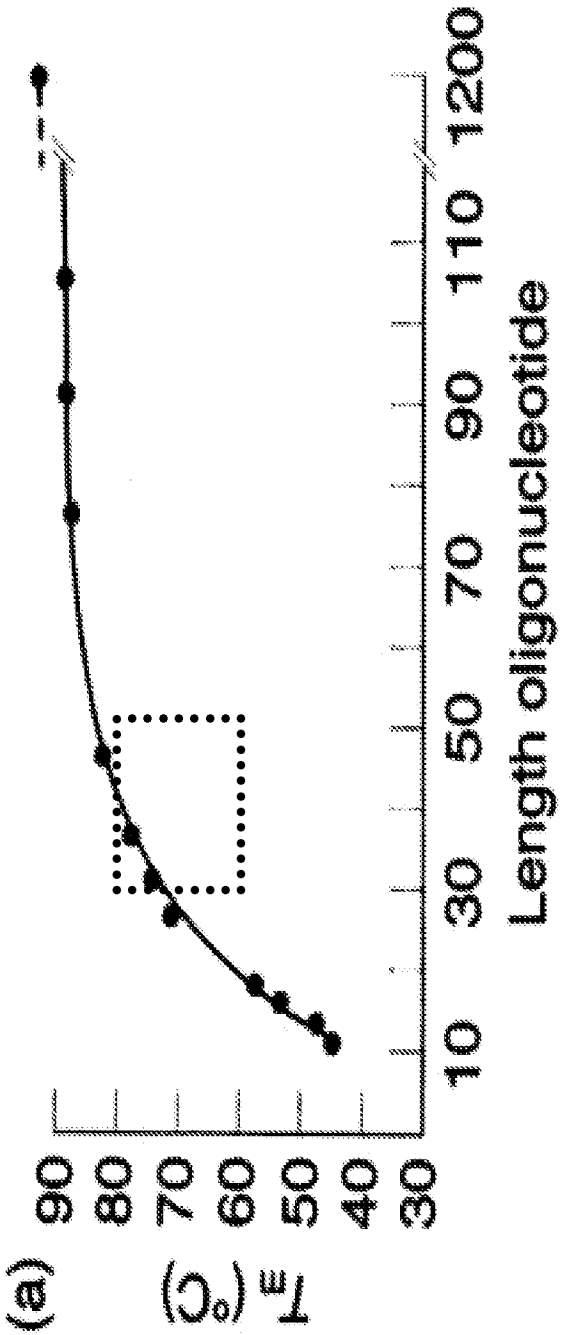

(a)
T_m (°C)
90
80
70
60
50
40
30
10
30
50
70
90
110
1200
Length oligonucleotide

Figure 18

Figure 19

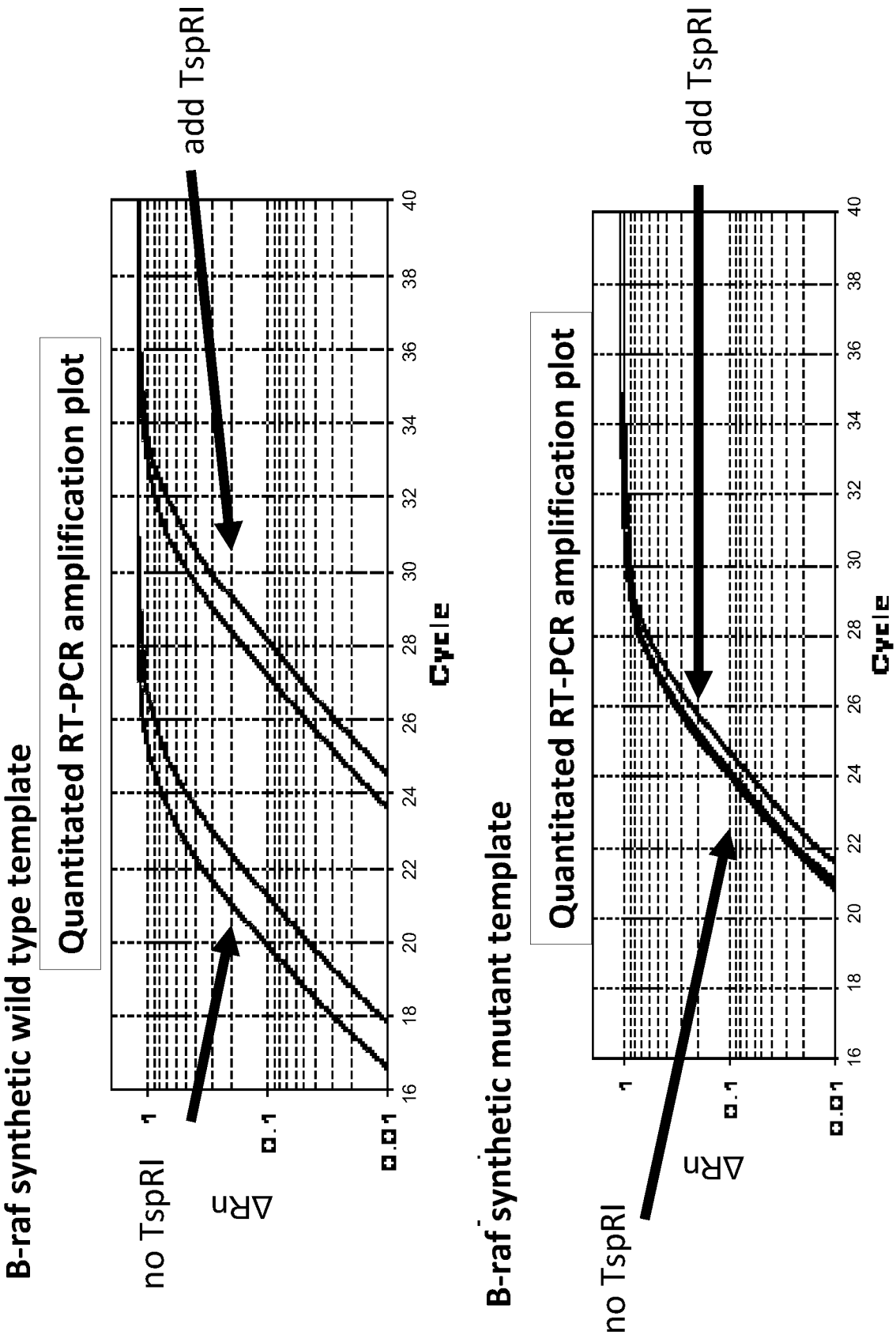

B-raf synthetic wild type template
Quantitated RT-PCR amplification plot
add TspRI
no TspRI
ΔRn
Cycle
B-raf synthetic mutant template
Quantitated RT-PCR amplification plot
add TspRI
no TspRI
ΔRn
Cycle

Figure 20

DNA sequencing detection of mutant product (red arrow) following PCR despite 1,000 fold greater input of wild type template.

