COMPOSITIONS AND METHODS FOR ANALYSIS OF NUCLEIC ACID MOLECULES DURING AMPLIFICATION REACTIONS

The present application claims priority to U.S. Provisional Application Serial No. 61/036,953, filed March 15, 2008, which is incorporated herein by reference.

FIELD OF THE INVENTION

The present invention provides systems, methods and kits for performing amplification reactions in combination with a detection assay (*e.g.,* cleavage assay), where the detection assay employs probes with relatively short (*e.g.*, 6-12 bases) analyte-specific regions ("ASRs") to provide a minimum amount of recognizable duplex for a nucleic acid modifying enzyme (*e.g.*, a FEN-1 endonuclease). In some embodiments, such assays are used for target quantification, and in other embodiments, such assays are used for genotyping. In certain embodiments, the use of such short probes allows for assays with increased dynamic range.

BACKGROUND

The quantification of nucleic acids plays an important role in the fields of biology and medicine. For example, quantification of nucleic acid is important in cancer diagnosis and prognosis and viral diagnosis and judgments of therapeutic effects (*e.g.*, for HCV and HIV). HCV RNA quantification is important for patients taking IFN. The effect of IFN therapy can be directly found by monitoring the amount of virus during IFN therapy. This enables more effective IFN therapy that is tailored to clinical conditions of each patient. Quantification of target nucleic acid is important for diagnosis of diseases in the future. For example, earlier diagnosis can be effected by examining the expression level of mRNA that responds to exogenous stimuli in the case of a disease that results from exogenous stimuli.

The polymerase chain reaction can be employed for nucleic acid quantification. However, when PCR is employed, the absolute amount of the amplified nucleic acids does not accurately reflect the amount of the target nucleic acid that had existed when amplification was initiated. At first, the amount of the product amplified by PCR generally exponentially increases every cycle, however, the rate of increase slows down and then stops when the amount of the amplified product exceeds a certain level. Thus, the final amount of the amplified product is constant

regardless of the amount of the target nucleic acid when the reaction was initiated. This phenomenon is referred to as the plateau effect, which should be taken into consideration when quantifying the product amplified by PCR.

A technique known as "real time" PCR is widely employed for target sequence quantification (see, *e.g.*, Bustin, Journal of Molecular Endocrinology (2000) 25, 169–193; Bustin and Nolan, Journal of Biomolecular Techniques 15:155–166; ABI "Essentials of Real Time PCR" Part Number 4371089 Revision A; ABI Primer Express Softare 3.0 "Getting Started Guide" Part Number 4362460 Rev. B, January 2005). In this technique, a calibration curve for each amplification target is first prepared. A serial dilution of the target nucleic acid is prepared, each sample is subjected to PCR, and the accumulation of product is detected in real time, *i.e.*, during the course of the amplification, generally by the detection of a signal (*e.g.*, fluorescence) that accumulates in proportion to the amount of amplified material. The threshold cycle (the Ct value), the cycle at which the signal specific to the amplified product is first detected above background fluorescence or the "baseline" is determined. The Ct values for the different dilutions in the series is plotted on a vertical axis, and the initial copy number of the target nucleic acids in each dilution is plotted on a horizontal axis to produce the calibration curve for that target material. An unknown sample of interest (*e.g.*, a sample suspected of containing the same target material in an unknown amount) is subjected to PCR under the same conditions and the Ct value for the unknown is determined. The calibration curve for that target is then used to determine the initial copy number for the unknown sample.

There are two types of homogeneous fluorescent reporting methods generally used. "Nonspecific" detection indicates the accumulation of amplified product but is not specific for a correct or intended amplicon, while "specific" detection methods verify the accumulation of a particular intended product. Nonspecific detection can use intercalating dyes, such as ethidium bromide or SYBR Green, which bind to any double-stranded DNA generated during the PCR reaction and emit enhanced fluorescence. Such dyes are added to the PCR reagents prior to thermocycling. Although intrinsically nonspecific, DNA melt curves can used to check the identity of the amplification products The advantages of using dye intercalation methods include that 1) the dyes an be used to monitor the amplification of any double-stranded DNA sequence, and 2) no probe is required, which reduces assay setup and running costs.

The primary disadvantage of using intercalating dyes as the reaction reporting method is that they dyes may generate false positive signals; *i.e.*, because the dye binds to any double-stranded DNA without sequence selectivity, they give signal in the presence of nonspecific double-stranded DNA. Non-specific DNA may include contaminating DNA, "primer dimer" artifact DNA, or any DNA other than the intended target that was able to amplify using the primers provided in the reaction.

In addition to the non-specificity, intercalating dyes can make quantification of even correct target more complex because the amount of signal is dependent on the *mass* of double-stranded DNA produced in the reaction amplification, not the number of copies of the product. A longer amplified product has greater mass and can bind more dye molecules and thus can generate more signal than the same number of copies of a shorter product. In contrast, when a fluorogenic probe is used, a single fluorophore is released from quenching for each copy of the amplified molecule, regardless of the length of the amplicon.

Template or target-specific analysis generally requires the design and synthesis of one or more custom-made fluorescent probes for each PCR assay. Because the primer sequences are incorporated into correct and incorrect amplification product alike, probes are generally designed to hybridize to a region of the target sequence that is between the primers, such that successful probe hybridization confirms that the intended target sequence was amplified. Most reporting systems utilize fluorescent resonance energy transfer (FRET) or similar interactions between donor and quencher molecules as the basis of detection. Generally, a fluorescent signal is only generated if the amplicon-specific probe hybridizes to its complementary target. In common embodiments such as the TAQMAN and other 5' nuclease detection assays, the 5' nuclease cleaves the hybridized probe to separate the fluorophore from the quencher molecule. In some reaction designs, though, the probes are not cleaved, but change conformation in the presence of the correct target. Such a change in probe conformation separates the fluorophore from the quencher molecule, providing the increased fluorescence that is indicative of successful amplification of the specific target.

Specific chemistries are advantageous in that nonspecific amplification, *e.g.*, due to mispriming or primer–dimer artifacts, does not generate a signal and is ignored by the fluorescence detector. This obviates the need for post-PCR Southern blotting, sequence analysis, or melt curves to confirm the identity of the amplicon. Another

advantage over intercalating dyes is that the probes can be labeled with different, distinguishable reporter dyes that allow the detection of amplification products from several distinct sequences in a single PCR reaction (multiplex). One primary disadvantage of probe-based specific detection chemistries are that synthesis of different probes is required for each different sequence to be detected. (ABI "Essentials of Real Time PCR" Part Number 4371089 Revision A). The need to use different custom probes labeled with expensive dye, and quencher moieties, and optional with minor groove binders as well, adds a significant level of additional expense to probe based, amplicon-specific real time PCR analysis. Another disadvantage is the difficulty in distinguishing closely related sequences. Allele discrimination using PCR and hydrolysis probes generally involves the use of either a primer that has a mismatched 3' end when hybridized to the 'wrong' target (*e.g.* a primer intended to amplify one allele, when hybridized to an allele that it is not intended to amplify) such that amplification of the allele is impeded, or hydrolysis probes designed to anneal poorly to the unintended allele. Both approaches offer imperfect discrimination, as both primer extension and probe cleavage can occur even when such mismatches are present.

In light of the above, what is needed are relatively simple and inexpensive homogeneous methods for quantitating nucleic acids in a target-specific fashion, in real time.

SUMMARY OF THE INVENTION

In some embodiments, the present invention provides detection technologies for analyzing a target nucleic acid of interest. As described herein, the detection technologies include real time detection capability that, if desired, can be quantitative or semi-quantitative. Systems, compositions (*e.g.*, kits, reaction mixtures, and the like), and methods are provided. The systems, compositions, and methods may be used in combination with other known or future developed technologies. In some embodiments, the present invention provides detection systems comprising the use of footprint probes, wherein the footprint probe provides, *e.g.*, a minimum structure (*e.g.*, duplex) needed for detection using a detection enzyme.

In some embodiments, the invention provides a method of analyzing a target nucleic acid, comprising selecting a 5' nuclease, wherein the 5' nuclease recognizes a structure comprising a nucleic acid duplex, determining a footprint duplex length for

the 5' nuclease, amplifying a target nucleic acid in the presence of a synthetic footprint probe and the 5' nuclease under conditions such that the synthetic footprint probe is cleaved during a target amplification reaction to generate cleaved fragments, wherein the synthetic footprint probe comprises an analyte-specific portion and a non-target portion, wherein the non-target potion is substantially non-complementary to said target nucleic acid and wherein the analyte-specific portion forms a probe-target duplex with said target nucleic acid that is no longer than the footprint duplex length for the 5' nuclease enzyme. In preferred embodiments, the method further comprises detecting the cleaved fragments during the amplification reaction. In particularly preferred embodiments, the target amplification reaction is a polymerase chain reaction. In preferred embodiments, the 5' nuclease is selected from the group consisting of a natural FEN-1 endonuclease, a modified FEN-1 endonuclease, and a chimerical protein comprising at least a portion of at least one FEN-1 endonuclease.

In some embodiments, the present invention provides a method of analyzing a target nucleic acid, comprising: a) amplifying a target nucleic acid in the presence of a synthetic probe and a FEN-1 endonuclease under conditions such that the synthetic probe is cleaved during an amplification reaction to generate cleaved fragments; wherein the synthetic probe is a footprint probe for the FEN-1 endonuclease, the footprint probe comprising an analyte-specific portion and a non-target portion, wherein the non-target potion is substantially non-complementary to said target nucleic acid and wherein, if the amplification reaction is an isothermal reaction, the analyte-specific portion of the footprint probe has a calculated T_m with the target that is at least 5° C below, more preferably 8° degrees below, and still more preferably 10°C below the temperature at which said isothermal reaction is conducted, or in embodiments in which the amplification reaction is a thermal cycling reaction, the analyte-specific portion of the footprint probe has a calculated T_m with said target that is at least 5° C below, more preferably 8° degrees below, and still more preferably 10°C below the lowest temperature used in said thermal cycling, and wherein the footprint probe does not comprise a minor groove binder moiety. In preferred embodiments, the method further comprises detecting the cleaved fragments during said amplification reaction.

In particularly preferred embodiments, the invention provides methods comprising the use of a nucleic acid amplification assay and a detection assay, wherein at least one of the amplification assay or the detection assay comprises the

use of a footprint probe having an analyte-specific region of 12 or fewer nucleotides, and further comprises the use of a 5' nuclease (*e.g.*, a FEN-1 nuclease of an archaeon or eukaryote, or 5' nuclease of virus or of a eubacterial DNA polymerase). In some embodiments, the ASR of the footprint probe contains 12 nucleotides that are completely complementary to a target nucleic acid, while in some embodiments, the ASR of the probe contains 12 nucleotides, one or more of which is not complementary to a corresponding nucleotide in a target nucleic acid strand.

In some embodiments, the present invention provides methods of analyzing a target nucleic acid, comprising: a) amplifying a target nucleic acid in the presence of a probe and an endonuclease under conditions such that the probe is cleaved during an amplification reaction to generate cleaved fragments; wherein the probe comprises an analyte-specific portion, *i.e.*, a target binding region, and a non-target portion, wherein the non-target potion is substantially non-complementary to the target nucleic acid and wherein the target binding portion contains no more than 12 nucleotides that are complementary to the target nucleic acid; and b) detecting the cleaved fragments during said amplification reaction. In some embodiments, the probe is a synthetic probe. In some embodiments, the endonuclease is a FEN-1 endonuclease (*e.g.*, a thermostable FEN-1 endonuclease, including, but not limited to FEN-1 endonucleases from an archaeal species).

The methods are not limited by the nature of the analysis that is conducted. In some embodiments, the analyzing comprises detecting the presence of the target nucleic acid by detecting the cleaved fragments during the amplification reaction. In some embodiments, the analyzing comprises identifying the presence of a polymorphism (*e.g.*, a single-nucleotide polymorphism, a deletion, an insertion, a repeat sequence, and the like) in the target nucleic acid. In some embodiments, the analyzing comprises determining an identity of an organism from which the target nucleic acid is derived. For example, in some embodiments the organism is identified at the level of the kingdom, phylum, class, order, family, genus, species, sub-species, or individual levels. In some embodiments, the analyzing comprises detecting the amount of the target nucleic acid. In some embodiments, the detected amount is the amount of the target nucleic acid initially present in a sample.

The present invention is not limited by the source of or nature of the target nucleic acid. In some embodiments, the target nucleic acid is isolated from a sample. The present invention is not limited by the nature of the sample. Samples include, but

are not limited to, cell samples (*e.g.*, from an organism, cultured cells, stem cells, tumor cells, pathogens, single-celled organisms), tissue samples, fluid samples (*e.g.*, blood, serum, plasma, urine, saliva, and the like), a culture sample, and an environmental sample. Target nucleic acids may be derived from an organism, including, but not limited to, an animal (*e.g.*, a mammal such as a human), a plant, a bacteria, a virus, and a fungi. The target nucleic may comprise DNA or RNA or combinations thereof. The target nucleic acid may comprise natural nucleic acid molecules or synthetic nucleic acid molecules, or combinations thereof. In some embodiments, where the target nucleic is or comprises RNA, the RNA is converted to DNA, for example, by reverse transcription with a reverse transcriptase enzyme. The target nucleic acid may include covalent or non-covalent modifications to one or more nucleotides or other parts of its structure. For example, in some embodiments, the nucleic acid is methylated at one or more nucleotides and the analysis includes determining the existence of, degree of, or location or methylated nucleotides in the target nucleic acid.

In some embodiments, the amplification reaction employed by the methods is a polymerase chain reaction, although other temperature cycling or isothermal amplification technologies may be employed. Many of the amplification technologies employ a polymerase. In some embodiments, the polymerase is a thermostable polymerase. In some embodiments, the polymerase lacks 5' to 3' exonuclease activity. Many of the amplification technologies employ one or more primers. Accordingly, in some embodiments, the methods utilize first and second primer oligonucleotides. Additional primer oligonucleotides may also be used, as needed or desired.

In some embodiments, the probe cleavage is achieved by forming cleavage structure and cleaving the cleavage structure. In some embodiments, a cleavage structure is formed before the probe is cleaved, wherein the cleavage structure is formed by association of the target nucleic acid with: a) the probe at a first region of the target nucleic acid; and b) a second oligonucleotide that associates with a second region of the target nucleic, wherein the second region is 3' of the first region along the length of the target nucleic acid. In some embodiments, the first and second regions of the target nucleic acid are contiguous with one another. In some embodiments, at least one nucleotide at the 3' end of the second oligonucleotide, in the cleavage structure, overlaps with a region of hybridization between the probe and the target nucleic acid. In some embodiments, the 3' terminal nucleotide of the

second oligonucleotide is not complementary to the target nucleic acid, in the cleavage structure. In some embodiments, the second oligonucleotide is also a primer used in the amplifying step.

In some embodiments, the cleavage structure is a structure or employs reagents or approaches described in one or more of: U.S. Pat. Nos.: 7,312,033, 7,306,917, 7,297,780, 7,273,696, 7,256,020, 7,195,871, 7,150,982, 7,101,672, 7,087,381, 7,067,643, 7,060,436, 7,045,289, 7,011,944, 6,932,943, 6,913,881, 6,875,572, 6,872,816, 6,780,982, 6,780,585, 6,759,226, 6,709,819, 6,709,815, 6,706,471, 6,692,917, 6,673,616, 6,635,463, 6,562,611, 6,555,357, 6,458,535, 6,372,424, 6,358,691, 6,355,437, 6,348,314, 6,214,545, 6,194,149, 6,090,606, 6,090,543, 6,001,567, 5,994,069, 5,985,557, 5,888,780, 5,846,717, 5,843,669, 5,843,654, 5,837,450, 5,719,028, 5,614,402, and 5,541,311 and U.S. Publ. Nos.: 20080015349, 20080014124, 20070292856, 20070207455, 20070202517, 20070111200, 20070087345, 20070049745, 20060252032, 20060246475, 20060240452, 20060199202, 20060183207, 20060160074, 20060147955, 20060147938, 20050277138, 20050196750, 20050186588, 20050181435, 20050164177, 20050158716, 20050130179, 20050106596, 20050074788, 20050048527, 20040219576, 20040203035, 20040096874, 20040014067, 20030219784, 20030143535, 20030134349, 20030124526, 20030113237, 20030113236, 20030104470, 20030104378, 20030092039, 20030082544, 20030072689, 20020156255, 20020142454, and 20020128465, each of which is herein incorporated by reference in its entirety. These patents and published applications also describe enzymes, design, manufacture, and detection systems, and other components useful in the methods, compositions, and systems of the invention.

