# DNA POLYMERASE MUTANTS HAVING ENHANCED TEMPLATE DISCRIMINATION ACTIVITY

## CROSS-REFERENCE TO RELATED APPLICATIONS

**[01]** This application claims benefit of priority under 35 U.S.C. 119 to U.S. provisional patent application serial number 61/904,335, filed November 14, 2013, and entitled "DNA POLYMERASE MUTANTS HAVING ENHANCED TEMPLATE DISCRIMINATION ACTIVITY," the contents of which are herein incorporated by reference in its entirety.

## FIELD

**[02]** This invention relates to mutant DNA polymerases having enhanced primer and/or template discrimination activities and uses of the same for polymerase-based assays for genetic diagnostic analysis.

## BACKGROUND

**[03]** The ability to accurately diagnose a given genetic condition and to predictably treat a genetically-based disorder requires reliable methods for accurately determining genetic sequence information. Many genetically-based disorders are associated with single nucleotide polymorphisms (SNP's) in protein coding genes. The presence of SNP's associated with a genetically-based disorder, such as a cancer, can be difficult to detect owing to the small numbers of genetically altered cells in the population that encode the allele(s) ("rare alleles").

**[04]** Polymerase-based assays, such as the polymerase chain reaction (PCR), have important impact and widespread use in genetic diagnostics and molecular medicine. Polymerases synthesize DNA sequences by the addition of nucleotides to the 3' end of a short oligonucleotide (abbreviated to "primer" in the following). The primer is hybridized to the single stranded sequence that is going to be amplified ("template"). DNA polymerases catalyze formation of a phosphodiester bond between the 3'-oxygen at the 3'-terminus of the primer and the incoming deoxynucleoside triphosphate ("dNTP"). This chemical reaction ("primer extension") adds a nucleotide to the primer (e.g., to the nascent growing DNA chain). Primer extension is base specific, in that the deoxynucleoside triphosphate that is complementary to the base in the template is added to the primer. The fidelity of DNA polymerase enzymes is very high and the rate of mutations introduced into the replicated DNA strand is low; however, the

precise error rate varies between different DNA polymerase enzymes and these rates have been well characterized. The extension reaction can be repeated until the end of the template is reached.

**[05]** The majority of polymerase-based assays for detecting SNP's rely upon having the polymerase enzyme discriminate between at least two different substrates. The first substrate contains the desired SNP that is to be detected; the second substrate contains the normal nucleotide that is not to be detected. Polymerase-based discrimination can be achieved by providing the first substrate as the preferred polymerase-competent substrate for assay. This discrimination can be maximized to the extent that the first substrate is the only polymerase-competent substrate present for assay.

**[06]** Many strategies are known in the art for establishing conditions that favor polymerase-based discrimination among substrates having minimal nucleotide differences, such as those containing only single nucleotide differences. One strategy relies on a polymerase's inability to efficiently initiate synthesis on substrates lacking a 3'-paired nucleotide on the primer. An allele-specific primer design is a primer in which the 3'-nucleotide forms a perfect match with the complementary base at the location containing the SNP-containing allele and forms a mismatched pairing when annealed to an allele lacking the SNP. The primer:template for the SNP-containing allele serves as the preferred polymerase-competent substrate since the polymerase can efficiently initiate primed synthesis from such substrates. Examples of these strategies are described in Chen *et al.*, "Single nucleotide polymorphism genotyping: biochemistry, protocol, cost and throughput" Pharmacogenomics J. 3(2):77-96 (2003); Kwok *et al.*, "Detection of single nucleotide polymorphisms" Curr. Issues Mol. Biol. 5(2):43-60 (April 2003); Shi, "Technologies for individual genotyping: detection of genetic polymorphisms in drug targets and disease genes" Am. J. Pharmacogenomics 2(3):197-205 (2002); and Kwok, "Methods for genotyping single nucleotide polymorphisms" Annu. Rev. Genomics Hum. Genet. 2:235-58 (2001). A strategy to improve selectivity for this class of allele-specific PCR primers is to introduce a second mutation at the penultimate base, next to the 3'-terminal nucleotide of the primer (i.e., next to the SNP site). As before, the 3'-terminal residue will either be a match or mismatch to the base under interrogation in the sample nucleic acid (SNP), but now the primer will either have two adjacent mismatches to the target (both 3'-terminal and penultimate base) or a single mismatch to the target (at only the penultimate base, with the 3'-terminal base being a

match). See, for example, Newton *et al.*, "Analysis of any point mutation in DNA. The amplification refractory mutation systems (ARMS)" Nucleic Acids Res. 17(7):2503-15 (1989). Yet another strategy to improve selectivity for this class of allele-specific PCR primers is to employ a chemically modified nucleic acid residue at the 3'-end of the primer, such as a locked nucleic acid (LNA), which reduces the ability of DNA polymerase to initiate DNA synthesis from a 3'-terminal mismatch. See, for example, Latorra et al., "SNP genotyping using 3' locked nucleic acid (LNA) primers" Human Mut. 22(1):79-85 (2003).

**[07]** Template substrate discrimination can be enhanced in polymerase-based assays by requiring a second nucleic acid enzyme catalyze formation of one or more primers for use in the polymerase-based assay. In one such assay, the ligase chain reaction assay, a DNA ligase is used with a polymerase to detect a template containing a SNP. Since polymerase-based assays require primers having a minimum length to hybridize to the template substrate, a DNA ligase can be used to generate polymerase primers from sub-optimal length oligonucleotides. The assay relies upon hybridizing two probes directly over the SNP polymorphic site, whereby ligation can occur if the probes are identical to the target DNA. Two probes are designed; an allele-specific probe which hybridizes to the target DNA so that its 3' base is situated directly over the SNP nucleotide and a second probe that hybridizes the template downstream in the complementary strand of the SNP polymorphic site providing a 5' end for the ligation reaction. If the allele-specific probe matches the target DNA, it will fully hybridize to the target DNA and ligation can occur. Ligation does not generally occur in the presence of a mismatched 3'-base. Once the oligonucleotide product is formed, it can serve as a primer or as a template for polymerase-based assays. Examples of this strategy are described in Barany F. "Genetic disease detection and DNA amplification using cloned thermostable ligase." *Proc Natl Acad Sci U S A.* 1991 Jan 1;88(1):189-93 and Wiedmann M., Wilson W.J., Czajka J., Luo J., Barany F., Batt C.A. "Ligase chain reaction (LCR)--overview and applications." *PCR Methods and Applications* 1994 Feb;3(4):S51-64.

**[08]** Since a polymerase-competent substrate requires a primer:template having an available 3'-hydroxyl group on the primer, another strategy known in the art, RNase H-based PCR (rhPCR), can be used for improving polymerase-based discrimination. The rhPCR method makes use of RNase H enzymes to convert a primer lacking a 3'-hydroxyl group ("blocked primer") or a primer that is otherwise disabled and cannot support PCR to a primer containing a

3'-hydroxyl group ("unblocked primer") that can support PCR. A blocked primer in rhPCR includes an oligonucleotide having a blocked 3'-end or other modification which prevents either priming or template function of the oligonucleotide and an internal RNA residue, which serves as a cleavage site. Type II RNase H recognizes annealed primer:template duplexes containing these blocked primers and cleaves the primer strand 5' of the RNA residue to generate a 3'-hydroxyl group at the adjacent DNA residue. Since RNase H enzymes do not cleave substrates containing an unpaired RNA reside at a mismatched site, allele-specific template discrimination can be achieved by placement of the RNA residue at the location complementary to the SNP on the selected allele template. The resultant Type II RNase H cleavage product can serve as a polymerase competent substrate. Examples of this enzyme cleaving strategy, similar RNase H strategies, and methods of blocking primer extension or inhibiting template function and thereby disabling PCR are described in U.S. Pat. No. 7,112,406 to Behlke et al., entitled POLYNOMIAL AMPLIFICATION OF NUCLEIC ACIDS, U.S. Pat. No. 5,763,181 to Han *et al.*, entitled CONTINOUS FLUOROMETRIC ASSAY FOR DETECTING NUCLEIC ACID CLEAVAGE, U.S. Pat. No. 7,135,291 to Sagawa *et al.*, entitled METHOD OF DETECTING NUCLEOTIDE POLYMORPHISM; U.S. Pat. App. No. 20090068643 to Behlke and Walder, entitled DUAL FUNCTION PRIMERS FOR AMPLIFYING DNA AND METHODS OF USE; and U.S. Pat. App. No. 20100167353 to Walder *et al.*, entitled RNASE H-BASED ASSAYS UTILIZING MODIFIED RNA MONOMERS.

**[09]** The field has focused on substrate-based approaches, such as those exemplified above, for improving detection of genetic differences and rare alleles. Yet the sensitivity of polymerase-based assays remains limited by the formation of non-specific amplification products that arise from ectopic or aberrant primer-related extension products independent of the desired templates. The inherent reactivity of the polymerase appears largely responsible for producing such artifacts during amplification.

**[010]** Any further improvements in mismatch discrimination may therefore require a modified polymerase enzyme endowed with inherently better 3'-nucleotide discrimination when used with one or more of the described strategies. A modified polymerase enzyme having activity differing from the unmodified form can be prepared by chemical or enzymatic modification of the protein or mutagenesis of corresponding gene that encodes the protein. The latter approach is generally preferred owing to the fact that genetically altered genes encoding a

given mutant protein can be stably maintained, expressed and purified to yield enzyme preparations having well-characterized properties.

**[011]** While an unbiased mutagenesis strategy can be used to generate a library of mutant polymerase genes, this approach has certain disadvantages. Many millions of mutant enzymes must be screened for activity and success is often dependent upon the chance that effective mutations are present in the limited pool generated by random mutagenesis. Direct genetic selection methods are not sufficiently sensitive for identifying mutations that pertain to secondary functions falling outside of an essential polymerase activity. The vast majority of mutant polymerase genes in a positive selection assay will likely encode protein that retains the functional attributes of the normal polymerase enzyme. Thus, secondary screening procedures that use biochemical assays must be done to identify whether any mutant polymerases have the desired activity. Notwithstanding the technical difficulties of setting up the initial selection process, the attendant costs associated with performing the secondary screens using biochemical assays is prohibitive if more than one hundred clones need to be purified and assayed.

**[012]** An alternative approach is to apply a biased mutagenesis strategy that is specifically targeted to a preselected region of a gene implicated in function. In this approach, one first identifies genetic regions by a selection method. One such selection method is a comparative phylogenetic analysis of a particular gene that is required for organisms of diverse origins. The principle of comparative phylogenetic analysis is premised on the hypothesis that diverse organisms will not share protein coding sequences in essential genes unless those sequences are evolutionary constrained for reasons related to essential protein function.

**[013]** A phylogenetic comparative analysis of genes encoding DNA polymerases can provide insights about possible amino acid residues important to polymerase functions. The overall folding pattern of DNA polymerases resembles the human right hand and contains three distinct subdomains of palm, fingers, and thumb. (See, for example. Beese *et al.*, Science 260:352-355, 1993; Patel *et al.*, Biochemistry 34:5351-5363, 1995). While the structure of the fingers and thumb subdomains vary greatly between polymerases that differ in size and in cellular functions, the catalytic palm subdomains are all superimposable. For example, motif A, which interacts with the incoming dNTP and stabilizes the transition state during chemical catalysis, is superimposable with a root mean deviation of about one Ångström among mammalian pol α and prokaryotic pol I family DNA polymerases (Wang et al., Cell 89:1087-

1099, 1997). Motif A begins structurally at an antiparallel β–strand containing predominantly hydrophobic residues and continues to an α–helix. The primary amino acid sequence of DNA polymerase active sites is exceptionally conserved.

**[014]** In addition to being well-conserved, the active site of DNA polymerases has also been shown to be relatively mutable, capable of accommodating certain amino acid substitutions without reducing DNA polymerase activity significantly. (See, e.g., U.S. Pat. No. 6,602,695). Such mutant DNA polymerases can offer various selective advantages in, e.g., diagnostic and research applications comprising nucleic acid synthesis reactions. We identify mutations in protein sequence using the single-letter amino acid codes and an integer number that indicates location of the mutation from the beginning of the protein sequence. The single-letter amino acids codes are well known in the art, e.g., Stryer et al., Biochemistry, 5.sup.th ed., Freeman and Company (2002). As an example, aspartic acid ("D") is changed to glycine ("G") in D580G mutant and the change is located 580 amino acids from the beginning of the protein sequence.

**[015]** Reichert *et al.* conducted a comparative phylogenetic analysis of thermoactive DNA polymerases from thermophilic bacteria, wherein the protein coding sequences of DNA Polymerase I enzymes were aligned for thirteen phylogenetically distinct species. The analysis revealed that eight amino acid positions within a 15-amino acid long motif located at amino acid positions 645-685 (in reference to *Thermus* sp. Z05 DNA polymerase ("Z05 DNA Polymerase") might tolerate alterations without compromising core enzyme function.

**[016]** Comparative phylogenetic analysis does not provide specific functional information pertaining non-conserved amino acids, other than to suggest that non-conserved amino acids are not likely critical to core enzyme functions. For that reason, specific mutations were introduced into a recombinant gene encoding a variant of the Z05 DNA Polymerase ("Z05 D580G polymerase") and the resultant Z05 D580G polymerase mutants were screened for their ability to provide a reduced ability to extend an oligonucleotide primer with a 3'-mismatch to a template. Reichert *et al.* found that one such mutant, Z05 D580G V667E polymerase, displayed better discrimination (~2-fold) than the parental Z05 D580G polymerase. See U.S. Pat. App. No. 2012/0015405 to Reichert *et al.*, entitled DNA POLYMERASES WITH INCREASED 3'-MISMATCH DISCRIMINATION.

**[017]** The comparative phylogenetic analysis has limitations with respect to identifying DNA polymerase activities that display improved 3'-nucleotide discrimination. This is due to the

fact that all DNA polymerases of a given enzyme class are confronted with similar template substrates and nucleotide pools across the spectrum of phylogenetically diverse organisms. Given the fact that all DNA polymerases must display 3'-nucleotide mismatch discrimination to preserve high fidelity replication of daughter template strands, it is not surprising that one can apply comparative phylogenetic analysis to identify possible amino acid positions that might affect mismatch discrimination. For template substrates having different 3'-end modifications that are presented to a polymerase only in a biochemical assay, such as those used in several PCR-based assays for rare allele detection, there is a need for identifying DNA polymerases having improved 3'-nucleotide discrimination.

**[018]** Taq DNA Polymerase is an enzyme that was discovered in *Thermus aquaticus* bacterium (Chien, A., et al., *J Bacteriol.* 1976, 127: 1550–1557). The enzyme is classified as deoxyribonucleic acid polymerase, class I (enzyme code, EC 2.7.7.7). Its catalytic activity is to amplify DNA sequences. The peptide and gene sequences of Taq DNA polymerase enzyme isolated from nature are well known in prior art and are listed in Table 1 (Lawyer, F.C., et al., *J. Biol. Chem.* 1989, 264: 6427-6437; Genbank database ID J04639.1).

**Table 1. DNA and amino acid sequence of native Taq DNA polymerase.**

| Type | Sequence |
|---|---|
| Protein sequence (N to C terminus)<br><br>[SEQ ID NO:1] | MRGMLPLFEPKGRVLLVDGHHLAYRTFHALKGLTTSRGEPVQAVYGFAKSLLKALKED<br>GDAVIVVFDAKAPSFRHEAYGGYKAGRAPTPEDFPRQLALIKELVDLLGLARLEVPGY<br>EADDVLASLAKKAEKEGYEVRILTADKDLYQLLSDRIHVLHPEGYLITPAWLWEKYGL<br>RPDQWADYRALTGDESDNLPGVKGIGEKTARKLLEEWGSLEALLKNLDRLKPAIREKI<br>LAHMDDLKLSWDLAKVRTDLPLEVDFAKRREPDRERLRAFLERLEFGSLLHEFGLLES<br>PKALEEAPWPPPEGAFVGFVLSRKEPMWADLLALAAARGGRVHRAPEPYKALRDLKEA<br>RGLLAKDLSVLALREGLGLPPGDDPMLLAYLLDPSNTTPEGVARRYGGEWTEEAGERA<br>ALSERLFANLWGRLEGEERLLWLYREVERPLSAVLAHMEATGVRLDVAYLRALSLEVA<br>EEIARLEAEVFRLAGHPFNLNSRDQLERVLFDELGLPAIGKTEKTGKRSTSAAVLEAL<br>REAHPIVEKILQYRELTKLKSTYIDPLPDLIHPRTGRLHTRFNQTATATGRLSSSDPN<br>LQNIPVRTPLGQRIRRAFIAEEGWLLVALDYSQIELRVLAHLSGDENLIRVFQEGRDI<br>HTETASWMFGVPREAVDPLMRRAAKTINFGVLYGMSAHRLSQELAIPYEEAQAFIERY<br>FQSFPKVRAWIEKTLEEGRRRGYVETLFGRRRYVPDLEARVKSVREAAERMAFNMPVQ<br>GTAADLMKLAMVKLFPRLEEMGARMLLQVHDELVLEAPKERAEAVARLAKEVMEGVYP<br>LAVPLEVEVGIGEDWLSAKE |

| Type | Sequence |
|---|---|
| DNA sequence (5' to 3')<br><br>[SEQ ID NO:2] | aagctcagat ctacctgcct gagggcgtcc ggttccagct ggcccttccc<br>gaggggggaga gggaggcgtt tctaaaagcc cttcaggacg ctacccgggg<br>gcgggtggtg gaagggtaac atgaggggga tgctgcccct ctttgagccc<br>aagggccggg tcctcctggt ggacggccac cacctggcct accgcacctt<br>ccacgccctg aagggcctca ccaccagccg gggggagccg gtgcaggcgg<br>tctacggctt cgccaagagc ctcctcaagg ccctcaagga ggacggggac<br>gcggtgatcg tggtctttga cgccaaggcc ccctccttcc gccacgaggc<br>ctacgggggg tacaaggcgg gccgggcccc cacgccggag gactttcccc<br>ggcaactcgc cctcatcaag gagctggtgg acctcctggg gctggcgcgc<br>ctcgaggtcc cgggctacga ggcggacgac gtcctggcca gcctggccaa<br>gaaggcggaa aaggagggct acgaggtccg catcctcacc gccgacaaag<br>acctttacca gctcctttcc gaccgcatcc acgtcctcca ccccgagggg<br>tacctcatca ccccggcctg gctttgggaa aagtacggcc tgaggcccga<br>ccagtgggcc gactaccggg ccctgaccgg ggacgagtcc gacaaccttc<br>ccggggtcaa gggcatcggg gagaagacgg cgaggaagct tctggaggag<br>tgggggagcc tggaagccct cctcaagaac ctggaccggc tgaagcccgc<br>catccgggag aagatcctgg cccacatgga cgatctgaag ctctcctggg<br>acctggccaa ggtgcgcacc gacctgcccc tggaggtgga cttcgccaaa<br>aggcgggagc ccgaccggga gaggcttagg gcctttctgg agaggcttga<br>gtttggcagc ctcctccacg agttcggcct tctggaaagc cccaaggccc<br>tggaggaggc cccctggccc ccgccggaag gggccttcgt gggctttgtg<br>ctttcccgca aggagcccat gtgggccgat cttctggccc tggccgccgc<br>caggggggggc cgggtccacc gggcccccga gccttataaa gccctcaggg<br>acctgaagga ggcgcggggg cttctcgcca aagacctgag cgttctggcc<br>ctgagggaag gccttggcct cccgcccggc gacgacccca tgctcctcgc<br>ctacctcctg gacccttcca acaccacccc cgaggggtg gcccggcgct<br>acggcgggga gtggacggag gaggcggggg agcgggccgc cctttccgag<br>aggctcttcg ccaacctgtg ggggaggctt gaggggggagg agaggctcct<br>ttggctttac cgggaggtgg agaggcccct ttccgctgtc ctggcccaca<br>tggaggccac gggggtgcgc ctggacgtgg cctatctcag ggccttgtcc<br>ctggaggtgg ccgaggagat cgcccgcctc gaggccgagg tcttccgcct<br>ggccggccac cccttcaacc tcaactcccg ggaccagctg gaaagggtcc<br>tctttgacga gctagggctt cccgccatcg gcaagacgga gaagaccggc<br>aagcgctcca ccagcgccgc cgtcctggag gccctccgcg aggcccaccc<br>catcgtggag aagatcctgc agtaccggga gctcaccaag ctgaagagca<br>cctacattga ccccttgccg gacctcatcc accccaggac gggccgcctc<br>cacacccgct tcaaccagac ggccacggcc acgggcaggc taagtagctc<br>cgatcccaac ctccagaaca tccccgtccg caccccgctt gggcagagga<br>tccgccgggc cttcatcgcc gaggaggggt ggctattggt ggccctggac<br>tatagccaga tagagctcag ggtgctggcc cacctctccg gcgacgagaa<br>cctgatccgg gtcttccagg aggggcggga catccacacg gagaccgcca<br>gctggatgtt cggcgtcccc cgggaggccg tggaccccct gatgcgccgg<br>gcggccaaga ccatcaactt cggggtcctc tacggcatgt cggcccaccg<br>cctctcccag gagctagcca tcccttacga ggaggcccag gccttcattg<br>agcgctactt tcagagcttc cccaaggtgc gggcctggat tgagaagacc<br>ctggaggagg gcaggaggcg ggggtacgtg gagaccctct tcggccgccg<br>ccgctacgtg ccagacctag aggcccgggt gaagagcgtg cgggaggcgg<br>ccgagcgcat ggccttcaac atgcccgtcc agggcaccgc cgccgacctc<br>atgaagctgg ctatggtgaa gctcttcccc aggctggagg aaatgggggc<br>caggatgctc cttcaggtcc acgacgagct ggtcctcgag gccccaaaag<br>agagggcgga ggccgtggcc cggctggcca aggaggtcat ggaggggtg<br>tatcccctgg ccgtgccccct ggaggtggag gtggggatag gggaggactg<br>gctctccgcc aaggagtgat accacc |

**[019]** Taq DNA polymerase extends primers composed from deoxyribonucleotides, however, some chemical modifications of the primer are tolerated and do not decrease much the efficiency of primer extension reactions. For example, when the nucleotide at 3' primer terminus is ribonucleotide instead of deoxyribonucleotide, Taq DNA polymerase can extend such primer with significant efficiency and speed.

## BRIEF SUMMARY OF THE INVENTION

**[020]** In one aspect, a mutant Taq DNA polymerase having an enhanced template discrimination activity compared with an unmodified Taq DNA polymerase is provided. The amino acid sequence of the mutant Taq DNA polymerase includes at least one substitution at residue positions 783 or 784 of the unmodified Taq DNA polymerase.

**[021]** In another aspect, a mutant thermostable DNA polymerase having an enhanced template discrimination activity compared with an unmodified thermostable DNA polymerase is provided. The amino acid sequence of the mutant thermostable DNA polymerase includes at least one substitution at residue positions orthologous to positions 783 or 784 of the unmodified Taq DNA polymerase.

**[022]** In another aspect, a mutant DNA polymerase having an enhanced template discrimination activity compared with the corresponding unmodified DNA polymerase is provided, wherein the mutant DNA polymerase includes a thermostable polymerase. The amino acid sequence of the mutant DNA polymerase peptide includes at least one substitution at residue positions orthologous to positions 783 or 784 of the unmodified Taq DNA polymerase, wherein the mutant DNA polymerase is selected from the group of species consisting of *E. coli*, *Eubacterium siraeum, Clostridium leptum, Enterococcus, Facklamia hominis, Bacillus anthracis and Bacillus cereus ATCC 10987.*

**[023]** In another aspect, a mutant non-VH-related DNA polymerase having an enhanced template discrimination activity compared with its unmodified non-VH-related DNA polymerase counterpart is provided, wherein the mutant non-VH-related DNA polymerase includes a thermostable polymerase. The amino acid sequence of the mutant non-VH-related DNA polymerase includes at least one substitution at residue positions orthologous to reside positions 783 and/or 784 of the unmodified Taq DNA polymerase.

**[024]** In another aspect, recombinant nucleic acid encoding any of the mutant DNA polymerases of described above is provided.

**[025]** In another aspect, a method for conducting primer extension is provided. The method includes the step of contacting a mutant DNA polymerase as described above with a primer, a polynucleotide template, and nucleoside triphosphates under conditions suitable for a primer extension method, thereby producing an extended primer.

**[026]** In another aspect, a kit for producing an extended primer, comprising: at least one container providing a mutant DNA polymerase as described above.

**[027]** In another aspect, a reaction mixture is provided that includes a mutant DNA polymerase as described above, at least one primer, a polynucleotide template, and nucleoside triphosphates.

**[028]** In another aspect, a method for performing rhPCR is provided that includes the step of performing primer extension with a mutant DNA polymerase as described above.

**[029]** In another aspect, a mutant Taq DNA polymerase having an enhanced template discrimination activity compared with an unmodified Taq DNA polymerase is provided. The amino acid sequence of the mutant Taq DNA polymerase comprises one of following selected substitutions: (1) A661E; I665W; F667L [SEQ ID NO:87]; (2) V783F [SEQ ID NO:83]; (3) H784Q [SEQ ID NO:85]; (4) V783L;H784Q [SEQ ID NO:89]; (5) H784A [SEQ ID NO:147]; (6) H784S [SEQ ID NO:149]; (7) H784I [SEQ ID NO:155]; (8) H784T [SEQ ID NO:151], (9) H784V [SEQ ID NO:153]; (10) H784M [SEQ ID NO:157]; (11) H784F [SEQ ID NO:159]; or (12) H784Y [SEQ ID NO:161].

**[030]** In another aspect, a mutant Taq DNA polymerase having a deleted 5' exonuclease domain (KlenTaq) and containing additional mutations, having an enhanced template discrimination activity compared with an unmodified Taq DNA polymerase is provided. The amino acid sequence of the mutant Taq DNA polymerase comprises one of following selected substitutions: (1) A661E; I665W; F667L [SEQ ID NO: 170]; (2) V783F [SEQ ID NO: 172]; (3) H784Q [SEQ ID NO: 174]; (4) V783L;H784Q [SEQ ID NO: 176]; (5) H784S [SEQ ID NO: 178]; or (6) H784Y [SEQ ID NO: 180].

## BRIEF DESCRIPTION OF THE DRAWINGS

**[031]** **FIG. 1** depicts a model of the active site constructed from the known Taq DNA polymerase PDB ID 2KTQ crystal structure. The polymerase backbone is displayed using ribbons. The "C2'" label indicates the 2' carbon atom at the primer terminal nucleotide. The dNTP is binding in the pocket above the primer.

**[032]** **FIG. 2A** shows gel images depicting purified protein for the Taq DNA polymerase mutants and wild type Taq DNA polymerase. Legend: Aliquots of purified recombinant proteins were separated by polyacrylamide gel electrophoresis (PAGE) and stained with Coomassie Brilliant Blue. The Marker lane (M) is indicated, showing protein size markers identified in kilodaltons (kDa).

**[033]** **FIG. 2B** shows gel images depicting purified protein for the Taq DNA polymerase mutants and wild type Taq DNA polymerase. Legend as in **FIG. 2A**.

**[034]** **FIG. 2C** shows gel images depicting purified protein for the Taq DNA polymerase mutants and wild type Taq DNA polymerase. Legend as in **FIG. 2A**.

**[035]** **FIG. 2D** shows gel images depicting purified protein for the Taq DNA polymerase mutants and wild type Taq DNA polymerase. Legend as in **FIG. 2A**.

**[036]** **FIG. 3A** shows graphical representations of the allele-specific PCR (AS-PCR) data from Tables 10 and 31 for wild type OptiTaq (sub-panel (i)), Mutant ID 2 (sub-panel (ii)) and Mutant ID 3 (sub-panel (iii)). Legend: Average ΔCq values obtained from AS-PCR reactions plus/minus standard deviation (error bars) are shown (ΔCq = Cq mismatch – Cq match) comparing mismatch discrimination of the wild-type OptiTaq with the mutant Taq DNA polymerases. All possible pairwise mismatch base combinations are included. The base identity of the SNP site in the target nucleic acid is indicated on the X-axis (A, G, C, T) along with the 3'-DNA residue of the AS-PCR reverse primer employed (dA, dG, dC, dT).

**[037]** **FIG. 3B** shows graphical representations of the allele-specific PCR (AS-PCR) data from Tables 10 and 31 for wild type OptiTaq (sub-panel (i)), Mutant ID 10 (sub-panel (ii)) and Mutant ID 18 (sub-panel (iii)). Legend as in **FIG. 3A**.

**[038]** **FIG. 3C** shows graphical representations of the allele-specific PCR (AS-PCR) data from Tables 10 and 31 for wild type OptiTaq (sub-panel (i)), Mutant ID 3 (sub-panel (ii)) and Mutant ID 20 (sub-panel (iii)). Legend as in **FIG. 3A**.

**[039]** **FIG. 3D** shows graphical representations of the allele-specific PCR (AS-PCR) data from Tables 10 and 31 for wild type OptiTaq (sub-panel (i)), Mutant ID21 (sub-panel (ii)) and Mutant ID 22 (sub-panel (iii)). Legend as in **FIG. 3A**.

**[040]** **FIG. 3E.** shows graphical representations of the allele-specific PCR (AS-PCR) data from Tables 10 and 31 for wild type OptiTaq (sub-panel (i)), Mutant ID 24 (sub-panel (ii)) and Mutant ID 26 (sub-panel (iii)). Legend as in **FIG. 3A**.

**[041]** **FIG. 3F** shows graphical representations of the allele-specific PCR (AS-PCR) data from Tables 10 and 31 for wild type OptiTaq (sub-panel (i)), Mutant ID 27 (sub-panel (ii)) and Mutant ID 29 (sub-panel (iii)). Legend as in **FIG. 3A**.

**[042]** **FIG. 3G** shows graphical representations of the allele-specific PCR (AS-PCR) data from Tables 10 and 31 for wild type OptiTaq (sub-panel (i)) and Mutant ID 30 (sub-panel (ii)). Legend as in **FIG. 3A**.

**[043]** **FIG. 4** shows a graphical representation of the ΔCq values (Table 13) obtained from comparing qPCR results using primers ending with a 3'-RNA residue and primers ending with a 3'-DNA residue for wild type OptiTaq with four mutant Taq DNA polymerases, where ΔCq = Cq 3'-RNA – Cq 3'-DNA; rA is compared with dA, rC is compared with dC, rG is compared with dG, and rU is compared with dT. Identity of each DNA polymerase studied is shown on the X-axis.

**[044]** **FIG. 5A** shows graphical representations of the ΔCq values (Tables 20 and 34) obtained from comparing mismatch discrimination between wild type OptiTaq with Mutant ID 2, Mutant ID 3, Mutant ID 10, and Mutant ID 18 Taq DNA polymerases detecting a human genomic DNA SNP in the SMAD7 gene (NM_005904, C/T SNP, rs4939827) using Gen1 RDDDDx blocked-cleavable primers in a quantitative rhPCR assay. ΔCq = [Cq mismatch – Cq match]. Legend: Identity of each DNA polymerase studied is shown on the X-axis. The RDDDDx blocked-cleavable primer contained either a rC or rU residue as the cleavable base, specific for the "C" or "T" allele, as indicated.

**[045]** **FIG. 5B** shows graphical representations of the ΔCq values (Tables 20 and 34) obtained from comparing mismatch discrimination between wild type OptiTaq with Mutant ID 3,

Mutant ID 20, Mutant ID 21, Mutant ID 22, and Mutant ID 24 Taq DNA polymerases detecting a human genomic DNA SNP in the SMAD7 gene (NM_005904, C/T SNP, rs4939827) using Gen1 RDDDDx blocked-cleavable primers in a quantitative rhPCR assay. ΔCq = [Cq mismatch – Cq match]. Legend as in **FIG. 5A**.

**[046]** **FIG. 5C** shows graphical representations of the ΔCq values (Tables 20 and 34) obtained from comparing mismatch discrimination between wild type OptiTaq with Mutant ID 3, Mutant ID 26, Mutant ID 27, Mutant ID 29, and Mutant ID 30 Taq DNA polymerases detecting a human genomic DNA SNP in the SMAD7 gene (NM_005904, C/T SNP, rs4939827) using Gen1 RDDDDx blocked-cleavable primers in a quantitative rhPCR assay. ΔCq = [Cq mismatch – Cq match]. Legend as in **FIG. 5A**.

**[047]** **FIG. 6** shows a graphical representation of the ΔCq values (Tables 21 and 35) obtained from comparing mismatch discrimination between wild type OptiTaq with Mutant ID 2, Mutant ID 3, Mutant ID 10, and Mutant ID 18 Taq DNA polymerases detecting a human genomic DNA SNP in the SMAD7 gene (NM_005904, C/T SNP, rs4939827) using Gen2 RDxxD blocked-cleavable primers in a quantitative rhPCR assay. ΔCq = [Cq mismatch – Cq match]. Legend: Identity of each DNA polymerase studied is shown on the X-axis. The RDxxD blocked-cleavable primer contained either a rC or rU residue as the cleavable base, specific for the "C" or "T" allele, as indicated.

**[048]** **FIG. 6B** shows a graphical representation of the ΔCq values (Tables 21 and 35) obtained from comparing mismatch discrimination between wild type OptiTaq with Mutant ID 3, Mutant ID 20, Mutant ID 21, Mutant ID 22, and Mutant ID 24 Taq DNA polymerases detecting a human genomic DNA SNP in the SMAD7 gene (NM_005904, C/T SNP, rs4939827) using Gen2 RDxxD blocked-cleavable primers in a quantitative rhPCR assay. ΔCq = [Cq mismatch – Cq match]. Legend as in **FIG. 6A**.

**[049]** **FIG. 6C** shows a graphical representation of the ΔCq values (Tables 21 and 35) obtained from comparing mismatch discrimination between wild type OptiTaq with Mutant ID 3, Mutant ID 26, Mutant ID 27, Mutant ID 29, and Mutant ID 30 Taq DNA polymerases detecting a human genomic DNA SNP in the SMAD7 gene (NM_005904, C/T SNP, rs4939827) using Gen2 RDxxD blocked-cleavable primers in a quantitative rhPCR assay. ΔCq = [Cq mismatch – Cq match]. Legend as in **FIG. 6A**.

**[050]** **FIG. 7A** shows graphical representations of the allele-specific PCR (AS-PCR) data from Tables 10 and 31 for OptiTaq KlenTaq (Mutant ID 37) (sub-panel (i)), Mutant ID 38 (sub-panel (ii)) and Mutant ID 39 (sub-panel (iii)). Legend: Average ΔCq values obtained from AS-PCR reactions plus/minus standard deviation (error bars) are shown (ΔCq = Cq mismatch – Cq match) comparing mismatch discrimination of the wild-type OptiTaq with four mutant Taq DNA polymerases. All possible pairwise mismatch base combinations are included. The base identity of the SNP site in the target nucleic acid is indicated on the X-axis (A, G, C, T) along with the 3'-DNA residue of the AS-PCR reverse primer employed (dA, dG, dC, dT).

**[051]** **FIG. 7B.** shows graphical representations of the allele-specific PCR (AS-PCR) data from Tables 10 and 31 for OptiTaq KlenTaq (Mutant ID 37) (sub-panel (i)), Mutant ID 40 (sub-panel (ii)) and Mutant ID 41 (sub-panel (iii)). Legend as in **FIG. 7A**.

**[052]** **FIG. 7C**. shows graphical representations of the allele-specific PCR (AS-PCR) data from Tables 10 and 31 for OptiTaq KlenTaq (Mutant ID 37) (sub-panel (i)), Mutant ID 42 (sub-panel (ii)) and Mutant ID 43 (sub-panel (iii)). Legend as in **FIG. 7A**.

## DETAILED DESCRIPTION

**[053]** The current invention provides novel thermostable DNA polymerases, including specific examples derived from *Thermus aquaticus* (Taq) DNA polymerase. These polymerases offer improvements to existing methods for nucleic acid amplification, genotyping, and detection of rare alleles. New assay formats comprising the use of these novel thermostable DNA polymerases are also provided.

### DEFINITIONS

**[054]** To aid in understanding the invention, several terms are defined below.

**[055]** Terms used herein are intended as "open" terms (for example, the term "including" should be interpreted as "including but not limited to," the term "having" should be interpreted as "having at least," the term "includes" should be interpreted as "includes but is not limited to," etc.).

**[056]** The articles "a" and "an" refer to one or to more than one (for example, to at least one) of the grammatical object of the article.

**[057]** The terms "about" and "approximately" shall generally mean an acceptable degree of error for the quantity measured given the nature or precision of the measurements. Exemplary degrees of error are within 20-25 percent (%), typically, within 10%, and more typically, within 5% of a given value or range of values.

**[058]** Furthermore, in those instances where a convention analogous to "at least one of A,B and C, etc." is used, in general such a construction is intended in the sense of one having ordinary skill in the art would understand the convention (for example, "a system having at least one of A, B and C" would include but not be limited to systems that have A alone, B alone, C alone, A and B together, A and C together, B and C together, and/or A, B, and C together.). It will be further understood by those within the art that virtually any disjunctive word and/or phrase presenting two or more alternative terms, whether in the description or figures, should be understood to contemplate the possibilities of including one of the terms, either of the terms, or both terms. For example, the phrase "A or B" will be understood to include the possibilities of "A" or 'B or "A and B."

**[059]** All language such as "from," "to," "up to," "at least," "greater than," "less than," and the like, include the number recited and refer to ranges which can subsequently be broken down into sub-ranges.

**[060]** A range includes each individual member. Thus, for example, a group having 1-3 members refers to groups having 1, 2, or 3 members. Similarly, a group having 6 members refers to groups having 1, 2, 3, 4, or 6 members, and so forth.

**[061]** The modal verb "may" refers to the preferred use or selection of one or more options or choices among the several described embodiments or features contained within the same. Where no options or choices are disclosed regarding a particular embodiment or feature contained in the same, the modal verb "may" refers to an affirmative act regarding how to make or use and aspect of a described embodiment or feature contained in the same, or a definitive decision to use a specific skill regarding a described embodiment or feature contained in the same. In this latter context, the modal verb "may" has the same meaning and connotation as the auxiliary verb "can."

**[062]** The term "conventional" or "natural" when referring to nucleic acid bases, nucleoside triphosphates, or nucleotides refers to those which occur naturally in the polynucleotide being described (i.e., for DNA these are dATP, dGTP, dCTP and dTTP). Additionally, dITP, and

7-deaza-dGTP are frequently utilized in place of dGTP and 7-deaza-dATP can be utilized in place of dATP in in vitro DNA synthesis reactions, such as sequencing. Collectively, these may be referred to as dNTPs.

**[063]** The term "unconventional" or "modified" when referring to a nucleic acid base, nucleoside, or nucleotide includes modification, derivations, or analogues of conventional bases, nucleosides, or nucleotides that naturally occur in a particular polynucleotide. Certain unconventional nucleotides are modified at the 2' position of the ribose sugar in comparison to conventional dNTPs. Thus, although for RNA the naturally occurring nucleotides are ribonucleotides (i.e., ATP, GTP, CTP, UTP, collectively rNTPs), because these nucleotides have a hydroxyl group at the 2' position of the sugar, which, by comparison is absent in dNTPs, as used herein, ribonucleotides are unconventional nucleotides as substrates for DNA polymerases. As used herein, unconventional nucleotides include, but are not limited to, compounds used as terminators for nucleic acid sequencing. Exemplary terminator compounds include but are not limited to those compounds that have a 2',3' dideoxy structure and are referred to as dideoxynucleoside triphosphates. The dideoxynucleoside triphosphates ddATP, ddTTP, ddCTP and ddGTP are referred to collectively as ddNTPs.

**[064]** The term "nucleotide," in addition to referring to the naturally occurring ribonucleotide or deoxyribonucleotide monomers, shall herein be understood to refer to related structural variants thereof, including derivatives and analogs, that are functionally equivalent with respect to the particular context in which the nucleotide is being used (e.g., hybridization to a complementary base), unless the context clearly indicates otherwise.

**[065]** The terms "nucleic acid" and "oligonucleotide," as used herein, refer to polydeoxyribonucleotides (containing 2-deoxy-D-ribose), polyribonucleotides (containing D-ribose), and to any other type of polynucleotide which is an N glycoside of a purine or pyrimidine base. There is no intended distinction in length between the terms "nucleic acid", "oligonucleotide" and "polynucleotide", and these terms will be used interchangeably. These terms refer only to the primary structure of the molecule. Thus, these terms include double- and single-stranded DNA, as well as double- and single-stranded RNA. For use in the present invention, an oligonucleotide also can comprise nucleotide analogs in which the base, sugar or phosphate backbone is modified as well as non-purine or non-pyrimidine nucleotide analogs.

**[066]** Oligonucleotides can be prepared by any suitable method, including direct chemical synthesis by a method such as the phosphotriester method of Narang et al., 1979, Meth. Enzymol. 68:90-99; the phosphodiester method of Brown et al., 1979, Meth. Enzymol. 68:109-151; the diethylphosphoramidite method of Beaucage et al., 1981, Tetrahedron Lett. 22:1859-1862; and the solid support method of U.S. Pat. No. 4,458,066, each incorporated herein by reference. A review of synthesis methods of conjugates of oligonucleotides and modified nucleotides is provided in Goodchild, 1990, Bioconjugate Chemistry 1(3): 165-187, incorporated herein by reference.

**[067]** The term "primer," as used herein, refers to an oligonucleotide capable of acting as a point of initiation of DNA synthesis under suitable conditions. Such conditions include those in which synthesis of a primer extension product complementary to a nucleic acid strand is induced in the presence of four different nucleoside triphosphates and an agent for extension (e.g., a DNA polymerase or reverse transcriptase) in an appropriate buffer and at a suitable temperature. Primer extension can also be carried out in the absence of one or more of the nucleoside triphosphates in which case an extension product of limited length is produced. As used herein, the term "primer" is intended to encompass the oligonucleotides used in ligation-mediated reactions, in which one oligonucleotide is "extended" by ligation to a second oligonucleotide which hybridizes at an adjacent position. Thus, the term "primer extension", as used herein, refers to both the polymerization of individual nucleoside triphosphates using the primer as a point of initiation of DNA synthesis and to the ligation of two oligonucleotides to form an extended product.

**[068]** A primer is preferably a single-stranded DNA. The appropriate length of a primer depends on the intended use of the primer but typically ranges from 6 to 50 nucleotides, preferably from 15-35 nucleotides. Short primer molecules generally require cooler temperatures to form sufficiently stable hybrid complexes with the template. A primer need not reflect the exact sequence of the template nucleic acid, but must be sufficiently complementary to hybridize with the template. The design of suitable primers for the amplification of a given target sequence is well known in the art and described in the literature cited herein.

**[069]** Primers can incorporate additional features which allow for the detection or immobilization of the primer but do not alter the basic property of the primer, that of acting as a point of initiation of DNA synthesis. For example, primers may contain an additional nucleic

acid sequence at the 5' end which does not hybridize to the target nucleic acid, but which facilitates cloning or detection of the amplified product. The region of the primer which is sufficiently complementary to the template to hybridize is referred to herein as the hybridizing region. Primers may incorporate modified residues other than DNA, so long as these alternations do not impede priming or template functionality.

**[070]** The phrase "3'-nucleotide discrimination" refers to a property of a DNA polymerase to catalyze a primer extension reaction with greater specificity for deoxyribonucleotides and less efficiently when the nucleotide at the primer 3' terminus was chemically modified. For example, a mutated Taq DNA polymerase that displays 3'-nucleotide discrimination exhibits selectivity for deoxyribonucleotide primer and suppressed catalytic activity when the primer is for example modified with ribonucleotides.

**[071]** The term "3'-mismatch discrimination" refers to a property of a DNA polymerase to distinguish a fully complementary sequence from a mismatch-containing (nearly complementary) sequence where the nucleic acid to be extended (for example, a primer or other oligonucleotide) has a mismatch at the 3' terminus of the nucleic acid compared to the template to which the nucleic acid hybridizes. In some embodiments, the nucleic acid to be extended comprises a mismatch at the 3' end relative to the fully complementary sequence.

**[072]** The term "rare allele discrimination" refers to a property of a DNA polymerase to preferentially replicate a first nucleic acid in a population of nucleic acids that includes a plurality of a second nucleic acid, wherein the first nucleic acid is under-represented in the population of nucleic acids relative to the plurality of a second nucleic acid. Typically, the first nucleic acid may be under-represented in the population of nucleic acids that contain a plurality of a second nucleic acids by a ratio of the first nucleic acid to the second nucleic acid in the range from about 1:10 to about 1:1,000,000, including 1:100, 1:1,000; 1:10,000 and 1:100,000, among other ratios. Typically, though not exclusively, a polymerase having rare allele discrimination can be used to detect a SNP difference between a first nucleic acid and a second nucleic acid, as further elaborated herein.

**[073]** The phrase "template discrimination activity" refers to a DNA polymerase having at least one of 3'-nucleotide discrimination, 3'-mismatch discrimination, rare allele discrimination and combinations thereof.

**[074]** The phrase "enhanced template discrimination activity" refers to a DNA polymerase having at least one of 3'-nucleotide discrimination, 3'-mismatch discrimination and rare allele discrimination, or combinations thereof, wherein the DNA polymerase displays greater activity than a reference DNA polymerase. For example, a DNA polymerase mutant having "enhanced template discrimination activity" displays at least one of 3'-nucleotide discrimination, 3'-mismatch discrimination, rare allele discrimination and combinations thereof that is greater than the corresponding activity of the naturally-occurring, wild-type DNA polymerase from which the DNA polymerase mutant was derived.

**[075]** A "template discrimination activity assay" refers to an assay for assessing the ability of a polymerase to discriminate between two templates that differ in one or more variables. Assays designed to reveal 3'-nucleotide discrimination, 3'-mismatch discrimination or rare allele discrimination are examples of template discrimination activity assays.

**[076]** The term "quantification cycle value," denoted as Cq, refers to the amplification cycle number at which positive signal is first detected.

**[077]** The term "discrimination quantification cycle value," denoted as ΔCq, refers to a calculated difference between a first reference state and a second reference state, wherein both the first and second reference states differ in terms of only one variable. For examples, a first and second reference states can refer to identical polymerase reactions that differ in polymerases, such as a wild-type polymerase and a polymerase mutant, that differ in primer template nucleotide sequence, such as a mismatched primer template and a matched primer template, or that differ in primer template 3'-nucleotide ribose structure, such as a primer template containing a 3'-deoxyribose moiety and a primer template containing a 3'-ribose moiety.

**[078]** The term "differential discrimination quantification cycle value," denoted as ΔΔCq, refers to a calculated difference between a first discrimination quantification cycle value and a second discrimination quantification cycle value for polymerase reactions that differ in two variables. In the context of the present disclosure, the ΔΔCq value is a measure of the improvement that a given polymerase mutant displays relative to the wild-type polymerase in a template discrimination activity assay. A preferred ΔΔCq value depends upon the nature of the assay, but generally a preferred ΔΔCq value is at least 1.0 and is typically greater than 1.0.

**[079]** The terms "target, "target sequence", "target region", and "target nucleic acid," as used herein, are synonymous and refer to a region or sequence of a nucleic acid which is to be amplified, sequenced or detected.

**[080]** The term “template” refers to a nucleic acid that includes at least one single stranded region. The term “template” as it modifies “substrate” refers to a nucleic acid that is used in a hybridization reaction to anneal with a primer and/or an extension reaction with a polymerase.

**[081]** The term "hybridization," as used herein, refers to the formation of a duplex structure by two single-stranded nucleic acids due to complementary base pairing. Hybridization can occur between fully complementary nucleic acid strands or between "substantially complementary" nucleic acid strands that contain minor regions of mismatch. Conditions under which hybridization of fully complementary nucleic acid strands is strongly preferred are referred to as "stringent hybridization conditions" or "sequence-specific hybridization conditions". Stable duplexes of substantially complementary sequences can be achieved under less stringent hybridization conditions; the degree of mismatch tolerated can be controlled by suitable adjustment of the hybridization conditions. Those skilled in the art of nucleic acid technology can determine duplex stability empirically considering a number of variables including, for example, the length and base pair composition of the oligonucleotides, ionic strength, and incidence of mismatched base pairs, following the guidance provided by the art (see, e.g., Sambrook et al., 1989, Molecular Cloning--A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.; Wetmur, 1991, Critical Review in Biochem. and Mol. Biol. 26(3/4):227-259; and Owczarzy et al., 2008, Biochemistry, 47: 5336-5353, which are incorporated herein by reference).

**[082]** The term "amplification reaction" refers to any chemical reaction, including an enzymatic reaction, which results in increased copies of a template nucleic acid sequence or results in transcription of a template nucleic acid. Amplification reactions include reverse transcription, the polymerase chain reaction (PCR), including Real Time PCR (see U.S. Pat. Nos. 4,683,195 and 4,683,202; PCR Protocols: A Guide to Methods and Applications (Innis et al., eds, 1990)), and the ligase chain reaction (LCR) (see Barany et al., U.S. Pat. No. 5,494,810). Exemplary "amplification reactions conditions" or "amplification conditions" typically comprise either two or three step cycles. Two step cycles have a high temperature denaturation step followed by a hybridization/elongation (or ligation) step. Three step cycles comprise a

denaturation step followed by a hybridization step followed by a separate elongation or ligation step.

**[083]** An "amino acid" refers to any monomer unit that can be incorporated into a peptide, polypeptide, or protein. As used herein, the term "amino acid" includes the following twenty natural or genetically encoded alpha-amino acids: alanine (Ala or A), arginine (Arg or R), asparagine (Asn or N), aspartic acid (Asp or D), cysteine (Cys or C), glutamine (Gln or Q), glutamic acid (Glu or E), glycine (Gly or G), histidine (His or H), isoleucine (Ile or I), leucine (Leu or L), lysine (Lys or K), methionine (Met or M), phenylalanine (Phe or F), proline (Pro or P), serine (Ser or S), threonine (Thr or T), tryptophan (Trp or W), tyrosine (Tyr or Y), and valine (Val or V). In cases where "X" residues are undefined, these should be defined as "any amino acid." The structures of these twenty natural amino acids are shown in, e.g., Stryer et al., Biochemistry, 5.sup.th ed., Freeman and Company (2002), which is incorporated by reference. Additional amino acids, such as selenocysteine and pyrrolysine, can also be genetically coded for (Stadtman (1996) "Selenocysteine," Annu Rev Biochem. 65:83-100 and Ibba et al. (2002) "Genetic code: introducing pyrrolysine," Curr Biol. 12(13):R464-R466, which are both incorporated by reference). The term "amino acid" also includes unnatural amino acids, modified amino acids (e.g., having modified side chains and/or backbones), and amino acid analogs. See, e.g., Zhang et al. (2004) "Selective incorporation of 5-hydroxytryptophan into proteins in mammalian cells," Proc. Natl. Acad. Sci. U.S.A. 101(24):8882-8887, Anderson et al. (2004) "An expanded genetic code with a functional quadruplet codon" Proc. Natl. Acad. Sci. U.S.A. 101(20):7566-7571, Ikeda et al. (2003) "Synthesis of a novel histidine analogue and its efficient incorporation into a protein in vivo," Protein Eng. Des. Sel. 16(9):699-706, Chin et al. (2003) "An Expanded Eukaryotic Genetic Code," Science 301(5635):964-967, James et al. (2001) "Kinetic characterization of ribonuclease S mutants containing photoisomerizable phenylazophenylalanine residues," Protein Eng. Des. Sel. 14(12):983-991, Kohrer et al. (2001) "Import of amber and ochre suppressor tRNAs into mammalian cells: A general approach to site-specific insertion of amino acid analogues into proteins," Proc. Natl. Acad. Sci. U.S.A. 98(25):14310-14315, Bacher et al. (2001) "Selection and Characterization of Escherichia coli Variants Capable of Growth on an Otherwise Toxic Tryptophan Analogue," J. Bacteriol. 183(18):5414-5425, Hamano-Takaku et al. (2000) "A Mutant Escherichia coli Tyrosyl-tRNA Synthetase Utilizes the Unnatural Amino Acid Azatyrosine More Efficiently than Tyrosine," J.

Biol. Chem. 275(51):40324-40328, and Budisa et al. (2001) "Proteins with {beta}-(thienopyrrolyl)alanines as alternative chromophores and pharmaceutically active amino acids," Protein Sci. 10(7):1281-1292, which are each incorporated by reference.

**[084]** The term "residue" is synonymous and interchangeable with "amino acid" or "nucleotide" depending upon context.

**[085]** As used herein, a "polymerase" refers to an enzyme that catalyzes the polymerization of nucleotides. Generally, the enzyme will initiate synthesis at the 3'-end of the primer annealed to a nucleic acid template sequence. "DNA polymerase" catalyzes the polymerization of deoxyribonucleotides. Known DNA polymerases include, for example, *Pyrococcus furiosus* (Pfu) DNA polymerase (Lundberg et al., 1991, Gene, 108:1), *E. coli* DNA polymerase I (Lecomte and Doubleday, 1983, Nucleic Acids Res. 11:7505), T7 DNA polymerase (Nordstrom et al., 1981, J. Biol. Chem. 256:3112), *Thermus thermophilus* (Tth) DNA polymerase (Myers and Gelfand 1991, Biochemistry 30:7661), *Bacillus stearothermophilus* DNA polymerase (Stenesh and McGowan, 1977, Biochim Biophys Acta 475:32), *Thermococcus litoralis* (Tli) DNA polymerase (also referred to as Vent DNA polymerase, Cariello et al., 1991, Nucleic Acids Res, 19: 4193), *Thermotoga maritima* (Tma) DNA polymerase (Diaz and Sabino, 1998 Braz J. Med. Res, 31:1239), *Thermus aquaticus* (Taq) DNA polymerase (Chien et al., 1976, J. Bacteoriol, 127: 1550), *Pyrococcus kodakaraensis* KOD DNA polymerase (Takagi et al., 1997, Appl. Environ. Microbiol. 63:4504), JDF-3 DNA polymerase (Patent application WO 0132887), and *Pyrococcus GB-D* (PGB-D) DNA polymerase (Juncosa-Ginesta et al., 1994, Biotechniques, 16:820). The polymerase activity of any of the above enzymes can be determined by means well known in the art.

**[086]** The term "thermostable polymerase," refers to an enzyme that is stable to heat, is heat resistant, and retains sufficient activity to effect subsequent polynucleotide extension reactions and does not become irreversibly denatured (inactivated) when subjected to the elevated temperatures for the time necessary to effect denaturation of double-stranded nucleic acids. The heating conditions necessary for nucleic acid denaturation are well known in the art and are exemplified in, e.g., U.S. Pat. Nos. 4,683,202, 4,683,195, and 4,965,188, which are incorporated herein by reference. As used herein, a thermostable polymerase is suitable for use in a temperature cycling reaction such as the polymerase chain reaction ("PCR"). Irreversible denaturation for purposes herein refers to permanent and complete loss of enzymatic activity. For

a thermostable polymerase, enzymatic activity refers to the catalysis of the combination of the nucleotides in the proper manner to form polynucleotide extension products that are complementary to a template nucleic acid strand. Thermostable DNA polymerases from thermophilic bacteria include, e.g., DNA polymerases from *Thermus aquaticus*, among others.

**[087]** The term "thermoactive" refers to an enzyme that maintains catalytic properties at temperatures commonly used for reverse transcription or anneal/extension steps in RT-PCR and/or PCR reactions (i.e., 45-80° C.). Thermostable enzymes are those which are not irreversibly inactivated or denatured when subjected to elevated temperatures necessary for nucleic acid denaturation. Thermoactive enzymes may or may not be thermostable. Thermoactive DNA polymerases can be DNA or RNA dependent from thermophilic species or from mesophilic species including, but not limited to, *Escherichia coli*, Moloney murine leukemia viruses, and Avian myoblastosis virus.

**[088]** As used herein, a primer is "specific," for a target sequence if, when used in an amplification reaction under sufficiently stringent conditions, the primer hybridizes primarily to the target nucleic acid. Typically, a primer is specific for a target sequence if the primer-target duplex stability is greater than the stability of a duplex formed between the primer and any other sequence found in the sample. One of skill in the art will recognize that various factors, such as salt conditions as well as base composition of the primer and the location of the mismatches, will affect the specificity of the primer, and that routine experimental confirmation of the primer specificity will be needed in many cases. Hybridization conditions can be chosen under which the primer can form stable duplexes only with a target sequence. Thus, the use of target-specific primers under suitably stringent amplification conditions enables the selective amplification of those target sequences which contain the target primer binding sites.

**[089]** The term "non-specific amplification," as used herein, refers to the amplification of nucleic acid sequences other than the target sequence which results from primers hybridizing to sequences other than the target sequence and then serving as a substrate for primer extension. The hybridization of a primer to a non-target sequence is referred to as "non-specific hybridization" and is apt to occur especially during the lower temperature, reduced stringency, pre-amplification conditions, or in situations where there is a variant allele in the sample having a very closely related sequence to the true target as in the case of a single nucleotide polymorphism (SNP).