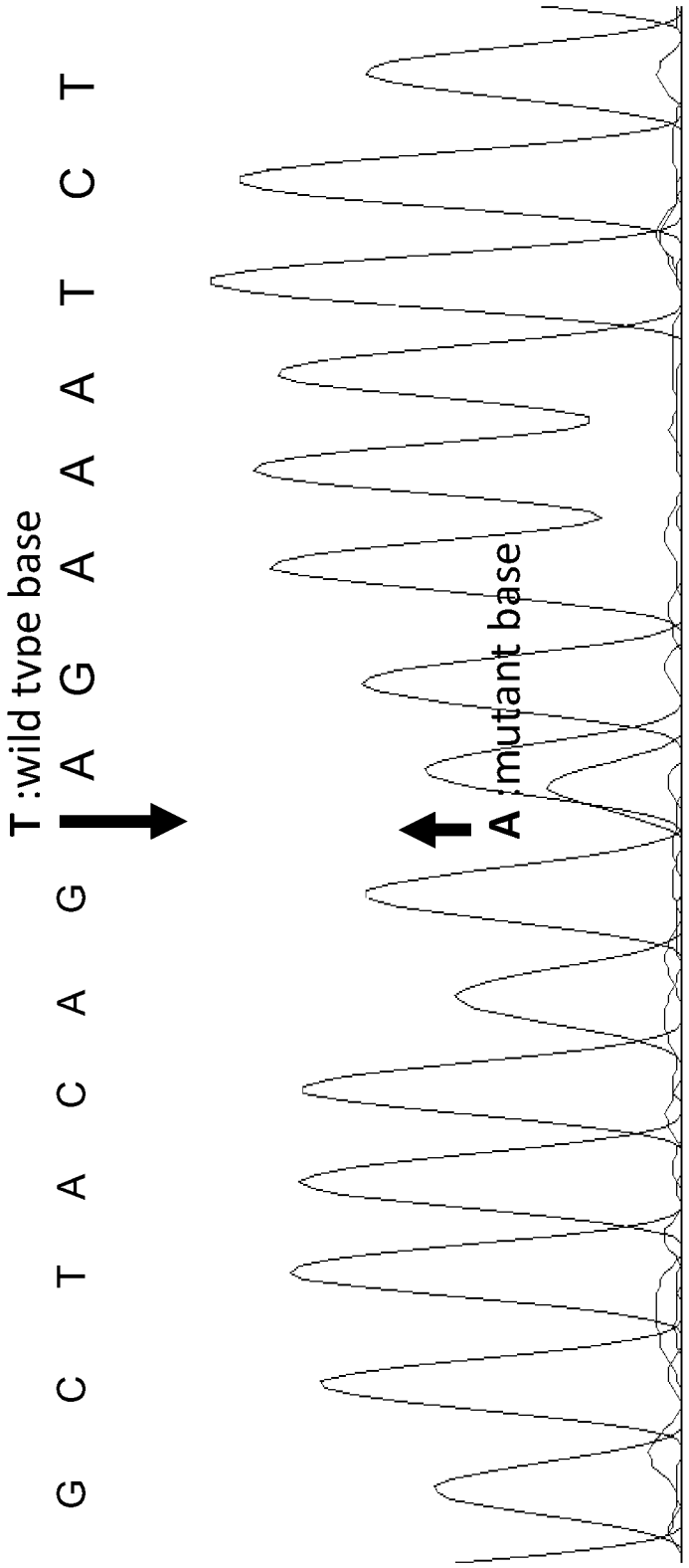


Figure 21

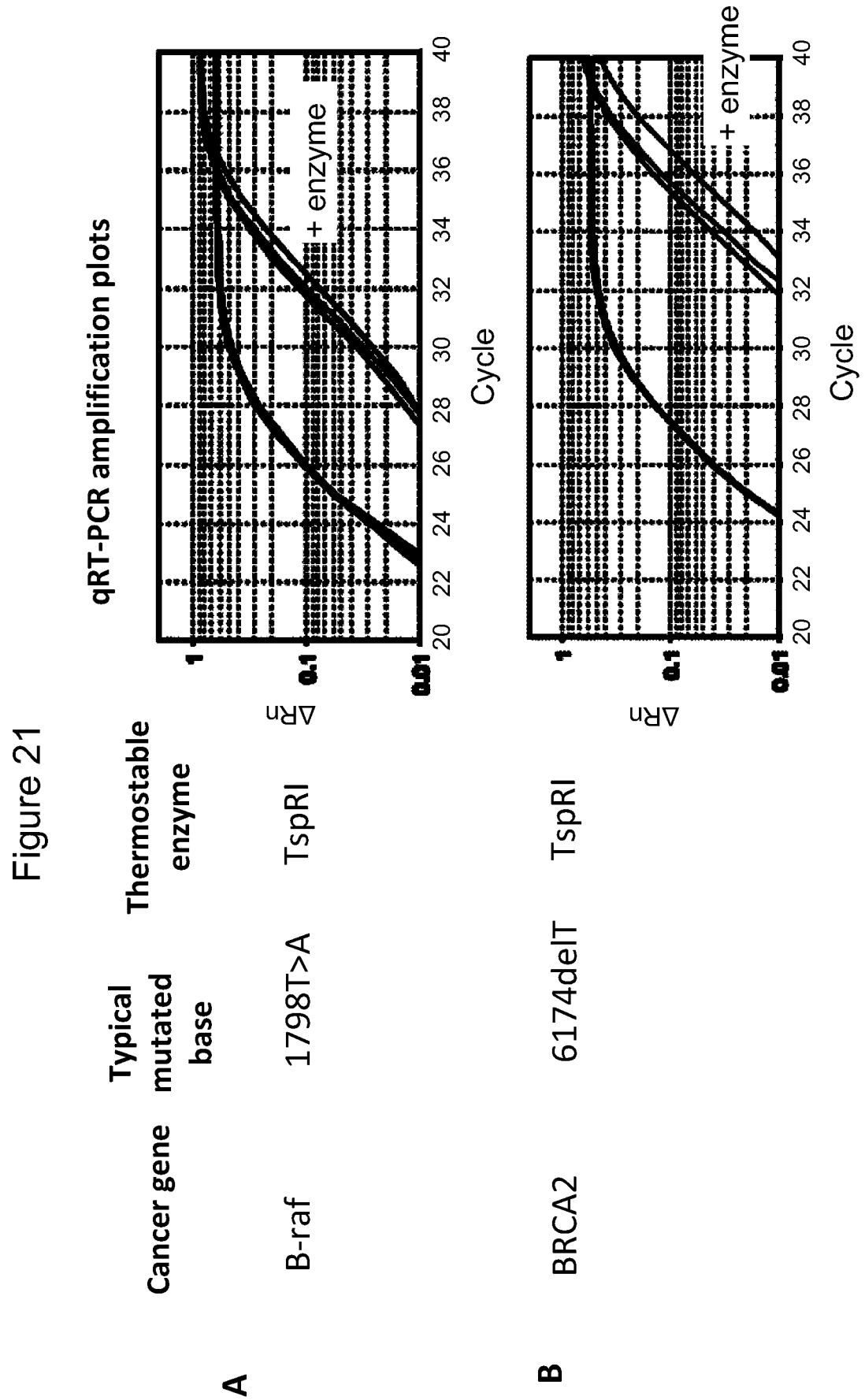

qRT-PCR amplification plots
Cancer gene
Typical mutated base
Thermostable enzyme
A
B-raf
1798T>A
TspRI
B
BRCA2
6174delT
TspRI
ΔRn
Cycle
+ enzyme
1
0.1
0.01
20
22
24
26
28
30
32
34
36
38
40