In some embodiments, the target binding portion of the probe contains no more than 11 (*e.g.*, 10, 9, 8, 7, 6, *etc.*) nucleotides that are complementary to said target nucleic acid. In some embodiments, the probe is labeled. In other embodiments, the probe is unlabeled. In some embodiments, the probe does not contain non-natural nucleotides. In some embodiments, the probe consists of naturally occurring nucleotides. In some embodiments, the probe has a moiety at its 3' end that prevents extension of the probe by a polymerase. The non-target portion of the probe may be any desired length. In some embodiments, it is a single nucleotide. In other embodiments, it is two or more (*e.g.*, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12,

13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, . . ., 40, . . . 50, . . ., *etc.*) nucleotides in length.

In some embodiments, the detecting step utilizes a detection oligonucleotide. For example, in some embodiments, the detecting of the cleaved fragments comprises associating one or more of said cleaved fragments with a synthetic detection oligonucleotide. In some embodiments, the synthetic detection oligonucleotide has a region of self-complementarity that forms a hairpin structure. In some embodiments, the synthetic detection oligonucleotide comprises a label (*e.g.*, a fluorescent label). In some embodiments, the synthetic detection oligonucleotide further comprises a fluorescent quencher moiety. In some embodiments, the cleaved flaps are detected by extension of the cleaved flaps, using the detection oligonucleotide as a template. In some embodiments, the cleaved flaps are detected by ligation of the cleaved flaps to another molecule, using the detection oligonucleotide as a template. In some embodiments, the cleaved fragments, when associated with the synthetic detection oligonucleotide, form a cleavage structure that is cleavable by the FEN-1 endonuclease. In some embodiments, the detecting comprises cleaving the cleavage structure (that comprises the synthetic detection oligonucleotide) to generate a detectable signal.

In some embodiments, an unknown target nucleic acid is analyzed in combination with a known synthetic control target nucleic acid, to, for example, determine an amount of the unknown target nucleic acid.

The present invention also provides compositions and systems containing one or more components useful, necessary, or sufficient for conducting any of the methods described herein. For example, in some embodiments, a composition comprises: a) a target nucleic acid; b) amplification primers; c) a polymerase; d) a FEN-1 endonuclease; and e) an unlabeled synthetic probe comprising an analyte-specific portion and a non-target portion, wherein the non-target potion is substantially non-complementary to the target nucleic acid and wherein the target binding portion contains no more than 12 nucleotides that are complementary to the target nucleic acid. In some embodiments, the composition is a reaction mixture. In some embodiments, the composition is a kit (*e.g.*, containing one or more containers, each housing one or more of the components). In some embodiments, a system of the invention comprises the composition and one or more additional components such as

sample purification or processing reagents or equipment, detection equipment, control software, and data analysis systems.

In some embodiments, the composition comprises: a) a target nucleic acid; b) amplification primers; c) a polymerase; d) a FEN-1 endonuclease; and e) an unlabeled synthetic probe comprising an analyte-specific portion and a non-target portion, wherein the non-target potion is substantially non-complementary to the target nucleic acid. The present invention also provides methods of analyzing a target nucleic acid comprising: providing such a composition, forming a cleavage structure between the probe and the target nucleic acid during an amplification reaction, cleaving the probe with the FEN-1 endonuclease to generate a cleavage product containing the non-target portion of the probe, and detecting the cleavage product.

DESCRIPTION OF THE DRAWINGS

Figure 1 provides a schematic diagram of an embodiment of the present invention comprising a combined PCR and invasive cleavage reaction using footprint probes for invasive cleavage.

Figure 2A provides a diagram showing use in an assay according to Figure 1 of footprint probes having ASRs of 10 and 11 nucleotides (calculated Tms of 34.2 and 36.2°C, respectively, calculated using the nearest-neighbor model and published parameters for DNA duplex formation, Allawi and SantaLucia, Biochemistry, 36:10581 (1997), and SantaLucia, Proc Natl Acad Sci U S A., 95(4):1460 (1998)) in assays according to Figure 1. For the assays shown in Figures 2A and 2B, target nucleic acid was amplified by PCR in a separate reaction. The polymerase enzyme was heat killed and an aliquot of the PCR was used in INVADER assay reactions lacking FRET cassettes and using the diagrammed invasive cleavage structures. The reactions were run using Pfu FEN-1 endonuclease at the indicated temperatures (in a gradient thermal cycler). Appropriate FRET cassettes were then added to the reactions and the amounts of probe cleaved in the initial INVADER assay reactions were determined by measuring cleavage of the FRET cassettes. These data show that probes comprising ASRs of 10 or 11 nucleotides have similar broad curves of performance over a range of reaction temperatures, and similar performance independence from reaction temperature.

Figure 2B shows the performance of a footprint probe having an ASR of 9 nucleotides (T_m of 45.6°C calculated according to the methods described in the

description of Figure 2A) in assays according to Figure 1. These data show that a probe comprising an ASR of 9 nucleotides also performs over a range of reaction temperatures, and that performance decreases modestly as the reaction temperature is increased. While not limiting the present invention to any particular mechanism of action, the observations that this probe has a higher T_m than the 10 and 11 ASR probes, yet shows a decrease in performance as temperature is increased suggests that the decrease in performance is linked to the enzyme's decreased ability to stabilize the probe as the ASR of the probe

Figure 3 shows a Table of footprint probes showing variations in calculated T_m of analyte-specific regions. None of these probes contained duplex stabilizing moieties such as MGBs. The indicated Tms were calculated according to the methods described in the description of Figure 2A. Each of the probes shown in Figure 3 performed in invasive cleavage reactions at 50°C (tests at 52°C showed the same results), demonstrating that footprint probes comprising ASRs of about 12 or few nucleotides can be designed and used without the need to match the Tms to the reaction temperature(s).

Figure 4 compares results using either the real time PCR+invasive cleavage using a footprint probe (Panel A) with a TAQMAN assay (Panel B) for detection of miR 155 microRNA. These data demonstrate that signal accumulation is faster in the PCR+invasive cleavage assays, and that background in a no-target control starts to appear in the TAQMAN assays but not in the reactions using the footprint probes. Reactions were performed as described in Example 3.

Figure 5 shows oligonucleotide sequences for amplifying and genotyping a SNP in a Factor V target sequence using PCR+invasive cleavage with footprint probes.

Figure 6 compares the results of PCR+invasive cleavage assays using the assay designs shown in Figure 8, with genotyping of the same alleles using the TAQMAN assay. Figures 6A-C show the PCR+invasive cleavage results (performed as described in Example 3), with Figure 6A showing a detection plot for the mutant Factor V target sequence; Figure 6B showing a detection plot for the wild-type Factor V target sequence; and Figure 6C showing a detection plot for heterogeneous Factor V target sequences. Figures 6D-F show the TAQMAN assay results, with Figure 6D showing an amplification plot for the mutant Factor V target sequence; Figure 6E

showing an amplification plot for the wild-type Factor V target sequence; and Figure 6F showing an amplification plot for heterogeneous Factor V target sequences.

Figure 7 compares scatter plots for SNP detection in the Factor V gene by PCR+invasive cleavage with footprint probes (as described in Example 3)(7B) and by the TAQMAN assay (7A).

Figure 8A-8D compares the results of detecting single base variations using the "INVADER Plus" assay combining PCR amplification with post-amplification detection using the INVADER assay (panel 8A) with detection of wild type or Oka mutation of VZV using real time PCR+ invasive cleavage using footprint probes (panels B and C, with D providing a no target control).

Figure 9A-B shows results achieved (using conditions as described in Example 3) in the detection of Human miR-21 microRNA with a footprint probe comprising 12 complementary nucleotides in the ASR compared with a footprint probe comprising 12 nucleotides, in which 1 nucleotide is mismatched to the target, and a footprint probe having only 11 nucleotides in the ASR. A probe having 11 complementary nucleotides plus 2 mismatched nucleotides (for a total of 13 nucleotides in the ASR) was also tested. Panel A shows a schematic diagram of the test molecules, and panel B shows data from samples having 600 or 6000 copies of miR-21 RNA.

Figure 10A-B shows results achieved in the detection of hsa-miR-17-3p microRNA with a footprint probe comprising 11 complementary nucleotides in the ASR compared with a footprint probe comprising 11 nucleotides, in which 1 nucleotide is mismatched to the target, and a footprint probe having only 10 nucleotides in the ASR, (using conditions as described in Example 3). Panel A shows a schematic diagram of the test molecules, and panel B shows data from samples having 6×10^6 copies of hsa-miR-17-3p microRNA. These examples show that, while having an ASR that is both near the optimal length for a FEN-1 enzyme (*e.g.*,11 or 12 nucleotides) provides the strongest detection signal, using a probe having an ASR of the proper length but comprising a mismatch provides stronger signal than is achieved using a probe having an ASR that is below the optimal length.

Figure 11A shows oligonucleotide sequences for amplifying and detecting the human miR-21 target; Figure 11B shows oligonucleotide sequences for amplifying and detecting the human miR-155 target; Figure 11C shows oligonucleotide sequences for amplifying and detecting the human miR-126 target; Figure 11D shows

oligonucleotide sequences for amplifying and detecting the human U6 snRNA target; and Figure 11E shows oligonucleotide sequences for amplifying and detecting the human U24 snRNA target.

Figure 12 shows the results of the combined PCR-invasive cleavage assay using the assay designs shown in Figure 11, which includes the use of footprint probes. The data for each target was plotted as fluorescence versus cycle number. For each target, a threshold was assigned that gave the most linear fit of copy number versus target level (see bottom panel in each figure). The results for each target are shown in the following figures: miR-155 (Figure 12A); miR-21 (Figure 112B); miR-126 (Figure 12C); U6 (Figure 12D); and U24 (Figure 12E).

Figure 13A shows the oligonucleotide sequences for amplifying and detecting the U6 DNA target; Figure 13B shows the oligonucleotide sequences for amplifying and detecting the factor V DNA target; Figure 13C shows the oligonucleotide sequences for amplifying and detecting the factor II DNA target; Figure 13D shows the oligonucleotide sequences for amplifying and detecting the GA-21-R DNA target; and Figure 13E shows the oligonucleotide sequences for amplifying and detecting the human U6 RNA target.

Figure 14 shows the results of detecting Factor II with the combination of PCR and an invasive cleavage assay and footprint probes from Example 2. Figures 14A-B show the results of Example 2 with both the invasive oligonucleotide and the Afu FEN-1 enzyme present, while Figures 14C-D shows the results with the invasive oligonucleotide and Afu FEN-1 enzyme not present.

Figure 15 shows the results of detecting Factor V with the combination of PCR and an invasive cleavage assay using a footprint probe from Example 2. Figures 15A-B show the results of Example 2 with all the reactions components present. Figures 15C-D show the results with the invasive oligonucleotide and Afu FEN-1 enzyme not present. Figures 15E-F show the same results as Figures 15C-D, except the y-axis maximum is 10,000 rather than 60,000.

Figure 16 shows the results of detecting GA-21-R with the combination of PCR and an invasive cleavage assay using a footprint probe from Example 2. Figures 16A-B show the results of this Example with both the invasive oligonucleotide and the Afu FEN-1 enzyme present, while Figures 16C-D show the results with the invasive oligonucleotide and Afu FEN-1 enzyme not present.

Figure 17 shows the results of detecting U6 RNA with the combination of PCR and an invasive cleavage assay using a footprint probes from Example 2. Figures 17A-B show the results of Example 2 with both the invasive oligonucleotide and the Afu FEN-1 enzyme present, while Figures 17-C-D show the results with the invasive oligonucleotide and Afu FEN-1 enzyme not present.

DEFINITIONS

To facilitate an understanding of the present invention, a number of terms and phrases are defined below:

The term "enzyme footprint probe" or "footprint probe" as used herein in reference to nucleic acid probes configured to form a structure, *e.g.*, a duplex, with, for example, a target nucleic acid, refers to probes selected to form a minimal structure necessary for a selected level of enzyme response to the structure. In some embodiments, a footprint probe is selected to provide the minimal structure necessary for optimal performance (*e.g.*, recognition, binding, cleavage, *etc.*), while in some embodiments, a footprint probe is selected to provide less than the minimal structure necessary for optimal performance, such that the performance is, for example, restricted, reduced, or otherwise made more stringent in its application.

By way of example and not limiting the invention to any particular nucleic acid modifying enzyme, a particular 5' nuclease, *e.g.*, a FEN-1 endonuclease, may have been determined to favor a duplex of at least about 12 base pairs between a probe and target strand for optimal recognition and cleavage of the probe annealed to the target nucleic acid. Generally, a longer probe-target duplex, while not necessarily reducing the activities of the enzyme, does not provide any further increase in activity (*e.g.*, in binding or cleavage activity of the FEN-1 endonuclease). A probe that provides additional duplex length without an increase in the activity of interest of the enzyme (*e.g.*, probe cleavage by the FEN-1 enzyme) would generally fall outside the definition of a footprint probe for this enzyme. For example, for a FEN-1 endonuclease having maximum activity when the probe-target duplex is at least 12 base pairs long, a probe providing more base pairs, *e.g.*, 15 or 16, or more base pairs of probe/target duplex, would not generally be considered a footprint probe for this particular enzyme. If a different FEN-1 endonuclease were to have maximum activity only when the probe-target duplex is at least 15 base pairs long (*e.g.*, a FEN from a different organism, or modified to have a larger footprint, or used in conditions in

which the footprint is determined to be enlarged from a smaller footprint), a footprint probe for the second FEN-1 would be a probe having an ASR of 15 or fewer nucleotides.

The term "footprint duplex length" as used herein refers to the minimum length of duplex at which a duplex-recognizing, nucleic acid modifying enzyme, *e.g.*, a FEN-1 endonuclease, demonstrates full activity in the conditions of a reaction. For example, for a FEN-1 endonuclease having maximum activity when the probe-target duplex is at least 12 base pairs long, as discussed above, the footprint duplex length of the FEN-1 enzyme is 12 base pairs. A footprint probe generally provides an ASR that has a length up to but not exceeding the footprint duplex length of the enzyme with which the probe is to be used.

As used herein, the term "dynamic range" refers to the quantitative range of usefulness in a detection assay (*e.g.*, a nucleic acid detection assay). For example, the dynamic range of a viral detection assay is the range between the smallest number of viral particles (*e.g.*, copy number) and the largest number of viral particles that the assay can distinguish between.

As used herein, the terms "subject" and "patient" refer to any organisms including plants, microorganisms and animals (*e.g.*, mammals such as dogs, cats, livestock, and humans).

The term "primer" refers to an oligonucleotide that is capable of acting as a point of initiation of synthesis when placed under conditions in which primer extension is initiated. An oligonucleotide "primer" may occur naturally, as in a purified restriction digest or may be produced synthetically. In some embodiments, an oligonucleotide primer is used with a template nucleic acid, extension of the primer is template dependent, such that a complement of the template is formed.

The term "cleavage structure" as used herein, refers to a structure that is formed by the interaction of at least one probe oligonucleotide and a target nucleic acid, forming a structure comprising a duplex, the resulting structure being cleavable by a cleavage means, including but not limited to an enzyme. The cleavage structure is a substrate for specific cleavage by the cleavage agent, in contrast to a nucleic acid molecule that is a substrate for non-specific cleavage by agents such as phosphodiesterases, which cleave nucleic acid molecules without regard to secondary structure (*i.e.*, no formation of a duplexed structure is required).

The term "invasive cleavage structure" as used herein refers to a cleavage structure comprising i) a target nucleic acid, ii) an upstream nucleic acid (*e.g.*, an INVADER oligonucleotide), and iii) a downstream nucleic acid (*e.g.*, a probe), where the upstream and downstream nucleic acids anneal to contiguous regions of the target nucleic acid, and where an overlap forms between the a 3' portion of the upstream nucleic acid and duplex formed between the downstream nucleic acid and the target nucleic acid. An overlap occurs where one or more bases from the upstream and downstream nucleic acids occupy the same position with respect to a target nucleic acid base, whether or not the overlapping base(s) of the upstream nucleic acid are complementary with the target nucleic acid, and whether or not those bases are natural bases or non-natural bases. In some embodiments, the 3' portion of the upstream nucleic acid that overlaps with the downstream duplex is a non-base chemical moiety such as an aromatic ring structure, *e.g.*, as disclosed, for example, in U.S. Patent No. 6,090,543, incorporated herein by reference in its entirety. In some embodiments, one or more of the nucleic acids may be attached to each other, *e.g.*, through a covalent linkage such as nucleic acid stem-loop, or through a non-nucleic acid chemical linkage (*e.g.*, a multi-carbon chain).

The term "cleavage means" or "cleavage agent" as used herein refers to any means that is capable of cleaving a cleavage structure, including but not limited to enzymes. "Structure-specific nucleases" or "structure-specific enzymes" are enzymes that recognize specific secondary structures in a nucleic molecule and cleave these structures. The cleavage agent of the invention cleaves a nucleic acid molecule in response to the presence of a cleavage structure; it is not necessary that the cleavage agent cleave the cleavage structure at any particular location within the cleavage structure.

