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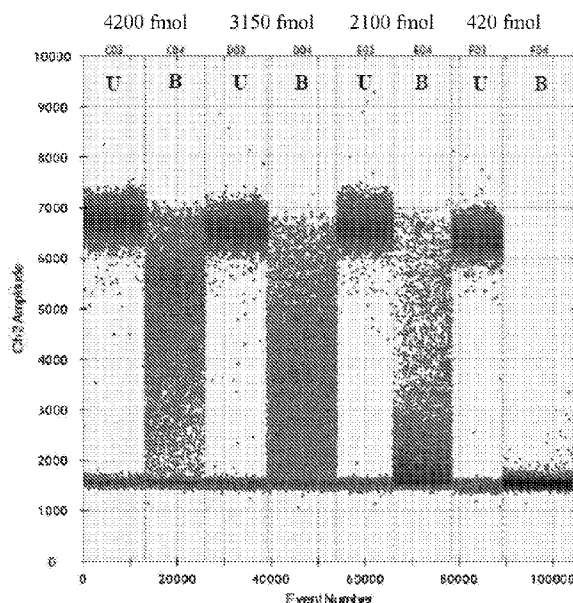
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(54) Title: RNASE H MUTANTS IN AN EMULSION

Figure 1



(57) Abstract: The invention is directed to methods and kits for performing an RNase H2 -mediated cleavage reaction on a sample in an emulsion.



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RNASE H MUTANTS IN AN EMULSION

BRIEF DESCRIPTION OF THE INVENTION

[0001] This invention relates to the use of mutant RNase H2 enzymes in an emulsion system.

CROSS-REFERENCE TO RELATED APPLICATIONS

[0002] This patent application claims the benefit of U.S. Provisional Patent Application No. 62/372,436, filed August 9, 2016.

INCORPORATION-BY-REFERENCE OF MATERIAL SUBMITTED ELECTRONICALLY

[0003] Incorporated by reference in its entirety herein is a computer-readable nucleotide/amino acid sequence listing submitted concurrently herewith and identified as follows: One 151,351 Byte ASCII (Text) file named “Sequence Listing Updated_ST25 – 21486972v1.txt” created on August 1, 2017.

BACKGROUND OF THE INVENTION

[0004] Polymerase chain reaction (PCR) is a widely used biotechnology that allows researchers to amplify a single copy of DNA to generate large quantities of a desired product. Like many technologies, the PCR methodologies are continuously being modified to improve their efficacy. One of the challenges of PCR is the formation of “primer dimers,” wherein the primers in the reaction unintentionally hybridize to each other along complementary bases. Primers that have dimerized are unable to hybridize to the target sequence on DNA, preventing amplification. Carefully designing primers reduces the likelihood of primer dimer formation; however, it is occasionally necessary to position primers at suboptimal locations in a target DNA sequence.

[0005] RNase H-dependent PCR (rhPCR) was developed in part to reduce primer dimer formation in PCR. In an rhPCR system, the primers are “blocked” with the inclusion of non-extendable groups at the 3’-end of the primer, such as a label or a C3 spacer. The primers include

one or more ribonucleotide residues near the 3'-end of the primer, and an RNase H is used to cleave the primer at the RNA base region if there is perfect complementary between the primer and target. The cleaving of the primer with RNase H removes the blocking group and allows extension with a DNA polymerase. RhPCR therefore enhances the specificity of the priming/extension and reduces unwanted extension products such as the products that may result from primer-dimers.

[0006] Walder et al., described the use of an RNase H2 enzyme derived from the hyperthermophilic archaeon *Pyrococcus abyssi* (*P.a.*) (see, e.g., International Patent Application Publication WO 2013/142364). RNase H enzymes are endoribonucleases that cleave the phosphodiester bond in an RNA strand when it is part of an RNA:DNA duplex. *P.a.* RNase H2 is active at high temperatures with reduced activity at lower temperatures, allowing the PCR reaction to be setup at room temperature without premature cleaving of the ribonucleotide. RNase H2 is compatible with most PCR buffers, and when added directly to the PCR master mix, cleavage of the ribonucleotide residue occurs seamlessly with PCR amplification by the DNA polymerase (see, e.g., U.S. Patent 8,911,948).

[0007] Improving the sensitivity and accurate quantification of amplified targets are additional areas of development in PCR technology. In particular, the combination of digital PCR and emulsion-based technologies has yielded a highly sensitive and quantitative PCR platform. In digital PCR (also known as “single molecule PCR” or “limiting dilution PCR”), a sample is sufficiently diluted and partitioned such that each partition contains on average a single copy or less of the target nucleic acid; the amplified target can additionally be quantified by including a probe in the reaction (see, e.g., Vogelstein et al., *Proc. Natl. Acad. Sci. USA*, 96: 9236-9241 (1999) and U.S. Patent 6,440,706). In emulsion PCR, partitions are generated by creating a water-in-oil emulsion, wherein each droplet of water contains on average a single copy or less of a nucleic acid and the necessary reagents for performing PCR. Reaction volumes can be significantly reduced as compared to conventional 96-, 384-, or even 1536-well reactions. Emulsion PCR also permits high-throughput analysis of amplification products.

[0008] The combination of digital and emulsion based PCR platforms has yielded digital droplet PCR, which allows a nucleic acid sample to be partitioned into tens of thousands of droplets in an emulsion, with PCR amplification and quantification of the desired target

occurring in each individual droplet. Digital droplet PCR provides sensitive and specific detection of single template molecules. As with all types of PCR, however, digital droplet PCR is also susceptible to primer dimer formation (see Hindson et al., *Anal Chem*, 83:8604-8610 (2011)).

[0009] The potential utility of rhPCR in a droplet digital format is extensive (see, e.g., U.S. Patent Application Publication 2013/0310269). There is a need, however for digital rhPCR methods with reduced side reactions and increased specificity. The present invention provides such methods.

BRIEF SUMMARY OF THE INVENTION

[0010] The present disclosure provides a method of performing an RNase H2-mediated cleavage of one or more nucleic acid sequences of interest, which comprises providing a sample comprising one or more nucleic acid sequences of interest; performing an RNase H2-mediated cleavage reaction on the one or more nucleic acid sequences, wherein the RNase H2-mediated cleavage reaction comprises: (i) a mutant *Pyrococcus abyssi* (*P.a.*) RNase H2 enzyme comprising an amino acid sequence selected from SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 70, SEQ ID NO: 71, SEQ ID NO: 73, SEQ ID NO: 74, SEQ ID NO: 76, SEQ ID NO: 77, SEQ ID NO: 78, SEQ ID NO: 79, SEQ ID NO: 80, SEQ ID NO: 81, SEQ ID NO: 82, SEQ ID NO: 84, SEQ ID NO: 85, SEQ ID NO: 86, SEQ ID NO: 87, SEQ ID NO: 88, SEQ ID NO: 89, SEQ ID NO: 90, and SEQ ID NO: 165; (ii) a mutant *Pyrococcus furiosus* (*P.fur*) RNase H2 enzyme comprising an amino acid sequence selected from SEQ ID NO: 126, SEQ ID NO: 127, SEQ ID NO: 128, SEQ ID NO: 129, and SEQ ID NO: 130; (iii) a mutant *Pyrococcus horikoshii* (*P.hori*) RNase H2 enzyme comprising an amino acid sequence selected from SEQ ID NO: 131, SEQ ID NO: 132, and SEQ ID NO: 133; (iv) a mutant *Thermococcus kodakarensis* (*T.kod*) RNase H2 enzyme comprising an amino acid sequence selected from SEQ ID NO: 134, SEQ ID NO: 135, and SEQ ID NO: 136; or (v) a mutant *Thermococcus litoralis* (*T.lit*) RNase H2 enzyme comprising the amino acid sequence of SEQ ID NO: 137 or SEQ ID NO: 138; whereupon one or more nucleic acid sequences of interest are cleaved.

[0011] The present disclosure also provides a kit for cleaving one or more nucleic acid sequences of interest comprising (a) one or more reagents for performing an RNase H2-mediated cleavage reaction in an emulsion on a sample comprising one or more nucleic acid sequences of interest, (b) one or more mutant RNase H2 enzymes selected from: (i) a mutant *Pyrococcus abyssi* (*P.a.*) RNase H2 enzyme comprising an amino acid sequence selected from SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 70, SEQ ID NO: 71, SEQ ID NO: 73, SEQ ID NO: 74, SEQ ID NO: 76, SEQ ID NO: 77, SEQ ID NO: 78, SEQ ID NO: 79, SEQ ID NO: 80, SEQ ID NO: 81, SEQ ID NO: 82, SEQ ID NO: 84, SEQ ID NO: 85, SEQ ID NO: 86, SEQ ID NO: 87, SEQ ID NO: 88, SEQ ID NO: 89, SEQ ID NO: 90, and SEQ ID NO: 165; (ii) a mutant *Pyrococcus furiosus* (*P.fur*) RNase H2 enzyme comprising an amino acid sequence selected from SEQ ID NO: 126, SEQ ID NO: 127, SEQ ID NO: 128, SEQ ID NO: 129, and SEQ ID NO: 130; (iii) a mutant *Pyrococcus horikoshii* (*P.hori*) RNase H2 enzyme comprising an amino acid sequence selected from SEQ ID NO: 131, SEQ ID NO: 132, and SEQ ID NO: 133; (iv) a mutant *Thermococcus kodakarensis* (*T.kod*) RNase H2 enzyme comprising an amino acid sequence selected from SEQ ID NO: 134, SEQ ID NO: 135, and SEQ ID NO: 136; and (v) a mutant *Thermococcus litoralis* (*T.lit*) RNase H2 enzyme comprising the amino acid sequence of SEQ ID NO: 137 or SEQ ID NO: 138, and (c) instructions for performing the RNase H2-mediated cleavage reaction.

[0012] The disclosure also provides a mutant RNase H2 enzyme selected from: (a) a mutant *Pyrococcus abyssi* (*P.a.*) RNase H2 enzyme comprising the amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, or SEQ ID NO: 22, SEQ ID NO: 70, SEQ ID NO: 71, SEQ ID NO: 73, SEQ ID NO: 74, SEQ ID NO: 76, SEQ ID NO: 77, SEQ ID NO: 78, SEQ ID NO: 79, SEQ ID NO: 80, SEQ ID NO: 81, SEQ ID NO: 82, SEQ ID NO: 84, SEQ ID NO: 85, SEQ ID NO: 86, SEQ ID NO: 87, SEQ ID NO: 88, SEQ ID NO: 89, SEQ ID NO: 90, or SEQ ID NO: 165; (b) a mutant *Pyrococcus furiosus* (*P.fur*) RNase H2 enzyme comprising the amino acid sequence of SEQ ID NO: 126, SEQ ID NO: 127, SEQ ID NO: 128, SEQ ID NO:

129, or SEQ ID NO: 130; (c) a mutant *Pyrococcus horikoshii* (*P.hori*) RNase H2 enzyme comprising the amino acid sequence of SEQ ID NO: 131, SEQ ID NO: 132, or SEQ ID NO: 133; (d) a mutant *Thermococcus kodakarensis* (*T.kod*) RNase H2 enzyme comprising the amino acid sequence of SEQ ID NO: 134, SEQ ID NO: 135, or SEQ ID NO: 136; and (e) a mutant *Thermococcus litoralis* (*T.lit*) RNase H2 enzyme comprising the amino acid sequence of SEQ ID NO: 137 or SEQ ID NO: 138.

[0013] The present disclosure also provides mutant RNase H2 enzymes comprising one of the following: (a) an amino acid sequence comprising SEQ ID NO: 95 except for one or more of the following amino acid substitutions: A9S, R11K, P13S, E48R, M80L, A107V, P171G, S172I, D173E, E199Y, E199G, and/or F220L; (b) an amino acid sequence comprising SEQ ID NO: 96 except for one or more of the following amino acid substitutions: A9S, R11K, P13S, Q48R, M80L, A107V, P171G, S172I, D173E, E199Y, E199G, and/or F218L; (c) an amino acid sequence comprising SEQ ID NO: 97 except for one or more of the following amino acid substitutions: A9S, R11K, P13S, A107V, P171G, S172I, D173E, K199Y, K199G, K199E, and/or Y224L; or (d) an amino acid sequence comprising SEQ ID NO: 98 except for one or more of the following amino acid substitutions: A9S, R11K, P13S, M80L, A107V, P171G, S172I, D173E, K199Y, K199G, K199E, and/or F220L. The present disclosure also provides a kit for cleaving one or more nucleic acid sequences of interest comprising (a) one or more reagents for performing an RNase H2-mediated cleavage reaction in an emulsion on a sample comprising one or more nucleic acid sequences of interest, (b) one or more of the aforementioned mutant RNase H2 enzymes, and (c) instructions for performing the RNase H-mediated cleavage reaction.

[0014] The disclosure also provides a method of performing an RNase H2-mediated cleavage of one or more nucleic acid sequences of interest comprising (a) providing a sample comprising one or more nucleic acid sequences of interest and (b) performing an RNase H2-mediated cleavage reaction on the one or more nucleic acid sequences, wherein the RNase H2-mediated cleavage reaction comprises a *Thermococcus kodakarensis* (*T.kod*) RNase H2 enzyme comprising the amino acid sequence of SEQ ID NO: 97, whereupon one or more nucleic acid sequences of interest are cleaved.

BRIEF DESCRIPTION OF THE DRAWINGS

[0015] Figure 1 is a plot from a QX-200™ DROPLET DIGITAL™ (DDPCR™) system (Bio-Rad Laboratories, Inc., Hercules, CA) showing a wild-type *Pyrococcus abyssi* (*P.a.*) RNase H2 titration in a droplet digital rhPCR system. An RNase P (RNP) assay was run using either unblocked (U) primers, or blocked (B) rhPrimers with 4200, 3150, 2100, or 420 fmol of wild-type *P.a.* RNase H2. Fluorescence intensity (Y-axis) was plotted for each droplet (event number, X-axis).

[0016] Figure 2 is a plot showing wild-type (WT) and mutant RNase H2 enzyme activities in a digital droplet rhPCR system using the QX-200™ DROPLET DIGITAL™ (DDPCR™) system (Bio-Rad). An RNase P (RNP) assay was run using either unblocked or blocked rhPrimers, and 420 fmol (21 nM) of each RNase H2 enzyme. Only the results with the wild-type enzyme are shown for the unblocked primers (WT-U). The results with blocked rhPrimers are shown for both the wild-type (WT-B) and the mutant enzymes (numbered lanes).

[0017] Figure 3 is a plot showing the titration of P13S mutant RNase H2 in a digital droplet rhPCR system. The RNase H2 enzyme P13S was titrated from 420 fmoles to 26 fmoles and evaluated with an RNase P (RNP) assay with either standard unblocked (U) primers or blocked (B) rhPrimers.

[0018] Figure 4 compares the wild-type RNase H2 and emulsion-based evolution derived mutant RNase H2 enzymes in a digital drop PCR system using the QX-200™ Droplet Reader (Bio-Rad). An RNase P (RNP) assay was run using either standard unblocked (U) primers or blocked (B) rhPrimers with 420 fmol of each mutant. The unblocked sample with the wild-type mutant is shown as a reference.