**[090]** The term "primer dimer," as used herein, refers to a template-independent non-specific amplification product, which is believed to result from primer extensions wherein another primer serves as a template. Although primer dimers frequently appear to be a concatamer of two primers, i.e., a dimer, concatamers of more than two primers also occur. The term "primer dimer" is used herein generically to encompass a template-independent non-specific amplification product.

**[091]** The term "reaction mixture," as used herein, refers to a solution containing reagents necessary to carry out a given reaction. An "amplification reaction mixture", which refers to a solution containing reagents necessary to carry out an amplification reaction, typically contains oligonucleotide primers and a DNA polymerase or ligase in a suitable buffer. A "PCR reaction mixture" typically contains oligonucleotide primers, a DNA polymerase (most typically a thermostable DNA polymerase), dNTPs, and a divalent metal cation in a suitable buffer. A reaction mixture is referred to as complete if it contains all reagents necessary to enable the reaction, and incomplete if it contains only a subset of the necessary reagents. It will be understood by one of skill in the art that reaction components are routinely stored as separate solutions, each containing a subset of the total components, for reasons of convenience, storage stability, or to allow for application-dependent adjustment of the component concentrations, and that reaction components are combined prior to the reaction to create a complete reaction mixture. Furthermore, it will be understood by one of skill in the art that reaction components are packaged separately for commercialization and that useful commercial kits may contain any subset of the reaction components which includes the blocked primers of the invention.

**[092]** The terms "non-activated" or "inactivated," as used herein, refer to a primer or other oligonucleotide that is incapable of participating in a primer extension reaction or a ligation reaction because either DNA polymerase or DNA ligase cannot interact with the oligonucleotide for their intended purposes. In some embodiments when the oligonucleotide is a primer, the non-activated state occurs because the primer is blocked at or near the 3'-end so as to prevent primer extension. When specific groups are bound at or near the 3'-end of the primer, DNA polymerase cannot bind to the primer and extension cannot occur. A non-activated primer is, however, capable of hybridizing to a substantially complementary nucleotide sequence.

**[093]** The term "activated," as used herein, refers to a primer or other oligonucleotide that is capable of participating in a reaction with DNA polymerase or DNA ligase. A primer or other

oligonucleotide becomes activated after it hybridizes to a substantially complementary nucleic acid sequence and is cleaved to generate a functional 3'- or 5'-end so that it can interact with a DNA polymerase or a DNA ligase. For example, when the oligonucleotide is a primer, and the primer is hybridized to a template, a 3'-blocking group can be removed from the primer by, for example, a cleaving enzyme such that DNA polymerase can bind to the 3' end of the primer and promote primer extension.

**[094]** The term "cleavage domain" or "cleaving domain," as used herein, are synonymous and refer to a region located between the 5' and 3' end of a primer or other oligonucleotide that is recognized by a cleavage compound, for example a cleavage enzyme, that will cleave the primer or other oligonucleotide. For the purposes of this invention, the cleavage domain is designed such that the primer or other oligonucleotide is cleaved only when it is hybridized to a complementary nucleic acid sequence, but will not be cleaved when it is single-stranded. The cleavage domain or sequences flanking it may include a moiety that a) prevents or inhibits the extension or ligation of a primer or other oligonucleotide by a polymerase or a ligase, b) enhances discrimination to detect variant alleles, or c) suppresses undesired cleavage reactions. One or more such moieties may be included in the cleavage domain or the sequences flanking it.

**[095]** The term "RNase H cleavage domain," as used herein, is a type of cleavage domain that contains one or more ribonucleic acid residue or an alternative analog which provides a substrate for an RNase H. An RNase H cleavage domain can be located anywhere within a primer or oligonucleotide, and is preferably located at or near the 3'-end or the 5'-end of the molecule.

**[096]** An "RNase H1 cleavage domain" generally contains at least three consecutive RNA residues. An "RNase H2 cleavage domain" may contain one RNA residue, a sequence of contiguously linked RNA residues or RNA residues separated by DNA residues or other chemical groups. For example, an RNase H2 cleavage domain may include a 2'-fluoronucleoside residue, among others.

**[097]** The terms "cleavage compound," or "cleaving agent" as used herein, refers to any compound that can recognize a cleavage domain within a primer or other oligonucleotide, and selectively cleave the oligonucleotide based on the presence of the cleavage domain. The cleavage compounds utilized in the invention selectively cleave the primer or other oligonucleotide comprising the cleavage domain only when it is hybridized to a substantially

complementary nucleic acid sequence, but will not cleave the primer or other oligonucleotide when it is single stranded. The cleavage compound cleaves the primer or other oligonucleotide within or adjacent to the cleavage domain. The term "adjacent," as used herein, means that the cleavage compound cleaves the primer or other oligonucleotide at either the 5'-end or the 3' end of the cleavage domain. Cleavage reactions preferred in the invention yield a 5'-phosphate group and a 3'-OH group.

**[098]** In a preferred embodiment, the cleavage compound is a "cleaving enzyme." A cleaving enzyme is a protein or a ribozyme that is capable of recognizing the cleaving domain when a primer or other nucleotide is hybridized to a substantially complementary nucleic acid sequence, but that will not cleave the complementary nucleic acid sequence (i.e., it provides a single strand break in the duplex). The cleaving enzyme will also not cleave the primer or other oligonucleotide comprising the cleavage domain when it is single stranded. Examples of cleaving enzymes are RNase H enzymes and other nicking enzymes.

**[099]** The term "nicking," as used herein, refers to the cleavage of only one strand of the double-stranded portion of a fully or partially double-stranded nucleic acid. The position where the nucleic acid is nicked is referred to as the "nicking site" (NS). A "nicking agent" (NA) is an agent that nicks a partially or fully double-stranded nucleic acid. It may be an enzyme or any other chemical compound or composition. In certain embodiments, a nicking agent may recognize a particular nucleotide sequence of a fully or partially double-stranded nucleic acid and cleave only one strand of the fully or partially double-stranded nucleic acid at a specific position (i.e., the NS) relative to the location of the recognition sequence. Such nicking agents (referred to as "sequence specific nicking agents") include, but are not limited to, nicking endonucleases (e.g., *Nt.BstNBI*).

**[0100]** A "nicking endonuclease" (NE), as used herein, thus refers to an endonuclease that recognizes a nucleotide sequence of a completely or partially double-stranded nucleic acid molecule and cleaves only one strand of the nucleic acid molecule at a specific location relative to the recognition sequence. In such a case the entire sequence from the recognition site to the point of cleavage constitutes the "cleavage domain".

**[0101]** The term "blocking group," as used herein, refers to a chemical moiety that is bound to the primer or other oligonucleotide such that an amplification reaction does not occur. For example, primer extension and/or DNA ligation does not occur. Once the blocking group is

removed from the primer or other oligonucleotide, the oligonucleotide is capable of participating in the assay for which it was designed (PCR, ligation, sequencing, *etc.*). Thus, the "blocking group" can be any chemical moiety that inhibits recognition by a polymerase or DNA ligase. The blocking group may be incorporated into the cleavage domain but is generally located on either the 5'- or 3'-side of the cleavage domain. The blocking group can be comprised of more than one chemical moiety. In the present invention the "blocking group" is typically removed after hybridization of the oligonucleotide to its target sequence.

**[0102]** The term "blocked-cleavable primer" refers to a primer that is inactive or inactivated for priming DNA synthesis from a polymerase owing to the presence of a blocking group at or near the 3'-terminus of the primer. A blocked-cleavable primer can be converted into a competent primer by removing the blocking group at or near the 3'-terminus of the primer by a cleavage compound or a cleaving agent (for example, a cleaving enzyme) resulting in an active or activated primer.

**[0103]** An RDDDDx blocked-cleavable primer (also known as "generation 1" or "Gen 1" blocked-cleavable primer) refers to a blocked-cleavable primer having at its 3'-terminus the sequence RDDDDx, wherein R is an RNA base, D is a DNA base and x is a C3 spacer group.

**[0104]** An RDxxD blocked-cleavable primer (also known as "generation 2" or "Gen 2" blocked-cleavable primer) refers to a blocked-cleavable primer having at its 3'-terminus the sequence RDxxD, wherein R is an RNA base, D is a DNA base and x is a C3 spacer group.

**[0105]** The term "fluorescent generation probe" refers either to a) an oligonucleotide having an attached fluorophore and quencher, and optionally a minor groove binder or to b) a DNA binding reagent such as SYBR™ Green dye.

**[0106]** The terms "fluorescent label" or "fluorophore" refers to compounds with a fluorescent emission maximum between about 350 and 900 nm. A wide variety of fluorophores can be used, including but not limited to: 5-FAM (also called 5-carboxyfluorescein; also called Spiro(isobenzofuran-1(3H), 9'-(9H)xanthene)-5-carboxylic acid,3',6'-dihydroxy-3-oxo-6-carboxyfluorescein); 5-Hexachloro-Fluorescein; ([4,7,2',4',5',7'-hexachloro-(3',6'-dipivaloyl-fluoresceinyl)-6-carboxyli- c acid]); 6-Hexachloro-Fluorescein; ([4,7,2',4',5',7'-hexachloro-(3',6'-dipivaloylfluoresceinyl)-5-carboxylic acid]); 5-Tetrachloro-Fluorescein; ([4,7,2',7'-tetra-chloro-(3',6'-dipivaloylfluoresceinyl)-5-carboxylic acid]); 6-Tetrachloro-Fluorescein; ([4,7,2',7'-tetrachloro-(3',6'-dipivaloylfluoresceinyl)-6-carboxylic acid]); 5-TAMRA (5-

carboxytetramethylrhodamine); Xanthylium, 9-(2,4-dicarboxyphenyl)-3,6-bis(dimethyl-amino); 6-TAMRA (6-carboxytetramethylrhodamine); 9-(2,5-dicarboxyphenyl)-3,6-bis(dimethylamino); EDANS (5-((2-aminoethyl)amino)naphthalene-1-sulfonic acid); 1,5-IAEDANS (5-((((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid); Cy5 (Indodicarbocyanine-5); Cy3 (Indo-dicarbocyanine-3); and BODIPY FL (2,6-dibromo-4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-proprionic acid); Quasar®-670 dye (Biosearch Technologies); Cal Fluor® Orange dye (Biosearch Technologies); Rox dyes; Max dyes (Integrated DNA Technologies), as well as suitable derivatives thereof.

**[0107]** As used herein, the term "quencher" refers to a molecule or part of a compound, which is capable of reducing the emission from a fluorescent donor when attached to or in proximity to the donor. Quenching may occur by any of several mechanisms including fluorescence resonance energy transfer, photo-induced electron transfer, paramagnetic enhancement of intersystem crossing, Dexter exchange coupling, and exciton coupling such as the formation of dark complexes. Fluorescence is "quenched" when the fluorescence emitted by the fluorophore is reduced as compared with the fluorescence in the absence of the quencher by at least 10%, for example, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 98%, 99%, 99.9% or more. A number of commercially available quenchers are known in the art, and include but are not limited to DABCYL, Black Hole™ Quenchers (BHQ-1, BHQ-2, and BHQ-3), Iowa Black® FQ and Iowa Black® RQ. These are so-called dark quenchers. They have no intrinsic fluorescence in the wavelength range from 300 to 900 nm, virtually eliminating background problems seen with other quenchers such as TAMRA which is intrinsically fluorescent.

**[0108]** The term "ligation" as used herein refers to the covalent joining of two polynucleotide ends. In various embodiments, ligation involves the covalent joining of a 3' end of a first polynucleotide (the acceptor) to a 5' end of a second polynucleotide (the donor). Ligation results in a phosphodiester bond being formed between the polynucleotide ends. In various embodiments, ligation may be mediated by any enzyme, chemical, or process that results in a covalent joining of the polynucleotide ends. In certain embodiments, ligation is mediated by a ligase enzyme.

**[0109]** As used herein, "ligase" refers to an enzyme that is capable of covalently linking the 3'-hydroxyl group of one polynucleotide to the 5' phosphate group of a second polynucleotide. Examples of ligases include *E. coli* DNA ligase, T4 DNA ligase, *etc.*

**[0110]** The ligation reaction can be employed in DNA amplification methods such as the "ligase chain reaction" (LCR), also referred to as the "ligase amplification reaction" (LAR), see Barany, Proc. Natl. Acad. Sci., 88:189 (1991); and Wu and Wallace, Genomics 4:560 (1989) incorporated herein by reference. In LCR, four oligonucleotides, two adjacent oligonucleotides which uniquely hybridize to one strand of the target DNA, and a complementary set of adjacent oligonucleotides, that hybridize to the opposite strand are mixed and DNA ligase is added to the mixture. In the presence of the target sequence, DNA ligase will covalently link each set of hybridized molecules. Importantly, in LCR, two oligonucleotides are ligated together only when they base-pair with sequences without gaps. Repeated cycles of denaturation, hybridization and ligation amplify a short segment of DNA. A mismatch at the junction between adjacent oligonucleotides inhibits ligation. As in other oligonucleotide ligation assays this property allows LCR to be used to distinguish between variant alleles such as SNPs. LCR has also been used in combination with PCR to achieve enhanced detection of single-base changes, see Segev, PCT Public. No. WO9001069 (1990).

**[0111]** The term "unmodified form," in the context of the Taq DNA polymerase, is a term used herein for purposes of defining a host cell-specific, codon-optimized Taq DNA polymerase gene that expresses Taq DNA polymerase in the host cell. The term "unmodified form" refers to a functional DNA polymerase that has the amino acid sequence of the naturally occurring polymerase. The term "unmodified form" includes a functional DNA polymerase in a recombinant form.

**[0112]** The term "mutant", in the context of DNA polymerases disclosed, means a polypeptide, typically recombinant, that comprises one or more amino acid substitutions relative to a corresponding, naturally-occurring form or unmodified form of DNA polymerase.

**[0113]** "Recombinant", as used herein, refers to an amino acid sequence or a nucleotide sequence that has been intentionally modified by recombinant methods. By the term "recombinant nucleic acid" herein is meant a nucleic acid, originally formed in vitro, in general, by the manipulation of a nucleic acid by endonucleases, in a form not normally found in nature. Thus an isolated, mutant DNA polymerase nucleic acid, in a linear form, or an expression vector formed in vitro by ligating DNA molecules that are not normally joined, are both considered recombinant for the purposes of this invention. It is understood that once a recombinant nucleic acid is made and reintroduced into a host cell, it will replicate non-recombinantly, i.e., using the

in vivo cellular machinery of the host cell rather than in vitro manipulations; however, such nucleic acids, once produced recombinantly, although subsequently replicated non-recombinantly, are still considered recombinant for the purposes of the invention. A "recombinant protein" is a protein made using recombinant techniques, i.e., through the expression of a recombinant nucleic acid as depicted above.

**[0114]** A nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation.

**[0115]** The term "vector" refers to a piece of DNA, typically double-stranded, which may have inserted into it a piece of foreign DNA. The vector may be, for example, of plasmid origin. Vectors contain "replicon" polynucleotide sequences that facilitate the autonomous replication of the vector in a host cell. Foreign DNA is defined as heterologous DNA, which is DNA not naturally found in the host cell, which, for example, replicates the vector molecule, encodes a selectable or screenable marker, or encodes a transgene. The vector is used to transport the foreign or heterologous DNA into a suitable host cell. Once in the host cell, the vector can replicate independently of or coincidental with the host chromosomal DNA, and several copies of the vector and its inserted DNA can be generated. In addition, the vector can also contain the necessary elements that permit transcription of the inserted DNA into an mRNA molecule or otherwise cause replication of the inserted DNA into multiple copies of RNA. Some expression vectors additionally contain sequence elements adjacent to the inserted DNA that increase the half-life of the expressed mRNA and/or allow translation of the mRNA into a protein molecule. Many molecules of mRNA and polypeptide encoded by the inserted DNA can thus be rapidly synthesized.

**[0116]** The term “affinity tag” refers to a short polypeptide sequence that permits detection and/or selection of the polypeptide sequence. For the purposes of this disclosure, a recombinant gene that encodes a recombinant DNA polymerase may include an affinity tag. In particular, the affinity tag is positioned typically at either the N-terminus or C-terminus of the coding sequence for a DNA polymerase through the use of recombination technology. Exemplary affinity tags include polyhistine (for example,($His_6$),), glutathione-S-transferase (GST), HaloTag®, AviTag,

Calmodulin-tag, polyglutamate tag, FLAG-tag, HA-tag, Myc-tag, S-tag, SBP-tag, Softag 3, V5 tag, Xpress tag, among others.

**[0117]** The term "host cell" refers to both single-cellular prokaryote and eukaryote organisms (e.g., bacteria, yeast, and actinomycetes) and single cells from higher order plants or animals when being grown in cell culture. Exemplary suitable host cells include *E. coli*, *S. cerevisiae* and *S. frugiperda*.

**[0118]** As used herein, "percentage of sequence identity" is determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the sequence in the comparison window can comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity.

**[0119]** The terms "identical" or "identity", in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same. Sequences are "substantially identical" to each other if they have a specified percentage of nucleotides or amino acid residues that are the same (e.g., at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% identity over a specified region), when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. These definitions also refer to the complement of a test sequence. Optionally, the identity exists over a region that is at least about 50 nucleotides in length, or more typically over a region that is 100 to 500 or 1000 or more nucleotides in length.

**[0120]** The terms "similarity" or "percent similarity", in the context of two or more polypeptide sequences, refer to two or more sequences or subsequences that have a specified percentage of amino acid residues that are either the same or similar as defined by a conservative amino acid substitutions (e.g., 60% similarity, optionally 65%, 70%, 75%, 80%, 85%, 90%, or

95% similar over a specified region), when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. Sequences are "substantially similar" to each other if they are at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, or at least 55% similar to each other. Optionally, this similarly exists over a region that is at least about 50 amino acids in length, or more typically over a region that is at least about 100 to 500 or 1000 or more amino acids in length.

**[0121]** For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters are commonly used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities or similarities for the test sequences relative to the reference sequence, based on the program parameters.

**[0122]** A "comparison window", as used herein, includes reference to a segment of any one of the number of contiguous positions selected from the group consisting of from 20 to 600, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequences for comparison are well known in the art. Optimal alignment of sequences for comparison can be conducted, for example, by the local homology algorithm of Smith and Waterman (Adv. Appl. Math. 2:482, 1970), by the homology alignment algorithm of Needleman and Wunsch (J. Mol. Biol. 48:443, 1970), by the search for similarity method of Pearson and Lipman (Proc. Natl. Acad. Sci. USA 85:2444, 1988), by computerized implementations of these algorithms (e.g., GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, Wis.), or by manual alignment and visual inspection (see, e.g., Ausubel et al., Current Protocols in Molecular Biology (1995 supplement)).

**[0123]** Algorithms suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul et al. (Nuc. Acids Res. 25:3389-402, 1977), and Altschul et al. (J. Mol. Biol. 215:403-10, 1990), respectively. Software for performing BLAST analyses is publicly available through publicly

available online and internet databases and the National Center for Biotechnology Information within the National Library of Medicine of the U.S. National Institutes of Health (http://www.ncbi.nlm.nih.gov/).

**Rational design of Taq DNA polymerase mutants**

**[0124]** As outlined above, many strategies have been developed to improve discrimination of the polymerase chain reaction to selectively amplify a specific nucleic acid sequence based on the identity of a single nucleotide polymorphism, which in the past most often involved modifications introduced into the primer while using a naturally occurring DNA polymerase. The ability of DNA polymerases to discriminate between match and mismatch at the 3'-end of the primer nucleic acid is limited and varies greatly with the identity of the specific base pairs present. An alternative strategy to improve the selectivity of PCR amplification is to alter the properties of the DNA polymerase to improve discrimination between a primer that is a match versus one which has a terminal mismatch to the template nucleic acid. The present invention provides for DNA polymerase mutants having improved mismatch discrimination for base pairing at the 3'-terminus of the primer, leading to improved specificity of the ensuing amplification reaction.

**[0125]** The rhPCR method employs blocked-cleavable primers which must be unblocked by the action of RNase H2 before amplification can commence. The enzymatic unblocking step requires cleavage at a single internal RNA base within the primer, which is typically positioned at the SNP site. RNase H2 cleaves the RNA at the 5'-side, leaving a primer with a 3'-hydroxyl which is capable of priming PCR. Cleavage by RNase H2 occurs with high efficiency when the primer matches the template and with low efficiency when a mismatch is present due to a SNP. Therefore match templates are amplified with greater efficiency than mismatch templates. It is thought that the primary mechanism that permits amplification of mismatched templates begins with alternative cleavage of the substrate (i.e., the blocked-cleavable primer) at the 3'-side of the RNA residue, leading to inappropriate priming when a mismatch is present, retention of the RNA base in the primer, and conversion of the PCR product to primer sequence, which then faithfully replicates as a match in subsequent PCR cycles. Fidelity of the rhPCR process could be improved through improvements in the DNA polymerase which limit its ability to initiate DNA synthesis from a primer having a 3'-RNA residue. The present invention provides for DNA

polymerase mutants having a reduced ability to initiate DNA synthesis from 3'-RNA containing primers, leading to improved specificity of the ensuing amplification reaction.

**[0126]** The present invention includes novel DNA polymerase mutants having improved discrimination for base identity at the 3'-end of the primer nucleic acid and/or DNA polymerase mutants having decreased priming efficiency from a 3'-RNA residue.

**[0127]** A novel design strategy was developed to rationally design DNA polymerase mutants having improved discrimination at the 3'-terminal base of the primer compared to discrimination of the native DNA polymerase, limiting the ability to initiate DNA synthesis if a mismatch is present or if an RNA residue is present. The process described herein employed the Taq DNA polymerase as the parent enzyme; the approach can be applied to other DNA polymerases, especially if crystal structure is known. The design strategy includes a first component based upon theoretical analyses of biophysical, biochemical and genetic information relating to the native DNA polymerase and, to a lesser extent, related polymerases which differ in amino acid sequence. The design strategy includes a second component based upon molecular biological and biochemical analyses of known genetically-engineered mutant polymerases to assist as a guide in predicting the effects of novel mutations in an attempt to rationally engineer new properties into the mutant polymerase, in this case to improve 3'-nucleotide discrimination.

**[0128]** In the first stage, the mechanism of Taq DNA polymerase enzymatic reaction based upon published mutational structure-activity-relationship (SAR) studies was analyzed and correlated with protein structure, when known, and predicted using molecular dynamic simulations when not known. The mechanism of enzyme catalysis has been described in the prior art (Patel, P.H., *et al.*, J. Mol. Biol. 2001, 308:823-837; Li, Y. & Waksman, G., Protein Sci 2001, 10:1225-1233). Amino acid residues located at the C-terminus, from positions 424 to 832, are responsible for the primer extension catalytic activity of the protein. Taq DNA polymerase binds the primer-template duplex to form a binary complex. This allows an incoming substrate dNTP to bind in the pocket at the 3'-end of the primer to form an open ternary complex. If the dNTP is complementary to the template nucleotide, the active site changes conformation where the α-helix made from residues 659 to 671 rotates towards the site, and template base rotates towards the incoming dNTP, encouraging formation of a Watson-Crick base pair. This event "closes" the ternary complex, and brings the α-phosphate group of the dNTP close to the primer 3'-OH group. The oxygen of this hydroxyl group makes a nucleophilic attack on the phosphorus,

forms a covalent bond and pyrophosphate is released. Taq DNA polymerase catalytic activity requires the presence of magnesium ions, which are assumed to facilitate deprotonation of the attacking hydroxyl group.

**[0129]** One criteria for rational design of Taq DNA polymerase mutants having improved 3'-nucleotide discrimination is to provide for novel polymerase enzyme variants having normal or near-normal polymerase processivity compared with the native DNA polymerase. For this reason, the first step of analysis serves to narrow the sequence space of amino acids that are available for alteration that should not compromise core enzymatic functions. For example, residues D610, D785, and E786 form the catalytic core. Their carboxylate groups are assumed to bind divalent metal ions, which in turn bind and stabilize the incoming dNTP and the terminal nucleotide of the primer. Mutations of these three essential residues are likely to render the polymerase inactive. Mutant polymerases which include alterations of residues D610, D785 and E786 were therefore excluded from consideration. Likewise, mutations that affect fidelity of complementary base recognition, such as residues that facilitate open to closed ternary complex formation at a complementary dNTP and the template base, were excluded from consideration.

**[0130]** Additional criteria of the first stage of analysis were to identify the polymerase amino acid residues in the vicinity of the 3' terminal nucleotide of the primer. For this purpose, the atomic three-dimensional structures of Taq DNA polymerase that are available from prior art (Eom, S.H., *et al.*, Nature 1996, 382:278-281; Li, Y., *et al.*, EMBO J. 1998, 17: 7514-7525; Doublié, S., *et al.*, Structure 1999, 7:R31-R35; Li, Y., *et al.*, Protein Sci 1998, 7:1116-1123) were selected for analyses. The structures were downloaded from the Protein Data Bank (H.M. Berman, *et al.*, Nucleic Acids Research 2000, 28: 235-242). The structures of PDB ID 2KTQ and 3KTQ were thoroughly analyzed because they show the open and closed ternary complex of the large fragment of Taq DNA polymerase co-crystalized with primer and template nucleic acids (Li, Y., *et al.*, EMBO J. 1998, 17: 7514-7525). This structure shows the location of the primer 3'-terminus at the active site and its interaction with key amino acid residues (FIG. 1).

**[0131]** For structure visualization, the hydrogen atom attached to the 2' carbon is replaced with a hydroxyl group for primers modified with a 3'-ribonucleotide. Those amino acid residues that are in close proximity to the 2' carbon of the nucleotide at the 3' terminus of the primer were selected for additional analysis. These amino acid residues are listed in the Table 2 and are most likely to interact with the primer 3'-terminal nucleotide. Mutation at these sites may affect

catalytic activity of the polymerase when primer modifications, like OH, are attached to the 2' carbon atom of the ribose.

**Table 2. Chemical groups in close vicinity to the 2' carbon of the terminal 3' primer nucleotide.**

| Distance from C2' of the primer terminal residue[1] | Chemical group |
|---|---|
| ≤ 0.35 nm | dNTP to be added to primer |
| 0.35 - 0.40 nm | D785[2] |
| 0.40 - 0.45 nm | H784, V783 |
| 0.45 - 0.50 nm | R573 |
| 0.50 - 0.60 nm | E786[2] |

[1]The distances were measured in the PDB ID 2KTQ structure.
[2]Residues D785 and E786 are catalytic core residues.

**[0132]** A further aspect of this criterion relates to approaches to increase specificity while retaining catalytic activity of the polymerase. One approach to increasing specificity of Taq DNA polymerase would be to decrease the size of the binding pocket, so that a modified primer would not fit within it. Any additional chemical group will increases the volume of space occupied by the 3' nucleotide. To align atoms for effective catalysis and nucleophilic attack, the active site pocket must be flexible to accommodate additional atom(s), for example, the oxygen of the OH group of an RNA residue. The size of the active site can be decreased by substitution of neighboring amino acids with larger amino acids. Additional consideration is given to the amino acid properties and abilities of their side chains to engage in electrostatic and van der Waals interactions. Amino acid can be categorized into groups of positively charged (R, H, K), negatively charged (D, E), uncharged polar (S, T, N, Q), hydrophobic (A, V, I, L, M, F, Y, W), and special (C, G, P) side chains. Mutations within a group are conservative and are more likely to maintain existing properties while mutations across groups or within the special group amino acids are more likely to result in substantial changes of enzyme activity and/or specificity.

**[0133]** Another approach to increasing 3'-nucleotide discrimination of Taq DNA polymerase is to employ residue substitutions that decrease the flexibility of the binding pocket. Examples include substitution of amino acid aliphatic side chains with aromatic side chains, which lead to a

higher energetic barrier to change rotamer conformations. As explained above, the residues of the catalytic core are preferably unaltered, residues spatially near the catalytic core are given the greatest attention for change. For example, three non-catalytic core residues of Table 2, H784, V783, and R573 are herein proposed to be substituted for larger or less flexible amino acids while the maintaining the general physical characteristics of their side chains. These residues exhibit key interactions with the ribose moiety of the primer through a water-mediated hydrogen-bonding network. R573 also binds to the primer base in the minor groove of primer-template duplex. Mutants ID 1 to ID 4 were designed using this strategy (Table 3). The next mutant, ID 5, Q582K, was designed to alter interactions with and the position of the important H784 residue. It is seen from the known crystal structure that Q582 is situated on the opposite side of H784 from the oligonucleotide primer. Substitution of Q582H may shift H784 towards the terminal primer nucleotide, leading to a more constrained binding pocket. The interactions of residue 582 with the next-to-terminal nucleotide may also be affected.

**[0134]** Residues that stack above the incoming dNTP molecule can also influence the size of the binding pocket in the polymerase active site. For example, substitutions at F667, which is located at this position, are known to change selectivity towards the incoming dNTP. For example, the F667Y substitution significantly improves incorporation of dideoxynucleoside triphosphates by Taq DNA polymerase (Tabor, S. & Richardson, C.C., *Proc. Nat. Acad. Sci. USA* 1995, 92:6339-6343), a useful property for DNA polymerases employed in Sanger method terminator DNA sequencing. Mutant ID 6 increases the size of the aromatic side chain of F667 from phenylalanine to tryptophan, in an attempt to push the dNTP against the primer terminal ribonucleotide and decrease ability of binding pocket to accommodate a 2' hydroxyl group, thereby biasing this mutant against primers containing a 3'-RNA residue. Mutant ID7 was designed based on similar conceptual framework. The H639 interacts with F667 amino acid and H639W mutant might also push F667 towards the incoming dNTP.

**[0135]** Additional mutations were considered that can effectively reduce the binding pocket size of the polymerase. Mutants IDs 8 to 16 were designed from a negative inferential analysis based on published studies of "relaxed specificity" mutant polymerases. Mutations have been reported that can modify polymerase specificity towards the ribose of the incoming dNTP. The prior art Taq DNA polymerase variants described were evolved from large random libraries either through selection or screening. Chen *et al.* described mutations that allow Taq DNA

polymerase to incorporate a dNTP with large substituents on the ribose 3' carbon atom (Chen, F., *et al.*, Proc. Nat. Acad. Sci. USA 2010, 107:1948-1953). This residue was found to be important because it also interacts with F667. The substitution L616A decreases specificity by giving more space to the phenylalanine residue. Mutant ID 8 (L616M) was designed to produce the opposite effect. The methionine substitution may subtly increase the steric constraints at this site compared to leucine. This restriction may make the active site less likely to accommodate extra substituents in a dNTP or in a primer nucleotide, which could reduce activity of 3'-RNA containing primers or those having a mismatch to template, which presumably occupies more space than primers having a perfect match to the template nucleic acid.

**[0136]** A similar conceptual framework was applied to design Taq DNA polymerase mutant ID 9. Mutations I614E, E615G were reported to relax the active site pocket, so that the polymerase could extend a primer using 2'-O-methyl ribonucleoside triphosphates (Fa, M., *et al.*, J. Am. Chem. Soc. 2004, 126:1748-1754). The nature of these mutations is essentially the shift of glutamic acid from residue 615 to 614. A shift in the opposing direction, E615L, L616E may therefore impose constraints on the active site and produce a Taq DNA polymerase mutant that will not accept ribonucleotide residues.

**[0137]** Another approach to increasing 3'-nucleotide discrimination is to focus on sites of interest identified in Taq DNA polymerase studies that reported amino acid substitutions which increased base selection fidelity and decreased incorporation of mispaired base pairs (i.e., those mutation that improve replication fidelity). These changes could potentially affect selectivity of Taq DNA polymerase regarding to modifications of terminal primer nucleotide as well. One location that was reported to improve fidelity involves the F667 residue and neighboring amino acids (Suzuki, M., *et al.*, J. Biol. Chem. 2000, 275:32728-32735). Another site of potential interest includes residues 782 to 784, adjacent to an essential aspartic acid residue (Strerath, M., *et al.*, ChemBioChem 2007, 8:395-401). Mutants ID 10 to ID 13 were designed to alter amino acid character at these positions. F667 affects specificity as it interacts with the terminal base of the primer and stacks on the base of the incoming dNTP; this residue resides in the O-helix. Residues I665 and A661 are located on the opposite side of the helix. Mutation here to larger amino acids (A661E,I665W) may move the O-helix towards the active site, restricting the size of the active pocket and limiting ability of the polymerase to accept mispaired bases or RNA residues (Mutant ID 10: A661E,I665W,F667L).

**[0138]** Data derived from mutagenesis studies of different polymerases can also be used to help select positions for modification, but use of this data is more difficult in the absence of crystal structure or due to possible differences in effects between the polymerases. Polymerase I from *Escherichia coli* ("E. coli Pol") shows a somewhat similar structure at the active site when compared to Taq DNA polymerase and maintains identical essential catalytic residues. Both protein sequences exhibit high degree of homology (Li, Y., *et al.*, EMBO J. 1998, 17: 7514-7525). Thus, mutations reported for *Escherichia coli* DNA polymerase were also considered, by extrapolating amino acid position to the corresponding positions in the Taq DNA polymerase. For example, the triplet amino acid substitutions, Q879P, V880L, H881Q, improved base fidelity of *E. coli* DNA polymerase (Summerer, D., *et al.*, Angew. Chem. Int. Ed. 2005, 44:4712 -4715). Substitutions in mutant ID 14 includes substitutions at Q782, V783, H784 in the Taq DNA polymerase active site, which appear to correspond to this *E. coli* residue triplet.

**[0139]** A number of additional substitutions in the *E. coli* DNA polymerase are known which decrease or increase the specificity of primer extension (Minnick, D.T., *et al.*, J. Biol. Chem. 1999, 274:3067–3075). Mutants Q849A and R754A improved fidelity. These have locations equivalent to Q754 and R659 in the Taq DNA polymerase active site, respectively. Arginine 659 has a significant impact on selection of the base complementary to the template base. This appears to be general feature in the polymerase A family. For example, in *Thermotoga neapolitana* polymerase I, the equivalent residue is R722. Mutation of this residue to histidine increases fidelity of this polymerase (Yang, S.W., *et al.*, Nucleic Acids Res. 2002, 30:4314-4320). These two residues were also selected for study (mutants ID 15 and 16 of Table 3). Mutant ID 17 represents combination of the mutations studied in Mutants ID 2 and 3. Mutant ID 18 represents a modification of triple mutant ID 14 (Q782P, V783L, H784Q) reduced to a double mutant (V783L, H784Q) by eliminating the Q782P mutation; the substitution of a less flexible P for Q residue will likely cause significant structural perturbation which would alter function, and Mut ID 18 may avoid this problem. Initial testing indicated that more than one mutant at position H784 showed improved mismatch discrimination, suggesting that this position was generally important for determining primer specifcity. Therefore a comprehensive study of amino acid substitutions at this site was performed, comprising Mut IDs 19-36.

**Table 3. Novel Taq DNA polymerase mutants selected for study.**

| Mutant ID | Specific amino acid changes from sequence in Table I |
|---|---|
| 1 | V783I |
| 2 | V783F |
| 3 | H784Q |
| 4 | R573H |
| 5 | Q582K |
| 6 | F667W |
| 7 | H639W |
| 8 | L616M |
| 9 | E615L, L616E |
| 10 | A661E, I665W, F667L |
| 11 | Q782I, H784F |
| 12 | Q782I, V783L, H784L |
| 13 | Q782S, V783F, H784N |
| 14 | Q782P, V783L, H784Q |
| 15 | Q754A |
| 16 | R659H |
| 17 | V783F, H784Q |
| 18 | V783L, H784Q |
| 19 | H784G |
| 20 | H784A |
| 21 | H784S |
| 22 | H784T |
| 23 | H784C |
| 24 | H784V |
| 25 | H784L |
| 26 | H784I |
| 27 | H784M |

| Mutant ID | Specific amino acid changes from sequence in Table I |
|---|---|
| 28 | H784P |
| 29 | H784F |
| 30 | H784Y |
| 31 | H784W |
| 32 | H784D |
| 33 | H784E |
| 34 | H784N |
| 35 | H784K |
| 36 | H784R |

**[0140]** The second component of the design strategy includes molecular biological and biochemical analyses of genetically-engineered Taq DNA polymerase mutants to identify novel enzymes having improved 3'-nucleotide discrimination. This requires expression of native Taq DNA polymerase and the series of designed mutants in a suitable host, such as the bacterium *E. coli*. To maximize expression, the codons of the native gene sequence encoding Taq DNA polymerase were altered and optimized for expression in *E. coli* using standard codon usage tables for this organism (see: Codon usage tabulated from the international DNA sequence databases: status for the year 2000. Nakamura, Y., Gojobori, T. and Ikemura, T. (2000) *Nucleic Acids Res.* 28:292). Codon optimization does not alter the amino acid sequence of the expressed protein. A recombinant form of a codon-optimized gene encoding the unaltered Taq DNA polymerase peptide was assembled and cloned into a plasmid vector as an artificial gene made from synthetic oligonucleotides using standard methods (Example 1). The plasmid vector for this purpose can be any plasmid vector routinely available in the art. Synthetic recombinant forms of the series of identified desired Taq DNA polymerase mutants (Table 3, Mutant IDs 1-36) were prepared by site directed mutagenesis of the previously assembled codon-optimized recombinant native Taq DNA polymerase as the substrate for site directed mutagenesis (SDM), using techniques well known to those with skill in the art (Example 1). The unmodified and mutant Taq DNA polymerases were prepared from *E. coli* host cells following introduction of

expression vectors that contain the corresponding recombinant forms of the genes operably linked to suitable transcriptional and translational control elements.

**[0141]** The enzymatic properties of the unmodified Taq DNA polymerase and mutant Taq DNA polymerases were evaluated for primer extension assays, thermostability, PCR assays, allele-specific PCR assays, ability to employ primers having a 3'-ribonucleotide, as well as their suitability for use in rhPCR assays. The mutant Taq DNA polymerases displayed one of four categories of enzymatic properties: (1) inactivated polymerase activity; (2) normal polymerase activity; (3) improved 3'-nucleotide discrimination activity, but having reduced activity (for example, reduced processivity); and (4) improved 3'-nucleotide discrimination and having normal or near normal polymerase activity (for example, processivity comparable to the native polymerase).

**[0142]** Mutant Taq DNA polymerases having the fourth category of enzymatic properties displayed comparable or enhanced 3'-mismatch discrimination (that is, comparable or improved performance in standard primer extension assays and allele-specific PCR assays when compared to the wild-type Taq DNA polymerase); enhanced 3'-nucleotide discrimination (that is, reduced primer extension activity from templates containing RNA-containing primers when compared to the wild-type Taq DNA polymerase) and enhanced rare allele discrimination (for example, improved specificity in rhPCR assay when compared to the wild-type Taq DNA polymerase). These mutant Taq DNA polymerases include mutations at one of the following residue position(s): (1) A661E; I665W; F667L triple substitution mutant peptide (Mutant ID 10 of Table 3); (2) V783F single substitution mutant peptide (Mutant ID 2 of Table 3); H784Q single substitution mutant peptide (Mutant ID 3 of Table 3); and V783L; H784Q double substitution mutant peptide (Mutant ID 18 of Table 3), H784A, single substitution mutant peptide (Mutant ID 20 of Table 3); H784S, single substitution mutant peptide (Mutant ID 21 of Table 3); H784T, single substitution mutant peptide (Mutant ID 22 of Table 3); H784V, single substitution mutant peptide (Mutant ID 24 of Table 3); H784I, single substitution mutant peptide (Mutant ID 26 of Table 3); H784M, single substitution mutant peptide (Mutant ID 27 of Table 3); H784F, single substitution mutant peptide (Mutant ID 29 of Table 3); and H784Y single substitution mutant peptide (Mutant ID 30 of Table 3).

**[0143]** Thus, the novel design algorithm provides a robust approach to predict mutant DNA polymerases having improved 3'-nucleotide discrimination, as adjudged by their activity in allele-specific PCR, rare allele detection assays and rhPCR assays that utilize templates with or without a 3'-RNA residue in the primer. Specifically, residues V783 and H784 were identified as critical residues which influence the ability of the polymerase to interrogate the status of the 3'-base of the primer oligonucleotide (e.g., whether this residue is matched or mismatched with template and/or whether this residue is DNA or RNA). The significance of these residues in polymerase function was heretofore not appreciated. In addition to the mutations directly testing in the example, the present invention also contemplates other amino acid substitutions at these two positions, or double-mutants affecting both the V783 and H784 sites.

**[0144]** The properties of these mutants are further described in the Examples presented herein. Importantly, however, the design strategy employed herein enables access to functional space for novel Taq DNA polymerase mutants that were previously unrecognized or predicted or otherwise not obtained using other approaches (for example, phylogenetic comparative analysis or earlier attempts using random mutagenesis).

**Evaluation of Taq DNA polymerase mutants at residue positions 783 and 784**

**[0145]** The present disclosure demonstrates that mutation at residue positions 783 and/or 784 results in active Taq DNA polymerase mutants having enhanced template discrimination activity, as compared to unmodified Taq DNA polymerase. Thus, the entirety of the sequence space that includes every conceivable single amino acid substitution at the individual positions 783 or 784 as well as every conceivable double amino acid substitution at both positions 783 and 784 fall within the scope of the present disclosure as related to Taq DNA polymerase. Accordingly, those active Taq DNA polymerase mutants selected from the mutant sets of 19 single residue 783 mutants, 19 single residue 784 mutants (Table 3 Mut IDs 19-30) and 361 double residue 783/784 mutants that also possess enhanced template discrimination activity fall within the scope of the present disclosure.

**[0146]** Because Taq DNA polymerase is a thermostable enzyme, one facile approach to screening the candidate collection of 399 single- and double-substitution mutants at residue positions 783 and 784 is to perform a PCR assay with a pre-treated sample encoding a candidate Taq DNA polymerase mutant enzyme. The sample can be a selected individual colony or corresponding micro-cultures (for example, 50 μL to 1.0 mL cultures) obtained from the

individual colony transformed with recombinant DNA that expresses a desired recombinant Taq DNA polymerase mutant gene. The pre-treatment regimen can include the step of pre-incubating the sample at 70-95° C, followed by the step of clarifying the supernatant to remove the denatured cellular debris. For samples that express thermostable polymerase activity under standard PCR assay conditions, the corresponding recombination DNA can be further characterized to confirm the sequence of the desired recombinant Taq DNA polymerase mutant genotype and the polymerase protein purified for additional biochemical analysis. For the purposes of this disclosure, a Taq DNA polymerase mutant that expresses thermostable polymerase activity at a level of at least 0.01 of that expressed by wild-type Taq DNA polymerase under comparable PCR assay conditions can be adjudged as possessing thermostable polymerase activity.

**Evaluation of other select polymerase candidate mutants functionally homologous to Taq DNA polymerase at residue positions 783 and 784**

**[0147]** Comparative phylogenetic analysis tools can be used to identify the sequence space of other thermoactive polymerases having homologous sequence information relative to the unmodified Taq DNA polymerase at residue positions corresponding to V783 and H784. As explained supra, a strong prediction of the comparative phylogenetic analysis is that structural sequences shared among DNA polymerases across phylogenetically diverse species are conserved for functional reasons. If the identified V783/H784 residues of Taq DNA polymerase are invariant in sequence identity among wild-type polymerases from diverse species, that observation strongly supports the conclusion that nature selected against the specific variation of amino acid substitutions at those positions that result the observed enhanced template discrimination activity of the engineered Taq DNA polymerase mutants disclosed herein.

**[0148]** Example 11 provides an exemplary BLAST search using Taq DNA polymerase sequences encompassing positions V783 and H784 as a comparison window to identify candidate wild-type DNA polymerases from other species sharing extensive sequence identity with Taq DNA polymerase. As further elaborated in Example 11, the BLAST results revealed that virtually every identified DNA polymerase from diverse species has maintained Val and His at positions orthologous to V783 and H784 of Taq DNA polymerase. Thus, the BLAST results confirm a natural counter-selection against DNA polymerases having enhanced template discrimination activity and provide strong evidence that the disclosed engineered Taq DNA

polymerase mutants having these properties are novel and non-obvious. Like that observed with the engineered Taq DNA polymerase mutants, each of the identified non-Taq DNA polymerases represent a sequence space from which engineered mutant enzymes can be generated having enhanced template discrimination activity, as compared to their respective unmodified counterparts.

**[0149]** In those cases where comparative phylogenetic analysis cannot access the sequence space of more evolutionary distant organisms, a comparative biophysical crystallographic analysis can provide clues to the relevant sequence residues having functional homology to Taq DNA polymerase resides V783 and H784. As explained *supra*, the Q782, V783 and H784 residue triplet of Taq DNA polymerase was selected for analysis based upon the corresponding triplet amino acid substitutions Q879P, V880L and H881Q of *E. coli* DNA polymerase having improved base fidelity and a similar active site architecture to that of Taq DNA polymerase. Conversely, based upon the noted enhanced template discrimination activity of V783 and H784 Taq DNA polymerase mutants relative to wild-type Taq DNA polymerase, the present disclosure contemplates that the corresponding substitutions at V880 and H881 of the *E. coli* DNA polymerase will possess enhanced template discrimination activity relative to wild-type *E. coli* DNA polymerase.

**Identification and characterization of non-VH-related polymerase mutants having enhanced template discrimination activity.**

**[0150]** The foregoing collection of DNA polymerases share extensively conserved sequences in the region corresponding to V783 and H784 of Taq DNA polymerase ("VH-related polymerases"). Comparative biophysical analysis is useful for identifying wild-type DNA polymerases having different amino acid sequences in the functionally homologous positions as V783 and H784 of Taq DNA polymerase ("non-VH-related DNA polymerases"). The instant disclosure contemplates engineering mutant polymerases having enhanced template discrimination activity from these non-VH-related DNA polymerases in the same manner as disclosed for the VH-related DNA polymerases. Candidate non-VH resides for directed mutagenesis and analysis by enhanced template discrimination activity assays include those resides within 0.40 – 0.45 nm of the C2' of the primer terminal residue, as revealed in the polymerase:template co-crystal structure.

**Combination of site-specific Taq DNA Mutants with deletion of the 5'-exonuclease domain.**

**[0151]** The present invention discloses novel Taq DNA Polymerase mutants that show improved discrimination of mismatches positioned at the 3'-residue of the primer oligonucleotide and/or discrimination against the presence of an RNA residue at the 3'-end of the primer oligonucleotide. Improved mismatch discrimination has been described for the "KlenTaq" deletion mutant of Taq DNA Polymerase, which entirely removes the domain having 5'-exonuclease activity (Barnes, W.M., Gene 112:29-35, 1992). Combination of the novel mutants of the present invention with the KlenTaq 5'-exonuclease domain deletion led to further improvements in mismatch discrimination (Examples 18-22), however this comination led to decreases in enzymatic activity which may reduce utility of this family of double-mutants. In some circumstances, particularly when amplicon size is small and limited processivity could be tolerated, the enhanced decrimination of these mutants will have benefit.

**Reaction Mixtures**

**[0152]** In another aspect, reaction mixtures are provided comprising the polymerases with increased 3'-nucleotide discrimination activity. The reaction mixtures can further comprise reagents for use in, for example, nucleic acid amplification procedures (for example, PCR, RT-PCR, rhPCR), DNA sequencing procedures, or DNA labeling procedures. For example, in certain embodiments, the reaction mixtures comprise a buffer suitable for a primer extension reaction. The reaction mixtures can also contain a template nucleic acid (DNA and/or RNA), one or more primer or probe polynucleotides, nucleoside triphosphates (including, for example, deoxyribonucleotides, ribonucleotides, labeled nucleotides, unconventional nucleotides), salts (for example, $Mn^{2+}$, $Mg^{2+}$), and labels (for example, fluorophores). In some embodiments, the reaction mixture further comprises double stranded DNA binding dyes, such as SYBR green, or double stranded DNA intercalating dyes, such as ethidium bromide. In some embodiments, the reaction mixtures contain a 5'-sense primer hybridizable, under primer extension conditions, to a predetermined polynucleotide template, or a primer pair comprising the 5'-sense primer and a corresponding 3'-antisense primer. In certain embodiments, the reaction mixture further comprises a fluorogenic FRET hydrolysis probe for detection of amplified template nucleic acids, for example a Taqman® or PrimeTime® probe. In some embodiments, the reaction mixture contains two or more primers that are fully complementary to single nucleotide polymorphisms or multiple nucleotide polymorphisms. In some embodiments, the reaction mixtures contain

alpha-phosphorothioate dNTPs, dUTP, dITP, and/or labeled dNTPs such as, for example, fluorescein- or cyanin-dye family dNTPs. In some embodiments, the reaction mixtures contain blocked-cleavable primers and RNase H2.

**Kits**

**[0153]** In another aspect, kits are provided for use in primer extension methods described herein. In some embodiments, the kit is compartmentalized for ease of use and contains at least one container providing a DNA polymerase of the invention having increased 3'-nucleotide discrimination in accordance with the present disclosure. One or more additional containers providing additional reagent(s) can also be included. Such additional containers can include any reagents or other elements recognized by the skilled artisan for use in primer extension procedures in accordance with the methods described above, including reagents for use in, for example, nucleic acid amplification procedures (for example, PCR, RT-PCR, rhPCR), DNA sequencing procedures, or DNA labeling procedures. For example, in certain embodiments, the kit further includes a container providing a 5'-sense primer hybridizable, under primer extension conditions, to a predetermined polynucleotide template, or a primer pair comprising the 5'-sense primer and a corresponding 3'-antisense primer. In some embodiments, the kit includes one or more containers containing one or more primers that are fully complementary to single nucleotide polymorphisms or multiple nucleotide polymorphisms, wherein the primers are useful for multiplex reactions, as described above. In some embodiments, the reaction mixtures contain one or more containers containing blocked-cleavable primers. In some embodiments, the reaction mixtures contain one or more containers containing RNase H2. In other, non-mutually exclusive variations, the kit includes one or more containers providing nucleoside triphosphates (conventional and/or unconventional). In specific embodiments, the kit includes alpha-phosphorothioate dNTPs, dUTP, dITP, and/or labeled dNTPs such as, for example, fluorescein- or cyanine-dye family dNTPs. In still other, non-mutually exclusive embodiments, the kit includes one or more containers providing a buffer suitable for a primer extension reaction. In some embodiments, the kit includes one or more labeled or unlabeled probes. Examples of probes include dual-labeled FRET (fluorescence resonance energy transfer) probes and molecular beacon probes. In another embodiment, the kit contains an aptamer, for example, for hot start PCR assays.

**[0154]** The present disclosure contemplates kits that provide novel DNA polymerases having enhanced template discrimination activity. As demonstrated in more detail in the examples, each DNA polymerase can display a unique signature of enhanced template discrimination activity. Certain DNA polymerases can display a relatively greater 3'-nucleotide discrimination, as compared to its other activities (3'-mismatch discrimination and rare allele discrimination), while other DNA polymerases can display a relatively greater 3'-mismatch discrimination, as compared to its other activities (3'-nucleotide discrimination and rare allele discrimination), and yet other DNA polymerases can display a relatively greater rare allele discrimination, as compared to its other activities (3'-nucleotide discrimination and 3'-mismatch discrimination). Accordingly, kits can include individual containers of specific DNA polymerases having an activity profile optimally tailored to a specific enhanced template discrimination activity for a specific assay platform. Alternatively, kits can include a single container that includes a plurality of DNA polymerases having an activity profile optimally tailored to accommodate enhanced template discrimination activity as may be needed for a plurality of assay platforms.

**EXAMPLES**

**[0155]** The present invention is further illustrated by reference to the following Examples. However, it should be noted that these Examples, like the embodiments described above, are illustrative and are not to be construed as restricting the enabled scope of the invention in any way.

**Example 1. Cloning and expression of a codon optimized DNA polymerase from *Thermus aquaticus*.**

**[0156]** The amino acid and gene sequences for Taq DNA polymerase are known (Table 1, SEQ ID NOs. 1 and 2). Because codon usage differs among organisms, the codons of the native gene sequence encoding Taq DNA polymerase were optimized for expression in *E. coli* using standard codon usage tables (see: Codon usage tabulated from the international DNA sequence databases: status for the year 2000. Nakamura, Y., Gojobori, T. and Ikemura, T. (2000) *Nucleic Acids Res.* 28:292); synonymous codon changes were introduced to avoid repeated use of identical codons over a 20 amino acid stretch. A recombinant codon-optimized gene encoding the Taq DNA polymerase unmodified peptide was assembled from synthetic oligonucleotides using standard methods. The gene was made in three fragments, each of which was subcloned in a plasmid vector; sequences are shown in Table 4 (SEQ ID NOs. 3-5). Sequence identity was

verified by Sanger DNA sequencing. The three Taq DNA polymerase subfragments were assembled together using the Gibson assembly method (Gibson, D.G. *et al.* Nature Methods, 343–345 (2009)) and cloned into a the plasmid expression vector pET-27b(+) using terminal Nde I and Not I restrictions sites to create a final, full-length codon optimized Taq DNA polymerase gene (designated "OptiTaq"). Sequence was verified by Sanger DNA sequencing; sequence is shown in Table 4 (SEQ ID NO. 6). The translated amino acid sequence of the new codon optimized gene is identical to native Taq DNA polymerase (Table 1, SEQ ID NO. 1).