The cleavage agent may include nuclease activity provided from a variety of sources including the CLEAVASE enzymes from Third Wave Technologies, Inc. (Madison, WI), the FEN-1 endonucleases (including RAD2 and XPG proteins, and natural or modified FEN-1 enzymes or chimerical enzymes comprising at least a portion of one or more FEN-1 enzymes), and enzymes comprising a 5' nuclease activity, such as eubacterial PolA polymerases including but not limited to *Taq* DNA polymerase, *Tth* DNA polymerase and *E. coli* DNA polymerase I. The cleavage agent may also include modified DNA polymerases having 5' nuclease activity but lacking synthetic activity. Examples of cleavage agents suitable for use in the method and

kits of the present invention are provided in U.S. Patent Nos. 5,614,402; 5,795,763; 5,843,669; 7,122,364, 7,150,982, and PCT Appln. Nos WO 98/23774; WO 02/070755A2; and WO0190337A2, each of which is herein incorporated by reference it its entirety.

The term "thermostable" when used in reference to an enzyme, such as a 5' nuclease, indicates that the enzyme is functional or active (*i.e.*, can perform catalysis) at an elevated temperature, *i.e.*, at about 55°C or higher. In some embodiments the enzyme is functional or active at an elevated temperature of 65°C or higher (*e.g.*, 75°C, 85°C, 95°C, *etc.*).

The term "cleavage products" as used herein, refers to products generated by the reaction of a cleavage agent with a cleavage structure (*i.e.*, the treatment of a cleavage structure with a cleavage agent).

As used herein, the term "specifically hybridizes" means that under given hybridization conditions a probe or primer detectably hybridizes to substantially only the target sequence in a sample comprising the target sequence (*i.e.*, there is little or no detectable hybridization to non-target sequences). In an amplification method that comprises cycles of denaturation and annealing of nucleic acid, *e.g.*, targets and primers or probes, given hybridization conditions include the conditions for the annealing step in the amplification method, *i.e.,* an annealing temperature selected on the basis of predicted T_m, and salt conditions suitable for the polymerase enzyme of choice.

The term "amplified" as used herein refers to an increase in the abundance of molecule, moiety or effect. A target nucleic acid may be amplified, *e.g.*, by *in vitro* replication such as by PCR. A signal, *e.g.*, a detectable event or product that indicates the presence of a target nucleic acid

As used herein, the term "amplification method" as used in reference to nucleic acid amplification means a process of specifically amplifying the abundance of a nucleic acid of interest. Some amplification methods (*e.g.*, polymerase chain reaction, or PCR) comprise iterative cycles of thermal denaturation, oligonucleotide primer annealing to template molecules, and nucleic acid polymerase extension of the annealed primers. Conditions and times necessary for each of these steps are well known in the art. Some amplification methods are conducted at a single temperature and are deemed "isothermal." Accumulation of the products of amplification may be exponential or linear. Some amplification methods ("target amplification" methods)

amplify the abundance of a target sequence, *e.g.*, by copying it many times (*e.g.*, PCR, NASBA, TMA, strand displacement amplification, ligase chain reaction, LAMP, ICAN, RPA, SPIA, HAD, *etc.*), while some amplification methods amplify the abundance of a nucleic acid species that may or may not contain the target sequence, but the amplification of which indicates the presence of a particular target sequence in the reaction (*e.g.*, rolling circle amplification, RAM amplification). The latter methods are sometimes referred to as "signal amplification" methods. Some signal amplification methods may increase the abundance of a species of nucleic acid by converting a starting nucleic acid, *e.g.*, by cleaving the starting nucleic acid to form cleavage products, or by extending it by, *e.g.*, polymerization or ligation. A target amplification method may be applied to a signal molecule (*e.g.*, PCR may be used to produce more copies of the product of a ligation, cleavage, or non-target copying reaction), or vice versa.

As used herein, the terms "polymerase chain reaction" and "PCR" refer to an enzymatic reaction in which a segment of DNA is replicated from a target nucleic acid *in vitro*. The reaction generally involves extension of a primer on each strand of a target nucleic acid with a template dependent DNA polymerase to produce a complementary copy of a portion of that strand. The chain reaction comprises iterative cycles of denaturation of the DNA strands, *e.g.*, by heating, followed by cooling to allow primer annealing and extension, resulting in an exponential accumulation of copies of the region of the target nucleic acid that is flanked by and that includes the primer binding sites. When an RNA target nucleic acid is amplified by PCR, it is generally first reverse transcribed to produce a DNA copy strand.

As used herein, the term "primer annealing" refers to conditions that permit oligonucleotide primers to hybridize to template nucleic acid strands. Conditions for primer annealing vary with the length and sequence of the primer and are generally based upon the T_m that is determined or calculated for the primer. For example, an annealing step in an amplification method that involves thermocycling involves reducing the temperature after a heat denaturation step to a temperature based on the T_m of the primer sequence, for a time sufficient to permit such annealing.

As used herein, the term "amplifiable nucleic acid" is used in reference to nucleic acids that may be amplified by any amplification method. It is contemplated that "amplifiable nucleic acid" will usually comprise "sample template."

The term "real time" as used herein in reference to detection of nucleic acid amplification or signal amplification refers to the detection or measurement of the accumulation of products or signal in the reaction while the reaction is in progress, *e.g.*, during incubation or thermal cycling. Such detection or measurement may occur continuously, or it may occur at a plurality of discrete points during the progress of the amplification reaction, or it may be a combination. For example, in a polymerase chain reaction, detection (*e.g.*, of fluorescence) may occur continuously during all or part of thermal cycling, or it may occur transiently, at one or more points during one or more cycles. In some embodiments, real time detection of PCR is accomplished by determining a level of fluorescence at the same point (*e.g.*, a time point in the cycle, or temperature step in the cycle) in each of a plurality of cycles, or in every cycle. Real time detection of amplification may also be referred to as detection "during" the amplification reaction.

As used herein, the terms "reverse transcription" and "reverse transcribe" refer to the use of a template-dependent polymerase to produce a DNA strand complementary to an RNA template.

As used herein, the term "abundance of nucleic acid" refers to the amount of a particular target nucleic acid sequence present in a sample or aliquot. The amount is generally referred to in terms of mass (*e.g.*, µgs), mass per unit of volume (*e.g.*, µgs per µl); copy number (*e.g.*, 1000 copies, 1 attomole), or copy number per unit of volume (*e.g.*, 1000 copies per ml, 1 attomole per µl). Abundance of a nucleic acid can also be expressed as an amount relative to the amount of a standard of known concentration or copy number. Measurement of abundance of a nucleic acid may be on any basis understood by those of skill in the art as being a suitable quantitative representation of nucleic acid abundance, including physical density or the sample, optical density, refractive property, staining properties, or on the basis of the intensity of a detectable label, *e.g.* a fluorescent label.

The term "amplicon" or "amplified product" refers to a segment of nucleic acid, generally DNA, generated by an amplification process such as the PCR process. The terms are also used in reference to RNA segments produced by amplification methods that employ RNA polymerases, such as NASBA, TMA, *etc.*

The term "amplification plot" as used in reference to a thermal cycling amplification reaction refers to the plot of signal that is indicative of amplification, *e.g.*, fluorescence signal, versus cycle number. When used in reference to a non-

thermal cycling amplification method, an amplification plot generally refers to a plot of the accumulation of signal as a function of time.

The term "baseline" as used in reference to an amplification plot refers to the detected signal coming from assembled amplification reactions at prior to incubation or, in the case of PCR, in the initial cycles, in which there is little change in signal.

The term "Ct" or "threshold cycle" as used herein in reference to real time detection during an amplification reaction that is thermal cycled refers to the fractional cycle number at which the detected signal (*e.g.*, fluorescence) passes the fixed threshold.

The term "no template control" and "no target control" (or "NTC") as used herein in reference to a control reaction refers to a reaction or sample that does not contain template or target nucleic acid. It is used to verify amplification quality.

The term "passive reference" as used in reference to a detection reaction refers to a reference material, such as a dye, that provides an internal reference to which a reporter signal (*e.g.*, another dye) can be normalized during data analysis. Normalization is generally necessary to correct for fluctuations caused by changes in concentration or volume.

"Rn" or "normalized reporter" refers to the fluorescence emission intensity of the reporter dye divided by the fluorescence emission intensity of the passive reference dye.

"Rn+" refers to the Rn value of a reaction containing all components, including the template or target.

Rn- refers to the Rn value of an un-reacted sample. The Rn-value can be obtained from the early cycles of a real time reaction, *e.g.*, a real time PCR run (those cycles prior to a detectable increase in fluorescence), or from a reaction that does not contain any template.

"ΔRn" or "delta Rn" refers to the magnitude of the signal generated by the given set of amplification conditions, *e.g.*, PCR conditions. The ΔRn value is determined by the following formula: (Rn+) – (Rn-) Standard A sample of known concentration used to construct a standard curve. By running standards of varying concentrations, one creates a standard curve from which one can extrapolate the quantity of an unknown sample.

The term "threshold" as used in reference to real time detection of an amplification reaction refers to the average standard deviation of Rn for the early PCR

cycles, multiplied by an adjustable factor. The threshold should be set in the region associated with an exponential growth of PCR product.

The term "unknown" as used in reference to a quantitative assay refers to a sample containing an unknown quantity of template, generally a sample whose quantity one wants to determine, *e.g.*, by performance of a quantitative assay such as a real time PCR and/or INVADER assay reaction.

As used herein, the term "sample template" refers to nucleic acid originating from a sample that is analyzed for the presence of "target." In contrast, "background template" is used in reference to nucleic acid other than sample template that may or may not be present in a sample. The presence of background template is most often inadvertent. It may be the result of carryover, or it may be due to the presence of nucleic acid contaminants sought to be purified away from the sample. For example, nucleic acids from organisms other than those to be detected may be present as background in a test sample.

The terms "analyte-specific region" or "ASR" and "analyte-specific portion" as used in reference to an oligonucleotide, such as a primer, a probe oligonucleotide, or an INVADER oligonucleotide, are used interchangeably and refer to a region/portion of an oligonucleotide selected to specifically hybridize to a particular nucleic acid sequence in a target nucleic acid or set of target nucleic acids. In some embodiments, an analyte-specific region of an oligonucleotide may be completely complementary to the segment of a target nucleic acid to which it hybridizes, while in other embodiments, an analyte-specific region may comprise one or more mismatches to the segment of a target nucleic acid to which it hybridizes. In yet other embodiments, an analyte-specific region may comprise one or more base analogs, *e.g.*, compounds that have altered hydrogen bonding, or that do not hydrogen bond, to the bases in the target strand. In some embodiments, the entire sequence of an oligonucleotide is an analyte-specific region, while in other embodiments an oligonucleotide comprises an analyte-specific region and one or more regions not complementary the target sequence (*e.g.*, non-complementary flap regions).

The term "substantially single-stranded" when used in reference to a nucleic acid substrate means that the substrate molecule exists primarily as a single strand of nucleic acid in contrast to a double-stranded substrate which exists as two strands of nucleic acid which are held together by inter-strand base pairing interactions.

The term "liberating" as used herein refers to the release of a nucleic acid fragment from a larger nucleic acid fragment, such as an oligonucleotide, by the action of, for example, a 5' nuclease such that the released fragment is no longer covalently attached to the remainder of the oligonucleotide.

The term "microorganism" as used herein means an organism too small to be observed with the unaided eye and includes, but is not limited to bacteria, virus, protozoans, fungi, and ciliates.

The term "microbial gene sequences" refers to gene sequences derived from a microorganism.

The term "bacteria" refers to any bacterial species.

The terms "archaea," "archaeal species," "archaean" and "archaebacteria" are used interchangeably refer to any organisms classified as a member of the Archaea domain or kingdom of life.

The term "virus" refers to obligate, ultramicroscopic, intracellular parasites incapable of autonomous replication (*i.e.*, replication requires the use of the host cell's machinery).

The term "multi-drug resistant" or multiple-drug resistant" refers to a microorganism that is resistant to more than one of the antibiotics or antimicrobial agents used in the treatment of said microorganism.

The term "source of target nucleic acid" refers to any sample that contains nucleic acids (RNA or DNA). Particularly preferred sources of target nucleic acids are biological samples including, but not limited to blood, saliva, cerebral spinal fluid, pleural fluid, milk, lymph, sputum and semen.

A sample "suspected of containing" a first and a second target nucleic acid may contain either, both or neither target nucleic acid molecule.

The term "reactant" is used herein in its broadest sense. The reactant can comprise, for example, an enzymatic reactant, a chemical reactant or light (*e.g.*, ultraviolet light, particularly short wavelength ultraviolet light is known to break oligonucleotide chains). Any agent capable of reacting with an oligonucleotide to either shorten (*i.e.*, cleave) or elongate the oligonucleotide is encompassed within the term "reactant."

As used herein the term "portion" when in reference to a protein (as in "a portion of a given protein") refers to a fragment of that protein or a sequence of amino acids that is less than the complete chain of amino acids of the polypeptide.

Similarly, when in reference to a nucleic acid (as in "a portion of a given nucleic acid or oligonucleotide"), the term refers to a fragment of a nucleic acid, or it refers to a sequence of nucleotides that is less than the complete chain of nucleotides of the nucleic acid or oligonucleotide. A portion may range in size from 1 amino acid or nucleotide residues, to the entire amino acid or nucleotide sequence.

The term "duplex" refers to the state of nucleic acids in which the base portions of the nucleotides on one strand are bound through hydrogen bonding the their complementary bases arrayed on a second strand. The condition of being in a duplex form reflects on the state of the bases of a nucleic acid. By virtue of base pairing, the strands of nucleic acid also generally assume the tertiary structure of a double helix, having a major and a minor groove. The assumption of the helical form is implicit in the act of becoming duplexed.

The term "template" refers to a strand of nucleic acid on which a complementary copy is built from nucleoside triphosphates through the activity of a template-dependent nucleic acid polymerase. Within a duplex the template strand is, by convention, depicted and described as the "bottom" strand. Similarly, the non-template strand is often depicted and described as the "top" strand.

As used herein, the term "sample" is used in its broadest sense. For example, in some embodiments, it is meant to include a specimen or culture (*e.g.*, microbiological culture), whereas in other embodiments, it is meant to include both biological and environmental samples (*e.g.*, suspected of comprising a target sequence, gene or template). In some embodiments, a sample may include a specimen of synthetic origin. Samples may be unpurifed or may be partially or completely purified or otherwise processed.

The present invention is not limited by the type of biological sample used or analyzed. The present invention is useful with a variety of biological samples including, but are not limited to, tissue (*e.g.*, organ (*e.g.*, heart, liver, brain, lung, stomach, intestine, spleen, kidney, pancreas, and reproductive (*e.g.*, ovaries) organs), glandular, skin, and muscle tissue), cell (*e.g.*, blood cell (*e.g.*, lymphocyte or erythrocyte), muscle cell, tumor cell, and skin cell), gas, bodily fluid (*e.g.*, blood or portion thereof, serum, plasma, urine, semen, saliva, etc), or solid (*e.g.*, stool) samples obtained from a human (*e.g.*, adult, infant, or embryo) or animal (*e.g.*, cattle, poultry, mouse, rat, dog, pig, cat, horse, and the like). In some embodiments, biological samples may be solid food and/or feed products and/or ingredients such as dairy

items, vegetables, meat and meat by-products, and waste. Biological samples may be obtained from all of the various families of domestic animals, as well as feral or wild animals, including, but not limited to, such animals as ungulates, bear, fish, lagomorphs, rodents, *etc.*

Biological samples also include biopsies and tissue sections (*e.g.*, biopsy or section of tumor, growth, rash, infection, or paraffin-embedded sections), medical or hospital samples (*e.g.*, including, but not limited to, blood samples, saliva, buccal swab, cerebrospinal fluid, pleural fluid, milk, colostrum, lymph, sputum, vomitus, bile, semen, oocytes, cervical cells, amniotic fluid, urine, stool, hair and sweat), laboratory samples (*e.g.*, subcellular fractions), and forensic samples (*e.g.*, blood or tissue (*e.g.*, spatter or residue), hair and skin cells containing nucleic acids), and archeological samples (*e.g.*, fossilized organisms, tissue, or cells).

Environmental samples include, but are not limited to, environmental material such as surface matter, soil, water (*e.g.*, freshwater or seawater), algae, lichens, geological samples, air containing materials containing nucleic acids, crystals, and industrial samples, as well as samples obtained from food and dairy processing instruments, apparatus, equipment, utensils, disposable and non-disposable items.