[0019] Figure 5 is an alignment of the wild-type RNase H2 amino acid sequences from *P. furiosus*, *P. abyssi*, *P. horikoshii*, *T. kodakarensis*, and *T. literalis*. Mutations of interest that were identified in *P.a.* are listed above the sequences. Natural occurrences of these mutations in *T. kodakarensis* and *T. literalis* are indicated in bold and underline

[0020] Figure 6 is a plot from a QX-200™ DROPLET DIGITAL™ (DDPCR™) system (Bio-Rad Laboratories, Inc., Hercules, CA) showing a comparison of wild-type RNase H2 isolated from *P. abyssi*, *T. kodakarensis*, *P. horikoshii*, *T. literalis*, and *P. furiosus*. An RNase P

assay was run using either standard unblocked primers (U) or blocked (B) rhPrimers with 420 fmol (equivalent of 200 mU wild-type *P.a.*) of the indicated wild-type RNase H2.

[0021] Figure 7 is a plot from a QX-200™ DROPLET DIGITAL™ (DDPCR™) system (Bio-Rad Laboratories, Inc., Hercules, CA) showing a comparison of *P.a.* and *T. kod* wild-type RNase H2 titrations. An RNase P assay was run using either standard unblocked primers (U) or blocked (B) rhPrimers. The wild-type *P.a.* RNase H2 enzyme was titrated from 840 fmol to 210 fmol (400 mU to 100 mU) and the wild-type *T. kod.* was titrated from 840 fmol to 52.5 fmol (equivalent of 400 mU to 25 mU of wild-type *P.a.*).

[0022] Figure 8 is a plot from a QX-200™ DROPLET DIGITAL™ (DDPCR™) system (Bio-Rad Laboratories, Inc., Hercules, CA) showing a comparison of wild-type and mutant RNase H2 enzymes from *T. litoralis*. An RNase P assay was run using either standard unblocked primers (U) or blocked (B) rhPrimers and 420 fmol (equivalent of 200 mU wild-type *P.a.*) of the indicated mutant *T. litoralis* RNase H2 enzyme.

[0023] Figure 9 is a plot from a QX-200™ DROPLET DIGITAL™ (DDPCR™) system (Bio-Rad Laboratories, Inc., Hercules, CA) showing a comparison of wild-type and mutant RNase H2 enzymes from *P. furiosis*. An RNase P assay was run using either standard unblocked primers (U) or blocked (B) rhPrimers and 420 fmol (equivalent of 200 mU wild-type *P.a.*) of the indicated mutant *P. furiosis* RNase H2 enzyme.

[0024] Figure 10 is a plot from a QX-200™ DROPLET DIGITAL™ (DDPCR™) system (Bio-Rad Laboratories, Inc., Hercules, CA) showing a comparison of wild-type and mutant RNase H2 enzymes from *P. horikoshii*. An RNase P assay was run using either standard unblocked primers (U) or blocked (B) rhPrimers and 420 fmol (equivalent of 200 mU wild-type *P.a.*) of the indicated mutant *P. horikoshii* RNase H2 enzyme.

[0025] Figure 11 is a plot from a QX-200™ DROPLET DIGITAL™ (DDPCR™) system (Bio-Rad Laboratories, Inc., Hercules, CA) showing a comparison of wild-type and mutant RNase H2 enzymes from *T. kodakarensis*. An RNase P assay was run using either standard unblocked primers (U) or blocked (B) rhPrimers and 4.2 fmol (equivalent of 2 mU wild-type *P.a.*) of the indicated mutant *T. kodakarensis* RNase H2 enzyme.

[0026] Figure 12 is a plot from a QX-200™ DROPLET DIGITAL™ (DDPCR™) system (Bio-Rad Laboratories, Inc., Hercules, CA) showing a comparison of wild-type *P.a.* and P13S

Q48R M80L A107V mutant *P.a.* RNase H2-titrations. An RNase P assay was run using either standard unblocked primers (U) or blocked (B) rhPrimers and 4200 fmol or 420 fmol (2000 mU to 200 mU) of the wild-type *P.a.* RNase H2 enzyme or a titration from 4200 fmol to 4.2 fmol (equivalent of 2000 mU to 2 mU of wild-type *P.a.*) of the *P.a.* P13S Q48R M80L A107V mutant RNase H2 enzyme.

DETAILED DESCRIPTION OF THE INVENTION

[0027] Before any embodiments of the invention are explained in detail, it is to be understood that the invention is not limited in its application to the details of construction and the arrangement of components set forth in the following description or illustrated in the following drawings. The invention is capable of other embodiments and of being practiced or of being carried out in various ways.

1. Definitions

[0028] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art. In case of conflict, the present document, including definitions, will control. Preferred methods and materials are described below, although methods and materials similar or equivalent to those described herein can be used in practice or testing of the present invention.

[0029] “Complement” or “complementary” as used herein means a nucleic acid, and can mean Watson-Crick (e.g., A-T/U and C-G) or Hoogsteen base pairing between nucleotides or nucleotide analogs of nucleic acid molecules.

[0030] “Fluorophore” or “fluorescent label” refers to compounds with a fluorescent emission maximum between about 350 and 900 nm.

[0031] “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. “Primer dimers” refers to the hybridization of two oligonucleotide primers. “Stringent hybridization conditions” as used herein means conditions under which hybridization of fully complementary nucleic acid strands is

strongly preferred. Under stringent hybridization conditions, a first nucleic acid sequence (for example, a primer) will hybridize to a second nucleic acid sequence (for example, a target sequence), such as in a complex mixture of nucleic acids. Stringent conditions are sequence-dependent and will be different in different circumstances. Stringent conditions can be selected to be about 5-10°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength pH. The T_m can be the temperature (under defined ionic strength, pH, and nucleic concentration) at which 50% of an oligonucleotide complementary to a target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at T_m , 50% of the probes are occupied at equilibrium). Stringent conditions can be those in which the salt concentration is less than about 1.0 M sodium ion, such as about 0.01-1.0 M sodium ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g., about 10-50 nucleotides) and at least about 60°C for long probes (e.g., greater than about 50 nucleotides). Stringent conditions can also be achieved with the addition of destabilizing agents such as formamide. For selective or specific hybridization, a positive signal can be at least 2 to 10 times background hybridization. Exemplary stringent hybridization conditions include the following: 50% formamide, 5x SSC, and 1% SDS, incubating at 42°C, or, 5x SSC, 1% SDS, incubating at 65°C, with wash in 0.2x SSC, and 0.1% SDS at 65°C.

[0032] The terms “nucleic acid,” “oligonucleotide,” or “polynucleotide,” as used herein, refer to at least two nucleotides covalently linked together. The depiction of a single strand also defines the sequence of the complementary strand. Thus, a nucleic acid also encompasses the complementary strand of a depicted single strand. Many variants of a nucleic acid can be used for the same purpose as a given nucleic acid. Thus, a nucleic acid also encompasses substantially identical nucleic acids and complements thereof. A single strand provides a probe that can hybridize to a target sequence under stringent hybridization conditions. Thus, a nucleic acid also encompasses a probe that hybridizes under stringent hybridization conditions.

[0033] Nucleic acids can be single stranded or double stranded, or can contain portions of both double stranded and single stranded sequences. The nucleic acid can be DNA, both genomic and cDNA, RNA, or a hybrid, where the nucleic acid can contain combinations of deoxyribo- and ribonucleotides, and combinations of bases including uracil, adenine, thymine, cytosine, guanine, inosine, xanthine hypoxanthine, isocytosine and isoguanine. Nucleic acids

can be obtained by chemical synthesis methods or by recombinant methods and can contain non-nucleotide modifications such as spacers or labels. A particular nucleic acid sequence can encompass conservatively modified variants thereof (e.g., codon substitutions), alleles, orthologs, single nucleotide polymorphisms (SNPs), and complementary sequences as well as the sequence explicitly indicated.

[0034] “Polymerase Chain Reaction (PCR)” refers to the enzymatic reaction in which DNA fragments are synthesized and amplified from a substrate DNA *in vitro*. The reaction typically involves the use of two synthetic oligonucleotide primers, which are complementary to nucleotide sequences in the substrate DNA which are separated by a short distance of a few hundred to a few thousand base pairs, and the use of a thermostable DNA polymerase. The chain reaction consists of a series of 10 to 40 cycles. In each cycle, the substrate DNA is first denatured at high temperature. After cooling down, synthetic primers which are present in vast excess, hybridize to the substrate DNA to form double-stranded structures along complementary nucleotide sequences. The primer-substrate DNA complexes will then serve as initiation sites for a DNA synthesis reaction catalyzed by a DNA polymerase, resulting in the synthesis of a new DNA strand complementary to the substrate DNA strand. The synthesis process is repeated with each additional cycle, creating an amplified product of the substrate DNA.

[0035] A “cycling probe” reaction or “cycling probe technology (CPT)” is an isothermal signal amplification method for the detection of specific target DNA sequences. A chimeric probe DNA-RNA-DNA, and a thermostable RNase H enzyme, are the two main components of this assay. In the presence of a target sequence, a DNA/RNA hybrid is formed and RNase H specifically catalyzes the cleavage of the RNA portion of the hybrid. Since cleaved fragments are small, they dissociate spontaneously from the target sequence at the reaction temperature. The target is then recycled and available for hybridization with another probe; the reaction is inherently cyclic without external manipulations (see, e.g., U.S. Patent 5,403,711, Warnon et al., *BioTechniques*, 28: 1152-1160 (2000); and Duck et al., *BioTechniques*, 9: 142-147 (1990)). Unlike PCR, products accumulate in a linear fashion.

[0036] “Loop-mediated isothermal amplification” or “LAMP” is an isothermal nucleic acid amplification method that is carried out at a constant temperature and does not require a thermal cycler, in contrast to PCR. In LAMP, a large amount of DNA is synthesized, yielding a large

pyrophosphate ion by-product. Pyrophosphate ion combines with divalent metallic ion to form an insoluble salt. Adding manganous ion and calcein, a fluorescent metal indicator, to the reaction solution allows a visualization of substantial alteration of the fluorescence during the one-step amplification reaction, which takes approximately 30-60 minutes (see, e.g., Tomita et al., *Nature Protocols*, 3: 877-882 (2008)).

[0037] “Primer,” as used herein, refers to an oligonucleotide capable of acting as a point of initiation for DNA synthesis under suitable conditions. Suitable conditions include those in which hybridization of the oligonucleotide to a template nucleic acid occurs, and synthesis or amplification of the target sequence occurs, in the presence of four different nucleoside triphosphates and an agent for extension (e.g., a DNA polymerase) in an appropriate buffer and at a suitable temperature.

[0038] “Probe” and “fluorescent generation probe” are synonymous and refer to either a) a sequence-specific oligonucleotide having an attached fluorophore and/or a quencher, and optionally a minor groove binder or b) a DNA binding reagent, such as, but not limited to, SYBR® Green dye.

[0039] “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.

2. RNaseH2 Cleavage Reaction

[0040] Described herein are methods for performing an RNase-mediated cleavage of one or more nucleic acid sequences of interest, which comprise providing a sample comprising one or more nucleic acid sequences of interest; and performing an RNase-mediated cleavage reaction on the one or more nucleic acid sequences. The term “RNase-mediated cleavage reaction” refers to a reaction in which an RNase enzyme catalyzes the breakage of at least one of the covalent sugar-phosphate linkages that forms the sugar-phosphate backbone of RNA. The RNase-mediated cleavage reaction may be performed as part of any method which requires RNase-mediated nucleic acid cleavage, such as, for example, an RNase H-dependent PCR (rhPCR)

reaction, a loop-mediated isothermal amplification (LAMP) reaction, cycling probe technology (CPT), or any emulsion-based assay. An RNase-mediated cleavage reaction may also be used to detect homology directed repair (HDR) of double-strand DNA breaks (e.g, as a result of targeted endonuclease activity) in an RNase H-dependent PCR reaction.

3. Digital PCR System

[0041] In one embodiment, the RNase H2-mediated cleavage reaction is performed as part of a method for amplifying one or more nucleic acids of interest which comprises performing an RNase H-dependent PCR (rhPCR) reaction on a sample that contains one or more nucleic acids of interest in a digital PCR system. The one or more nucleic acid sequences of interest to be amplified also can be referred to as a “target,” “target sequence,” “target region,” or “target nucleic acid,” all of which are synonymous and refer to a region or sequence of a nucleic acid which is to be amplified, sequenced, or detected.

[0042] The term “RNase H PCR (rhPCR)” refers to a PCR reaction which utilizes “blocked” oligonucleotide primers and an RNase H enzyme. “Blocked” primers contain a blocking group that prevents extension of the primer by a polymerase, and blocked primers contain at least one chemical moiety (such as, but not limited to, a ribonucleic acid residue or a 2’fluoro base) within the primer or other oligonucleotide, such that when the blocked primer hybridizes to the template or target nucleic acid, the blocking group is removed by cleavage by an RNase H enzyme that recognizes and cleaves at the chemical moiety. Following RNase H cleavage, amplification of the target DNA can occur.

[0043] In one embodiment, the 3’ end of a blocked primer can comprise the blocking group rDDDDMx, wherein relative to the target nucleic acid sequence, “r” is an RNA residue, “D” is a complementary DNA residue, “M” is a mismatched DNA residue, and “x” is a C3 spacer. A C3 spacer is a short 3-carbon chain attached to the terminal 3’ hydroxyl group of the oligonucleotide, which further inhibits the DNA polymerase from binding before cleavage of the RNA residue.

[0044] The methods described herein can be performed using any suitable RNase H enzyme that is derived or obtained from any organism. Typically, RNase H-dependent PCR reactions are performed using an RNase H enzyme obtained or derived from the hyperthermophilic archaeon

Pyrococcus abyssi (*P.a.*), such as RNase H2. Thus, in one embodiment, the RNase H enzyme employed in the methods described herein desirably is obtained or derived from *Pyrococcus abyssi*, preferably an RNase H2 obtained or derived from *Pyrococcus abyssi*. In other embodiments, the RNase H enzyme employed in the methods described herein can be obtained or derived from other species, for example, *Pyrococcus furiosus*, *Pyrococcus horikoshii*, *Thermococcus kodakarensis*, or *Thermococcus litoralis*.

[0045] In one embodiment, the RNase H-dependent PCR reaction is performed in a digital PCR system (i.e., digital rhPCR). “Digital PCR” refers to a PCR reaction which is carried out on a single selected starting template, wherein a number of individual templates are each isolated into separate reaction areas (see, e.g., Vogelstein et al., *PNAS*, 96: 9236-9241 (1999) and U.S. Patent 6,440,706). As such, digital PCR allows for the detection and precise quantification of, for example, low-level pathogens, rare genetic sequences, quantification of copy number variants, and rare mutations (see, e.g., Manoj, P., *Mitochondrial DNA*, 27(1): 742-6 (2016)). In digital PCR, the reaction area can be, for example, a well, chamber, bead, or water-in-oil emulsion. Digital PCR reactions will yield a negative result if no starting molecule is present or a positive result if the targeted starting template is present. Analyzing the number of positive reactions allows for the quantification of the starting template. Accordingly, a large number of reaction areas can be used for a single digital PCR experiment. Any suitable digital PCR system can be used in the inventive method, many of which are known in the art and commercially available from sources such as, for example, the QX200™ DROPLET DIGITAL™ PCR system (Bio-Rad Laboratories Inc., Hercules, CA), the RAINDROP™ Digital PCR system (Raindance Technologies, Inc., Billerica, MA), the QUANTSTUDIO® 3D digital PCR system (ThermoFisher Scientific, Waltham, MA), and the DIGITAL ARRAY™ integrated fluid circuit (IFC) system (Fluidigm Corporation, South San Francisco, CA).