**Table 4: DNA sequence of Taq DNA Polymerase codon-optimized for expression in *E. coli.***

| Name | Sequence |
|---|---|
| SEQ ID NO. 3<br><br>Taq subfragment #1 | CATATGCGTGGTATGCTGCCGTTGTTCGAGCCTAAAGGCCGCGTACTGTTAGTCGATGGTCA<br>TCACTTGGCCTATCGGACGTTCCATGCACTCAAAGGTCTGACGACCAGTCGTGGCGAACCGG<br>TCCAGGCTGTTTATGGTTTCGCTAAGTCTTTGCTCAAAGCACTGAAAGAAGACGGGGACGCG<br>GTAATTGTTGTATTTGATGCCAAAGCACCGAGCTTCCGCCACGAAGCTTATGGTGGCTACAA<br>GGCAGGACGCGCCCCTACCCCAGAAGATTTCCCCCGTCAGCTGGCATTAATTAAGGAGTTAG<br>TAGACCTTCTCGGCTTAGCGCGTCTGGAAGTTCCGGGTTATGAGGCGGACGATGTCCTTGCA<br>TCCTTGGCTAAAAAGGCCGAAAAAGAGGGCTACGAAGTCCGCATCTTGACGGCAGACAAAGA<br>TCTGTACCAGCTTCTGTCTGACCGTATTCATGTTTTGCACCCTGAAGGCTACTTAATCACTC<br>CGGCCTGGCTCTGGGAAAAGTACGGTCTGCGTCCCGATCAGTGGGCGGATTATCGGGCTTTG<br>ACGGGAGATGAGAGCGACAACCTGCCAGGAGTTAAGGGCATTGGTGAAAAAACCGCACGTAA<br>GCTGCTTGAAGAGTGGGGTTCCCTGGAAGCCTTGTTAAAAAATCTGGATCGTCTCAAGCCCG<br>CAATTCGTGAAAAGATCCTGGCTCATATGGACGATCTTAAATTAAGTTGGGACCTGGCCAAG<br>GTGCGCACCGATTTACCGCTTGAAGTGGATTTTGCAAAACGCCGTGAGCCGGACCGGGAACG<br>TTTACGCGCTTTCTTAGAGCGTCTGGAATTCGGTTCACTGCTTCATGAATTCGGTCTGT |
| SEQ ID NO. 4<br><br>Taq subfragment #2 | TCGGTTCACTGCTTCATGAATTCGGTCTGTTAGAGTCTCCTAAAGCACTCGAAGAGGCACCG<br>TGGCCGCCCCCAGAAGGTGCTTTTGTTGGCTTCGTACTTTCCCGTAAGGAGCCTATGTGGGC<br>AGATCTTCTGGCTTTAGCGGCTGCACGCGGTGGCCGTGTTCACCGGGCCCCTGAGCCATACA<br>AAGCGTTACGTGATCTGAAGGAAGCACGTGGCTTGCTGGCAAAAGACCTTTCTGTTTTGGCC<br>CTGCGCGAGGGTCTTGGACTGCCGCCAGGCGACGATCCCATGTTATTGGCCTATCTGTTAGA<br>CCCTAGCAATACCACACCTGAAGGGGTCGCTCGTCGGTATGGCGGTGAATGGACTGAGGAAG<br>CCGGAGAGCGCGCCGCATTGTCCGAACGGCTCTTTGCAAACTTATGGGGTCGTCTGGAAGGG<br>GAGGAACGTCTGTTATGGTTGTATCGGGAAGTCGAACGTCCTCTTTCGGCCGTATTAGCGCA<br>TATGGAGGCAACAGGTGTGCGTTTAGATGTCGCGTACCTTCGGGCCTTATCACTGGAAGTTG<br>CAGAGGAAATCGCCCGTCTCGAGGCTGAAGTGTTCCGGTTGGCCGGTCACCCGTTTAACCTC<br>AACTCCCGTGACCAGCTGGAACGCGTTTTATTCGATGAGCTTGGGCTTCCCGCAATTGGCAA<br>AACCGAAAAGACTGGCAAACGCAGTACGAGCGCTGCCGTCCTTGAGGCACTCCGCGAGGCTC<br>ACCCTATTGTAGAAAAGATCCTGCAATACCGTGAGTTGACGAAGCTTAAAAGCACTTATATT<br>GATCCTCTCCCGGATCTGATCCATCCTCGTACCGGCCGCTTGCACACACGTTTCAACCAGAC<br>GGCGACTGCAAC |

| Name | Sequence |
|---|---|
| SEQ ID NO. 5<br><br>Taq subfragment #3 | CACACGTTTCAACCAGACGGCGACTGCAACCGGCCGTCTGTCTAGCTCGGATCCAAATCTCC<br>AGAACATTCCGGTCCGTACACCCTTGGGCCAACGTATCCGCCGGGCGTTTATCGCTGAGGAA<br>GGATGGTTACTGGTCGCATTGGACTACTCGCAGATTGAGCTGCGCGTCCTCGCACATCTCTC<br>TGGTGACGAAAATTTAATCCGCGTGTTTCAAGAGGGGCGTGATATTCACACAGAAACTGCCT<br>CATGGATGTTCGGTGTCCCACGTGAAGCAGTGGATCCTTTGATGCGCCGTGCAGCTAAAACA<br>ATTAATTTTGGAGTGCTGTACGGAATGAGCGCTCATCGCTTGAGTCAGGAACTGGCAATCCC<br>CTACGAGGAAGCGCAGGCATTCATCGAACGTTACTTTCAATCGTTTCCGAAAGTTCGCGCAT<br>GGATCGAGAAGACGCTCGAGGAAGGTCGTCGTCGGGGCTATGTCGAAACTCTGTTTGGTCGC<br>CGTCGGTACGTACCAGATCTTGAAGCCCGCGTCAAATCGGTACGGGAGGCTGCGGAGCGTAT<br>GGCATTTAATATGCCTGTACAGGGTACTGCAGCTGACCTCATGAAACTGGCAATGGTCAAGC<br>TTTTCCCGCGCTTGGAGGAAATGGGCGCACGTATGCTTCTGCAGGTCCATGACGAGCTGGTG<br>TTAGAAGCCCCTAAGGAGCGCGCCGAAGCTGTCGCGCGCCTCGCTAAAGAAGTGATGGAGGG<br>CGTTTACCCATTGGCCGTACCCCTCGAAGTGGAGGTCGGTATTGGAGAAGATTGGTTATCTG<br>CAAAGGAAGCGGCCGC |

| Name | Sequence |
|---|---|
| SEQ ID NO. 6<br><br>Complete codon-optimized Taq DNA polymerase<br><br>"OptiTaq" | CAT**ATG**CGTGGTATGCTGCCGTTGTTCGAGCCTAAAGGCCGCGTACTGTTAGTCGATGGTCA<br>TCACTTGGCCTATCGGACGTTCCATGCACTCAAAGGTCTGACGACCAGTCGTGGCGAACCGG<br>TCCAGGCTGTTTATGGTTTCGCTAAGTCTTTGCTCAAAGCACTGAAAGAAGACGGGGACGCG<br>GTAATTGTTGTATTTGATGCCAAAGCACCGAGCTTCCGCCACGAAGCTTATGGTGGCTACAA<br>GGCAGGACGCGCCCCTACCCCAGAAGATTTCCCCCGTCAGCTGGCATTAATTAAGGAGTTAG<br>TAGACCTTCTCGGCTTAGCGCGTCTGGAAGTTCCGGGTTATGAGGCGGACGATGTCCTTGCA<br>TCCTTGGCTAAAAAGGCCGAAAAAGAGGGCTACGAAGTCCGCATCTTGACGGCAGACAAAGA<br>TCTGTACCAGCTTCTGTCTGACCGTATTCATGTTTTGCACCCTGAAGGCTACTTAATCACTC<br>CGGCCTGGCTCTGGGAAAAGTACGGTCTGCGTCCCGATCAGTGGGCGGATTATCGGGCTTTG<br>ACGGGAGATGAGAGCGACAACCTGCCAGGAGTTAAGGGCATTGGTGAAAAAACCGCACGTAA<br>GCTGCTTGAAGAGTGGGGTTCCCTGGAAGCCTTGTTAAAAAATCTGGATCGTCTCAAGCCCG<br>CAATTCGTGAAAAGATCCTGGCTCATATGGACGATCTTAAATTAAGTTGGGACCTGGCCAAG<br>GTGCGCACCGATTTACCGCTTGAAGTGGATTTTGCAAAACGCCGTGAGCCGGACCGGGAACG<br>TTTACGCGCTTTCTTAGAGCGTCTGGAATTCGGTTCACTGCTTCATGAATTCGGTCTGTTAG<br>AGTCTCCTAAAGCACTCGAAGAGGCACCGTGGCCGCCCCCAGAAGGTGCTTTTGTTGGCTTC<br>GTACTTTCCCGTAAGGAGCCTATGTGGGCAGATCTTCTGGCTTTAGCGGCTGCACGCGGTGG<br>CCGTGTTCACCGGGCCCCTGAGCCATACAAAGCGTTACGTGATCTGAAGGAAGCACGTGGCT<br>TGCTGGCAAAAGACCTTTCTGTTTTGGCCCTGCGCGAGGGTCTTGGACTGCCGCCAGGCGAC<br>GATCCCATGTTATTGGCCTATCTGTTAGACCCTAGCAATACCACACCTGAAGGGGTCGCTCG<br>TCGGTATGGCGGTGAATGGACTGAGGAAGCCGGAGAGCGCGCCGCATTGTCCGAACGGCTCT<br>TTGCAAACTTATGGGGTCGTCTGGAAGGGGAGGAACGTCTGTTATGGTTGTATCGGGAAGTC<br>GAACGTCCTCTTTCGGCCGTATTAGCGCATATGGAGGCAACAGGTGTGCGTTTAGATGTCGC<br>GTACCTTCGGGCCTTATCACTGGAAGTTGCAGAGGAAATCGCCCGTCTCGAGGCTGAAGTGT<br>TCCGGTTGGCCGGTCACCCGTTTAACCTCAACTCCCGTGACCAGCTGGAACGCGTTTTATTC<br>GATGAGCTTGGGCTTCCCGCAATTGGCAAAACCGAAAAGACTGGCAAACGCAGTACGAGCGC<br>TGCCGTCCTTGAGGCACTCCGCGAGGCTCACCCTATTGTAGAAAAGATCCTGCAATACCGTG<br>AGTTGACGAAGCTTAAAAGCACTTATATTGATCCTCTCCCGGATCTGATCCATCCTCGTACC<br>GGCCGCTTGCACACACGTTTCAACCAGACGGCGACTGCAACCGGCCGTCTGTCTAGCTCGGA<br>TCCAAATCTCCAGAACATTCCGGTCCGTACACCCTTGGGCCAACGTATCCGCCGGGCGTTTA<br>TCGCTGAGGAAGGATGGTTACTGGTCGCATTGGACTACTCGCAGATTGAGCTGCGCGTCCTC<br>GCACATCTCTCTGGTGACGAAAATTTAATCCGCGTGTTTCAAGAGGGGCGTGATATTCACAC<br>AGAAACTGCCTCATGGATGTTCGGTGTCCCACGTGAAGCAGTGGATCCTTTGATGCGCCGTG<br>CAGCTAAAACAATTAATTTTGGAGTGCTGTACGGAATGAGCGCTCATCGCTTGAGTCAGGAA<br>CTGGCAATCCCCTACGAGGAAGCGCAGGCATTCATCGAACGTTACTTTCAATCGTTTCCGAA<br>AGTTCGCGCATGGATCGAGAAGACGCTCGAGGAAGGTCGTCGTCGGGGCTATGTCGAAACTC<br>TGTTTGGTCGCCGTCGGTACGTACCAGATCTTGAAGCCCGCGTCAAATCGGTACGGGAGGCT<br>GCGGAGCGTATGGCATTTAATATGCCTGTACAGGGTACTGCAGCTGACCTCATGAAACTGGC<br>AATGGTCAAGCTTTTCCCGCGCTTGGAGGAAATGGGCGCACGTATGCTTCTGCAGGTCCATG<br>ACGAGCTGGTGTTAGAAGCCCCTAAGGAGCGCGCCGAAGCTGTCGCGCGCCTCGCTAAAGAA<br>GTGATGGAGGGCGTTTACCCATTGGCCGTACCCCTCGAAGTGGAGGTCGGTATTGGAGAAGA<br>TTGGTTATCTGCAAAGGAAGCGGCCGC |

For the final completed "OptiTaq" clone, Nde I and Not I restrictions sites are underlined. The ATG start codon is identified in bold font.

**Example 2. Production of Codon Optimized Taq DNA Polymerase mutants.**

**[0157]** Eighteen mutant versions of Taq DNA polymerase (Table 3, Mut IDs 1-18) were made by site directed mutagenesis of the cloned OptiTaq codon-optimized Taq DNA polymerase. Specific mutations were introduced into the OptiTaq sequence using the method of

PCR site-directed mutagenesis (Weiner MP, *et al.*, Gene. 151(1-2):119-23 (1994)). Each mutagenesis reaction employed 10 pmoles of two complementary oligonucleotides (Table 5) containing the desired base changes, annealed to the double-stranded OptiTaq plasmid (20 ng), 5 U KOD DNA polymerase (Novagen-EMD Chemicals, San Diego, CA), 1.5 mM $MgSO_4$, in 1X KOD PCR buffer. Thermal cycling parameters were 95°C for 3 minutes (95°C for 20 sec-55°C for 20 sec-70°C for 2.5 minutes) for 16 cycles followed by a 70°C soak for 4 minutes. After PCR site-directed mutagenesis, the amplified product was treated with 10 U of Dpn I (NEB, Ipswich, MA), at 37°C for 1 hour, followed by inactivation at 80°C for 20 minutes. $1/110^{th}$ of the digestion material was transformed into XL-1 Blue competent bacteria. Bacterial clones were isolated, plasmid DNA prepared, and individual mutations were confirmed by Sanger DNA sequencing. All mutants remained in the pET-27b(+) expression vector, which is suitable for expressing the recombinant proteins in *E. coli.*

**Table 5: Oligonucleotides used for site-directed mutagenesis to produce 18 Taq DNA Polymerase mutants.**

| Mutant ID | Amino acid changes | Sequence" Sense mutagenesis oligonucleotide | SEQ ID No. | Sequence" Antisense mutagenesis oligonucleotide | SEQ ID No. |
|---|---|---|---|---|---|
| 1 | V783I | aatgggcgcacgtatgcttct gcagATTcatgacgagctggt gttagaagccc | 7 | gggcttctaacaccagctcgtca tgAATctgcagaagcatacgtgc gcccatt | 8 |
| 2 | V783F | aatgggcgcacgtatgcttct gcagTTCcatgacgagctggt gttagaagccc | 9 | gggcttctaacaccagctcgtca tgGAActgcagaagcatacgtgc gcccatt | 10 |
| 3 | H784Q | gggcgcacgtatgcttctgca ggtcCAGgacgagctggtgtt agaagccccta | 11 | taggggcttctaacaccagctcg tcCTGgacctgcagaagcatacg tgcgccc | 12 |
| 4 | R573H | caaccagacggcgactgcaac cggcCATctgtctagctcgga tccaaatctcc | 13 | ggagatttggatccgagctagac agATGgccggttgcagtcgccgt ctggttg | 14 |
| 5 | Q582K | tctgtctagctcggatccaaa tctcAAAaacattccggtccg tacacccttgg | 15 | ccaagggtgtacggaccggaatg ttTTTgagatttggatccgagct agacaga | 16 |
| 6 | F667W | gcgccgtgcagctaaaacaat taatTGGggagtgctgtacgg aatgagcgctc | 17 | gagcgctcattccgtacagcact ccCCAattaattgttttagctgc acggcgc | 18 |
| 7 | H639W | cgtgtttcaagaggggcgtga tattTGGacagaaactgcctc atggatgttcg | 19 | cgaacatccatgaggcagtttct gtCCAaatatcacgcccctcttg aaacacg | 20 |

| Mutant ID | Amino acid changes | Sequence" Sense mutagenesis oligonucleotide | SEQ ID No. | Sequence" Antisense mutagenesis oligonucleotide | SEQ ID No. |
|---|---|---|---|---|---|
| 8 | L616M | cgcattggactactcgcagat tgagATGcgcgtcctcgcaca tctctctggtg | 21 | caccagagagatgtgcgaggacg cgCATctcaatctgcgagtagtc caatgcg | 22 |
| 9 | E615L L616E | ggtcgcattggactactcgca gattCTGGAGcgcgtcctcgc acatctctctggtg | 23 | caccagagagatgtgcgaggacg cgCTCCAGaatctgcgagtagtc caatgcgacc | 24 |
| 10 | A661E I665W F667L | cgtgaagcagtggatcctttg atgcgccgtGAAgctaaaaca TGGaatTTGggagtgctgtac ggaatgagcgctcatcgc | 25 | gcgatgagcgctcattccgtaca gcactccCAAattCCAtgtttta gcTTCacggcgcatcaaaggatc cactgcttcacg | 26 |
| 11 | Q782I H784F | ggaaatgggcgcacgtatgct tctgATCgtcTTCgacgagct ggtgttagaagccccta | 27 | taggggcttctaacaccagctcg tcGAAgacGATcagaagcatacg tgcgcccatttcc | 28 |
| 12 | Q782I V783L H784L | ggaaatgggcgcacgtatgct tctgATTTTGCTGgacgagct ggtgttagaagccccta | 29 | taggggcttctaacaccagctcg tcCAGCAAAATcagaagcatacg tgcgcccatttcc | 30 |
| 13 | Q782S V783F H784N | ggaaatgggcgcacgtatgct tctgTCCTTCAACgacgagct ggtgttagaagccccta | 31 | taggggcttctaacaccagctcg tcGTTGAAGGAcagaagcatacg tgcgcccatttcc | 32 |
| 14 | Q782P V783L H784Q | ggaaatgggcgcacgtatgct tctgCCGTTACAGgacgagct ggtgttagaagccccta | 33 | taggggcttctaacaccagctcg tcCTGTAACGGcagaagcatacg tgcgcccatttcc | 34 |
| 15 | Q754A | gcgtatggcatttaatatgcc tgtaGCGggtactgcagctga cctcatgaaac | 35 | gtttcatgaggtcagctgcagta ccCGCtacaggcatattaaatgc catacgc | 36 |
| 16 | R659H | acgtgaagcagtggatccttt gatgCACcgtgcagctaaaac aattaattttg | 37 | caaaattaattgttttagctgca cgGTGcatcaaaggatccactgc ttcacgt | 38 |
| 17 | V783F H784Q | aatgggcgcacgtatgcttct gcagTTCCAGgacgagctggt gttagaagc | 39 | GcttctaacaccagctcgtcCTG GAActgcagaagcatacgtgcgc ccatt | 40 |
| 18 | V783L H784Q | aatgggcgcacgtatgcttct gcagCTGCAGgacgagctggt gttagaagccc | 41 | gggcttctaacaccagctcgtcC TGCAGctgcagaagcatacgtgc gcccatt | 42 |

DNA bases identical to codon optimized OptiTaq are shown in lower case; those specific for the mutations introduced by site-directed mutagenesis are shown in upper case.

**Example 3. Expression of Recombinant Taq DNA Polymerases.**

**[0158]** The following example demonstrates the expression of recombinant Taq DNA polymerase unmodified and mutant peptides. The synthetic gene sequences from Examples 1, 2

and 12 were cloned in the pET-27b(+) expression vector (Novagen, EMD Biosciences, La Jolla, CA). This vector places six histidine residues (which together comprise a "His-tag") at the carboxy terminus of the expressed peptide, followed by a stop codon. A "His-tag" permits use of rapid, simple purification of recombinant proteins using $Ni^{2+}$ affinity chromatography methods which are well known to those with skill in the art. Alternatively, the synthetic genes could be expressed in native form without the His-tag and purified using size exclusion chromatography, anion-exchange chromatography, or other such methods, which are also well known to a person of ordinary skill in the art.

**[0159]** BL21(DE3) competent *E. coli* cells (Novagen) were transformed with ~1 ng of each plasmid. Briefly, plasmids were added to the cells on ice and gently stirred. After a 5 minute incubation on ice, cells were heat shocked at 42°C for 30 seconds, then returned to ice for 2 minutes. Room temperature SOC (80 µL) was added to the transformed cells, followed by a 1 hour outgrow period at 37°C, with agitation at 250rpm. Cells were plated (20 µL) on 37°C pre-warmed LB/Kan plates (Luria Broth agar plates supplemented with 50 µg/mL kanamycin) and were placed at 37°C overnight. The next morning, one colony was picked and grown (37°C, 250 rpm) in 10 mL LB/Kan broth (50 µg/mL) to log phase ($OD_{600}$ 0.3-0.9). Cells were then induced with Overnight Express ™ Autoinduction System 1 (Novagen) in Terrific Broth at 37°C, 250 rpm following the protocol recommended by the manufacturer. Culture volumes were 100 mL for wild type OptiTaq and 200 mL for mutants. Growth saturation was reached after 18 hours, and the culture was pelleted at 10,000 x g for 10 minutes in a Beckman Avanti™ J-25 Centrifuge. The pellet (~6 g) was lysed using 30 mL BugBuster® Protein Extraction Reagent (Novagen), 30 kU rLysozyme™ Solution (Novagen) and 1500 U DNase I (Life Technologies, Grand Island, NY) to release soluble proteins and degrade nucleic acids according to the manufacturer's instructions. Following centrifugation at 15,000 x g for 20 minutes to remove cell debris, the lysates were heated at 75°C for 15 minutes to inactive DNase I and other cellular nucleases. The lysates were then spun at 15,000 x g for 20 minutes to sediment denatured protein. The heat denaturation and centrifugation steps provide significant purity enrichment of the recombinant enzymes. Both "total" and "soluble" fractions of the bacterial lysates were analyzed using SDS 4-20% polyacrylamide gel electrophoresis for 1 hour at 125 V. Proteins were visualized with Coomassie Blue staining for 1 hour, followed by 3-4 rounds of destaining until protein bands were clear.

**[0160]** The recovered soluble protein was passed over a $Ni^{2+}$ affinity column containing His·Bind Resin (Novagen) and eluted with a buffer containing 200 mM imidazole (200 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9). The purified protein (~6 mL) was then concentrated at 3210 x g in a Beckman Coulter 6R tabletop centrifuge swinging bucket rotor using an Amicon Ultra-15, PLGC Ultracel-PL Membrane, 10 KDa concentrator (EMD Millipore, Billerica, MA) to ~ 200 μL and stored at -20°C until dialysis. The concentrated protein was then dialyzed against storage buffer (20 mM Tris pH7.5, 100mM NaCl, 1 mM DTT, 0.1 mM EDTA, 50% glycerol, 0.1% Triton X-100) at 4°C overnight, followed by 3x2 hours (at 1000 fold ratio of protein solution to dialysis buffer each time). The final purified protein was stored at -20°C. Using this protocol, 100 mL of an autoinduced culture yielded ~1.2 mg/67.6 μM/12,168 pmol of purified soluble protein for OptiTaq. Similar yields were obtained for the mutant DNA polymerases.

**[0161]** To determine protein concentration, samples were examined alongside known quantities of BSA (bovine serum albumin) using SDS 4-20% polyacrylamide gel electrophoresis for 1 hour. Proteins were visualized with Coomassie Blue staining for 1 hour, followed by 3-4 rounds of destaining until protein bands were clear. Band intensity was analyzed using ImageJ software (National Institutes of Health, Bethesda, MD).

**[0162]** To evaluate purity and quality of the recombinant protein preparations, 500 ng of each recombinant protein (wild type OptiTaq and each mutant) were separated on a 4-20% SDS-PAGE gel, stained with Coomassie Blue, and visualized. The recombinant proteins all migrate at the appropriate position on the gel for proteins having a molecular weight of 97.1 kDa. The preparations show relatively high purity with few additional species detected. Gel images are shown in Figures 2A, 2B, 2C, and 2D. Similar gels were run for MUT IDs 22 (H784T), 24 (H784V), 30 (H784Y), 31 (H784W), and 35 (H784K), and single bands corresponding to the desired recombinant protein were visualized (data not shown).

**[0163]** The purified enzymes were tested for nuclease contamination using DNaseAlert™ and RNaseAlert® nuclease detection kits (Integrated DNA Technologies, Coralville, IA) following protocols recommended by the manufacturer. All enzyme preparations were determined to be free of contaminating nucleases.

**Example 4. Characterization of properties of 18 mutant Taq DNA Polymerases in PCR.**

**[0164]** The 18 mutant Taq DNA polymerase enzymes described in Example 3 were characterized for polymerase activity and the ability to discriminate a 3'-RNA residue in the primer oligonucleotide.

**[0165]** The unit activity of the purified wild-type protein was determined by comparing performance in qPCR of known quantities of OptiTaq and each mutant compared to a commercial non-hot-start Taq DNA polymerase, Taq-B DNA Polymerase (Enzymatics, Beverly, MA). Quantification cycle values (Cq, the amplification cycle number at which positive signal is first detected) and amplification curve shapes were analyzed to determine the nanogram amounts at which both enzymes performed similarly in the suboptimal range for each. Using these nanogram amounts and known unit values of Taq-B DNA polymerase, relative activity unit values could be extrapolated for all of the mutant DNA polymerase enzymes having sufficient activity to support PCR.

**[0166]** The following reaction conditions were employed: 1x qPCR buffer (20 mM Tris pH 8.4, 50 mM KCl, 3 mM $MgCl_2$, 0.01% Triton-X100), 800 μM dNTPs (200 μM each), 500 nM For primer (Hs HPRT F517, SEQ ID NO. 43), 500 nM Rev primer (Hs HPRT R591, SEQ ID NO. 44), 250 nM probe (Hs HPRT P554, SEQ ID NO. 45), 2 x $10^3$ copies of linearized cloned plasmid template (HPRT-targ, SEQ ID NO. 46), in 10 μL final volume. The amount of DNA polymerase added to each reaction was varied as follows: for wild type (OptiTaq), reactions were set using 10, 1, 0.1, 0.01, and 0.001 U/μL (220, 22, 2.2, 0.22, or 0.022 ng of protein per 10 μL reaction). Mutant polymerases were run in similar concentrations. In addition, those mutant enzymes showing polymerase activity were more finely titrated testing 220, 22, 10.6, 4.8, 2.2, 1.1, 0.48, and 0.22 ng of protein per 10 μL reaction. Enzyme dilutions were made in enzyme dilution buffer (20 mM Tris pH7.5, 100 mM NaCl, 1 mM DTT, 0.1% Triton-X100, 1 mg/mL BSA, 10% glycerol). Reactions were run in 384 well format on a BIO-RAD CFX384™ Real-Time System (BIO-RAD, Hercules, CA) using cycling parameters 95°C for 30 seconds followed by 60 cycles of [95°C for 15 seconds followed by 60°C for 1 minutes]. Detection was achieved using a fluorescence-quenched probe (5'-nuclease assay format, note that the mutations introduced into the present series of Taq mutants do not lie in the 5'-nuclease domain). Sequences of the primers, probe, and template (plasmid insert) are shown in Table 6.

**Table 6. Sequence of oligonucleotides employed in Taq DNA polymerase activity assay.**

| Name | Sequence | SEQ ID NO. |
|---|---|---|
| Hs HPRT F517 | GACTTTGCTTTCCTTGGTCAG | SEQ ID NO. 43 |
| Hs HPRT R591 | GGCTTATATCCAACACTTCGTG | SEQ ID NO. 44 |
| Hs HPRT P554 | FAM-ATGGTCAAG(ZEN)GTCGCAAGCTTGCTGGT-IBFQ | SEQ ID NO. 45 |
| HPRT-targ | GACTTTGCTTTCCTTGGTCAGGCAGTATAATCCAAAGATGGTCAAGGTC GCAAGCTTGCTGGTGAAAAGGACCCCACGAAGTGTTGGATATAAGCC | SEQ ID NO. 46 |

Nucleic acid sequences are shown 5'-3'. FAM = 6-carboxyfluorescein, IBFQ = Iowa Black FQ (fluorescence quencher), and ZEN = ZEN internal fluorescence quencher.

**[0167]** These 18 Taq DNA polymerase mutants were characterized as outlined above. Results are summarized in Table 7. Six mutants, including Mutant IDs 4, 5, 9 12, 13, and 17, did not show detectable DNA polymerase activity and were not studied further. Six mutants, Mutant IDs 6, 7, 11, 14, 15, and 16 had DNA polymerase activity; however, processivity was reduced from 4-50 fold relative to the wild type enzyme. Six mutants, Mutant IDs 1, 2, 3, 8, 10, and 18, showed DNA polymerase activity similar to wild type OptiTaq.

**Table 7. Novel Taq DNA polymerase mutants selected for initial study.**

| Mutant ID | Amino acid changes from wild-type Taq | Polymerase Activity | Relative activity* | ΔCq Delay in priming from an RNA base** |
|---|---|---|---|---|
| 1 | V783I | Yes | 1 | 0 |
| 2 | V783F | Yes | 1 | 1 |
| 3 | H784Q | Yes | 1 | 1 |
| 4 | R573H | No | - | - |
| 5 | Q582K | No | - | - |
| 6 | F667W | Yes | 0.25 | 9 |
| 7 | H639W | Yes | 0.02 | 20 |
| 8 | L616M | Yes | 1 | 0 |
| 9 | E615L, L616E | No | - | - |

| Mutant ID | Amino acid changes from wild-type Taq | Polymerase Activity | Relative activity* | ΔCq Delay in priming from an RNA base** |
|---|---|---|---|---|
| 10 | A661E, I665W, F667L | Yes | 1 | 2.9 |
| 11 | Q782I, H784F | Yes | 0.20 | 2 |
| 12 | Q782I, V783L, H784L | No | - | - |
| 13 | Q782S, V783F, H784N | No | - | - |
| 14 | Q782P, V783L, H784Q | Yes | 0.02 | 2.5 |
| 15 | Q754A | Yes | 0.2 | >35 |
| 16 | R659H | Yes | 0.1 | >35 |
| 17 | V783F, H784Q | No | - | - |
| 18 | V783L, H784Q | Yes | 1 | 1 |

*Wild-type OptiTaq was set to "1" and the relative activity of each of the mutant polymerases was normalized to this amplification efficiency, with 1 as the maximum.
**ΔCq = [Cq Mutant ID X] – [Cq OptiTaq] when qPCR reactions are run using primers having a 3'-RNA residue.

**[0168]** The subset of these mutant Taq DNA polymerases which showed DNA polymerase activity were studied for their ability to discriminate between primers having a 3'-DNA versus a 3'-RNA residue relative to the wild type OptiTaq enzyme. Real-time PCR was performed as before, employing in the reactions the amount of each mutant DNA polymerase equal to 0.5 units of wild-type OptiTaq per 10 μL reaction. The following reaction conditions were employed: 1x qPCR buffer (20 mM Tris pH 8.4, 50 mM KCl, 3 mM $MgCl_2$, 0.01% Triton-X100), 800 μM dNTPs (200 μM each), 500 nM For primer (Hs SFRS9 F569 rU, SEQ ID NO. 47), 500 nM Rev primer (Hs SFRS9 R712 rA, SEQ ID NO. 48), 250 nM probe (Hs SFRS9 P644, SEQ ID NO. 49), 2 x $10^3$ copies of linearized cloned plasmid template (SFRS9-targ, SEQ ID NO. 50), in 10 μL final volume. Reactions were run in 384 well format on a BIO-RAD CFX384™ Real-Time System (BIO-RAD, Hercules, CA) using cycling parameters 95°C for 30 seconds followed by 60 cycles of [95°C for 15 seconds followed by 60°C for 1 minutes]. Detection was achieved using a fluorescence-quenched probe (5'-nuclease assay format). Sequences of the primers, probe, and template (plasmid insert) are shown in Table 8.

**Table 8. Sequence of oligonucleotides employed in the primer 3'-RNA discrimination assay.**

| Name | Sequence | SEQ ID NO. |
|---|---|---|
| Hs SFRS9 F569 rU | TGTGCAGAAGGATGGAGu | SEQ ID NO. 47 |
| Hs SFRS9 R712 rA | CTGGTGCTTCTCTCAGGATa | SEQ ID NO. 48 |
| Hs SFRS9 P644 | HEX-TGGAATATG(ZEN)CCCTGCGTAAACTGGA-IBFQ | SEQ ID NO. 48 |
| SFRS9-targ | TGTGCAGAAGGATGGAGTGGGGATGGTCGAGTATCTCAGAAAAGAAGA CATGGAATATGCCCTGCGTAAACTGGATGACACCAAATTCCGCTCTCA TGAGGGTGAAACTTCCTACATCCGAGTTTATCCTGAGAGAAGCACCAG | SEQ ID NO. 50 |

Nucleic acid sequences are shown 5'-3' with DNA uppercase and RNA lowercase. HEX = hexachlorofluorescein, IBFQ = Iowa Black FQ (fluorescence quencher), and ZEN = ZEN fluorescence quencher.

**[0169]** The 12 Taq DNA polymerase mutants that supported PCR were tested for the ability to use a 3'-RNA modified primer as outlined above. Results are summarized in Table 7. Mutant IDs 1 and 8 did not show any difference between primers having a 3'-DNA versus a 3'-RNA residue. Mutant IDs 2, 3, 6, 7, 10, 11, 14, 15, 16, and 18 showed an amplification delay using 3'-RNA primers. Thus the rational design strategy employed herein was successful and Taq DNA polymerase mutants were identified which discriminated against priming from a 3'-RNA residue. Those mutants which showed some delay with RNA priming and showed high processivity were studied for improvements in primer 3'-residue mismatch discrimination.

**Example 5: Improved mismatch discrimination in allele-specific PCR using mutant Taq DNA polymerases.**

**[0170]** Of the 18 mutant enzymes studied in Example 4, Mutant IDs 2, 3, 10, and 18 showed the ability to discriminate against a 3'-RNA residue in the primer and retained high enzymatic activity/processivity. These four mutants were studied for the ability to discriminate against a 3'-terminal DNA mismatch compared with wild type OptiTaq DNA polymerase using an allele-specific qPCR assay. Amplification reactions were performed against a synthetic oligonucleotide template where a single base was varied (SNP) which was positioned to lie at the 3'-end of the reverse primer. Synthetic templates were employed having each of the 4 possible bases at this

position. Reverse primers were employed having each of the 4 possible bases at the 3'-end. Relative amplification efficiency for all pairwise combinations was assessed using qPCR.

**[0171]** Quantitative allele-specific real-time PCR (AS-qPCR) was performed in 10 μL reaction volumes in 384 well format with 2 x $10^3$ copies of a 103 bp synthetic template (SEQ ID NOs. 51-4). Final reaction conditions used were 20 mM Tris-HCL (pH 8.4 at 25°C), 50 mM KCL, and 3 mM $MgCl_2$, 0.01% Triton X-100, 800 μM total dNTPs, and 200 nM of the universal forward primer (SEQ ID NO. 60), 200 nM of a reverse primer (separate reactions were set up for each of the allele-specific primers SEQ ID NOs. 55-58 or the control universal primer SEQ ID NO. 59) and 200 nM of the 5' nuclease detection probe (SEQ ID NO. 61). Each allele-specific primer was tested on each SNP template. Reactions utilized either 0.5 U (10.8 ng/11.1 nM/111 fmol) of the wild type OptiTaq DNA polymerase or 0.5 U of one of the 4 Taq DNA polymerase mutants studied (MUT ID No. 2 V783F, MUT ID NO. 3 H784Q, MUT ID NO. 10 A661E I665W F667L, or MUT ID NO. 18 V783L H784Q). Amplification was performed on a CFX384™ C1000™ Thermo Cycler system (Bio-Rad, Hercules, CA) using the following cycling parameters: 95°C for 30 seconds initial denaturation followed by 60 cycles of 95°C for 10 seconds, then 60°C for 30 seconds. Oligonucleotide reagents used in this example are shown in Table 9.

**Table 9. Synthetic oligonucleotides employed in Example 5.**

| Name | Sequence (5'-3') | SEQ ID NO. |
|---|---|---|
| A Template | AGCTCTGCCCAAAGATTACCCTGACAGCTAAGTGGCAGTGGAA GTTGGCCTCAGAAGTAGTGGCCAGCTGTGTGTCGGGGAACAGT AAAGGCATGAAGCTCAG | SEQ ID NO. 51 |
| C Template | AGCTCTGCCCAAAGATTACCCTGACAGCTAAGTGGCAGTGGAA GTTGGCCTCAGAAGTAGTGGCCAGCTGTGTGTCGGGGCACAGT AAAGGCATGAAGCTCAG | SEQ ID NO. 52 |
| G Template | AGCTCTGCCCAAAGATTACCCTGACAGCTAAGTGGCAGTGGAA GTTGGCCTCAGAAGTAGTGGCCAGCTGTGTGTCGGGGGACAGT AAAGGCATGAAGCTCAG | SEQ ID NO. 53 |
| T Template | AGCTCTGCCCAAAGATTACCCTGACAGCTAAGTGGCAGTGGAA GTTGGCCTCAGAAGTAGTGGCCAGCTGTGTGTCGGGGTACAGT AAAGGCATGAAGCTCAG | SEQ ID NO. 54 |
| Syn Rev T | CTGAGCTTCATGCCTTTACTGTT | SEQ ID NO. 55 |
| Syn Rev C | CTGAGCTTCATGCCTTTACTGTC | SEQ ID NO. 56 |

| Name | Sequence (5'-3') | SEQ ID NO. |
|---|---|---|
| Syn Rev A | CTGAGCTTCATGCCTTTACTGTA | SEQ ID NO. 57 |
| Syn Rev G | CTGAGCTTCATGCCTTTACTGTG | SEQ ID NO. 58 |
| Syn Rev | CTGAGCTTCATGCCTTTACTGT | SEQ ID NO. 59 |
| Syn For | AGCTCTGCCCAAAGATTACCCTG | SEQ ID NO. 60 |
| Syn Probe | FAM-TTCTGAGGC(ZEN)CAACTTCCACTGCCACTTA-IBFQ | SEQ ID NO. 61 |

DNA bases are uppercase; FAM = 6-carboxyfluorescein; IBFQ = Iowa Black™ FQ fluorescence quencher; ZEN = internal ZEN fluorescence quencher; underlined base indicates the SNP site in the synthetic template DNA.

**[0172]** Initially all reactions were run in triplicate. Similar results were obtained for all replicates when using the wild type OptiTaq. However, results showed greater variation for the mutant polymerases. To obtain statistically meaningful results, each reaction was therefore performed 96 times for the mutant polymerases and 81 times for the wild type enzyme. ΔCq values were calculated as the Cq value obtained for each mismatched base pair minus the Cq value obtained for the matched base pair (ΔCq = Cq mismatch – Cq match). The ΔCq values for all 96 replicates were averaged and standard deviations were calculated. Results are shown in Table 10 and are graphically summarized in Figures 3A and 3B. Note that the reverse primer is the allele-specific primer, so the "Syn Rev T" primer (SEQ ID NO. 55) is the perfect match to the Template A (SEQ ID NO. 51), etc.

**Table 10: ΔCq values for AS-qPCR reactions using WT OptiTaq and mutant Taq DNA polymerases.**

| DNA Polymerase | Reverse Primer | | Template | | | |
|---|---|---|---|---|---|---|
| | Name | SEQ ID NO. | A SEQ ID NO. 51 | C SEQ ID NO. 52 | G SEQ ID NO. 53 | T SEQ ID NO. 54 |
| OptiTaq | Syn Rev T | 55 | - | 2.3 +/- 0.2 | 1.4 +/- 0.2 | 3.8 +/- 0.2 |
| | Syn Rev G | 58 | 7.6 +/- 0.6 | - | 5.6 +/- 0.3 | 1.9 +/- 0.2 |

| DNA Polymerase | Reverse Primer | | Template | | | |
|---|---|---|---|---|---|---|
| | Name | SEQ ID NO. | A SEQ ID NO. 51 | C SEQ ID NO. 52 | G SEQ ID NO. 53 | T SEQ ID NO. 54 |
| | Syn Rev C | 56 | 1.8 +/- 0.2 | 7.6 +/- 0.6 | - | 2.0 +/- 0.2 |
| | Syn Rev A | 57 | 6.6 +/- 0.4 | 1.5 +/- 0.2 | 8.0 +/- 0.6 | - |
| MUT ID 2 V783F | Syn Rev T | 55 | - | 7.3 +/- 2.9 | 4.5 +/- 0.5 | 9.5 +/- 1.8 |
| | Syn Rev G | 58 | 17.9 +/- 8.3 | - | 16.4 +/- 7.5 | 4.1 +/- 0.2 |
| | Syn Rev C | 56 | 6.5 +/- 1.2 | 15.0 +/- 8.9 | - | 5.3 +/- 0.5 |
| | Syn Rev A | 57 | 7.8 +/- 4.0 | 3.5 +/- 0.4 | 14.6 +/- 9.7 | - |
| MUT ID 3 H784Q | Syn Rev T | 55 | - | 7.5 +/- 0.8 | 7.0 +/- 0.6 | 10.4 +/- 2.3 |
| | Syn Rev G | 58 | 13.3 +/- 7.6 | - | 10.1 +/- 4.8 | 4.6 +/- 0.2 |
| | Syn Rev C | 56 | 6.9 +/- 0.5 | 8.6 +/- 2.6 | - | 5.6 +/- 0.4 |
| | Syn Rev A | 57 | 17.1 +/- 7.2 | 6.3 +/- 0.5 | 21.2 +/- 8.7 | - |
| MUT ID 10 A661E I665W F667L | Syn Rev T | 55 | - | 9.0 +/- 0.9 | 5.7 +/- 0.3 | 11.2 +/- 2.6 |
| | Syn Rev G | 58 | 19.9 +/- 8.4 | - | 13.9 +/- 5.3 | 3.9 +/- 0.3 |
| | Syn Rev C | 56 | 8.7 +/- 4.3 | 19.2 +/- 9.7 | - | 7.4 +/- 0.8 |
| | Syn Rev A | 57 | 13.3 +/- 8.2 | 6.1 +/- 0.8 | 13.1 +/- 8.6 | - |
| MUT ID 18 V783L | Syn Rev T | 55 | - | 5.8 +/- 1.3 | 6.0 +/- 0.4 | 9.4 +/- 1.2 |

| DNA Polymerase | Reverse Primer | | Template | | | |
|---|---|---|---|---|---|---|
| | Name | SEQ ID NO. | A SEQ ID NO. 51 | C SEQ ID NO. 52 | G SEQ ID NO. 53 | T SEQ ID NO. 54 |
| H784Q | Syn Rev G | 58 | 22.7 +/- 8.0 | - | 18.9 +/- 8.4 | 4.9 +/- 0.3 |
| | Syn Rev C | 56 | 6.8 +/- 0.5 | 17.6 +/- 9.6 | - | 4.8 +/- 0.4 |
| | Syn Rev A | 57 | 19.3 +/- 8.2 | 6.1 +/- 0.4 | 26.6 +/- 6.4 | - |

Average ΔCq values are shown, where ΔCq = [Cq mismatch – Cq match], +/- standard deviation calculated from 96 replicates.

**[0173]** The wild type OptiTaq showed an average ΔCq for AS-qPCR in this synthetic amplicon system of 4.2 with a range of 1.4 to 8.0. Mutant ID 2 (V783F) showed an average ΔCq of 9.4 with a range of 3.5 to 17.9. Mutant ID 3 (H784Q) showed an average ΔCq of 9.9 with a range of 4.6 to 21.2. Mutant ID 10 (A661E, I665W, F667L) showed an average ΔCq of 10.9 with a range of 3.9 to 19.9. Mutant ID 18 (V783L, H784Q) showed an average ΔCq of 12.4 with a range of 4.9 to 26.6. Therefore in all pairwise combinations of 4 template bases and 4 3'-terminal primer bases the mutant Taq DNA polymerases of the present invention showed greater discrimination to mismatch than did the wild type OptiTaq DNA polymerase. The magnitude of improvement for each mismatch pair is defined by the ΔΔCq, which is the difference of discrimination between the mutant and wild type enzymes (ΔΔCq = ΔCq mutant – ΔCq wild type). The ΔΔCq values were calculated and are shown in Table 11.

**Table 11: ΔΔCq values for AS-qPCR reactions for the mutant Taq DNA polymerases compared with wild type OptiTaq.**

| DNA Polymerase | Reverse Primer | | Template | | | |
|---|---|---|---|---|---|---|
| | Name | SEQ ID NO. | A SEQ ID NO. 51 | C SEQ ID NO. 52 | G SEQ ID NO. 53 | T SEQ ID NO. 54 |
| MUT ID NO. 2 | Syn Rev T | 55 | - | 5.0 | 3.1 | 5.7 |

| DNA Polymerase | Reverse Primer | | Template | | | |
|---|---|---|---|---|---|---|
| | Name | SEQ ID NO. | A SEQ ID NO. 51 | C SEQ ID NO. 52 | G SEQ ID NO. 53 | T SEQ ID NO. 54 |
| V783F | Syn Rev G | 58 | 10.3 | - | 10.8 | 2.2 |
| | Syn Rev C | 56 | 4.7 | 7.4 | - | 3.3 |
| | Syn Rev A | 57 | 1.2 | 2.0 | 6.6 | - |
| MUT ID NO. 3 H784Q | Syn Rev T | 55 | - | 5.2 | 5.6 | 6.6 |
| | Syn Rev G | 58 | 5.7 | - | 4.5 | 2.7 |
| | Syn Rev C | 56 | 5.1 | 1.0 | - | 3.6 |
| | Syn Rev A | 57 | 10.5 | 4.8 | 13.2 | - |
| MUT ID NO. 10 A661E I665W F667L | Syn Rev T | 55 | - | 6.7 | 4.3 | 7.4 |
| | Syn Rev G | 58 | 12.3 | - | 8.3 | 2.0 |
| | Syn Rev C | 56 | 6.9 | 11.6 | - | 5.4 |
| | Syn Rev A | 57 | 6.7 | 4.6 | 5.1 | - |
| MUT ID NO. 18 V783L H784Q | Syn Rev T | 55 | - | 3.5 | 4.6 | 5.6 |
| | Syn Rev G | 58 | 15.1 | - | 13.3 | 3.0 |
| | Syn Rev C | 56 | 5.0 | 10.0 | - | 2.8 |
| | Syn Rev A | 57 | 12.7 | 4.6 | 18.6 | - |

Average ΔΔCq values are shown, where ΔΔCq = [ΔCq mutant – ΔCq wild type], from data in Table 10.

**[0174]** Mutant ID 2 (V783F) showed an average ΔΔCq of 5.2 compared to wild type OptiTaq. Mutant ID 3 (H784Q) showed an average ΔΔCq of 5.7 compared to wild type OptiTaq. Mutant ID 10 (A661E, I665W, F667L) showed an average ΔΔCq of 6.7 compared to wild type OptiTaq. Mutant ID 18 (V783L, H784Q) showed an average ΔΔCq of 8.2 compared to wild type OptiTaq. Therefore each of the mutant Taq DNA polymerases of the present invention showed a significant improvement over wild type OptiTaq in mismatch discrimination, and, importantly, mismatch discrimination was improved for every possible mismatch base pair combination. Overall, mutant ID 18 (V783L, H784Q) showed the best SNP discrimination within the set of 4 mutant enzymes studied in this example using an AS-PCR assay.

**Example 6: Discrimination against a primer 3'-RNA residue by Taq DNA polymerase mutants.**

**[0175]** All 18 Taq DNA polymerase mutants were screened for the ability to discriminate against priming from a 3'-RNA residue in Example 4. The four mutants studied in AS-PCR in Example 5 (MUT IDs 2, 3, 10, and 18) which showed good 3'-mismatch discrimination were studied in greater detail in the present example for the ability to discriminate against the presence of a 3'-terminal RNA residue in the primer, examining for possible base-specific effects. Amplification reactions were performed against a synthetic oligonucleotide template where a single base was varied (SNP) which was positioned to lie at the 3'-end of the reverse primer. Synthetic templates were employed having each of the 4 possible bases at this position. Reverse primers were employed having each of the 4 possible RNA bases at the 3'-end and results were compared to control reactions using primers having each of the 4 possible DNA bases at the 3'-end. Relative amplification efficiency was assessed using qPCR.

**[0176]** Quantitative real-time PCR (qPCR) was performed in 10 μL reaction volumes in 384 well format with 2 x $10^3$ copies of a 103 bp synthetic template (SEQ ID NOs. 51-54). Final reaction conditions used were 20 mM Tris-HCL (pH 8.4 at 25°C), 50 mM KCL, and 3 mM $MgCl_2$, 0.01% Triton X-100, 800 μM total dNTPs, and 200 nM of the universal forward primer (SEQ ID NO. 60), 200 nM of a reverse primer (separate reactions were set up for each of the

four 3'-RNA primers SEQ ID NOs. 62-65, each of the four 3'DNA primers SEQ ID NOs. 55-58, or the control universal primer SEQ ID NO. 59) and 200 nM of the 5' nuclease detection probe (SEQ ID NO. 61). Each primer was tested only on the complementary template (mismatch conditions were not tested). Reactions utilized either 0.5 U (10.8 ng/11.1 nM/111 fmol) of the wild type OptiTaq DNA polymerase or 0.5 U of one of the 4 Taq DNA polymerase mutants studied (MUT ID No. 2 V783F, MUT ID NO. 3 H784Q, MUT ID NO. 10 A661E I665W F667L, or MUT ID NO. 18 V783L H784Q). Amplification was performed on a CFX384™ C1000™ Thermo Cycler system (Bio-Rad, Hercules, CA) using the following cycling parameters: 95°C for 30 seconds initial denaturation followed by 60 cycles of 95°C for 10 seconds, then 60°C for 30 seconds. Oligonucleotide reagents used in this example are shown in Table 12. A total of 96 replicates were performed for each pairwise combination.

**Table 12. Synthetic oligonucleotides employed in Example 6.**

| Name | Sequence (5'-3') | SEQ ID NO. |
|---|---|---|
| A Template | AGCTCTGCCCAAAGATTACCCTGACAGCTAAGTGGCAGTGGAA GTTGGCCTCAGAAGTAGTGGCCAGCTGTGTGTCGGGGAACAGT AAAGGCATGAAGCTCAG | SEQ ID NO. 51 |
| C Template | AGCTCTGCCCAAAGATTACCCTGACAGCTAAGTGGCAGTGGAA GTTGGCCTCAGAAGTAGTGGCCAGCTGTGTGTCGGGGCACAGT AAAGGCATGAAGCTCAG | SEQ ID NO. 52 |
| G Template | AGCTCTGCCCAAAGATTACCCTGACAGCTAAGTGGCAGTGGAA GTTGGCCTCAGAAGTAGTGGCCAGCTGTGTGTCGGGGGACAGT AAAGGCATGAAGCTCAG | SEQ ID NO. 53 |
| T Template | AGCTCTGCCCAAAGATTACCCTGACAGCTAAGTGGCAGTGGAA GTTGGCCTCAGAAGTAGTGGCCAGCTGTGTGTCGGGGTACAGT AAAGGCATGAAGCTCAG | SEQ ID NO. 54 |
| Syn Rev T | CTGAGCTTCATGCCTTTACTGTT | SEQ ID NO. 55 |
| Syn Rev C | CTGAGCTTCATGCCTTTACTGTC | SEQ ID NO. 56 |
| Syn Rev A | CTGAGCTTCATGCCTTTACTGTA | SEQ ID NO. 57 |
| Syn Rev G | CTGAGCTTCATGCCTTTACTGTG | SEQ ID NO. 58 |
| Syn Rev rU | CTGAGCTTCATGCCTTTACTGTu | SEQ ID NO. 62 |
| Syn Rev rC | CTGAGCTTCATGCCTTTACTGTc | SEQ ID NO. 63 |
| Syn Rev rA | CTGAGCTTCATGCCTTTACTGTa | SEQ ID NO. 64 |

| Name | Sequence (5'-3') | SEQ ID NO. |
|---|---|---|
| Syn Rev rG | CTGAGCTTCATGCCTTTACTGTg | SEQ ID NO. 65 |
| Syn Rev | CTGAGCTTCATGCCTTTACTGT | SEQ ID NO. 59 |
| Syn For | AGCTCTGCCCAAAGATTACCCTG | SEQ ID NO. 60 |
| Syn Probe | FAM-TTCTGAGGC(ZEN)CAACTTCCACTGCCACTTA-IBFQ | SEQ ID NO. 61 |

DNA bases are uppercase and RNA bases are lowercase; FAM = 6-carboxyfluorescein; IBFQ = Iowa Black™ FQ fluorescence quencher; ZEN = internal ZEN fluorescence quencher; underlined base indicates the SNP site in the synthetic template DNA.

**[0177]** Average Cq values were calculated for the 96-replicate sets. ΔCq values were calculated as the difference between the average Cq values for the 3'-RNA primer reactions from the average Cq values for the 3'-DNA primer reactions (ΔCq = Cq 3'-RNA – Cq 3'-DNA). Higher ΔCq values indicate a greater degree of discrimination against priming from a 3'-RNA primer. Results are shown in Table 13 and are graphically summarized in Figure 4.

**Table 13: ΔCq values for qPCR reactions using WT OptiTaq and mutant Taq DNA polymerases comparing 3'-DNA vs. 3'-RNA primers.**

| DNA Polymerase | Reverse Primers compared | | | |
|---|---|---|---|---|
| | Name | SEQ ID NO. | Template | ΔCq |
| OptiTaq | Syn Rev T<br>Syn Rev rU | 55<br>62 | A<br>SEQ ID NO. 51 | 0.1 |
| | Syn Rev G<br>Syn Rev rG | 58<br>65 | C<br>SEQ ID NO. 52 | 0.2 |
| | Syn Rev C<br>Syn Rev rC | 56<br>63 | G<br>SEQ ID NO. 53 | 0.0 |
| | Syn Rev A<br>Syn Rev rA | 57<br>64 | T<br>SEQ ID NO. 54 | 0.1 |
| MUT ID 2<br>V783F | Syn Rev T<br>Syn Rev rU | 55<br>62 | A<br>SEQ ID NO. 51 | 5.4 |

| DNA Polymerase | Reverse Primers compared | | | |
|---|---|---|---|---|
| | **Name** | **SEQ ID NO.** | **Template** | **ΔCq** |
| | Syn Rev G<br>Syn Rev rG | 58<br>65 | C<br>SEQ ID NO. 52 | 1.5 |
| | Syn Rev C<br>Syn Rev rC | 56<br>63 | G<br>SEQ ID NO. 53 | 4.8 |
| | Syn Rev A<br>Syn Rev rA | 57<br>64 | T<br>SEQ ID NO. 54 | 2.2 |
| MUT ID 3<br>H784Q | Syn Rev T<br>Syn Rev rU | 55<br>62 | A<br>SEQ ID NO. 51 | 6.9 |
| | Syn Rev G<br>Syn Rev rG | 58<br>65 | C<br>SEQ ID NO. 52 | 2.0 |
| | Syn Rev C<br>Syn Rev rC | 56<br>63 | G<br>SEQ ID NO. 53 | 9.8 |
| | Syn Rev A<br>Syn Rev rA | 57<br>64 | T<br>SEQ ID NO. 54 | 1.4 |
| MUT ID 10<br>A661E<br>I665W<br>F667L | Syn Rev T<br>Syn Rev rU | 55<br>62 | A<br>SEQ ID NO. 51 | 5.5 |
| | Syn Rev G<br>Syn Rev rG | 58<br>65 | C<br>SEQ ID NO. 52 | 1.3 |
| | Syn Rev C<br>Syn Rev rC | 56<br>63 | G<br>SEQ ID NO. 53 | 4.2 |
| | Syn Rev A<br>Syn Rev rA | 57<br>64 | T<br>SEQ ID NO. 54 | 0.8 |
| MUT ID 18<br>V783L<br>H784Q | Syn Rev T<br>Syn Rev rU | 55<br>62 | A<br>SEQ ID NO. 51 | 9.3 |
| | Syn Rev G<br>Syn Rev rG | 58<br>65 | C<br>SEQ ID NO. 52 | 2.4 |
| | Syn Rev C<br>Syn Rev rC | 56<br>63 | G<br>SEQ ID NO. 53 | 9.5 |
| | Syn Rev A<br>Syn Rev rA | 57<br>64 | T<br>SEQ ID NO. 54 | 2.3 |

Average ΔCq values are shown, where ΔCq = Cq 3'-RNA primer – Cq 3'-DNA primer.

**[0178]** Wild type OptiTaq did not show any significant discrimination between a 3'-DNA and a 3'-RNA primer. All four mutant Taq DNA polymerases, however, showed reduced priming efficiency when using a 3'-RNA primer. Thus the goal of creating novel polymerases which discriminate against a 3'-RNA residue in a primer was achieved using the intelligent mutagenesis design strategy described herein. Interestingly, the magnitude of discrimination was much greater for RNA pyrimidine residues (rC or rU) than for RNA purine residues (rA or rG).

**Example 7: Improved mismatch discrimination in rhPCR using mutant Taq DNA polymerases.**

**[0179]** RNase H-based PCR (rhPCR) employs the enzyme RNase H2 to convert a blocked-cleavable oligonucleotide which cannot prime DNA synthesis into a form that can prime DNA synthesis and initiate PCR. The blocked-cleavable oligonucleotide, or blocked-cleavable primer, contains a single RNA residue near the 3'-end of the oligonucleotide (which comprises the cleavage site) and is modified at or near the 3'-end so that the primer cannot prime DNA synthesis and/or has lost template function and so is incompetent to support PCR even if primer extension can occur. This method can be used for genotyping (SNP discrimination) and relies on the ability of RNase H2 to distinguish between base-pair match vs. mismatch at the RNA base cleavage site when hybridized to the target nucleic acid. In rhPCR, SNP discrimination occurs at the primer unblocking step, not at the primer extension step (in AS-PCR, discrimination occurs at the primer extension step). Examples of this enzyme cleaving strategy, similar RNase H strategies, and methods of blocking primer extension or inhibiting template function and thereby disabling PCR are described in U.S. Pat. No. 7,112,406 to Behlke et al., entitled POLYNOMIAL AMPLIFICATION OF NUCLEIC ACIDS, U.S. Pat. No. 5,763,181 to Han *et al.*, entitled CONTINOUS FLUOROMETRIC ASSAY FOR DETECTING NUCLEIC ACID CLEAVAGE, U.S. Pat. No. 7,135,291 to Sagawa *et al.*, entitled METHOD OF DETECTING NUCLEOTIDE POLYMORPHISM; U.S. Pat. App. No. 20090068643 to Behlke and Walder, entitled DUAL FUNCTION PRIMERS FOR AMPLIFYING DNA AND METHODS OF USE; and U.S. Pat. App. No. 20100167353 to Walder *et al.*, entitled RNASE H-BASED ASSAYS UTILIZING MODIFIED RNA MONOMERS and in Dobosy et al., RNase H-dependent PCR (rhPCR): improved specificity and single nucleotide polymorphism detection using blocked cleavable primers, BMC Biotechnology., 11:e80 (2011).

**[0180]** In AS-PCR the SNP is positioned at the 3'-end of the primer. In this configuration, a mispriming event (where DNA synthesis is initiated in the presence of a 3'-terminal mismatch) leads to incorporation of the base present in the primer into the nascent DNA strand and thereby into the PCR amplicon. This event converts the PCR product to the primer sequence so that the amplified DNA now matches primer and no longer matches the original input sample nucleic acid sequence. Since the amplicon sequence now matches the primer and not the input sample, amplification proceeds at high efficiency.