Figure 21 Continued

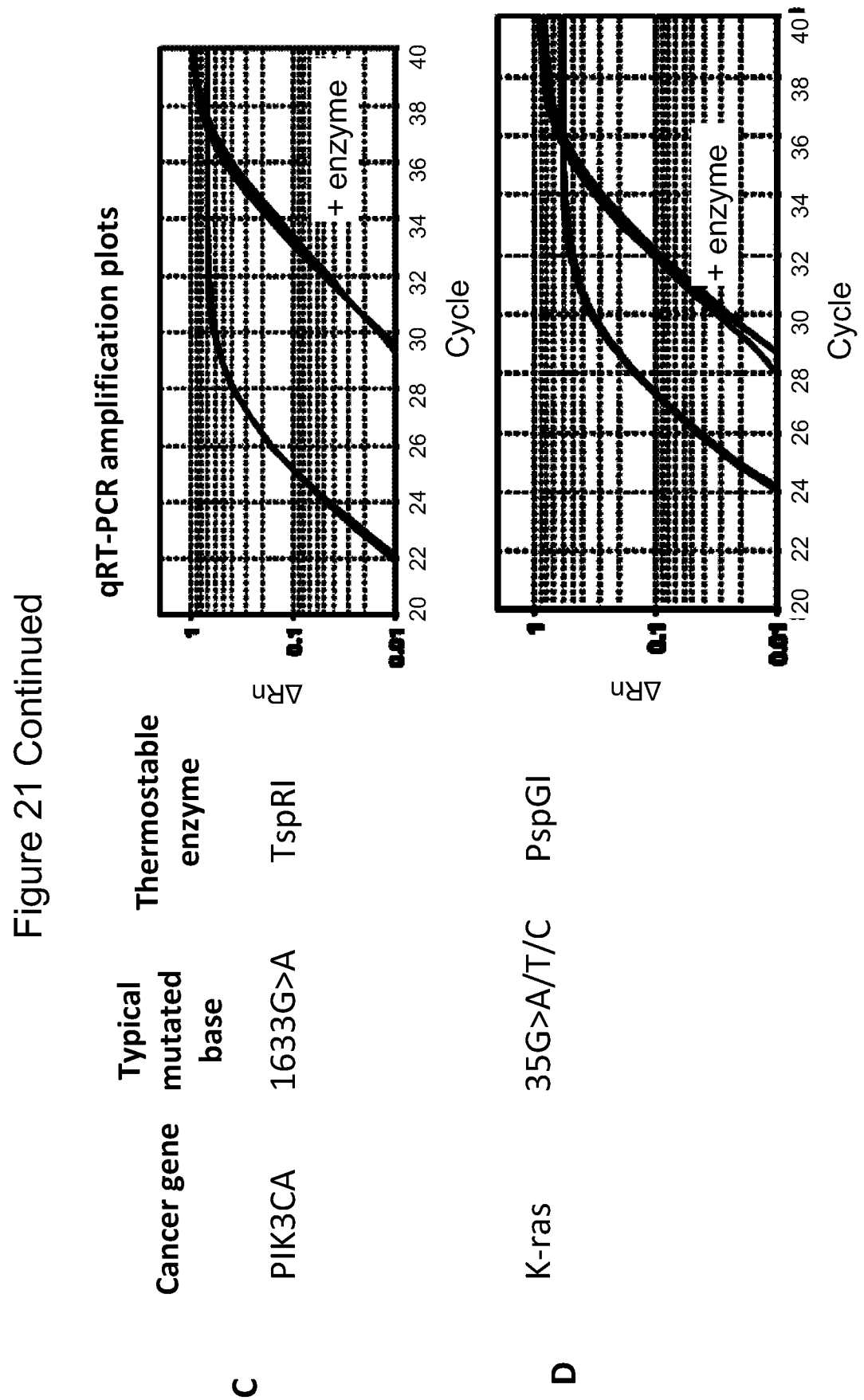


Figure 21 Continued

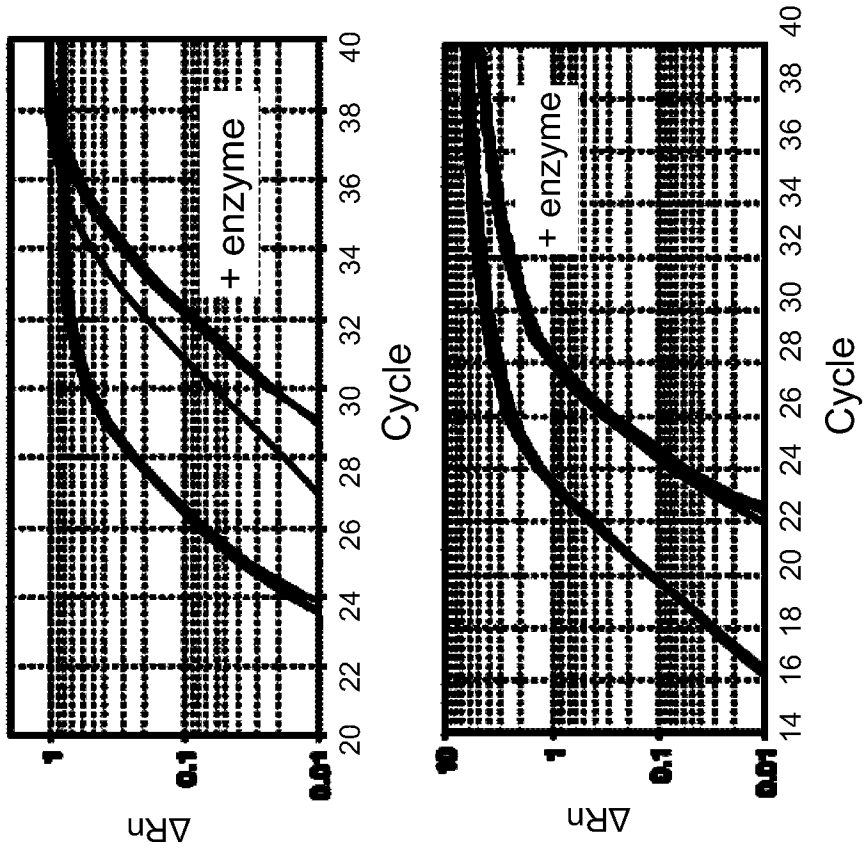


Figure 21 Continued

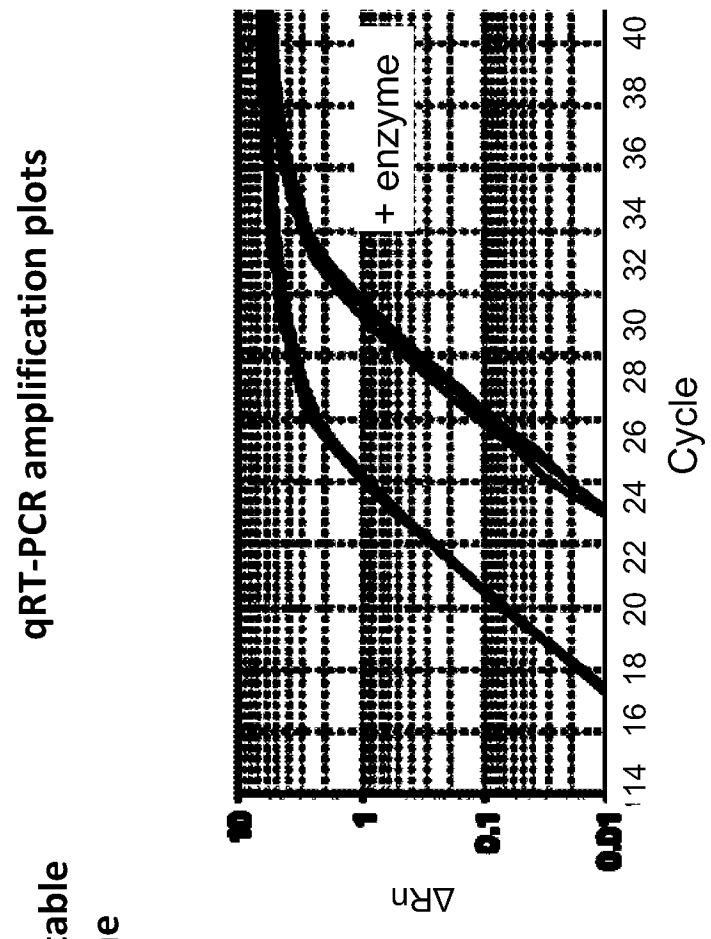
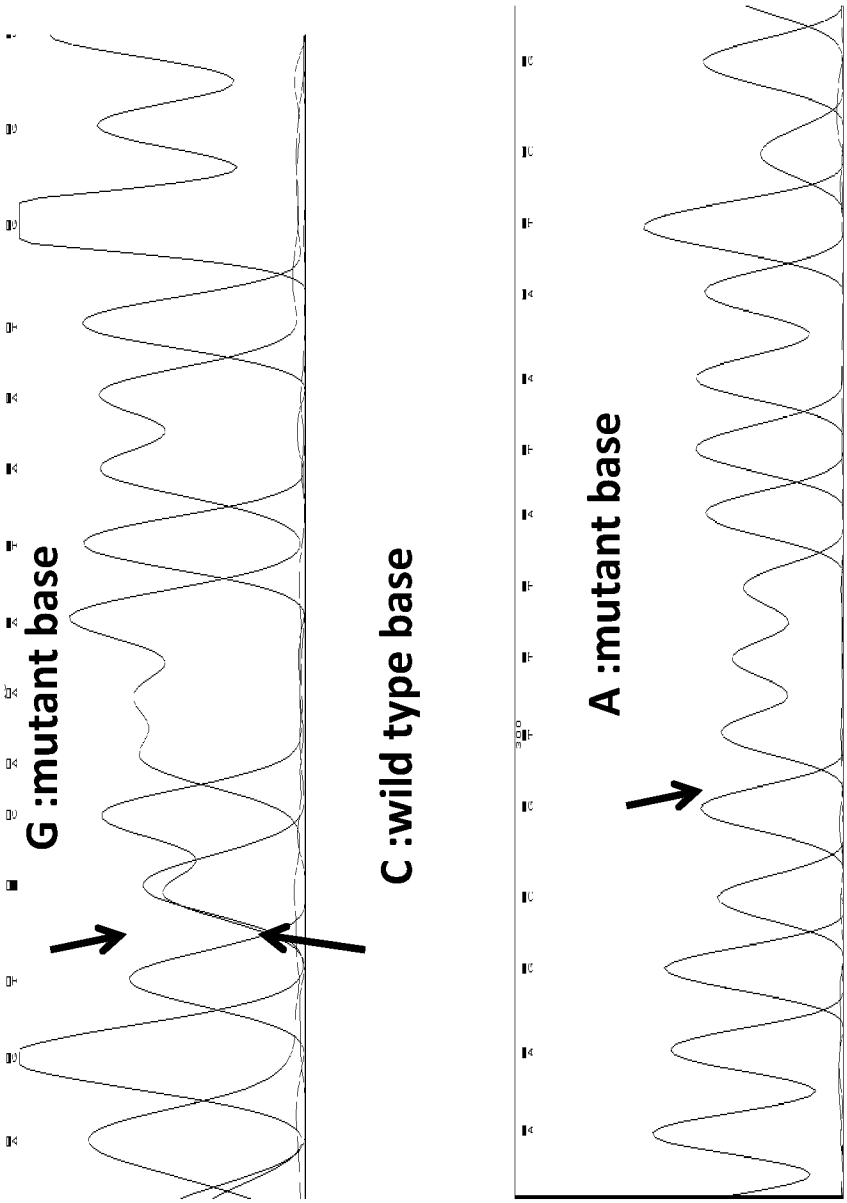


Figure 22

DNA sequencing results following enriched PCR amplification of a synthetic mutant site in a custom synthesized universal template



Equal detection of wild type and mutant products despite 100-fold excess of wild type template in presence of BseLI thermostable enzyme

Selective detection of mutant products despite 100-fold excess of wild type template in presence of BseJI thermostable enzyme