[0046] In one embodiment, the digital PCR system can be a droplet digital PCR system. The term “droplet digital PCR” refers to a digital PCR system in which the reaction area is a droplet of water in a well. Preferably, the digital PCR system is an emulsion droplet digital PCR system. The term “emulsion droplet digital PCR” refers to a digital PCR system in which the reaction area is a droplet that is formed in a water-oil emulsion. Techniques for performing droplet digital PCR and emulsion droplet digital PCR are known in the art and include, but are not

limited to, those described in Hindson et al., *Anal Chem*, 83:8604-8610 (2011); Pinheiro et al., *Anal Chem*, 84:1003-1011 (2012); and Jones et al., *J. Virological Methods*, 202: 46-53 (2014). Droplet digital PCR systems and emulsion droplet digital PCR systems also are commercially available from sources such as, for example, the QX200™ DROPLET DIGITAL™ PCR system (Bio-Rad Laboratories, Inc., Hercules, CA).

[0047] The RNase H-dependent PCR reaction described herein can be performed using any suitable combination of primer and probe oligonucleotide sequences, the choice of which will depend on the sequence of the target nucleic acid to be amplified. In one embodiment, the RNase H-dependent PCR reaction is performed using one set of blocked primers and one set of unblocked primers. Suitable probes include, for example, dual-labeled probes, multi-fluorophore or multi-quencher (or combinations thereof) such as dual-quencher ZEN probes (Integrated DNA Technologies, Inc.), MGB TaqMan probes, and dual-labeled non-MGB TaqMan probes (see Hindson et al., *Anal Chem*, 83:8604-8610 (2011)), and molecular beacon probes (see U.S. Patent 6,440,706). The dual labeled probes are cleaved by polymerases with 5'-exonuclease activity. While the aforementioned probes typically are longer than 16 nucleotides, a probe used in connection with the inventive method can be of any suitable size. For example, a probe can be about 25-30 nucleotides in length, about 50-80 nucleotides in length, or about 100-150 nucleotides in length. Examples of specific primer and probe sequences that can be used in the inventive method are set forth below in the Examples.

[0048] In one embodiment, a combination of two or more probes can be used when two or more target nucleic acid sequences are amplified by the inventive method. For example, two probes can be used to identify two targets in droplets, such that four populations can exist within a particular PCR reaction: no target present (Probe1-/Probe2-), one of two targets present (Probe1+/Probe2- or Probe1-/Probe2+), or both of targets present (Probe1+/Probe2+). The choice of an appropriate combination of probes specific for a particular combination of target nucleic acid sequences is well within the skill in the art, and such probes can be designed and generated using routine methods known in the art.

[0049] As discussed above, a skilled artisan will appreciate that a probe oligonucleotide can comprise a fluorophore and/or a quencher attached thereto. The probe used in the inventive method can comprise any suitable fluorophore and/or quencher attached thereto. Suitable

fluorophores include, for example, 5-FAM (also called 5-carboxyfluorescein, also known as 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'-dipivaloylfluoresceinyl)-6-carboxylic 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); Xanthylum, 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-propionic acid), Quasar®-670 dye (Biosearch Technologies), Cal Fluor® Orange dye (Biosearch Technologies), ATTO Dyes (Atto-Tec GmbH), Rox dyes, Max dyes (Integrated DNA Technologies), and derivatives thereof.

[0050] Prior to amplification of a target nucleic acid, a quencher prevents the fluorophore signal from being detected. Any suitable quencher can be used in the invention, such as, for example, DABCYL, BLACK HOLE™ Quenchers (such as, BHQ-1®, BHQ-2®, and BHQ-3®), IOWA BLACK® FQ, and IOWA BLACK® RQ, all of which are commercially available from a variety of sources. During amplification of the target nucleic acid by DNA polymerase, the fluorophore and/or quencher can be cleaved and separated, allowing the detection of the fluorophore signal. The fluorescence intensity is proportional to the amount of amplified product and allows for quantification of a target nucleic acid.

4. Mutant RNase H Enzyme

[0051] The method described herein involves an RNase H2-dependent cleavage reaction which comprises a mutant RNase H enzyme, such as a mutant RNase H2 enzyme obtained or derived from *Pyrococcus abyssi* (*P.a.*), *Pyrococcus furiosus* (*P. fur.*), *Pyrococcus horikoshii* (*P. hori.*), *Thermococcus kodakarensis* (*T. kod.*), or *Thermococcus litoralis* (*T. lit.*). By “mutant” is meant that the amino acid sequence of the RNase H2 described herein comprises a deletion,

insertion, or substitution of one or more amino acid residues as compared to a wild-type or naturally occurring RNase H2 amino acid sequence. Mutant enzymes may be designed by suitable techniques known in the art, such as by standard site-directed mutagenesis methods (also called site-specific mutagenesis or oligonucleotide-directed mutagenesis) (see, e.g., Weiner M. et al., *Gene*, 151:119-123 (1994)). Site-directed mutagenesis may be performed using one or more oligonucleotide primers, with each primer bearing at least one mutated nucleic acid residue relative to the wild-type RNase H2 sequence. In one embodiment, each primer for site-specific mutagenesis can contain at least three nucleic acid mutations. In another embodiment, the mutations occur at consecutive nucleic acid residues. Mutant enzymes may be expressed in a suitable host, such as bacteria, and purified by any suitable technique known in the art, such as those described in, for example, Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 4th ed., Cold Spring Harbor Press, Cold Spring Harbor, New York (2012); and Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley & Sons, New York (2016). In one embodiment, for example, a nucleic acid sequence encoding a mutant RNase H enzyme generated by site-specific mutagenesis can be expressed in *E. coli*, purified by NiNTA chromatography, and re-suspended in Buffer F (20 mM Tris-HCl pH 8.4, 100 mM KCl, 0.1 M EDTA, 0.1% TRITON X-100™, and 50% glycerol), as described in, e.g., U.S. Patent 8,911,948.

[0052] The activity of a mutant RNase H enzyme can be evaluated based on the amplification efficiency of a known starting quantity of a template in an RNase H2-dependant PCR reaction (rhPCR). In one embodiment, for example, the starting template can be the human *RNASE P* gene, and activity of a particular mutant RNase H enzyme can be measured using an RNase P (RNP) assay. An “RNase P (RNP) assay” is a method of evaluating enzymatic activity by quantifying amplification of *RNASE P*. An RNP assay utilizes two oligonucleotide primers in combination with a probe, and allows for the addition of an enzyme, such as, for example, RNase H2. Other RNase H2 assay methods are well known to those of skill in the art.

[0053] Examples of mutant *P.a.* RNase H2 enzyme amino acid sequences that can be used in connection with the methods described herein include, but are not limited to, SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ

ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 70, SEQ ID NO: 71, SEQ ID NO: 73, SEQ ID NO: 74, SEQ ID NO: 76, SEQ ID NO: 77, SEQ ID NO: 78, SEQ ID NO: 79, SEQ ID NO: 80, SEQ ID NO: 81, SEQ ID NO: 82, SEQ ID NO: 84, SEQ ID NO: 85, SEQ ID NO: 86, SEQ ID NO: 87, SEQ ID NO: 88, SEQ ID NO: 89, SEQ ID NO: 90, and SEQ ID NO: 165, or an amino acid sequence that is at least 90% identical (e.g., 90%, 91%, 92%, 93%, 94% identical), and preferably at least 95% identical (e.g., 95%, 96%, 97%, 98%, 99%, or 100% identical) to any of the foregoing amino acid sequences. More preferably, the mutant *P.a.* RNase H2 enzyme comprises the amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, or SEQ ID NO: 20, SEQ ID NO: 70, SEQ ID NO: 71, SEQ ID NO: 73, SEQ ID NO: 74, SEQ ID NO: 76, SEQ ID NO: 77, SEQ ID NO: 78, SEQ ID NO: 79, SEQ ID NO: 80, SEQ ID NO: 81, SEQ ID NO: 82, SEQ ID NO: 84, SEQ ID NO: 85, SEQ ID NO: 86, SEQ ID NO: 87, SEQ ID NO: 88, SEQ ID NO: 89, SEQ ID NO: 90, and SEQ ID NO: 165.

[0054] Examples of mutant *Pyrococcus furiosus* RNase H2 enzyme amino acid sequences that can be used in connection with the methods described herein include, but are not limited to, SEQ ID NO: 126, SEQ ID NO: 127, SEQ ID NO: 128, SEQ ID NO: 129, and SEQ ID NO: 130. Examples of mutant *Pyrococcus horikoshii* RNase H2 enzyme amino acid sequences that can be used in connection with the methods described herein include, but are not limited to, SEQ ID NO: 131, SEQ ID NO: 132, and SEQ ID NO: 133. Examples of mutant *Thermococcus kodakarensis* RNase H2 enzyme amino acid sequences that can be used in connection with the methods described herein include, but are not limited to, SEQ ID NO: 134, SEQ ID NO: 135, and SEQ ID NO: 136. Examples of mutant *Thermococcus litoralis* RNase H2 enzyme amino acid sequences that can be used in connection with the methods described herein include, but are not limited to, SEQ ID NO: 137 and SEQ ID NO: 138.

[0055] In another embodiment, a mutant *Pyrococcus furiosus* RNase H2 enzyme can comprise an amino acid sequence comprising SEQ ID NO: 95 except for one or more of the following amino acid substitutions: A9S, R11K, P13S, E48R, M80L, A107V, P171G, S172I, D173E, E199Y, E199G, and/or F220L. In another embodiment, a mutant *Pyrococcus horikoshii* RNase H2 enzyme can comprise an amino acid sequence comprising SEQ ID NO: 96 except for one or more of the following amino acid substitutions: A9S, R11K, P13S, Q48R, M80L,

A107V, P171G, S172I, D173E, E199Y, E199G, and/or F218L. In another embodiment, a mutant *Thermococcus kodakarensis* RNase H2 enzyme can comprise an amino acid sequence comprising SEQ ID NO: 97 except for one or more of the following amino acid substitutions: A9S, R11K, P13S, A107V, P171G, S172I, D173E, K199Y, K199G, K199E, and/or Y224L. In a further embodiment, a mutant *Thermococcus litoralis* RNase H2 enzyme can comprise an amino acid sequence comprising SEQ ID NO: 98 except for one or more of the following amino acid substitutions: A9S, R11K, P13S, M80L, A107V, P171G, S172I, D173E, K199Y, K199G, K199E, and/or F220L. As discussed above, the mutant RNase H2 enzyme also can comprise an amino acid sequence that is at least 90% identical (e.g., 90%, 91%, 92%, 93%, 94% identical), and preferably at least 95% identical (e.g., 95%, 96%, 97%, 98%, 99%, or 100% identical) to any of the foregoing amino acid sequences.

[0056] In addition to digital PCR systems, such as digital droplet PCR (ddPCR) and emulsion droplet digital PCR, the mutant RNase H enzymes described herein can be used in other nucleic acid amplification and detection assays, including, but not limited to, “hot start” assays (e.g., hot start PCR), Cycling Probe Technology (CPT), rolling circle amplification (RCA), helicase dependent amplification (HDA), oligonucleotide ligation assay (OLA), ligation chain reaction (LCR), polynomial amplification, and DNA sequencing.

[0057] “Hot start” PCR is a modified form of PCR which avoids a non-specific amplification of DNA by inactivating the Taq polymerase at lower temperatures. Specifically, hot start assays employ a modified oligonucleotide that is unable to participate in the PCR reaction until it hybridizes to a complementary nucleic acid sequence, and is cleaved to generate a functional 5’- or 3’-end. Hot start protocols provide enhanced specificity as compared to corresponding assays which utilize standard unmodified DNA oligonucleotides. In addition, the requirement for reversibly inactivated DNA polymerases or DNA ligases is eliminated. In one embodiment, the hot start component can be a thermostable RNase H or other nicking enzyme that gains activity at the elevated temperatures employed in the reaction. Other assays that can be performed with the mutant RNase H enzymes described herein include, e.g., primer extension assays (including PCR, DNA sequencing, and polynomial amplification), cycling probe reactions, sequencing by ligation, and sequencing by generation of end-labeled fragments.

[0058] In OLA, a set of two or more oligonucleotides in combination with a thermostable Taq DNA ligase are used to discriminate single-nucleotide polymorphism alleles. One probe is an allele-specific probe that hybridizes to a target DNA so that the 3' base is situated directly over the SNP nucleotide, and the other probe hybridizes to the template upstream of the SNP polymorphic site providing a 5' end for the ligation reaction. Only when the allele-specific probe matches the target DNA can ligation occur. In LCR, for each of the two DNA strands, two partial probes are ligated to form one probe; thus LCR uses two enzymes: a DNA polymerase and a thermostable DNA ligase. In other embodiments, where readout depends upon a PCR assay to amplify the product of a ligation event, any blocking group may be placed in the domain of the oligonucleotide of the invention that is removed by RNase H cleavage. In such embodiments, the precise position of the blocking group in the RNase H cleavable domain may be adjusted to alter specificity for cleavage and precise placement of the blocking group relative to the cleavable RNA bases may alter the amount of enzyme needed to achieve optimal cleavage rates.

[0059] For ligation assays (e.g., OLA and LCR), a modification which inhibits polymerase extension and/or ligation activity may be located at or near either the 3' - or 5' -end of the oligonucleotide. In other embodiments, a modification which inhibits polymerase extension and/or ligation activity, if used, is preferably placed within the domain that is 3' to the cleavable RNA base in the region that is removed by probe cleavage. In other embodiments, C3 spacers may be positioned close to the RNA base in the oligonucleotide probes described herein to improve specificity that is helpful for improving mismatch discrimination.

5. Sample

[0060] The methods described herein desirably are practiced on a sample comprising one or more nucleic acid sequences. "Sample" or "biological sample" or "specimen" are synonymous and refer to a sample obtained from a subject. The sample can comprise a nucleic acid, for example a deoxyribonucleic acid (DNA) and/or a ribonucleic acid (RNA). The sample may be obtained from a subject as defined herein, which is preferably a mammal, and more preferably a human (e.g., a human comprising a rare allele or mutation). The subject may also include non-human animals, for example, all mammalian and non-mammalian vertebrates (such as, but not

limited to, non-human primates, sheep, dogs, cats, cows, pigs, horses, rodents, poultry, amphibians, and reptiles). The sample can be, for example, a biological fluid (e.g., blood, urine, semen, saliva, cerebrospinal fluid, amniotic fluid, etc.), a tissue biopsy, curettage, fine needle aspirate, and *ex vivo* cell or tissue culture.

6. Kit for Cleaving a Target Sequence(s)

[0061] The invention described herein also comprises a kit for cleaving a target sequence in an RNase H2-dependent cleavage reaction in an emulsion, such as an RNase H-dependent PCR (rhPCR) reaction (e.g., an emulsion droplet digital PCR system), a loop-mediated isothermal amplification (LAMP) reaction, or cycling probe technology (CPT) as described herein. The kit desirably comprises a mutant RNase H2 enzyme, such as those described herein, in combination with one or more reagents for performing an RNase H2-mediated cleavage reaction on a sample. Examples of suitable reagents for inclusion in the kit include, for example, a wild-type RNase H2 enzyme (for a control), a blocking agent, a labeling agent, one or more primers, a buffer, a metaphase spread, and the like. Many such reagents are described herein or otherwise known in the art and commercially available.

7. Examples

[0062] The following examples further illustrate the invention but should not be construed as in any way limiting its scope.

EXAMPLE 1

[0063] This example demonstrates the failure of wild-type *P.a.* RNase H2 enzyme to perform in an emulsion droplet digital PCR (ddPCR) assay.