**[0181]** In rhPCR, cleavage of the blocked-cleavable primer by RNase H2 occurs at the 5'-side of the RNA residue; if the SNP is positioned at the RNA residue (e.g., the RNA base pairs with the SNP), then the first base incorporated by DNA polymerase during primer extension and PCR is the SNP site and results in daughter products which remain identical to the input nucleic acid sequence. Rarely, non-canonical RNase H2 cleavage occurs at the 3'-side of the RNA base, which leaves the RNA residue at the 3'-end of the primer positioned overlying the SNP. In this case, the rhPCR reaction proceeds like AS-PCR, where the 3'-end of the primer is positioned at the SNP site and is either a match or mismatch to the target nucleic acid. Like AS-PCR, in the case of a mismatch, the sequence of the DNA extension product and PCR amplicon converts to the sequence of the primer and thus might not faithfully replicate the sequence of the sample during amplification. Any method which reduces the frequency of this undesired mispriming event will improve mismatch discrimination in the rhPCR assay. Therefore, although base discrimination in rhPCR is primarily mediated by RNase H2 at the primer cleavage stage, use of a DNA polymerase that has an improved ability to discriminate against a 3'-terminal mismatch and/or a 3'-terminal RNA residue may improve the overall mismatch discrimination capacity of rhPCR by preventing extension when undesired 3'-cleavage events occur. The DNA polymerase mutants described herein both reduce priming efficiency when a 3'-mismatch is present (improve mismatch discrimination) and reduce priming efficiency when a 3'-terminal RNA residue is present in the primer (discriminate against a primer 3'-RNA residue) compared with wild type Taq DNA polymerase. The present example demonstrates that the novel mutant Taq DNA polymerases of the present invention improve specificity and SNP discrimination of rhPCR.

**[0182]** Quantitative real-time rhPCR was performed comparing performance of wild type OptiTaq DNA polymerase with mutant Taq DNA polymerases Mutant IDs 2, 3, 10, and 18. Two different blocked-cleavable primer designs were tested, including the generation 1 (Gen1)

"RDDDDx" primers and the generation 2 (Gen2) "RDxxD" primers (see: US Patent Application 2012/0258455 by Behlke et al., entitled, RNASE H-BASED ASSAYS UTILIZING MODIFIED RNA MONOMERS). Amplification reactions were performed using the same synthetic oligonucleotide template employed in Example 5 where a single base was varied (the SNP site) which was positioned to lie at the RNA residue in both Gen1 and Gen2 blocked-cleavable (rhPCR) primers. Synthetic templates were employed having each of the 4 possible bases at this position. Reverse primers were employed having each of the 4 possible complementary bases at this position (the RNA base). The same forward primer was used for all reactions. Relative amplification efficiency was assessed using real-time PCR.

**[0183]** Quantitative rhPCR was performed in 10 µL reaction volumes in 384 well format with 2 x $10^6$ copies of a 103 bp synthetic template (SEQ ID NOs. 51-4). Final reaction conditions used were 20 mM Tris-HCL (pH 8.4 at 25°C), 50 mM KCL, 3 mM $MgCl_2$, 0.01% Triton X-100, 800 µM total dNTPs, 200 nM of the universal forward primer (SEQ ID NO. 60), 200 nM of a reverse primer, and 200 nM of the 5' nuclease detection probe (SEQ ID NO. 61). Reverse primers included Gen1 RDDDDx configuration allele-specific rhPCR primers (SEQ ID NOs. 66-69), Gen2 RDxxD configuration allele-specific rhPCR primers (SEQ ID NOs. 70-73) and a control universal reverse primer (SEQ ID NO.59). Each of the rhPCR blocked-cleavable reverse primers were tested on each of the four SNP templates. Reactions utilized either 0.5 U (10.8 ng/11.1 nM/111 fmol) of the wild type OptiTaq DNA polymerase or 0.5 U of one of the four Taq DNA polymerase mutants (MUT ID 2, V783F; MUT ID 3, H784Q; MUT ID 10, A661E I665W F667L; or MUT ID 18, V783L H784Q). *P.abyssi* RNase H2 was added to each reaction in 1 µL volume. Reactions using the control and Gen1 blocked-cleavable RDDDDx rhPCR primers employed 2.6 mU RNase H2 per 10 µL reaction (5 fmoles, 0.5 nM enzyme). Reactions using the Gen2 blocked-cleavable RDxxD rhPCR primers employed 25 mU RNase H2 10 µL reaction (48 fmoles, 4.8 nM enzyme) for the rC and rA primers (SEQ ID NOs. 71 and 72) and 200 mU RNase H2 per 10 µL reaction (384 fmoles, 38 nM enzyme) for the rG and rU primers (SEQ ID NOs. 70 and 73). Cycling was performed on a Roche LightCycler® 480 (Roche Applied Science, Indianapolis, IN, USA) as follows: 95°C for 3 minutes followed by 75 cycles of 95°C for 10 seconds and 60°C for 30 seconds. All reactions were performed in triplicate. Oligonucleotide reagents used in this example are shown in Table 14.

**Table 14. Synthetic oligonucleotides employed in Example 7.**

| Name | Sequence (5'-3') | SEQ ID NO. |
|---|---|---|
| A Template | AGCTCTGCCCAAAGATTACCCTGACAGCTAAGTGGCAGTGGAA GTTGGCCTCAGAAGTAGTGGCCAGCTGTGTGTCGGGGAACAGT AAAGGCATGAAGCTCAG | SEQ ID NO. 51 |
| C Template | AGCTCTGCCCAAAGATTACCCTGACAGCTAAGTGGCAGTGGAA GTTGGCCTCAGAAGTAGTGGCCAGCTGTGTGTCGGGGCACAGT AAAGGCATGAAGCTCAG | SEQ ID NO. 52 |
| G Template | AGCTCTGCCCAAAGATTACCCTGACAGCTAAGTGGCAGTGGAA GTTGGCCTCAGAAGTAGTGGCCAGCTGTGTGTCGGGGGACAGT AAAGGCATGAAGCTCAG | SEQ ID NO. 53 |
| T Template | AGCTCTGCCCAAAGATTACCCTGACAGCTAAGTGGCAGTGGAA GTTGGCCTCAGAAGTAGTGGCCAGCTGTGTGTCGGGGTACAGT AAAGGCATGAAGCTCAG | SEQ ID NO. 54 |
| Syn Rev | CTGAGCTTCATGCCTTTACTGT | SEQ ID NO. 59 |
| Syn For | AGCTCTGCCCAAAGATTACCCTG | SEQ ID NO. 60 |
| Syn Probe | FAM-TTCTGAGGC(ZEN)CAACTTCCACTGCCACTTA-IBFQ | SEQ ID NO. 61 |
| Syn Rev rU DDDDx | CTGAGCTTCATGCCTTTACTGTuCCCCx | SEQ ID NO. 66 |
| Syn Rev rC DDDDx | CTGAGCTTCATGCCTTTACTGTcCCCCx | SEQ ID NO. 67 |
| Syn Rev rA DDDDx | CTGAGCTTCATGCCTTTACTGTaCCCCx | SEQ ID NO. 68 |
| Syn Rev rG DDDDx | CTGAGCTTCATGCCTTTACTGTgCCCCx | SEQ ID NO. 69 |
| Syn Rev rU DxxD | CTGAGCTTCATGCCTTTACTGTuCxxC | SEQ ID NO. 70 |
| Syn Rev rC DxxD | CTGAGCTTCATGCCTTTACTGTcCxxC | SEQ ID NO. 71 |
| Syn Rev rA DxxD | CTGAGCTTCATGCCTTTACTGTaCxxC | SEQ ID NO. 72 |
| Syn Rev rG DxxD | CTGAGCTTCATGCCTTTACTGTgCxxC | SEQ ID NO. 73 |

DNA bases are uppercase and RNA bases are lowercase; FAM = 6-carboxyfluorescein; IBFQ = Iowa Black™ FQ fluorescence quencher; ZEN = internal ZEN fluorescence quencher; underlined base indicates the SNP site in the synthetic template DNA; "x" = C3 Spacer (propanediol).

**[0184]** MUT ID 10 (A661E, I665W, F667L) unexpectedly showed large amplification delays when the primers matched the SNP site in the target in the rhPCR reactions using this synthetic amplicon system. This polymerase, however, did not show any delays when using a human genomic DNA system for rhPCR (see Examples 8 and 9). MUT ID 10 was therefore excluded from analysis in the synthetic system experiments. Data generated using the other three mutant polymerases were analyzed and ΔCq values were calculated comparing matched versus mismatched primer/template pairs, where ΔCq = Cq mismatch – Cq match. Results are shown in Table 15 for the Gen1 RDDDDx blocked-cleavable rhPCR primers and in Table 16 for the Gen2 RDxxD blocked-cleavable rhPCR primers.

**Table 15: ΔCq values for rhPCR reactions using WT OptiTaq and mutant Taq DNA polymerases with Gen1 RDDDDx blocked-cleavable rhPCR primers.**

| DNA Polymerase | Reverse Primer | | Template | | | |
|---|---|---|---|---|---|---|
| | Name | SEQ ID NO. | A SEQ ID NO. 51 | G SEQ ID NO. 53 | T SEQ ID NO. 54 | C SEQ ID NO. 52 |
| OptiTaq | Syn Rev rU DDDDx | 66 | - | 10.5 | 3.4 | 6.6 |
| | Syn Rev rC DDDDx | 67 | 3.3 | - | 1.3 | 2.2 |
| | Syn Rev rA DDDDx | 68 | 9.5 | 10.5 | - | 3.5 |
| | Syn Rev rG DDDDx | 69 | 9.5 | 10.8 | 11.8 | - |
| MUT ID 2 V783F | Syn Rev rU DDDDx | 66 | - | 11.1 | 5.1 | 9.0 |
| | Syn Rev rC DDDDx | 67 | 4.0 | - | 1.9 | 3.6 |
| | Syn Rev rA DDDDx | 68 | 10.4 | 11.1 | - | 5.6 |
| | Syn Rev rG DDDDx | 69 | 10.2 | 10.5 | 10.7 | - |

| DNA Polymerase | Reverse Primer | | Template | | | |
|---|---|---|---|---|---|---|
| | Name | SEQ ID NO. | A SEQ ID NO. 51 | G SEQ ID NO. 53 | T SEQ ID NO. 54 | C SEQ ID NO. 52 |
| MUT ID 3 H784Q | Syn Rev rU DDDDx | 66 | - | 11.3 | 5.0 | 10.0 |
| | Syn Rev rC DDDDx | 67 | 7.6 | - | 4.3 | 5.9 |
| | Syn Rev rA DDDDx | 68 | 10.8 | 11.3 | - | 7.6 |
| | Syn Rev rG DDDDx | 69 | 10.9 | 10.9 | 11.0 | - |
| MUT ID 18 V783L H784Q | Syn Rev rU DDDDx | 66 | - | 12.3 | 6.7 | 11.5 |
| | Syn Rev rC DDDDx | 67 | 9.9 | - | 8.3 | 10.4 |
| | Syn Rev rA DDDDx | 68 | 11.5 | 13.2 | - | 6.8 |
| | Syn Rev rG DDDDx | 69 | 11.3 | 12.0 | 12.6 | - |

Average ΔCq values are shown, where ΔCq = [Cq mismatch – Cq match].

**Table 16: ΔCq values for rhPCR reactions using WT OptiTaq and mutant Taq DNA polymerases with Gen2 RDxxD blocked-cleavable rhPCR primers.**

| DNA Polymerase | Reverse Primer | | Template | | | |
|---|---|---|---|---|---|---|
| | Name | SEQ ID NO. | A SEQ ID NO. 51 | G SEQ ID NO. 53 | T SEQ ID NO. 54 | C SEQ ID NO. 52 |
| OptiTaq | Syn Rev rU DxxD | 70 | - | 11.6 | 15.1 | 12.8 |
| | Syn Rev rC DxxD | 71 | 6.3 | - | 6.7 | 4.6 |
| | Syn Rev rA DxxD | 72 | 13.7 | 15.6 | - | 14.3 |

| DNA Polymerase | Reverse Primer | | Template | | | |
|---|---|---|---|---|---|---|
| | Name | SEQ ID NO. | A SEQ ID NO. 51 | G SEQ ID NO. 53 | T SEQ ID NO. 54 | C SEQ ID NO. 52 |
| | Syn Rev rG DxxD | 73 | 13.2 | 11.4 | 10.2 | - |
| MUT ID 2 V783F | Syn Rev rU DxxD | 70 | - | 12.2 | 15.0 | 14.0 |
| | Syn Rev rC DxxD | 71 | 8.3 | - | 6.5 | 4.4 |
| | Syn Rev rA DxxD | 72 | 14.1 | 15.9 | - | 14.2 |
| | Syn Rev rG DxxD | 73 | 13.8 | 12.2 | 11.6 | - |
| MUT ID 3 H784Q | Syn Rev rU DxxD | 70 | - | 12.4 | 15.0 | 14.1 |
| | Syn Rev rC DxxD | 71 | 9.5 | - | 7.8 | 6.4 |
| | Syn Rev rA DxxD | 72 | 16.9 | 19.1 | - | 18.4 |
| | Syn Rev rG DxxD | 73 | 15.0 | 13.0 | 12.7 | - |
| MUT ID 18 V783L H784Q | Syn Rev rU DxxD | 70 | - | 13.0 | 15.3 | 14.3 |
| | Syn Rev rC DxxD | 71 | 6.9 | - | 9.6 | 3.6 |
| | Syn Rev rA DxxD | 72 | 15.8 | 15.3 | - | 14.5 |
| | Syn Rev rG DxxD | 73 | 15.0 | 13.4 | 13.7 | - |

Average ΔCq values are shown, where ΔCq = [Cq mismatch – Cq match].

**[0185]** In almost all cases, mismatch discrimination was superior for rhPCR reactions run using the mutant Taq DNA polymerases than wild type OptiTaq. The magnitude of improvement

is best seen by examining the ΔΔCq values, which is the difference of discrimination seen using wild type OptiTaq and the mutants (ΔΔCq = ΔCq mutant – ΔCq wild type). These results are shown in Table 17 for the Gen1 RDDDDx primers and in Table 18 for the Gen2 RDxxD primers. When using the Gen1 RDDDDx primers, the overall greatest benefit was seen when the mismatched base was a "C" in the target nucleic acid and least benefit was seen when the blocked-cleavable primer contained a rG paired with a mismatched T in the target. The greatest improvements were obtained using the mutant Taq DNA polymerase MUT ID 18 (V783L H784Q). The average ΔΔCq for MUT ID 2 (V783F) was 1.0. The average ΔΔCq for MUT ID 3 (H784Q) was 2.0. The average ΔΔCq for MUT ID 18 (V783L, H784Q) was 3.6. Benefits obtained using mutant Taq DNA polymerases was lower for the Gen2 RDxxD primers, which already showed high ΔCq values using wild type OptiTaq. Average ΔΔCq for the three mutant polymerases studied in the Example were 0.6, 2.1, and 1.2. Therefore greatest benefit when using the Gen2 RDxxD primers was seen with MUT ID 3 (H784Q).

**Table 17: ΔΔCq values for rhPCR reactions using mutant Taq DNA polymerases compared with wild type OptiTaq for Gen1 RDDDDx blocked-cleavable rhPCR primers.**

| DNA Polymerase | Reverse Primer | | Template | | | |
|---|---|---|---|---|---|---|
| | Name | SEQ ID NO. | A SEQ ID NO. 51 | G SEQ ID NO. 53 | T SEQ ID NO. 54 | C SEQ ID NO. 52 |
| MUT ID 2 V783F | Syn Rev rU DDDDx | 66 | - | 0.6 | 1.7 | 2.4 |
| | Syn Rev rC DDDDx | 67 | 0.7 | - | 0.6 | 1.4 |
| | Syn Rev rA DDDDx | 68 | 0.9 | 0.6 | - | 2.1 |
| | Syn Rev rG DDDDx | 69 | 0.7 | -0.3 | 1.1 | - |
| MUT ID 3 H784Q | Syn Rev rU DDDDx | 66 | - | 0.8 | 1.6 | 3.4 |
| | Syn Rev rC DDDDx | 67 | 4.3 | - | 3.0 | 3.7 |

| DNA Polymerase | Reverse Primer | | Template | | | |
|---|---|---|---|---|---|---|
| | Name | SEQ ID NO. | A SEQ ID NO. 51 | G SEQ ID NO. 53 | T SEQ ID NO. 54 | C SEQ ID NO. 52 |
| | Syn Rev rA DDDDx | 68 | 1.3 | 0.8 | - | 4.1 |
| | Syn Rev rG DDDDx | 69 | 1.4 | 0.1 | -0.8 | - |
| MUT ID 18 V783L H784Q | Syn Rev rU DDDDx | 66 | - | 1.8 | 3.3 | 4.9 |
| | Syn Rev rC DDDDx | 67 | 6.6 | - | 7.0 | 8.2 |
| | Syn Rev rA DDDDx | 68 | 2.0 | 2.7 | - | 3.3 |
| | Syn Rev rG DDDDx | 69 | 1.8 | 1.2 | 0.8 | - |

ΔΔCq values are shown, where ΔΔCq = [ΔCq mutant – ΔCq wild type polymerase].

**Table 18: ΔΔCq values for rhPCR reactions using mutant Taq DNA polymerases compared with wild type OptiTaq for Gen2 RDxxD blocked-cleavable rhPCR primers.**

| DNA Polymerase | Reverse Primer | | Template | | | |
|---|---|---|---|---|---|---|
| | Name | SEQ ID NO. | A SEQ ID NO. 51 | G SEQ ID NO. 53 | T SEQ ID NO. 54 | C SEQ ID NO. 52 |
| MUT ID 2 V783F | Syn Rev rU DxxD | 70 | - | 0.6 | -0.1 | 1.2 |
| | Syn Rev rC DxxD | 71 | 2.0 | - | -0.2 | -0.2 |
| | Syn Rev rA DxxD | 72 | 0.4 | 0.3 | - | -0.1 |
| | Syn Rev rG DxxD | 73 | 0.6 | 0.8 | 1.4 | - |
| MUT ID 3 H784Q | Syn Rev rU DxxD | 70 | - | 0.8 | -0.1 | 1.3 |

| DNA Polymerase | Reverse Primer | | Template | | | |
|---|---|---|---|---|---|---|
| | Name | SEQ ID NO. | A SEQ ID NO. 51 | G SEQ ID NO. 53 | T SEQ ID NO. 54 | C SEQ ID NO. 52 |
| | Syn Rev rC DxxD | 71 | 3.2 | - | 1.1 | 1.8 |
| | Syn Rev rA DxxD | 72 | 3.2 | 3.5 | - | 4.1 |
| | Syn Rev rG DxxD | 73 | 2.8 | 1.6 | 2.5 | - |
| MUT ID 18 V783L H784Q | Syn Rev rU DxxD | 70 | - | 1.4 | 0.2 | 1.5 |
| | Syn Rev rC DxxD | 71 | 0.6 | - | 2.9 | -1.0 |
| | Syn Rev rA DxxD | 72 | 2.1 | -0.3 | - | 0.2 |
| | Syn Rev rG DxxD | 73 | 1.8 | 2.0 | 3.5 | - |

ΔΔCq values are shown, where ΔΔCq = [ΔCq mutant – ΔCq wild type polymerase].

**Example 8: Improved mismatch discrimination in rhPCR using mutant Taq DNA polymerases in a human genomic DNA SNP assay.**

**[0186]** Example 7 demonstrated utility of the novel mutant Taq DNA polymerases of the present invention in a synthetic amplicon rhPCR SNP discrimination assay system. The present Example demonstrates utility of the novel mutant Taq DNA polymerases in a human genomic DNA rhPCR SNP discrimination assays system, examining a SNP site in the SMAD7 gene (NM_005904, C/T SNP, rs4939827). The assays employed target DNAs GM18562 (homozygous C/C) and GM18537 (homozygous T/T) from the Coriell Institute for Medical Research (Camden, NJ, USA). Two different blocked-cleavable primer designs were tested, including the generation 1 (Gen1) "RDDDDx" primers and the generation 2 (Gen2) "RDxxD" primers (see: US Patent Application 2012/0258455 by Behlke et al., entitled, RNASE H-BASED ASSAYS UTILIZING MODIFIED RNA MONOMERS).

**[0187]** Quantitative real-time rhPCR was performed in 10 μL reaction volumes in 384 well format with 20 ng (the equivalent of 6600 copies of target) of human genomic DNA (GM18562 or GM18537). Reactions utilized either 0.5 U (10.8 ng/11.1 nM/111 fmol) of wild type OptiTaq DNA polymerase or 0.5 U of one of the four Taq DNA polymerase mutants (MUT ID 2, V783F; MUT ID 3, H784Q; MUT ID 10, A661E I665W F667L; or MUT ID 18, V783L H784Q). Final reaction conditions used were 20 mM Tris-HCL (pH 8.4 at 25°C), 50 mM KCL, 3 mM $MgCl_2$, 0.01% Triton X-100, 800 μM total dNTPs, 200 nM of a forward primer (SEQ ID NOs. 75-79), 200 nM of the universal reverse primer (SEQ ID NO. 74), and 200 nM of the SMAD7 probe (SEQ ID NO. 80). Sequence of the 85 bp SMAD7 amplicon is shown as SEQ ID NO. 81. Forward primers included RDDDDx configuration Gen1 allele-specific rhPCR primers (SEQ ID NOs. 76 and 77), RDxxD configuration Gen2 allele-specific rhPCR primers (SEQ ID NOs. 78 and 79) and the control universal forward primer (SEQ ID NO.75) which is not allele specific. Oligonucleotide reagents employed in this Example are shown in Table 19. Reactions included 1 μL of *P.a.* RNase H2 at a concentration of 2.6 mU per 10 μL reaction (5 fmoles, 0.5 nM) for the Gen1 RDDDDx primers and control primer (SEQ ID NOs. 75-77) or 200 mU per 10 μL reaction (384 fmoles, 38.4 nM) for the Gen2 RDxxD primers (SEQ ID NOs. 78 and 79). Amplification was performed on a Roche LightCycler® 480 (Roche Applied Science, Indianapolis, IN, USA) as follows: 95°C for 3 minutes followed by 75 cycles of 95°C for 10 seconds and 60°C for 30 seconds. All reactions were performed in triplicate.

**Table 19. Synthetic oligonucleotides employed in Example 8.**

| Name | Sequence (5'-3') | SEQ ID NO. |
|---|---|---|
| SMAD7 Rev | CTCACTCTAAACCCCAGCATT | 74 |
| SMAD7 For | CAGCCTCATCCAAAAGAGGAAA | 75 |
| SMAD7 For rC DDDDx | CAGCCTCATCCAAAAGAGGAAAcAGGAx | 76 |
| SMAD7 For rU DDDDx | CAGCCTCATCCAAAAGAGGAAAuAGGAx | 77 |

| Name | Sequence (5'-3') | SEQ ID NO. |
|---|---|---|
| SMAD7 For rC DxxD | CAGCCTCATCCAAAAGAGGAAAcAxxA | 78 |
| SMAD7 For rU DxxD | CAGCCTCATCCAAAAGAGGAAAuAxxA | 79 |
| SMAD7 probe | FAM-CCCAGAGCTCCCTCAGACTCCT-IBFQ | 80 |
| SMAD7 target | CAGCCTCATCCAAAAGAGGAAATAGGACCCCAGAGCTCCCTCA GACTCCTCAGGAAACACAGACAATGCTGGGGTTTAGAGTGAG | 81 |

DNA bases are uppercase and RNA bases are lowercase; FAM = 6-carboxyfluorescein; IBFQ = Iowa Black™ FQ fluorescence quencher; "x" = C3 Spacer (propanediol). Primer and probe binding sites in the SMAD7 target are underlined.

**[0188]** Results using the Gen1 RDDDDx rhPCR primers are shown in Table 20 and using the Gen2 RDxxD rhPCR primers are shown in Table 21. Overall, use of the mutant Taq DNA polymerases showed small but real improvements in SNP discrimination in this human genomic DNA rhPCR assay using the Gen1 RDDDDx primers. However, large improvements in discrimination were seen using the Gen2 RDxxD primers. The Gen2 RDxxD primers inherently show greater SNP discrimination and these levels were increased so that ΔCq values are in some cases were greater than 40 amplification cycles between match and mismatch; this level of discrimination would be "greater than assay" for most users, as qPCR reactions are seldom run for over 45-50 cycles and positive signal was not detected in these cases until after 70 cycles (Table 21). Therefore use of the new mutant Taq DNA polymerases improves SNP discrimination in rhPCR genotyping assays.

**Table 20: SNP discrimination of a site in the SMAD7 gene using Gen1 RDDDDx primers comparing wild type OptiTaq with four mutant Taq DNA polymerases.**

| DNA Polymerase | For Primer | SEQ ID NO. | mU RNase H2 per 10 µL rxn | Cq Value C/C DNA | Cq Value T/T DNA | ΔCq |
|---|---|---|---|---|---|---|
| Wild type OptiTaq | SMAD7 For | 75 | 2.6 | 24.3 | 25.3 | - |
| | SMAD7 For rC DDDDx | 76 | 2.6 | 26.1 | 38.1 | 11.9 |

| DNA Polymerase | For Primer | SEQ ID NO. | mU RNase H2 per 10 μL rxn | Cq Value C/C DNA | Cq Value T/T DNA | ΔCq |
|---|---|---|---|---|---|---|
| | SMAD7 For rU DDDDx | 77 | 2.6 | 36.6 | 26.8 | 9.8 |
| MUT ID 2 V783F | SMAD7 For | 75 | 2.6 | 24.7 | 25.5 | - |
| | SMAD7 For rC DDDDx | 76 | 2.6 | 26.2 | 40.3 | 14.1 |
| | SMAD7 For rU DDDDx | 77 | 2.6 | 37.8 | 27.6 | 10.1 |
| MUT ID 3 H784Q | SMAD7 For | 75 | 2.6 | 25.3 | 27.1 | - |
| | SMAD7 For rC DDDDx | 76 | 2.6 | 26.2 | 46.1 | 19.9 |
| | SMAD7 For rU DDDDx | 77 | 2.6 | 38.9 | 32.4 | 6.5 |
| MUT ID 10 A661E I665W F667L | SMAD7 For | 75 | 2.6 | 24.3 | 25.8 | - |
| | SMAD7 For rC DDDDx | 76 | 2.6 | 25.6 | 43.9 | 18.3 |
| | SMAD7 For rU DDDDx | 77 | 2.6 | 42.6 | 28.5 | 14.1 |
| MUT ID 18 V783L H784Q | SMAD7 For | 75 | 50 | 24.6 | 25.6 | - |
| | SMAD7 For rC DDDDx | 76 | 50 | 25.2 | 35.7 | 10.5 |
| | SMAD7 For rU DDDDx | 77 | 50 | 37.9 | 26.4 | 11.5 |

DNA targets included GM18562 (homozygous C/C) and GM18537 (homozygous T/T) from the Coriell Institute for Medical Research. ΔCq = [Cq mismatch – Cq match].

**Table 21: SNP discrimination of a site in the SMAD7 gene using Gen2 RDxxD primers comparing wild type OptiTaq with four mutant Taq DNA polymerases.**

| DNA Polymerase | For Primer | SEQ ID NO. | mU RNase H2 per 10 µL rxn | Cq Value C/C DNA | Cq Value T/T DNA | ΔCq |
|---|---|---|---|---|---|---|
| Wild type OptiTaq | SMAD7 For | 75 | 2.6 | 24.3 | 25.3 | - |
| | SMAD7 For rC DxxD | 78 | 200 | 25.9 | 40.4 | 14.5 |
| | SMAD7 For rU DxxD | 79 | 200 | 47.9 | 26.6 | 21.3 |
| MUT ID 2 V783F | SMAD7 For | 75 | 2.6 | 24.7 | 25.5 | - |
| | SMAD7 For rC DxxD | 78 | 200 | 26.6 | 64.4 | 37.7 |
| | SMAD7 For rU DxxD | 79 | 200 | 59.7 | 28.0 | 31.6 |
| MUT ID 3 H784Q | SMAD7 For | 75 | 2.6 | 25.3 | 27.1 | - |
| | SMAD7 For rC DxxD | 78 | 200 | 26.7 | 71.7 | 45.0 |
| | SMAD7 For rU DxxD | 79 | 200 | 62.5 | 28.9 | 33.7 |
| MUT ID 10 A661E I665W F667L | SMAD7 For | 75 | 2.6 | 24.3 | 25.8 | - |
| | SMAD7 For rC DxxD | 78 | 200 | 25.6 | 74.4 | 48.8 |
| | SMAD7 For rU DxxD | 79 | 200 | 54.3 | 28.2 | 26.0 |
| MUT ID 18 V783L H784Q | SMAD7 For | 75 | 50 | 24.6 | 25.6 | - |
| | SMAD7 For rC DxxD | 78 | 200 | 25.1 | 52.7 | 27.6 |
| | SMAD7 For rU DxxD | 79 | 200 | 43.0 | 27.6 | 15.3 |

DNA targets included GM18562 (homozygous C/C) and GM18537 (homozygous T/T) from the Coriell Institute for Medical Research. ΔCq = [Cq mismatch – Cq match].

**[0189]** The ΔCq values for the SMAD7 SNP genotyping assays are graphically summarized in Figure 5A for the Gen1 RDDDDx primers and in Figure 6A for the Gen2 RDxxD primers. It is interesting to note that, for the rhPCR genotyping assays studied in Example 8, MUT ID 10 (A661E I665W F667L) showed the greatest improvement compare with wild type OptiTaq, especially when using the Gen2 RDxxD primers. Example 5 demonstrated utility of the mutant Taq DNA polymerases in AS-PCR, and in this case use of MUT ID 18 (V783L H784Q) showed the greatest benefit and MUT ID 3 (H784Q) showed the next greatest relative benefit. It is clear that not only do the different mutant Taq DNA polymerases of the present invention have utility in different amplification assays but that the different mutants show varying levels of benefit depending on the nature of the assay used. It is therefore useful to have a collection of mutant polymerases whose properties can be matched to different assays/applications so that maximal benefit is obtained.

**Example 9: Improved discrimination of rare alleles in genomic DNA using rhPCR with mutant Taq DNA polymerases.**

**[0190]** Use of the Gen2 RDxxD blocked-cleavable primers in rhPCR can detect the presence of a SNP at a level of 1:1,000 to 1:10,000 in the background of wild type genomic DNA using native (wild type) Taq DNA polymerase (see: US Patent Application 2012/0258455 by Behlke et al., entitled, RNASE H-BASED ASSAYS UTILIZING MODIFIED RNA MONOMERS). The present example demonstrates that the mutant Taq DNA polymerases of the present invention improve rare allele discrimination in the rhPCR assay.

**[0191]** Rare allele detection experiments were designed to detect the base identity of a SNP site in the SMAD7 gene (NM_005904, C/T SNP, rs4939827) and employed target DNAs GM18562 (homozygous C/C) and GM18537 (homozygous T/T) (Coriell Institute for Medical Research, Camden, NJ, USA). Control reactions were set up using 2 ng (660 copies), 0.2 ng (66 copies), or 0.02 ng (6.6 copies) of input matched target DNA. Rare allele detection reactions were set up using 2 ng (660 copies), 0.2 ng (66 copies), or 0.02 ng (6.6 copies) of input matched target DNA of one allele plus 200 ng (66,000 copies) of the other (mismatched) allele. Background was established in reactions that contained 0 copies of matched target DNA plus

200 ng (66,000 copies) of the mismatched target DNA. Both combinations were tested: GM18562 (C/C) as the rare allele in the presence of excess GM18537 (T/T) and GM18537 (T/T) as the rare allele in the presence of excess GM18562 (C/C).

**[0192]** Quantitative real-time rhPCR was performed in 10 μL reaction volumes in 384 well format. Final reaction conditions used were 10 mM Tris-HCL (pH 8.4 at 25°C), 50 mM KCL, 3.5 mM $MgCl_2$, 0.01% Triton-X100, 0.8 mM dNTPs, 200 nM of one of the SMAD7 forward primers (SEQ ID NOs. 75, 78, and 79), 200 nM of the SMAD7 reverse primer (SEQ ID NO. 74), and 200 nM of the SMAD7 probe (SEQ ID NO. 80). The 85 bp SMAD7 amplicon defined by these primers is shown as SEQ ID NO. 81. Note that the forward primers were either unmodified (control, SEQ ID NO. 75) or were specific for the SMAD7 C-allele (SEQ ID NO. 78) or the SMAD7 T-allele (SEQ ID NO. 79) using blocked-cleavable rhPCR Gen2 RDxxD design. Reactions utilized either 0.5 U of the wild type OptiTaq DNA polymerase or 0.5 U of one of the four Taq DNA polymerase mutants studied (MUT ID No. 2, V783F; MUT ID NO. 3, H784Q; MUT ID NO. 10, A661E I665W F667L; or MUT ID NO. 18, V783L H784Q). Reactions included *P.abyssi* RNase H2 at a concentration of 200 mU per 10 μL reaction (384 fmoles) when using the SMAD7 For rC DxxD (SEQ ID NO. 78) primer and control reactions or 500-600 mU per 10 μL reaction (960-1152 fmoles) when using the SMAD7 For rU DxxD (SEQ ID NO. 79) primer. Oligonucleotide reagents used in this Example are shown in Table 22. Cycling was performed on a Roche LightCycler® 480 (Roche Applied Science, Indianapolis, IN, USA) as follows: 95° C for 3 minutes followed by 65 cycles of 95° C for 10 seconds and 60° C for 30 seconds. All reactions were performed in triplicate.

**Table 22. Synthetic oligonucleotides employed in Example 9.**

| **Name** | **Sequence (5'-3')** | **SEQ ID NO.** |
|---|---|---|
| SMAD7 Rev | CTCACTCTAAACCCCAGCATT | 74 |
| SMAD7 For | CAGCCTCATCCAAAAGAGGAAA | 75 |
| SMAD7 For rC DxxD | CAGCCTCATCCAAAAGAGGAAAcAxxA | 78 |
| SMAD7 For rU DxxD | CAGCCTCATCCAAAAGAGGAAAuAxxA | 79 |

| Name | Sequence (5'-3') | SEQ ID NO. |
|---|---|---|
| SMAD7 probe | FAM-CCCAGAGCTCCCTCAGACTCCT-IBFQ | 80 |
| SMAD7 target | CAGCCTCATCCAAAAGAGGAAATAGGACCCCAGAGCTCCCTCA GACTCCTCAGGAAACACAGACAATGCTGGGGTTTAGAGTGAG | 81 |

DNA bases are uppercase and RNA bases are lowercase; FAM = 6-carboxyfluorescein; IBFQ = Iowa Black™ FQ fluorescence quencher; "x" = C3 Spacer (propanediol). Primer and probe binding sites in the SMAD7 target are underlined.

**[0193]** Results were analyzed and are shown in Table 23. The control columns show Cq values for matched primer/target reactions with no mismatched target present and establish a quantification standard curve. The rare allele detection columns show Cq values for detection of 660, 66, 6, or 0 (background control) copies of matched primer/target in the presence of 66,000 copies of mismatched target. It is generally assumed that at least a 3 cycle difference ($\Delta$Cq = 3.0 or greater) between background and positive signal is needed to call a reaction "positive" for rare allele detection; a 5 cycle difference ($\Delta$Cq = 5.0 or greater) is preferred. In this system, background is the signal observed when amplification is done using no input target that is matched to the primer, so signal arises solely from amplification originating off the mismatched target.

**[0194]** Using wild type OptiTaq DNA polymerase, detection of the "C" allele in an excess of "T" background and detection of the "T" allele in an excess of "C" background both met the $\Delta$Cq 3.0 and $\Delta$Cq 5.0 levels of stringency to call a 1:1000 rare allele detection event (66 copies of match target in the presence of 66,000 copies of mismatch target). The 1:10,000 reactions (6 copies of match target in the presence of 66,000 copies of mismatch target) did not meet either of these criteria. Thus rhPCR had a 1:1000 rare allele detection limit using wild type OptiTaq in this genomic DNA SNP system.

**[0195]** In contrast, rhPCR using each of the four mutants showed a 1:10,000 rare allele detection limit for both the "C" and "T" allele targets with a $\Delta$Cq stringency cutoff of 3.0. MUT ID 3 (H784Q) showed a 1:10,000 rare allele detection limit for both the "C" and "T" targets in this genomic SNP system for the higher $\Delta$Cq stringency cutoff of 5.0. The other three mutant Taq DNA polymerases (MUT ID No. 2, V783F; MUT ID NO. 10, A661E I665W F667L; and MUT ID NO. 18, V783L H784Q) showed a 1:10,000 rare allele detection limit for the "C" allele

target with a ΔCq stringency cutoff of 5.0 and a 1:10,000 rare allele detection limit for the "T" allele target with a ΔCq stringency cutoff of 3.0. We therefore conclude that the new mutant Taq DNA polymerases of the present invention provide for improved rare allele detection reactions using blocked-cleavable primers in rhPCR compared with use of the wild type DNA polymerase.

**Table 23. Rare allele detection using Gen2 RDxxD rhPCR primers comparing wild type OptiTaq with new mutant Taq DNA polymerases**

| DNA Polymerase | For Primer | SEQ ID NO. | RNase H2 per 10 µL rxn | 200 ng mismatched template (66,000 copies of "wild type") | | | | Control (No mismatched template) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 660 Match (1:100) | 66 Match (1:1,000) | 6 Match (1:10,000) | 0 Match (background) | 660 Match | 66 Match | 6 Match | 0 Match |
| Wild type OptiTaq | SMAD7 For | 75 | 200 mU | 22.1 | 21.2 | 21.2 | 21.8 | 27.9 | 31.3 | 34.4 | >65 |
| | SMAD7 For rC DxxD | 78 | 200 mU | **28.2** | **31.5** | 35.1 | 37.0 | 28.8 | 33.3 | 37.3 | >65 |
| | SMAD7 For rU DxxD | 79 | 500 mU | **31.0** | **34.7** | 37.7 | 39.7 | 31.2 | 34.6 | 41.0 | >65 |
| MUT ID 2 (V783F) | SMAD7 For | 75 | 200 mU | 22.2 | 22.2 | 22.1 | 22.2 | 28.9 | 32.7 | 35.7 | >65 |
| | SMAD7 For rC DxxD | 78 | 200 mU | **28.2** | **31.7** | **35.4** | 45.4 | 29.0 | 33.3 | 37.5 | >65 |
| | SMAD7 For rU DxxD | 79 | 500 mU | **28.6** | **32.5** | **36.7** | 41.3 | 28.2 | 34.0 | 42.0 | >65 |
| MUT ID 3 (H784Q) | SMAD7 For | 75 | 200 mU | 23.5 | 23.6 | 24.5 | 24.1 | 30.5 | 33.4 | 38.0 | >65 |
| | SMAD7 For rC DxxD | 78 | 200 mU | **29.8** | **33.8** | **37.6** | >65 | 30.5 | 35.5 | 39.6 | >65 |
| | SMAD7 For rU DxxD | 79 | 500 mU | **32.9** | **37.7** | **44.0** | 52.3 | 30.1 | 35.9 | 44.9 | >65 |
| MUT ID 10 (A661E I665W F667L) | SMAD7 For | 75 | 200 mU | 22.2 | 22.4 | 22.5 | 22.8 | 28.3 | 31.9 | 35.5 | >65 |
| | SMAD7 For rC DxxD | 78 | 200 mU | **31.8** | **34.7** | **38.5** | 59.3 | 30.0 | 33.9 | 37.8 | >65 |
| | SMAD7 For rU DxxD | 79 | 600 mU | **33.5** | **38.4** | **43.2** | 46.2 | 31.9 | 36.5 | 41.0 | >65 |

| DNA Polymerase | For Primer | SEQ ID NO. | RNase H2 per 10 µL rxn | 200 ng mismatched template (66,000 copies of "wild type") | | | | Control (No mismatched template) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 660 Match (1:100) | 66 Match (1:1,000) | 6 Match (1:10,000) | 0 Match (background) | 660 Match | 66 Match | 6 Match | 0 Match |
| MUT ID 18 (V783L H784Q) | SMAD7 For | 75 | 200 mU | 22.4 | 22.4 | 22.7 | 22.5 | 27.8 | 31.5 | 34.8 | >65 |
| | SMAD7 For rC DxxD | 78 | 200 mU | **28.8** | **32.9** | **37.5** | 46.5 | 29.5 | 33.4 | 37.8 | >65 |
| | SMAD7 For rU DxxD | 79 | 500 mU | **30.1** | **34.0** | **38.4** | 41.8 | 29.4 | 36.0 | 44.7 | >65 |

Cq values are shown. For the rare allele detection series (selective detection of 6-660 copes one genotype in the presence of 66,000 copies of the other genotype), those reactions having a ΔCq of 3.0 or better are highlighted in bold font and those having a ΔCq of 5.0 or better are highlighted in bold font with underline. ΔCq = [(Cq 0 copies match) – (Cq 6 copies match)], or ΔCq = [(Cq 0 copies match) – (Cq 66 copies match)], or ΔCq = [(Cq 0 copies match) – (Cq 660 copies match)].

**Example 10. Sequence of Taq DNA polymerase mutants showing improved discrimination for mismatch or the presence of an RNA residue at the 3'-end of the primer.**

**[0196]** The complete amino acid and nucleotide sequences of the codon optimized mutant enzymes employed in Examples 5-9 are shown below. Although these sequences are easily derived from information provided in Tables 1, 3, 4 and 5 by one with skill in the art, the final assembled sequences are provided below for clarity. Base changes are identified in bold underlined font for the nucleic acid and amino acid substitutions.

**[0197]** SEQ ID NO. 82, nucleotide sequence of Mutant ID 2 (V783F).

CATATGCGTGGTATGCTGCCGTTGTTCGAGCCTAAAGGCCGCGTACTGTTAGTCGATGGTCATCACTTGGCCTATCG
GACGTTCCATGCACTCAAAGGTCTGACGACCAGTCGTGGCGAACCGGTCCAGGCTGTTTATGGTTTCGCTAAGTCTT
TGCTCAAAGCACTGAAAGAAGACGGGGACGCGGTAATTGTTGTATTTGATGCCAAAGCACCGAGCTTCCGCCACGAA
GCTTATGGTGGCTACAAGGCAGGACGCGCCCCTACCCCAGAAGATTTCCCCCGTCAGCTGGCATTAATTAAGGAGTT
AGTAGACCTTCTCGGCTTAGCGCGTCTGGAAGTTCCGGGTTATGAGGCGGACGATGTCCTTGCATCCTTGGCTAAAA
AGGCCGAAAAAGAGGGCTACGAAGTCCGCATCTTGACGGCAGACAAAGATCTGTACCAGCTTCTGTCTGACCGTATT
CATGTTTTGCACCCTGAAGGCTACTTAATCACTCCGGCCTGGCTCTGGGAAAAGTACGGTCTGCGTCCCGATCAGTG
GGCGGATTATCGGGCTTTGACGGGAGATGAGAGCGACAACCTGCCAGGAGTTAAGGGCATTGGTGAAAAAACCGCAC
GTAAGCTGCTTGAAGAGTGGGGTTCCCTGGAAGCCTTGTTAAAAAATCTGGATCGTCTCAAGCCCGCAATTCGTGAA
AAGATCCTGGCTCATATGGACGATCTTAAATTAAGTTGGGACCTGGCCAAGGTGCGCACCGATTTACCGCTTGAAGT
GGATTTTGCAAAACGCCGTGAGCCGGACCGGGAACGTTTACGCGCTTTCTTAGAGCGTCTGGAATTCGGTTCACTGC
TTCATGAATTCGGTCTGTTAGAGTCTCCTAAAGCACTCGAAGAGGCACCGTGGCCGCCCCCAGAAGGTGCTTTTGTT
GGCTTCGTACTTTCCCGTAAGGAGCCTATGTGGGCAGATCTTCTGGCTTTAGCGGCTGCACGCGGTGGCCGTGTTCA
CCGGGCCCCTGAGCCATACAAAGCGTTACGTGATCTGAAGGAAGCACGTGGCTTGCTGGCAAAAGACCTTTCTGTTT
TGGCCCTGCGCGAGGGTCTTGGACTGCCGCCAGGCGACGATCCCATGTTATTGGCCTATCTGTTAGACCCTAGCAAT
ACCACACCTGAAGGGGTCGCTCGTCGGTATGGCGGTGAATGGACTGAGGAAGCCGGAGAGCGCGCCGCATTGTCCGA
ACGGCTCTTTGCAAACTTATGGGGTCGTCTGGAAGGGGAGGAACGTCTGTTATGGTTGTATCGGGAAGTCGAACGTC
CTCTTTCGGCCGTATTAGCGCATATGGAGGCAACAGGTGTGCGTTTAGATGTCGCGTACCTTCGGGCCTTATCACTG
GAAGTTGCAGAGGAAATCGCCCGTCTCGAGGCTGAAGTGTTCCGGTTGGCCGGTCACCCGTTTAACCTCAACTCCCG
TGACCAGCTGGAACGCGTTTTATTCGATGAGCTTGGGCTTCCCGCAATTGGCAAAACCGAAAAGACTGGCAAACGCA
GTACGAGCGCTGCCGTCCTTGAGGCACTCCGCGAGGCTCACCCTATTGTAGAAAAGATCCTGCAATACCGTGAGTTG
ACGAAGCTTAAAAGCACTTATATTGATCCTCTCCCGGATCTGATCCATCCTCGTACCGGCCGCTTGCACACACGTTT
CAACCAGACGGCGACTGCAACCGGCCGTCTGTCTAGCTCGGATCCAAATCTCCAGAACATTCCGGTCCGTACACCCT
TGGGCCAACGTATCCGCCGGGCGTTTATCGCTGAGGAAGGATGGTTACTGGTCGCATTGGACTACTCGCAGATTGAG
CTGCGCGTCCTCGCACATCTCTCTGGTGACGAAAATTTAATCCGCGTGTTTCAAGAGGGGCGTGATATTCACACAGA
AACTGCCTCATGGATGTTCGGTGTCCCACGTGAAGCAGTGGATCCTTTGATGCGCCGTGCAGCTAAAACAATTAATT
TTGGAGTGCTGTACGGAATGAGCGCTCATCGCTTGAGTCAGGAACTGGCAATCCCCTACGAGGAAGCGCAGGCATTC
ATCGAACGTTACTTTCAATCGTTTCCGAAAGTTCGCGCATGGATCGAGAAGACGCTCGAGGAAGGTCGTCGTCGGGG
CTATGTCGAAACTCTGTTTGGTCGCCGTCGGTACGTACCAGATCTTGAAGCCCGCGTCAAATCGGTACGGGAGGCTG
CGGAGCGTATGGCATTTAATATGCCTGTACAGGGTACTGCAGCTGACCTCATGAAACTGGCAATGGTCAAGCTTTTC
CCGCGCTTGGAGGAAATGGGCGCACGTATGCTTCTGCAG**TTC**CATGACGAGCTGGTGTTAGAAGCCCCTAAGGAGCG
CGCCGAAGCTGTCGCGCGCCTCGCTAAAGAAGTGATGGAGGGCGTTTACCCATTGGCCGTACCCCTCGAAGTGGAGG
TCGGTATTGGAGAAGATTGGTTATCTGCAAAGGAAGCGGCCGC

**[0198]** SEQ ID NO. 83, amino acid sequence of Mutant ID 2 (V783F).

MRGMLPLFEPKGRVLLVDGHHLAYRTFHALKGLTTSRGEPVQAVYGFAKSLLKALKEDGDAVIVVFDAKAPSFRHEA
YGGYKAGRAPTPEDFPRQLALIKELVDLLGLARLEVPGYEADDVLASLAKKAEKEGYEVRILTADKDLYQLLSDRIH
VLHPEGYLITPAWLWEKYGLRPDQWADYRALTGDESDNLPGVKGIGEKTARKLLEEWGSLEALLKNLDRLKPAIREK
ILAHMDDLKLSWDLAKVRTDLPLEVDFAKRREPDRERLRAFLERLEFGSLLHEFGLLESPKALEEAPWPPPEGAFVG
FVLSRKEPMWADLLALAAARGGRVHRAPEPYKALRDLKEARGLLAKDLSVLALREGLGLPPGDDPMLLAYLLDPSNT
TPEGVARRYGGEWTEEAGERAALSERLFANLWGRLEGEERLLWLYREVERPLSAVLAHMEATGVRLDVAYLRALSLE
VAEEIARLEAEVFRLAGHPFNLNSRDQLERVLFDELGLPAIGKTEKTGKRSTSAAVLEALREAHPIVEKILQYRELT
KLKSTYIDPLPDLIHPRTGRLHTRFNQTATATGRLSSSDPNLQNIPVRTPLGQRIRRAFIAEEGWLLVALDYSQIEL
RVLAHLSGDENLIRVFQEGRDIHTETASWMFGVPREAVDPLMRRAAKTINFGVLYGMSAHRLSQELAIPYEEAQAFI
ERYFQSFPKVRAWIEKTLEEGRRRGYVETLFGRRRYVPDLEARVKSVREAAERMAFNMPVQGTAADLMKLAMVKLFP
RLEEMGARMLLQ**F**HDELVLEAPKERAEAVARLAKEVMEGVYPLAVPLEVEVGIGEDWLSAKEAA

**[0199]** SEQ ID NO. 84, nucleotide sequence of Mutant ID 3 (H784Q).

CATATGCGTGGTATGCTGCCGTTGTTCGAGCCTAAAGGCCGCGTACTGTTAGTCGATGGTCATCACTTGGCCTATCG
GACGTTCCATGCACTCAAAGGTCTGACGACCAGTCGTGGCGAACCGGTCCAGGCTGTTTATGGTTTCGCTAAGTCTT
TGCTCAAAGCACTGAAAGAAGACGGGGACGCGGTAATTGTTGTATTTGATGCCAAAGCACCGAGCTTCCGCCACGAA
GCTTATGGTGGCTACAAGGCAGGACGCGCCCCTACCCCAGAAGATTTCCCCCGTCAGCTGGCATTAATTAAGGAGTT
AGTAGACCTTCTCGGCTTAGCGCGTCTGGAAGTTCCGGGTTATGAGGCGGACGATGTCCTTGCATCCTTGGCTAAAA
AGGCCGAAAAAGAGGGCTACGAAGTCCGCATCTTGACGGCAGACAAAGATCTGTACCAGCTTCTGTCTGACCGTATT
CATGTTTTGCACCCTGAAGGCTACTTAATCACTCCGGCCTGGCTCTGGGAAAAGTACGGTCTGCGTCCCGATCAGTG
GGCGGATTATCGGGCTTTGACGGGAGATGAGAGCGACAACCTGCCAGGAGTTAAGGGCATTGGTGAAAAAACCGCAC
GTAAGCTGCTTGAAGAGTGGGGTTCCCTGGAAGCCTTGTTAAAAAATCTGGATCGTCTCAAGCCCGCAATTCGTGAA
AAGATCCTGGCTCATATGGACGATCTTAAATTAAGTTGGGACCTGGCCAAGGTGCGCACCGATTTACCGCTTGAAGT
GGATTTTGCAAAACGCCGTGAGCCGGACCGGGAACGTTTACGCGCTTTCTTAGAGCGTCTGGAATTCGGTTCACTGC
TTCATGAATTCGGTCTGTTAGAGTCTCCTAAAGCACTCGAAGAGGCACCGTGGCCGCCCCCAGAAGGTGCTTTTGTT
GGCTTCGTACTTTCCCGTAAGGAGCCTATGTGGGCAGATCTTCTGGCTTTAGCGGCTGCACGCGGTGGCCGTGTTCA
CCGGGCCCCTGAGCCATACAAAGCGTTACGTGATCTGAAGGAAGCACGTGGCTTGCTGGCAAAAGACCTTTCTGTTT
TGGCCCTGCGCGAGGGTCTTGGACTGCCGCCAGGCGACGATCCCATGTTATTGGCCTATCTGTTAGACCCTAGCAAT
ACCACACCTGAAGGGGTCGCTCGTCGGTATGGCGGTGAATGGACTGAGGAAGCCGGAGAGCGCGCCGCATTGTCCGA
ACGGCTCTTTGCAAACTTATGGGGTCGTCTGGAAGGGGAGGAACGTCTGTTATGGTTGTATCGGGAAGTCGAACGTC
CTCTTTCGGCCGTATTAGCGCATATGGAGGCAACAGGTGTGCGTTTAGATGTCGCGTACCTTCGGGCCTTATCACTG
GAAGTTGCAGAGGAAATCGCCCGTCTCGAGGCTGAAGTGTTCCGGTTGGCCGGTCACCCGTTTAACCTCAACTCCCG
TGACCAGCTGGAACGCGTTTTATTCGATGAGCTTGGGCTTCCCGCAATTGGCAAAACCGAAAAGACTGGCAAACGCA
GTACGAGCGCTGCCGTCCTTGAGGCACTCCGCGAGGCTCACCCTATTGTAGAAAAGATCCTGCAATACCGTGAGTTG
ACGAAGCTTAAAAGCACTTATATTGATCCTCTCCCGGATCTGATCCATCCTCGTACCGGCCGCTTGCACACACGTTT
CAACCAGACGGCGACTGCAACCGGCCGTCTGTCTAGCTCGGATCCAAATCTCCAGAACATTCCGGTCCGTACACCCT
TGGGCCAACGTATCCGCCGGGCGTTTATCGCTGAGGAAGGATGGTTACTGGTCGCATTGGACTACTCGCAGATTGAG
CTGCGCGTCCTCGCACATCTCTCTGGTGACGAAAATTTAATCCGCGTGTTTCAAGAGGGGCGTGATATTCACACAGA
AACTGCCTCATGGATGTTCGGTGTCCCACGTGAAGCAGTGGATCCTTTGATGCGCCGTGCAGCTAAAACAATTAATT
TTGGAGTGCTGTACGGAATGAGCGCTCATCGCTTGAGTCAGGAACTGGCAATCCCCTACGAGGAAGCGCAGGCATTC
ATCGAACGTTACTTTCAATCGTTTCCGAAAGTTCGCGCATGGATCGAGAAGACGCTCGAGGAAGGTCGTCGTCGGGG
CTATGTCGAAACTCTGTTTGGTCGCCGTCGGTACGTACCAGATCTTGAAGCCCGCGTCAAATCGGTACGGGAGGCTG
CGGAGCGTATGGCATTTAATATGCCTGTACAGGGTACTGCAGCTGACCTCATGAAACTGGCAATGGTCAAGCTTTTC
CCGCGCTTGGAGGAAATGGGCGCACGTATGCTTCTGCAGGTC**CAG**GACGAGCTGGTGTTAGAAGCCCCTAAGGAGCG
CGCCGAAGCTGTCGCGCGCCTCGCTAAAGAAGTGATGGAGGGCGTTTACCCATTGGCCGTACCCCTCGAAGTGGAGG
TCGGTATTGGAGAAGATTGGTTATCTGCAAAGGAAGCGGCCGC

**[0200]** SEQ ID NO. 85, amino acid sequence of Mutant ID 3 (H784Q).

MRGMLPLFEPKGRVLLVDGHHLAYRTFHALKGLTTSRGEPVQAVYGFAKSLLKALKEDGDAVIVVFDAKAPSFRHEA
YGGYKAGRAPTPEDFPRQLALIKELVDLLGLARLEVPGYEADDVLASLAKKAEKEGYEVRILTADKDLYQLLSDRIH
VLHPEGYLITPAWLWEKYGLRPDQWADYRALTGDESDNLPGVKGIGEKTARKLLEEWGSLEALLKNLDRLKPAIREK
ILAHMDDLKLSWDLAKVRTDLPLEVDFAKRREPDRERLRAFLERLEFGSLLHEFGLLESPKALEEAPWPPPEGAFVG
FVLSRKEPMWADLLALAAARGGRVHRAPEPYKALRDLKEARGLLAKDLSVLALREGLGLPPGDDPMLLAYLLDPSNT
TPEGVARRYGGEWTEEAGERAALSERLFANLWGRLEGEERLLWLYREVERPLSAVLAHMEATGVRLDVAYLRALSLE
VAEEIARLEAEVFRLAGHPFNLNSRDQLERVLFDELGLPAIGKTEKTGKRSTSAAVLEALREAHPIVEKILQYRELT
KLKSTYIDPLPDLIHPRTGRLHTRFNQTATATGRLSSSDPNLQNIPVRTPLGQRIRRAFIAEEGWLLVALDYSQIEL
RVLAHLSGDENLIRVFQEGRDIHTETASWMFGVPREAVDPLMRRAAKTINFGVLYGMSAHRLSQELAIPYEEAQAFI
ERYFQSFPKVRAWIEKTLEEGRRRGYVETLFGRRRYVPDLEARVKSVREAAERMAFNMPVQGTAADLMKLAMVKLFP
RLEEMGARMLLQV**Q**DELVLEAPKERAEAVARLAKEVMEGVYPLAVPLEVEVGIGEDWLSAKEAA