Other types of biological samples include bacteria (*e.g.*, *Actinobacteria* (*e.g.*, *Actinomyces*, *Arthrobacter*, *Corynebacterium* (*e.g.*, *C. diphtheriae*)), *Mycobacterium* (*e.g.*, *M. tuberculosis* and *M. leprae*), *Propionibacterium* (*e.g.*, *P. acnes*), *Streptomyces*, *hlamydiae* (*e.g.*, *C. trachomatis* and *C. pneumoniae*), *Cyanobacteria*, *Deinococcus* (*e.g.*, *Thermus* (*e.g.*, *T. aquaticus)*), *Firmicutes* (*e.g.*, *Bacilli* (*e.g.*, *B. anthracis*, *B. cereus*, *B. thuringiensis*, and *B. subtilis*)), *Listeria* (*e.g.*, *L. monocytogenes*), *Staphylococcus* (*e.g.*, *S. aureus*, *S. epidermidis*, and *S. haemolyticus*), *Fusobacteria*, *Proteobacteria* (*e.g.*, *Rickettsiales*, *Sphingomonadales*, *Bordtella* (*e.g.*, *B. pertussis*), *Neisserisales* (*e.g.*, *N. gonorrhoeae* and *N. meningitidis*), *Enterobacteriales* (*e.g.*, *Escherichia* (*e.g.*, *E. coli*), *Klebsiella*, *Plesiomonas*, *Proteus*, *Salmonella*, *Shigella*, and *Yersinia*), *Legionellales*, *Pasteurellales* (*e.g.*, *Haemophilus influenzae*), *Pseudomonas*, *Vibrio* (*e.g.*, *V. cholerae* and *V. vulnificus*), *Campylobacterales* (*e.g.*, *Campylobacteria* (*e.g.*, *C. jejuni*), and *Helicobacter* (*e.g.*, *H. pylori*)), and *Spirochaetes* (*e.g.*, *Leptospira, B. bergdorferi*, and *T. pallidum*)); Archaea (*e.g.*, *Halobacteria* and *Methanobacteria*); Eucarya (*e.g.*, *Animalia* (*e.g.*, *Annelidia*, *Arthropoda* (*e.g.*, *Chelicerata*, *Myriapoda*, *Insecta*, and *Crustacea*), *Mollusca*, *Nematoda*,(*e.g.*, *C. elegans*, and *T. spiralis*) and *Chordata* (*e.g.*,

Actinopterygii, *Amphibia*, *Aves*, *Chondrichthyes*, *Reptilia*, and *Mammalia* (*e.g.*, *Primates*, *Rodentia*, *Lagomorpha*, and *Carnivora*)))); Fungi (*e.g.*, *Dermatophytes*, *Fusarium*, *Penicillum*, and *Saccharomyces*); *Plantae* (*e.g.*, *Magnoliophyta* (*e.g.*, *Magnoliopsida* and *Liliopsida*)), and *Protista* (*e.g.*, *Apicomplexa* (*e.g.*, *Cryptosporidium*, *Plasmodium* (*e.g.*, *P. falciparum*, and *Toxoplasma*), and *Metamonada* (*e.g.*, *G. lambia*))); and Viruses (*e.g.*, dsDNA viruses (*e.g.*, *Bacteriophage*, *Adenoviridae*, *Herpesviridiae*, *Papillomaviridae*, *Polyomaviridae*, and *Poxviridae*), ssDNA virues (*e.g.*, *Parvoviridae*), dsRNA viruses (including *Reoviridae*), (+)ssRNA viruses (*e.g.*, *Coronaviridae*, *Astroviridae*, *Bromoviridae*, *Comoviridae*, *Flaviviridae*, *Picornaviridae*, and *Togaviridae*), (-) ssRNA viruses (*e.g.*, *Bornaviridae*, *Filoviridae*, *Paramyxoviridae*, *Rhabdoviridae*, *Bunyaviridae*, and *Orthomyxovirdiae*), ssRNA-reverse transcribing viruses (*e.g.*, *Retroviridae*), and dsDNA-reverse transcribing viruses (*e.g.*, *Hepadnaviridae* and *Caulomoviridae*)).

Sample may be prepared by any desired or suitable method. In some embodiments, nucleic acids are analyzed directly from bodily fluids or other samples using the methods described in U.S. Pat. Pub. Serial No. 20050186588, herein incorporated by reference in its entirety.

The above described examples are not, however, to be construed as limiting the sample (*e.g.*, suspected of comprising a target sequence, gene or template (*e.g.*, the presence or absence of which can be determined using the compositions and methods of the present invention)) types applicable to the present invention.

The term "nucleotide analog", "non-natural", or "non-naturally occurring" as used herein refers to nucleotides other than the natural nucleotides and bases. Such analogs and non-natural bases and nucleotides include modified natural nucleotides and non-naturally occurring nucleotides, including but not limited to analogs that have altered stacking interactions such as 7-deaza purines (*i.e.*, 7-deaza-dATP and 7-deaza-dGTP); base analogs with alternative hydrogen bonding configurations (*e.g.*, such as iso-C and iso-G and other non-standard base pairs described in U.S. Patent No. 6,001,983 to S. Benner, and the selectively binding base analogs described in U.S. Patent No. 5,912,340 to Igor V. Kutyavin, *et al.*); non-hydrogen bonding analogs (*e.g.*, non-polar, aromatic nucleoside analogs such as 2,4-difluorotoluene, described by B.A. Schweitzer and E.T. Kool, J. Org. Chem., 1994, 59, 7238-7242, B.A. Schweitzer and E.T. Kool, J. Am. Chem. Soc., 1995, 117, 1863-1872); "universal" bases such as 5-nitroindole and 3-nitropyrrole; and universal purines and pyrimidines

(such as "K" and "P" nucleotides, respectively; P. Kong, *et al.*, Nucleic Acids Res., 1989, 17, 10373-10383, P. Kong *et al.*, Nucleic Acids Res., 1992, 20, 5149-5152). Nucleotide analogs include modified forms of deoxyribonucleotides as well as ribonucleotides. "Non-natural" and "non-naturally occurring" bases and nucleotides are specifically not limited to such bases as are *never* found in nature. Natural processes such as nucleic acid damage can give rise to "natural" occurrence of bases that are nonetheless not generally considered to be part of the set of "natural" nucleotides as defined herein. For example, iso-G can be found in oxidatively damaged DNA. Such non-natural bases and their behaviors in replication and other nucleic acid syntheses have been extensively studied in contexts such as DNA damage studies, although the compounds are sometimes described using different nomenclature. For example, the ribonucleoside comprising the isoguanosine base has been referred to in the literature variously as: iG; isoG; iso-G; isoguanosine; 2-hydroxyadenine; 2-oxoadenine; 2-hydroxy A; and 2-OH-A. The deoxyribonucleoside comprising the isoguanosine base has been referred to variously as: iG; isoG; iso dG; deoxyiso-G; deoxyisoguanosine; 2-hydroxydeoxyadenosine; 2-hydroxy dA; and 2-OH-Ade.

Still other nucleotide analogs include modified forms of deoxyribonucleotides as well as ribonucleotides. Various oligonucleotides of the present invention (*e.g.*, a primary probe or INVADER oligo) may contain nucleotide analogs.

The terms "nucleic acid sequence" and "nucleic acid molecule" as used herein refer to an oligonucleotide, nucleotide or polynucleotide, and fragments or portions thereof. The terms encompasses sequences that include analogs of DNA and RNA nucleotides, including those listed above, and also including, but not limited to, 4-acetylcytosine, 8-hydroxy-N6-methyladenosine, aziridinylcytosine, pseudoisocytosine, 5-(carboxyhydroxyl-methyl) uracil, 5-fluorouracil, 5-bromouracil, 5-carboxymethylaminomethyl-2-thiouracil, 5-carboxymethyl-aminomethyluracil, dihydrouracil, inosine, N6-isopentenyladenine, 1-methyladenine, 1-methylpseudo-uracil, 1-methylguanine, 1-methylinosine, 2,2-dimethyl-guanine, 2-methyladenine, 2-methylguanine, 3-methyl-cytosine, 5-methylcytosine, N6-methyladenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxy-amino-methyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarbonylmethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, oxybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-

thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, N-uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, pseudouracil, queosine, 2-thiocytosine, 2,6-diaminopurine, and pyrazolo[3,4-d]pyrimidines such as guanine analogue 6 amino 1H-pyrazolo[3,4d]pyrimidin 4(5H) one (ppG or PPG, also Super G) and the adenine analogue 4 amino 1H-pyrazolo[3,4d]pyrimidine (ppA or PPA). The xanthine analogue 1H-pyrazolo[5,4d]pyrimidin 4(5H)-6(7H)-dione (ppX) can also be used. These base analogues, when present in an oligonucleotide, strengthen hybridization and improve mismatch discrimination. All tautomeric forms of naturally-occurring bases, modified bases and base analogues may be included in the oligonucleotide conjugates of the invention. Other modified bases useful in the present invention include 6-amino-3-prop-1-ynyl-5-hydropyrazolo[3,4-d]pyrimidine-4-one, PPPG; 6-amino-3-(3-hydroxyprop-1-yny)l-5-hydropyrazolo[3,4-d]pyrimidine-4-one, HOPPPG; 6-amino-3-(3-aminoprop-1-ynyl)-5-hydropyrazolo[3,4-d]pyrimidine-4- -one, NH2PPPG; 4-amino-3-(prop-1-ynyl)pyrazolo[3,4-d]pyrimidine, PPPA; 4-amino-3-(3-hydroxyprop-1-ynyl)pyrazolo[3,4-d]pyrimidine, HOPPPA; 4-amino-3-(3-aminoprop-1-ynyl)pyrazolo[3,4-d]pyrimidine, NH_2 PPPA; 3-prop-1-ynylpyrazolo[3,4-d]pyrimidine-4,6-diamino, $(NH_2)_2$ PPPA; 2-(4,6-diaminopyrazolo[3,4-d]pyrimidin-3-yl)ethyn-1-ol, $(NH_2)_2$ PPPAOH; 3-(2-aminoethynyl)pyrazolo[3,4-d]pyrimidine-4,6-diamine, $(NH_2)_2$ $PPPANH_2$; 5-prop-1-ynyl-1,3-dihydropyrimidine-2,4-dione, PU; 5-(3-hydroxyprop-1-ynyl)-1,3-dihydropyrimidine-2,4-dione, HOPU; 6-amino-5-prop-1-ynyl-3-dihydropyrimidine-2-one, PC; 6-amino-5-(3-hydroxyprop-1-yny)-1,3-dihydropyrimidine-2-one, HOPC; and 6-amino-5-(3-aminoprop-1-yny)-1,3-dihydropyrimidine-2-one, NH_2PC; 5-[4-amino-3-(3-methoxyprop-1-ynyl)pyrazol[3,4-d]pyrimidinyl]-2-(hydroxym-ethyl)oxolan-3-ol, CH_3 OPPPA; 6-amino-1-[4-hydroxy-5-(hydroxymethyl)oxolan-2-yl]-3-(3-methoxyprop-1-yny- l)-5-hydropyrazolo[3,4-d]pyrimidin-4-one, CH_3 OPPPG; 4,(4,6-Diamino-1H-pyrazolo[3,4-d]pyrimidin-3-yl)-but-3-yn-1-ol, Super A; 6-Amino-3-(4-hydroxy-but-1-ynyl)-1,5-dihydro-pyrazolo[3,4-d]pyrimidin-4-o- ne; 5-(4-hydroxy-but-1-ynyl)-1H-pyrimidine-2,4-dione, Super T; 3-iodo-1H-pyrazolo[3,4-d]pyrimidine-4,6-diamine ($(NH_2)_2$PPAI); 3-bromo-1H-pyrazolo[3,4-d]pyrimidine-4,6-diamine ($(NH_2)_2$ PPABr); 3-chloro-1H-pyrazolo[3,4-d]pyrimidine-4,6-diamine ($(NH_2)_2$PPACl); 3-Iodo-1H-pyrazolo[3,4-d]pyrimidin-4-ylamine (PPAI); 3-Bromo-1H-pyrazolo[3,4-d]pyrimidin-4-ylamine (PPABr); and 3-chloro-1H-pyrazolo[3,4-d]pyrimidin-4-ylamine (PPACl).

In addition to the modified bases noted above, oligonucleotides of the invention can have a backbone of sugar or glycosidic moieties, preferably 2-deoxyribofuranosides wherein all internucleotide linkages are the naturally occurring phosphodiester linkages. In alternative embodiments however, the 2-deoxy-β-D-ribofuranose groups are replaced with other sugars, for example, β-D-ribofuranose. In addition, β-D-ribofuranose may be present wherein the 2-OH of the ribose moiety is alkylated with a C_{1-6} alkyl group (2-(O--C_{1-6} alkyl) ribose) or with a C_{2-6} alkenyl group (2-(O—C_{2-6} alkenyl) ribose), or is replaced by a fluoro group (2-fluororibose). Related oligomer-forming sugars useful in the present invention are those that are "locked", *i.e.*, contain a methylene bridge between C-4' and an oxygen atom at C-2'. Other sugar moieties compatible with hybridization of the oligonucleotide can also be used, and are known to those of skill in the art, including, but not limited to, α-D-arabinofuranosides, α-2'-deoxyribofuranosides or 2',3'-dideoxy-3'-aminoribofuranosides. Oligonucleotides containing α-D-arabinofuranosides can be prepared as described in U.S. Pat. No. 5,177,196. Oligonucleotides containing 2', 3'-dideoxy-3'-aminoribofuranosides are described in Chen et al. Nucleic Acids Res. 23:2661-2668 (1995). Synthetic procedures for locked nucleic acids (Singh et al, Chem. Comm., 455-456 (1998); Wengel J., Acc. Chem. Res., 32:301-310 (1998)) and oligonucleotides containing 2'-halogen-2'-deoxyribofuranosides (Palissa et al., Z. Chem., 27:216 (1987)) have also been described. The phosphate backbone of the modified oligonucleotides described herein can also be modified so that the oligonucleotides contain phosphorothioate linkages and/or methylphosphonates and/or phosphoroamidates (Chen et al., Nucl. Acids Res., 23:2662-2668 (1995)). Combinations of oligonucleotide linkages are also within the scope of the present invention. Still other backbone modifications are known to those of skill in the art.

In some embodiments, the modified bases described herein are incorporated into PNA and DNA/PNA chimeras to balance T_ms and provide modified oligonucleotides having improved mismatch discrimination. Various modified forms of DNA and DNA analogues have been used in attempts to overcome some of the disadvantages of the use of DNA molecules as probes and primers. Among these are peptide nucleic acids (PNAs, also known as polyamide nucleic acids). Nielsen et al. Science 254:1497-1500 (1991). PNAs contain heterocyclic base units, as found in DNA and RNA, that are linked by a polyamide backbone, instead of the sugar-phosphate backbone characteristic of DNA and RNA. PNAs are capable of

hybridization to complementary DNA and RNA target sequences and, in fact, hybridize more strongly than a corresponding nucleic acid probe. The synthesis of PNA oligomers and reactive monomers used in the synthesis of PNA oligomers have been described in U.S. Pat. Nos. 5,539,082; 5,714,331; 5,773,571; 5,736,336 and 5,766,855. Alternate approaches to PNA and DNA/PNA chimera synthesis and monomers for PNA synthesis have been summarized. Uhlmann et al. Angew. Chem. Int. Ed. 37:2796-2823 (1998). Accordingly, the use of any combination of normal bases, unsubstituted pyrazolo[3,4-d]pyrimidine bases (*e.g.*, PPG and PPA), 3-substituted pyrazolo[3,4-d]pyrimidines, modified purine, modified pyrimidine, 5-substituted pyrimidines, universal bases, sugar modification, backbone modification or a minor groove binder to balance the T_m of a DNA, PNA or DNA/PNA chimera is in the scope of this invention. The synthetic methods necessary for the synthesis of modified base monomeric units required for nucleic acid, PNA and PNA/DNA chimeras synthesis are available in the art, see methods in this application and Uhlmann et al. Angew. Chem. Int. Ed. 37:2796-2823 (1998).

A nucleic acid sequence or molecule may be DNA or RNA, of either genomic or synthetic origin, that may be single or double stranded, and represent the sense or antisense strand. Thus, nucleic acid sequence may be dsDNA, ssDNA, mixed ssDNA, mixed dsDNA, dsDNA made into ssDNA (*e.g.*, through melting, denaturing, helicases, *etc.*), A-, B-, or Z- DNA, triple-stranded DNA, RNA, ssRNA, dsRNA, mixed ss and dsRNA, dsRNA made into ssRNA (*e.g.*, via melting, denaturing, helicases, *etc.*), messenger RNA (mRNA), ribosomal RNA (rRNA), transfer RNA (tRNA), catalytic RNA, snRNA, microRNA, or protein nucleic acid (PNA).