[0064] An RNase P assay (RNP assay) was first used to evaluate the activity of the wild-type *P.a.* RNase H2 enzyme in a digital rhPCR system. In an RNP assay, the target nucleic acid is a known quantity of the human *RNASE P* gene. The RNP assay was conducted with a dilution series of the wild-type RNase H2 enzyme using the QX-200™ DROPLET DIGITAL™ (DDPCR™) system (Bio-Rad Laboratories, Inc., Hercules, CA). This system provides absolute

quantification of target nucleic acid molecules (DNA or RNA) for EVAGREEN® (Bio-Rad Laboratories) or probe-based digital PCR applications.

[0065] *RNASE P* nucleic acid samples were prepped for PCR by adding a master mix containing a probe and either unblocked or blocked RNP primers. Unblocked primers, which do not require cleavage by RNase H2 to become functional, were used as controls. Blocked primers included a ribonucleotide residue that prevents primer dimer formation, and this residue must be cleaved by RNase H2 in order for product amplification to occur in a PCR system. The specific probe and primer nucleotide sequences used in the assay are set forth in Table 1. In Table 1, DNA nucleotides are shown in uppercase, and RNA nucleotides are shown in lowercase. HEX (6-carboxy-2',4,4',5',7,7'-hexachlorofluorescein) is a fluorescent dye, and FQ is IOWA BLACK® FQ fluorescent quencher. X is a C3 propanediol spacer utilized to prevent primer extension prior to RNase H2 cleavage of the primer.

Table 1. RNase P Primers and Probe Sequences

Oligonucleotide	Oligonucleotide Sequence	SEQ ID NO.
Unblocked RNP Primer1	GCGGAGGGAAGCTCATCAG	23
Unblocked RNP Primer2	CCCTAGTCTCAGACCTTCCCAA	24
RNP Probe (HEX)	HEX-CCACGAGCTGAGTTCGTCCTGTCA-FQ	25
Blocked RNP Primer1	GCGGAGGGAAGCTCATCAGuGGGGG-x	26
Blocked RNP Primer2	CCCTAGTCTCAGACCTTCCCAAgGGACA-x	27

[0066] For each 20 µl reaction volume, either 4200 fmol (210 nM or 2000 mU), 3150 fmol (157.5 nM or 1500 mU), 2100 fmol (105 nM or 1000 mU), or 420 fmol (21 nM or 200 mU) of the wild-type RNase H2 enzyme was added to a mix containing 1x ddPCR Supermix for Probes (no dUTP) (Bio-Rad), 900 nM of blocked or unblocked RNP primers, 250 nM probe, and 5×10⁴ copies GBLOCK® RNase P template (Integrated DNA Technologies (IDT), Coralville, IA). Reactions containing unblocked primers served as controls to ensure viability of the digital rhPCR system.

[0067] The samples were then emulsified using the BIO-RAD® AUTOMATED DROPLET GENERATOR™ (Bio-Rad) system, which generates up to 20,000 uniform nanoliter-sized water-in-oil droplets within each sample. Within the droplets, the target DNA was randomly distributed, and each droplet served to partition the reactions. The fraction of PCR-positive

droplets was used to quantify the target nucleic acid according to the Poisson distribution. Digital rhPCR was then performed using the primers and probes listed in Table 1, and each of the 20,000 droplets was analyzed for an increase in fluorescence intensity on a BIO-RAD® QX-200™ DROPLET DIGITAL™ Reader. An increase in fluorescence intensity indicated a positive reaction, while no fluorescence indicated that amplification of the target nucleic acid did not occur. The fluorescence intensity was then plotted for each droplet (event number), using the QUANTASOFT™ v1.7 software (Bio-Rad). The results are shown in Figure 1. Reactions with unblocked primers (U) exhibited tight bands with high fluorescent expression, indicative of successful amplification of the template DNA. In contrast, reactions with blocked primers (B) exhibited a characteristic “rain” effect with 4200 fmol, 3150 fmol, and 2100 fmol of wild-type RNase H2, indicative of poor amplification of the template DNA. This was a direct result of poor wild-type RNase H2 activity in the emulsion, which causes the primers to remain blocked and reduces the efficacy of the amplification reaction. Amplification did not occur at all with blocked primers at 420fmol of wild-type *P.a.* RNase H2.

[0068] These results confirm that wild-type *P.a.* RNase H2 is not effective in an emulsion droplet digital PCR system.

EXAMPLE 2

[0069] This example describes the use of mutant *P.a.* RNase H2 enzymes in an emulsion droplet digital PCR (ddPCR) assay.

[0070] To determine whether a mutated *P.a.* RNase H2 enzyme could exhibit improved performance as compared to the wild-type *P.a.* RNase H2 in an emulsion-based digital PCR assay, a series of 22 mutants were created with respect to the wild-type enzyme by standard site-directed mutagenesis methods (see Weiner et al., *Gene*, 151:119-123 (1994)) using the oligonucleotide primers in Table 2. Mutant RNase H enzyme sequences were codon optimized for expression in *E. coli* using the Integrated DNA Technologies Codon Optimization web tool (www.idtdna.com/CodonOpt). Each mutant enzyme contained at least one amino acid substitution. As shown in Table 2, each mutant was designated according to the amino acid residue number where the substitution occurred, preceded by the original amino acid, and proceeded by the substituted amino acid.

Table 2

Mutant ID	Amino acid change	Mutant Amino Acid Sequence SEQ ID NO:	Sense Mutagenesis Oligonucleotide SEQ ID NO:	Antisense Mutagenesis Oligonucleotide SEQ ID NO:
1	A9S	1	28	29
2	R11K	2	30	31
3	R11Q	3	32	33
4	G12A	4	34	35
5	G12T	5	36	37
6	G12C	6	38	39
7	P13S	7	40	41
8	P13T	8	42	43
9	P13E	9	44	45
10	V14L	10	46	47
11	V14F	11	48	49
12	G169A	12	50	51
13	P171G	13	52	53
14	S172T	14	54	55
15	S172G	15	56	57
16	S172I	16	58	59
17	S172H	17	60	61
18	D173E	18	62	63
19	K149R	19	64	65
20	A9S R11K	20	28, 30	29, 31
21	R11A	21	66	67
22	S172Q	22	68	69

[0071] The RNase H mutants were expressed in *E. coli*, purified by NiNTA chromatography, and re-suspended in Buffer F (20 mM Tris-HCl pH 8.4, 100 mM KCl, 0.1 M EDTA, 0.1% Triton X-100, and 50% glycerol). Techniques for purification were identical to those previously described for purifying HIS-tagged wild-type *P.a.* RNase H2 (see U.S. Patent 8,911,948).

[0072] The mutant enzymes were characterized to determine their functional activity using a previously described synthetic rhPCR assay (see U.S. Patent 8,911,948). The enzymatic activity of each mutant relative to wild-type *P.a.* RNase H2 is shown in Table 3.

Table 3

Mut ID #	Amino acid change	Mutant Amino Acid Sequence SEQ ID NO:	U/μg	% of wild-type activity	1 mU = x fmol
	Wild-type RNase H2	125	17.3	100.0%	2.1
1	A9S	1	5.2	30.1%	7
2	R11K	2	5.2	30.1%	7
3	R11Q	3	2.6	15.0%	14
4	G12A	4	0.65	3.8%	55.9
5	G12T	5	2.6	15.0%	14
6	G12C	6	2.6	15.0%	14
7	P13S	7	2.6	15.0%	14
8	P13T	8	2.6	15.0%	14
9	P13E	9	2.6	15.0%	14
10	V14L	10	1.3	7.5%	27.9
11	V14F	11	0.65	3.8%	55.9
12	G169A	12	0.27	1.6%	134.6
13	P171G	13	2.6	15.0%	14
14	S172T	14	2.5	14.5%	14.5
15	S172G	15	1.3	7.5%	27.9
16	S172I	16	2.6	15.0%	14
17	S172H	17	1.3	7.5%	27.9
18	D173E	18	2.6	15.0%	14
19	K149R	19	2.6	15.0%	14
20	A9S R11K	20	5.2	30.1%	7
21	R11A	21	0.028	0.2%	1297.5
22	S172Q	22	0.65	3.8%	55.9

[0073] Once activity was determined, the mutant RNase enzymes were further tested using the previous described digital rhPCR RNP assay.

[0074] For each 20 μ l reaction volume, 420 fmol (21 nM) of each RNase H2 enzyme was added to a mix containing 1x ddPCR Supermix for Probes (No dUTP) (Bio-Rad Laboratories, Inc., Hercules, CA), 900 nM of blocked or unblocked RNP primers, 250 nM probe, and 5×10^4 copies GBLOCK® RNase P template (Integrated DNA Technologies (IDT), Coralville,

IA). Reactions containing unblocked primers served as controls to ensure viability of the digital rhPCR system.

[0075] The samples were emulsified using the Bio-Rad® Automated Droplet Generator™ (Bio-Rad® Laboratories, Inc., Hercules, CA) system, which generated 20,000 uniform nanoliter-sized water-in-oil droplets within each sample. The target DNA was randomly distributed among the droplets, and each droplet served to partition the reactions. The fraction of PCR-positive droplets was used to quantify the target nucleic acid according to the Poisson distribution. Digital rhPCR was then performed using the primers and probes listed in Table 1, and each of the 20,000 droplets was analyzed for an increase in fluorescence intensity on a Bio-Rad® QX-200™ Droplet Digital™ Reader. An increase in fluorescence intensity indicated a positive reaction, while no fluorescence indicated that amplification of the target nucleic acid did not occur. The fluorescence intensity was then plotted for each droplet (event number) using the QUANTASOFT™ v1.7 software (Bio-Rad® Laboratories, Inc., Hercules, CA). Reactions with unblocked primers (U) exhibited tight bands with high fluorescent expression, indicative of successful amplification of the template DNA. The results of this assay are shown in Figure 2. Almost no amplification of the *RNASE P* template occurred in the reaction mixtures containing blocked primers and wild-type RNase H2 (WT-B, relative to reactions that contained unblocked primers), consistent with the results shown in Figure 1. However, several reactions containing mutated RNase H2 enzymes showed an improvement in amplification efficacy; in particular, mutants A9S (SEQ ID NO: 1), R11K (SEQ ID NO: 2), P13S (SEQ ID NO: 7), P13T (SEQ ID NO: 8), P171G (SEQ ID NO: 13), S172G (SEQ ID NO: 15), S172I (SEQ ID NO: 16), S172H (SEQ ID NO: 17), D173E (SEQ ID NO: 18), and A9S R11K (SEQ ID NO: 20) exhibited greater amplification of the *RNASE P* template as compared to reactions containing the wild-type RNase H2 enzyme.

[0076] Similar to the results shown in Figure 1, successful reactions with mutant RNase H2 enzymes manifested as a band of positive reactions with high fluorescence intensities. Of the mutant enzymes tested, P13S (SEQ ID NO: 7) exhibited the highest level of activity, producing a distinct band that exhibited a fluorescent signal intensity nearing that of the unblocked control reactions. Additionally, the quantitative value obtained for the *RNASE P* template was similar between the P13S mutant and that of the wild-type RNase H2 enzyme (2450 copies/μl, data not

shown), indicating that successful amplification of the product was achieved by both the wild-type and the mutant enzymes.

EXAMPLE 3

[0077] This example describes the effect of different concentrations of a mutant *P.a.* RNase H2 enzyme in an emulsion droplet digital PCR (ddPCR) assay.

[0078] To further evaluate the functionality of the P13S RNase H2 mutant, a titration of this enzyme ranging from 420 fmoles (21 nM) to 26 fmoles (1.3 nM) was performed, and the activity of each dilution was evaluated using an RNP assay in a digital rhPCR system.

[0079] For each 20 μ l reaction volume, either 420 fmol (21 nM), 210 fmol (10.5 nM), 105 fmol (5.25 nM), 52 fmol (2.6 nM), or 26 fmol (1.3 nM) of the P13S mutant RNase H2 enzyme was added to a mix containing 1x ddPCR Supermix for Probes (No dUTP) (Bio-Rad Laboratories, Inc., Hercules, CA), 900 nM of blocked or unblocked RNP primers, 250 nM probe, and 5×10^4 copies GBLOCK® RNase P template (Integrated DNA Technologies (IDT), Coralville, IA). Reactions containing unblocked primers served as controls to ensure viability of the digital rhPCR system.

[0080] The samples were then emulsified using the Bio-Rad® Automated Droplet Generator™ (Bio-Rad® Laboratories, Inc., Hercules, CA) system, which generated up to 20,000 uniform nanoliter-sized water-in-oil droplets within each sample. The target DNA was randomly distributed among the droplets, and each droplet served to partition the reactions. The fraction of PCR-positive droplets was used to quantify the target nucleic acid according to the Poisson distribution. Digital rhPCR was then performed using the primers and probes listed in Table 1, and each of the 20,000 droplets was analyzed for an increase in fluorescence intensity on a Bio-Rad® QX-200™ Droplet Digital™ Reader. An increase in fluorescence intensity indicated a positive reaction, while no fluorescence indicated that amplification of the target nucleic acid did not occur. The fluorescence intensity was then plotted for each droplet (event number) using the QUANTASOFT® v1.7 software (Bio-Rad® Laboratories, Inc., Hercules, CA).

[0081] The results of this experiment are shown in Figure 3. The signal intensity of the PCR reaction was decreased in samples containing blocked RNP primers (B) compared to those with unblocked primers (U); however, the P13S mutant (SEQ ID NO: 7) exhibited successful

amplification of the RNP template with 420 and 210 fmoles (21 and 10.5 nM, respectively), and good concentration calls were obtained down to 105 fmoles (5.25 nM) of enzyme. Beginning at 105 fmoles of the P13S mutant enzyme, the amplification efficacy of the reaction began to decline, as indicated by a widening band of fluorescent intensities. Efficacy continued to decline at 52 fmoles and 26 fmoles. These results demonstrate that P13S performs ideally when present in a reaction at greater than 210 fmoles.

[0082] The results of this example demonstrate that nucleic acid sequences can be amplified in an RNase H-dependent PCR reaction using a mutant *Pyrococcus abyssi* (*P.a.*) RNase H2 enzyme in accordance with the inventive method. Additionally, the P13S mutant (SEQ ID NO: 7) is effective at lower concentrations.

EXAMPLE 4

[0083] This example demonstrates the use of additional *P.a.* RNase H2 mutant enzymes in an emulsion-based digital droplet PCR assay.

[0084] A total of 26 mutant RNase H2 enzymes were created by using standard site-directed mutagenesis in an emulsion system (see Weiner M., et al., *Gene*, 151:119-123 (1994)) and oligonucleotide primers as listed in Table 4. Sequences were codon optimized for *E. coli* using the Integrated DNA Technologies Codon Optimization web tool. These mutants were expressed in *E.coli*, purified by NiNTA chromatography, and re-suspended in Buffer F, as described above. Each mutant enzyme contained at least one amino acid substitution. In Table 4, the site of the substitution is designated by the residue number, preceded by the original amino acid, and proceeded by the substituted amino acid. The corresponding nucleic acid codon of the substituted amino acid is shown in uppercase, while all other DNA bases are shown in lower case.