**[0201]** SEQ ID NO. 86, nucleotide sequence of Mutant ID 10 (A661E, I665W, F667L).

CATATGCGTGGTATGCTGCCGTTGTTCGAGCCTAAAGGCCGCGTACTGTTAGTCGATGGTCATCACTTGGCCTATCG
GACGTTCCATGCACTCAAAGGTCTGACGACCAGTCGTGGCGAACCGGTCCAGGCTGTTTATGGTTTCGCTAAGTCTT
TGCTCAAAGCACTGAAAGAAGACGGGGACGCGGTAATTGTTGTATTTGATGCCAAAGCACCGAGCTTCCGCCACGAA
GCTTATGGTGGCTACAAGGCAGGACGCGCCCCTACCCCAGAAGATTTCCCCCGTCAGCTGGCATTAATTAAGGAGTT
AGTAGACCTTCTCGGCTTAGCGCGTCTGGAAGTTCCGGGTTATGAGGCGGACGATGTCCTTGCATCCTTGGCTAAAA
AGGCCGAAAAAGAGGGCTACGAAGTCCGCATCTTGACGGCAGACAAAGATCTGTACCAGCTTCTGTCTGACCGTATT
CATGTTTTGCACCCTGAAGGCTACTTAATCACTCCGGCCTGGCTCTGGGAAAAGTACGGTCTGCGTCCCGATCAGTG
GGCGGATTATCGGGCTTTGACGGGAGATGAGAGCGACAACCTGCCAGGAGTTAAGGGCATTGGTGAAAAAACCGCAC
GTAAGCTGCTTGAAGAGTGGGGTTCCCTGGAAGCCTTGTTAAAAAATCTGGATCGTCTCAAGCCCGCAATTCGTGAA
AAGATCCTGGCTCATATGGACGATCTTAAATTAAGTTGGGACCTGGCCAAGGTGCGCACCGATTTACCGCTTGAAGT
GGATTTTGCAAAACGCCGTGAGCCGGACCGGGAACGTTTACGCGCTTTCTTAGAGCGTCTGGAATTCGGTTCACTGC
TTCATGAATTCGGTCTGTTAGAGTCTCCTAAAGCACTCGAAGAGGCACCGTGGCCGCCCCCAGAAGGTGCTTTTGTT
GGCTTCGTACTTTCCCGTAAGGAGCCTATGTGGGCAGATCTTCTGGCTTTAGCGGCTGCACGCGGTGGCCGTGTTCA
CCGGGCCCCTGAGCCATACAAAGCGTTACGTGATCTGAAGGAAGCACGTGGCTTGCTGGCAAAAGACCTTTCTGTTT
TGGCCCTGCGCGAGGGTCTTGGACTGCCGCCAGGCGACGATCCCATGTTATTGGCCTATCTGTTAGACCCTAGCAAT
ACCACACCTGAAGGGGTCGCTCGTCGGTATGGCGGTGAATGGACTGAGGAAGCCGGAGAGCGCGCCGCATTGTCCGA
ACGGCTCTTTGCAAACTTATGGGGTCGTCTGGAAGGGGAGGAACGTCTGTTATGGTTGTATCGGGAAGTCGAACGTC
CTCTTTCGGCCGTATTAGCGCATATGGAGGCAACAGGTGTGCGTTTAGATGTCGCGTACCTTCGGGCCTTATCACTG
GAAGTTGCAGAGGAAATCGCCCGTCTCGAGGCTGAAGTGTTCCGGTTGGCCGGTCACCCGTTTAACCTCAACTCCCG
TGACCAGCTGGAACGCGTTTTATTCGATGAGCTTGGGCTTCCCGCAATTGGCAAAACCGAAAAGACTGGCAAACGCA
GTACGAGCGCTGCCGTCCTTGAGGCACTCCGCGAGGCTCACCCTATTGTAGAAAAGATCCTGCAATACCGTGAGTTG
ACGAAGCTTAAAAGCACTTATATTGATCCTCTCCCGGATCTGATCCATCCTCGTACCGGCCGCTTGCACACACGTTT
CAACCAGACGGCGACTGCAACCGGCCGTCTGTCTAGCTCGGATCCAAATCTCCAGAACATTCCGGTCCGTACACCCT
TGGGCCAACGTATCCGCCGGGCGTTTATCGCTGAGGAAGGATGGTTACTGGTCGCATTGGACTACTCGCAGATTGAG
CTGCGCGTCCTCGCACATCTCTCTGGTGACGAAAATTTAATCCGCGTGTTTCAAGAGGGGCGTGATATTCACACAGA
AACTGCCTCATGGATGTTCGGTGTCCCACGTGAAGCAGTGGATCCTTTGATGCGCCGT**GAA**GCTAAAACA**TGG**AAT**T**
**TG**GGAGTGCTGTACGGAATGAGCGCTCATCGCTTGAGTCAGGAACTGGCAATCCCCTACGAGGAAGCGCAGGCATTC
ATCGAACGTTACTTTCAATCGTTTCCGAAAGTTCGCGCATGGATCGAGAAGACGCTCGAGGAAGGTCGTCGTCGGGG
CTATGTCGAAACTCTGTTTGGTCGCCGTCGGTACGTACCAGATCTTGAAGCCCGCGTCAAATCGGTACGGGAGGCTG
CGGAGCGTATGGCATTTAATATGCCTGTACAGGGTACTGCAGCTGACCTCATGAAACTGGCAATGGTCAAGCTTTTC
CCGCGCTTGGAGGAAATGGGCGCACGTATGCTTCTGCAGGTCCATGACGAGCTGGTGTTAGAAGCCCCTAAGGAGCG
CGCCGAAGCTGTCGCGCGCCTCGCTAAAGAAGTGATGGAGGGCGTTTACCCATTGGCCGTACCCCTCGAAGTGGAGG
TCGGTATTGGAGAAGATTGGTTATCTGCAAAGGAAGCGGCCGC

**[0202]** SEQ ID NO. 87, amino acid sequence of Mutant ID 10 (A661E, I665W, F667L).

MRGMLPLFEPKGRVLLVDGHHLAYRTFHALKGLTTSRGEPVQAVYGFAKSLLKALKEDGDAVIVVFDAKAPSFRHEA
YGGYKAGRAPTPEDFPRQLALIKELVDLLGLARLEVPGYEADDVLASLAKKAEKEGYEVRILTADKDLYQLLSDRIH
VLHPEGYLITPAWLWEKYGLRPDQWADYRALTGDESDNLPGVKGIGEKTARKLLEEWGSLEALLKNLDRLKPAIREK
ILAHMDDLKLSWDLAKVRTDLPLEVDFAKRREPDRERLRAFLERLEFGSLLHEFGLLESPKALEEAPWPPPEGAFVG
FVLSRKEPMWADLLALAAARGGRVHRAPEPYKALRDLKEARGLLAKDLSVLALREGLGLPPGDDPMLLAYLLDPSNT
TPEGVARRYGGEWTEEAGERAALSERLFANLWGRLEGEERLLWLYREVERPLSAVLAHMEATGVRLDVAYLRALSLE
VAEEIARLEAEVFRLAGHPFNLNSRDQLERVLFDELGLPAIGKTEKTGKRSTSAAVLEALREAHPIVEKILQYRELT
KLKSTYIDPLPDLIHPRTGRLHTRFNQTATATGRLSSSDPNLQNIPVRTPLGQRIRRAFIAEEGWLLVALDYSQIEL
RVLAHLSGDENLIRVFQEGRDIHTETASWMFGVPREAVDPLMRR**E**AKT**W**N**L**GVLYGMSAHRLSQELAIPYEEAQAFI
ERYFQSFPKVRAWIEKTLEEGRRRGYVETLFGRRRYVPDLEARVKSVREAAERMAFNMPVQGTAADLMKLAMVKLFP
RLEEMGARMLLQVHDELVLEAPKERAEAVARLAKEVMEGVYPLAVPLEVEVGIGEDWLSAKEAA

**[0203]** SEQ ID NO. 88, nucleotide sequence of Mutant ID 18 (V783L, H784Q).

CATATGCGTGGTATGCTGCCGTTGTTCGAGCCTAAAGGCCGCGTACTGTTAGTCGATGGTCATCACTTGGCCTATCG
GACGTTCCATGCACTCAAAGGTCTGACGACCAGTCGTGGCGAACCGGTCCAGGCTGTTTATGGTTTCGCTAAGTCTT
TGCTCAAAGCACTGAAAGAAGACGGGGACGCGGTAATTGTTGTATTTGATGCCAAAGCACCGAGCTTCCGCCACGAA
GCTTATGGTGGCTACAAGGCAGGACGCGCCCCTACCCCAGAAGATTTCCCCCGTCAGCTGGCATTAATTAAGGAGTT
AGTAGACCTTCTCGGCTTAGCGCGTCTGGAAGTTCCGGGTTATGAGGCGGACGATGTCCTTGCATCCTTGGCTAAAA
AGGCCGAAAAAGAGGGCTACGAAGTCCGCATCTTGACGGCAGACAAAGATCTGTACCAGCTTCTGTCTGACCGTATT
CATGTTTTGCACCCTGAAGGCTACTTAATCACTCCGGCCTGGCTCTGGGAAAAGTACGGTCTGCGTCCCGATCAGTG
GGCGGATTATCGGGCTTTGACGGGAGATGAGAGCGACAACCTGCCAGGAGTTAAGGGCATTGGTGAAAAAACCGCAC
GTAAGCTGCTTGAAGAGTGGGGTTCCCTGGAAGCCTTGTTAAAAAATCTGGATCGTCTCAAGCCCGCAATTCGTGAA
AAGATCCTGGCTCATATGGACGATCTTAAATTAAGTTGGGACCTGGCCAAGGTGCGCACCGATTTACCGCTTGAAGT
GGATTTTGCAAAACGCCGTGAGCCGGACCGGGAACGTTTACGCGCTTTCTTAGAGCGTCTGGAATTCGGTTCACTGC
TTCATGAATTCGGTCTGTTAGAGTCTCCTAAAGCACTCGAAGAGGCACCGTGGCCGCCCCCAGAAGGTGCTTTTGTT
GGCTTCGTACTTTCCCGTAAGGAGCCTATGTGGGCAGATCTTCTGGCTTTAGCGGCTGCACGCGGTGGCCGTGTTCA
CCGGGCCCCTGAGCCATACAAAGCGTTACGTGATCTGAAGGAAGCACGTGGCTTGCTGGCAAAAGACCTTTCTGTTT
TGGCCCTGCGCGAGGGTCTTGGACTGCCGCCAGGCGACGATCCCATGTTATTGGCCTATCTGTTAGACCCTAGCAAT
ACCACACCTGAAGGGGTCGCTCGTCGGTATGGCGGTGAATGGACTGAGGAAGCCGGAGAGCGCGCCGCATTGTCCGA
ACGGCTCTTTGCAAACTTATGGGGTCGTCTGGAAGGGGAGGAACGTCTGTTATGGTTGTATCGGGAAGTCGAACGTC
CTCTTTCGGCCGTATTAGCGCATATGGAGGCAACAGGTGTGCGTTTAGATGTCGCGTACCTTCGGGCCTTATCACTG
GAAGTTGCAGAGGAAATCGCCCGTCTCGAGGCTGAAGTGTTCCGGTTGGCCGGTCACCCGTTTAACCTCAACTCCCG
TGACCAGCTGGAACGCGTTTTATTCGATGAGCTTGGGCTTCCCGCAATTGGCAAAACCGAAAAGACTGGCAAACGCA
GTACGAGCGCTGCCGTCCTTGAGGCACTCCGCGAGGCTCACCCTATTGTAGAAAAGATCCTGCAATACCGTGAGTTG
ACGAAGCTTAAAAGCACTTATATTGATCCTCTCCCGGATCTGATCCATCCTCGTACCGGCCGCTTGCACACACGTTT
CAACCAGACGGCGACTGCAACCGGCCGTCTGTCTAGCTCGGATCCAAATCTCCAGAACATTCCGGTCCGTACACCCT
TGGGCCAACGTATCCGCCGGGCGTTTATCGCTGAGGAAGGATGGTTACTGGTCGCATTGGACTACTCGCAGATTGAG
CTGCGCGTCCTCGCACATCTCTCTGGTGACGAAAATTTAATCCGCGTGTTTCAAGAGGGGCGTGATATTCACACAGA
AACTGCCTCATGGATGTTCGGTGTCCCACGTGAAGCAGTGGATCCTTTGATGCGCCGTGCAGCTAAAACAATTAATT
TTGGAGTGCTGTACGGAATGAGCGCTCATCGCTTGAGTCAGGAACTGGCAATCCCCTACGAGGAAGCGCAGGCATTC
ATCGAACGTTACTTTCAATCGTTTCCGAAAGTTCGCGCATGGATCGAGAAGACGCTCGAGGAAGGTCGTCGTCGGGG
CTATGTCGAAACTCTGTTTGGTCGCCGTCGGTACGTACCAGATCTTGAAGCCCGCGTCAAATCGGTACGGGAGGCTG
CGGAGCGTATGGCATTTAATATGCCTGTACAGGGTACTGCAGCTGACCTCATGAAACTGGCAATGGTCAAGCTTTTC
CCGCGCTTGGAGGAAATGGGCGCACGTATGCTTCTGCAG**CTGCAG**GACGAGCTGGTGTTAGAAGCCCCTAAGGAGCG
CGCCGAAGCTGTCGCGCGCCTCGCTAAAGAAGTGATGGAGGGCGTTTACCCATTGGCCGTACCCCTCGAAGTGGAGG
TCGGTATTGGAGAAGATTGGTTATCTGCAAAGGAAGCGGCCGC

**[0204]** SEQ ID NO. 89, amino acid sequence of Mutant ID 18 (V783L, H784Q).

MRGMLPLFEPKGRVLLVDGHHLAYRTFHALKGLTTSRGEPVQAVYGFAKSLLKALKEDGDAVIVVFDAKAPSFRHEA
YGGYKAGRAPTPEDFPRQLALIKELVDLLGLARLEVPGYEADDVLASLAKKAEKEGYEVRILTADKDLYQLLSDRIH
VLHPEGYLITPAWLWEKYGLRPDQWADYRALTGDESDNLPGVKGIGEKTARKLLEEWGSLEALLKNLDRLKPAIREK
ILAHMDDLKLSWDLAKVRTDLPLEVDFAKRREPDRERLRAFLERLEFGSLLHEFGLLESPKALEEAPWPPPEGAFVG
FVLSRKEPMWADLLALAAARGGRVHRAPEPYKALRDLKEARGLLAKDLSVLALREGLGLPPGDDPMLLAYLLDPSNT
TPEGVARRYGGEWTEEAGERAALSERLFANLWGRLEGEERLLWLYREVERPLSAVLAHMEATGVRLDVAYLRALSLE
VAEEIARLEAEVFRLAGHPFNLNSRDQLERVLFDELGLPAIGKTEKTGKRSTSAAVLEALREAHPIVEKILQYRELT
KLKSTYIDPLPDLIHPRTGRLHTRFNQTATATGRLSSSDPNLQNIPVRTPLGQRIRRAFIAEEGWLLVALDYSQIEL
RVLAHLSGDENLIRVFQEGRDIHTETASWMFGVPREAVDPLMRRAAKTINFGVLYGMSAHRLSQELAIPYEEAQAFI
ERYFQSFPKVRAWIEKTLEEGRRRGYVETLFGRRRYVPDLEARVKSVREAAERMAFNMPVQGTAADLMKLAMVKLFP
RLEEMGARMLLQ**LQ**DELVLEAPKERAEAVARLAKEVMEGVYPLAVPLEVEVGIGEDWLSAKEAA

**Example 11. BLAST search for additional wild-type VH-related DNA polymerases**

**[0205]** A BLAST search using Taq DNA polymerase sequences G755 through P812 (SEQ ID NO. 90) as a comparison window was performed using available on-line databases through the National Center for Biotechnology Information of the National Library of Medicine of the National Institutes of Health (http://www.ncbi.nlm.nih.gov). The BLAST search revealed numerous wild-type DNA polymerase from other species sharing extensive sequence identity with Taq DNA polymerase, including identity at positions V783 and H784 of Taq DNA polymerase ("VH-related DNA polymerases"). An exemplary listing of these thermostable polymerases is illustrated in Table 24 and similar listing of putatively thermosenstive polymerases is illustrated in Table 25. In all the identified wild-type polymerase genes except one (*Facklamia hominis*), the amino acids corresponding to V783 and H784 of Taq DNA polymerase are preserved. In the exceptional case, however, namely, *Facklamia hominis*, an Ile naturally occurs at the residue position of the Taq DNA polymerase corresponding to V783. However, the Taq DNA polymerase mutant corresponding to Mutant ID 1 that includes this particular substitution behaves like the wild-type Taq DNA polymerase. Thus, the DNA polymerase of *Facklamia hominis* apparently deviates from the strong selection of Val at this position is postulated to maintain wild-type activity if either a Val or Ile residue is present. These BLAST results confirm a natural counter-selection against DNA polymerases having enhanced template discrimination activity and provide strong evidence that the disclosed engineered Taq DNA polymerase mutants having these properties are novel and non-obvious.

**[0206]** These identified DNA polymerases share extensive sequence homology with Taq DNA polymerase in the region that includes residues V783 and V784 of Taq DNA polymerase.

Like that observed with the engineered Taq DNA polymerase mutants, each of the identified non-Taq DNA polymerases represent a sequence space from which engineered mutant enzymes can be generated having enhanced template discrimination activity, as compared to their respective unmodified counterparts. The magnitude of the enhanced template discrimination activity obtained for identical amino acid substitutions for non-Taq DNA polymerases may not be identical when compared to the respective unmodified non-Taq DNA polymerases or even when compared to the magnitude of enhanced template discrimination activity observed for the corresponding Taq DNA polymerase mutant. Nevertheless, a strong prediction of this disclosure is that at least some amino acid substitutions in non-Taq DNA polymerases having homology to residues V783 and/or H784 of Taq DNA polymerase will display enhanced template discrimination activity relative to their respective unmodified counterparts.

**[0207] Table 24. Non-Taq thermostable DNA polymerases having homology to Taq sequences in region of V783 and H784**

| Accession No. | Species | Alignment (Query: Taq; Sbjct: Species) | SEQ Identity* |
|---|---|---|---|
| ref\|WP_018111631.1\| SEQ ID NO. 91 | Thermus igniterrae | Query 1 GTAADLMKLAMVKLFPRLEEMGARMLLQ**VH**DELVLEAPKERAEAVARLAKEVMEGVYP 58<br>GTAADLMKLAMV+LFPRL+E+GARMLLQ**VH**DELVLEAPK+RAE VA LAKEVMEGV+P<br>Sbjct 752 GTAADLMKLAMVRLFPRLQELGARMLLQ**VH**DELVLEAPKDRAERVAALAKEVMEGVWP 809 | 88% |
| ref\|WP_022798807.1\| SEQ ID NO. 92 | Thermus islandicus | Query 1 GTAADLMKLAMVKLFPRLEEMGARMLLQ**VH**DELVLEAPKERAEAVARLAKEVMEGVYP 58<br>GTAADLMKLAMVKLFPRL E GARMLLQ**VH**DEL+LEAPK+RAE VA LAKEVMEGVYP<br>Sbjct 752 GTAADLMKLAMVKLFPRLREAGARMLLQ**VH**DELLLEAPKDRAEEVAALAKEVMEGVYP 809 | 90% |
| ref\|YP_005654546.1\| SEQ ID NO. 93 | Thermus sp. CCB_US3_UF1 | Query 1 GTAADLMKLAMVKLFPRLEEMGARMLLQ**VH**DELVLEAPKERAEAVARLAKEVMEGVYP 58<br>GTAADLMKLAMV+LFP L +GARMLLQ**VH**DEL+LEAPKERAE VARLA+EVMEGV+P<br>Sbjct 756 GTAADLMKLAMVRLFPLLPGVGARMLLQ**VH**DELLLEAPKERAEEVARLAREVMEGVWP 813 | 84% |
| ref\|WP_018461567.1\| SEQ ID NO. 94 | Thermus oshimai | Query 1 GTAADLMKLAMVKLFPRLEEMGARMLLQ**VH**DELVLEAPKERAEAVARLAKEVMEGVYP 58<br>GTAADLMKLAMVKLFPRL +G R+LLQ**VH**DELVLEAPK RAE A+LAKE MEGVYP<br>Sbjct 753 GTAADLMKLAMVKLFPRLRPLGVRILLQ**VH**DELVLEAPKARAEEAAQLAKETMEGVYP 810 | 83% |
| ref\|WP_008632471.1\| SEQ ID NO. 95 | Thermus sp. RL | Query 1 GTAADLMKLAMVKLFPRLEEMGARMLLQ**VH**DELVLEAPKERAEAVARLAKEVMEGVYP 58<br>GTAADLMKLAMVKLFPRL EMGARMLLQ**VH**DEL+LEAP+ RAE VA LAKE ME YP<br>Sbjct 754 GTAADLMKLAMVKLFPRLREMGARMLLQ**VH**DELLLEAPQARAEEVAALAKEAMEKAYP 811 | 84% |
| ref\|YP_005640602.1\| SEQ ID NO: 96 | Thermus thermophilus SG0.5JP17-16 | Query 1 GTAADLMKLAMVKLFPRLEEMGARMLLQ**VH**DELVLEAPKERAEAVARLAKEVMEGVYP 58<br>GTAADLMKLAMVKLFPRL EMGARMLLQ**VH**DEL+LEAP+ RAE VA LAKE ME YP<br>Sbjct 754 GTAADLMKLAMVKLFPRLREMGARMLLQ**VH**DELLLEAPQARAEEVAALAKEAMEKAYP 811 | 84% |
| ref\|WP_019550117.1\|\ SEQ ID NO. 97 | Thermus scotoductus | Query 1 GTAADLMKLAMVKLFPRLEEMGARMLLQ**VH**DELVLEAPKERAEAVARLAKEVMEGVYP 58<br>GTAADLMKLAMVKLFPRL+E+GARMLLQ**VH**DELVLEAPKE+AE VA+ AK ME V+P<br>Sbjct 753 GTAADLMKLAMVKLFPRLQELGARMLLQ**VH**DELVLEAPKEQAEEVAQEAKRTMEEVWP 810 | 83% |
| gb\|AAB81398.1\| SEQ ID NO. 98 | Thermus caldophilus | Query 1 GTAADLMKLAMVKLFPRLEEMGARMLLQ**VH**DELVLEAPKERAEAVARLAKEVMEGVYP 58<br>GTAADLMKLAMVKLFPRL EMGARMLLQ**VH**DEL+LEAP+ AE VA LAKE ME YP<br>Sbjct 757 GTAADLMKLAMVKLFPRLREMGARMLLQ**VH**DELLLEAPQAGAEEVAALAKEAMEKAYP 814 | 83% |
| ref\|YP_004367987.1\| SEQ ID NO. 99 | Marini-thermus hydro- | Query 1 GTAADLMKLAMVKLFPRLEEMGARMLLQ**VH**DELVLEAPKERAEAVARLAKEVMEGVY 57<br>GTAADLMKLAMVKL P + +GAR++LQ**VH**DELVLEAP+ERAEAVAR+ +EVMEG +<br>Sbjct 758 GTAADLMKLAMVKLAPEIRSLGARLILQ**VH**DELVLEAPQERAEAVARVVREVMEGAW 814 | 75% |

| Accession No. | Species | Alignment (Query: Taq; Sbjct: Species) | SEQ Identity* |
|---|---|---|---|
| | thermalis DSM 14884 | | |
| gb\|AAR11876.1\| SEQ ID NO. 100 | Thermus filiformis | Query 1 GTAADLMKLAMVKLFPRLEEMGARMLLQ**VH**DELVLEAPKERAEAVARLAKEVMEGVYP 58<br>GTAADLMK+AMVKLFPRL+ +GA +LLQ**VH**DELVLE P++RAE L KEVME YP<br>Sbjct 755 GTAADLMKIAMVKLFPRLKPLGAHLLLQ**VH**DELVLEVPEDRAEEAKALVKEVMENTYP 812 | 72% |
| ref\|WP_018465880.1\| SEQ ID NO. 101 | Meiothermus timidus | Query 1 GTAADLMKLAMVKLFPRLEEMGARMLLQ**VH**DELVLEAPKERAEAVARLAKEVMEGVY 57<br>GTAADLMKLAMVKL P+LE + A ++LQ**VH**DELV+EAP+ERAE VA LA+E M +<br>Sbjct 774 GTAADLMKLAMVKLGPKLEPLDAHLVLQ**VH**DELVIEAPRERAEEVAELARETMRTAW 830 | 68% |
| ref\|WP_013637959.1\| SEQ ID NO. 102 | Desulfuro-bacterium thermolithotrophum | Query 1 GTAADLMKLAMVKLFPRLEEMGARMLLQ**VH**DELVLEAPKERAEAVARLAKEVMEGV 56<br>GTAAD+MKLAMVKL+ +LE++GA M+LQ**VH**DE+V+EA +E+ E + ++ KE ME V<br>Sbjct 765 GTAADIMKLAMVKLYKKLEKLGAYMVLQ**VH**DEIVIEALEEKTEEIMKIVKETMENV 820 | 61% |
| ref\|YP_005442159.1\| SEQ ID NO. 103 | Caldilinea aerophila | Query 1 GTAADLMKLAMVKLFPRLEEMG--ARMLLQ**VH**DELVLEAPKERAEAVARLAKEVMEGVY 57<br>GTAAD+MK+AM++L+ RL+ G R+L+Q**VH**DELVLEAP E E+ L +E M Y<br>Sbjct 875 GTAADIMKIAMIRLYERLQNDGYRTRLLIQ**VH**DELVLEAPPEELESATHLVRETMANAY 933 | 54% |

*Sequence identity refers to the percent identity of the query sequence with wild type Taq DNA polymerase.

**[0208] Table 25. Non-Taq putatively thermosensitive DNA polymerasess having homology to Taq sequence in the region of V783 and H784**

| Accession No. | Species | Alignment (Query: Taq; Sbjct: Species) | SEQ Identity* |
|---|---|---|---|
| ref\|WP_015519435.1\| SEQ ID NO: 104 | Eubacterium siraeum | Query 1 GTAADLMKLAMVKLFPRLEEMG--ARMLLQ**VH**DELVLEAPKERAEAVARLAKEVMEG 55<br>GTAAD++K+AM+K++ RLEE G AR++LQ**VH**DEL++EA ++ AE VA L KE ME<br>Sbjct 751 GTAADIIKIAMIKVYNRLEESGLDARLILQ**VH**DELIVEAKEDCAEKVALLLKEEMEN 807 | 60% |
| ref\|WP_022236670.1\| SEQ ID NO: 105 | Clostridium leptum | Query 1 GTAADLMKLAMVKLFPRL--EEMGARMLLQ**VH**DELVLEAPKERAEAVARLAKEVMEG 55<br>GTAAD++K+AMV++ RL E M AR++LQ**VH**DEL++EAP++ AE AR+ E MEG<br>Sbjct 320 GTAADIIKIAMVRVDRRLKRENMRARLILQ**VH**DELIVEAPEDEAEQAARILTEEMEG 376 | 58% |
| ref\|WP_002333048.1\| SEQ ID NO: 106 | Enterococcus | Query 1 GTAADLMKLAMVKLFPRLEEMG--ARMLLQ**VH**DELVLEAPKERAEAVARLAKEVME 54<br>G+AAD++K+AM++L RL+E G A MLLQ**VH**DELV E PK+ E++ +L KEVME<br>Sbjct 803 GSAADILKIAMIELDKRLKETGLQATMLLQ**VH**DELVFEVPKKELESLDKLVKEVME 858 | 59% |
| ref\|WP_016648372.1\| SEQ ID NO: 107 | Facklamia hominis | Query 1 GTAADLMKLAMVKLFPRLEEMG--ARMLLQ**VH**DELVLEAPKERAEAVARLAKEVMEG 55<br>GTAAD++KLAMV+L RLEE G +R+LLQ**+H**DEL+LE PKE + +L EVME<br>Sbjct 803 GTAADIIKLAMVRLQARLEEAGLSSRLLLQ**IH**DELILEGPKEEMPQLQKLVVEVMES 859 | 60% |
| ef\|WP_000412792\| SEQ ID NO: 108 | Bacillus anthracis | Query 1 GTAADLMKLAMVKLFPRLEEMG--ARMLLQ**VH**DELVLEAPKERAEAVARLAKEVME 54<br>GTAAD++K AM+ + RLEE G AR+LLQ**VH**DEL+ EAPKE E + +L EVME<br>Sbjct 799 GTAADIIKKAMIIMADRLEEEGLQARLLLQ**VH**DELIFEAPKEEVEKLEKLVPEVME 854 | 61% |
| ref\|NP_981011.1\| SEQ ID NO: 109 | Bacillus cereus ATCC 10987 | Query 1 GTAADLMKLAMVKLFPRLEEMG--ARMLLQ**VH**DELVLEAPKERAEAVARLAKEVME 54<br>GTAAD++K AM+ + RLEE G AR+LLQ**VH**DEL+ EAPKE E + +L EVME<br>Sbjct 799 GTAADIIKKAMIIMADRLEEEGLQARLLLQ**VH**DELIFEAPKEEIEKLEKLVPEVME 854 | 61% |

*Sequence identity refers to the percent identity of the query sequence with wild type Taq DNA polymerase.

**Example 12. Production of Additional Codon Optimized Taq DNA Polymerase mutants at position H784.**

**[0209]** After determining the properties of the first eighteen mutant versions of the Taq polymerase (Table 3, Mut IDs 1-18), an additional eighteen mutant versions of Taq DNA polymerase (Table 3, Mut IDs 19-30) were made by site directed mutagenesis of the cloned OptiTaq codon-optimized WT Taq DNA polymerase. The full set represents all possible amino acid variations at position 784 in Taq polymerase. Specific mutations were introduced into the OptiTaq sequence using the method of PCR site-directed mutagenesis (Weiner MP, *et al.*, Gene. 151(1-2):119-23 (1994)). Each mutagenesis reaction employed 10 pmoles of two complementary oligonucleotides (Table 26) containing the desired base changes, annealed to the double-stranded OptiTaq plasmid (20 ng), 5 U KOD DNA polymerase (Novagen-EMD Chemicals, San Diego, CA), 1.5 mM $MgSO_4$, in 1X KOD PCR buffer. Thermal cycling parameters were 95°C for 3 minutes (95°C for 20 sec-55°C for 20 sec-70°C for 2.5 minutes) for 16 cycles followed by a 70°C soak for 4 minutes. After PCR site-directed mutagenesis, the amplified product was treated with 10 U of Dpn I (NEB, Ipswich, MA), at 37°C for 1 hour, followed by inactivation at 80°C for 20 minutes. $1/110^{th}$ of the digestion material was transformed into XL-1 Blue competent bacteria. Bacterial clones were isolated, plasmid DNA prepared, and individual mutations were confirmed by Sanger DNA sequencing. All mutants remained in the pET-27b(+) expression vector, which is suitable for expressing the recombinant proteins in *E. coli.* Expression and purification of the recombinant mutants of the Taq polymerase were performed as described in Example 3.

**Table 26: Oligonucleotides used for site-directed mutagenesis to produce 18 Taq DNA Polymerase mutants at position 784.**

| Mutant ID | Amino acid changes | Sequence" Sense mutagenesis oligonucleotide | SEQ ID No. | Sequence" Antisense mutagenesis oligonucleotide | SEQ ID No. |
|---|---|---|---|---|---|
| 19 | H784G | gggcgcacgtatgcttctgcaggtcGGTgacgagctggtgttagaagccccta | 110 | taggggcttctaacaccagctcgtcACCgacctgcagaagcatacgtgcgccc | 111 |
| 20 | H784A | gggcgcacgtatgcttctgcaggtcGCGgacgagctggtgttagaagccccta | 112 | taggggcttctaacaccagctcgtcCGCgacctgcagaagcatacgtgcgccc | 113 |

| Mutant ID | Amino acid changes | Sequence" Sense mutagenesis oligonucleotide | SEQ ID No. | Sequence" Antisense mutagenesis oligonucleotide | SEQ ID No. |
|---|---|---|---|---|---|
| 21 | H784S | gggcgcacgtatgcttctgca ggtcAGCgacgagctggtgtt agaagccccta | 114 | taggggcttctaacaccagctcg tcGCTgacctgcagaagcatacg tgcgccc | 115 |
| 22 | H784T | gggcgcacgtatgcttctgca ggtcACGgacgagctggtgtt agaagccccta | 116 | taggggcttctaacaccagctcg tcCGTgacctgcagaagcatacg tgcgccc | 117 |
| 23 | H784C | gggcgcacgtatgcttctgca ggtcTGCgacgagctggtgtt agaagccccta | 118 | taggggcttctaacaccagctcg tcGCAgacctgcagaagcatacg tgcgccc | 119 |
| 24 | H784V | gggcgcacgtatgcttctgca ggtcGTAgacgagctggtgtt agaagccccta | 120 | taggggcttctaacaccagctcg tcTACgacctgcagaagcatacg tgcgccc | 121 |
| 25 | H784L | gggcgcacgtatgcttctgca ggtcTTGgacgagctggtgtt agaagccccta | 122 | taggggcttctaacaccagctcg tcCAAgacctgcagaagcatacg tgcgccc | 123 |
| 26 | H784I | gggcgcacgtatgcttctgca ggtcATTgacgagctggtgtt agaagccccta | 124 | taggggcttctaacaccagctcg tcAATgacctgcagaagcatacg tgcgccc | 125 |
| 27 | H784M | gggcgcacgtatgcttctgca ggtcATGgacgagctggtgtt agaagccccta | 126 | taggggcttctaacaccagctcg tcCATgacctgcagaagcatacg tgcgccc | 127 |
| 28 | H784P | gggcgcacgtatgcttctgca ggtcCCAgacgagctggtgtt agaagccccta | 128 | taggggcttctaacaccagctcg tcTGGgacctgcagaagcatacg tgcgccc | 129 |
| 29 | H784F | gggcgcacgtatgcttctgca ggtcTTTgacgagctggtgtt agaagccccta | 130 | taggggcttctaacaccagctcg tcAAAgacctgcagaagcatacg tgcgccc | 131 |
| 30 | H784Y | gggcgcacgtatgcttctgca ggtcTATgacgagctggtgtt agaagccccta | 132 | taggggcttctaacaccagctcg tcATAgacctgcagaagcatacg tgcgccc | 133 |
| 31 | H784W | gggcgcacgtatgcttctgca ggtcTGGgacgagctggtgtt agaagccccta | 134 | taggggcttctaacaccagctcg tcCCAgacctgcagaagcatacg tgcgccc | 135 |
| 32 | H784D | gggcgcacgtatgcttctgca ggtcGATgacgagctggtgtt agaagccccta | 136 | taggggcttctaacaccagctcg tcATCgacctgcagaagcatacg tgcgccc | 137 |
| 33 | H784E | gggcgcacgtatgcttctg caggtcGAAgacgagctgg tgttagaagccccta | 138 | taggggcttctaacaccagctcg tcTTCgacctgcagaagcatacg tgcgccc | 139 |

| Mutant ID | Amino acid changes | Sequence" Sense mutagenesis oligonucleotide | SEQ ID No. | Sequence" Antisense mutagenesis oligonucleotide | SEQ ID No. |
|---|---|---|---|---|---|
| 34 | H784N | gggcgcacgtatgcttctgca ggtcAACgacgagctggtgtt agaagccccta | 140 | taggggcttctaacaccagctcg tcGTTgacctgcagaagcatacg tgcgccc | 141 |
| 35 | H784K | gggcgcacgtatgcttctgca ggtcAAAgacgagctggtgtt agaagccccta | 142 | taggggcttctaacaccagctcg tcTTTgacctgcagaagcatacg tgcgccc | 143 |
| 36 | H784R | gggcgcacgtatgcttctgca ggtcCGGgacgagctggtgtt agaagccccta | 144 | taggggcttctaacaccagctcg tcCCGgacctgcagaagcatacg tgcgccc | 145 |

DNA bases identical to codon optimized OptiTaq are shown in lower case; those specific for the mutations introduced by site-directed mutagenesis are shown in upper case.

**Example 13. Characterization of properties of 18 mutant Taq DNA Polymerases altered at position H784 in PCR.**

**[0210]** The 18 mutant Taq DNA polymerase enzymes described in Example 12 were characterized for polymerase activity and ability to discriminate a 3'-RNA residue in the primer oligonucleotide.

**[0211]** The unit activity of the purified wild-type protein was determined by comparing performance in qPCR of known quantities of OptiTaq and each mutant compared to a commercial native non-hot-start Taq DNA polymerase, Taq-B DNA Polymerase (Enzymatics, Beverly, MA). Quantification cycle values (Cq, the amplification cycle number at which positive signal is first detected) and amplification curve shapes were analyzed to determine the nanogram amounts at which both enzymes performed similarly in the suboptimal range for each. Using these nanogram amounts and known unit values of Taq-B DNA polymerase, relative activity unit values could be extrapolated for all of the mutant DNA polymerase enzymes having sufficient activity to support PCR.

**[0212]** The following reaction conditions were employed: 1x qPCR buffer (20 mM Tris pH 8.4, 50 mM KCl, 3 mM $MgCl_2$, 0.01% Triton-X100), 800 μM dNTPs (200 μM each), 500 nM For primer (Hs HPRT F517, SEQ ID NO. 43), 500 nM Rev primer (Hs HPRT R591, SEQ ID NO. 44), 250 nM probe (Hs HPRT P554, SEQ ID NO. 45), 2 x $10^3$ copies of linearized cloned plasmid template (HPRT-targ, SEQ ID NO. 46), in 10 μL final volume. The amount of DNA polymerase added to each reaction was varied as follows: for wild type (OptiTaq), reactions were

set using 10, 1, 0.1, 0.01, and 0.001 U/μL (220, 22, 2.2, 0.22, or 0.022 ng of protein per 10 μL reaction). Mutant polymerases were run in similar concentrations. In addition, those mutant enzymes showing polymerase activity were more finely titrated testing 220, 22, 10.6, 4.8, 2.2, 1.1, 0.48, and 0.22 ng of protein per 10 μL reaction. Enzyme dilutions were made in enzyme dilution buffer (20 mM Tris pH7.5, 100 mM NaCl, 1 mM DTT, 0.1% Triton-X100, 1 mg/mL BSA, 10% glycerol). Reactions were run in 384 well format on a BIO-RAD CFX384™ Real-Time System (BIO-RAD, Hercules, CA) using cycling parameters 95°C for 30 seconds followed by 60 cycles of [95°C for 15 seconds followed by 60°C for 1 minutes]. Detection was achieved using a fluorescence-quenched probe (5'-nuclease assay format, note that the mutations introduced into the present series of Taq mutants do not lie in the 5'-nuclease domain). Sequences of the primers, probe, and template (plasmid insert) are shown in Table 27.

**Table 27. Sequence of oligonucleotides employed in Taq DNA polymerase activity assay.**

| Name | Sequence | SEQ ID NO. |
|---|---|---|
| Hs HPRT F517 | GACTTTGCTTTCCTTGGTCAG | SEQ ID NO. 43 |
| Hs HPRT R591 | GGCTTATATCCAACACTTCGTG | SEQ ID NO. 44 |
| Hs HPRT P554 | FAM-ATGGTCAAG(ZEN)GTCGCAAGCTTGCTGGT-IBFQ | SEQ ID NO. 45 |
| HPRT-targ | GACTTTGCTTTCCTTGGTCAGGCAGTATAATCCAAAGATGGTCAAGGTC GCAAGCTTGCTGGTGAAAAGGACCCCACGAAGTGTTGGATATAAGCC | SEQ ID NO. 46 |

Nucleic acid sequences are shown 5'-3'. FAM = 6-carboxyfluorescein, IBFQ = Iowa Black FQ (fluorescence quencher), and ZEN = ZEN internal fluorescence quencher.

**[0213]** These 18 Taq DNA polymerase mutants were characterized as outlined above. Results are summarized in Table 28. Ten mutants, including Mutant IDs 19, 23, 25, 28, and 31 to 36, did not show detectable DNA polymerase activity and were not studied further. Four mutants, Mutant IDs 20, 21, 27 and 29 had DNA polymerase activity; however, processivity was reduced from 4-6 fold relative to the wild type enzyme. Three mutants, Mutant IDs 24, 26, and 30, showed DNA polymerase activity similar to wild type OptiTaq.

**Table 28. Activity of novel Taq DNA polymerase mutants.**

| Mutant ID | Amino acid changes from wild-type Taq | Polymerase Activity | Relative activity* | ΔCq Delay in priming from an RNA base** |
|---|---|---|---|---|
| 19 | H784G | No | - | - |
| 20 | H784A | Yes | 0.2 | 1 |
| 21 | H784S | Yes | 0.16 | 2 |
| 22 | H784T | Yes | 1 | 0 |
| 23 | H784C | No | - | - |
| 24 | H784V | Yes | 0.45 | >35 |
| 25 | H784L | No | - | - |
| 26 | H784I | Yes | 0.5 | 6 |
| 27 | H784M | Yes | 0.22 | >35 |
| 28 | H784P | No | - | - |
| 29 | H784F | Yes | 0.22 | 3 |
| 30 | H784Y | Yes | 0.45 | 5 |
| 31 | H784W | No | - | - |
| 32 | H784D | No | - | - |
| 33 | H784E | No | - | - |
| 34 | H784N | No | - | - |
| 35 | H784K | No | - | - |
| 36 | H784R | No | - | - |

*Wild-type OptiTaq was set to "1" and the relative activity of each of the mutant polymerases was normalized to this amplification efficiency, with 1 as the maximum.
**ΔCq = [Cq Mutant ID X] – [Cq OptiTaq] when qPCR reactions are run using primers having a 3'-RNA residue.

**[0214]** The subset of these mutant Taq DNA polymerases which showed suitable levels of DNA polymerase activity were studied for their ability to discriminate between primers have a 3'-DNA versus a 3'-RNA residue relative to the wild type OptiTaq enzyme. Real-time PCR was performed as before, employing in the reactions the amount of each mutant DNA polymerase equal to 0.5 units of wild-type OptiTaq per 10 μL reaction. The following reaction conditions

were employed: 1x qPCR buffer (20 mM Tris pH 8.4, 50 mM KCl, 3 mM $MgCl_2$, 0.01% Triton-X100), 800 μM dNTPs (200 μM each), 500 nM For primer (Hs SFRS9 F569 rU, SEQ ID NO. 47), 500 nM Rev primer (Hs SFRS9 R712 rA, SEQ ID NO. 48), 250 nM probe (Hs SFRS9 P644, SEQ ID NO. 49), 2 x $10^3$ copies of linearized cloned plasmid template (SFRS9-targ, SEQ ID NO. 50), in 10 μL final volume. Reactions were run in 384 well format on a BIO-RAD CFX384™ Real-Time System (BIO-RAD, Hercules, CA) using cycling parameters 95°C for 30 seconds followed by 60 cycles of [95°C for 15 seconds followed by 60°C for 1 minutes]. Detection was achieved using a fluorescence-quenched probe (5'-nuclease assay format). Sequences of the primers, probe, and template (plasmid insert) are shown in Table 29.

**Table 29. Sequence of oligonucleotides employed in the primer 3'-RNA discrimination assay.**

| Name | Sequence | SEQ ID NO. |
|---|---|---|
| Hs SFRS9 F569 rU | TGTGCAGAAGGATGGAGu | SEQ ID NO. 47 |
| Hs SFRS9 R712 rA | CTGGTGCTTCTCTCAGGATa | SEQ ID NO. 48 |
| Hs SFRS9 P644 | HEX-TGGAATATG(ZEN)CCCTGCGTAAACTGGA-IBFQ | SEQ ID NO. 48 |
| SFRS9-targ | TGTGCAGAAGGATGGAGTGGGGATGGTCGAGTATCTCAGAAAAGAAGA CATGGAATATGCCCTGCGTAAACTGGATGACACCAAATTCCGCTCTCA TGAGGGTGAAACTTCCTACATCCGAGTTTATCCTGAGAGAAGCACCAG | SEQ ID NO. 50 |

Nucleic acid sequences are shown 5'-3' with DNA uppercase and RNA lowercase. HEX = hexachlorofluorescein, IBFQ = Iowa Black FQ (fluorescence quencher), and ZEN = ZEN fluorescence quencher.

**[0215]** The eight Taq DNA polymerase mutants that supported PCR were tested for the ability to use a 3'-RNA modified primer as outlined above. Results are summarized in Table 28. Mutant IDs 20 and 22 did not show any significant difference between primers having a 3'-DNA versus a 3'-RNA residue. Mutant IDs 21, 24, 26, 27, 29, and 30 showed an amplification delay using 3'-RNA primers. Thus, additional Taq DNA polymerase mutants were identified which discriminate against priming from a 3'-RNA residue. Those mutants which showed some delay with RNA priming and showed high processivity were further studied for improvements in primer 3'-residue mismatch discrimination.

**Example 14: Improved mismatch discrimination in allele-specific PCR using mutant Taq DNA polymerases altered at position H784.**

**[0216]** Of the 18 mutant enzymes studied in Example 12 and 13, Mutant IDs 21, 24, 26, 27, 29, and 30 showed the ability to discriminate against a 3'-RNA residue in the primer and retained high enzymatic activity/processivity. These six mutants and additionally Mutant IDs 20 and 22 were studied for the ability to discriminate against a 3'-terminal DNA mismatch compared with wild type OptiTaq DNA polymerase using an allele-specific qPCR assay. Amplification reactions were performed against a synthetic oligonucleotide template where a single base was varied (SNP) which was positioned to lie at the 3'-end of the reverse primer. Synthetic templates were employed having each of the 4 possible bases at this position. Reverse primers were employed having each of the 4 possible bases at the 3'-end. Relative amplification efficiencies of all pairwise primer/template combinations were assessed using qPCR.

**[0217]** Quantitative allele-specific real-time PCR (AS-qPCR) was performed in 10 μL reaction volumes in 384 well format with 2 x $10^5$ copies of a 103 bp synthetic template (SEQ ID NOs. 51-4). Final reaction conditions used were 20 mM Tris-HCL (pH 8.4 at 25°C), 50 mM KCL, and 3 mM $MgCl_2$, 0.01% Triton X-100, 800 μM total dNTPs, and 200 nM of the universal forward primer (SEQ ID NO. 60), 200 nM of a reverse primer (separate reactions were set up for each of the allele-specific primers SEQ ID NOs. 55-58 or the control universal primer SEQ ID NO. 59) and 200 nM of the 5' nuclease detection probe (SEQ ID NO. 61). Each allele-specific primer was tested on each SNP template. Reactions utilized either 0.5 U (10.8 ng/11.1 nM/111 fmol) of the wild type OptiTaq DNA polymerase or 0.5 U of one of the nine Taq DNA polymerase mutants studied (Mutant ID 3 (H784Q) (10.8 ng/11.1 nM/111 fmol); Mutant ID 20 H784A (54 ng/55.5 nM/555 fmol); Mutant ID22 H784T (10.8 ng/11.1 nM/111 fmol); Mutant ID 24 H784V (24 ng/24.7 nM/246.7 fmol); Mutant ID 26 H784I (21.6 ng/22.2 nM/222 fmol); Mutant ID 27 H784M (10.8 ng/11.1 nM/111 fmol); Mutant ID 29 H784F (49.1 ng/49.4 nM/494.5 fmol); Mutant ID 30 H784Y) (24 ng/24.7 nM/246.7 fmol). Amplification was performed on a CFX384™ C1000™ Thermo Cycler system (Bio-Rad, Hercules, CA) using the following cycling parameters: 95°C for 30 seconds initial denaturation followed by 60 cycles of 95°C for 10 seconds, then 60°C for 30 seconds. Oligonucleotide reagents used in this example are shown in Table 30.

**Table 30. Synthetic oligonucleotides employed in Example 13.**

| Name | Sequence (5'-3') | SEQ ID NO. |
|---|---|---|
| A Template | AGCTCTGCCCAAAGATTACCCTGACAGCTAAGTGGCAGTGGAA GTTGGCCTCAGAAGTAGTGGCCAGCTGTGTGTCGGGGAACAGT AAAGGCATGAAGCTCAG | SEQ ID NO. 51 |
| C Template | AGCTCTGCCCAAAGATTACCCTGACAGCTAAGTGGCAGTGGAA GTTGGCCTCAGAAGTAGTGGCCAGCTGTGTGTCGGGGCACAGT AAAGGCATGAAGCTCAG | SEQ ID NO. 52 |
| G Template | AGCTCTGCCCAAAGATTACCCTGACAGCTAAGTGGCAGTGGAA GTTGGCCTCAGAAGTAGTGGCCAGCTGTGTGTCGGGGGACAGT AAAGGCATGAAGCTCAG | SEQ ID NO. 53 |
| T Template | AGCTCTGCCCAAAGATTACCCTGACAGCTAAGTGGCAGTGGAA GTTGGCCTCAGAAGTAGTGGCCAGCTGTGTGTCGGGGTACAGT AAAGGCATGAAGCTCAG | SEQ ID NO. 54 |
| Syn Rev T | CTGAGCTTCATGCCTTTACTGTT | SEQ ID NO. 55 |
| Syn Rev C | CTGAGCTTCATGCCTTTACTGTC | SEQ ID NO. 56 |
| Syn Rev A | CTGAGCTTCATGCCTTTACTGTA | SEQ ID NO. 57 |
| Syn Rev G | CTGAGCTTCATGCCTTTACTGTG | SEQ ID NO. 58 |
| Syn Rev | CTGAGCTTCATGCCTTTACTGT | SEQ ID NO. 59 |
| Syn For | AGCTCTGCCCAAAGATTACCCTG | SEQ ID NO. 60 |
| Syn Probe | FAM-TTCTGAGGC(ZEN)CAACTTCCACTGCCACTTA-IBFQ | SEQ ID NO. 61 |

DNA bases are uppercase; FAM = 6-carboxyfluorescein; IBFQ = Iowa Black™ FQ fluorescence quencher; ZEN = internal ZEN fluorescence quencher; underlined base indicates the SNP site in the synthetic template DNA.

**[0218]** Initially all reactions were run in triplicate. Similar results were obtained for all replicates when using the wild type OptiTaq. However, results showed greater variation for the mutant polymerases. To obtain statistically meaningful results, each reaction was therefore performed 24 times for the mutant polymerases and 21 times for the wild type enzyme. ΔCq values were calculated as the Cq value obtained for each mismatched base pair minus the Cq value obtained for the matched base pair (ΔCq = Cq mismatch – Cq match). The ΔCq values for all 24 replicates were averaged and standard deviations were calculated. Results are shown in Table 31 and are graphically summarized in Figures 3C, 3D, 3E, 3F, and 3G. Note that the

reverse primer is the allele-specific primer, so the "Syn Rev T" primer (SEQ ID NO. 55) is the perfect match to the Template A (SEQ ID NO. 51), etc.

**Table 31: ΔCq values for AS-qPCR reactions using WT OptiTaq and H784 mutant Taq DNA polymerases.**

| DNA Polymerase | Reverse Primer | | Template | | | |
|---|---|---|---|---|---|---|
| | Name | SEQ ID NO. | A<br>SEQ ID NO. 51 | C<br>SEQ ID NO. 52 | G<br>SEQ ID NO. 53 | T<br>SEQ ID NO. 54 |
| OptiTaq | Syn Rev T | 55 | - | 1.4 +/- 0.1 | 0.6 +/- 0.2 | 4.8 +/- 0.2 |
| | Syn Rev G | 58 | 8.5 +/- 0.2 | - | 5.9 +/- 0.2 | 3.5 +/- 0.2 |
| | Syn Rev C | 56 | 2.8 +/- 0.2 | 7.7 +/- 0.1 | - | 3.9 +/- 0.1 |
| | Syn Rev A | 57 | 5.3 +/- 0.2 | -0.8 +/- 0.1 | 6.1 +/- 0.1 | - |
| MUT ID 3 H784Q | Syn Rev T | 55 | - | 5.7 +/- 0.2 | 5.7 +/- 0.3 | 10.8 +/- 0.4 |
| | Syn Rev G | 58 | 14.5 +/- 0.6 | - | 12.5 +/- 0.5 | 6.8 +/- 0.2 |
| | Syn Rev C | 56 | 8.5 +/- 1.5 | 10.6 +/- 0.2 | - | 7.7 +/- 0.3 |
| | Syn Rev A | 57 | 10.3 +/- 0.5 | 4.1 +/- 0.1 | 11.0 +/- 0.7 | - |
| MUT ID 20 H784A | Syn Rev T | 55 | - | 7.6 +/- 0.3 | 7.7 +/- 0.4 | 12.3 +/- 0.8 |
| | Syn Rev G | 58 | 19.1 +/- 6.0 | - | 14.8 +/- 1.4 | 6.3 +/- 0.5 |
| | Syn Rev C | 56 | 9.6 +/- 0.5 | 12.4 +/- 4.7 | - | 8.2 +/- 0.4 |
| | Syn Rev A | 57 | 14.9 +/- 4.4 | 7.6 +/- 0.2 | 14.2 +/- 1.9 | - |

| DNA Polymerase | Reverse Primer | | Template | | | |
|---|---|---|---|---|---|---|
| | Name | SEQ ID NO. | A<br>SEQ ID NO. 51 | C<br>SEQ ID NO. 52 | G<br>SEQ ID NO. 53 | T<br>SEQ ID NO. 54 |
| MUT ID 21 H784S | Syn Rev T | 55 | - | 7.9 +/- 0.5 | 19.6 +/- 8.5 | 8.6 +/- 0.8 |
| | Syn Rev G | 58 | 25.8 +/- 9.2 | - | 23.9 +/- 9.6 | 6.6 +/- 0.3 |
| | Syn Rev C | 56 | 11.4 +/- 4.0 | 16.4 +/- 9.2 | - | 9.1 +/- 1.5 |
| | Syn Rev A | 57 | 23.1 +/- 8.2 | 8.4 +/- 0.4 | 22.9 +/- 8.6 | - |
| MUT ID 22 H784T | Syn Rev T | 55 | - | 1.5 +/- 0.3 | 3.7 +/- 0.3 | 5.6 +/- 0.3 |
| | Syn Rev G | 58 | 13.3 +/- 0.6 | - | 10.7 +/- 0.5 | 3.9 +/- 0.2 |
| | Syn Rev C | 56 | 5.2 +/- 0.3 | 9.3 +/- 0.5 | - | 3.3 +/- 0.3 |
| | Syn Rev A | 57 | 9.8 +/- 0.3 | 2.4 +/- 0.2 | 11.0 +/- 0.4 | - |
| MUT ID 24 H784V | Syn Rev T | 55 | - | -0.3 +/- 0.2 | 1.8 +/- 0.2 | 2.6 +/- 0.2 |
| | Syn Rev G | 58 | 10.2 +/- 0.2 | - | 8.4 +/- 0.1 | 2.8 +/- 0.2 |
| | Syn Rev C | 56 | 2.8 +/- 0.1 | 4.6 +/- 0.1 | - | 1.8 +/- 0.1 |
| | Syn Rev A | 57 | 5.4 +/- 0.1 | 0.2 +/- 0.1 | 9.2 +/- 0.2 | - |
| MUT ID 26 H784I | Syn Rev T | 55 | - | 0.3 +/- 0.2 | 3.1 +/- 0.1 | 2.4 +/- 0.2 |
| | Syn Rev G | 58 | 11.3 +/- 0.2 | - | 9.0 +/- 0.2 | 3.4 +/- 0.2 |
| | Syn Rev C | 56 | 4.3 +/- 0.2 | 6.7 +/- 0.1 | - | 2.5 +/- 0.2 |

| DNA Polymerase | Reverse Primer | | Template | | | |
|---|---|---|---|---|---|---|
| | Name | SEQ ID NO. | A<br>SEQ ID NO. 51 | C<br>SEQ ID NO. 52 | G<br>SEQ ID NO. 53 | T<br>SEQ ID NO. 54 |
| | Syn Rev A | 57 | 6.3 +/- 0.1 | 0.7 +/- 0.1 | 10.0 +/- 0.2 | - |
| MUT ID 27 H784M | Syn Rev T | 55 | - | 4.5 +/- 0.2 | 6.9 +/- 0.2 | 9.6 +/- 0.5 |
| | Syn Rev G | 58 | 16.7 +/- 3.9 | - | 13.7 +/- 0.7 | 6.5 +/- 0.3 |
| | Syn Rev C | 56 | 9.5 +/- 0.3 | 11.0 +/- 0.4 | - | 7.4 +/- 0.3 |
| | Syn Rev A | 57 | 12.7 +/- 0.1 | 5.1 +/- 0.1 | 14.0 +/- 2.0 | - |
| MUT ID 29 H784F | Syn Rev T | 55 | - | 5.6 +/- 0.2 | 3.5 +/- 0.1 | 7.0 +/- 0.2 |
| | Syn Rev G | 58 | 13.3 +/- 0.6 | - | 10.3 +/- 0.3 | 3.0 +/- 0.2 |
| | Syn Rev C | 56 | 8.1 +/- 0.2 | 9.7 +/- 0.3 | - | 5.7 +/- 0.2 |
| | Syn Rev A | 57 | 10.9 +/- 0.3 | 4.6 +/- 0.2 | 11.3 +/- 0.4 | - |
| MUT ID 30 H784Y | Syn Rev T | 55 | - | 5.3 +/- 0.2 | 4.9 +/- 0.2 | 8.8 +/- 0.2 |
| | Syn Rev G | 58 | 15.7 +/- 4.6 | - | 11.8 +/- 0.5 | 5.5 +/- 0.3 |
| | Syn Rev C | 56 | 7.3 +/- 0.2 | 9.9 +/- 0.3 | - | 6.0 +/- 0.2 |
| | Syn Rev A | 57 | 10.2 +/- 0.2 | 4.5 +/- 0.2 | 10.5 +/- 0.3 | - |

Average ΔCq values are shown, where ΔCq = [Cq mismatch – Cq match], +/- standard deviation calculated from 96 replicates.