The present invention is not limited by the type or source of nucleic acid (*e.g.*, sequence or molecule (*e.g.* target sequence and/or oligonucleotide)) utilized. For example, the nucleic acid sequence may be amplified or created sequence (*e.g.*, amplification or creation of nucleic acid sequence via synthesis (*e.g.*, polymerization (*e.g.*, primer extension (*e.g.*, RNA-DNA hybrid primer technology)) and reverse transcription (*e.g.*, of RNA into DNA)) and/or amplification (*e.g.*, polymerase chain reaction (PCR), rolling circle amplification (RCA), nucleic acid sequence based amplification (NASBA), transcription mediated amplification (TMA), ligase chain reaction (LCR), cycling probe technology, Q-beta replicase, strand displacement amplification (SDA), branched-DNA signal amplification (bDNA), hybrid capture, and helicase dependent amplification).

The terms "nucleotide" and "base" are used interchangeably when used in reference to a nucleic acid sequence, unless indicated otherwise herein.

The term "oligonucleotide" as used herein is defined as a molecule comprising two or more nucleotides (*e.g.*, deoxyribonucleotides or ribonucleotides), preferably at least 5 nucleotides, more preferably at least about 10-15 nucleotides and more preferably at least about 15 to 30 nucleotides, or longer (*e.g.*, oligonucleotides are typically less than 200 residues long (*e.g.*, between 15 and 100 nucleotides), however, as used herein, the term is also intended to encompass longer polynucleotide chains). The exact size will depend on many factors, which in turn depend on the ultimate function or use of the oligonucleotide. Oligonucleotides are often referred to by their length. For example a 24 residue oligonucleotide is referred to as a "24-mer". Oligonucleotides can form secondary and tertiary structures by self-hybridizing or by hybridizing to other polynucleotides. Such structures can include, but are not limited to, duplexes, hairpins, cruciforms, bends, and triplexes. Oligonucleotides may be generated in any manner, including chemical synthesis, DNA replication, reverse transcription, PCR, or a combination thereof. In some embodiments, oligonucleotides that form invasive cleavage structures are generated in a reaction (*e.g.*, by extension of a primer in an enzymatic extension reaction).

Because mononucleotides are reacted to make oligonucleotides in a manner such that the 5' phosphate of one mononucleotide pentose ring is attached to the 3' oxygen of its neighbor in one direction via a phosphodiester linkage, an end of an oligonucleotide is referred to as the "5' end" if its 5' phosphate is not linked to the 3' oxygen of a mononucleotide pentose ring and as the "3' end" if its 3' oxygen is not linked to a 5' phosphate of a subsequent mononucleotide pentose ring. As used herein, a nucleic acid sequence, even if internal to a larger oligonucleotide, also may be said to have 5' and 3' ends. A first region along a nucleic acid strand is said to be upstream of another region if the 3' end of the first region is before the 5' end of the second region when moving along a strand of nucleic acid in a 5' to 3' direction.

When two different, non-overlapping oligonucleotides anneal to different regions of the same linear complementary nucleic acid sequence, and the 3' end of one oligonucleotide points towards the 5' end of the other, the former may be called the "upstream" oligonucleotide and the latter the "downstream" oligonucleotide. Similarly, when two overlapping oligonucleotides are hybridized to the same linear complementary nucleic acid sequence, with the first oligonucleotide positioned such

that its 5' end is upstream of the 5' end of the second oligonucleotide, and the 3' end of the first oligonucleotide is upstream of the 3' end of the second oligonucleotide, the first oligonucleotide may be called the "upstream" oligonucleotide and the second oligonucleotide may be called the "downstream" oligonucleotide.

As used herein, the terms "complementary" or "complementarity" are used in reference to polynucleotides (*e.g.*, a sequence of two or more nucleotides (*e.g.*, an oligonucleotide or a target nucleic acid)) related by the base-pairing rules. For example, the sequence "5'-A-G-T-3'," is complementary to the sequence "3'-T-C-A-5'." Complementarity may be "partial," in which only some of the nucleic acid bases are matched according to the base pairing rules. Or, there may be "complete" or "total" complementarity between the nucleic acid bases. The degree of complementarity between nucleic acid strands has significant effects on the efficiency and strength of hybridization between nucleic acid strands. This is of particular importance in amplification reactions, as well as detection methods that depend upon the association of two or more nucleic acid strands. Either term may also be used in reference to individual nucleotides, especially within the context of polynucleotides. For example, a particular nucleotide within an oligonucleotide may be noted for its complementarity, or lack thereof, to a nucleotide within another nucleic acid sequence (*e.g.*, a target sequence), in contrast or comparison to the complementarity between the rest of the oligonucleotide and the nucleic acid sequence.

The complement of a nucleic acid sequence as used herein refers to an oligonucleotide which, when aligned with the nucleic acid sequence such that the 5' end of one sequence is paired with the 3' end of the other, is in "antiparallel association." Nucleotide analogs, as discussed above, may be included in the nucleic acids of the present invention and include. Complementarity need not be perfect; stable duplexes may contain mismatched base pairs or unmatched bases. Those skilled in the art of nucleic acid technology can determine duplex stability empirically considering a number of variables including, for example, the length of the oligonucleotide, base composition and sequence of the oligonucleotide, ionic strength and incidence of mismatched base pairs.

The term "homology" refers to a degree of complementarity. There may be partial homology or complete homology (*i.e.*, identity). A partially homologous sequence is one that is less than 100% identical to another sequence. A partially complementary sequence that is "substantially homologous" is a nucleic acid

molecule that at least partially inhibits a completely complementary nucleic acid molecule from hybridizing to a target nucleic acid. The inhibition of hybridization of the completely complementary sequence to the target sequence may be examined using a hybridization assay (*e.g.*, Southern or Northern blot, solution hybridization and the like) under conditions of low stringency. A substantially homologous sequence or probe will compete for and inhibit the binding (*e.g.*, the hybridization) of a completely homologous nucleic acid molecule to a target under conditions of low stringency. This is not to say that conditions of low stringency are such that non-specific binding is permitted (*e.g.*, the low stringency conditions may be such that the binding of two sequences to one another be a specific (*e.g.*, selective) interaction). The absence of non-specific binding may be tested by the use of a second target that is substantially non-complementary (*e.g.*, less than about 30% identity); in the absence of non-specific binding the probe will not hybridize to the second non-complementary target.

When used in reference to a double-stranded nucleic acid sequence such as a cDNA or genomic clone, the term "substantially homologous" refers to any probe that can hybridize to either or both strands of the double-stranded nucleic acid sequence under conditions of low stringency as described above.

When used in reference to a single-stranded nucleic acid sequence, the term "substantially homologous" refers to any probe that can hybridize (*e.g.*, is complementary to) the single-stranded nucleic acid sequence under conditions of low stringency as described above.

The terms "target nucleic acid" and "target sequence," refers to a nucleic acid of to be detected or analyzed. Thus, the "target" is sought to be distinguished from other nucleic acids or nucleic acid sequences. For example, when used in reference to an amplification reaction, these terms may refer to the nucleic acid or portion of nucleic acid that will be amplified by the reaction, while when used in reference to a polymorphism, they may refer to the portion of an containing a suspected polymorphism. When used in reference to an invasive cleavage reaction, these terms refer to a nucleic acid molecule containing a sequence that has at least partial complementarity with at least a first nucleic acid molecule (*e.g.* probe oligonucleotide) and may also have at least partial complementarity with a second nucleic acid molecule (*e.g.* INVADER oligonucleotide). Generally, the target nucleic acid (*e.g.*, present within, isolated from, enriched from, or amplified from or within a

sample (*e.g.*, a biological or environmental sample)) is located within a target region and is identifiable via the successful formation of an invasive cleavage structure in combination with a first and second nucleic acid molecule (*e.g.*, probe oligonucleotide and INVADER oligonucleotide) that is cleavable by a cleavage agent. Target nucleic acids from an organism are not limited to genomic DNA and RNA. Target nucleic acids from an organism may comprise any nucleic acid species, including but not limited to genomic DNAs and RNAs, messenger RNAs, structural RNAs, ribosomal and tRNAs, and small RNAs such as snRNAs, siRNAs and microRNAs (miRNAs). See, *e.g.*, co-pending U.S. Patent Application Ser. No. 10/740,256, filed 12/18/03, which is incorporated herein by reference in its entirety. A "segment" is defined as a region of nucleic acid within the target sequence.

As used herein, the term "probe oligonucleotide," refers to an oligonucleotide that interacts with a target nucleic acid to form a detectable complex. In 5' nuclease cleavage assays such as the TAQMAN assay and the INVADER assay, the probe oligonucleotide hybridizes to the target nucleic acid and cleavage occurs within the probe oligonucleotide. In some embodiments, the complex between a probe and target is detected while it exists, while in some embodiments, the formation of the complex may be detected when it no longer exits, *e.g.*, by detection of an event (*e.g.*, a cleavage event) that occurred as a result of formation of the probe/target complex.

The term "INVADER oligonucleotide" refers to an oligonucleotide that hybridizes to a target nucleic acid at a location near the region of hybridization between a probe and the target nucleic acid, wherein the INVADER oligonucleotide comprises a portion (*e.g.*, a chemical moiety, or nucleotide, whether complementary to that target or not) that overlaps with the region of hybridization between the probe and target. In some embodiments, the INVADER oligonucleotide contains sequences at its 3' end that are substantially the same as sequences located at the 5' end of a probe oligonucleotide.

The term "cassette," as used herein refers to an oligonucleotide or combination of oligonucleotides configured to generate a detectable signal in response to cleavage of a probe oligonucleotide. In preferred embodiments, the cassette hybridizes to a cleavage product from cleavage of the probe oligonucleotide to form a second invasive cleavage structure, such that the cassette can then be cleaved.

In some embodiments, the cassette is a single oligonucleotide comprising a hairpin portion (*i.e.*, a region wherein one portion of the cassette oligonucleotide

hybridizes to a second portion of the same oligonucleotide under reaction conditions, to form a duplex). In other embodiments, a cassette comprises at least two oligonucleotides comprising complementary portions that can form a duplex under reaction conditions. In preferred embodiments, the cassette comprises a label. In particularly preferred embodiments, the cassette comprises labeled moieties that produce a fluorescence resonance energy transfer (FRET) effect.

An oligonucleotide is said to be present in "excess" relative to another oligonucleotide (or target nucleic acid sequence) if that oligonucleotide is present at a higher molar concentration than the other oligonucleotide (or target nucleic acid sequence). When an oligonucleotide such as a probe oligonucleotide is present in a cleavage reaction in excess relative to the concentration of the complementary target nucleic acid sequence, the reaction may be used to indicate the amount of the target nucleic acid present. Typically, when present in excess, the probe oligonucleotide will be present in at least a 100-fold molar excess; typically at least 1 pmole of each probe oligonucleotide would be used when the target nucleic acid sequence was present at about 10 fmoles or less.

As used herein, the term "gene" refers to a nucleic acid (*e.g.*, DNA) sequence that comprises coding sequences necessary for the production of a polypeptide, precursor, or RNA (*e.g.*, rRNA, tRNA). The polypeptide can be encoded by a full length coding sequence or by any portion of the coding sequence so long as the desired activity or functional properties (*e.g.*, enzymatic activity, ligand binding, signal transduction, immunogenicity, *etc.*) of the full-length or fragment polypeptide are retained. The term also encompasses the coding region of a structural gene and the sequences located adjacent to the coding region on both the 5' and 3' ends for a distance of about 1 kb or more on either end such that the gene corresponds to the length of the full-length mRNA. Sequences located 5' of the coding region and present on the mRNA are referred to as 5' non-translated sequences. Sequences located 3' or downstream of the coding region and present on the mRNA are referred to as 3' non-translated sequences. The term "gene" encompasses both cDNA and genomic forms of a gene. A genomic form or clone of a gene contains the coding region interrupted with non-coding sequences termed "introns" or "intervening regions" or "intervening sequences." Introns are segments of a gene that are transcribed into nuclear RNA (*e.g.*, hnRNA); introns may contain regulatory elements (*e.g.*, enhancers). Introns are removed or "spliced out" from the nuclear or primary

transcript; introns therefore are absent in the messenger RNA (mRNA) transcript. The mRNA functions during translation to specify the sequence or order of amino acids in a nascent polypeptide.

As used herein, the term "heterologous gene" refers to a gene that is not in its natural environment. For example, a heterologous gene includes a gene from one species introduced into another species (*e.g.*, a viral or bacterial gene present within a human host (*e.g.*, extrachromosomally or integrated into the host's DNA)). A heterologous gene also includes a gene native to an organism that has been altered in some way (*e.g.*, mutated, added in multiple copies, linked to non-native regulatory sequences, etc). In some embodiments, a heterologous gene can be distinguished from endogenous genes in that the heterologous gene sequences are typically joined to DNA sequences that are not found naturally associated with the gene sequences in the chromosome or are associated with portions of the chromosome not found in nature (*e.g.*, genes expressed in loci where the gene is not normally expressed).

As used herein, the term "gene expression" refers to the process of converting genetic information encoded in a gene into RNA (*e.g.*, mRNA, rRNA, tRNA, or snRNA) through "transcription" of the gene (*e.g.*, via the enzymatic action of an RNA polymerase), and for protein encoding genes, into protein through "translation" of mRNA. Gene expression can be regulated at many stages in the process. "Up-regulation" or "activation" refers to regulation that increases the production of gene expression products (*e.g.*, RNA or protein), while "down-regulation" or "repression" refers to regulation that decrease production. Molecules (*e.g.*, transcription factors) that are involved in up-regulation or down-regulation are often called "activators" and "repressors," respectively.

In addition to containing introns, genomic forms of a gene may also include sequences located on both the 5' and 3' end of the sequences that are present on the RNA transcript. These sequences are referred to as "flanking" sequences or regions (*e.g.*, these flanking sequences can be located 5' or 3' to the non-translated sequences present on the mRNA transcript). The 5' flanking region may contain regulatory sequences such as promoters and enhancers that control or influence the transcription of the gene. The 3' flanking region may contain sequences that direct the termination of transcription, post-transcriptional cleavage and polyadenylation.

The term "wild-type" refers to a gene or gene product isolated from a naturally occurring source. A wild-type gene is that which is most frequently observed in a

population and is thus arbitrarily designed the "normal" or "wild-type" form of the gene. In contrast, the term "modified" or "mutant" refers to a gene or gene product that displays modifications in sequence and or functional properties (*e.g.*, altered characteristics) when compared to the wild-type gene or gene product. It is noted that naturally occurring mutants can be isolated (*e.g.*, identified by the fact that they have altered characteristics (*e.g.*, altered nucleic acid sequences) when compared to the wild-type gene or gene product).

The term "isolated" when used in relation to a nucleic acid (*e.g.*, "an isolated oligonucleotide" or "isolated polynucleotide" or "an isolated nucleic acid sequence") refers to a nucleic acid sequence that is separated from at least one component or contaminant with which it is ordinarily associated in its natural source. Thus, an isolated nucleic acid is present in a form or setting that is different from that in which it is found in nature. In contrast, non-isolated nucleic acids are nucleic acids such as DNA and RNA found in the state they exist in nature. For example, a given DNA sequence (*e.g.*, a gene) is found on the host cell chromosome in proximity to neighboring genes; RNA sequences, such as a specific mRNA sequence encoding a specific protein, are found in the cell as a mixture with numerous other mRNAs that encode a multitude of proteins. However, isolated nucleic acid encoding a given protein includes, by way of example, such nucleic acid in cells ordinarily expressing the given protein where the nucleic acid is in a chromosomal location different from that of natural cells, or is otherwise flanked by a different nucleic acid sequence than that found in nature. The isolated nucleic acid, oligonucleotide, or polynucleotide may be present in single-stranded or double-stranded form. When an isolated nucleic acid, oligonucleotide or polynucleotide is to be utilized to express a protein, the oligonucleotide or polynucleotide will contain at a minimum the sense or coding strand (*e.g.*, the oligonucleotide or polynucleotide may be single-stranded), but may contain both the sense and anti-sense strands (*e.g.*, the oligonucleotide or polynucleotide may be double-stranded).

As used herein, the terms "purified" or "to purify" when used in reference to a sample (*e.g.*, a molecule (*e.g.*, a nucleic acid or amino acid sequence)) refers to removal (*e.g.*, isolation and/or separation) of the sample from its natural environment. The term "substantially purified" refers to a sample (*e.g.*, molecule (*e.g.* a nucleic acid or amino acid sequence) that has been removed (*e.g.*, isolated and/or purified) from its natural environment and is at least 60% free, preferably 75% free, or most preferably

90% or more free from other components with which it is naturally associated. An "isolated polynucleotide" or "isolated oligonucleotide" may therefore be substantially purified if it is rendered free (*e.g.*, 60%, 75% or more preferably 90% or more) from other components with which it is naturally associated.

The present invention is not limited to any particular means of purification (*e.g.*, to generate purified or substantially purified molecules (*e.g.*, nucleic acid sequences)). Indeed, a variety of purification techniques may be utilized including, but not limited to, centrifugation (*e.g.*, isopycnic, rate-zonal, gradient, and differential centrifugation), electrophoresis (*e.g.*, gel and capillary electrophoresis), gel filtration, matrix capture, charge capture, mass capture, antibody capture, magnetic separation, flow cytometry, and sequence-specific hybridization array capture.