Table 4

Mutant ID #	Amino acid changes	Mutant Amino Acid Sequence SEQ ID NO:	Sense Mutagenesis Oligonucleotide SEQ ID NO:	Antisense Mutagenesis Oligonucleotide SEQ ID NO:
7	P13S	7	40	41
23	Q48R	70	99	100
24	K149T	71	101	102

Mutant ID #	Amino acid changes	Mutant Amino Acid Sequence SEQ ID NO:	Sense Mutagenesis Oligonucleotide SEQ ID NO:	Antisense Mutagenesis Oligonucleotide SEQ ID NO:
25	D199Y	72	103	104
26	D199N	73	105	106
27	D199G	74	107	108
28	D199E	75	109	110
29	D199V	76	111	112
30	M80L	77	113	114
31	F218L	78	115	116
32	P13S A107V	79	40, 117	41, 118
33	P13S D199Y	80	40, 103	41, 104
34	Q48R D199N	81	98, 105	99, 106
35	Q48R A107V	82	98, 117	99, 118
36	A107V K149T	83	117, 101	118, 102
37	A107V D199Y	84	117, 103	118, 104
38	A107V D199N	85	117, 105	118, 106
39	A107V D199G	86	117, 107	118, 108
40	A107V D199E	87	117, 109	118, 110
41	M80L F218L	88	113, 115	114, 116
42	M80L A107V F218L	89	113, 117, 115	114, 118, 116
43	A107V	90	117	118
44	G12S	91	119	120
45	G10S	92	121	122
46	E157K	93	123	124
47	G12S A107V	94	119, 117	120, 118

[0085] The functional activity of these mutants was first characterized in a digital rhPCR system using an RNP assay. Each of the mutants was tested in a reaction that contained 420 fmol of enzyme and either blocked or unblocked RNP primers. For each 20 μ l reaction volume, either 420 fmol (21 nM) of each mutant RNase H2 enzyme was added to a separate reaction mix also containing 1x ddPCR Supermix for Probes (No dUTP) (Bio-Rad Laboratories, Inc., Hercules, CA), 900 nM of blocked or unblocked RNP primers, 250 nM probe, and 5×10^4 copies

GBLOCK® RNase P template (Integrated DNA Technologies (IDT), Coralville, IA). Reactions containing unblocked primers served as controls to ensure viability of the digital rhPCR system.

[0086] The samples were then emulsified using the Bio-Rad® Automated Droplet Generator™ (Bio-Rad® Laboratories, Inc., Hercules, CA) system, which generated 20,000 uniform nanoliter-sized water-in-oil droplets within each sample. The target DNA was randomly distributed among the droplets, and each droplet served to partition the reactions. The fraction of PCR-positive droplets was used to quantify the target nucleic acid according to the Poisson distribution. Digital rhPCR was then performed using the primers and probes listed in Table 1, and each of the 20,000 droplets was analyzed for an increase in fluorescence intensity on a Bio-Rad® QX-200™ Droplet Digital™ Reader. An increase in fluorescence intensity indicated a positive reaction, while no fluorescence indicated that amplification of the target nucleic acid did not occur. The fluorescence intensity was then plotted for each droplet (event number) using the QUANTASOFT™ v1.7 software (Bio-Rad® Laboratories, Inc., Hercules, CA). The results are shown in Figure 4.

[0087] The activity of each mutant is listed in Table 5, and is presented relative to the activity level of wild-type *P.a.* RNase H2. From these experiments, the RNase H2 mutants P13S (SEQ ID NO: 7), Q48R (SEQ ID NO: 70), M80L (SEQ ID NO: 77), P13S_A107V (SEQ ID NO: 79), P13S_D199Y (SEQ ID NO: 80), A107V_D199G (SEQ ID NO: 86) and A107V_D199E (SEQ ID NO: 87) were identified as top performers.

Table 5

Mutant ID #	Specific AA changes	SEQ ID NO:	U/μg	% of WT activity	1 mU = x fmol
--	Wild-type RNase H2	125	17.3	100.0%	2.1
7	P13S	7	5.2	30.1%	7.0
23	Q48R	70	17.3	100.0%	2.1
24	K149T	71	0.059	0.3%	615.8
25	D199Y	72	17.3	100.0%	2.1
26	D199N	73	26	150.3%	1.4
27	D199G	74	26	150.3%	1.4
28	D199E	75	17.3	100.0%	2.1
29	D199V	76	20	115.6%	1.8

Mutant ID #	Specific AA changes	SEQ ID NO:	U/ μ g	% of WT activity	1 mU = x fmol
30	M80L	77	26.2	151.4%	1.4
31	F218L	78	40.4	233.5%	0.9
32	P13S A107V	79	13	75.1%	2.8
33	P13S D199Y	80	13	75.1%	2.8
34	Q48R D199N	81	26	150.3%	1.4
35	Q48R A107V	82	26	150.3%	1.4
36	A107V K149T	83	0.65	3.8%	55.9
37	A107V D199Y	84	26	150.3%	1.4
38	A107V D199N	85	26	150.3%	1.4
39	A107V D199G	86	17.3	100.0%	2.1
40	A107V D199E	87	17.3	100.0%	2.1
41	M80L F218L	88	13	75.1%	2.8
42	M80L A107V F218L	89	20	115.6%	1.8
43	A107V	90	20.00	115.6%	1.8
44	G12S	91	0.15	0.9%	238.9
45	G10S	92	0.22	1.3%	166.3
46	E157K	93	20.00	115.6%	1.8
47	G12S A107V	94	2.60	15.0%	14.0

[0088] The results of this example confirm that *P.a.* RNase H2 mutant enzymes can be used in an emulsion-based digital droplet PCR assay.

EXAMPLE 5

[0089] This example demonstrates the activity of wild-type RNase H2 enzymes obtained or derived from additional species in an emulsion-based digital droplet PCR assay.

[0090] Wild-type RNase H2 enzymes were synthesized and/or purified from *Pyrococcus furiosus* (SEQ ID NO: 95), *Pyrococcus horikoshii* (SEQ ID NO: 96), and the archaeal species *Thermococcus kodakarensis* (SEQ ID NO: 97) and *Thermococcus litoralis* (SEQ ID NO: 98), which have greater sequence divergence from *Pyrococcus*. A comparison of the RNase H2 amino acid sequences (see Figure 5) revealed that both *Thermococcus* species have a native

Q48R amino acid sequence and that *T. kodakarensis* has an additional native leucine amino acid at position 80 (equivalent to M80L in *P.a.* RNase H2).

[0091] Wild-type RNase H2 sequences for each enzyme were obtained as synthetic sequences and codon optimized for *E. coli* using the Integrated DNA Technologies (IDT) Codon Optimization web tool (www.idtdna.com/CodonOpt). The wild-type RNase H2 sequences were expressed in *E. coli*, purified by Ni-NTA affinity chromatography, and dialyzed in Buffer F (20 mM Tris-HCl pH 8.4, 100 mM KCl, 0.1 mM EDTA, 0.1% Triton X-100, and 50% glycerol) as previously described for purification of wild-type *P.a.* RNase H2 (see U.S. Patent 8,911,948). Protein concentrations were independently verified using Bradford and bicinchoninic acid (BCA) assays, using standard protocols described by the manufacturers. The recombinant proteins were tested for RNase and DNase contamination using DNASE ALERT® and RNASE ALERT® kits (Integrated DNA Technologies, Inc., Coralville, IA).

[0092] To determine whether the wild-type proteins from *Pyrococcus furiosus*, *Pyrococcus horikoshii*, *Thermococcus kodakarensis*, and *Thermococcus litoralis* showed improved activity in rh-ddPCR, functionality was assessed using an RNase P assay as described above (see Table 1, SEQ ID Nos: 23-27). Reactions were carried out in 96-well plate format and contained 1x ddPCR™ Supermix (Bio-Rad Laboratories, Inc., Hercules, CA) for probes (no dUTP), 900 nM blocked or unblocked primers, 250 nM HEX probe, and 5x10⁴ copies RNase P GBLOCK® template (Integrated DNA Technologies, Inc., Coralville, IA). RNase H2 enzymes were diluted in Buffer D and 420 fmol were added to a final volume of 20 µl. Emulsions were generated with the Bio-Rad Automated Droplet Generator™ system (Bio-Rad Laboratories, Inc., Hercules, CA). Droplets were analyzed using the Bio-Rad QX200™ Digital Drop Reader (Bio-Rad Laboratories, Inc., Hercules, CA) following PCR amplification.

[0093] As demonstrated above, the wild-type *P.a.* did not support amplification from the blocked primers at 420 fmol (see Figure 6). The closely related wild-type *P.fur* and *P.hori* also did not support amplification at 420 fmol. While the wild-type *T.lit* did not demonstrate activity at 420 fmol, the wild-type *T.kod* (containing native Q48R and M80L mutations) displayed activity at 420 fmol, with a signal intensity similar to the unblocked primer control, as shown in Figure 6.

[0094] A titration of *T.kod* RNase H2 from 840 fmol to 52.5 fmol (the equivalent of 400 mU to 25 mU of wild-type *P.a.*) was tested in rh ddPCR to further assess the enzyme activity, as shown in Figure 7. A titration of *P.a.* RNase H2 from 840 fmol to 210 fmol (400 mU to 100 mU) was included. While the wild-type *P.a.* enzyme was inactive at 210 fmol, the wild-type *T.kod* enzyme supported efficient amplification with signal intensity similar to the unblocked control at the tested enzyme concentrations.

[0095] These results confirm that wild-type RNase H2 enzymes from *P.fur*, *P.hori*, and *T.lit* are not effective in an emulsion droplet digital PCR system, and that wild-type *T.kod* is effective at lowered concentrations.

EXAMPLE 6

[0096] This example demonstrates the use of RNase H2 mutant enzymes derived from *P.fur*, *P.hori*, *T.kod*, and *T.lit* in an emulsion-based digital droplet PCR assay.

[0097] To determine whether the equivalents of *P.a.* P13S, Q48R, M80L, A107V and P13S A107V mutations improved activity in an emulsion in alternative RNase H2 enzymes, these mutations were introduced into the *P.fur*, *P.hori*, *T.lit*, and *T.kod* RNase H2 proteins by site-directed mutagenesis (see Weiner et al., *Gene*, 151:119-123 (1994)) using the primers listed in Table 6. Mutant RNase H enzyme sequences were codon optimized for expression in *E. coli* using the Integrated DNA Technologies Codon Optimization web tool (www.idtdna.com/CodonOpt). Each mutant enzyme contained at least one amino acid substitution. As shown in Table 6, each mutant was designated according to the amino acid residue number where the substitution occurred, preceded by the original amino acid, and proceeded by the substituted amino acid.

Table 6

Mutant ID	Mutant Species	Amino acid change	Mutant Amino Acid Sequence SEQ ID NO:	Sense Mutagenesis Oligonucleotide SEQ ID NO:	Antisense Mutagenesis Oligonucleotide SEQ ID NO:
48	<i>P. fur</i>	P13S	126	139	140
49	<i>P. fur</i>	E48R	127	141	142

Mutant ID	Mutant Species	Amino acid change	Mutant Amino Acid Sequence SEQ ID NO:	Sense Mutagenesis Oligonucleotide SEQ ID NO:	Antisense Mutagenesis Oligonucleotide SEQ ID NO:
50	<i>P. fur</i>	M80L	128	143	144
51	<i>P. fur</i>	A107V	129	145	146
52	<i>P. fur</i>	P13S A107V	130	139, 145	140, 146
53	<i>P. hori</i>	Q48R	131	149	150
54	<i>P. hori</i>	M80L	132	151	152
55	<i>P. hori</i>	A107V	133	153	154
56	<i>T. kod</i>	P13S	134	155	156
57	<i>T. kod</i>	A107V	135	157	158
58	<i>T. kod</i>	P13S A107V	136	155, 157	156, 158
59	<i>T. lit</i>	M80L	137	161	162
60	<i>T. lit</i>	A107V	138	163	164
61	<i>P. abs</i>	P13S Q48R M80L A107V	165	40, 99, 113, 117	41, 100, 114, 118

[0098] The RNase H mutants were expressed in *E. coli*, purified by NiNTA chromatography, and re-suspended in Buffer F (20 mM Tris-HCl pH 8.4, 100 mM KCl, 0.1 M EDTA, 0.1% Triton X-100, and 50% glycerol). Techniques for purification were identical to those previously described for purifying HIS-tagged wild-type *P.a.* RNase H2 (see U.S. Patent 8,911,948).

[0099] Protein concentrations were independently verified using Bradford and BCA assays according to the manufacturer's instructions. Proteins were also tested for RNase and DNase contamination using DNASE ALERT® and RNASE ALERT® kits (Integrated DNA Technologies, Inc., Coralville, IA). Some mutant proteins exhibited low levels of RNase contamination. As a result, 5U of SUPERASE-IN™ RNase Inhibitor (Thermo Fisher Scientific, Waltham, MA) was included in all rh ddPCR reactions. SUPERASE-IN™ does not inhibit RNase H2 activity and blocks background RNase activity.

[00100] For each 20 µl reaction volume, 420 fmol (21 nM) of each RNase H2 enzyme was added to a mix containing 1x ddPCR Supermix for Probes (No dUTP) (Bio-Rad Laboratories, Inc., Hercules, CA), 900 nM of blocked or unblocked RNP primers, 250 nM probe, 5U of SUPERASE-IN™, and 5x10⁴ copies GBLOCK® RNase P template (Integrated DNA

Technologies (IDT), Coralville, IA). Reactions containing unblocked primers served as controls to ensure viability of the digital rhPCR system.

[00101] The samples were emulsified using the Bio-Rad AUTODG™ (Bio-Rad Laboratories, Inc., Hercules, CA) system, which generated 20,000 uniform nanoliter-sized water-in-oil droplets within each sample. The target DNA was randomly distributed among the droplets, and each droplet served to partition the reactions. The fraction of PCR-positive droplets was used to quantify the target nucleic acid according to the Poisson distribution. Digital rhPCR was then performed using the primers and probes listed in Table 1, and each of the 20,000 droplets was analyzed for an increase in fluorescence intensity on a QX-200™ DROPLET DIGITAL™ Reader (Bio-Rad Laboratories, Inc., Hercules, CA). An increase in fluorescence intensity indicated a positive reaction, while no fluorescence indicated that amplification of the target nucleic acid did not occur. The fluorescence intensity was then plotted for each droplet (event number) using the QUANTASOFT™ v1.7 software (Bio-Rad® Laboratories, Inc., Hercules, CA). Reactions with unblocked primers (U) exhibited tight bands with high fluorescent expression, indicative of successful amplification of the template DNA.

[00102] Comparisons were made with the wild-type RNase H2 for each mutant set to assess any improvement conferred by the *P.a.* mutations. With the exception of *T.lit* (see Figure 8), enhanced activity was observed in each wild-type enzyme background. In particular, improved activity was observed in the *P.fur* P13S (SEQ ID NO: 126) and P13S A107V (SEQ ID NO: 130) mutants, as shown in Figure 9. Cleavage and subsequent amplification activity was observed in the tested *P.hori* mutants Q48R (SEQ ID NO: 131), M80L (SEQ ID NO: 132), and A107V (SEQ ID NO: 133), with signal intensities similar to the unblocked primer controls, as shown in Figure 10. Cleavage and subsequent amplification activity was observed in *T.kod* mutants P13S (SEQ ID NO: 134), A107V (SEQ ID NO: 135), and P13S A107 (SEQ ID NO: 136), with signal intensities greater than wild-type mutants, and amplification occurring with reduced amounts of enzyme input, as shown in Figure 11.

[00103] The results of this example confirm that *P.fur*, *P.hori*, and *T.kod*. RNase H2 mutant enzymes can be used in an emulsion-based digital droplet PCR assay.