**[0219]** The wild type OptiTaq showed an average ΔCq for AS-qPCR in this synthetic amplicon system of 4.1 with a range of -0.8 to 8.5. Mutant ID 3 (H784Q) showed an average ΔCq of 9.9 with a range of 4.6 to 21.2. Mutant ID 20 (H784A) showed an average ΔCq of 11.2 with a range of 6.3 to 14.9. Mutant ID 21 (H784S) showed an average ΔCq of 15.3 with a range of 6.6 to 25.8. Mutant ID 22 (H784T) showed an average ΔCq of 6.6 with a range of 1.5 to 13.3. Mutant ID 24 (H784V) showed an average ΔCq of 4.1 with a range of -0.3 to 10.2. Mutant ID 26 (H784I) showed an average ΔCq of 5.0 with a range of 0.3 to 11.3. Mutant ID 27 (H784M) showed an average ΔCq of 9.8 with a range of 4.5 to 16.7. Mutant ID 29 (H784F) showed an average ΔCq of 7.8 with a range of 3.5 to 13.3. Mutant ID 30 (H784Y) showed an average ΔCq of 8.3 with a range of 5.3 to 15.7. Therefore, in nearly all pairwise combinations of 4 template bases and 4 3'-terminal primer bases, the mutant Taq DNA polymerases of the present invention showed greater discrimination to mismatch than did the wild type OptiTaq DNA polymerase. The magnitude of improvement for each mismatch pair is defined by the ΔΔCq, which is the difference of discrimination between the mutant and wild type enzymes (ΔΔCq = ΔCq mutant – ΔCq wild type). The ΔΔCq values were calculated and are shown in Table 32.

**Table 32: ΔΔCq values for AS-qPCR reactions for the H784 mutant Taq DNA polymerases compared with wild type OptiTaq.**

| DNA Polymerase | Reverse Primer | | Template | | | |
|---|---|---|---|---|---|---|
| | Name | SEQ ID NO. | A<br>SEQ ID NO. 51 | C<br>SEQ ID NO. 52 | G<br>SEQ ID NO. 53 | T<br>SEQ ID NO. 54 |
| MUT ID NO. 3<br>H784Q | Syn Rev T | 55 | - | 4.3 | 5.1 | 6.0 |
| | Syn Rev G | 58 | 6.0 | - | 6.6 | 3.3 |
| | Syn Rev C | 56 | 5.7 | 2.9 | - | 3.8 |
| | Syn Rev A | 57 | 5.0 | 4.9 | 4.9 | - |

| DNA Polymerase | Reverse Primer | | Template | | | |
|---|---|---|---|---|---|---|
| | Name | SEQ ID NO. | A<br>SEQ ID NO. 51 | C<br>SEQ ID NO. 52 | G<br>SEQ ID NO. 53 | T<br>SEQ ID NO. 54 |
| MUT ID 20 H784A | Syn Rev T | 55 | - | 6.2 | 7.1 | 7.5 |
| | Syn Rev G | 58 | 10.6 | - | 8.9 | 2.8 |
| | Syn Rev C | 56 | 6.8 | 4.7 | - | 4.3 |
| | Syn Rev A | 57 | 9.6 | 8.4 | 8.1 | - |
| MUT ID 21 H784S | Syn Rev T | 55 | - | 6.5 | 19.0 | 3.8 |
| | Syn Rev G | 58 | 17.3 | - | 18.0 | 3.1 |
| | Syn Rev C | 56 | 8.6 | 8.7 | - | 5.2 |
| | Syn Rev A | 57 | 17.8 | 9.2 | 16.8 | - |
| MUT ID 22 H784T | Syn Rev T | 55 | - | 0.1 | 3.1 | 0.8 |
| | Syn Rev G | 58 | 4.8 | - | 4.8 | 0.4 |
| | Syn Rev C | 56 | 2.4 | 1.6 | - | -0.6 |
| | Syn Rev A | 57 | 4.5 | 3.2 | 4.9 | - |
| MUT ID 24 H784V | Syn Rev T | 55 | - | -1.7 | 1.2 | -2.2 |
| | Syn Rev G | 58 | 1.7 | - | 2.5 | -0.7 |
| | Syn Rev C | 56 | 0.0 | -3.1 | - | -2.1 |

| DNA Polymerase | Reverse Primer | | Template | | | |
|---|---|---|---|---|---|---|
| | Name | SEQ ID NO. | A<br>SEQ ID NO. 51 | C<br>SEQ ID NO. 52 | G<br>SEQ ID NO. 53 | T<br>SEQ ID NO. 54 |
| | Syn Rev A | 57 | 0.1 | 1.0 | 3.1 | - |
| MUT ID 26 H784I | Syn Rev T | 55 | - | -1.1 | 2.5 | -2.4 |
| | Syn Rev G | 58 | 2.8 | - | 3.1 | -0.1 |
| | Syn Rev C | 56 | 1.5 | -1.0 | - | -1.4 |
| | Syn Rev A | 57 | 1.0 | 1.5 | 3.9 | - |
| MUT ID 27 H784M | Syn Rev T | 55 | - | 3.1 | 6.3 | 4.8 |
| | Syn Rev G | 58 | 8.2 | - | 7.8 | 3.0 |
| | Syn Rev C | 56 | 6.7 | 3.3 | - | 3.5 |
| | Syn Rev A | 57 | 7.4 | 5.9 | 7.9 | - |
| MUT ID 29 H784F | Syn Rev T | 55 | - | 4.2 | 2.9 | 2.2 |
| | Syn Rev G | 58 | 4.8 | - | 4.4 | -0.5 |
| | Syn Rev C | 56 | 5.3 | 2.0 | - | 1.8 |
| | Syn Rev A | 57 | 5.6 | 5.4 | 5.2 | - |
| MUT ID 30 H784Y | Syn Rev T | 55 | - | 3.9 | 4.3 | 4.0 |
| | Syn Rev G | 58 | 7.2 | - | 5.9 | 2.0 |

| DNA Polymerase | Reverse Primer | | Template | | | |
|---|---|---|---|---|---|---|
| | Name | SEQ ID NO. | A<br>SEQ ID NO. 51 | C<br>SEQ ID NO. 52 | G<br>SEQ ID NO. 53 | T<br>SEQ ID NO. 54 |
| | Syn Rev C | 56 | 4.5 | 2.2 | - | 2.1 |
| | Syn Rev A | 57 | 4.9 | 5.3 | 4.4 | - |

Average ΔΔCq values are shown, where ΔΔCq = [ΔCq mutant – ΔCq wild type], from data in Table 17.

**[0220]** Mutant ID 3 (H784Q) showed an average ΔΔCq of 4.9 compared to wild type OptiTaq. Mutant ID 20 (H784A) showed an average ΔΔCq of 7.1 compared to wild type OptiTaq. Mutant ID 21 (H784S) showed an average ΔΔCq of 11.2 compared to wild type OptiTaq. Mutant ID 22 (H784T) showed an average ΔΔCq of 2.5 compared to wild type OptiTaq. Mutant ID 24 (H784V) showed an average ΔΔCq of -0.2 compared to wild type OptiTaq. Mutant ID 26 (H784I) showed an average ΔΔCq of 1.0 compared to wild type OptiTaq. Mutant ID 27 (H784M) showed an average ΔΔCq of 5.7 compared to wild type OptiTaq. Mutant ID 29 (H784F) showed an average ΔΔCq of 3.6 compared to wild type OptiTaq. Mutant ID 30 (H784Y) showed an average ΔΔCq of 4.2 compared to wild type OptiTaq. Therefore, with the exception of Mutant ID 24 (H784V), each of the mutant Taq DNA polymerases of the present invention showed a significant improvement over wild type OptiTaq in mismatch discrimination. Overall, mutant ID 21 (H784S) showed the best SNP discrimination within the set of 9 mutant enzymes studied in this example using this AS-PCR assay.

**Example 15: Improved mismatch discrimination in rhPCR using mutant Taq DNA polymerases in a human genomic DNA SNP assay.**

**[0221]** Example 14 demonstrated utility of the novel mutant Taq DNA polymerases of the present invention in a synthetic amplicon rhPCR SNP discrimination assay system. The present Example demonstrates utility of the novel mutant Taq DNA polymerases in a human genomic DNA rhPCR SNP discrimination assays system, examining a SNP site in the SMAD7 gene

(NM_005904, C/T SNP, rs4939827). The assays employed target DNAs GM18562 (homozygous C/C) and GM18537 (homozygous T/T) from the Coriell Institute for Medical Research (Camden, NJ, USA). Two different blocked-cleavable primer designs were tested, including the generation 1 (Gen1) "RDDDDx" primers and the generation 2 (Gen2) "RDxxD" primers (see: US Patent Application 2012/0258455 by Behlke et al., entitled, RNASE H-BASED ASSAYS UTILIZING MODIFIED RNA MONOMERS).

**[0222]** Quantitative real-time rhPCR was performed in 10 μL reaction volumes in 384 well format with 20 ng (the equivalent of 6600 copies of target) of human genomic DNA (GM18562 or GM18537). Reactions utilized either 0.5 U (10.8 ng/11.1 nM/111 fmol) of wild type OptiTaq DNA polymerase or 0.5 U of one of the nine Taq DNA polymerase mutants (MUT ID 3, H784Q; MUT ID 20, H784A; MUT ID 21, H784S; MUT ID 22, H784T; MUT ID 24, H784V; MUT ID 26, H784I; MUT ID 27 H784M MUT ID 29, H784F; MUT ID 30, H784Y). Final reaction conditions used were 20 mM Tris-HCL (pH 8.4 at 25°C), 50 mM KCL, 3 mM $MgCl_2$, 0.01% Triton X-100, 800 μM total dNTPs, 200 nM of a forward primer (SEQ ID NOs. 75-79), 200 nM of the universal reverse primer (SEQ ID NO. 74), and 200 nM of the SMAD7 probe (SEQ ID NO. 80). Sequence of the 85 bp SMAD7 amplicon is shown as SEQ ID NO. 81. Forward primers included RDDDDx configuration Gen1 allele-specific rhPCR primers (SEQ ID NOs. 76 and 77), RDxxD configuration Gen2 allele-specific rhPCR primers (SEQ ID NOs. 78 and 79) and the control universal forward primer (SEQ ID NO.75) which is not allele specific. Oligonucleotide reagents employed in this Example are shown in Table 33. Reactions included 1 μL of *P.a.* RNase H2 at a concentration of 2.6 mU per 10 μL reaction (5 fmoles, 0.5 nM) with the exception of MUT ID 21 (H784S) for which 200 mU per 10 μL (384 fmoles, 38.4 nM) was used for the Gen1 RDDDDx primers and control primer (SEQ ID NOs. 75-77) or 200 mU per 10 μL reaction (384 fmoles, 38.4 nM) for the Gen2 RDxxD primers (SEQ ID NOs. 78 and 79). Amplification was performed on a Roche LightCycler® 480 (Roche Applied Science, Indianapolis, IN, USA) as follows: 95°C for 3 minutes followed by 95 cycles of 95°C for 10 seconds and 60°C for 30 seconds. All reactions were performed in triplicate.

**Table 33. Synthetic oligonucleotides employed in Example 14.**

| Name | Sequence (5'-3') | SEQ ID NO. |
|---|---|---|
| SMAD7 Rev | CTCACTCTAAACCCCAGCATT | 74 |
| SMAD7 For | CAGCCTCATCCAAAAGAGGAAA | 75 |
| SMAD7 For rC DDDDx | CAGCCTCATCCAAAAGAGGAAAcAGGAx | 76 |
| SMAD7 For rU DDDDx | CAGCCTCATCCAAAAGAGGAAAuAGGAx | 77 |
| SMAD7 For rC DxxD | CAGCCTCATCCAAAAGAGGAAAcAxxA | 78 |
| SMAD7 For rU DxxD | CAGCCTCATCCAAAAGAGGAAAuAxxA | 79 |
| SMAD7 probe | FAM-CCCAGAGCTCCCTCAGACTCCT-IBFQ | 80 |
| SMAD7 target | CAGCCTCATCCAAAAGAGGAAATAGGACCCCAGAGCTCCCTCA GACTCCTCAGGAAACACAGACAATGCTGGGGTTTAGAGTGAG | 81 |

DNA bases are uppercase and RNA bases are lowercase; FAM = 6-carboxyfluorescein; IBFQ = Iowa Black™ FQ fluorescence quencher; "x" = C3 Spacer (propanediol). Primer and probe binding sites in the SMAD7 target are underlined.

**[0223]** Results using the Gen1 RDDDDx rhPCR primers are shown in Table 34 using the Gen2 RDxxD rhPCR primers are shown in Table 35. Use of the mutant Taq DNA polymerases showed significant improvements in SNP discrimination in this human genomic DNA rhPCR assay using the Gen1 RDDDDx primers, although amplification efficiency was often reduced, as shown by the increases in the match Cqs. Large improvements in discrimination were seen using the Gen2 RDxxD primers, although amplification efficiency was often lost here as well. The Gen2 RDxxD primers inherently show greater SNP discrimination and these levels were increased so that ΔCq values are in some cases were greater than 40 amplification cycles between match and mismatch; this level of discrimination would be "greater than assay" for most users, as qPCR reactions are seldom run for over 45-50 cycles and positive signal was not

detected in these cases until after 70 cycles (Table 35). Therefore use of the new mutant Taq DNA polymerases improves SNP discrimination in rhPCR genotyping assays.

**Table 34: SNP discrimination of a site in the SMAD7 gene using Gen1 RDDDDx primers comparing wild type OptiTaq with four mutant Taq DNA polymerases.**

| DNA Polymerase | For Primer | SEQ ID NO. | mU RNase H2 per 10 µL rxn | Cq Value C/C DNA | Cq Value T/T DNA | ΔCq |
|---|---|---|---|---|---|---|
| Wild type OptiTaq | SMAD7 For | 75 | 2.6 | 24.1 | 24.9 | - |
| | SMAD7 For rC DDDDx | 76 | 2.6 | 24.3 | 36.3 | 11.9 |
| | SMAD7 For rU DDDDx | 77 | 2.6 | 35.1 | 27.5 | 7.6 |
| MUT ID 3 H784Q | SMAD7 For | 75 | 2.6 | 26.0 | 28.1 | - |
| | SMAD7 For rC DDDDx | 76 | 2.6 | 29.4 | 49.1 | 19.7 |
| | SMAD7 For rU DDDDx | 77 | 2.6 | 48.1 | 37.6 | 10.4 |
| MUT ID 20 H784A | SMAD7 For | 75 | 2.6 | 31.4 | 33.9 | - |
| | SMAD7 For rC DDDDx | 76 | 2.6 | 37.4 | 75.9 | 38.4 |
| | SMAD7 For rU DDDDx | 77 | 2.6 | 65.4 | 46.2 | 19.3 |
| MUT ID 21 H784S | SMAD7 For | 75 | 200 | 29.7 | 30.4 | - |
| | SMAD7 For rC DDDDx | 76 | 200 | 32.0 | 46.1 | 14.1 |
| | SMAD7 For rU DDDDx | 77 | 200 | 47.1 | 32.8 | 14.3 |
| MUT ID 22 H784T | SMAD7 For | 75 | 2.6 | 24.2 | 24.9 | - |
| | SMAD7 For rC DDDDx | 76 | 2.6 | 25.8 | 39.0 | 13.3 |

| DNA Polymerase | For Primer | SEQ ID NO. | mU RNase H2 per 10 μL rxn | Cq Value C/C DNA | Cq Value T/T DNA | ΔCq |
|---|---|---|---|---|---|---|
| | SMAD7 For rU DDDDx | 77 | 2.6 | 37.6 | 28.8 | 8.8 |
| MUT ID 24 H784V | SMAD7 For | 75 | 2.6 | 24.4 | 24.1 | |
| | SMAD7 For rC DDDDx | 76 | 2.6 | 24.7 | 34.5 | 9.8 |
| | SMAD7 For rU DDDDx | 77 | 2.6 | 36.0 | 25.6 | 10.4 |
| MUT ID 26 H784I | SMAD7 For | 75 | 2.6 | 24.4 | 24.9 | |
| | SMAD7 For rC DDDDx | 76 | 2.6 | 28.5 | 40.5 | 12.0 |
| | SMAD7 For rU DDDDx | 77 | 2.6 | 42.5 | 30.6 | 11.8 |
| MUT ID 27 H784M | SMAD7 For | 75 | 2.6 | 30.9 | 30.5 | |
| | SMAD7 For rC DDDDx | 76 | 2.6 | 36.1 | 58.8 | 22.7 |
| | SMAD7 For rU DDDDx | 77 | 2.6 | 51.7 | 37.7 | 14.0 |
| MUT ID 29 H784F | SMAD7 For | 75 | 2.6 | 25.8 | 26.5 | |
| | SMAD7 For rC DDDDx | 76 | 2.6 | 30.9 | 50.9 | 19.9 |
| | SMAD7 For rU DDDDx | 77 | 2.6 | 46.9 | 36.2 | 10.7 |
| MUT ID 29 H784Y | SMAD7 For | 75 | 2.6 | 27.3 | 26.7 | |
| | SMAD7 For rC DDDDx | 76 | 2.6 | 31.4 | 46.6 | 15.3 |
| | SMAD7 For rU DDDDx | 77 | 2.6 | 50.2 | 37.1 | 13.1 |

DNA targets included GM18562 (homozygous C/C) and GM18537 (homozygous T/T) from the Coriell Institute for Medical Research. ΔCq = [Cq mismatch – Cq match].

**Table 35: SNP discrimination of a site in the SMAD7 gene using Gen2 RDxxD primers comparing wild type OptiTaq with four mutant Taq DNA polymerases.**

| DNA Polymerase | For Primer | SEQ ID NO. | mU RNase H2 per 10 µL rxn | Cq Value C/C DNA | Cq Value T/T DNA | ΔCq |
|---|---|---|---|---|---|---|
| Wild type OptiTaq | SMAD7 For | 75 | 200 | 24.7 | 25.1 | - |
| | SMAD7 For rC DxxD | 78 | 200 | 24.9 | 39.6 | 14.7 |
| | SMAD7 For rU DxxD | 79 | 200 | 43.4 | 26.0 | 17.4 |
| MUT ID 3 H784Q | SMAD7 For | 75 | 200 | 26.5 | 27.5 | - |
| | SMAD7 For rC DxxD | 78 | 200 | 27.2 | 56.0 | 28.8 |
| | SMAD7 For rU DxxD | 79 | 200 | 73.4 | 37.1 | 36.3 |
| MUT ID 20 H784A | SMAD7 For | 75 | 200 | 26.0 | 26.7 | - |
| | SMAD7 For rC DxxD | 78 | 200 | 26.1 | 58.7 | 32.6 |
| | SMAD7 For rU DxxD | 79 | 200 | 64.1 | 33.2 | 31.2 |
| MUT ID 21 H784S | SMAD7 For | 75 | 200 | 27.0 | 27.3 | - |
| | SMAD7 For rC DxxD | 78 | 200 | 29.9 | 69.1 | 39.3 |
| | SMAD7 For rU DxxD | 79 | 200 | >95 | 62.6 | >32.4 |
| MUT ID 22 H784T | SMAD7 For | 75 | 200 | 24.8 | 25.2 | - |
| | SMAD7 For rC DxxD | 78 | 200 | 24.8 | 45.2 | 20.4 |

| DNA Polymerase | For Primer | SEQ ID NO. | mU RNase H2 per 10 μL rxn | Cq Value C/C DNA | Cq Value T/T DNA | ΔCq |
|---|---|---|---|---|---|---|
| | SMAD7 For rU DxxD | 79 | 200 | 57.5 | 26.8 | 30.8 |
| MUT ID 24 H784V | SMAD7 For | 75 | 200 | 25.3 | 24.8 | |
| | SMAD7 For rC DxxD | 78 | 200 | 25.3 | 39.9 | 14.6 |
| | SMAD7 For rU DxxD | 79 | 200 | 39.3 | 24.8 | 39.3 |
| MUT ID 26 H784I | SMAD7 For | 75 | 200 | 24.6 | 24.8 | |
| | SMAD7 For rC DxxD | 78 | 200 | 24.8 | 44.0 | 19.2 |
| | SMAD7 For rU DxxD | 79 | 200 | 46.2 | 26.9 | 46.2 |
| MUT ID 27 H784M | SMAD7 For | 75 | 200 | 30.0 | 29.7 | |
| | SMAD7 For rC DxxD | 78 | 200 | 31.9 | 80.1 | 48.2 |
| | SMAD7 For rU DxxD | 79 | 200 | 83.1 | 40.6 | 83.1 |
| MUT ID 29 H784F | SMAD7 For | 75 | 200 | 27.3 | 26.1 | |
| | SMAD7 For rC DxxD | 78 | 200 | 27.8 | 51.3 | 23.6 |
| | SMAD7 For rU DxxD | 79 | 200 | 56.3 | 29.1 | 56.3 |
| MUT ID 30 H784Y | SMAD7 For | 75 | 200 | 29.0 | 28.7 | |
| | SMAD7 For rC DxxD | 78 | 200 | 29.2 | 71.8 | 42.5 |
| | SMAD7 For rU DxxD | 79 | 200 | 73.5 | 30.4 | 73.5 |

DNA targets included GM18562 (homozygous C/C) and GM18537 (homozygous T/T) from the Coriell Institute for Medical Research. ΔCq = [Cq mismatch – Cq match].

**[0224]** The ΔCq values for the SMAD7 SNP genotyping assays are graphically summarized in Figure 5B and 5C for the Gen1 RDDDDx primers and in Figure 6B and 6C for the Gen2 RDxxD primers. It is clear that not only do the different mutant Taq DNA polymerases of the present invention have utility in different amplification assays but that the different mutants show varying levels of benefit depending on the nature of the assay used. It is therefore useful to have a collection of mutant polymerases whose properties can be matched to different assays/applications so that maximal benefit is obtained.

**Example 16: Improved discrimination of rare alleles in genomic DNA using rhPCR with mutant Taq DNA polymerases.**

**[0225]** Use of the Gen2 RDxxD blocked-cleavable primers in rhPCR can detect the presence of a SNP at a level of 1:1,000 to 1:10,000 in the background of wild type genomic DNA using native (wild type) Taq DNA polymerase (see: US Patent Application 2012/0258455 by Behlke et al., entitled, RNASE H-BASED ASSAYS UTILIZING MODIFIED RNA MONOMERS). The present example demonstrates that the mutant Taq DNA polymerases of the present invention improve rare allele discrimination in the rhPCR assay.

**[0226]** Rare allele detection experiments were designed to detect the base identity of a SNP site in the SMAD7 gene (NM_005904, C/T SNP, rs4939827) and employed target DNAs GM18562 (homozygous C/C) and GM18537 (homozygous T/T) (Coriell Institute for Medical Research, Camden, NJ, USA). Control reactions were set up using 2 ng (660 copies), 0.2 ng (66 copies), or 0.02 ng (6.6 copies) of input matched target DNA. Rare allele detection reactions were set up using 2 ng (660 copies), 0.2 ng (66 copies), or 0.02 ng (6.6 copies) of input matched target DNA of one allele plus 200 ng (66,000 copies) of the other (mismatched) allele. Background was established in reactions that contained 0 copies of matched target DNA plus 200 ng (66,000 copies) of the mismatched target DNA. Both combinations were tested: GM18562 (C/C) as the rare allele in the presence of excess GM18537 (T/T) and GM18537 (T/T) as the rare allele in the presence of excess GM18562 (C/C).

**[0227]** Quantitative real-time rhPCR was performed in 10 μL reaction volumes in 384 well format. Final reaction conditions used were 10 mM Tris-HCL (pH 8.4 at 25°C), 50 mM KCL,

3.5 mM $MgCl_2$, 0.01% Triton-X100, 0.8 mM dNTPs, 200 nM of one of the SMAD7 forward primers (SEQ ID NOs. 75, 78, and 79), 200 nM of the SMAD7 reverse primer (SEQ ID NO. 74), and 200 nM of the SMAD7 probe (SEQ ID NO. 80). The 85 bp SMAD7 amplicon defined by these primers is shown as SEQ ID NO. 81. Note that the forward primers were either unmodified (control, SEQ ID NO. 75) or were specific for the SMAD7 C-allele (SEQ ID NO. 78) or the SMAD7 T-allele (SEQ ID NO. 79) using blocked-cleavable rhPCR Gen2 RDxxD design. Reactions utilized either 0.5 U of the wild type OptiTaq DNA polymerase or 0.5 U of one of three example Taq DNA polymerase mutants studied (MUT ID 20(H784A); MUT ID 27(H784M); MUT ID 30(H784Y)). Reactions included *P.abyssi* RNase H2 at a concentration of 200 mU per 10 μL reaction (384 fmoles) when using the SMAD7 For rC DxxD (SEQ ID NO. 78) primer and control reactions or 500-600 mU per 10 μL reaction (960-1152 fmoles) when using the SMAD7 For rU DxxD (SEQ ID NO. 79) primer. Oligonucleotide reagents used in this Example are shown in Table 36. Cycling was performed on a Roche LightCycler® 480 (Roche Applied Science, Indianapolis, IN, USA) as follows: 95° C for 3 minutes followed by 65 cycles of 95° C for 10 seconds and 60° C for 30 seconds. All reactions were performed in triplicate.

**Table 36. Synthetic oligonucleotides employed in Example 16.**

| Name | Sequence (5'-3') | SEQ ID NO. |
|---|---|---|
| SMAD7 Rev | CTCACTCTAAACCCCAGCATT | 74 |
| SMAD7 For | CAGCCTCATCCAAAAGAGGAAA | 75 |
| SMAD7 For rC DxxD | CAGCCTCATCCAAAAGAGGAAAcAxxA | 78 |
| SMAD7 For rU DxxD | CAGCCTCATCCAAAAGAGGAAAuAxxA | 79 |
| SMAD7 probe | FAM-CCCAGAGCTCCCTCAGACTCCT-IBFQ | 80 |
| SMAD7 target | CAGCCTCATCCAAAAGAGGAAATAGGACCCCAGAGCTCCCTCA GACTCCTCAGGAAACACAGACAATGCTGGGGTTTAGAGTGAG | 81 |

DNA bases are uppercase and RNA bases are lowercase; FAM = 6-carboxyfluorescein; IBFQ = Iowa Black™ FQ fluorescence quencher; "x" = C3 Spacer (propanediol). Primer and probe binding sites in the SMAD7 target are underlined.

**[0228]** Results were analyzed and are shown in Table 37. The control columns show Cq values for matched primer/target reactions with no mismatched target present and establish a quantification standard curve. MUT ID NO. 3, H784Q is included in data analysis for comparison. The rare allele detection columns show Cq values for detection of 660, 66, 6, or 0 (background control) copies of matched primer/target in the presence of 66,000 copies of mismatched target. It is generally assumed that at least a 3 cycle difference ($\Delta$Cq = 3.0 or greater) between background and positive signal is needed to call a reaction "positive" for rare allele detection; a 5 cycle difference ($\Delta$Cq = 5.0 or greater) is preferred. In this system, background is the signal observed when amplification is done using no input target that is matched to the primer, so signal arises solely from amplification originating off the mismatched target.

**[0229]** Using wild type OptiTaq DNA polymerase, detection of the "C" allele in an excess of "T" background and detection of the "T" allele in an excess of "C" background both met the $\Delta$Cq 3.0 and $\Delta$Cq 5.0 levels of stringency to call a 1:1000 rare allele detection event (66 copies of match target in the presence of 66,000 copies of mismatch target). The 1:10,000 reactions (6 copies of match target in the presence of 66,000 copies of mismatch target) did not meet either of these criteria. Thus rhPCR had a 1:1000 rare allele detection limit using wild type OptiTaq in this genomic DNA SNP system.

**[0230]** In contrast, rhPCR using each of the four mutants showed a 1:10,000 rare allele detection limit for both the "C" and "T" allele targets with a $\Delta$Cq stringency cutoff of 3.0. MUT ID 3 (H784Q) showed a 1:10,000 rare allele detection limit for both the "C" and "T" targets in this genomic SNP system for the higher $\Delta$Cq stringency cutoff of 5.0. The other three mutant Taq DNA polymerases (MUT ID 20(H784A); MUT ID 27(H784M); MUT ID 30(H784Y)) showed a 1:10,000 rare allele detection limit for the "C" allele target with a $\Delta$Cq stringency cutoff of 5.0 and a 1:10,000 rare allele detection limit for the "T" allele target with a $\Delta$Cq stringency cutoff of 3.0. We therefore conclude that the new mutant Taq DNA polymerases of the present invention provide for improved rare allele detection reactions using blocked-cleavable primers in rhPCR compared with use of the wild type DNA polymerase.

**Table 37. Rare allele detection using Gen2 RDxxD rhPCR primers comparing wild type OptiTaq with new mutant Taq DNA polymerases**

| DNA Polymerase | For Primer | SEQ ID NO. | RNase H2 per 10 µL rxn | 200 ng mismatched template (66,000 copies of "wild type") | | | | Control (No mismatched template) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 660 Match (1:100) | 66 Match (1:1,000) | 6 Match (1:10,000) | 0 Match (background) | 660 Match | 66 Match | 6 Match | 0 Match |
| Wild type OptiTaq | SMAD7 For | 75 | 200 mU | 22.1 | 21.2 | 21.2 | 21.8 | 27.9 | 31.3 | 34.4 | >65 |
| | SMAD7 For rC DxxD | 78 | 200 mU | **28.2** | **31.5** | 35.1 | 37.0 | 28.8 | 33.3 | 37.3 | >65 |
| | SMAD7 For rU DxxD | 79 | 500 mU | **31.0** | **34.7** | 37.7 | 39.7 | 31.2 | 34.6 | 41.0 | >65 |
| MUT ID 3 (H784Q) | SMAD7 For | 75 | 200 mU | 23.5 | 23.6 | 24.5 | 24.1 | 30.5 | 33.4 | 38.0 | >65 |
| | SMAD7 For rC DxxD | 78 | 200 mU | **29.8** | **33.8** | **37.6** | >65 | 30.5 | 35.5 | 39.6 | >65 |
| | SMAD7 For rU DxxD | 79 | 500 mU | **32.9** | **37.7** | **44.0** | 52.3 | 30.1 | 35.9 | 44.9 | >65 |
| MUT ID 20 (H784A) | SMAD7 For | 75 | 200 mU | 23.5 | 24.1 | 24.7 | 24.9 | 31.6 | 36.1 | 40.2 | >65 |
| | SMAD7 For rC DxxD | 78 | 200 mU | **31.3** | **36.7** | **43.2** | 55.3 | 33.5 | 39.5 | 44.7 | >65 |
| | SMAD7 For rU DxxD | 79 | 500 mU | **35.7** | **40.0** | **43.8** | 54.7 | 33.3 | 38.8 | 41.5 | >65 |
| MUT ID 27 (H784M) | SMAD7 For | 75 | 200 mU | 24.2 | 24.8 | 25.4 | 26.4 | 31.6 | 36.2 | 40.2 | >65 |

| DNA Polymerase | For Primer | SEQ ID NO. | RNase H2 per 10 µL rxn | 200 ng mismatched template (66,000 copies of "wild type") | | | | Control (No mismatched template) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 660 Match (1:100) | 66 Match (1:1,000) | 6 Match (1:10,000) | 0 Match (background) | 660 Match | 66 Match | 6 Match | 0 Match |
| | SMAD7 For rC DxxD | 78 | 200 mU | **33.7** | **38.5** | **42.0** | **54.4** | 33.6 | 37.8 | 41.7 | >65 |
| | SMAD7 For rU DxxD | 79 | 600 mU | **39.7** | **43.2** | **45.8** | **50.0** | 31.9 | 36.5 | 41.0 | >65 |
| MUT ID 30 (H784Y) | SMAD7 For | 75 | 200 mU | 24.8 | 24.9 | 24.8 | 26.3 | 38.6 | 38.9 | 46.9 | >65 |
| | SMAD7 For rC DxxD | 78 | 200 mU | **28.8** | **32.9** | **37.5** | 46.5 | 29.5 | 33.4 | 37.8 | >65 |
| | SMAD7 For rU DxxD | 79 | 500 mU | **39.9** | **45.8** | **54.6** | 57.0 | 37.7 | 44.6 | 53.1 | >65 |

Cq values are shown. For the rare allele detection series (selective detection of 6-660 copes one genotype in the presence of 66,000 copies of the other genotype), those reactions having a ΔCq of 3.0 or better are highlighted in bold font and those having a ΔCq of 5.0 or better are highlighted in bold font with underline. ΔCq = [(Cq 0 copies match) – (Cq 6 copies match)], or ΔCq = [(Cq 0 copies match) – (Cq 66 copies match)], or ΔCq = [(Cq 0 copies match) – (Cq 660 copies match)].

**Example 17. Sequence of Taq DNA polymerase mutants showing improved discrimination for mismatch or the presence of an RNA residue at the 3'-end of the primer.**

**[0231]** The complete amino acid and nucleotide sequences of the codon optimized mutant enzymes employed in Examples 11-15 are shown below. Although these sequences are easily derived from information provided in Tables 1, 3, 4 and 26 by one with skill in the art, the final assembled sequences are provided below for clarity. Base changes are identified in bold underlined font for the nucleic acid and amino acid substitutions.

**[0232]** SEQ ID NO. 146, nucleotide sequence of Mutant ID 20 (H784A).

CATATGCGTGGTATGCTGCCGTTGTTCGAGCCTAAAGGCCGCGTACTGTTAGTCGATGGTCATCACTTGGCCTAT
CGGACGTTCCATGCACTCAAAGGTCTGACGACCAGTCGTGGCGAACCGGTCCAGGCTGTTTATGGTTTCGCTAAG
TCTTTGCTCAAAGCACTGAAAGAAGACGGGGACGCGGTAATTGTTGTATTTGATGCCAAAGCACCGAGCTTCCGC
CACGAAGCTTATGGTGGCTACAAGGCAGGACGCGCCCCTACCCCAGAAGATTTCCCCCGTCAGCTGGCATTAATT
AAGGAGTTAGTAGACCTTCTCGGCTTAGCGCGTCTGGAAGTTCCGGGTTATGAGGCGGACGATGTCCTTGCATCC
TTGGCTAAAAAGGCCGAAAAAGAGGGCTACGAAGTCCGCATCTTGACGGCAGACAAAGATCTGTACCAGCTTCTG
TCTGACCGTATTCATGTTTTGCACCCTGAAGGCTACTTAATCACTCCGGCCTGGCTCTGGGAAAAGTACGGTCTG
CGTCCCGATCAGTGGGCGGATTATCGGGCTTTGACGGGAGATGAGAGCGACAACCTGCCAGGAGTTAAGGGCATT
GGTGAAAAAACCGCACGTAAGCTGCTTGAAGAGTGGGGTTCCCTGGAAGCCTTGTTAAAAAATCTGGATCGTCTC
AAGCCCGCAATTCGTGAAAAGATCCTGGCTCATATGGACGATCTTAAATTAAGTTGGGACCTGGCCAAGGTGCGC
ACCGATTTACCGCTTGAAGTGGATTTTGCAAAACGCCGTGAGCCGGACCGGGAACGTTTACGCGCTTTCTTAGAG
CGTCTGGAATTCGGTTCACTGCTTCATGAATTCGGTCTGTTAGAGTCTCCTAAAGCACTCGAAGAGGCACCGTGG
CCGCCCCCAGAAGGTGCTTTTGTTGGCTTCGTACTTTCCCGTAAGGAGCCTATGTGGGCAGATCTTCTGGCTTTA
GCGGCTGCACGCGGTGGCCGTGTTCACCGGGCCCCTGAGCCATACAAAGCGTTACGTGATCTGAAGGAAGCACGT
GGCTTGCTGGCAAAAGACCTTTCTGTTTTGGCCCTGCGCGAGGGTCTTGGACTGCCGCCAGGCGACGATCCCATG
TTATTGGCCTATCTGTTAGACCCTAGCAATACCACACCTGAAGGGGTCGCTCGTCGGTATGGCGGTGAATGGACT
GAGGAAGCCGGAGAGCGCGCCGCATTGTCCGAACGGCTCTTTGCAAACTTATGGGGTCGTCTGGAAGGGGAGGAA
CGTCTGTTATGGTTGTATCGGGAAGTCGAACGTCCTCTTTCGGCCGTATTAGCGCATATGGAGGCAACAGGTGTG
CGTTTAGATGTCGCGTACCTTCGGGCCTTATCACTGGAAGTTGCAGAGGAAATCGCCCGTCTCGAGGCTGAAGTG
TTCCGGTTGGCCGGTCACCCGTTTAACCTCAACTCCCGTGACCAGCTGGAACGCGTTTTATTCGATGAGCTTGGG
CTTCCCGCAATTGGCAAAACCGAAAAGACTGGCAAACGCAGTACGAGCGCTGCCGTCCTTGAGGCACTCCGCGAG
GCTCACCCTATTGTAGAAAAGATCCTGCAATACCGTGAGTTGACGAAGCTTAAAAGCACTTATATTGATCCTCTC
CCGGATCTGATCCATCCTCGTACCGGCCGCTTGCACACACGTTTCAACCAGACGGCGACTGCAACCGGCCGTCTG
TCTAGCTCGGATCCAAATCTCCAGAACATTCCGGTCCGTACACCCTTGGGCCAACGTATCCGCCGGGCGTTTATC
GCTGAGGAAGGATGGTTACTGGTCGCATTGGACTACTCGCAGATTGAGCTGCGCGTCCTCGCACATCTCTCTGGT
GACGAAAATTTAATCCGCGTGTTTCAAGAGGGGCGTGATATTCACACAGAAACTGCCTCATGGATGTTCGGTGTC
CCACGTGAAGCAGTGGATCCTTTGATGCGCCGTGCAGCTAAAACAATTAATTTTGGAGTGCTGTACGGAATGAGC
GCTCATCGCTTGAGTCAGGAACTGGCAATCCCCTACGAGGAAGCGCAGGCATTCATCGAACGTTACTTTCAATCG
TTTCCGAAAGTTCGCGCATGGATCGAGAAGACGCTCGAGGAAGGTCGTCGTCGGGGCTATGTCGAAACTCTGTTT
GGTCGCCGTCGGTACGTACCAGATCTTGAAGCCCGCGTCAAATCGGTACGGGAGGCTGCGGAGCGTATGGCATTT
AATATGCCTGTACAGGGTACTGCAGCTGACCTCATGAAACTGGCAATGGTCAAGCTTTTCCCGCGCTTGGAGGAA
ATGGGCGCACGTATGCTTCTGCAGGTC**GCG**GACGAGCTGGTGTTAGAAGCCCCTAAGGAGCGCGCCGAAGCTGTC
GCGCGCCTCGCTAAAGAAGTGATGGAGGGCGTTTACCCATTGGCCGTACCCCTCGAAGTGGAGGTCGGTATTGGA
GAAGATTGGTTATCTGCAAAGGAAGCGGCCGC

**[0233]** SEQ ID NO. 147, amino acid sequence of Mutant ID 20 (H784A).

MRGMLPLFEPKGRVLLVDGHHLAYRTFHALKGLTTSRGEPVQAVYGFAKSLLKALKEDGDAVIVVFDAKAPSFRH
EAYGGYKAGRAPTPEDFPRQLALIKELVDLLGLARLEVPGYEADDVLASLAKKAEKEGYEVRILTADKDLYQLLS
DRIHVLHPEGYLITPAWLWEKYGLRPDQWADYRALTGDESDNLPGVKGIGEKTARKLLEEWGSLEALLKNLDRLK
PAIREKILAHMDDLKLSWDLAKVRTDLPLEVDFAKRREPDRERLRAFLERLEFGSLLHEFGLLESPKALEEAPWP
PPEGAFVGFVLSRKEPMWADLLALAAARGGRVHRAPEPYKALRDLKEARGLLAKDLSVLALREGLGLPPGDDPML
LAYLLDPSNTTPEGVARRYGGEWTEEAGERAALSERLFANLWGRLEGEERLLWLYREVERPLSAVLAHMEATGVR
LDVAYLRALSLEVAEEIARLEAEVFRLAGHPFNLNSRDQLERVLFDELGLPAIGKTEKTGKRSTSAAVLEALREA
HPIVEKILQYRELTKLKSTYIDPLPDLIHPRTGRLHTRFNQTATATGRLSSSDPNLQNIPVRTPLGQRIRRAFIA
EEGWLLVALDYSQIELRVLAHLSGDENLIRVFQEGRDIHTETASWMFGVPREAVDPLMRRAAKTINFGVLYGMSA
HRLSQELAIPYEEAQAFIERYFQSFPKVRAWIEKTLEEGRRRGYVETLFGRRRYVPDLEARVKSVREAAERMAFN
MPVQGTAADLMKLAMVKLFPRLEEMGARMLLQV**A**DELVLEAPKERAEAVARLAKEVMEGVYPLAVPLEVEVGIGE
DWLSAKEAA

**[0234]** SEQ ID NO. 148, nucleotide sequence of Mutant ID 21 (H784S).

CATATGCGTGGTATGCTGCCGTTGTTCGAGCCTAAAGGCCGCGTACTGTTAGTCGATGGTCATCACTTGGCCTAT
CGGACGTTCCATGCACTCAAAGGTCTGACGACCAGTCGTGGCGAACCGGTCCAGGCTGTTTATGGTTTCGCTAAG
TCTTTGCTCAAAGCACTGAAAGAAGACGGGGACGCGGTAATTGTTGTATTTGATGCCAAAGCACCGAGCTTCCGC
CACGAAGCTTATGGTGGCTACAAGGCAGGACGCGCCCCTACCCCAGAAGATTTCCCCCGTCAGCTGGCATTAATT
AAGGAGTTAGTAGACCTTCTCGGCTTAGCGCGTCTGGAAGTTCCGGGTTATGAGGCGGACGATGTCCTTGCATCC
TTGGCTAAAAAGGCCGAAAAAGAGGGCTACGAAGTCCGCATCTTGACGGCAGACAAAGATCTGTACCAGCTTCTG
TCTGACCGTATTCATGTTTTGCACCCTGAAGGCTACTTAATCACTCCGGCCTGGCTCTGGGAAAAGTACGGTCTG
CGTCCCGATCAGTGGGCGGATTATCGGGCTTTGACGGGAGATGAGAGCGACAACCTGCCAGGAGTTAAGGGCATT
GGTGAAAAAACCGCACGTAAGCTGCTTGAAGAGTGGGGTTCCCTGGAAGCCTTGTTAAAAAATCTGGATCGTCTC
AAGCCCGCAATTCGTGAAAAGATCCTGGCTCATATGGACGATCTTAAATTAAGTTGGGACCTGGCCAAGGTGCGC
ACCGATTTACCGCTTGAAGTGGATTTTGCAAAACGCCGTGAGCCGGACCGGGAACGTTTACGCGCTTTCTTAGAG
CGTCTGGAATTCGGTTCACTGCTTCATGAATTCGGTCTGTTAGAGTCTCCTAAAGCACTCGAAGAGGCACCGTGG
CCGCCCCCAGAAGGTGCTTTTGTTGGCTTCGTACTTTCCCGTAAGGAGCCTATGTGGGCAGATCTTCTGGCTTTA
GCGGCTGCACGCGGTGGCCGTGTTCACCGGGCCCCTGAGCCATACAAAGCGTTACGTGATCTGAAGGAAGCACGT
GGCTTGCTGGCAAAAGACCTTTCTGTTTTGGCCCTGCGCGAGGGTCTTGGACTGCCGCCAGGCGACGATCCCATG
TTATTGGCCTATCTGTTAGACCCTAGCAATACCACACCTGAAGGGGTCGCTCGTCGGTATGGCGGTGAATGGACT
GAGGAAGCCGGAGAGCGCGCCGCATTGTCCGAACGGCTCTTTGCAAACTTATGGGGTCGTCTGGAAGGGGAGGAA
CGTCTGTTATGGTTGTATCGGGAAGTCGAACGTCCTCTTTCGGCCGTATTAGCGCATATGGAGGCAACAGGTGTG
CGTTTAGATGTCGCGTACCTTCGGGCCTTATCACTGGAAGTTGCAGAGGAAATCGCCCGTCTCGAGGCTGAAGTG
TTCCGGTTGGCCGGTCACCCGTTTAACCTCAACTCCCGTGACCAGCTGGAACGCGTTTTATTCGATGAGCTTGGG
CTTCCCGCAATTGGCAAAACCGAAAAGACTGGCAAACGCAGTACGAGCGCTGCCGTCCTTGAGGCACTCCGCGAG
GCTCACCCTATTGTAGAAAAGATCCTGCAATACCGTGAGTTGACGAAGCTTAAAAGCACTTATATTGATCCTCTC
CCGGATCTGATCCATCCTCGTACCGGCCGCTTGCACACACGTTTCAACCAGACGGCGACTGCAACCGGCCGTCTG
TCTAGCTCGGATCCAAATCTCCAGAACATTCCGGTCCGTACACCCTTGGGCCAACGTATCCGCCGGGCGTTTATC
GCTGAGGAAGGATGGTTACTGGTCGCATTGGACTACTCGCAGATTGAGCTGCGCGTCCTCGCACATCTCTCTGGT
GACGAAAATTTAATCCGCGTGTTTCAAGAGGGGCGTGATATTCACACAGAAACTGCCTCATGGATGTTCGGTGTC
CCACGTGAAGCAGTGGATCCTTTGATGCGCCGTGCAGCTAAAACAATTAATTTTGGAGTGCTGTACGGAATGAGC
GCTCATCGCTTGAGTCAGGAACTGGCAATCCCCTACGAGGAAGCGCAGGCATTCATCGAACGTTACTTTCAATCG
TTTCCGAAAGTTCGCGCATGGATCGAGAAGACGCTCGAGGAAGGTCGTCGTCGGGGCTATGTCGAAACTCTGTTT
GGTCGCCGTCGGTACGTACCAGATCTTGAAGCCCGCGTCAAATCGGTACGGGAGGCTGCGGAGCGTATGGCATTT
AATATGCCTGTACAGGGTACTGCAGCTGACCTCATGAAACTGGCAATGGTCAAGCTTTTCCCGCGCTTGGAGGAA
ATGGGCGCACGTATGCTTCTGCAGGTC**AGC**GACGAGCTGGTGTTAGAAGCCCCTAAGGAGCGCGCCGAAGCTGTC
GCGCGCCTCGCTAAAGAAGTGATGGAGGGCGTTTACCCATTGGCCGTACCCCTCGAAGTGGAGGTCGGTATTGGA
GAAGATTGGTTATCTGCAAAGGAAGCGGCCGC

**[0235]** SEQ ID NO. 149, amino acid sequence of Mutant ID 21 (H784S).

MRGMLPLFEPKGRVLLVDGHHLAYRTFHALKGLTTSRGEPVQAVYGFAKSLLKALKEDGDAVIVVFDAKAPSFRH
EAYGGYKAGRAPTPEDFPRQLALIKELVDLLGLARLEVPGYEADDVLASLAKKAEKEGYEVRILTADKDLYQLLS
DRIHVLHPEGYLITPAWLWEKYGLRPDQWADYRALTGDESDNLPGVKGIGEKTARKLLEEWGSLEALLKNLDRLK
PAIREKILAHMDDLKLSWDLAKVRTDLPLEVDFAKRREPDRERLRAFLERLEFGSLLHEFGLLESPKALEEAPWP
PPEGAFVGFVLSRKEPMWADLLALAAARGGRVHRAPEPYKALRDLKEARGLLAKDLSVLALREGLGLPPGDDPML
LAYLLDPSNTTPEGVARRYGGEWTEEAGERAALSERLFANLWGRLEGEERLLWLYREVERPLSAVLAHMEATGVR
LDVAYLRALSLEVAEEIARLEAEVFRLAGHPFNLNSRDQLERVLFDELGLPAIGKTEKTGKRSTSAAVLEALREA
HPIVEKILQYRELTKLKSTYIDPLPDLIHPRTGRLHTRFNQTATATGRLSSSDPNLQNIPVRTPLGQRIRRAFIA
EEGWLLVALDYSQIELRVLAHLSGDENLIRVFQEGRDIHTETASWMFGVPREAVDPLMRRAAKTINFGVLYGMSA
HRLSQELAIPYEEAQAFIERYFQSFPKVRAWIEKTLEEGRRRGYVETLFGRRRYVPDLEARVKSVREAAERMAFN
MPVQGTAADLMKLAMVKLFPRLEEMGARMLLQV**S**DELVLEAPKERAEAVARLAKEVMEGVYPLAVPLEVEVGIGE
DWLSAKEAA

**[0236]** SEQ ID NO. 150, nucleotide sequence of Mutant ID 22 (H784T).

CATATGCGTGGTATGCTGCCGTTGTTCGAGCCTAAAGGCCGCGTACTGTTAGTCGATGGTCATCACTTGGCCTAT
CGGACGTTCCATGCACTCAAAGGTCTGACGACCAGTCGTGGCGAACCGGTCCAGGCTGTTTATGGTTTCGCTAAG
TCTTTGCTCAAAGCACTGAAAGAAGACGGGGACGCGGTAATTGTTGTATTTGATGCCAAAGCACCGAGCTTCCGC
CACGAAGCTTATGGTGGCTACAAGGCAGGACGCGCCCCTACCCCAGAAGATTTCCCCCGTCAGCTGGCATTAATT
AAGGAGTTAGTAGACCTTCTCGGCTTAGCGCGTCTGGAAGTTCCGGGTTATGAGGCGGACGATGTCCTTGCATCC
TTGGCTAAAAAGGCCGAAAAAGAGGGCTACGAAGTCCGCATCTTGACGGCAGACAAAGATCTGTACCAGCTTCTG
TCTGACCGTATTCATGTTTTGCACCCTGAAGGCTACTTAATCACTCCGGCCTGGCTCTGGGAAAAGTACGGTCTG
CGTCCCGATCAGTGGGCGGATTATCGGGCTTTGACGGGAGATGAGAGCGACAACCTGCCAGGAGTTAAGGGCATT
GGTGAAAAAACCGCACGTAAGCTGCTTGAAGAGTGGGGTTCCCTGGAAGCCTTGTTAAAAAATCTGGATCGTCTC
AAGCCCGCAATTCGTGAAAAGATCCTGGCTCATATGGACGATCTTAAATTAAGTTGGGACCTGGCCAAGGTGCGC
ACCGATTTACCGCTTGAAGTGGATTTTGCAAAACGCCGTGAGCCGGACCGGGAACGTTTACGCGCTTTCTTAGAG
CGTCTGGAATTCGGTTCACTGCTTCATGAATTCGGTCTGTTAGAGTCTCCTAAAGCACTCGAAGAGGCACCGTGG
CCGCCCCCAGAAGGTGCTTTTGTTGGCTTCGTACTTTCCCGTAAGGAGCCTATGTGGGCAGATCTTCTGGCTTTA
GCGGCTGCACGCGGTGGCCGTGTTCACCGGGCCCCTGAGCCATACAAAGCGTTACGTGATCTGAAGGAAGCACGT
GGCTTGCTGGCAAAAGACCTTTCTGTTTTGGCCCTGCGCGAGGGTCTTGGACTGCCGCCAGGCGACGATCCCATG
TTATTGGCCTATCTGTTAGACCCTAGCAATACCACACCTGAAGGGGTCGCTCGTCGGTATGGCGGTGAATGGACT
GAGGAAGCCGGAGAGCGCGCCGCATTGTCCGAACGGCTCTTTGCAAACTTATGGGGTCGTCTGGAAGGGGAGGAA
CGTCTGTTATGGTTGTATCGGGAAGTCGAACGTCCTCTTTCGGCCGTATTAGCGCATATGGAGGCAACAGGTGTG
CGTTTAGATGTCGCGTACCTTCGGGCCTTATCACTGGAAGTTGCAGAGGAAATCGCCCGTCTCGAGGCTGAAGTG
TTCCGGTTGGCCGGTCACCCGTTTAACCTCAACTCCCGTGACCAGCTGGAACGCGTTTTATTCGATGAGCTTGGG
CTTCCCGCAATTGGCAAAACCGAAAAGACTGGCAAACGCAGTACGAGCGCTGCCGTCCTTGAGGCACTCCGCGAG
GCTCACCCTATTGTAGAAAAGATCCTGCAATACCGTGAGTTGACGAAGCTTAAAAGCACTTATATTGATCCTCTC
CCGGATCTGATCCATCCTCGTACCGGCCGCTTGCACACACGTTTCAACCAGACGGCGACTGCAACCGGCCGTCTG
TCTAGCTCGGATCCAAATCTCCAGAACATTCCGGTCCGTACACCCTTGGGCCAACGTATCCGCCGGGCGTTTATC
GCTGAGGAAGGATGGTTACTGGTCGCATTGGACTACTCGCAGATTGAGCTGCGCGTCCTCGCACATCTCTCTGGT
GACGAAAATTTAATCCGCGTGTTTCAAGAGGGGCGTGATATTCACACAGAAACTGCCTCATGGATGTTCGGTGTC
CCACGTGAAGCAGTGGATCCTTTGATGCGCCGTGCAGCTAAAACAATTAATTTTGGAGTGCTGTACGGAATGAGC
GCTCATCGCTTGAGTCAGGAACTGGCAATCCCCTACGAGGAAGCGCAGGCATTCATCGAACGTTACTTTCAATCG
TTTCCGAAAGTTCGCGCATGGATCGAGAAGACGCTCGAGGAAGGTCGTCGTCGGGGCTATGTCGAAACTCTGTTT
GGTCGCCGTCGGTACGTACCAGATCTTGAAGCCCGCGTCAAATCGGTACGGGAGGCTGCGGAGCGTATGGCATTT
AATATGCCTGTACAGGGTACTGCAGCTGACCTCATGAAACTGGCAATGGTCAAGCTTTTCCCGCGCTTGGAGGAA
ATGGGCGCACGTATGCTTCTGCAGGTC**ACG**GACGAGCTGGTGTTAGAAGCCCCTAAGGAGCGCGCCGAAGCTGTC
GCGCGCCTCGCTAAAGAAGTGATGGAGGGCGTTTACCCATTGGCCGTACCCCTCGAAGTGGAGGTCGGTATTGGA
GAAGATTGGTTATCTGCAAAGGAAGCGGCCGC

**[0237]** SEQ ID NO. 151, amino acid sequence of Mutant ID 22 (H784T).

MRGMLPLFEPKGRVLLVDGHHLAYRTFHALKGLTTSRGEPVQAVYGFAKSLLKALKEDGDAVIVVFDAKAPSFRH
EAYGGYKAGRAPTPEDFPRQLALIKELVDLLGLARLEVPGYEADDVLASLAKKAEKEGYEVRILTADKDLYQLLS
DRIHVLHPEGYLITPAWLWEKYGLRPDQWADYRALTGDESDNLPGVKGIGEKTARKLLEEWGSLEALLKNLDRLK
PAIREKILAHMDDLKLSWDLAKVRTDLPLEVDFAKRREPDRERLRAFLERLEFGSLLHEFGLLESPKALEEAPWP
PPEGAFVGFVLSRKEPMWADLLALAAARGGRVHRAPEPYKALRDLKEARGLLAKDLSVLALREGLGLPPGDDPML
LAYLLDPSNTTPEGVARRYGGEWTEEAGERAALSERLFANLWGRLEGEERLLWLYREVERPLSAVLAHMEATGVR
LDVAYLRALSLEVAEEIARLEAEVFRLAGHPFNLNSRDQLERVLFDELGLPAIGKTEKTGKRSTSAAVLEALREA
HPIVEKILQYRELTKLKSTYIDPLPDLIHPRTGRLHTRFNQTATATGRLSSSDPNLQNIPVRTPLGQRIRRAFIA
EEGWLLVALDYSQIELRVLAHLSGDENLIRVFQEGRDIHTETASWMFGVPREAVDPLMRRAAKTINFGVLYGMSA
HRLSQELAIPYEEAQAFIERYFQSFPKVRAWIEKTLEEGRRRGYVETLFGRRRYVPDLEARVKSVREAAERMAFN
MPVQGTAADLMKLAMVKLFPRLEEMGARMLLQV**T**DELVLEAPKERAEAVARLAKEVMEGVYPLAVPLEVEVGIGE
DWLSAKEAA