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 12/65606

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12Q 1/68 (2013.01)
USPC - 435/6.11, 435/6.12
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8) - C12Q 1/68 (2013.01)
USPC - 435/6.11, 435/6.12

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 435/6.1

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PaBase; Google Scholar Search terms: allele, normal, wildtype, wild-type, mutant, variant, degrade, cleave, digest, destroy, enrich, restriction endonuclease, thermostable restriction endonuclease, amplify, PCR, primer, BRCA-1, BRCA1, BRCA-2, BRCA2, peKI, BsaXI, BseJI, BseLI, BsmI, BsrI, BstBI, BstHHI, Hyp1881, MwoI, PhoI, PspGI, TaqI, TasI, TfiI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y --- A	US 2009/0298085 A1 (GOCKE et al.) 3 December 2009 (03.12.2009) para [0015], [0026]-[0027], [0056], [0059], [0062]-[0065], [0075], [0080], [0081], [0110], [0114], [0127]-[0128]	1-10, 12-13, 15-17, 21-24, 26-28, 30-35 --- 11, 14, 18, 20, 25, 29, 36, 38 --- 19, 37
Y	MILBURY et al., PCR-Based Methods for the Enrichment of Minority Alleles and Mutations. Clinical Chemistry, 2009, Vol 55, No 4, pages 632-640. Especially p 634, col 2, para 3	11, 25
Y	MCPHERSON et al., Breast cancer--epidemiology, risk factors, and genetics. British Medical Journal, 9 September 2000, Vol 321, No 7261, pages 624-628. Especially p 625, col 1, para 4 to p 626, col 1, para 1	14, 20, 29, 38
Y	US 2009/0142752 A1 (HALL et al.) 4 June 2009 (04.06.2009) para [0006], [0085], [0108]	18, 36
A	US 2003/0051270 A1 (KMIEC et al.) 13 March 2003 (13.03.2003) SEQ ID NOs: 989, 653, 1085	19, 37

☒ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 March 2013 (06.03.2013)	08 APR 2013
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 12/65606

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	AC098118, Rattus norvegicus clone CH230-127H9. GenBank Accession No. AC098118, 22 September 2002 [online]. [Retrieved on 6 March 2013]. Retrieved from the internet: <URL: http://www.ncbi.nlm.nih.gov/nuccore/AC098118>	19, 37
A	US 2004/0181048 A1 (WANG) 16 September 2004 (16.09.2004) SEQ ID NO: 356882	19, 37

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 12/65606

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I: Claims 1-38, drawn to methods for selectively amplifying and detecting a mutant allele

Group II: Claims 39-56, drawn to a template tool for use in selecting optimal PCR conditions

---please see extra sheet---

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-38

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 12/65606

Continuation of:
Box No. III Observations where unity of invention is lacking

The inventions listed as Groups I though III do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Group I does not require a template tool as required by Group II.

The feature shared by Groups I and II is a method of detecting a mutant allele of a gene in a sample, which method selectively degrades a normal allele of the gene in the sample by a thermostable restriction endonuclease. However, this shared technical feature does not represent a contribution over prior art, because the shared technical feature is anticipated by US 2009/0298085 A1 to Gocke et al. (published 3 December 2009; hereinafter 'Gocke'). Gocke discloses a method of detecting a mutant allele of a gene in a sample (para [0015]), which method selectively degrades a normal allele of the gene in the sample (para [0015] - "an endonuclease, most preferably a restriction enzyme, specifically cleaves wildtype but not mutant DNA in the portion of the sequence between the positions of the oligonucleotide primers used to amplify the DNA. Thus, wildtype DNA in the sample cannot be amplified after restriction enzyme digestion, whereas mutant DNA can be amplified, and is preferentially amplified using the methods of the invention") by a thermostable restriction endonuclease (para [0015] - "thermostable restriction endonuclease"). As the shared technical feature was known in the art at the time of the invention, it cannot be considered a special technical feature that would otherwise unify the groups.

Groups I and II therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.