EXAMPLE 7

[00104] This example demonstrates the use of additional *P.a.* mutants in an emulsion-based digital droplet PCR assay.

[00105] A *P.a.* P13S Q48R M80L A107V (SEQ ID NO: 165) quadruple mutant was generated to compare activity to the *T.kod* P13S A107V (SEQ ID NO 136) mutant described in Example 6. The *P.a.* quadruple mutant was created using standard site-directed mutagenesis of the *P.a.* P13S A107V mutant described above using the primers listed in Table 6 to introduce Q48R and M80L, for a total of four rounds of site-directed mutagenesis. The protein was purified as described above. Protein concentration was independently verified using Bradford and BCA assays, and RNase and DNase contamination was tested using DNASE ALERT® and RNASE ALERT® kits (Integrated DNA Technologies, Inc., Coralville, IA).

[00106] The activity of the *P.a.* P13S Q48R M80L A107V mutant (SEQ ID NO: 136) was tested using the rh-ddPCR RNase P assay as previously described, including 5U of SUPERASE-IN™. A titration of the mutant enzymes from 4200 fmol to 4.2 fmol (the equivalent of 2000 mU to 2 mU of wild-type *P.a.*) was compared to the activity of the wild-type *P.a.* at 4200 and 420 fmol, as shown in Figure 12. The *P.a.* P13S Q48R M80L A107V mutant (SEQ ID NO: 136) displayed activity down to 42 fmol, but no activity at 4.2 fmol.

[00107] The results of this example demonstrate that the *P.a.* P13S Q48R M80L A107V mutant can support cleavage in a ddPCR environment.

EXAMPLE 8

[00108] This example describes a method of detecting homology directed repair (HDR) using RNase H mutants described herein.

[00109] To detect HDR events generated from a targeted endonuclease using RNase H-dependent ddPCR, a total of three rhPCR assays targeting a desired modification site utilizing rhPrimers, one specific for the desired changes, one for the sequence of the wild type sequence, and one specific for the product of a blunt insertion of the HDR template, are designed. A locus-specific reverse primer that will amplify both the wild type and edited template also is designed. In addition, an emulsion-competent RNase H2 cleavable cycling probe (CpT) is designed to serve as a control assay for the detection of both edited and non-edited DNA.

[00110] The first set of rhPrimers are designed to detect the presence of the wild type template in an emulsion reaction. These rhPrimers are designed with the cleavable moiety located one or two bases after the most common cleavage site for the targetable endonuclease (usually 3 bases before the PAM recognition domain in Cas9). The rhPrimers are cleaved by an emulsion-competent RNase H2 enzyme, such as those described herein, when they successfully bind to a wild type template, but not when they bind to an HDR or non-homologous end joining (NHEJ) edited template. This allows for easy discrimination against editing events which may occur, while still retaining specificity for the wild type template.

[00111] The second set of rhPrimers are designed to detect HDR editing events. These rhPrimers are specific for the HDR donor template and are designed, like the wild type specific primers, with the cleavable moiety located one or two bases after the start of the edited sequence, to most accurately distinguish between the HDR and wild type templates.

[00112] The third set of rhPrimers are designed to detect a blunt insertion of the HDR template. These rhPrimers are specifically designed to generate additional signal intensity from the HDR editing signal channel via recognition of sequence duplication created by the blunt insertion.

[00113] Each of the wild type or insert-specific primers are designed to possess a universal forward primer and one of two different fluorescent probe binding domains located on the 5' end of the primer. These universal domains allow for complementary probes to bind to the amplicon upon removal of the 3' blocks by the RNase H2 enzyme and amplification by the DNA polymerase. The 5' exonuclease activity of the polymerase will degrade the detection probe generating the fluorescent signal for the wild type or HDR determination.

[00114] The CpT assay is designed to detect the presence of the template whether or not the other primers amplify. This will serve to detect the presence or absence of DNA in each emulsion droplet. If template is present, the RNase H2 enzyme will cleave the probe, generating a fluorescent signal. The probe is designed so that its T_m is high enough for it to bind to the template while whole, but denatures when cleaved by the emulsion-competent RNase H2 enzyme. The desired T_m for this probe therefore is about 60°C in most reaction settings.

[00115] The described primers and probes are combined in a suitable emulsion PCR mix, and emulsion droplets are made using methods known to those of skill in the art. Thermal cycling

and fluorescence quantification is then performed, allowing for template amplification and RNase H2 cleavage. In the reaction mix during amplification, the cycling probe binds to each template in the reaction, allowing for the separation of the droplets that contain templates from the droplets that do not. The droplets that contain template will allow for cleavage of the cycling probe, and generation of fluorescent signal. While this is occurring, wild type or target-specific rhPrimers bind to the template present in each individual emulsion droplet. The rhPrimers are then cleaved by the RNase H2 enzyme, which is present in the droplet as well. The DNA polymerase extends from the newly unblocked primers, incorporating the 5' universal forward primer and probe binding domains into the newly generated amplicons. The universal forward primers will then bind to their complementary binding domains, and degrade the detection probes. This will provide a fluorescent signal corresponding to either the wild type or the desired HDR template, depending on which is present in the droplet. If a blunt insertion of the HDR template occurs by the NHEJ machinery, additional fluorescence intensity will distinguish this event from a correct HDR insertion. This signal will be combined with the fluorescence obtained from the cycling probe to generate the final signal intensity. Finally, if an NHEJ event occurs rather than the desired HDR event, then only the cycling probe will bind, and be cleaved. Using this technique, desired HDR events can be separated from NHEJ events and HDR template blunt insertion in a single reaction.

[00116] All references, including publications, patent applications, and patents, cited herein, are hereby incorporated by reference to the same extent as if each reference were individually and specifically indicated to be incorporated by reference and were set forth in its entirety herein.

[00117] The use of the terms “a” and “an” and “the” and “at least one” and similar referents in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. The use of the term “at least one” followed by a list of one or more items (for example, “at least one of A and B”) is to be construed to mean one item selected from the listed items (A or B) or any combination of two or more of the listed items (A and B), unless otherwise indicated herein or clearly contradicted by context. The terms “comprising,” “having,” “including,” and “containing” are to be construed as open-ended terms (i.e., meaning

“including, but not limited to,”) unless otherwise noted. The present disclosure also contemplates other embodiments “comprising,” “consisting of,” and “consisting essentially of,” the embodiments or elements presented herein, whether explicitly set forth or not. Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., “such as”) provided herein, is intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

[00118] Preferred embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Variations of those preferred embodiments may become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventors expect skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

CLAIMS:

1. A method of performing an RNase H2-mediated cleavage of one or more nucleic acid sequences of interest comprising:

(a) providing a sample comprising one or more nucleic acid sequences of interest;

(b) performing an RNase H2-mediated cleavage reaction on the one or more nucleic acid sequences, wherein the RNase H2-mediated cleavage reaction comprises:

(i) a mutant *Pyrococcus abyssi* (*P.a.*) RNase H2 enzyme comprising an amino acid sequence selected from SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 70, SEQ ID NO: 71, SEQ ID NO: 73, SEQ ID NO: 74, SEQ ID NO: 76, SEQ ID NO: 77, SEQ ID NO: 78, SEQ ID NO: 79, SEQ ID NO: 80, SEQ ID NO: 81, SEQ ID NO: 82, SEQ ID NO: 84, SEQ ID NO: 85, SEQ ID NO: 86, SEQ ID NO: 87, SEQ ID NO: 88, SEQ ID NO: 89, SEQ ID NO: 90, and SEQ ID NO: 165;

(ii) a mutant *Pyrococcus furiosus* (*P.fur*) RNase H2 enzyme comprising an amino acid sequence selected from SEQ ID NO: 126, SEQ ID NO: 127, SEQ ID NO: 128, SEQ ID NO: 129, and SEQ ID NO: 130;

(iii) a mutant *Pyrococcus horikoshii* (*P.hori*) RNase H2 enzyme comprising an amino acid sequence selected from SEQ ID NO: 131, SEQ ID NO: 132, and SEQ ID NO: 133;

(iv) a mutant *Thermococcus kodakarensis* (*T.kod*) RNase H2 enzyme comprising an amino acid sequence selected from SEQ ID NO: 134, SEQ ID NO: 135, and SEQ ID NO: 136; or

(v) a mutant *Thermococcus litoralis* (*T.lit*) RNase H2 enzyme comprising the amino acid sequence of SEQ ID NO: 137 or SEQ ID NO: 138;

whereupon one or more nucleic acid sequences of interest are cleaved.

2. The method of claim 1, wherein the RNase H2-mediated cleavage reaction is performed as part of an RNase H-dependent PCR (rhPCR) reaction, a loop-mediated isothermal amplification (LAMP) reaction, or cycling probe technology (CPT).

3. The method of claim 1 or claim 2, wherein the RNase H2-mediated cleavage reaction is performed as part of an RNase H-dependent PCR (rhPCR) reaction in a digital PCR system.

4. The method of claim 3, wherein the digital PCR system is an emulsion droplet digital PCR (ddPCR) system.

5. The method of any one of claims 1-4, wherein the RNase H2-mediated cleavage reaction comprises a mutant *Pyrococcus abyssi* (*P.a.*) RNase H2 enzyme comprising an amino acid sequence selected from SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO: 16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:20, SEQ ID NO: 70, SEQ ID NO: 71, SEQ ID NO: 73, SEQ ID NO: 74, SEQ ID NO: 76, SEQ ID NO: 77, SEQ ID NO: 78, SEQ ID NO: 79, SEQ ID NO: 80, SEQ ID NO: 81, SEQ ID NO: 82, SEQ ID NO: 84, SEQ ID NO: 85, SEQ ID NO: 86, SEQ ID NO: 87, SEQ ID NO: 88, SEQ ID NO: 89, SEQ ID NO: 90, and SEQ ID NO: 165.

6. The method of any one of claims 1-4, wherein the RNase H2-mediated cleavage reaction comprises a mutant *Pyrococcus furiosus* (*P.fur*) RNase H2 enzyme comprising an amino acid sequence selected from SEQ ID NO: 126, SEQ ID NO: 127, SEQ ID NO: 128, SEQ ID NO: 129, and SEQ ID NO: 130.

7. The method of any one of claims 1-4, wherein the RNase H2-mediated cleavage reaction comprises a mutant *Pyrococcus horikoshii* (*P.hori*) RNase H2 enzyme comprising an amino acid sequence selected from SEQ ID NO: 131, SEQ ID NO: 132, and SEQ ID NO: 133.

8. The method of any one of claims 1-4, wherein the RNase H2-mediated cleavage reaction comprises a mutant *Thermococcus kodakarensis* (*T.kod*) RNase H2 enzyme comprising an amino acid sequence selected from SEQ ID NO: 134, SEQ ID NO: 135, and SEQ ID NO:136.

9. The method of any one of claims 1-4, wherein the RNase H2-mediated cleavage reaction comprises a mutant *Thermococcus litoralis* (*T.lit*) RNase H2 enzyme comprising the amino acid sequence of SEQ ID NO: 137 or SEQ ID NO: 138.
10. The method of any one of claims 1-9, wherein the sample comprises one or more cells.
11. The method of claim 10, wherein the one or more cells are animal cells.
12. The method of claim 11, wherein the one or more cells are mammalian cells.
13. The method of any one of claims 10-12, wherein the one or more cells are *in vivo*.
14. The method of any one of claims 10-12, wherein the one or more cells are *in vitro*.
15. The method of any one of claims 1-14, wherein the sample is blood, plasma, serum, cerebrospinal fluid, semen, urine, or amniotic fluid.
16. The method of any one of claims 1-15, wherein the one or more nucleic acids are synthetically generated.
17. A kit for cleaving one or more nucleic acid sequences of interest comprising:
(a) one or more reagents for performing an RNase H2-mediated cleavage reaction in an emulsion on a sample comprising one or more nucleic acid sequences of interest,
(b) one or more mutant RNase H2 enzymes selected from:
(i) a mutant *Pyrococcus abyssi* (*P.a.*) RNase H2 enzyme comprising an amino acid sequence selected from SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:13, SEQ ID NO:15, SEQ ID NO: 16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:20, SEQ ID NO: 70, SEQ ID NO: 71, SEQ ID NO: 73, SEQ ID NO: 74, SEQ ID NO: 76, SEQ ID NO: 77, SEQ ID NO: 78, SEQ ID NO: 79, SEQ ID NO: 80, SEQ ID NO: 81, SEQ ID NO: 82, SEQ ID NO: 84,

SEQ ID NO: 85, SEQ ID NO: 86, SEQ ID NO: 87, SEQ ID NO: 88, SEQ ID NO: 89, SEQ ID NO: 90, and SEQ ID NO: 165;

(ii) a mutant *Pyrococcus furiosus* (*P.fur*) RNase H2 enzyme comprising an amino acid sequence selected from SEQ ID NO: 126, SEQ ID NO: 127, SEQ ID NO: 128, SEQ ID NO: 129, and SEQ ID NO: 130;

(iii) a mutant *Pyrococcus horikoshii* (*P.hori*) RNase H2 enzyme comprising an amino acid sequence selected from SEQ ID NO: 131, SEQ ID NO: 132, and SEQ ID NO: 133;

(iv) a mutant *Thermococcus kodakarensis* (*T.kod*) RNase H2 enzyme comprising an amino acid sequence selected from SEQ ID NO: 134, SEQ ID NO: 135, and SEQ ID NO: 136; and

(v) a mutant *Thermococcus litoralis* (*T.lit*) RNase H2 enzyme comprising the amino acid sequence of SEQ ID NO: 137 or SEQ ID NO: 138; and
c. instructions for performing the RNase H2-mediated cleavage reaction.

18. The kit of claim 17, wherein the RNase H2-mediated cleavage reaction is performed as part of an RNase H2-dependent PCR (rhPCR) reaction, a loop-mediated isothermal amplification (LAMP) reaction, or cycling probe technology (CPT).

19. The kit of claim 17 or claim 18, which comprises a mutant *Pyrococcus abyssi* (*P.a.*) RNase H2 enzyme comprising an amino acid sequence selected from SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 13, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 70, SEQ ID NO: 71, SEQ ID NO: 73, SEQ ID NO: 74, SEQ ID NO: 76, SEQ ID NO: 77, SEQ ID NO: 78, SEQ ID NO: 79, SEQ ID NO: 80, SEQ ID NO: 81, SEQ ID NO: 82, SEQ ID NO: 84, SEQ ID NO: 85, SEQ ID NO: 86, SEQ ID NO: 87, SEQ ID NO: 88, SEQ ID NO: 89, SEQ ID NO: 90, and SEQ ID NO: 165.

20. The kit of claim 17 or claim 18, which comprises a mutant *Pyrococcus furiosus* (*P.fur*) RNase H2 enzyme comprising an amino acid sequence selected from SEQ ID NO: 126, SEQ ID NO: 127, SEQ ID NO: 128, SEQ ID NO: 129, and SEQ ID NO: 130.

21. The kit of claim 17 or claim 18, which comprises a mutant *Pyrococcus horikoshii* (*P.hori*) RNase H2 enzyme comprising an amino acid sequence selected from SEQ ID NO: 131, SEQ ID NO: 132, and SEQ ID NO: 133.

22. The kit of claim 17 or claim 18, which comprises a mutant *Thermococcus kodakarensis* (*T.kod*) RNase H2 enzyme comprising an amino acid sequence selected from SEQ ID NO: 134, SEQ ID NO: 135, and SEQ ID NO: 136.