**[0238]** SEQ ID NO. 152, nucleotide sequence of Mutant ID 24 (H784V).

CATATGCGTGGTATGCTGCCGTTGTTCGAGCCTAAAGGCCGCGTACTGTTAGTCGATGGTCATCACTTGGCCTAT
CGGACGTTCCATGCACTCAAAGGTCTGACGACCAGTCGTGGCGAACCGGTCCAGGCTGTTTATGGTTTCGCTAAG
TCTTTGCTCAAAGCACTGAAAGAAGACGGGGACGCGGTAATTGTTGTATTTGATGCCAAAGCACCGAGCTTCCGC
CACGAAGCTTATGGTGGCTACAAGGCAGGACGCGCCCCTACCCCAGAAGATTTCCCCCGTCAGCTGGCATTAATT
AAGGAGTTAGTAGACCTTCTCGGCTTAGCGCGTCTGGAAGTTCCGGGTTATGAGGCGGACGATGTCCTTGCATCC
TTGGCTAAAAAGGCCGAAAAAGAGGGCTACGAAGTCCGCATCTTGACGGCAGACAAAGATCTGTACCAGCTTCTG
TCTGACCGTATTCATGTTTTGCACCCTGAAGGCTACTTAATCACTCCGGCCTGGCTCTGGGAAAAGTACGGTCTG
CGTCCCGATCAGTGGGCGGATTATCGGGCTTTGACGGGAGATGAGAGCGACAACCTGCCAGGAGTTAAGGGCATT
GGTGAAAAAACCGCACGTAAGCTGCTTGAAGAGTGGGGTTCCCTGGAAGCCTTGTTAAAAAATCTGGATCGTCTC
AAGCCCGCAATTCGTGAAAAGATCCTGGCTCATATGGACGATCTTAAATTAAGTTGGGACCTGGCCAAGGTGCGC
ACCGATTTACCGCTTGAAGTGGATTTTGCAAAACGCCGTGAGCCGGACCGGGAACGTTTACGCGCTTTCTTAGAG
CGTCTGGAATTCGGTTCACTGCTTCATGAATTCGGTCTGTTAGAGTCTCCTAAAGCACTCGAAGAGGCACCGTGG
CCGCCCCCAGAAGGTGCTTTTGTTGGCTTCGTACTTTCCCGTAAGGAGCCTATGTGGGCAGATCTTCTGGCTTTA
GCGGCTGCACGCGGTGGCCGTGTTCACCGGGCCCCTGAGCCATACAAAGCGTTACGTGATCTGAAGGAAGCACGT
GGCTTGCTGGCAAAAGACCTTTCTGTTTTGGCCCTGCGCGAGGGTCTTGGACTGCCGCCAGGCGACGATCCCATG
TTATTGGCCTATCTGTTAGACCCTAGCAATACCACACCTGAAGGGGTCGCTCGTCGGTATGGCGGTGAATGGACT
GAGGAAGCCGGAGAGCGCGCCGCATTGTCCGAACGGCTCTTTGCAAACTTATGGGGTCGTCTGGAAGGGGAGGAA
CGTCTGTTATGGTTGTATCGGGAAGTCGAACGTCCTCTTTCGGCCGTATTAGCGCATATGGAGGCAACAGGTGTG
CGTTTAGATGTCGCGTACCTTCGGGCCTTATCACTGGAAGTTGCAGAGGAAATCGCCCGTCTCGAGGCTGAAGTG
TTCCGGTTGGCCGGTCACCCGTTTAACCTCAACTCCCGTGACCAGCTGGAACGCGTTTTATTCGATGAGCTTGGG
CTTCCCGCAATTGGCAAAACCGAAAAGACTGGCAAACGCAGTACGAGCGCTGCCGTCCTTGAGGCACTCCGCGAG
GCTCACCCTATTGTAGAAAAGATCCTGCAATACCGTGAGTTGACGAAGCTTAAAAGCACTTATATTGATCCTCTC
CCGGATCTGATCCATCCTCGTACCGGCCGCTTGCACACACGTTTCAACCAGACGGCGACTGCAACCGGCCGTCTG
TCTAGCTCGGATCCAAATCTCCAGAACATTCCGGTCCGTACACCCTTGGGCCAACGTATCCGCCGGGCGTTTATC
GCTGAGGAAGGATGGTTACTGGTCGCATTGGACTACTCGCAGATTGAGCTGCGCGTCCTCGCACATCTCTCTGGT
GACGAAAATTTAATCCGCGTGTTTCAAGAGGGGCGTGATATTCACACAGAAACTGCCTCATGGATGTTCGGTGTC
CCACGTGAAGCAGTGGATCCTTTGATGCGCCGTGCAGCTAAAACAATTAATTTTGGAGTGCTGTACGGAATGAGC
GCTCATCGCTTGAGTCAGGAACTGGCAATCCCCTACGAGGAAGCGCAGGCATTCATCGAACGTTACTTTCAATCG
TTTCCGAAAGTTCGCGCATGGATCGAGAAGACGCTCGAGGAAGGTCGTCGTCGGGGCTATGTCGAAACTCTGTTT
GGTCGCCGTCGGTACGTACCAGATCTTGAAGCCCGCGTCAAATCGGTACGGGAGGCTGCGGAGCGTATGGCATTT
AATATGCCTGTACAGGGTACTGCAGCTGACCTCATGAAACTGGCAATGGTCAAGCTTTTCCCGCGCTTGGAGGAA
ATGGGCGCACGTATGCTTCTGCAGGTC**GTA**GACGAGCTGGTGTTAGAAGCCCCTAAGGAGCGCGCCGAAGCTGTC
GCGCGCCTCGCTAAAGAAGTGATGGAGGGCGTTTACCCATTGGCCGTACCCCTCGAAGTGGAGGTCGGTATTGGA
GAAGATTGGTTATCTGCAAAGGAAGCGGCCGC

**[0239]** SEQ ID NO. 153, amino acid sequence of Mutant ID 24 (H784V).

MRGMLPLFEPKGRVLLVDGHHLAYRTFHALKGLTTSRGEPVQAVYGFAKSLLKALKEDGDAVIVVFDAKAPSFRH
EAYGGYKAGRAPTPEDFPRQLALIKELVDLLGLARLEVPGYEADDVLASLAKKAEKEGYEVRILTADKDLYQLLS
DRIHVLHPEGYLITPAWLWEKYGLRPDQWADYRALTGDESDNLPGVKGIGEKTARKLLEEWGSLEALLKNLDRLK
PAIREKILAHMDDLKLSWDLAKVRTDLPLEVDFAKRREPDRERLRAFLERLEFGSLLHEFGLLESPKALEEAPWP
PPEGAFVGFVLSRKEPMWADLLALAAARGGRVHRAPEPYKALRDLKEARGLLAKDLSVLALREGLGLPPGDDPML
LAYLLDPSNTTPEGVARRYGGEWTEEAGERAALSERLFANLWGRLEGEERLLWLYREVERPLSAVLAHMEATGVR
LDVAYLRALSLEVAEEIARLEAEVFRLAGHPFNLNSRDQLERVLFDELGLPAIGKTEKTGKRSTSAAVLEALREA
HPIVEKILQYRELTKLKSTYIDPLPDLIHPRTGRLHTRFNQTATATGRLSSSDPNLQNIPVRTPLGQRIRRAFIA
EEGWLLVALDYSQIELRVLAHLSGDENLIRVFQEGRDIHTETASWMFGVPREAVDPLMRRAAKTINFGVLYGMSA
HRLSQELAIPYEEAQAFIERYFQSFPKVRAWIEKTLEEGRRRGYVETLFGRRRYVPDLEARVKSVREAAERMAFN
MPVQGTAADLMKLAMVKLFPRLEEMGARMLLQV**V**DELVLEAPKERAEAVARLAKEVMEGVYPLAVPLEVEVGIGE
DWLSAKEAA

**[0240]** SEQ ID NO. 154, nucleotide sequence of Mutant ID 26 (H784I).

CATATGCGTGGTATGCTGCCGTTGTTCGAGCCTAAAGGCCGCGTACTGTTAGTCGATGGTCATCACTTGGCCTAT
CGGACGTTCCATGCACTCAAAGGTCTGACGACCAGTCGTGGCGAACCGGTCCAGGCTGTTTATGGTTTCGCTAAG
TCTTTGCTCAAAGCACTGAAAGAAGACGGGGACGCGGTAATTGTTGTATTTGATGCCAAAGCACCGAGCTTCCGC
CACGAAGCTTATGGTGGCTACAAGGCAGGACGCGCCCCTACCCCAGAAGATTTCCCCCGTCAGCTGGCATTAATT
AAGGAGTTAGTAGACCTTCTCGGCTTAGCGCGTCTGGAAGTTCCGGGTTATGAGGCGGACGATGTCCTTGCATCC
TTGGCTAAAAAGGCCGAAAAAGAGGGCTACGAAGTCCGCATCTTGACGGCAGACAAAGATCTGTACCAGCTTCTG
TCTGACCGTATTCATGTTTTGCACCCTGAAGGCTACTTAATCACTCCGGCCTGGCTCTGGGAAAAGTACGGTCTG
CGTCCCGATCAGTGGGCGGATTATCGGGCTTTGACGGGAGATGAGAGCGACAACCTGCCAGGAGTTAAGGGCATT
GGTGAAAAAACCGCACGTAAGCTGCTTGAAGAGTGGGGTTCCCTGGAAGCCTTGTTAAAAAATCTGGATCGTCTC
AAGCCCGCAATTCGTGAAAAGATCCTGGCTCATATGGACGATCTTAAATTAAGTTGGGACCTGGCCAAGGTGCGC
ACCGATTTACCGCTTGAAGTGGATTTTGCAAAACGCCGTGAGCCGGACCGGGAACGTTTACGCGCTTTCTTAGAG
CGTCTGGAATTCGGTTCACTGCTTCATGAATTCGGTCTGTTAGAGTCTCCTAAAGCACTCGAAGAGGCACCGTGG
CCGCCCCCAGAAGGTGCTTTTGTTGGCTTCGTACTTTCCCGTAAGGAGCCTATGTGGGCAGATCTTCTGGCTTTA
GCGGCTGCACGCGGTGGCCGTGTTCACCGGGCCCCTGAGCCATACAAAGCGTTACGTGATCTGAAGGAAGCACGT
GGCTTGCTGGCAAAAGACCTTTCTGTTTTGGCCCTGCGCGAGGGTCTTGGACTGCCGCCAGGCGACGATCCCATG
TTATTGGCCTATCTGTTAGACCCTAGCAATACCACACCTGAAGGGGTCGCTCGTCGGTATGGCGGTGAATGGACT
GAGGAAGCCGGAGAGCGCGCCGCATTGTCCGAACGGCTCTTTGCAAACTTATGGGGTCGTCTGGAAGGGGAGGAA
CGTCTGTTATGGTTGTATCGGGAAGTCGAACGTCCTCTTTCGGCCGTATTAGCGCATATGGAGGCAACAGGTGTG
CGTTTAGATGTCGCGTACCTTCGGGCCTTATCACTGGAAGTTGCAGAGGAAATCGCCCGTCTCGAGGCTGAAGTG
TTCCGGTTGGCCGGTCACCCGTTTAACCTCAACTCCCGTGACCAGCTGGAACGCGTTTTATTCGATGAGCTTGGG
CTTCCCGCAATTGGCAAAACCGAAAAGACTGGCAAACGCAGTACGAGCGCTGCCGTCCTTGAGGCACTCCGCGAG
GCTCACCCTATTGTAGAAAAGATCCTGCAATACCGTGAGTTGACGAAGCTTAAAAGCACTTATATTGATCCTCTC
CCGGATCTGATCCATCCTCGTACCGGCCGCTTGCACACACGTTTCAACCAGACGGCGACTGCAACCGGCCGTCTG
TCTAGCTCGGATCCAAATCTCCAGAACATTCCGGTCCGTACACCCTTGGGCCAACGTATCCGCCGGGCGTTTATC
GCTGAGGAAGGATGGTTACTGGTCGCATTGGACTACTCGCAGATTGAGCTGCGCGTCCTCGCACATCTCTCTGGT
GACGAAAATTTAATCCGCGTGTTTCAAGAGGGGCGTGATATTCACACAGAAACTGCCTCATGGATGTTCGGTGTC
CCACGTGAAGCAGTGGATCCTTTGATGCGCCGTGCAGCTAAAACAATTAATTTTGGAGTGCTGTACGGAATGAGC
GCTCATCGCTTGAGTCAGGAACTGGCAATCCCCTACGAGGAAGCGCAGGCATTCATCGAACGTTACTTTCAATCG
TTTCCGAAAGTTCGCGCATGGATCGAGAAGACGCTCGAGGAAGGTCGTCGTCGGGGCTATGTCGAAACTCTGTTT
GGTCGCCGTCGGTACGTACCAGATCTTGAAGCCCGCGTCAAATCGGTACGGGAGGCTGCGGAGCGTATGGCATTT
AATATGCCTGTACAGGGTACTGCAGCTGACCTCATGAAACTGGCAATGGTCAAGCTTTTCCCGCGCTTGGAGGAA
ATGGGCGCACGTATGCTTCTGCAGGTC**ATT**GACGAGCTGGTGTTAGAAGCCCCTAAGGAGCGCGCCGAAGCTGTC
GCGCGCCTCGCTAAAGAAGTGATGGAGGGCGTTTACCCATTGGCCGTACCCCTCGAAGTGGAGGTCGGTATTGGA
GAAGATTGGTTATCTGCAAAGGAAGCGGCCGC

**[0241]** SEQ ID NO. 155, amino acid sequence of Mutant ID 26 (H784I).

MRGMLPLFEPKGRVLLVDGHHLAYRTFHALKGLTTSRGEPVQAVYGFAKSLLKALKEDGDAVIVVFDAKAPSFRH
EAYGGYKAGRAPTPEDFPRQLALIKELVDLLGLARLEVPGYEADDVLASLAKKAEKEGYEVRILTADKDLYQLLS
DRIHVLHPEGYLITPAWLWEKYGLRPDQWADYRALTGDESDNLPGVKGIGEKTARKLLEEWGSLEALLKNLDRLK
PAIREKILAHMDDLKLSWDLAKVRTDLPLEVDFAKRREPDRERLRAFLERLEFGSLLHEFGLLESPKALEEAPWP
PPEGAFVGFVLSRKEPMWADLLALAAARGGRVHRAPEPYKALRDLKEARGLLAKDLSVLALREGLGLPPGDDPML
LAYLLDPSNTTPEGVARRYGGEWTEEAGERAALSERLFANLWGRLEGEERLLWLYREVERPLSAVLAHMEATGVR
LDVAYLRALSLEVAEEIARLEAEVFRLAGHPFNLNSRDQLERVLFDELGLPAIGKTEKTGKRSTSAAVLEALREA
HPIVEKILQYRELTKLKSTYIDPLPDLIHPRTGRLHTRFNQTATATGRLSSSDPNLQNIPVRTPLGQRIRRAFIA
EEGWLLVALDYSQIELRVLAHLSGDENLIRVFQEGRDIHTETASWMFGVPREAVDPLMRRAAKTINFGVLYGMSA
HRLSQELAIPYEEAQAFIERYFQSFPKVRAWIEKTLEEGRRRGYVETLFGRRRYVPDLEARVKSVREAAERMAFN
MPVQGTAADLMKLAMVKLFPRLEEMGARMLLQV**V**DELVLEAPKERAEAVARLAKEVMEGVYPLAVPLEVEVGIGE
DWLSAKEAA

**[0242]** SEQ ID NO. 156, nucleotide sequence of Mutant ID 27 (H784M).

CATATGCGTGGTATGCTGCCGTTGTTCGAGCCTAAAGGCCGCGTACTGTTAGTCGATGGTCATCACTTGGCCTAT
CGGACGTTCCATGCACTCAAAGGTCTGACGACCAGTCGTGGCGAACCGGTCCAGGCTGTTTATGGTTTCGCTAAG
TCTTTGCTCAAAGCACTGAAAGAAGACGGGGACGCGGTAATTGTTGTATTTGATGCCAAAGCACCGAGCTTCCGC
CACGAAGCTTATGGTGGCTACAAGGCAGGACGCGCCCCTACCCCAGAAGATTTCCCCCGTCAGCTGGCATTAATT
AAGGAGTTAGTAGACCTTCTCGGCTTAGCGCGTCTGGAAGTTCCGGGTTATGAGGCGGACGATGTCCTTGCATCC
TTGGCTAAAAAGGCCGAAAAAGAGGGCTACGAAGTCCGCATCTTGACGGCAGACAAAGATCTGTACCAGCTTCTG
TCTGACCGTATTCATGTTTTGCACCCTGAAGGCTACTTAATCACTCCGGCCTGGCTCTGGGAAAAGTACGGTCTG
CGTCCCGATCAGTGGGCGGATTATCGGGCTTTGACGGGAGATGAGAGCGACAACCTGCCAGGAGTTAAGGGCATT
GGTGAAAAAACCGCACGTAAGCTGCTTGAAGAGTGGGGTTCCCTGGAAGCCTTGTTAAAAAATCTGGATCGTCTC
AAGCCCGCAATTCGTGAAAAGATCCTGGCTCATATGGACGATCTTAAATTAAGTTGGGACCTGGCCAAGGTGCGC
ACCGATTTACCGCTTGAAGTGGATTTTGCAAAACGCCGTGAGCCGGACCGGGAACGTTTACGCGCTTTCTTAGAG
CGTCTGGAATTCGGTTCACTGCTTCATGAATTCGGTCTGTTAGAGTCTCCTAAAGCACTCGAAGAGGCACCGTGG
CCGCCCCCAGAAGGTGCTTTTGTTGGCTTCGTACTTTCCCGTAAGGAGCCTATGTGGGCAGATCTTCTGGCTTTA
GCGGCTGCACGCGGTGGCCGTGTTCACCGGGCCCCTGAGCCATACAAAGCGTTACGTGATCTGAAGGAAGCACGT
GGCTTGCTGGCAAAAGACCTTTCTGTTTTGGCCCTGCGCGAGGGTCTTGGACTGCCGCCAGGCGACGATCCCATG
TTATTGGCCTATCTGTTAGACCCTAGCAATACCACACCTGAAGGGGTCGCTCGTCGGTATGGCGGTGAATGGACT
GAGGAAGCCGGAGAGCGCGCCGCATTGTCCGAACGGCTCTTTGCAAACTTATGGGGTCGTCTGGAAGGGGAGGAA
CGTCTGTTATGGTTGTATCGGGAAGTCGAACGTCCTCTTTCGGCCGTATTAGCGCATATGGAGGCAACAGGTGTG
CGTTTAGATGTCGCGTACCTTCGGGCCTTATCACTGGAAGTTGCAGAGGAAATCGCCCGTCTCGAGGCTGAAGTG
TTCCGGTTGGCCGGTCACCCGTTTAACCTCAACTCCCGTGACCAGCTGGAACGCGTTTTATTCGATGAGCTTGGG
CTTCCCGCAATTGGCAAAACCGAAAAGACTGGCAAACGCAGTACGAGCGCTGCCGTCCTTGAGGCACTCCGCGAG
GCTCACCCTATTGTAGAAAAGATCCTGCAATACCGTGAGTTGACGAAGCTTAAAAGCACTTATATTGATCCTCTC
CCGGATCTGATCCATCCTCGTACCGGCCGCTTGCACACACGTTTCAACCAGACGGCGACTGCAACCGGCCGTCTG
TCTAGCTCGGATCCAAATCTCCAGAACATTCCGGTCCGTACACCCTTGGGCCAACGTATCCGCCGGGCGTTTATC
GCTGAGGAAGGATGGTTACTGGTCGCATTGGACTACTCGCAGATTGAGCTGCGCGTCCTCGCACATCTCTCTGGT
GACGAAAATTTAATCCGCGTGTTTCAAGAGGGGCGTGATATTCACACAGAAACTGCCTCATGGATGTTCGGTGTC
CCACGTGAAGCAGTGGATCCTTTGATGCGCCGTGCAGCTAAAACAATTAATTTTGGAGTGCTGTACGGAATGAGC
GCTCATCGCTTGAGTCAGGAACTGGCAATCCCCTACGAGGAAGCGCAGGCATTCATCGAACGTTACTTTCAATCG
TTTCCGAAAGTTCGCGCATGGATCGAGAAGACGCTCGAGGAAGGTCGTCGTCGGGGCTATGTCGAAACTCTGTTT
GGTCGCCGTCGGTACGTACCAGATCTTGAAGCCCGCGTCAAATCGGTACGGGAGGCTGCGGAGCGTATGGCATTT
AATATGCCTGTACAGGGTACTGCAGCTGACCTCATGAAACTGGCAATGGTCAAGCTTTTCCCGCGCTTGGAGGAA
ATGGGCGCACGTATGCTTCTGCAGGTC**ATG**GACGAGCTGGTGTTAGAAGCCCCTAAGGAGCGCGCCGAAGCTGTC
GCGCGCCTCGCTAAAGAAGTGATGGAGGGCGTTTACCCATTGGCCGTACCCCTCGAAGTGGAGGTCGGTATTGGA
GAAGATTGGTTATCTGCAAAGGAAGCGGCCGC

**[0243]** SEQ ID NO. 157, amino acid sequence of Mutant ID 27 (H784M).

MRGMLPLFEPKGRVLLVDGHHLAYRTFHALKGLTTSRGEPVQAVYGFAKSLLKALKEDGDAVIVVFDAKAPSFRH
EAYGGYKAGRAPTPEDFPRQLALIKELVDLLGLARLEVPGYEADDVLASLAKKAEKEGYEVRILTADKDLYQLLS
DRIHVLHPEGYLITPAWLWEKYGLRPDQWADYRALTGDESDNLPGVKGIGEKTARKLLEEWGSLEALLKNLDRLK
PAIREKILAHMDDLKLSWDLAKVRTDLPLEVDFAKRREPDRERLRAFLERLEFGSLLHEFGLLESPKALEEAPWP
PPEGAFVGFVLSRKEPMWADLLALAAARGGRVHRAPEPYKALRDLKEARGLLAKDLSVLALREGLGLPPGDDPML
LAYLLDPSNTTPEGVARRYGGEWTEEAGERAALSERLFANLWGRLEGEERLLWLYREVERPLSAVLAHMEATGVR
LDVAYLRALSLEVAEEIARLEAEVFRLAGHPFNLNSRDQLERVLFDELGLPAIGKTEKTGKRSTSAAVLEALREA
HPIVEKILQYRELTKLKSTYIDPLPDLIHPRTGRLHTRFNQTATATGRLSSSDPNLQNIPVRTPLGQRIRRAFIA
EEGWLLVALDYSQIELRVLAHLSGDENLIRVFQEGRDIHTETASWMFGVPREAVDPLMRRAAKTINFGVLYGMSA
HRLSQELAIPYEEAQAFIERYFQSFPKVRAWIEKTLEEGRRRGYVETLFGRRRYVPDLEARVKSVREAAERMAFN
MPVQGTAADLMKLAMVKLFPRLEEMGARMLLQV**M**DELVLEAPKERAEAVARLAKEVMEGVYPLAVPLEVEVGIGE
DWLSAKEAA

**[0244]** SEQ ID NO. 158, nucleotide sequence of Mutant ID 29 (H784F).

CATATGCGTGGTATGCTGCCGTTGTTCGAGCCTAAAGGCCGCGTACTGTTAGTCGATGGTCATCACTTGGCCTAT
CGGACGTTCCATGCACTCAAAGGTCTGACGACCAGTCGTGGCGAACCGGTCCAGGCTGTTTATGGTTTCGCTAAG
TCTTTGCTCAAAGCACTGAAAGAAGACGGGGACGCGGTAATTGTTGTATTTGATGCCAAAGCACCGAGCTTCCGC
CACGAAGCTTATGGTGGCTACAAGGCAGGACGCGCCCCTACCCCAGAAGATTTCCCCCGTCAGCTGGCATTAATT
AAGGAGTTAGTAGACCTTCTCGGCTTAGCGCGTCTGGAAGTTCCGGGTTATGAGGCGGACGATGTCCTTGCATCC
TTGGCTAAAAAGGCCGAAAAAGAGGGCTACGAAGTCCGCATCTTGACGGCAGACAAAGATCTGTACCAGCTTCTG
TCTGACCGTATTCATGTTTTGCACCCTGAAGGCTACTTAATCACTCCGGCCTGGCTCTGGGAAAAGTACGGTCTG
CGTCCCGATCAGTGGGCGGATTATCGGGCTTTGACGGGAGATGAGAGCGACAACCTGCCAGGAGTTAAGGGCATT
GGTGAAAAAACCGCACGTAAGCTGCTTGAAGAGTGGGGTTCCCTGGAAGCCTTGTTAAAAAATCTGGATCGTCTC
AAGCCCGCAATTCGTGAAAAGATCCTGGCTCATATGGACGATCTTAAATTAAGTTGGGACCTGGCCAAGGTGCGC
ACCGATTTACCGCTTGAAGTGGATTTTGCAAAACGCCGTGAGCCGGACCGGGAACGTTTACGCGCTTTCTTAGAG
CGTCTGGAATTCGGTTCACTGCTTCATGAATTCGGTCTGTTAGAGTCTCCTAAAGCACTCGAAGAGGCACCGTGG
CCGCCCCCAGAAGGTGCTTTTGTTGGCTTCGTACTTTCCCGTAAGGAGCCTATGTGGGCAGATCTTCTGGCTTTA
GCGGCTGCACGCGGTGGCCGTGTTCACCGGGCCCCTGAGCCATACAAAGCGTTACGTGATCTGAAGGAAGCACGT
GGCTTGCTGGCAAAAGACCTTTCTGTTTTGGCCCTGCGCGAGGGTCTTGGACTGCCGCCAGGCGACGATCCCATG
TTATTGGCCTATCTGTTAGACCCTAGCAATACCACACCTGAAGGGGTCGCTCGTCGGTATGGCGGTGAATGGACT
GAGGAAGCCGGAGAGCGCGCCGCATTGTCCGAACGGCTCTTTGCAAACTTATGGGGTCGTCTGGAAGGGGAGGAA
CGTCTGTTATGGTTGTATCGGGAAGTCGAACGTCCTCTTTCGGCCGTATTAGCGCATATGGAGGCAACAGGTGTG
CGTTTAGATGTCGCGTACCTTCGGGCCTTATCACTGGAAGTTGCAGAGGAAATCGCCCGTCTCGAGGCTGAAGTG
TTCCGGTTGGCCGGTCACCCGTTTAACCTCAACTCCCGTGACCAGCTGGAACGCGTTTTATTCGATGAGCTTGGG
CTTCCCGCAATTGGCAAAACCGAAAAGACTGGCAAACGCAGTACGAGCGCTGCCGTCCTTGAGGCACTCCGCGAG
GCTCACCCTATTGTAGAAAAGATCCTGCAATACCGTGAGTTGACGAAGCTTAAAAGCACTTATATTGATCCTCTC
CCGGATCTGATCCATCCTCGTACCGGCCGCTTGCACACACGTTTCAACCAGACGGCGACTGCAACCGGCCGTCTG
TCTAGCTCGGATCCAAATCTCCAGAACATTCCGGTCCGTACACCCTTGGGCCAACGTATCCGCCGGGCGTTTATC
GCTGAGGAAGGATGGTTACTGGTCGCATTGGACTACTCGCAGATTGAGCTGCGCGTCCTCGCACATCTCTCTGGT
GACGAAAATTTAATCCGCGTGTTTCAAGAGGGGCGTGATATTCACACAGAAACTGCCTCATGGATGTTCGGTGTC
CCACGTGAAGCAGTGGATCCTTTGATGCGCCGTGCAGCTAAAACAATTAATTTTGGAGTGCTGTACGGAATGAGC
GCTCATCGCTTGAGTCAGGAACTGGCAATCCCCTACGAGGAAGCGCAGGCATTCATCGAACGTTACTTTCAATCG
TTTCCGAAAGTTCGCGCATGGATCGAGAAGACGCTCGAGGAAGGTCGTCGTCGGGGCTATGTCGAAACTCTGTTT
GGTCGCCGTCGGTACGTACCAGATCTTGAAGCCCGCGTCAAATCGGTACGGGAGGCTGCGGAGCGTATGGCATTT
AATATGCCTGTACAGGGTACTGCAGCTGACCTCATGAAACTGGCAATGGTCAAGCTTTTCCCGCGCTTGGAGGAA
ATGGGCGCACGTATGCTTCTGCAGGTC**TTT**GACGAGCTGGTGTTAGAAGCCCCTAAGGAGCGCGCCGAAGCTGTC
GCGCGCCTCGCTAAAGAAGTGATGGAGGGCGTTTACCCATTGGCCGTACCCCTCGAAGTGGAGGTCGGTATTGGA
GAAGATTGGTTATCTGCAAAGGAAGCGGCCGC

**[0245]** SEQ ID NO. 159, amino acid sequence of Mutant ID 29 (H784F).

MRGMLPLFEPKGRVLLVDGHHLAYRTFHALKGLTTSRGEPVQAVYGFAKSLLKALKEDGDAVIVVFDAKAPSFRH
EAYGGYKAGRAPTPEDFPRQLALIKELVDLLGLARLEVPGYEADDVLASLAKKAEKEGYEVRILTADKDLYQLLS
DRIHVLHPEGYLITPAWLWEKYGLRPDQWADYRALTGDESDNLPGVKGIGEKTARKLLEEWGSLEALLKNLDRLK
PAIREKILAHMDDLKLSWDLAKVRTDLPLEVDFAKRREPDRERLRAFLERLEFGSLLHEFGLLESPKALEEAPWP
PPEGAFVGFVLSRKEPMWADLLALAAARGGRVHRAPEPYKALRDLKEARGLLAKDLSVLALREGLGLPPGDDPML
LAYLLDPSNTTPEGVARRYGGEWTEEAGERAALSERLFANLWGRLEGEERLLWLYREVERPLSAVLAHMEATGVR
LDVAYLRALSLEVAEEIARLEAEVFRLAGHPFNLNSRDQLERVLFDELGLPAIGKTEKTGKRSTSAAVLEALREA
HPIVEKILQYRELTKLKSTYIDPLPDLIHPRTGRLHTRFNQTATATGRLSSSDPNLQNIPVRTPLGQRIRRAFIA
EEGWLLVALDYSQIELRVLAHLSGDENLIRVFQEGRDIHTETASWMFGVPREAVDPLMRRAAKTINFGVLYGMSA
HRLSQELAIPYEEAQAFIERYFQSFPKVRAWIEKTLEEGRRRGYVETLFGRRRYVPDLEARVKSVREAAERMAFN
MPVQGTAADLMKLAMVKLFPRLEEMGARMLLQV**F**DELVLEAPKERAEAVARLAKEVMEGVYPLAVPLEVEVGIGE
DWLSAKEAA

**[0246]** SEQ ID NO. 160, nucleotide sequence of Mutant ID 30 (H784Y).

CATATGCGTGGTATGCTGCCGTTGTTCGAGCCTAAAGGCCGCGTACTGTTAGTCGATGGTCATCACTTGGCCTAT
CGGACGTTCCATGCACTCAAAGGTCTGACGACCAGTCGTGGCGAACCGGTCCAGGCTGTTTATGGTTTCGCTAAG
TCTTTGCTCAAAGCACTGAAAGAAGACGGGGACGCGGTAATTGTTGTATTTGATGCCAAAGCACCGAGCTTCCGC
CACGAAGCTTATGGTGGCTACAAGGCAGGACGCGCCCCTACCCCAGAAGATTTCCCCCGTCAGCTGGCATTAATT
AAGGAGTTAGTAGACCTTCTCGGCTTAGCGCGTCTGGAAGTTCCGGGTTATGAGGCGGACGATGTCCTTGCATCC
TTGGCTAAAAAGGCCGAAAAAGAGGGCTACGAAGTCCGCATCTTGACGGCAGACAAAGATCTGTACCAGCTTCTG
TCTGACCGTATTCATGTTTTGCACCCTGAAGGCTACTTAATCACTCCGGCCTGGCTCTGGGAAAAGTACGGTCTG
CGTCCCGATCAGTGGGCGGATTATCGGGCTTTGACGGGAGATGAGAGCGACAACCTGCCAGGAGTTAAGGGCATT
GGTGAAAAAACCGCACGTAAGCTGCTTGAAGAGTGGGGTTCCCTGGAAGCCTTGTTAAAAAATCTGGATCGTCTC
AAGCCCGCAATTCGTGAAAAGATCCTGGCTCATATGGACGATCTTAAATTAAGTTGGGACCTGGCCAAGGTGCGC
ACCGATTTACCGCTTGAAGTGGATTTTGCAAAACGCCGTGAGCCGGACCGGGAACGTTTACGCGCTTTCTTAGAG
CGTCTGGAATTCGGTTCACTGCTTCATGAATTCGGTCTGTTAGAGTCTCCTAAAGCACTCGAAGAGGCACCGTGG
CCGCCCCCAGAAGGTGCTTTTGTTGGCTTCGTACTTTCCCGTAAGGAGCCTATGTGGGCAGATCTTCTGGCTTTA
GCGGCTGCACGCGGTGGCCGTGTTCACCGGGCCCCTGAGCCATACAAAGCGTTACGTGATCTGAAGGAAGCACGT
GGCTTGCTGGCAAAAGACCTTTCTGTTTTGGCCCTGCGCGAGGGTCTTGGACTGCCGCCAGGCGACGATCCCATG
TTATTGGCCTATCTGTTAGACCCTAGCAATACCACACCTGAAGGGGTCGCTCGTCGGTATGGCGGTGAATGGACT
GAGGAAGCCGGAGAGCGCGCCGCATTGTCCGAACGGCTCTTTGCAAACTTATGGGGTCGTCTGGAAGGGGAGGAA
CGTCTGTTATGGTTGTATCGGGAAGTCGAACGTCCTCTTTCGGCCGTATTAGCGCATATGGAGGCAACAGGTGTG
CGTTTAGATGTCGCGTACCTTCGGGCCTTATCACTGGAAGTTGCAGAGGAAATCGCCCGTCTCGAGGCTGAAGTG
TTCCGGTTGGCCGGTCACCCGTTTAACCTCAACTCCCGTGACCAGCTGGAACGCGTTTTATTCGATGAGCTTGGG
CTTCCCGCAATTGGCAAAACCGAAAAGACTGGCAAACGCAGTACGAGCGCTGCCGTCCTTGAGGCACTCCGCGAG
GCTCACCCTATTGTAGAAAAGATCCTGCAATACCGTGAGTTGACGAAGCTTAAAAGCACTTATATTGATCCTCTC
CCGGATCTGATCCATCCTCGTACCGGCCGCTTGCACACACGTTTCAACCAGACGGCGACTGCAACCGGCCGTCTG
TCTAGCTCGGATCCAAATCTCCAGAACATTCCGGTCCGTACACCCTTGGGCCAACGTATCCGCCGGGCGTTTATC
GCTGAGGAAGGATGGTTACTGGTCGCATTGGACTACTCGCAGATTGAGCTGCGCGTCCTCGCACATCTCTCTGGT
GACGAAAATTTAATCCGCGTGTTTCAAGAGGGGCGTGATATTCACACAGAAACTGCCTCATGGATGTTCGGTGTC
CCACGTGAAGCAGTGGATCCTTTGATGCGCCGTGCAGCTAAAACAATTAATTTTGGAGTGCTGTACGGAATGAGC
GCTCATCGCTTGAGTCAGGAACTGGCAATCCCCTACGAGGAAGCGCAGGCATTCATCGAACGTTACTTTCAATCG
TTTCCGAAAGTTCGCGCATGGATCGAGAAGACGCTCGAGGAAGGTCGTCGTCGGGGCTATGTCGAAACTCTGTTT
GGTCGCCGTCGGTACGTACCAGATCTTGAAGCCCGCGTCAAATCGGTACGGGAGGCTGCGGAGCGTATGGCATTT
AATATGCCTGTACAGGGTACTGCAGCTGACCTCATGAAACTGGCAATGGTCAAGCTTTTCCCGCGCTTGGAGGAA
ATGGGCGCACGTATGCTTCTGCAGGTC**TAT**GACGAGCTGGTGTTAGAAGCCCCTAAGGAGCGCGCCGAAGCTGTC
GCGCGCCTCGCTAAAGAAGTGATGGAGGGCGTTTACCCATTGGCCGTACCCCTCGAAGTGGAGGTCGGTATTGGA
GAAGATTGGTTATCTGCAAAGGAAGCGGCCGC

**[0247]** SEQ ID NO. 161, amino acid sequence of Mutant ID 30 (H784Y).

MRGMLPLFEPKGRVLLVDGHHLAYRTFHALKGLTTSRGEPVQAVYGFAKSLLKALKEDGDAVIVVFDAKAPSFRH
EAYGGYKAGRAPTPEDFPRQLALIKELVDLLGLARLEVPGYEADDVLASLAKKAEKEGYEVRILTADKDLYQLLS
DRIHVLHPEGYLITPAWLWEKYGLRPDQWADYRALTGDESDNLPGVKGIGEKTARKLLEEWGSLEALLKNLDRLK
PAIREKILAHMDDLKLSWDLAKVRTDLPLEVDFAKRREPDRERLRAFLERLEFGSLLHEFGLLESPKALEEAPWP
PPEGAFVGFVLSRKEPMWADLLALAAARGGRVHRAPEPYKALRDLKEARGLLAKDLSVLALREGLGLPPGDDPML
LAYLLDPSNTTPEGVARRYGGEWTEEAGERAALSERLFANLWGRLEGEERLLWLYREVERPLSAVLAHMEATGVR
LDVAYLRALSLEVAEEIARLEAEVFRLAGHPFNLNSRDQLERVLFDELGLPAIGKTEKTGKRSTSAAVLEALREA
HPIVEKILQYRELTKLKSTYIDPLPDLIHPRTGRLHTRFNQTATATGRLSSSDPNLQNIPVRTPLGQRIRRAFIA
EEGWLLVALDYSQIELRVLAHLSGDENLIRVFQEGRDIHTETASWMFGVPREAVDPLMRRAAKTINFGVLYGMSA
HRLSQELAIPYEEAQAFIERYFQSFPKVRAWIEKTLEEGRRRGYVETLFGRRRYVPDLEARVKSVREAAERMAFN
MPVQGTAADLMKLAMVKLFPRLEEMGARMLLQV**Y**DELVLEAPKERAEAVARLAKEVMEGVYPLAVPLEVEVGIGE
DWLSAKEAA

**Example 18. Production of Codon Optimized Taq DNA Polymerase mutants modified to eliminate 5'exonuclease activity**

**[0248]** Additional Taq DNA Polymerase mutants were made that eliminated the 5' exonuclease activity of several of the mutants from Table 3. Taq DNA Polyermase missing the 5'-exonuclease activity was previously named "KlenTaq" (Barnes, W.M., Gene 112:29-35, 1992). Deletion of the N-terminal 5' exonuclease domain of Taq polymerase improves the mismatch discrimination properties of the enzyme (Barnes, W.M., Gene 112:29-35, 1992). The present study characterized whether specificity improvements seen in the Taq DNA Polymerase mutants of the present invention were combined with mutations which eliminated 5'-exonuclease acitivty. The examples shown here are meant to be exemplary, and in no way limit the range of the claims. Specific mutations were introduced into the OptiTaq sequence using the method of PCR site-directed mutagenesis (Weiner MP, *et al.*, Gene. 151(1-2):119-23 (1994)). Each mutagenesis reaction employed 10 pmoles of two oligonucleotides (Table 38) to amplify around the plasmid containing the DNA polymerase, excluding the 5' exonuclease domain. These primers were manufactured to contain a 5' phosphate, which allowed for re-ligation after amplification. Breifly, these primers were annealed to the double-stranded plasmid containing previously characterized mutant DNA polymerases (MUT IDs 2, 3, 10, 18, 21, and 30) (20 ng each), 5 U KOD DNA polymerase (Novagen-EMD Chemicals, San Diego, CA), 1.5 mM $MgSO_4$, in 1X KOD PCR buffer. Thermal cycling parameters were 95°C for 3 minutes (95°C for 20 sec-55°C for 20 sec-70°C for 2 minutes) for 25 cycles followed by a 70°C soak for 4 minutes. After PCR site-directed mutagenesis, the amplified product was treated with 10 U of Dpn I (NEB, Ipswich, MA), at 37°C for 1 hour, followed by inactivation at 80°C for 20 minutes. 1/6$^{th}$ of the digestion material was ligated together with T4 DNA ligase (NEB, Ipswich, MA) at 16°C for 20

minutes, followed by inactivation at 65°C for 10 minutes. 1/15$^{th}$ of the ligated material was transformed into XL-1 Blue competent bacteria. Bacterial clones were isolated, plasmid DNA prepared, and deletion of the 5' exonuclease domains were confirmed by Sanger DNA sequencing. All mutants remained in the pET-27b(+) expression vector, which is suitable for expressing the recombinant proteins in *E. coli.* Expression and purification of the recombinant mutants of the Taq polymerase were performed as described in Example 3.

**Table 38: Oligonucleotides used for site-directed mutagenesis to produce 18 Taq DNA Polymerase mutants.**

| Mutant ID | Mutant name | Sequence" Sense mutagenesis oligonucleotide | SEQ ID No. | Sequence" Antisense mutagenesis oligonucleotide | SEQ ID No. |
|---|---|---|---|---|---|
| 37 | OptiTaq KlenTaq | Phos-ggttcactgcttcatgaattcggtc | 162 | Phos-catatgtattctccttcttaaagttaaacaaa | 163 |
| 38 | A661E, I665W, F667L KlenTaq | Phos-ggttcactgcttcatgaattcggtc | 162 | Phos-catatgtattctccttcttaaagttaaacaaa | 163 |
| 39 | V783F KlenTaq | Phos-ggttcactgcttcatgaattcggtc | 162 | Phos-catatgtattctccttcttaaagttaaacaaa | 163 |
| 40 | H784Q KlenTaq | Phos-ggttcactgcttcatgaattcggtc | 162 | Phos-catatgtattctccttcttaaagttaaacaaa | 163 |
| 41 | V783L H784Q KlenTaq | Phos-ggttcactgcttcatgaattcggtc | 162 | Phos-catatgtattctccttcttaaagttaaacaaa | 163 |
| 42 | H784S KlenTaq | Phos-ggttcactgcttcatgaattcggtc | 162 | Phos-catatgtattctccttcttaaagttaaacaaa | 163 |
| 43 | H784Y KlenTaq | Phos-ggttcactgcttcatgaattcggtc | 162 | Phos-catatgtattctccttcttaaagttaaacaaa | 163 |

DNA bases identical to codon optimized OptiTaq are shown in lower case; those specific for the mutations introduced by site-directed mutagenesis are shown in upper case.

**Example 19. Characterization of properties of 7 5'-exonuclease-deficient mutant Taq DNA Polymerases in PCR.**

**[0249]** The 7 mutant Taq DNA polymerase enzymes described in Example 18 were characterized for polymerase activity.

**[0250]** The unit activity of the purified wild-type protein was determined by comparing performance in qPCR of known quantities of OptiTaq and each mutant compared to a commercial non-hot-start Taq DNA polymerase, Taq-B DNA Polymerase (Enzymatics, Beverly, MA). Quantification cycle values (Cq, the amplification cycle number at which positive signal is first detected) and amplification curve shapes were analyzed to determine the nanogram amounts at which both enzymes performed similarly in the suboptimal range for each. Using these nanogram amounts and known unit values of Taq-B DNA polymerase, relative activity unit values could be extrapolated for all of the mutant DNA polymerase enzymes having sufficient activity to support PCR. Testing was also done to determine the $MgCl_2$ concentrations at which the polymerases would show optimal activity.

**[0251]** The following reaction conditions were employed: 1x qPCR buffer (20 mM Tris pH 8.4, 50 mM KCl, 0.01% Triton-X100), 800 µM dNTPs (200 µM each), 500 nM For primer (Hs HPRT F517, SEQ ID NO. 43), 500 nM Rev primer (Hs HPRT R591, SEQ ID NO. 44), 250 nM RNase H2 cleavable probe (Hs HPRT RN2 Probe, SEQ ID NO. 164), 20 mU *Pyrococcus abyssi* RNase H2, 2 x $10^3$ copies of linearized cloned plasmid template (HPRT-targ, SEQ ID NO. 46), in 10 µL final volume. $MgC_2$ was tested at 3, 4, or 5 mM in each case. The amount of DNA polymerase added to each reaction was varied as follows: for wild type (OptiTaq), reactions were set using 10, 1, 0.1, 0.01, and 0.001 U/µL (220, 22, 2.2, 0.22, or 0.022 ng of protein per 10 µL reaction). Mutant polymerases were run in similar concentrations. In addition, those mutant enzymes showing polymerase activity were more finely titrated testing 220, 22, 10.6, 4.8, 2.2, 1.1, 0.48, and 0.22 ng of protein per 10 µL reaction. Polymerase dilutions were made in enzyme dilution buffer (20 mM Tris pH7.5, 100 mM NaCl, 1 mM DTT, 0.1% Triton-X100, 1 mg/mL BSA, 10% glycerol). Reactions were run in 384 well format on a BIO-RAD CFX384™ Real-Time System (BIO-RAD, Hercules, CA) using cycling parameters 95°C for 30 seconds followed by 60 cycles of [95°C for 15 seconds followed by 60°C for 1 minutes]. Detection was achieved using a fluorescence-quenched probe (cleaved by the action of the *P.a.* RNase H2 enzyme). Sequences of the primers, probe, and template (plasmid insert) are shown in Table 39.

**Table 39. Sequence of oligonucleotides employed in Taq DNA polymerase activity assay.**

| Name | Sequence | SEQ ID NO. |
|---|---|---|
| Hs HPRT F517 | GACTTTGCTTTCCTTGGTCAG | SEQ ID NO. 43 |

| Name | Sequence | SEQ ID NO. |
|---|---|---|
| Hs HPRT R591 | GGCTTATATCCAACACTTCGTG | SEQ ID NO. 44 |
| Hs HPRT RN2 Probe | FAM-ATGGTCAAGGTCGCAAGcTTGCTGGT-IBFQ | SEQ ID NO. 164 |
| HPRT-targ | GACTTTGCTTTCCTTGGTCAGGCAGTATAATCCAAAGATGGTCAAGGTC GCAAGCTTGCTGGTGAAAAGGACCCCACGAAGTGTTGGATATAAGCC | SEQ ID NO. 46 |

DNA bases are uppercase and RNA bases are lowercase; Nucleic acid sequences are shown 5'-3'. FAM = 6-carboxyfluorescein, IBFQ = Iowa Black FQ (fluorescence quencher), and ZEN = ZEN internal fluorescence quencher.

These 7 Taq DNA polymerase 5'-exonuclease-deficient mutants were characterized as outlined above. Results are summarized in Table 40. All seven mutants had DNA polymerase activity; however, processivity in Mutant IDs 38, 39, 40, 41, 42, and 43 was reduced from 10-50 fold relative to the wild type enzyme. One mutant, Mutant ID 37 (OptiTaq KlenTaq), showed DNA polymerase activity nearly identical to wild type OptiTaq. Therefore the combination of complete deletion of the 5'-exonuclease domain of Taq DNA Polymerase coupled with point mutations that improve polymerase specificity all significantly compromised enzyme activity and processivity.

**Table 40. Novel Taq DNA polymerase mutants selected for initial study.**

| Mutant ID | Amino acid changes from wild-type Taq | Polymerase Activity | Relative activity* | Optimal $MgCl_2$ concentration (mM) |
|---|---|---|---|---|
| 37 | OptiTaq KlenTaq | Yes | 1 | 3 |
| 38 | A661E, I665W, F667L KlenTaq | Yes | 0.1 | 5 |
| 39 | V783F KlenTaq | Yes | 0.05 | 4 |
| 40 | H784Q KlenTaq | Yes | 0.03 | 4 |
| 41 | V783L H784Q KlenTaq | Yes | 0.02 | 5 |
| 42 | H784S KlenTaq | Yes | 0.02 | 5 |
| 43 | H784Y KlenTaq | Yes | 0.05 | 5 |

*Wild-type OptiTaq was set to "1" and the relative activity of each of the mutant polymerases was normalized to this amplification efficiency, with 1 as the maximum.

**Example 20: Improved mismatch discrimination in allele-specific PCR using mutant Taq DNA polymerases also having deletion of the 5'-exonuclease domain.**

**[0252]** Of the 7 mutant enzymes studied in Example 18 and 19, Mutant IDs 37, 38, 39, 40, 41, 42, and 43 retained sufficient enzymatic activity/processivity to characterize. These seven mutants were studied for the ability to discriminate against a 3'-terminal DNA mismatch compared with wild type OptiTaq DNA polymerase using an allele-specific qPCR assay. Amplification reactions were performed against a synthetic oligonucleotide template where a single base was varied (SNP) which was positioned to lie at the 3'-end of the reverse primer. Synthetic templates were employed having each of the 4 possible bases at this position. Reverse primers were employed having each of the 4 possible bases at the 3'-end. Relative amplification efficiency was assessed using qPCR.

**[0253]** Quantitative allele-specific real-time PCR (AS-qPCR) was performed in 10 µL reaction volumes in 384 well format with 2 x $10^5$ copies of a 103 bp synthetic template (SEQ ID NOs. 51-4). Final reaction conditions used were 20 mM Tris-HCL (pH 8.4 at 25°C), 50 mM KCL, the amount of $MgCl_2$ which was determined to be optimal for each polymerase in Example 19, 0.01% Triton X-100, 800 µM total dNTPs, and 200 nM of the universal forward primer (SEQ ID NO. 60), 200 nM of a reverse primer (separate reactions were set up for each of the allele-specific primers SEQ ID NOs. 55-58 or the control universal primer SEQ ID NO. 59) and 200 nM of the RNase H2 cleavable probe (SEQ ID NO. 165). 20 mU *Pyrococcus abyssi* RNase H2 was also include in each reaction. Each allele-specific primer was tested on each SNP template. Reactions utilized either 0.5 U (10.8 ng/11.1 nM/111 fmol) of the OptiTaq KlenTaq DNA polymerase (Mutant ID 37) or 0.5 U of one of the six Taq DNA polymerase mutants studied (Mutant ID 38 (108 ng/111 nM/1110 fmol); Mutant ID 39 (216 ng/222 nM/2220 fmol); Mutant ID 40 (360 ng/370 nM/3700 fmol); Mutant ID 41 (1060 ng/555 nM/5550 fmol); Mutant ID 42 (1060 ng/555 nM/5550 fmol); Mutant ID 43 (216 ng/222 nM/2220 fmol)). Amplification was performed on a CFX384™ C1000™ Thermo Cycler system (Bio-Rad, Hercules, CA) using the following cycling parameters: 95°C for 30 seconds initial denaturation followed by 60 cycles of 95°C for 10 seconds, then 60°C for 30 seconds. Oligonucleotide reagents used in this example are shown in Table 41.

**Table 41. Synthetic oligonucleotides employed in Example 20.**

| Name | Sequence (5'-3') | SEQ ID NO. |
|---|---|---|
| A Template | AGCTCTGCCCAAAGATTACCCTGACAGCTAAGTGGCAGTGGAA GTTGGCCTCAGAAGTAGTGGCCAGCTGTGTGTCGGGGAACAGT AAAGGCATGAAGCTCAG | SEQ ID NO. 51 |

| Name | Sequence (5'-3') | SEQ ID NO. |
|---|---|---|
| C Template | AGCTCTGCCCAAAGATTACCCTGACAGCTAAGTGGCAGTGGAA GTTGGCCTCAGAAGTAGTGGCCAGCTGTGTGTCGGGGCACAGT AAAGGCATGAAGCTCAG | SEQ ID NO. 52 |
| G Template | AGCTCTGCCCAAAGATTACCCTGACAGCTAAGTGGCAGTGGAA GTTGGCCTCAGAAGTAGTGGCCAGCTGTGTGTCGGGGGACAGT AAAGGCATGAAGCTCAG | SEQ ID NO. 53 |
| T Template | AGCTCTGCCCAAAGATTACCCTGACAGCTAAGTGGCAGTGGAA GTTGGCCTCAGAAGTAGTGGCCAGCTGTGTGTCGGGGTACAGT AAAGGCATGAAGCTCAG | SEQ ID NO. 54 |
| Syn Rev T | CTGAGCTTCATGCCTTTACTGTT | SEQ ID NO. 55 |
| Syn Rev C | CTGAGCTTCATGCCTTTACTGTC | SEQ ID NO. 56 |
| Syn Rev A | CTGAGCTTCATGCCTTTACTGTA | SEQ ID NO. 57 |
| Syn Rev G | CTGAGCTTCATGCCTTTACTGTG | SEQ ID NO. 58 |
| Syn Rev | CTGAGCTTCATGCCTTTACTGT | SEQ ID NO. 59 |
| Syn For | AGCTCTGCCCAAAGATTACCCTG | SEQ ID NO. 60 |
| RN2 Probe | FAM-TTCTGAGGCCAACuCCACTGCCACTTA-IBFQ | SEQ ID NO. 165 |

DNA bases are uppercase and RNA bases are lowercase; FAM = 6-carboxyfluorescein; IBFQ = Iowa Black™ FQ fluorescence quencher; ZEN = internal ZEN fluorescence quencher; underlined base indicates the SNP site in the synthetic template DNA.

**[0254]** Initially all reactions were run in triplicate. Similar results were obtained for all replicates when using the wild type OptiTaq. However, results showed greater variation for the mutant polymerases. To obtain statistically meaningful results, each reaction was therefore performed 24 times for the mutant polymerases and 21 times for the wild type enzyme. ΔCq values were calculated as the Cq value obtained for each mismatched base pair minus the Cq value obtained for the matched base pair (ΔCq = Cq mismatch – Cq match). The ΔCq values for all 24 replicates were averaged and standard deviations were calculated. Results are shown in Table 42 and are graphically summarized in Figures 7A, 7B, and 7C. Note that the reverse primer is the allele-specific primer, so the "Syn Rev T" primer (SEQ ID NO. 55) is the perfect match to the Template A (SEQ ID NO. 51), etc.

**Table 42: ΔCq values for AS-qPCR reactions using KlenTaq mutant Taq DNA polymerases.**

| DNA Polymerase | Reverse Primer | | Template | | | |
|---|---|---|---|---|---|---|
| | Name | SEQ ID NO. | A<br>SEQ ID NO. 51 | C<br>SEQ ID NO. 52 | G<br>SEQ ID NO. 53 | T<br>SEQ ID NO. 54 |
| Mutant ID 37 OptiTaq KlenTaq | Syn Rev T | 55 | - | 9.2 +/- 0.3 | 6.9 +/- 0.4 | 10.0 +/- 0.4 |
| | Syn Rev G | 58 | 14.7 +/- 1.2 | - | 11.3 +/- 0.5 | 3.9 +/- 0.3 |
| | Syn Rev C | 56 | 9.4 +/- 0.2 | 10.4 +/- 0.2 | - | 7.5 +/- 0.2 |
| | Syn Rev A | 57 | 13.3 +/- 0.4 | 8.7 +/- 0.2 | 12.4 +/- 0.4 | - |
| Mutant ID 38 A661E, I665W, F667L KlenTaq | Syn Rev T | 55 | - | 11.8 +/- 0.5 | 9.9 +/- 0.6 | 11.7 +/- 0.7 |
| | Syn Rev G | 58 | 17.4 +/- 5.8 | - | 13.1 +/- 1.5 | 7.9 +/- 2.9 |
| | Syn Rev C | 56 | 10.8 +/- 0.4 | 11.0 +/- 0.5 | - | 10.5 +/- 0.2 |
| | Syn Rev A | 57 | 13.8 +/- 0.6 | 11.5 +/- 0.4 | 13.6 +/- 0.8 | - |
| Mutant ID 39 V783F KlenTaq | Syn Rev T | 55 | - | 10.9 +/- 0.3 | 8.7 +/- 0.3 | 11.3 +/- 0.4 |
| | Syn Rev G | 58 | 17.1 +/- 6.7 | - | 12.9 +/- 0.7 | 6.9 +/- 0.3 |
| | Syn Rev C | 56 | 10.5 +/- 0.2 | 10.5 +/- 0.6 | - | 10.0 +/- 0.2 |
| | Syn Rev A | 57 | 13.4 +/- 0.6 | 10.6 +/- 0.2 | 13.0 +/- 0.4 | - |
| Mutant ID 40 H784Q KlenTaq | Syn Rev T | 55 | - | 11.5 +/- 0.4 | 10.1 +/-0.3 | 12.0 +/- 0.9 |
| | Syn Rev G | 58 | 18.2 +/- 6.4 | - | 13.2 +/- 0.5 | 7.9 +/- 0.3 |
| | Syn Rev C | 56 | 10.7 +/- 0.4 | 10.5 +/- 0.6 | - | 10.2 +/- 0.3 |

| DNA Polymerase | Reverse Primer | | Template | | | |
|---|---|---|---|---|---|---|
| | Name | SEQ ID NO. | A<br>SEQ ID NO. 51 | C<br>SEQ ID NO. 52 | G<br>SEQ ID NO. 53 | T<br>SEQ ID NO. 54 |
| | Syn Rev A | 57 | 13.8 +/- 0.7 | 11.3 +/- 0.3 | 13.5 +/- 0.6 | - |
| Mutant ID 41 V783L H784Q KlenTaq | Syn Rev T | 55 | - | 8.9 +/- 0.3 | 7.8 +/- 0.3 | 10.5 +/- 0.4 |
| | Syn Rev G | 58 | 15.8 +/- 4.3 | - | 12.4 +/- 0.5 | 5.8 +/- 0.3 |
| | Syn Rev C | 56 | 10.1 +/- 0.5 | 10.2 +/- 0.2 | - | 8.5 +/- 0.4 |
| | Syn Rev A | 57 | 13.3 +/- 0.8 | 9.5 +/- 0.3 | 12.6 +/- 0.4 | - |
| Mutant ID 42 H784S KlenTaq | Syn Rev T | 55 | - | 12.1 +/- 0.7 | 10.2 +/- 0.4 | 11.4 +/- 0.6 |
| | Syn Rev G | 58 | 15.8 +/- 1.0 | - | 13.3 +/- 0.6 | 8.3 +/- 0.5 |
| | Syn Rev C | 56 | 10.3 +/- 0.3 | 10.6 +/- 0.5 | - | 10.1 +/- 0.4 |
| | Syn Rev A | 57 | 14.1 +/- 0.4 | 12.0 +/-0.4 | 14.1 +/- 0.3 | - |
| Mutant ID 43 H784Y KlenTaq | Syn Rev T | 55 | - | 11.3 +/- 0.4 | 8.5 +/- 0.4 | 11.0 +/- 0.4 |
| | Syn Rev G | 58 | 15.5 +/- 1.2 | - | 12.4 +/- 0.7 | 6.5 +/- 0.3 |
| | Syn Rev C | 56 | 9.9 +/- 0.3 | 10.3 +/- 0.5 | - | 9.3 +/- 0.4 |
| | Syn Rev A | 57 | 13.7 +/- 1.2 | 11.6 +/- 1.2 | 13.9 +/- 1.5 | - |

Average ΔCq values are shown, where ΔCq = [Cq mismatch – Cq match], +/- standard deviation calculated from 24 replicates.