As used herein, the term "hybridization" is used in reference to the pairing of complementary nucleic acids. Hybridization and the strength of hybridization (*e.g.*, the strength of the association between the nucleic acids) is impacted by such factors as the degree of complementary between the nucleic acids, stringency of the conditions involved, the T_m of the formed hybrid, and the G:C ratio within the nucleic acids. A single molecule that contains pairing of complementary nucleic acids within its structure is said to be "self-hybridized."

As used herein, the term " T_m" is used in reference to the "melting temperature." The melting temperature is the temperature at which a population of double-stranded nucleic acid molecules becomes half dissociated into single strands. A "calculated T_m" refers to a melting temperature determined by calculation from the physical sequence of complementary nucleic acids, along with factors of reaction conditions (*e.g.*, salt concentration, concentrations of the complementary strands in a mixture). Several equations for calculating the T_m of nucleic acids are well known in the art. As indicated by standard references, a simple estimate of the T_m value may be calculated by the equation: $T_m = 81.5 + 0.41(\% G + C)$, when a nucleic acid is in aqueous solution at 1 M NaCl (See, *e.g.*, Young and Anderson, (1985) in *Nucleic Acid Hybridisation*: *A Practical Approach* (Hames & Higgins, Eds.) pp 47-71, IRL Press, Oxford). Other computations for calculating T_m are known in the art and take structural and environmental, as well as sequence characteristics into account (See, *e.g.*, Allawi, H.T. and SantaLucia, J., Jr. Biochemistry 36, 10581-94 (1997)) and SantaLucia, Proc Natl Acad Sci U S A., 95(4):1460 (1998)).

As used herein, the term "INVADER assay reagents" refers to one or more reagents for detecting target sequences, said reagents comprising nucleic acid molecules capable of participating in formation of an invasive cleavage structure in the presence of the target sequence. In some embodiments, INVADER assay reagents comprise all of the nucleic acid molecules needed to form an invasive cleavage structure in preformed configuration, while in some embodiments, INVADER assay reagents provide or, are used in conjunction with one or more additional reagents (*e.g.*, primers, polymerizing enzymes, ligases, nucleases) that allow the formation of nucleic acid molecules used in forming an invasive cleavage structure.

In some embodiments, the INVADER assay reagents further comprise an agent for detecting the presence of an invasive cleavage structure (*e.g.*, a cleavage agent). In some embodiments, the nucleic acid molecules comprise first and second oligonucleotides, said first oligonucleotide comprising a 5' portion complementary to a first region of the target nucleic acid and said second oligonucleotide comprising a 3' portion and a 5' portion, said 5' portion complementary to a second region of the target nucleic acid downstream of and contiguous to the first portion. In some embodiments, the 3' portion of the second oligonucleotide comprises a 3' terminal nucleotide not complementary to the target nucleic acid. In preferred embodiments, the 3' portion of the second oligonucleotide consists of a single nucleotide not complementary to the target nucleic acid. INVADER assay reagents may be found, for example, in U.S. Patent Nos. 5,846,717; 5,985,557; 5,994,069; 6,001,567; 6,913,881; and 6,090,543, WO 97/27214, WO 98/42873, U.S. Pat. Publ. Nos. 20050014163, 20050074788, 2005016596, 20050186588, 20040203035, 20040018489, and 20050164177; U.S. Pat. Appln. Ser. No. 11/266,723; and Lyamichev et al., Nat. Biotech., 17:292 (1999), Hall et al., PNAS, USA, 97:8272 (2000), each of which is herein incorporated by reference in its entirety for all purposes.

In some embodiments, INVADER assay reagents are configured to detect a target nucleic acid sequence comprising first and second non-contiguous single-stranded regions separated by an intervening region comprising a double-stranded region. In certain embodiments, the INVADER assay reagents comprise a bridging oligonucleotide capable of binding to said first and second non-contiguous single-stranded regions of a target nucleic acid sequence. In particularly preferred

embodiments, either or both of said first and/or said second oligonucleotides of said INVADER assay reagents are bridging oligonucleotides.

In some embodiments, the INVADER assay reagents further comprise a solid support. For example, in some embodiments, the one or more oligonucleotides of the assay reagents (*e.g.*, first and/or second oligonucleotide, whether bridging or non-bridging) is attached to said solid support. The one or more oligonucleotides of the assay reagents may be linked to the solid support directly or indirectly (*e.g.*, via a spacer molecule (*e.g.*, an oligonucleotide)). Exemplary solid phase invasive cleavage reactions are described in U.S. Pat. Pub. Nos. 20050164177 and 20030143585, herein incorporated by reference in their entireties.

As used herein, a "solid support" is any material that maintains its shape under assay conditions, and that can be separated from a liquid phase. Supports that maintain their shape need not be rigid. Indeed, it is contemplated that flexible polymers such as carbohydrate chains, may be used as solid supports, so long as they can be separated from a liquid phase. The present invention is not limited by the type of solid support utilized. Indeed, a variety of solid supports are contemplated to be useful in the present invention including, but not limited to, a bead, planar surface, controlled pore glass (CPG), a wafer, glass, silicon, diamond, graphite, plastic, paramagnetic bead, magnetic bead, latex bead, superparamagnetic bead, plurality of beads, microfluidic chip, a silicon chip, a microscope slide, a microplate well, a silica gel, a polymeric membrane, a particle, a derivatized plastic film, a glass bead, cotton, a plastic bead, an alumina gel, a polysaccharide, polyvinylchloride, polypropylene, polyethylene, nylon, Sepharose, poly(acrylate), polystyrene, poly(acrylamide), polyol, agarose, agar, cellulose, dextran, starch, FICOLL, heparin, glycogen, amylopectin, mannan, inulin, nitrocellulose, diazocellulose or starch, polymeric microparticle, polymeric membrane, polymeric gel, glass slide, styrene, multi-well plate, column, microarray, latex, hydrogel, porous 3D hydrophilic polymer matrix (*e.g.*, HYDROGEL, Packard Instrument Company, Meriden, Conn.), fiber optic bundles and beads (*e.g.*, BEADARRAY (Illumina, San Diego, CA.), described in U.S. Pat. App. 20050164177), small particles, membranes, frits, slides, micromachined chips, alkanethiol-gold layers, non-porous surfaces, addressable arrays, and polynucleotide-immobilizing media (*e.g.*, described in U.S. Pat. App. 20050191660). In some embodiments, the solid support is coated with a binding layer or material (*e.g.*, gold, diamond, or streptavidin).

In some embodiments, the INVADER assay reagents further comprise a buffer solution. In some preferred embodiments, the buffer solution comprises a source of divalent cations (*e.g.*, Mn^{2+} and/or Mg^{2+} ions). Individual ingredients (*e.g.*, oligonucleotides, enzymes, buffers, target nucleic acids) that collectively make up INVADER assay reagents are termed "INVADER assay reagent components."

In some embodiments, the INVADER assay reagents further comprise a third oligonucleotide complementary to a third portion of the target nucleic acid upstream of the first portion of the first target nucleic acid (*e.g.*, a stacker oligonucleotides). In yet other embodiments, the INVADER assay reagents further comprise a target nucleic acid. In some embodiments, the INVADER assay reagents further comprise a second target nucleic acid. In yet other embodiments, the INVADER assay reagents further comprise a third oligonucleotide comprising a 5' portion complementary to a first region of the second target nucleic acid. In some specific embodiments, the 3' portion of the third oligonucleotide is covalently linked to the second target nucleic acid. In other specific embodiments, the second target nucleic acid further comprises a 5' portion, wherein the 5' portion of the second target nucleic acid is the third oligonucleotide. In still other embodiments, the INVADER assay reagents further comprise an ARRESTOR molecule (*e.g.*, ARRESTOR oligonucleotide).

In some embodiments one or more of the INVADER assay reagents or INVADER assay reagent components may be provided in a predispensed format (*e.g.*, premeasured for use in a step of the procedure without re-measurement or re-dispensing). In some embodiments, selected INVADER assay reagent components are mixed and predispensed together. In preferred embodiments, predispensed assay reagent components are predispensed and are provided in a reaction vessel (*e.g.*, including, but not limited to, a reaction tube or a well (*e.g.*, a microtiter plate)). In certain preferred embodiments, the INVADER assay reagents are provided in microfluidic devices such as those described in U.S. Pats., 6,627,159; 6,720,187; 6,734,401; and 6,814,935, as well as U.S. Pat. Pub. 2002/0064885, each of which is herein incorporated by reference in its entirety. In particularly preferred embodiments, predispensed INVADER assay reagent components are dried down (*e.g.*, desiccated or lyophilized) in a reaction vessel.

In some embodiments, the INVADER assay reagents or INVADER assay reagent components are provided as a kit. As used herein, the term "kit" refers to any delivery system for delivering materials. In the context of reaction assays, such

delivery systems include systems that allow for the storage, transport, or delivery of reaction reagents (*e.g.*, oligonucleotides, enzymes, *etc.* in the appropriate containers) and/or supporting materials (*e.g.*, buffers, written instructions for performing the assay *etc.*) from one location to another. For example, kits include one or more enclosures (*e.g.*, boxes) containing the relevant reaction reagents and/or supporting materials. As used herein, the term "fragmented kit" refers to delivery systems comprising two or more separate containers that each contains a subportion of the total kit components. The containers may be delivered to the intended recipient together or separately. For example, a first container may contain an enzyme for use in an assay, while a second container contains oligonucleotides. The term "fragmented kit" is intended to encompass kits containing Analyte-specific reagents ' regulated under section 520(e) of the Federal Food, Drug, and Cosmetic Act, but are not limited thereto. Indeed, any delivery system comprising two or more separate containers that each contains a subportion of the total kit components are included in the term "fragmented kit." In contrast, a "combined kit" refers to a delivery system containing all of the components of a reaction assay in a single container (*e.g.*, in a single box housing each of the desired components). The term "kit" includes both fragmented and combined kits.

In some embodiments, the present invention provides INVADER assay reagent kits comprising one or more of the components necessary for practicing the present invention. For example, the present invention provides kits for storing or delivering the enzymes and/or the reaction components necessary to practice an INVADER assay. The kit may include any and all components necessary or desired for assays including, but not limited to, the reagents themselves, buffers, control reagents (*e.g.*, tissue samples, positive and negative control target oligonucleotides, *etc.*), solid supports, labels, written and/or pictorial instructions and product information, inhibitors, labeling and/or detection reagents, package environmental controls (*e.g.*, ice, desiccants, *etc.*), and the like. In some embodiments, the kits provide a sub-set of the required components, wherein it is expected that the user will supply the remaining components. In some embodiments, the kits comprise two or more separate containers wherein each container houses a subset of the components to be delivered. For example, a first container (*e.g.*, box) may contain an enzyme (*e.g.*, structure-specific cleavage enzyme in a suitable storage buffer and container), while a

second box may contain oligonucleotides (*e.g.*, INVADER oligonucleotides, probe oligonucleotides, control target oligonucleotides, *etc.*).

In some preferred embodiments, the INVADER assay reagents further comprise reagents for detecting a nucleic acid cleavage product. In some embodiments, one or more oligonucleotides in the INVADER assay reagents comprise a label. In some preferred embodiments, said first oligonucleotide comprises a label. In other preferred embodiments, said third oligonucleotide comprises a label. In particularly preferred embodiments, the reagents comprise a first and/or a third oligonucleotide labeled with moieties that produce a fluorescence resonance energy transfer (FRET) effect.

As used herein, the term "label" refers to any moiety (*e.g.*, chemical species) that can be detected or can lead to a detectable response. In some preferred embodiments, detection of a label provides quantifiable information. Labels can be any known detectable moiety, such as, for example, a radioactive label (*e.g.*, radionuclides), a ligand (*e.g.*, biotin or avidin), a chromophore (*e.g.*, a dye or particle that imparts a detectable color), a hapten (*e.g.*, digoxgenin), a mass label, latex beads, metal particles, a paramagnetic label, a luminescent compound (*e.g.*, bioluminescent, phosphorescent or chemiluminescent labels) or a fluorescent compound.

A label may be joined, directly or indirectly, to an oligonucleotide or other biological molecule. Direct labeling can occur through bonds or interactions that link the label to the oligonucleotide, including covalent bonds or non-covalent interactions such as hydrogen bonding, hydrophobic and ionic interactions, or through formation of chelates or coordination complexes. Indirect labeling can occur through use of a bridging moiety or "linker", such as an antibody or additional oligonucleotide(s), which is/are either directly or indirectly labeled.

Labels can be used alone or in combination with moieties that can suppress (*e.g.*, quench), excite, or transfer (*e.g.*, shift) emission spectra (*e.g.*, fluorescence resonance energy transfer (FRET)) of a label (*e.g.*, a luminescent label).

As used herein, the term "FRET" refers to fluorescence resonance energy transfer, a process in which moieties (*e.g.*, fluorophores) transfer energy *e.g.*, among themselves, or, from a fluorophore to a non-fluorophore (*e.g.*, a quencher molecule). In some circumstances, FRET involves an excited donor fluorophore transferring energy to a lower-energy acceptor fluorophore via a short-range (*e.g.*, about 10 nm or less) dipole-dipole interaction. In other circumstances, FRET involves a loss of

fluorescence energy from a donor and an increase in fluorescence in an acceptor fluorophore. In still other forms of FRET, energy can be exchanged from an excited donor flurophore to a non-fluorescing molecule (*e.g.*, a quenching molecule). FRET is known to those of skill in the art and has been described (See, *e.g.*, Stryer et al., 1978, Ann. Rev. Biochem., 47:819; Selvin, 1995, Methods Enzymol., 246:300; Orpana, 2004 Biomol Eng 21, 45-50; Olivier, 2005 Mutant Res 573, 103-110, each of which is incorporated herein by reference in its entirety).

As used herein, the term "unlabeled" as used in reference to a probe oligonucleotide refers to a probe oligonucleotide that does not comprise any non-nucleic acid moiety, *e.g.*, a chromorphore or fluorophore, to facilitate detection. An unlabeled probe may comprise modifications, such as 3' blocking groups to prevent extension by a polymerase.

As used herein, the term "donor" refers to a moiety (*e.g.*, a fluorophore) that absorbs at a first wavelength and emits at a second, longer wavelength. The term "acceptor" refers to a moiety such as a fluorophore, chromophore, or quencher and that is able to absorb some or most of the emitted energy from the donor when it is near the donor group (typically between 1-100 nm). An acceptor may have an absorption spectrum that overlaps the donor's emission spectrum. Generally, if the acceptor is a fluorophore, it then re-emits at a third, still longer wavelength; if it is a chromophore or quencher, it releases the energy absorbed from the donor without emitting a photon. In some preferred embodiments, alteration in energy levels of donor and/or acceptor moieties are detected (*e.g.*, via measuring energy transfer (*e.g.*, by detecting light emission) between or from donors and/or acceptor moieties). In some preferred embodiments, the emission spectrum of an acceptor moeity is distinct from the emission spectrum of a donor moiety such that emissions (*e.g.*, of light and/or energy) from the moieties can be distinguished (*e.g.*, spectrally resolved) from each other.

In some embodiments, a donor moiety is used in combination with multiple acceptor moieties. In a preferred embodiment, a donor moiety is used in combination with a non-fluorescing quencher moiety and with an acceptor moiety, such that when the donor moiety is close (*e.g.*. between 1-100 nm, or more preferably, between 1-25 nm, or even more preferably around 10 nm or less) to the quencher, its excitation is transferred to the quencher moiety rather than the acceptor moiety, and when the quencher moiety is removed (*e.g.*, by cleavage of a probe), donor moiety excitation is

transferred to an acceptor moiety. In some preferred embodiments, emission from the acceptor moiety is detected (*e.g.*, using wavelength shifting molecular beacons) (See, *e.g.*, Tyagi, et al., Nature Biotechnology 18:1191 (2000); Mhlanga and Malmberg, 2001 Methods 25, 463-471; Olivier, 2005 Mutant Res 573, 103-110, and U.S. Pat. App. 20030228703, each of which is incorporated herein by reference in its entirety).