23. The kit of claim 17 or claim 18, which comprises a mutant *Thermococcus litoralis* (*T.lit*) RNase H2 enzyme comprising the amino acid sequence of SEQ ID NO: 137 or SEQ ID NO: 138.

24. The kit of any one of claims 17-23, wherein the one or more reagents comprise a blocking agent, a labeling agent, one or more primers, a buffer, a metaphase spread, or combinations thereof.

25. A mutant RNase H2 enzyme selected from:

(a) a mutant *Pyrococcus abyssi* (*P.a.*) RNase H2 enzyme comprising the amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, or SEQ ID NO: 22, SEQ ID NO: 70, SEQ ID NO: 71, SEQ ID NO: 73, SEQ ID NO: 74, SEQ ID NO: 76, SEQ ID NO: 77, SEQ ID NO: 78, SEQ ID NO: 79, SEQ ID NO: 80, SEQ ID NO: 81, SEQ ID NO: 82, SEQ ID NO: 84, SEQ ID NO: 85, SEQ ID NO: 86, SEQ ID NO: 87, SEQ ID NO: 88, SEQ ID NO: 89, SEQ ID NO: 90, or SEQ ID NO: 165;

(b) a mutant *Pyrococcus furiosus* (*P.fur*) RNase H2 enzyme comprising the amino acid sequence of SEQ ID NO: 126, SEQ ID NO: 127, SEQ ID NO: 128, SEQ ID NO: 129, or SEQ ID NO: 130;

(c) a mutant *Pyrococcus horikoshii* (*P.hori*) RNase H2 enzyme comprising the amino acid sequence of SEQ ID NO: 131, SEQ ID NO: 132, or SEQ ID NO: 133;

- (d) a mutant *Thermococcus kodakarensis* (*T.kod*) RNase H2 enzyme comprising the amino acid sequence of SEQ ID NO: 134, SEQ ID NO: 135, or SEQ ID NO: 136; and
- (e) a mutant *Thermococcus litoralis* (*T.lit*) RNase H2 enzyme comprising the amino acid sequence of SEQ ID NO: 137 or SEQ ID NO: 138.

26. A mutant RNase H2 enzyme comprising one of the following:

- (a) an amino acid sequence comprising SEQ ID NO: 95 except for one or more of the following amino acid substitutions: A9S, R11K, P13S, E48R, M80L, A107V, P171G, S172I, D173E, E199Y, E199G, and/or F220L;
- (b) an amino acid sequence comprising SEQ ID NO: 96 except for one or more of the following amino acid substitutions: A9S, R11K, P13S, Q48R, M80L, A107V, P171G, S172I, D173E, E199Y, E199G, and/or F218L;
- (c) an amino acid sequence comprising SEQ ID NO: 97 except for one or more of the following amino acid substitutions: A9S, R11K, P13S, A107V, P171G, S172I, D173E, K199Y, K199G, K199E, and/or Y224L; or
- (d) an amino acid sequence comprising SEQ ID NO: 98 except for one or more of the following amino acid substitutions: A9S, R11K, P13S, M80L, A107V, P171G, S172I, D173E, K199Y, K199G, K199E, and/or F220L.

27. A kit for cleaving one or more nucleic acid sequences of interest comprising:

- (a) one or more reagents for performing an RNase H2-mediated cleavage reaction in an emulsion on a sample comprising one or more nucleic acid sequences of interest,
- (b) one or more mutant RNase H2 enzymes of claim 26, and
- (c) instructions for performing the RNase H-mediated cleavage reaction.

28. The kit of claim 27, wherein the RNase H2-mediated cleavage reaction is performed as part of an RNase H-dependent PCR (rhPCR) reaction, a loop-mediated isothermal amplification (LAMP) reaction, or cycling probe technology (CPT).

29. A method of performing an RNase H2-mediated cleavage of one or more nucleic acid sequences of interest comprising:

- (a) providing a sample comprising one or more nucleic acid sequences of interest;
- (b) performing an RNase H2-mediated cleavage reaction on the one or more nucleic acid sequences, wherein the RNase H2-mediated cleavage reaction comprises a *Thermococcus kodakarensis* (*T.kod*) RNase H2 enzyme comprising the amino acid sequence of SEQ ID NO: 97, whereupon one or more nucleic acid sequences of interest are cleaved.