**[0255]** The OptiTaq KlenTaq Mutant ID 37 showed an average ΔCq for AS-qPCR in this synthetic amplicon system of 9.8 with a range of 3.9 to 14.7. Mutant ID 38 (A661E, I665W, F667L KlenTaq) showed an average ΔCq of 11.9 with a range of 7.9 to 17.4. Mutant ID 39 (V783F KlenTaq) showed an average ΔCq of 11.3 with a range of 6.9 to 17.1. Mutant ID 40

(H784Q KlenTaq) showed an average ΔCq of 11.9 with a range of 7.9 to 18.2. Mutant ID 41 (V783L H784Q KlenTaq) showed an average ΔCq of 10.5 with a range of 5.8 to 15.8. Mutant ID 42 (H784S KlenTaq) showed an average ΔCq of 11.9 with a range of 8.3 to 15.8. Mutant ID 43 (H784Y KlenTaq) showed an average ΔCq of 11.2 with a range of 6.5 to 15.5. Therefore, in all pairwise combinations of 4 template bases and 4 3'-terminal primer bases the mutant Taq DNA polymerases of the present invention showed greater discrimination to mismatch than did the OptiTaq or OptiTaq KlenTaq DNA polymerases. The magnitude of improvement for each mismatch pair is defined by the ΔΔCq, which is the difference of discrimination between the mutant and wild type KlenTaq enzymes (ΔΔCq = ΔCq mutant KlenTaq– ΔCq OptiTaq KlenTaq). The ΔΔCq values were calculated and are shown in Table 43.

**Table 43: ΔΔCq values for AS-qPCR reactions for the mutant KlenTaq DNA polymerases compared with OptiTaq KlenTaq.**

| DNA Polymerase | Reverse Primer | | Template | | | |
|---|---|---|---|---|---|---|
| | Name | SEQ ID NO. | A<br>SEQ ID NO. 51 | C<br>SEQ ID NO. 52 | G<br>SEQ ID NO. 53 | T<br>SEQ ID NO. 54 |
| Mutant ID 38 A661E, I665W, F667L KlenTaq | Syn Rev T | 55 | - | 2.6 | 3 | 1.7 |
| | Syn Rev G | 58 | 2.7 | - | 1.8 | 4 |
| | Syn Rev C | 56 | 1.4 | 0.6 | - | 3 |
| | Syn Rev A | 57 | 0.7 | 2.8 | 1.2 | - |
| Mutant ID 39 V783F KlenTaq | Syn Rev T | 55 | - | 1.7 | 1.8 | 1.3 |
| | Syn Rev G | 58 | 2.4 | - | 1.6 | 3 |
| | Syn Rev C | 56 | 1.1 | 0.1 | - | 2.5 |
| | Syn Rev A | 57 | 0.3 | 1.9 | 0.6 | - |

| DNA Polymerase | Reverse Primer | | Template | | | |
|---|---|---|---|---|---|---|
| | Name | SEQ ID NO. | A<br>SEQ ID NO. 51 | C<br>SEQ ID NO. 52 | G<br>SEQ ID NO. 53 | T<br>SEQ ID NO. 54 |
| Mutant ID 40 H784Q KlenTaq | Syn Rev T | 55 | - | 2.3 | 3.2 | 2 |
| | Syn Rev G | 58 | 3.5 | - | 1.9 | 4 |
| | Syn Rev C | 56 | 1.3 | 0.4 | - | 2.7 |
| | Syn Rev A | 57 | 0.7 | 2.6 | 1.1 | |
| Mutant ID 41 V783L H784Q KlenTaq | Syn Rev T | 55 | - | -0.3 | 0.9 | 0.5 |
| | Syn Rev G | 58 | 1.1 | - | 1.1 | 1.9 |
| | Syn Rev C | 56 | 0.7 | -0.2 | - | 1 |
| | Syn Rev A | 57 | 0.2 | 0.8 | 0.2 | - |
| Mutant ID 42 H784S KlenTaq | Syn Rev T | 55 | - | 2.9 | 3.3 | 1.4 |
| | Syn Rev G | 58 | 1.1 | - | 2 | 4.4 |
| | Syn Rev C | 56 | 0.9 | 0.2 | - | 2.6 |
| | Syn Rev A | 57 | 1 | 3.3 | 1.7 | - |
| Mutant ID 43 H784Y KlenTaq | Syn Rev T | 55 | - | 2.1 | 1.6 | 1 |
| | Syn Rev G | 58 | 0.8 | - | 1.1 | 2.6 |
| | Syn Rev C | 56 | 0.5 | -0.1 | - | 1.8 |
| | Syn Rev A | 57 | 0.6 | 2.9 | 1.5 | - |

Average ΔΔCq values are shown, where ΔΔCq = [ΔCq mutant KlenTaq– ΔCq OptiTaq KlenTaq], from data in Table 42.

**[0256]** Mutant ID 38 (A661E, I665W, F667L KlenTaq) showed an average ΔΔCq of 1.7 compared to OptiTaq KlenTaq. Mutant ID 39 (V783F KlenTaq) showed an average ΔΔCq of 2.0 compared to OptiTaq KlenTaq. Mutant ID 40 (H784Q KlenTaq) showed an average ΔΔCq of 2.1 compared to OptiTaq KlenTaq. Mutant ID 41 (V783L H784Q KlenTaq) showed an average ΔΔCq of 0.7 compared to OptiTaq KlenTaq. Mutant ID 42 (H784S KlenTaq) showed an average ΔΔCq of 2.1 compared to OptiTaq KlenTaq. Mutant ID 43 (H784Y KlenTaq) showed an average ΔΔCq of 2.0 compared to OptiTaq KlenTaq. Therefore, each of the mutant Taq DNA polymerases of the present invention showed a significant improvement in mismatch discrimination over OptiTaq KlenTaq which had complete deletion of the 5'-exonuclease domain but contained no other secondary mutations. Overall, mutant IDs 40 and 42 (H784Q KlenTaq and H784S KlenTaq) showed the best SNP discrimination within the set of mutant enzymes studied in this example using an AS-PCR assay.

**Example 21: Improved mismatch discrimination in rhPCR using mutant KlenTaq DNA polymerases in a human genomic DNA SNP assay.**

**[0257]** Example 20 demonstrated utility of the novel mutant Taq DNA polymerases of the present invention in a synthetic amplicon rhPCR SNP discrimination assay system. The present Example demonstrates utility of the novel mutant Taq DNA polymerases in a human genomic DNA rhPCR SNP discrimination assays system, examining a SNP site in the SMAD7 gene (NM_005904, C/T SNP, rs4939827). The assays employed target DNAs GM18562 (homozygous C/C) and GM18537 (homozygous T/T) from the Coriell Institute for Medical Research (Camden, NJ, USA). One blocked-cleavable primer design was tested, the generation 1 (Gen1) "RDDDDx" primers (see: US Patent Application 2012/0258455 by Behlke et al., entitled, RNASE H-BASED ASSAYS UTILIZING MODIFIED RNA MONOMERS).

**[0258]** Quantitative real-time rhPCR was performed in 10 µL reaction volumes in 384 well format with 20 ng (the equivalent of 6600 copies of target) of human genomic DNA (GM18562 or GM18537). Reactions utilized either 0.5 U (10.8 ng/11.1 nM/111 fmol) of OptiTaq KlenTaq DNA polymerase or 0.5 U of one of the three Taq DNA polymerase mutants (Mutant ID 40 (360 ng/370 nM/3700 fmol); Mutant ID 41 (1060 ng/555 nM/5550

fmol); Mutant ID 43 (216 ng/222 nM/2220 fmol)). Final reaction conditions used were 20 mM Tris-HCL (pH 8.4 at 25°C), 50 mM KCL, 3 mM $MgCl_2$, 0.01% Triton X-100, 800 µM total dNTPs, 200 nM of a forward primer (SEQ ID NOs. 75-79), 200 nM of the universal reverse primer (SEQ ID NO. 74), and 200 nM of the RNase H2 cleavable SMAD7 probe (SEQ ID NO. 166). Sequence of the 85 bp SMAD7 amplicon is shown as SEQ ID NO. 81. Forward primers included RDDDDx configuration Gen1 allele-specific rhPCR primers (SEQ ID NOs. 76 and 77), and the control universal forward primer (SEQ ID NO.75) which is not allele specific. Oligonucleotide reagents employed in this Example are shown in Table 44. Reactions included 1 µL of *P.a.* RNase H2 at a concentration of 2.6 mU per 10 µL reaction (5 fmoles, 0.5 nM). Amplification was performed on a Roche LightCycler® 480 (Roche Applied Science, Indianapolis, IN, USA) as follows: 95°C for 3 minutes followed by 95 cycles of 95°C for 10 seconds and 60°C for 30 seconds. All reactions were performed in triplicate.

**Table 44. Synthetic oligonucleotides employed in Example 21.**

| Name | Sequence (5'-3') | SEQ ID NO. |
|---|---|---|
| SMAD7 Rev | CTCACTCTAAACCCCAGCATT | 74 |
| SMAD7 For | CAGCCTCATCCAAAAGAGGAAA | 75 |
| SMAD7 For rC DDDDx | CAGCCTCATCCAAAAGAGGAAAcAGGAx | 76 |
| SMAD7 For rU DDDDx | CAGCCTCATCCAAAAGAGGAAAuAGGAx | 77 |
| SMAD7 RN2 probe | FAM-CCCAGAGCTCcCTCAGACTCCT-IBFQ | 166 |
| SMAD7 target | CAGCCTCATCCAAAAGAGGAAATAGGACCCCAGAGCTCCCTCA GACTCCTCAGGAAACACAGACAATGCTGGGGTTTAGAGTGAG | 81 |

DNA bases are uppercase and RNA bases are lowercase; FAM = 6-carboxyfluorescein; IBFQ = Iowa Black™ FQ fluorescence quencher; "x" = C3 Spacer (propanediol). Primer and probe binding sites in the SMAD7 target are underlined.

**[0259]** Results using the Gen1 RDDDDx rhPCR primers are shown in Table 45. Use of the mutant Taq DNA polymerases showed significant improvements in SNP discrimination in this human genomic DNA rhPCR assay using the Gen1 RDDDDx primers, although

amplification efficiency was often reduced, as shown by the increases in the match Cqs. Therefore use of the new mutant KlenTaq DNA polymerases improves SNP discrimination in rhPCR genotyping assays.

**Table 45: SNP discrimination of a site in the SMAD7 gene using Gen1 RDDDDx primers comparing wild type OptiTaq with four mutant Taq DNA polymerases.**

| DNA Polymerase | For Primer | SEQ ID NO. | mU RNase H2 per 10 µL rxn | Cq Value C/C DNA | Cq Value T/T DNA | ΔCq |
|---|---|---|---|---|---|---|
| MUT ID 37 OptiTaq KlenTaq | SMAD7 For | 75 | 2.6 | 24.4 | 23.5 | |
| | SMAD7 For rC DDDDx | 76 | 2.6 | 31.1 | 38.4 | 7.3 |
| | SMAD7 For rU DDDDx | 77 | 2.6 | 46.1 | 33.0 | 13.1 |
| MUT ID 40 H784Q KlenTaq | SMAD7 For | 75 | 2.6 | 24.6 | 24.5 | |
| | SMAD7 For rC DDDDx | 76 | 2.6 | 28.1 | 38.8 | 10.7 |
| | SMAD7 For rU DDDDx | 77 | 2.6 | 42.1 | 28.8 | 13.3 |
| MUT ID 41 V783L H784Q KlenTaq | SMAD7 For | 75 | 2.6 | 24.5 | 24.3 | |
| | SMAD7 For rC DDDDx | 76 | 2.6 | 26.2 | 37.6 | 11.4 |
| | SMAD7 For rU DDDDx | 77 | 2.6 | 41.2 | 27.8 | 13.4 |
| MUT ID 43 H784Y KlenTaq | SMAD7 For | 75 | 2.6 | 24.7 | 24.8 | |
| | SMAD7 For rC DDDDx | 76 | 2.6 | 33.8 | 45.1 | 11.2 |
| | SMAD7 For rU DDDDx | 77 | 2.6 | 50.4 | 35.2 | 15.2 |

DNA targets included GM18562 (homozygous C/C) and GM18537 (homozygous T/T) from the Coriell Institute for Medical Research. ΔCq = [Cq mismatch – Cq match].

**Example 22. Sequence of Taq DNA polymerase mutants showing improved discrimination for mismatch or the presence of an RNA residue at the 3'-end of the primer.**

**[0260]** The complete amino acid and nucleotide sequences of the codon optimized mutant enzymes employed in Examples 18-21 are shown below. Although these sequences are easily derived from information provided in Tables 1, 3, 4 26 and 38 by one with skill in the art, the final assembled sequences are provided below for clarity. Base changes are identified in bold underlined font for the nucleic acid and amino acid substitutions.

**[0261]** SEQ ID NO. 167, nucleotide sequence of Mutant ID 37 (OptiTaq KlenTaq).

CATATGGGTTCACTGCTTCATGAATTCGGTCTGTTAGAGTCTCCTAAAGCACTCGAAGAGGCACCGTGGCCGCCC
CCAGAAGGTGCTTTTGTTGGCTTCGTACTTTCCCGTAAGGAGCCTATGTGGGCAGATCTTCTGGCTTTAGCGGCT
GCACGCGGTGGCCGTGTTCACCGGGCCCCTGAGCCATACAAAGCGTTACGTGATCTGAAGGAAGCACGTGGCTTG
CTGGCAAAAGACCTTTCTGTTTTGGCCCTGCGCGAGGGTCTTGGACTGCCGCCAGGCGACGATCCCATGTTATTG
GCCTATCTGTTAGACCCTAGCAATACCACACCTGAAGGGGTCGCTCGTCGGTATGGCGGTGAATGGACTGAGGAA
GCCGGAGAGCGCGCCGCATTGTCCGAACGGCTCTTTGCAAACTTATGGGGTCGTCTGGAAGGGGAGGAACGTCTG
TTATGGTTGTATCGGGAAGTCGAACGTCCTCTTTCGGCCGTATTAGCGCATATGGAGGCAACAGGTGTGCGTTTA
GATGTCGCGTACCTTCGGGCCTTATCACTGGAAGTTGCAGAGGAAATCGCCCGTCTCGAGGCTGAAGTGTTCCGG
TTGGCCGGTCACCCGTTTAACCTCAACTCCCGTGACCAGCTGGAACGCGTTTTATTCGATGAGCTTGGGCTTCCC
GCAATTGGCAAAACCGAAAAGACTGGCAAACGCAGTACGAGCGCTGCCGTCCTTGAGGCACTCCGCGAGGCTCAC
CCTATTGTAGAAAAGATCCTGCAATACCGTGAGTTGACGAAGCTTAAAAGCACTTATATTGATCCTCTCCCGGAT
CTGATCCATCCTCGTACCGGCCGCTTGCACACACGTTTCAACCAGACGGCGACTGCAACCGGCCGTCTGTCTAGC
TCGGATCCAAATCTCCAGAACATTCCGGTCCGTACACCCTTGGGCCAACGTATCCGCCGGGCGTTTATCGCTGAG
GAAGGATGGTTACTGGTCGCATTGGACTACTCGCAGATTGAGCTGCGCGTCCTCGCACATCTCTCTGGTGACGAA
AATTTAATCCGCGTGTTTCAAGAGGGGCGTGATATTCACACAGAAACTGCCTCATGGATGTTCGGTGTCCCACGT
GAAGCAGTGGATCCTTTGATGCGCCGTGCAGCTAAAACAATTAATTTTGGAGTGCTGTACGGAATGAGCGCTCAT
CGCTTGAGTCAGGAACTGGCAATCCCCTACGAGGAAGCGCAGGCATTCATCGAACGTTACTTTCAATCGTTTCCG
AAAGTTCGCGCATGGATCGAGAAGACGCTCGAGGAAGGTCGTCGTCGGGGCTATGTCGAAACTCTGTTTGGTCGC
CGTCGGTACGTACCAGATCTTGAAGCCCGCGTCAAATCGGTACGGGAGGCTGCGGAGCGTATGGCATTTAATATG
CCTGTACAGGGTACTGCAGCTGACCTCATGAAACTGGCAATGGTCAAGCTTTTCCCGCGCTTGGAGGAAATGGGC
GCACGTATGCTTCTGCAGGTCCATGACGAGCTGGTGTTAGAAGCCCCTAAGGAGCGCGCCGAAGCTGTCGCGCGC
CTCGCTAAAGAAGTGATGGAGGGCGTTTACCCATTGGCCGTACCCCTCGAAGTGGAGGTCGGTATTGGAGAAGAT
TGGTTATCTGCAAAGGAAGCGGCCGC

**[0262]** SEQ ID NO. 168, amino acid sequence of Mutant ID 37 (OptiTaq KlenTaq).

MGSLLHEFGLLESPKALEEAPWPPPEGAFVGFVLSRKEPMWADLLALAAARGGRVHRAPEPYKALRDLKEARGLL
AKDLSVLALREGLGLPPGDDPMLLAYLLDPSNTTPEGVARRYGGEWTEEAGERAALSERLFANLWGRLEGEERLL
WLYREVERPLSAVLAHMEATGVRLDVAYLRALSLEVAEEIARLEAEVFRLAGHPFNLNSRDQLERVLFDELGLPA
IGKTEKTGKRSTSAAVLEALREAHPIVEKILQYRELTKLKSTYIDPLPDLIHPRTGRLHTRFNQTATATGRLSSS
DPNLQNIPVRTPLGQRIRRAFIAEEGWLLVALDYSQIELRVLAHLSGDENLIRVFQEGRDIHTETASWMFGVPRE
AVDPLMRRAAKTINFGVLYGMSAHRLSQELAIPYEEAQAFIERYFQSFPKVRAWIEKTLEEGRRRGYVETLFGRR
RYVPDLEARVKSVREAAERMAFNMPVQGTAADLMKLAMVKLFPRLEEMGARMLLQVHDELVLEAPKERAEAVARL
AKEVMEGVYPLAVPLEVEVGIGEDWLSAKEAA

**[0263]** SEQ ID NO. 169, nucleotide sequence of Mutant ID 38 (A661E, I665W, F667L KlenTaq).

CATATGGGTTCACTGCTTCATGAATTCGGTCTGTTAGAGTCTCCTAAAGCACTCGAAGAGGCACCGTGGCCGCCC
CCAGAAGGTGCTTTTGTTGGCTTCGTACTTTCCCGTAAGGAGCCTATGTGGGCAGATCTTCTGGCTTTAGCGGCT
GCACGCGGTGGCCGTGTTCACCGGGCCCCTGAGCCATACAAAGCGTTACGTGATCTGAAGGAAGCACGTGGCTTG
CTGGCAAAAGACCTTTCTGTTTTGGCCCTGCGCGAGGGTCTTGGACTGCCGCCAGGCGACGATCCCATGTTATTG
GCCTATCTGTTAGACCCTAGCAATACCACACCTGAAGGGGTCGCTCGTCGGTATGGCGGTGAATGGACTGAGGAA
GCCGGAGAGCGCGCCGCATTGTCCGAACGGCTCTTTGCAAACTTATGGGGTCGTCTGGAAGGGGAGGAACGTCTG
TTATGGTTGTATCGGGAAGTCGAACGTCCTCTTTCGGCCGTATTAGCGCATATGGAGGCAACAGGTGTGCGTTTA
GATGTCGCGTACCTTCGGGCCTTATCACTGGAAGTTGCAGAGGAAATCGCCCGTCTCGAGGCTGAAGTGTTCCGG
TTGGCCGGTCACCCGTTTAACCTCAACTCCCGTGACCAGCTGGAACGCGTTTTATTCGATGAGCTTGGGCTTCCC
GCAATTGGCAAAACCGAAAAGACTGGCAAACGCAGTACGAGCGCTGCCGTCCTTGAGGCACTCCGCGAGGCTCAC
CCTATTGTAGAAAAGATCCTGCAATACCGTGAGTTGACGAAGCTTAAAAGCACTTATATTGATCCTCTCCCGGAT
CTGATCCATCCTCGTACCGGCCGCTTGCACACACGTTTCAACCAGACGGCGACTGCAACCGGCCGTCTGTCTAGC
TCGGATCCAAATCTCCAGAACATTCCGGTCCGTACACCCTTGGGCCAACGTATCCGCCGGGCGTTTATCGCTGAG
GAAGGATGGTTACTGGTCGCATTGGACTACTCGCAGATTGAGCTGCGCGTCCTCGCACATCTCTCTGGTGACGAA
AATTTAATCCGCGTGTTTCAAGAGGGGCGTGATATTCACACAGAAACTGCCTCATGGATGTTCGGTGTCCCACGT
GAAGCAGTGGATCCTTTGATGCGCCGT**GAA**GCTAAAACA**TGG**AAT**TTG**GGAGTGCTGTACGGAATGAGCGCTCAT
CGCTTGAGTCAGGAACTGGCAATCCCCTACGAGGAAGCGCAGGCATTCATCGAACGTTACTTTCAATCGTTTCCG
AAAGTTCGCGCATGGATCGAGAAGACGCTCGAGGAAGGTCGTCGTCGGGGCTATGTCGAAACTCTGTTTGGTCGC
CGTCGGTACGTACCAGATCTTGAAGCCCGCGTCAAATCGGTACGGGAGGCTGCGGAGCGTATGGCATTTAATATG
CCTGTACAGGGTACTGCAGCTGACCTCATGAAACTGGCAATGGTCAAGCTTTTCCCGCGCTTGGAGGAAATGGGC
GCACGTATGCTTCTGCAGGTCCATGACGAGCTGGTGTTAGAAGCCCCTAAGGAGCGCGCCGAAGCTGTCGCGCGC
CTCGCTAAAGAAGTGATGGAGGGCGTTTACCCATTGGCCGTACCCCTCGAAGTGGAGGTCGGTATTGGAGAAGAT
TGGTTATCTGCAAAGGAAGCGGCCGC

**[0264]** SEQ ID NO. 170, amino acid sequence of Mutant ID 38 (A661E, I665W, F667L KlenTaq).

MGSLLHEFGLLESPKALEEAPWPPPEGAFVGFVLSRKEPMWADLLALAAARGGRVHRAPEPYKALRDLKEARGLL
AKDLSVLALREGLGLPPGDDPMLLAYLLDPSNTTPEGVARRYGGEWTEEAGERAALSERLFANLWGRLEGEERLL
WLYREVERPLSAVLAHMEATGVRLDVAYLRALSLEVAEEIARLEAEVFRLAGHPFNLNSRDQLERVLFDELGLPA
IGKTEKTGKRSTSAAVLEALREAHPIVEKILQYRELTKLKSTYIDPLPDLIHPRTGRLHTRFNQTATATGRLSSS
DPNLQNIPVRTPLGQRIRRAFIAEEGWLLVALDYSQIELRVLAHLSGDENLIRVFQEGRDIHTETASWMFGVPRE
AVDPLMRR**E**AKT**W**N**L**GVLYGMSAHRLSQELAIPYEEAQAFIERYFQSFPKVRAWIEKTLEEGRRRGYVETLFGRR
RYVPDLEARVKSVREAAERMAFNMPVQGTAADLMKLAMVKLFPRLEEMGARMLLQVHDELVLEAPKERAEAVARL
AKEVMEGVYPLAVPLEVEVGIGEDWLSAKEAA

**[0265]** SEQ ID NO. 171, nucleotide sequence of Mutant ID 39 (V783F KlenTaq).

CATATGGGTTCACTGCTTCATGAATTCGGTCTGTTAGAGTCTCCTAAAGCACTCGAAGAGGCACCGTGGCCGCCC
CCAGAAGGTGCTTTTGTTGGCTTCGTACTTTCCCGTAAGGAGCCTATGTGGGCAGATCTTCTGGCTTTAGCGGCT
GCACGCGGTGGCCGTGTTCACCGGGCCCCTGAGCCATACAAAGCGTTACGTGATCTGAAGGAAGCACGTGGCTTG
CTGGCAAAAGACCTTTCTGTTTTGGCCCTGCGCGAGGGTCTTGGACTGCCGCCAGGCGACGATCCCATGTTATTG
GCCTATCTGTTAGACCCTAGCAATACCACACCTGAAGGGGTCGCTCGTCGGTATGGCGGTGAATGGACTGAGGAA
GCCGGAGAGCGCGCCGCATTGTCCGAACGGCTCTTTGCAAACTTATGGGGTCGTCTGGAAGGGGAGGAACGTCTG
TTATGGTTGTATCGGGAAGTCGAACGTCCTCTTTCGGCCGTATTAGCGCATATGGAGGCAACAGGTGTGCGTTTA
GATGTCGCGTACCTTCGGGCCTTATCACTGGAAGTTGCAGAGGAAATCGCCCGTCTCGAGGCTGAAGTGTTCCGG
TTGGCCGGTCACCCGTTTAACCTCAACTCCCGTGACCAGCTGGAACGCGTTTTATTCGATGAGCTTGGGCTTCCC
GCAATTGGCAAAACCGAAAAGACTGGCAAACGCAGTACGAGCGCTGCCGTCCTTGAGGCACTCCGCGAGGCTCAC
CCTATTGTAGAAAAGATCCTGCAATACCGTGAGTTGACGAAGCTTAAAAGCACTTATATTGATCCTCTCCCGGAT
CTGATCCATCCTCGTACCGGCCGCTTGCACACACGTTTCAACCAGACGGCGACTGCAACCGGCCGTCTGTCTAGC

TCGGATCCAAATCTCCAGAACATTCCGGTCCGTACACCCTTGGGCCAACGTATCCGCCGGGCGTTTATCGCTGAG
GAAGGATGGTTACTGGTCGCATTGGACTACTCGCAGATTGAGCTGCGCGTCCTCGCACATCTCTCTGGTGACGAA
AATTTAATCCGCGTGTTTCAAGAGGGGCGTGATATTCACACAGAAACTGCCTCATGGATGTTCGGTGTCCCACGT
GAAGCAGTGGATCCTTTGATGCGCCGTGCAGCTAAAACAATTAATTTTGGAGTGCTGTACGGAATGAGCGCTCAT
CGCTTGAGTCAGGAACTGGCAATCCCCTACGAGGAAGCGCAGGCATTCATCGAACGTTACTTTCAATCGTTTCCG
AAAGTTCGCGCATGGATCGAGAAGACGCTCGAGGAAGGTCGTCGTCGGGGCTATGTCGAAACTCTGTTTGGTCGC
CGTCGGTACGTACCAGATCTTGAAGCCCGCGTCAAATCGGTACGGGAGGCTGCGGAGCGTATGGCATTTAATATG
CCTGTACAGGGTACTGCAGCTGACCTCATGAAACTGGCAATGGTCAAGCTTTTCCCGCGCTTGGAGGAAATGGGC
GCACGTATGCTTCTGCAG**TTC**CATGACGAGCTGGTGTTAGAAGCCCCTAAGGAGCGCGCCGAAGCTGTCGCGCGC
CTCGCTAAAGAAGTGATGGAGGGCGTTTACCCATTGGCCGTACCCCTCGAAGTGGAGGTCGGTATTGGAGAAGAT
TGGTTATCTGCAAAGGAAGCGGCCGC

**[0266]** SEQ ID NO. 172, amino acid sequence of Mutant ID 39 (V783F KlenTaq).

**[0267]** MGSLLHEFGLLESPKALEEAPWPPPEGAFVGFVLSRKEPMWADLLALAAARGGRVHRAPEPYKALR
DLKEARGLLAKDLSVLALREGLGLPPGDDPMLLAYLLDPSNTTPEGVARRYGGEWTEEAGERAALSERLFANLWG
RLEGEERLLWLYREVERPLSAVLAHMEATGVRLDVAYLRALSLEVAEEIARLEAEVFRLAGHPFNLNSRDQLERV
LFDELGLPAIGKTEKTGKRSTSAAVLEALREAHPIVEKILQYRELTKLKSTYIDPLPDLIHPRTGRLHTRFNQTA
TATGRLSSSDPNLQNIPVRTPLGQRIRRAFIAEEGWLLVALDYSQIELRVLAHLSGDENLIRVFQEGRDIHTETA
SWMFGVPREAVDPLMRRAAKTINFGVLYGMSAHRLSQELAIPYEEAQAFIERYFQSFPKVRAWIEKTLEEGRRRG
YVETLFGRRRYVPDLEARVKSVREAAERMAFNMPVQGTAADLMKLAMVKLFPRLEEMGARMLLQ**L**HDELVLEAPK
ERAEAVARLAKEVMEGVYPLAVPLEVEVGIGEDWLSAKEAA

**[0268]** SEQ ID NO. 173, nucleotide sequence of Mutant ID 40 (H784Q KlenTaq).

CATATGGGTTCACTGCTTCATGAATTCGGTCTGTTAGAGTCTCCTAAAGCACTCGAAGAGGCACCGTGGCCGCCC
CCAGAAGGTGCTTTTGTTGGCTTCGTACTTTCCCGTAAGGAGCCTATGTGGGCAGATCTTCTGGCTTTAGCGGCT
GCACGCGGTGGCCGTGTTCACCGGGCCCCTGAGCCATACAAAGCGTTACGTGATCTGAAGGAAGCACGTGGCTTG
CTGGCAAAAGACCTTTCTGTTTTGGCCCTGCGCGAGGGTCTTGGACTGCCGCCAGGCGACGATCCCATGTTATTG
GCCTATCTGTTAGACCCTAGCAATACCACACCTGAAGGGGTCGCTCGTCGGTATGGCGGTGAATGGACTGAGGAA
GCCGGAGAGCGCGCCGCATTGTCCGAACGGCTCTTTGCAAACTTATGGGGTCGTCTGGAAGGGGAGGAACGTCTG
TTATGGTTGTATCGGGAAGTCGAACGTCCTCTTTCGGCCGTATTAGCGCATATGGAGGCAACAGGTGTGCGTTTA
GATGTCGCGTACCTTCGGGCCTTATCACTGGAAGTTGCAGAGGAAATCGCCCGTCTCGAGGCTGAAGTGTTCCGG
TTGGCCGGTCACCCGTTTAACCTCAACTCCCGTGACCAGCTGGAACGCGTTTTATTCGATGAGCTTGGGCTTCCC
GCAATTGGCAAAACCGAAAAGACTGGCAAACGCAGTACGAGCGCTGCCGTCCTTGAGGCACTCCGCGAGGCTCAC
CCTATTGTAGAAAAGATCCTGCAATACCGTGAGTTGACGAAGCTTAAAAGCACTTATATTGATCCTCTCCCGGAT
CTGATCCATCCTCGTACCGGCCGCTTGCACACACGTTTCAACCAGACGGCGACTGCAACCGGCCGTCTGTCTAGC
TCGGATCCAAATCTCCAGAACATTCCGGTCCGTACACCCTTGGGCCAACGTATCCGCCGGGCGTTTATCGCTGAG
GAAGGATGGTTACTGGTCGCATTGGACTACTCGCAGATTGAGCTGCGCGTCCTCGCACATCTCTCTGGTGACGAA
AATTTAATCCGCGTGTTTCAAGAGGGGCGTGATATTCACACAGAAACTGCCTCATGGATGTTCGGTGTCCCACGT
GAAGCAGTGGATCCTTTGATGCGCCGTGCAGCTAAAACAATTAATTTTGGAGTGCTGTACGGAATGAGCGCTCAT
CGCTTGAGTCAGGAACTGGCAATCCCCTACGAGGAAGCGCAGGCATTCATCGAACGTTACTTTCAATCGTTTCCG
AAAGTTCGCGCATGGATCGAGAAGACGCTCGAGGAAGGTCGTCGTCGGGGCTATGTCGAAACTCTGTTTGGTCGC
CGTCGGTACGTACCAGATCTTGAAGCCCGCGTCAAATCGGTACGGGAGGCTGCGGAGCGTATGGCATTTAATATG
CCTGTACAGGGTACTGCAGCTGACCTCATGAAACTGGCAATGGTCAAGCTTTTCCCGCGCTTGGAGGAAATGGGC
GCACGTATGCTTCTGCAGGTC**CAG**GACGAGCTGGTGTTAGAAGCCCCTAAGGAGCGCGCCGAAGCTGTCGCGCGC
CTCGCTAAAGAAGTGATGGAGGGCGTTTACCCATTGGCCGTACCCCTCGAAGTGGAGGTCGGTATTGGAGAAGAT
TGGTTATCTGCAAAGGAAGCGGCCGC

**[0269]** SEQ ID NO. 174, amino acid sequence of Mutant ID 40 (H784Q KlenTaq).

MGSLLHEFGLLESPKALEEAPWPPPEGAFVGFVLSRKEPMWADLLALAAARGGRVHRAPEPYKALRDLKEARGLL
AKDLSVLALREGLGLPPGDDPMLLAYLLDPSNTTPEGVARRYGGEWTEEAGERAALSERLFANLWGRLEGEERLL
WLYREVERPLSAVLAHMEATGVRLDVAYLRALSLEVAEEIARLEAEVFRLAGHPFNLNSRDQLERVLFDELGLPA
IGKTEKTGKRSTSAAVLEALREAHPIVEKILQYRELTKLKSTYIDPLPDLIHPRTGRLHTRFNQTATATGRLSSS
DPNLQNIPVRTPLGQRIRRAFIAEEGWLLVALDYSQIELRVLAHLSGDENLIRVFQEGRDIHTETASWMFGVPRE
AVDPLMRRAAKTINFGVLYGMSAHRLSQELAIPYEEAQAFIERYFQSFPKVRAWIEKTLEEGRRRGYVETLFGRR
RYVPDLEARVKSVREAAERMAFNMPVQGTAADLMKLAMVKLFPRLEEMGARMLLQV**Q**DELVLEAPKERAEAVARL
AKEVMEGVYPLAVPLEVEVGIGEDWLSAKEAA

**[0270]** SEQ ID NO. 175, nucleotide sequence of Mutant ID 41 (V783L H784Q KlenTaq).

CATATGGGTTCACTGCTTCATGAATTCGGTCTGTTAGAGTCTCCTAAAGCACTCGAAGAGGCACCGTGGCCGCCC
CCAGAAGGTGCTTTTGTTGGCTTCGTACTTTCCCGTAAGGAGCCTATGTGGGCAGATCTTCTGGCTTTAGCGGCT
GCACGCGGTGGCCGTGTTCACCGGGCCCCTGAGCCATACAAAGCGTTACGTGATCTGAAGGAAGCACGTGGCTTG
CTGGCAAAAGACCTTTCTGTTTTGGCCCTGCGCGAGGGTCTTGGACTGCCGCCAGGCGACGATCCCATGTTATTG
GCCTATCTGTTAGACCCTAGCAATACCACACCTGAAGGGGTCGCTCGTCGGTATGGCGGTGAATGGACTGAGGAA
GCCGGAGAGCGCGCCGCATTGTCCGAACGGCTCTTTGCAAACTTATGGGGTCGTCTGGAAGGGGAGGAACGTCTG
TTATGGTTGTATCGGGAAGTCGAACGTCCTCTTTCGGCCGTATTAGCGCATATGGAGGCAACAGGTGTGCGTTTA
GATGTCGCGTACCTTCGGGCCTTATCACTGGAAGTTGCAGAGGAAATCGCCCGTCTCGAGGCTGAAGTGTTCCGG
TTGGCCGGTCACCCGTTTAACCTCAACTCCCGTGACCAGCTGGAACGCGTTTTATTCGATGAGCTTGGGCTTCCC
GCAATTGGCAAAACCGAAAAGACTGGCAAACGCAGTACGAGCGCTGCCGTCCTTGAGGCACTCCGCGAGGCTCAC
CCTATTGTAGAAAAGATCCTGCAATACCGTGAGTTGACGAAGCTTAAAAGCACTTATATTGATCCTCTCCCGGAT
CTGATCCATCCTCGTACCGGCCGCTTGCACACACGTTTCAACCAGACGGCGACTGCAACCGGCCGTCTGTCTAGC
TCGGATCCAAATCTCCAGAACATTCCGGTCCGTACACCCTTGGGCCAACGTATCCGCCGGGCGTTTATCGCTGAG
GAAGGATGGTTACTGGTCGCATTGGACTACTCGCAGATTGAGCTGCGCGTCCTCGCACATCTCTCTGGTGACGAA
AATTTAATCCGCGTGTTTCAAGAGGGGCGTGATATTCACACAGAAACTGCCTCATGGATGTTCGGTGTCCCACGT
GAAGCAGTGGATCCTTTGATGCGCCGTGCAGCTAAAACAATTAATTTTGGAGTGCTGTACGGAATGAGCGCTCAT
CGCTTGAGTCAGGAACTGGCAATCCCCTACGAGGAAGCGCAGGCATTCATCGAACGTTACTTTCAATCGTTTCCG
AAAGTTCGCGCATGGATCGAGAAGACGCTCGAGGAAGGTCGTCGTCGGGGCTATGTCGAAACTCTGTTTGGTCGC
CGTCGGTACGTACCAGATCTTGAAGCCCGCGTCAAATCGGTACGGGAGGCTGCGGAGCGTATGGCATTTAATATG
CCTGTACAGGGTACTGCAGCTGACCTCATGAAACTGGCAATGGTCAAGCTTTTCCCGCGCTTGGAGGAAATGGGC
GCACGTATGCTTCTGCAG**CTGCAG**GACGAGCTGGTGTTAGAAGCCCCTAAGGAGCGCGCCGAAGCTGTCGCGCGC
CTCGCTAAAGAAGTGATGGAGGGCGTTTACCCATTGGCCGTACCCCTCGAAGTGGAGGTCGGTATTGGAGAAGAT
TGGTTATCTGCAAAGGAAGCGGCCGC

**[0271]** SEQ ID NO. 176, amino acid sequence of Mutant ID 41 (V783L H784Q KlenTaq).

MGSLLHEFGLLESPKALEEAPWPPPEGAFVGFVLSRKEPMWADLLALAAARGGRVHRAPEPYKALRDLKEARGLL
AKDLSVLALREGLGLPPGDDPMLLAYLLDPSNTTPEGVARRYGGEWTEEAGERAALSERLFANLWGRLEGEERLL
WLYREVERPLSAVLAHMEATGVRLDVAYLRALSLEVAEEIARLEAEVFRLAGHPFNLNSRDQLERVLFDELGLPA
IGKTEKTGKRSTSAAVLEALREAHPIVEKILQYRELTKLKSTYIDPLPDLIHPRTGRLHTRFNQTATATGRLSSS
DPNLQNIPVRTPLGQRIRRAFIAEEGWLLVALDYSQIELRVLAHLSGDENLIRVFQEGRDIHTETASWMFGVPRE
AVDPLMRRAAKTINFGVLYGMSAHRLSQELAIPYEEAQAFIERYFQSFPKVRAWIEKTLEEGRRRGYVETLFGRR
RYVPDLEARVKSVREAAERMAFNMPVQGTAADLMKLAMVKLFPRLEEMGARMLLQ**LH**DELVLEAPKERAEAVARL
AKEVMEGVYPLAVPLEVEVGIGEDWLSAKEAA

**[0272]** SEQ ID NO. 177, nucleotide sequence of Mutant ID 42 (H784S KlenTaq).

CATATGGGTTCACTGCTTCATGAATTCGGTCTGTTAGAGTCTCCTAAAGCACTCGAAGAGGCACCGTGGCCGCCC
CCAGAAGGTGCTTTTGTTGGCTTCGTACTTTCCCGTAAGGAGCCTATGTGGGCAGATCTTCTGGCTTTAGCGGCT
GCACGCGGTGGCCGTGTTCACCGGGCCCCTGAGCCATACAAAGCGTTACGTGATCTGAAGGAAGCACGTGGCTTG
CTGGCAAAAGACCTTTCTGTTTTGGCCCTGCGCGAGGGTCTTGGACTGCCGCCAGGCGACGATCCCATGTTATTG
GCCTATCTGTTAGACCCTAGCAATACCACACCTGAAGGGGTCGCTCGTCGGTATGGCGGTGAATGGACTGAGGAA
GCCGGAGAGCGCGCCGCATTGTCCGAACGGCTCTTTGCAAACTTATGGGGTCGTCTGGAAGGGGAGGAACGTCTG
TTATGGTTGTATCGGGAAGTCGAACGTCCTCTTTCGGCCGTATTAGCGCATATGGAGGCAACAGGTGTGCGTTTA
GATGTCGCGTACCTTCGGGCCTTATCACTGGAAGTTGCAGAGGAAATCGCCCGTCTCGAGGCTGAAGTGTTCCGG
TTGGCCGGTCACCCGTTTAACCTCAACTCCCGTGACCAGCTGGAACGCGTTTTATTCGATGAGCTTGGGCTTCCC
GCAATTGGCAAAACCGAAAAGACTGGCAAACGCAGTACGAGCGCTGCCGTCCTTGAGGCACTCCGCGAGGCTCAC
CCTATTGTAGAAAAGATCCTGCAATACCGTGAGTTGACGAAGCTTAAAAGCACTTATATTGATCCTCTCCCGGAT
CTGATCCATCCTCGTACCGGCCGCTTGCACACACGTTTCAACCAGACGGCGACTGCAACCGGCCGTCTGTCTAGC
TCGGATCCAAATCTCCAGAACATTCCGGTCCGTACACCCTTGGGCCAACGTATCCGCCGGGCGTTTATCGCTGAG
GAAGGATGGTTACTGGTCGCATTGGACTACTCGCAGATTGAGCTGCGCGTCCTCGCACATCTCTCTGGTGACGAA
AATTTAATCCGCGTGTTTCAAGAGGGGCGTGATATTCACACAGAAACTGCCTCATGGATGTTCGGTGTCCCACGT
GAAGCAGTGGATCCTTTGATGCGCCGTGCAGCTAAAACAATTAATTTTGGAGTGCTGTACGGAATGAGCGCTCAT
CGCTTGAGTCAGGAACTGGCAATCCCCTACGAGGAAGCGCAGGCATTCATCGAACGTTACTTTCAATCGTTTCCG
AAAGTTCGCGCATGGATCGAGAAGACGCTCGAGGAAGGTCGTCGTCGGGGCTATGTCGAAACTCTGTTTGGTCGC
CGTCGGTACGTACCAGATCTTGAAGCCCGCGTCAAATCGGTACGGGAGGCTGCGGAGCGTATGGCATTTAATATG
CCTGTACAGGGTACTGCAGCTGACCTCATGAAACTGGCAATGGTCAAGCTTTTCCCGCGCTTGGAGGAAATGGGC
GCACGTATGCTTCTGCAGGTC**AGC**GACGAGCTGGTGTTAGAAGCCCCTAAGGAGCGCGCCGAAGCTGTCGCGCGC
CTCGCTAAAGAAGTGATGGAGGGCGTTTACCCATTGGCCGTACCCCTCGAAGTGGAGGTCGGTATTGGAGAAGAT
TGGTTATCTGCAAAGGAAGCGGCCGC

**[0273]** SEQ ID NO. 178, amino acid sequence of Mutant ID 42 (H784S KlenTaq).

MGSLLHEFGLLESPKALEEAPWPPPEGAFVGFVLSRKEPMWADLLALAAARGGRVHRAPEPYKALRDLKEARGLL
AKDLSVLALREGLGLPPGDDPMLLAYLLDPSNTTPEGVARRYGGEWTEEAGERAALSERLFANLWGRLEGEERLL
WLYREVERPLSAVLAHMEATGVRLDVAYLRALSLEVAEEIARLEAEVFRLAGHPFNLNSRDQLERVLFDELGLPA
IGKTEKTGKRSTSAAVLEALREAHPIVEKILQYRELTKLKSTYIDPLPDLIHPRTGRLHTRFNQTATATGRLSSS
DPNLQNIPVRTPLGQRIRRAFIAEEGWLLVALDYSQIELRVLAHLSGDENLIRVFQEGRDIHTETASWMFGVPRE
AVDPLMRRAAKTINFGVLYGMSAHRLSQELAIPYEEAQAFIERYFQSFPKVRAWIEKTLEEGRRRGYVETLFGRR
RYVPDLEARVKSVREAAERMAFNMPVQGTAADLMKLAMVKLFPRLEEMGARMLLQV**S**DELVLEAPKERAEAVARL
AKEVMEGVYPLAVPLEVEVGIGEDWLSAKEAA

**[0274]** SEQ ID NO. 179, nucleotide sequence of Mutant ID 43 (H784Y KlenTaq).

CATATGGGTTCACTGCTTCATGAATTCGGTCTGTTAGAGTCTCCTAAAGCACTCGAAGAGGCACCGTGGCCGCCC
CCAGAAGGTGCTTTTGTTGGCTTCGTACTTTCCCGTAAGGAGCCTATGTGGGCAGATCTTCTGGCTTTAGCGGCT
GCACGCGGTGGCCGTGTTCACCGGGCCCCTGAGCCATACAAAGCGTTACGTGATCTGAAGGAAGCACGTGGCTTG
CTGGCAAAAGACCTTTCTGTTTTGGCCCTGCGCGAGGGTCTTGGACTGCCGCCAGGCGACGATCCCATGTTATTG
GCCTATCTGTTAGACCCTAGCAATACCACACCTGAAGGGGTCGCTCGTCGGTATGGCGGTGAATGGACTGAGGAA
GCCGGAGAGCGCGCCGCATTGTCCGAACGGCTCTTTGCAAACTTATGGGGTCGTCTGGAAGGGGAGGAACGTCTG
TTATGGTTGTATCGGGAAGTCGAACGTCCTCTTTCGGCCGTATTAGCGCATATGGAGGCAACAGGTGTGCGTTTA
GATGTCGCGTACCTTCGGGCCTTATCACTGGAAGTTGCAGAGGAAATCGCCCGTCTCGAGGCTGAAGTGTTCCGG
TTGGCCGGTCACCCGTTTAACCTCAACTCCCGTGACCAGCTGGAACGCGTTTTATTCGATGAGCTTGGGCTTCCC

GCAATTGGCAAAACCGAAAAGACTGGCAAACGCAGTACGAGCGCTGCCGTCCTTGAGGCACTCCGCGAGGCTCAC
CCTATTGTAGAAAAGATCCTGCAATACCGTGAGTTGACGAAGCTTAAAAGCACTTATATTGATCCTCTCCCGGAT
CTGATCCATCCTCGTACCGGCCGCTTGCACACACGTTTCAACCAGACGGCGACTGCAACCGGCCGTCTGTCTAGC
TCGGATCCAAATCTCCAGAACATTCCGGTCCGTACACCCTTGGGCCAACGTATCCGCCGGGCGTTTATCGCTGAG
GAAGGATGGTTACTGGTCGCATTGGACTACTCGCAGATTGAGCTGCGCGTCCTCGCACATCTCTCTGGTGACGAA
AATTTAATCCGCGTGTTTCAAGAGGGGCGTGATATTCACACAGAAACTGCCTCATGGATGTTCGGTGTCCCACGT
GAAGCAGTGGATCCTTTGATGCGCCGTGCAGCTAAAACAATTAATTTTGGAGTGCTGTACGGAATGAGCGCTCAT
CGCTTGAGTCAGGAACTGGCAATCCCCTACGAGGAAGCGCAGGCATTCATCGAACGTTACTTTCAATCGTTTCCG
AAAGTTCGCGCATGGATCGAGAAGACGCTCGAGGAAGGTCGTCGTCGGGGCTATGTCGAAACTCTGTTTGGTCGC
CGTCGGTACGTACCAGATCTTGAAGCCCGCGTCAAATCGGTACGGGAGGCTGCGGAGCGTATGGCATTTAATATG
CCTGTACAGGGTACTGCAGCTGACCTCATGAAACTGGCAATGGTCAAGCTTTTCCCGCGCTTGGAGGAAATGGGC
GCACGTATGCTTCTGCAGGTC**TAT**GACGAGCTGGTGTTAGAAGCCCCTAAGGAGCGCGCCGAAGCTGTCGCGCGC
CTCGCTAAAGAAGTGATGGAGGGCGTTTACCCATTGGCCGTACCCCTCGAAGTGGAGGTCGGTATTGGAGAAGAT
TGGTTATCTGCAAAGGAAGCGGCCGC

**[0275]** SEQ ID NO. 180, amino acid sequence of Mutant ID 43 (H784Y KlenTaq).

MGSLLHEFGLLESPKALEEAPWPPPEGAFVGFVLSRKEPMWADLLALAAARGGRVHRAPEPYKALRDLKEARGLL
AKDLSVLALREGLGLPPGDDPMLLAYLLDPSNTTPEGVARRYGGEWTEEAGERAALSERLFANLWGRLEGEERLL
WLYREVERPLSAVLAHMEATGVRLDVAYLRALSLEVAEEIARLEAEVFRLAGHPFNLNSRDQLERVLFDELGLPA
IGKTEKTGKRSTSAAVLEALREAHPIVEKILQYRELTKLKSTYIDPLPDLIHPRTGRLHTRFNQTATATGRLSSS
DPNLQNIPVRTPLGQRIRRAFIAEEGWLLVALDYSQIELRVLAHLSGDENLIRVFQEGRDIHTETASWMFGVPRE
AVDPLMRRAAKTINFGVLYGMSAHRLSQELAIPYEEAQAFIERYFQSFPKVRAWIEKTLEEGRRRGYVETLFGRR
RYVPDLEARVKSVREAAERMAFNMPVQGTAADLMKLAMVKLFPRLEEMGARMLLQV**Y**DELVLEAPKERAEAVARL
AKEVMEGVYPLAVPLEVEVGIGEDWLSAKEAA

Incorporation by Reference

**[0276]** All publications, patents, patent applications, Accession No. data mentioned herein are hereby incorporated by reference in their entirety as if each individual publication, patent, patent application or Accession No. data was specifically and individually indicated to be incorporated by reference. In the case of Accession No. data citations and references, the corresponding DNA polymerase amino acid and nucleotide sequences are incorporated herein by reference as if such sequences are disclosed by way of a SEQ ID NO. In case of conflict, the present application, including any definitions herein, will control.

**[0277]** The terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting. With respect to the use of substantially, any plural and/or singular terms herein, those having skill in the art can translate from the plural as is appropriate to the context and/or application. The various singular/plural permutations may be expressly set forth herein for the sake of clarity.

**[0278]** While the present invention has been described with reference to certain embodiments, it will be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the scope of the present invention. In addition, many modifications may be made to adapt a particular situation or

material to the teachings of the present invention without departing from its scope. Therefore, it is intended that the present invention not be limited to the particular embodiments or examples disclosed, but that the present invention will include all embodiments falling within the scope of the appended claims.

**Claims**

1. A method for conducting primer extension, comprising: contacting a polynucleotide template with a DNA polymerase selected from the group consisting of SEQ ID NOS: 83, 85, 87, 89, 147, 149, 151, 153, 155, 157, 159, 161, 170, 172, 174, 176, 178 and 180, an RNase H2 enzyme, a blocked-cleavable rhPCR primer and nucleoside triphosphates; and performing a primer extension method under suitable conditions, thereby producing an extended primer.

2. The method of claim 1, wherein the primer extension method comprises a method for conducting polymerase chain reaction (PCR).

3. The method of claim 2, wherein the method for conducting PCR comprises allele-specific PCR.

4. The method of claim 2, wherein the method for conducting PCR comprises detecting a rare allele at a level of discrimination of > 1:10,000.

5. A mutant Taq DNA polymerase selected from the group consisting of SEQ ID NOS: 83, 85, 87, 89, 147, 149, 151, 153, 155, 157, 159, 161, 170, 172,174, 176, 178 and 180.

6. A kit for producing an extended primer, comprising: at least one container providing a DNA polymerase selected from the group consisting of SEQ ID NOS: 83, 85, 87, 89, 147, 149, 151, 153, 155, 157, 159, 161, 170, 172,174, 176, 178 and 180; a container containing an RNase H2; and a container containing a blocked-cleavable rhPCR primer.

7. A reaction mixture comprising:

a polynucleotide template;

a DNA polymerase selected from the group consisting of SEQ ID NOS: 83, 85, 87, 89, 147, 149, 151, 153, 155, 157, 159, 161, 170, 172,174, 176, 178 and 180;

an RNase H2 enzyme;

at least one block-cleavable rhPCR primer; and

nucleoside triphosphates.

8. A method for performing rhPCR, comprising performing primer extension with the reaction mixture according to claim 7.

9. The method according to claim 1 or 7, wherein the blocked-cleavable rhPCR primer is selected from the group consisting of a RDDDDx blocked-cleavable rhPCR primer and a RDxxD blocked-cleavable rhPCR primer,

wherein the RDDDDx blocked-cleavable rhPCR primer consists of an oligonucleotide complementary to the polynucleotide template and having at its 3'-terminus the sequence RDDDDx, wherein R is an RNA base, D is a DNA base and x is a C3 spacer group, and

wherein the RDxxD blocked-cleavable rhPCR primer consists of an oligonucleotide complementary to the polynucleotide template and having at its 3'-terminus the sequence RDxxD, wherein R is an RNA base, D is a DNA base and x is a C3 spacer group.

10**.** The kit according to claim 6, wherein the blocked-cleavable rhPCR primer is selected from the group consisting of a RDDDDx blocked-cleavable rhPCR primer and a RDxxD blocked-cleavable rhPCR primer,

wherein the RDDDDx blocked-cleavable rhPCR primer consists of an oligonucleotide complementary to the polynucleotide template and having at its 3'-terminus the sequence RDDDDx, wherein R is an RNA base, D is a DNA base and x is a C3 spacer group, and

wherein the RDxxD blocked-cleavable rhPCR primer consists of an oligonucleotide complementary to the polynucleotide template and having at its 3'-terminus the sequence RDxxD, wherein R is an RNA base, D is a DNA base and x is a C3 spacer group.

11. The reaction mixture according to claim 7, wherein the at least one blocked-cleavable rhPCR primer is selected from the group consisting of a RDDDDx blocked-cleavable rhPCR primer and a RDxxD blocked-cleavable rhPCR primer,

wherein the RDDDDx blocked-cleavable rhPCR primer consists of an oligonucleotide complementary to the polynucleotide template and having at its 3'-terminus the sequence RDDDDx, wherein R is an RNA base, D is a DNA base and x is a C3 spacer group, and

wherein the RDxxD blocked-cleavable rhPCR primer consists of an oligonucleotide complementary to the polynucleotide template and having at its 3'-terminus the sequence RDxxD, wherein R is an RNA base, D is a DNA base and x is a C3 spacer group.