Suitable fluorophores include but are not limited to fluorescein, rhodamine, REDMOND RED dye, YAKIMA YELLOW dye, hexachloro-fluorescein, TAMRA dye, ROX dye, Cy3, Cy3.5, Cy5, Cy5.5, and Cy7, 4,4-difluoro-5,7-diphenyl-4-bora-3a,4a-diaza- -s-indacene-3-propionic acid, 4,4-difluoro-5,p-methoxyphenyl-4-bora-3a,4a-- diaza-s-indacene-3-propionic acid, 4,4-difluoro-5-styryl-4-bora-3a,4-adiaz- a-S-indacene-propionic acid, 6-carboxy-X-rhodamine, N,N,N',N'-tetramethyl-6-carboxyrhodamine, Texas Red, eosin, fluorescein, 4,4-difluoro-5,7-diphenyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid, 4,4-difluoro-5,p-ethoxyphenyl-4-bora-3a,4a-diaza-s-indacene 3-propionic acid and 4,4-difluoro-5-styryl-4-bora-3a,4a-diaza-S-indacene-propionic acid, 6-carboxyfluorescein (6-FAM), 2',4',1,4,-tetrachlorofluorescein (TET), 2l,4',5l,7',l,4-hexachlorofluorescein (HEX), 2',7'-dimethoxy-4',5'-dichloro-6-carboxyrhodamine (JOE), 2'-chloro-5'-fluoro-7',8'-fused phenyl- 1,4-dichloro-6-carboxyfluorescein (NED), 2'-chloro-7'-phenyl-l,4-dichloro-6-carboxyfluorescein (VIC), fluorescein isothiocyanate (FITC), 5,6-carboxymethyl fluorescein, Texas red, nitrobenz-2- oxa-l,3-diazol-4-yl (NBD), coumarin, dansyl chloride, amino-methyl coumarin (AMCA), Erythrosin, BODIPY dye, CASCADE BLUE dye, OREGON GREEN dye, pyrene, lissamine, xanthenes, acridines, oxazines, phycoerythrin, QUANTUM DYE, thiazole orange-ethidium heterodimer, and the like. Suitable quenchers include, but are not limited to, cyanine dyes, *e.g.*, Cy3, Cy3.5, Cy5, Cy5.5, and Cy7, rhodamine dyes, *e.g.*, tetramethyl-6-carboxyrhodamine (TAMRA) and tetrapropano-6- carboxyrhodamine (ROX), DABSYL dye, DABCYL dye, cyanine dyes, nitrothiazole blue (NTB), anthraquinone, malachite green, nitrothiazole, or nitroimidazole compounds, QSY7 (Molecular Probes, Eugene, OR), ECLIPSE quencher (Nanogen, San Diego, CA), and the like. Analysis of factors such as absorbance and emission spectra of various molecules in selection of pairs or groups of moieties for use in FRET configurations is well known to those of skill in the art.

Detection of labels or a detectable response (*e.g.*, provided by the labels) can be measured using a multitude of techniques, systems and methods known in the art.

For example, a label may be detected because the label provides detectable fluorescence (*e.g.*, simple fluorescence, FRET, time-resolved fluorescence, fluorescence quenching, fluorescence polarization, *etc.*), radioactivity, chemiluminescence, electrochemiluminescence, RAMAN, colorimetry, gravimetry, hyrbridization (*e.g.*, to a sequence in a hybridization protection assay), X-ray diffraction or absorption, magnetism, enzymatic activity, characteristics of mass or behavior affected by mass (*e.g.*, MALDI time-of-flight mass spectrometry), and the like.

As used herein the term "interactive label" refers to a label having two or more components that interact so as to produce a detectable effect. The interaction is not limited to any particular nature of interaction. The interaction of the label components may be via direct contact, *e.g.*, a covalent or non-covalent contact between two moieties (*e.g.*, a protein-protein contact, or collisional energy transfer between proximal moieties); it may comprise resonance energy transfer (*e.g.*, between one or more dyes, or between a dye and a quencher moieties); it may comprise a diffusion effect, *e.g.*, wherein the product from a reaction occurring at the site of one label diffuses to the site of another label to create a detectable effect. The components of an interactive label may be the same (*e.g.*, two or more of the same molecule or atom) or they may be different.

A label may be a charged moiety (positive or negative charge) or alternatively, may be charge neutral. Labels can include or consist of nucleic acid or protein sequence, so long as the sequence comprising the label is detectable. In some embodiments, the label is not nucleic acid or protein.

In some embodiments, a label comprises a particle for detection. For example, in some embodiments, the particle is a phosphor particle. An example of a phosphor particle includes, but is not limited to, an up-converting phosphor particle (See, *e.g.*, Ostermayer, Preparation and properties of infrared-to-visible conversion phosphors. Metall. Trans. 752, 747–755 (1971)). In some embodiments, rare earth-doped ceramic particles are used as phosphor particles. Phosphor particles may be detected by any suitable method, including but not limited to up-converting phosphor technology (UPT), in which up-converting phosphors transfer low energy infrared (IR) radiation to high-energy visible light. Although an understanding of the mechanism is not necessary to practice the present invention and the present invention is not limited to any particular mechanism of action, in some embodiments the UPT

up-converts infrared light to visible light by multi-photon absorption and subsequent emission of dopant-dependant phosphorescence (See, *e.g.*, U.S. Patent No. 6,399,397; van De Rijke, et al., Nature Biotechnol. 19(3):273-6 (2001); Corstjens, et al., IEE Proc. Nanobiotechnol. 152(2):64 (2005), each incorporated by reference herein in its entirety.

As used herein, the term "distinct" in reference to signals (*e.g.*, of one or more labels) refers to signals that can be differentiated one from another, *e.g.*, by spectral properties such as fluorescence emission wavelength, color, absorbance, mass, size, fluorescence polarization properties, charge, *etc.*, or by capability of interaction with another moiety, such as with a chemical reagent, an enzyme, an antibody, *etc.*

As used herein, the term "synthetic" as used in reference to a polynucleotide or oligonucleotide (*e.g.,* a probe) refers to a nucleic acid created in a cell-free *in vitro* reaction, *e.g.*, an enzymatic or chemical synthesis reaction. Examples of enzymatic formation of a synthetic nucleic acid include formation by restriction digestion, polymerization (templated or non-templated), ligation, *etc.* Examples of chemical synthesis of nucleic acid include but are not limited to, *e.g.*, phosphodiester and phosphotriester chemistries, phosphoramidite and H-phosphonate, chemistries, *etc.* See *e.g.*, *Methods in Molecular Biology, Vol* 20 *: Protocols for Oligonucleotides and Analogs* pp. 165-189 (S. Agrawal, Ed., Humana Press, 1993).; *Oligonucleotides and Analogues: A Practical Approach* , pp. 87-108 (F. Eckstein, Ed., 1991); and Uhlmann and Peyman, supra. Agrawal and Iyer, Curr. Op. in Biotech. 6: 12 (1995); and *Anti-sense Research and Applications* (Crooke and Lebleu; Eds., CRC Press, Boca Raton, 1993), Beaucage and Caruthers, Tetrahedron Lett. 22: 1859-1862 (1981), and Agrawal and Zamecnik, U.S. Pat. No. 5,149,798 (1992). In some embodiments, synthetic oligonucleotides are introduced into a reaction pre-formed, while in some embodiments, synthetic oligonucleotides are formed or modified within the reaction, *e.g.*, by action of a polymerase, ligase, cleavage enzyme, or the like.

As used herein, the term "FEN-1" in reference to an enzyme refers to a non-polymerase flap endonuclease from a eukaryote or archaeal organism.

As used herein, the term FEN-1 activity refers to any enzymatic activity of a FEN-1 enzyme, including but not limited to flap endonuclease (FEN), nick exonuclease (EXO), and gap endonuclease (GEN) activities (see, *e.g.*, Shen, *et al.*, BioEssays Volume 27, Issue 7, Pages 717 – 729, incorporated herein by reference).

As used herein, the term "identifying the presence of a polymorphism" refers to any method of inferring the identity of a nucleotide at a position of a suspected point of genetic variation. In some embodiments, the presence of a particular polymorphism or mutation is directly detected, *e.g.*, the presence of the polymorphism causes a detectable event to occur (*e.g.*, probe hybridization, probe cleavage, nucleic acid target or signal amplification, *etc.*), while in other embodiments, the presence of a polymorphism or mutation may be inferred from the absence of a particular nucleotide or sequence of nucleotides (*e.g.*, the absence of a wild-type nucleotide in a position in a nucleic acid sequence as an indicator of the presence of a mutant or polymorphic nucleotide at that position.)

As used herein, the term "determining an identity of an organism" encompasses any manner of assigning identification to an organism of interest, including but not limited to identification of a unique, individual organism, *e.g.*, as a variant among a population of related organisms, and/or classification of an organism, *e.g.*, by species, genus, family order, *etc.* Identity of an organism may be by phenotype or genotype.

DESCRIPTION OF THE INVENTION

The present invention relates to homogenous, real time assays for the detection of amplified nucleic acid, *e.g.*, PCR amplified DNA. In some embodiments, the probes used comprise detectable moieties such as fluorophores, while in some embodiments, probes comprise an interactive detection system, such as fluorophore and quencher moieties. In yet other embodiments, the methods and systems of the present invention use unlabeled analyte-specific probes, coupled with a detection system configured to detect products of the cleavage of the analyte-specific probes.

The present invention provides real time detection methods using enzyme footprint probes having short analyte-specific regions. The footprint probes of the present invention are generally used in assay conditions that are at temperatures far in excess of the melting temperatures of the probes as calculated using standard methods, *e.g.*, the nearest neighbor model and published parameters for DNA duplex formation, Allawi and SantaLucia, Biochemistry, 36:10581 (1997), and SantaLucia, Proc Natl Acad Sci U S A., 95(4):1460 (1998).

Use of footprint probes in detection and characterization provides a more selective assay. While not limiting the invention to any particular mechanism of

action, data suggests that the selectivity comes not only from the use of stringent hybridization conditions (*e.g.*, elevated reaction temperatures), but also from the fact that any duplex formed must supply a successful footprint for enzyme recognition and cleavage.

One surprising result of the instant invention is that the functionality of the footprint probes has little or no dependence on the precise sequence of the probe, so long as the probe contains the correct length of ASR to form the probe-target duplex. An important aspect of this lack of dependence on sequence is that the T_m of the analyte-specific region (determined, *e.g.*, by the sequence content of the ASR) does not influence the performance of the probe in the detection reactions of the invention, even if the detection reaction is performed at a temperature substantially above the calculated T_m of the probe-target duplex. Thus, in preferred embodiments, one can choose a probe based almost solely on the length (*e.g.* a length selected to be the known footprint of the enzyme to be used in the detection assay; *e.g.* 12 or fewer nucleotides for Afu FEN-1 endonuclease) and one need not even calculate T_m of the analyte-specific region before proceeding with the detection assay.

This aspect of the instant invention is a dramatic departure from traditional probe based assays, in which elaborate software and probe modifications are used to select and construct probes that are operable at particular reaction temperatures. For example, in many prior art assays, *e.g.*, the TAQMAN 5' nuclease assay or some embodiments of the INVADER and INVADER PLUS assays, a particular operating temperature for the assay is chosen as a first step in designing an assay for a new target. Reaction temperature is often selected as the first step so that numerous different experiments can be designed to be performed together, *e.g.*, in the same thermal cycler or incubator. Once a temperature of operation is chosen, probes, primers and/or other nucleic acid components of the assay are designed for performance in the selected temperature conditions. For different target sequences, the probes and primers are adjusted, *e.g.*, in length, G-C basepair content, and/or by the attachment of stabilizing moieties such as MGBs or peptide nucleic acids. The length of probes selected to operate at the same temperature may be very different, depending on the sequence of the target nucleic acid (*e.g.*, whether it is G-C rich or A-T rich).

To successfully design large numbers of assays configured to operate at the same temperature (or in the same thermal cycling set of temperatures), complex

software is often used in the design process. For example, Applied Biosystems provides the "PrimerExpress" software package for the design of primers and primer/probe combinations for use in TAQMAN and similar 5' nuclease PCR assays (*e.g.* , the probe-based FULLVELOCITY assay by Stratagene, La Jolla, CA) to be run in preselected thermal cycling temperature profiles.. Third Wave Technologies provides the INVADERCreator software for selection or INVADER oligonucleotide /probe combinations for detection assays to be run at a preselected temperature (*e.g.*, 63°C).

The compositions, methods, systems, and kits of the present invention obviate the need for such complex assay design steps and software.

Identification of footprint size

As discussed above, the present invention makes use of enzyme footprint probes. Identification of a recognition footprint for a duplex-recognizing nucleic acid modification enzyme is described here in the context of 5' nucleases, such as FEN-1 endonucleases. However, the invention is not limited to footprint probes designed for use with these enzymes. One of skill in the art would be well able to apply the same principles to the analysis of the activity requirements of other nucleic acid modifying enzymes.

A three-dimensional model of the structure-specific 5' flap endonuclease (FEN-1) from *Pyrococcus furiosus* in its complex with DNA has been proposed (see, *e.g.*, U.S. Patent Appl. Ser. No. 10/783,557, and Allawi, *et al.*, Journal of Molecular Biology v. 328(3):537-554 (2003)). The model is based on the known X-ray structure of the enzyme and a variety of biochemical and molecular dynamics data utilized in the form of distance restraints between the enzyme and the DNA. Contacts between the 5' flap endonuclease and the sugar-phosphate backbone of the overlap flap substrate were identified using enzyme activity assays on substrates with methylphosphonate or 2'-O-methyl substitutions. The enzyme footprint extends 2-4 base pairs upstream and 8-9 base pairs downstream of the cleavage site, thus covering 10-13 base pairs of duplex DNA. The footprint data are consistent with a model in which the substrate is bound in the DNA-binding groove such that the downstream duplex interacts with the helix-hairpin-helix motif of the enzyme.

It had been found, though, that, when used in conjunction with a probe or hairpin providing an upstream duplex, probes that provide a downstream duplex in the

range of 6 to 12 nucleotides (*i.e.*, probes that have 6-12 nucleotides of target-specific sequence) demonstrate increases in the rates of enzyme recognition and/or cleavage as the length of analyte-specific sequence approaches about 12 nucleotides when tested with the FEN-1 and polymerase-derived 5' nucleases, while no improvement in cleavage is seen for duplexes beyond that length (see, *e.g.*, Kaiser, *et al.*, J Biol Chem, Vol. 274, Issue 30, 21387-21394, July 23, 1999. Thus, in preferred embodiments of the present invention, a footprint probe for use with the FEN-1 and polymerase-derived 5' nucleases provides an analyte-specific region of up to about 12 nucleotides in length. As noted above, in certain embodiments, one or more of the up to 12 nucleotides may be non-complementary to the target strand. In preferred embodiments, the footprint probes are used in conjunction with an oligonucleotide (*e.g.*, a primer or INVADER oligonucleotide) that provides an upstream duplex.

Use of footprint probes in amplification assays

It is shown here that short footprint probes can be used in amplification assays, even in the presence of primers having higher melting temperatures, and even in reactions in which the "annealing temperature" used in thermal cycling is well above the calculated T_m of the ASR of the footprint probe (calculated, *e.g.*, using standard methods in the art, such as the nearest neighbor model and published parameters for DNA duplex formation, Allawi and SantaLucia, Biochemistry, 36:10581 (1997), and SantaLucia, Proc Natl Acad Sci U S A., 95(4):1460 (1998). While not limiting the present invention to any particular mechanism of action, it appears that the FEN-1 enzyme may be a factor in facilitating the annealing and cleavage of short probes at temperatures well above their calculated T_ms. In fact, experimental data provided herewith shows that the temperatures at which the methods of the present invention can be practice have little or no dependence on the calculated T_ms of the ASRs of the probes used.

In some embodiments a probe that is to be cleaved in the course of the detection reaction and having a short analyte-specific region of, *e.g.*, 6-12 nts, is used in conjunction with an oligonucleotide that hybridizes immediately downstream of the probe (downstream along the target strand and upstream of the 5' end of the ASR of the probe). To form the cleavage structure, an upstream nucleic acid (*e.g.*, an INVADER oligonucleotide) is provided to promote cleavage of the short probe.

Although the methods, compositions, and systems of the present invention are described in this Description of the Invention, and in the Summary of the Invention (which is incorporated here by reference), in connection with certain specific embodiments, *e.g.*, in the context of the polymerase chain reaction method of target amplification, it is to be appreciated that the invention is not limited to these embodiments. The embodiments discussed below are provided by way of example, and not to limit the invention.

Several embodiments and aspects of the present invention are discussed below.

Assays Having Improved Specificity

An aspect of the present invention is to provide systems and methods for detecting target amplification assays, such as PCR assays, in real time. In some embodiments, the present invention provides systems, methods and kits for performing target amplification assays in combination with detection assays (*e.g.*, cleavage assays), where the detection assay employs probes with relatively short (*e.g.*, 6-12 bases) analyte-specific regions. In preferred embodiments, these probes are configured to provide a probe/analyte duplex at or near the minimum length of duplex that is efficiently recognizable by a detection agent, *e.g.*, a cleavage agent such as a FEN-1 nuclease, such that probes annealed to target with a mismatch in sequence to the analyte-specific portion of the probe are not cleaved with full efficiency or at all. In such embodiments, detectable cleavage from probes hybridized to mismatched target nucleic acids is reduced compared to detectable cleavage from probes hybridized to target nucleic acid that is perfectly complementary to the analyte-specific portion of the probes, and the assay thereby has greater specificity and/or lower background.