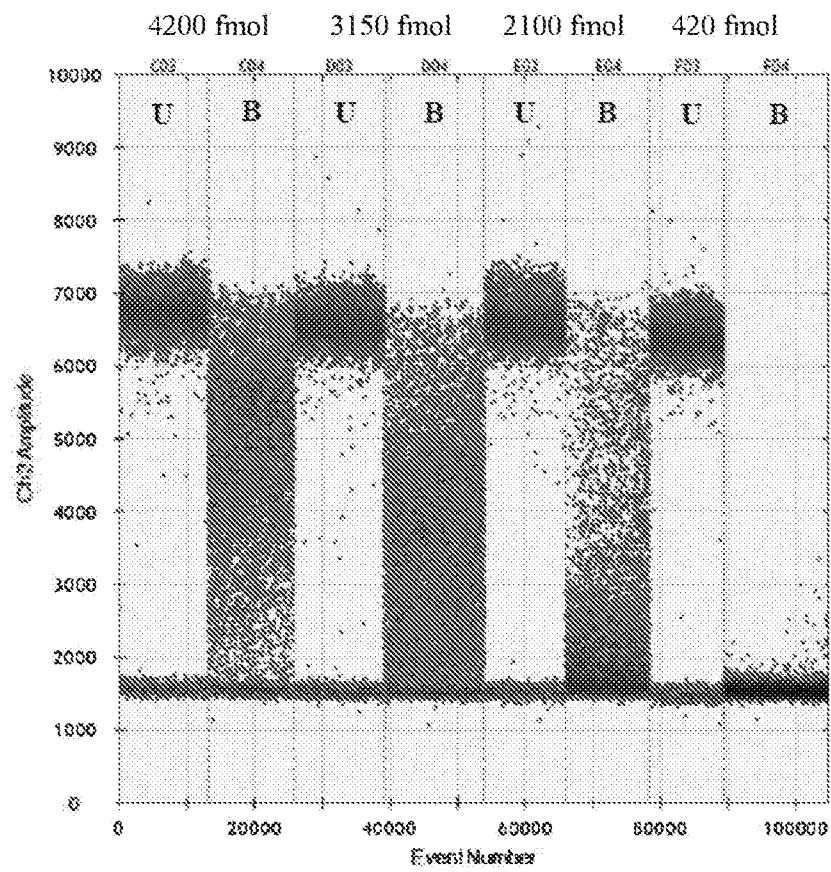
Figure 1

Figure 2

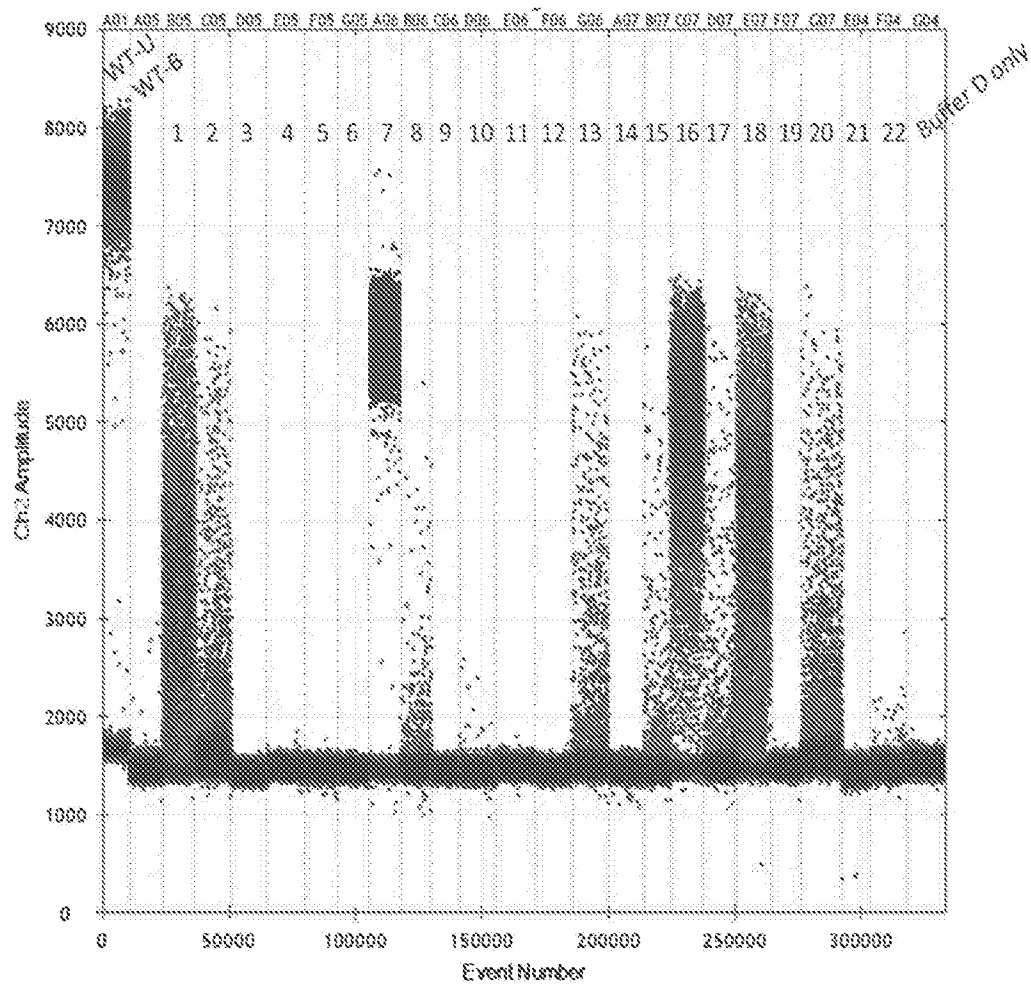


Figure 3

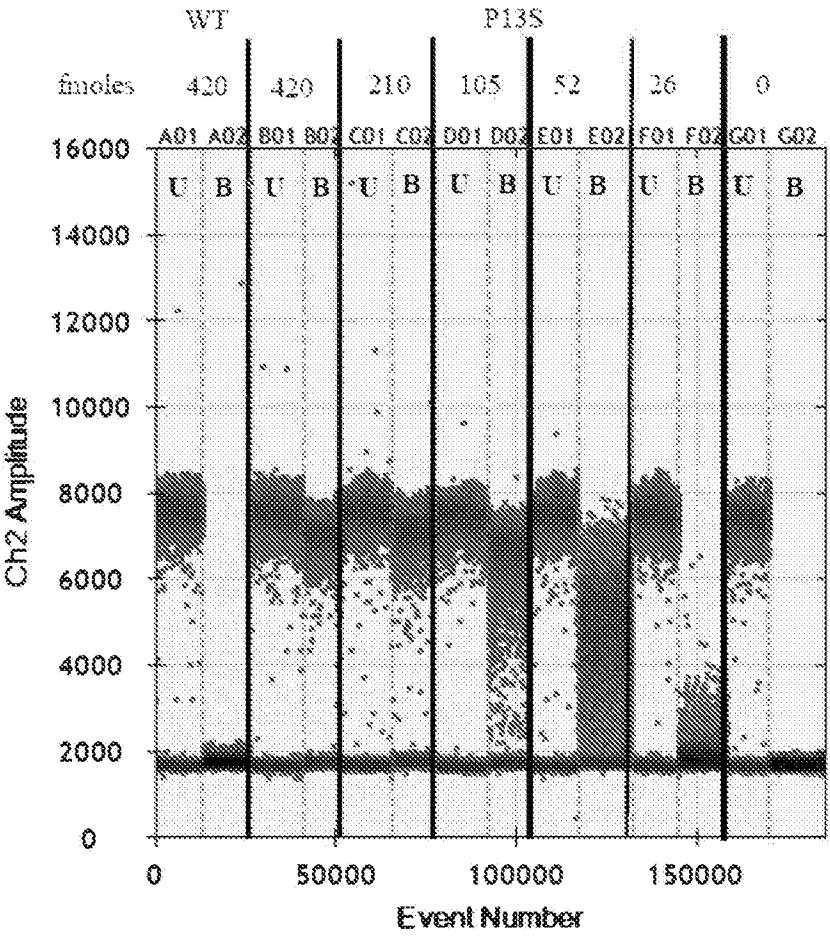


Figure 4

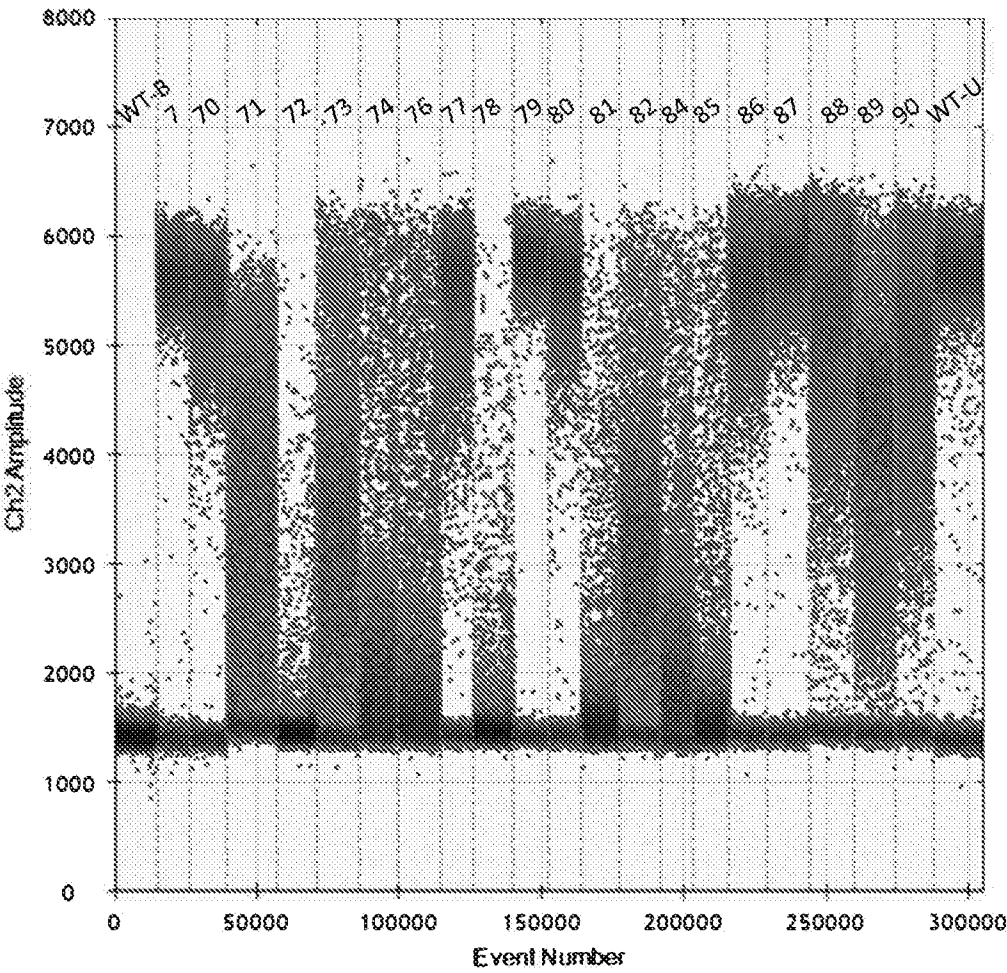


Figure 5

	S	R	
<i>P. fur</i>	MEIGGIDEAGRGPAIGFLVVATVVVDEERNIEKLRNIGVKDSKQLTPPREKKNLFSQITSIA	60	
<i>P. abyssi</i>	MKVAGADEAGRGPFVIGFLVIVAIVVEEDKIRSLTKLGVKDSKQLTEAQREKLFDEIVKVL	60	
<i>P. hori</i>	MEVAGVDEAGRGPFVIGFLVIGVAVIDEKNIERLRDVGKDSKQLTPGQREKLFSLIDIL	60	
<i>T. kod</i>	MKIAGIDEAGRGPFVIGPMVIAAVVVDENSLPFLPELKVVDSSKLTPEKPEKLFNEILGVL	60	
<i>T. lit</i>	MKLGIDEAGRGPFVIGFLVIAAVVVDENSLPFLPELKVVDSSKLTPEKPEKLFNEILGVL	60	
	L	V	
<i>P. fur</i>	DDYKIVIVSPEEIDNRSGMTMNELEVEKFPALALNSLQIKPALIYADAADVDANREASLIER	120	
<i>P. abyssi</i>	DDYSVVIVSPQDIDGRKXGSMNELEVENFVRALNSLKVKEPVIYIDSADVKAEREFAENIRS	120	
<i>P. hori</i>	DDYYVLLVTPKEIDEPHHEMNELEAEKFPVALNSLRKPKQKIYVDSADVDPKREASLIKA	120	
<i>T. kod</i>	DDYVILELFPDVGSRREGTLNFEFEVENFAKALNSLKVKEPVIYADAADVDEERFARELGE	120	
<i>T. lit</i>	DDHVITQLSPEEIDGRGDMNELEVENFAKALNSLKVKEPVIYADAADVDEERFARELGE	120	
<i>P. fur</i>	RLNYKAKIIAEHKADAKYPVVSAAASILAKVVRDEEIERLKKQYGDGFGSGYPSDPRTKKWL	180	
<i>P. abyssi</i>	RLAYEAEVVAEHKADAKYEIVSAAASILAKVIRDREIERLKAHYGDGFGSGYPSDPRTKKWL	180	
<i>P. hori</i>	GLKYEATVIAEHKADAKYEIVSAAASILAKVTRDREIERLQKQYGEFGSGYPSDPRTKKWL	180	
<i>T. kod</i>	RLNFEAEVVAEHKADAKYEIVSAAASILAKVTRDRAVEKLEEEYGEIGSGYPSDPRTKRAFL	180	
<i>T. lit</i>	RLSFSPKIIAEHKADSKYIPVAAASILAKVTRDRAIEKLELYGEIGSGYPSDPRTKRAFL	180	
<i>P. fur</i>	EEYVKHNSFPPIVRRRTWETVRKIEESIFAKKSQ-----LTLDKFFKRP--	224	SEQ ID NO: 95
<i>P. abyssi</i>	EEWYSKHGTFPIVRRRTWDTAKKIEEF--KRAQ-----LTLDNFKRRFRN	224	SEQ ID NO: 125
<i>P. hori</i>	EEYYEQYGFPPPIVRRRTWETVRKIEERF--RKNQ-----LTLDKFLK----	220	SEQ ID NO: 96
<i>T. kod</i>	ENYYRENGEFPPIVRKGTTLKKAIEKVESEKKAERQATLDRYFRKV--	228	SEQ ID NO: 97
<i>T. lit</i>	EEYYKARGEFPPPIVRKSWKTLKIEKRLKAKETQ-----ETILDFLKKE--	224	SEQ ID NO: 98

Figure 6

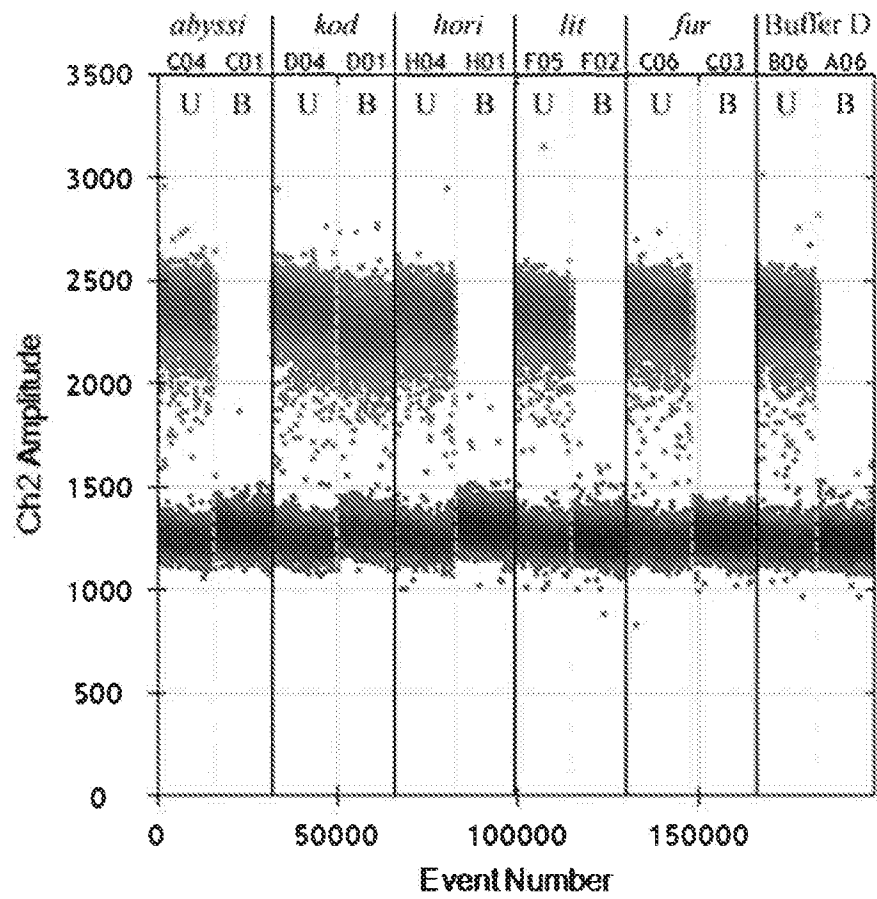


Figure 7

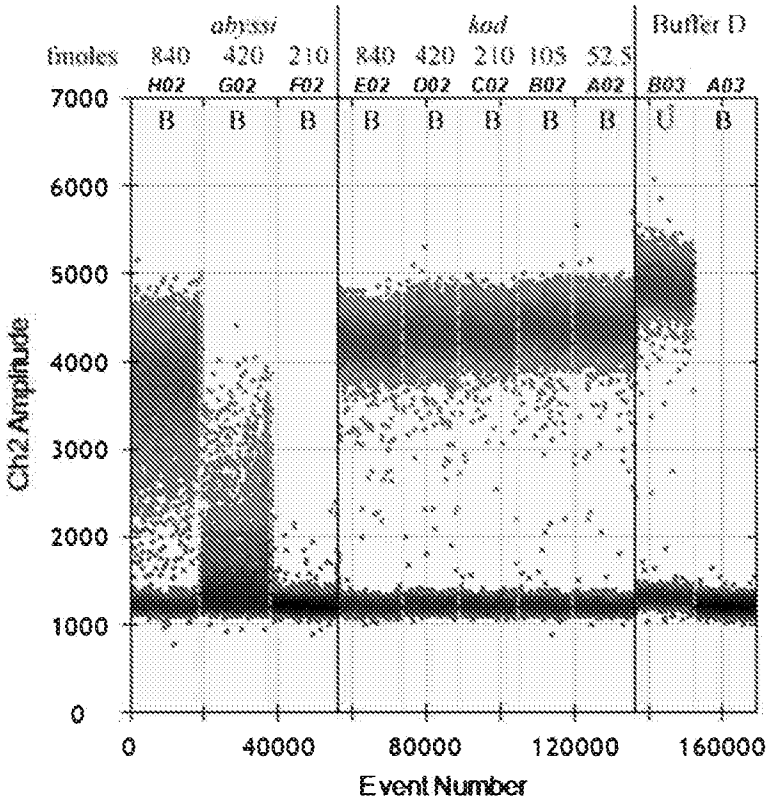


Figure 8

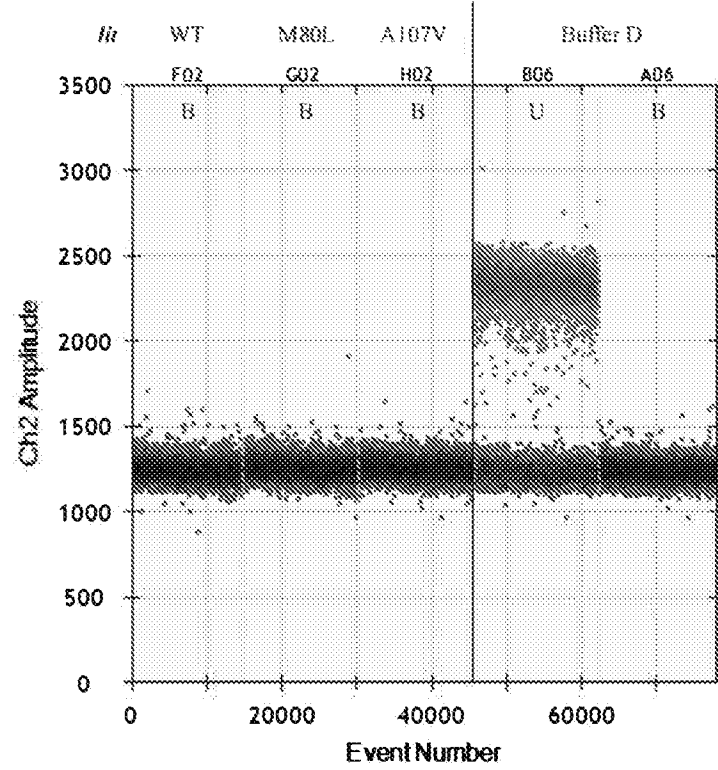


Figure 9

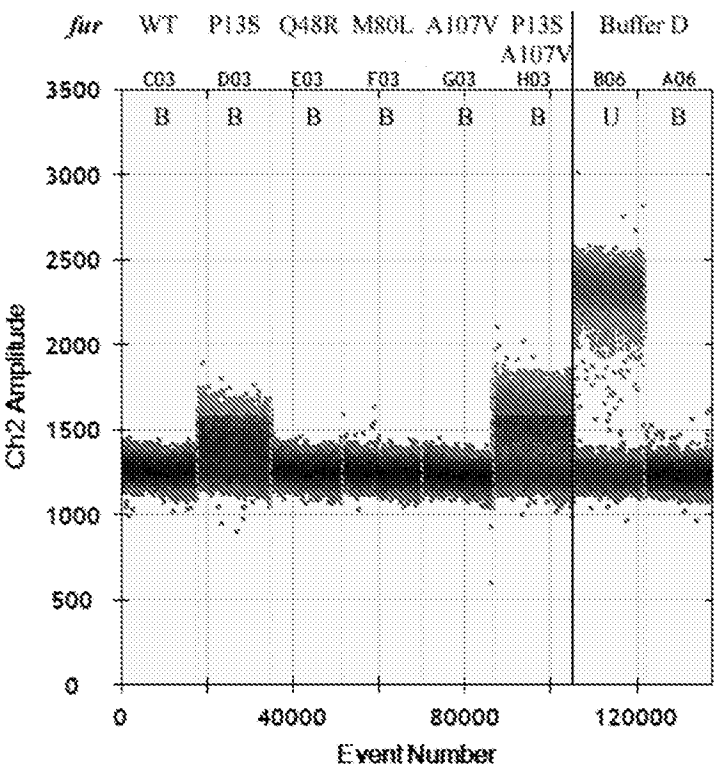


Figure 10

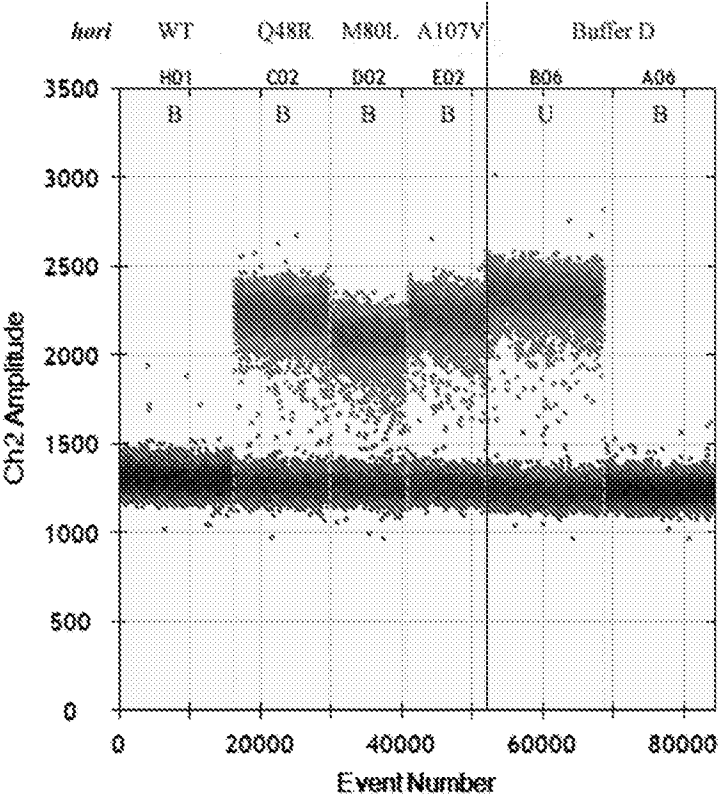


Figure 11

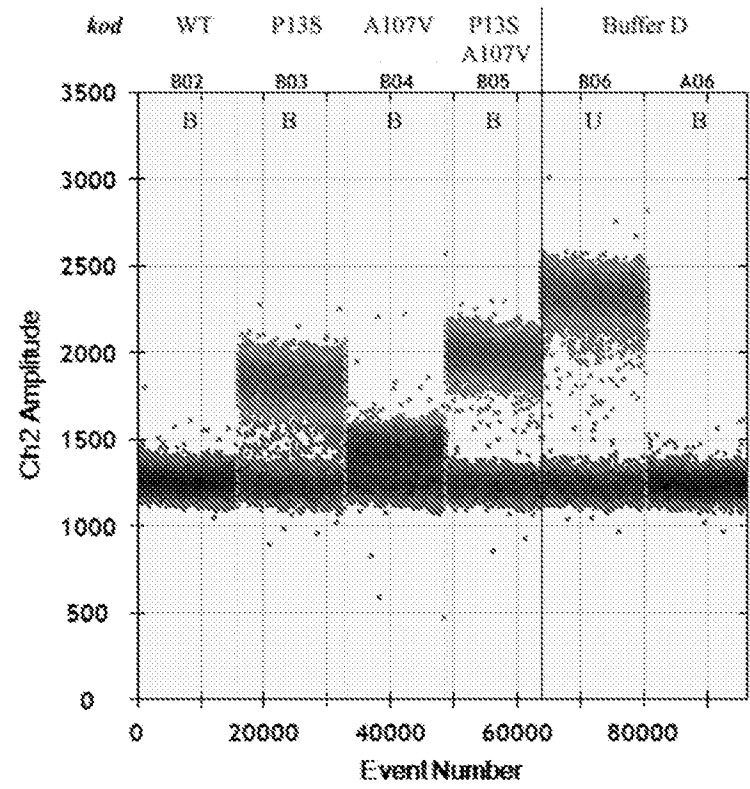


Figure 12