The invention is not limited to any particular mechanism of improved specificity and/or reduced background through the use of the footprint probes of the invention. One aspect of improved specificity is more selective hybridization afforded by short probes used in stringent hybridization conditions (*e.g.*, at temperatures greater than the calculated T_m of the analyte specific portion of the probe), such that only perfectly matched probes tend to anneal to a target strand, while in some embodiments, improved results may be due to discrimination by a cleavage enzyme between perfectly matched and imperfectly matched probe/target duplexes.

Another aspect of improved specificity is through the use of probes configured to provide a duplex with the intended target that is at or near the minimum duplex length for efficient cleavage of the probe by a 5' nuclease, *e.g.*, a FEN-1 enzyme. In such embodiments, if the analyte-specific portion of the probe is mismatched to the target in one or more nucleotide positions, the 5' nuclease cleavage of the duplex will be substantially reduced or absent. In particularly preferred embodiments, the FEN-1 is a thermostable FEN-1 from an archaeal species.

The effect of probe/target duplex length (also referred to as "downstream duplex length," in contrast to an "upstream duplex" such as is provided by, *e.g.*, an INVADER oligonucleotide or a primer oligonucleotide) on cleavage efficiency of cleavage structures (*e.g.*, invasive cleavage structures) has been studied for a variety of 5' nuclease enzymes. See, *e.g.*, Kaiser et al., J. Biol. Chem. Jul 23;274(30):21387-94 (1999), which is incorporated herein by reference. Kaiser examined the effects of probe/target duplex using substrates with downstream duplex lengths of between 6 and 16 base pairs for an overlapping flap substrate in which the upstream duplex (*e.g.*, an INVADER oligonucleotide/target duplex) was 6 base pairs in length. Experiments examined four archaeal FEN1 enzymes, from *A. fulgidus* (AfuFEN), *P. furiosus* (PfuFEN), *M. jannaschii* (MjaFEN), and *M. thermoautotrophicum* (MthFEN), two eubacterial polymerase I enzymes from *T. aquaticus* (TaqPol) and *T. thermophilus* (TthPol) and the 5' nuclease domain of TaqPol (referred to as TaqExo). All enzymes, except TaqPol, cleaved the overlapping flap substrates at rates independent of downstream duplex length in the range from 10 to 16 base pairs; TaqPol cleaved the 10 base pair substrate approximately five times slower than the 12 base pair substrate. For most enzymes, cleavage activities decreased for the 8 base pair downstream duplex substrate and significantly dropped when the duplex length was reduced to 6 bp, although the FEN-1 enzymes of MjaFEN and MthFEN were able to cleave the substrate with the 6-base pair downstream duplex. Thus, use of probes having short analyte-specific regions provides an additional level of discrimination beyond the mechanics of hybridization in that, even if a short probe anneals to a mismatched target nucleic acid, cleavage by a 5' nuclease will be reduced compared to the same probe hybridized to it matched target sequence. Thus, for these enzymes, the use of probes having 12 or fewer bases complementary to the target nucleic acid in the analyte-specific portion of the probe provides significant advantages over the use of

probes of more conventional design, when one of these enzymes is used in the cleavage reaction.

Probes comprising short analyte-specific regions have been used in certain embodiments of invasive cleavage assays, *e.g.* INVADER assays. See, *e.g.*, U.S. Patent No. 5,985,557, to Prudent *et al.* However, in view of long-established rules for probe design for real time detection methods in amplification reactions such as in PCR, it is surprising that probes having such short ASRs, especially probes lacking duplex stabilizing moieties such as MGBs, can be made to work for real time detection in assays performed at temperatures well above the melting temperatures of the probes. The performance of these probes is especially surprising with respect to reactions such as PCR amplifications, in which the extension of a primer having a higher T_m would be expected to occlude the probe binding site altogether. While not limiting the invention to any particular mechanism of action, in some embodiments an enzyme in the reaction of the present invention, *e.g.*, a FEN-1 enzyme, may stabilize the footprint probe-target duplex, such that short probes can anneal before extension of a primer occludes the binding sites, and can be used at temperatures that exceed their melting temperatures by several degrees or more.

As discussed in the Background section, one commonly used method for real time detection in PCR is the use of fluorescently labeled analyte-specific hydrolysis probes in the reaction. In the most commonly used methods, a polymerase is used that includes a 5' nuclease "nick translation" activity (*e.g.*, *Taq* DNA polymerase) and cleavage of the probe occurs as the polymerase extends the PCR primer and encounters the hybridized probe (see, *e.g.*, U.S. Patent No. 5,210,015, and Holland et al., Proc. Natl. Acad. Sci. USA 88:7276 (1991)). In some types of 5' nuclease PCR reactions, the polymerase and 5' nuclease are provided as separate proteins (*e.g.*, *Pfu* DNA polymerase and *Pfu* FEN-1 endonuclease). In the latter configuration, the polymerase extends the primer to the point where the probe is partially displaced so as to create a structure recognized by a FEN-1 enzyme and the probe molecule is then cleaved by the FEN-1 (see, *e.g.*, U.S. Patent Nos. 6,528,254 and 6,548,250, FULLVELOCITY Q-PCR Master Mix Instruction Manual Revision #114005, Stratagene Corp., FULLVELOCITY QRT-PCR Master Mix Instruction Manual Revision # Revision #114005, Stratagene Corp.).

In both methods described above, it is considered imperative in the assay design that the hydrolysis probe be annealed to the target strand before the primer is

extended. For this reason, hydrolysis probes for use in these real time PCR methods (including FULLVELOCITY methods) are selected to bind to the target nucleic acid more stably (*i.e.*, with a higher melting temperature, or T_m) than the primers used in the amplification reaction. See, *e.g.*, U.S. Patent No. 5,210,015, Primer Express® oligo design software from Applied Biosystems, Stratagene's "Introduction to Quantitative PCR" Methods and Applications Guide, IN #70200-00/Revision #084001 Copyright 2004 by Stratagene.

In some real time PCR assay designs, the stability of the probe is ensured by selecting an analyte-specific probe sequence having length and sequence composition selected to have a T_m of above that of the primers. In other assay designs, shorter probes are used, but the melting temperature of the probe is raised by attachment to the probe of a duplex stabilizing moiety. In particular, moieties that bind duplex DNA, such as minor groove binders ("MGBs") (see, *e.g.*, U.S. 6,312,894, Kutyavin, *et al.*, Nucleic Acids Research, 2000, Vol. 28, No. 2 655-661, and ABI Primer Express manual, *supra*) are commonly used on hydrolysis probes for use in real time PCR. Conjugation of an MGB to an oligonucleotide dramatically increases the stability of the hybrid formed between the oligonucleotide and its target (see, *e.g.*, U.S. 6,312,894, Kutyavin, *et al.*, *supra*). Increased stability (*i.e.,* increased degree of hybridization) is manifested in a higher melting temperature of hybrid duplexes formed by such MGB-oligonucleotide conjugates, compared to those formed by an unconjugated oligonucleotide of identical length and sequence. This effect is particularly pronounced for short oligonucleotides (*e.g.*, less than about 21 nucleotides in length), and allows such conjugated short oligonucleotide probes, down to about 12 nucleotides, meet the design imperatives of homogenous 5' nuclease PCR assays, *i.e.*, to bind the target nucleic acid more stably than the primers.

The Primer Express Software Version 3.0 "Getting Started" guide for designing probes and primers for real time PCR detection based on the TAQMAN 5' nuclease probe cleavage assay instructs that probes should always have a higher T_m than the primers. For example, a standard (non-MGB) probe is taught as optimally having a T_m of between 68 °C to 70 °C, while the primers are taught as optimally having T_ms of between 58 °C to 60 °C. When an MGB is used on the probe (*e.g.*, for allelic discrimination), the manual instructs that a probe T_m of 65 °C to 67 °C should be used with primers having T_ms of between 58 °C to 60 °C. With or without a stabilizing MGB, the manual instructs that a probe used in real time 5' nuclease assay

detection should have a T_m at least 5 to 10 °C higher than the T_ms of the primers it is to be used with. For assays in which the polymerase and 5' nuclease cleavage activities are provided as separate enzymes, the recommendation is the same (see, *e.g.*, FULLVELOCITY Q-PCR and QRT-PCR Master Mix Instruction Manuals, *supra*).

As discussed above, the present invention provides improved real time detection assays comprising hydrolysis probes having short analyte-specific regions selected to provide the minimal footprint duplex, *e.g.*, for a FEN-1 enzyme. In contrast to the hydrolysis probes typically used in TAQMAN assays and FULLVELOCITY methods, the footprint probes of the present invention are selected to provide the shortest length of probe/target duplex that permits suitable performance of the FEN-1 enzyme in the reaction. The footprint probes of the present invention have melting temperatures substantially below that of the PCR primers with which they are used. In some preferred embodiments, the probes have analyte-specific portions of 12 or fewer nucleotides. Although labels and other such components may influence the stability of a probe/target duplex, in particularly preferred embodiments, the probes of the I, nstant invention do not contain a non-nucleic acid moiety provided for the purpose of increasing the stability of the duplex (*e.g.*, a minor groove binder).

The examples provided herein demonstrate that the short footprint probes of the instant invention provide clean signal during real time PCR detection, with little background (*e.g.*, little accumulation of signal in no-target controls; see, *e.g.*, Figure 4).

Assays Providing Faster Detection

Another aspect of the present invention is to provide faster real time detection of amplification reactions, when compared to standard hydrolysis probe-based methods. As discussed above, TAQMAN-type assays cleave hydrolysis probes as a primer is extended along a target in a cycle of PCR. Thus, the rate of signal accumulation is tied to, and limited by, the temperature cycling of the PCR.

Assays have been described that provide a secondary cleavage event that is enabled by cleavage of the hydrolysis probe during PCR. See, *e.g.*, US Patent No. 6,893,819, in which a 5' flap from a hydrolysis probe, once cleaved, is used as a primer in a detection reaction. The flap-primer is extended to form a second cleavage structure comprising another probe, to release another flap, and so on. However, in

such an assay, the cleavage product from the original hydrolysis event, the 5' flap, is consumed in the second reaction and does not contribute to additional reactions.

An aspect of the present invention is to provide detection assays in which the accumulation of detectable signal is not dependent on or limited by the thermal cycling of the concurrent PCR assay. For example, in preferred embodiments of the present invention, a cleavage product is release from the analyte-specific probe when it is annealed to the target strand, and the cleavage product then participates in the formation of many subsequent cleavage structures, *e.g.*, with FRET cassettes as depicted in Figure 1. In some embodiments, the initial cleavage product is not further altered during cleavage of the second and subsequent cleavage structures, while in other embodiments, any alteration of the initial cleavage product (*e.g.*, further cleavage, primer extension) does not prevent the altered initial cleavage product from participating in the formation of second and subsequent cleavage structures.

In particularly preferred embodiments, the second and subsequent cleavage structures are configured such that they can form and dissociate without reliance on the PCR temperature cycling, such that many cleavage structures can form and be cleaved at all times that the temperature of the reaction is within the range at which at least some of the detection cleavage structures can form. When so configured, many copies of the detection cleavage structure may be cleaved for each copy of the initial cleavage product, even as the temperature of the reaction moves through the thermal cycles of the concurrent PCR. In this way, the signal amplification from the detection assay will accumulate more rapidly than accumulation of the amplicon, and more rapidly than is possible with standard hydrolysis probe cleavage, or with secondary detection assays in which the initial cleavage product is consumed rather than re-used. Rapid signal accumulation allows more sensitive detection, and earlier time-to-result.

Assays Providing Lower Costs

As noted above, one of the primary disadvantages of probe-based specific detection chemistries for real time PCR is the need to use different custom probes comprising expensive dyes, quenchers and, optionally, MGBs, for each different analyte sequence. In some embodiments, present invention provides real time detection methods that comprise use of an analyte-specific probe that is unlabeled, coupled with a secondary detection reaction using labeled oligonucleotides, such that

the need to produce analyte-specific probes labeled with expensive moieties is avoided.

Assays Providing Improved Dynamic Range

The methods of the present invention are not limited by the type of target nucleic acid. For example, the target nucleic acid may include, for example, nucleic acid material prepared from viruses having an RNA genome. Typically, the RNA target sequence will be converted to a cDNA molecule through the action of a reverse transcriptase, and then detected by the nucleic acid detection assay. Incorporation of the methods of the present invention will increase the dynamic range of detection of RNA target sequences to a breadth not previously feasible.

In some embodiments, the target sequence is a synthetic sequence. For example, a fragment generated in an enzymatic reaction (*e.g.*, a restriction fragment, a cleaved flap from an invasive cleavage reaction, *etc.*) can be considered a target sequence. In some such embodiments, the detection of such a molecule indirectly detects a separate target nucleic acid from which the synthetic sequence was generated. For example, in an invasive cleavage reaction, a cleaved flap from a primary reaction may be detected with first and second probes that are FRET cassettes. The FRET cassettes differ in some characteristic (*e.g.*, length, *etc.*) such that the cleaved flap differentially hybridizes to the first and second probes. By using both FRET cassettes (or a third, fourth, *etc.*), the dynamic range of the reaction is improved.

In some embodiments, the methods of the invention are used in conjunction with methods to further increase the dynamic range of detection. See, *e.g.*, U.S. Patent Application Ser. No, 11/338,244, which is incorporated here by reference. For example, in some embodiments, the present invention is used in conjunction with methods that achieve greater dynamic range of detection through the use of differential levels of amplification of regions of a target nucleic acid (e.g., no amplification, linear amplification at one or more efficiencies, and/or exponential amplification at one or more efficiencies). In some embodiments, the present invention is used in conjunction with methods that achieve greater dynamic range of detection through the use of probes with different hybridization properties to one or more analyte-specific regions of a target nucleic acid or target nucleic acids. In some preferred embodiments, the different probes have different ASRs, to provide a range

of optimal and/or sub-optimal footprints, *e.g.*, for cleavage by a detection reagent. In preferred embodiments, combinations of two or more of the methods are employed. For example, in some preferred embodiments, two or more probes (e.g., three, four, etc.) are contacted with first and second amplicons obtained via different levels of amplification. In some such embodiments, each probe generates the same type of signal so that one simply detects total signal generated by the reactions. The collective signal permits detection of target nucleic acid over a broad dynamic range. For example, experiments conducted during the development of the present invention have demonstrated the ability to detect target nucleic acid from samples differing in over eight logs of copy number of target nucleic acid originally present in the sample.

The compositions, methods, systems, and kits of the invention are useful for the detection and quantitation of a wide variety of nucleic acid targets. The compositions and methods of the present invention are particularly useful for the quantitation of viral target nucleic acids (*e.g.*, viral pathogens). Exemplary viral nucleic acids for which a clinical or research need for the detection of a large range of viral concentrations (*e.g.*, viral load) include, but are not limited to, human immunodeficiency virus (HIV) and other retroviruses, hepatitis C virus (HCV), hepatitis B virus (HBV), hepatitis A virus (HAV), human cytomegalovirus, (CMV), Epstein bar virus (EBV), human papilloma virus (HPV), herpes simplex virus (HSV), Varicella Zoster Virus (VZV), bacteriophages (*e.g.*, phage lambda), adenoviruses, and lentiviruses. In other embodiments, the compositions and methods of the present invention find use in the detection of bacteria (*e.g.*, pathogens or bacteria important in commercial and research applications). Examples include, but are not limited to, *Chlamydia sp.*, *N. gonorrhea*, and group B *streptococcus*.

The quantitation of target nucleic acids using the methods and compositions of the present invention are utilized in a variety of clinical and research applications. For example, in some embodiments, the detection assays with increased dynamic range of the present invention are utilized in the detection and quantitation of viral pathogens in human samples. The detection assays of the present invention are suitable for use with a variety of purified and unpurified samples including, but not limited to, urine, stool, lymph, whole blood, and serum. In preferred embodiments, the detection assays of the present invention are suitable for use in the presence of host cells.

In other embodiments, the detection assays of the present invention find use in research applications including, but not limited to, drug screening (*e.g.*, for drugs against viral pathogens), animal models of disease, and in vitro quantitation of target nucleic acid (*e.g.*, bacterial, viral, or genomic nucleic acids).

The probe oligonucleotides of the present invention find use in a variety of nucleic acid detection assays including, but not limited to, those described below. It should be understood that any nucleic acid detection method that employs hybridization can benefit from the systems and methods of the present invention.

EXPERIMENTAL

In the disclosure that follows, the following abbreviations apply: Ex. (Example); Fig. (Figure);°C (degrees Centigrade); g (gravitational field); hr (hour); min (minute); olio (oligonucleotide); rxn (reaction); vol (volume); w/v (weight to volume); v/v (volume to volume); BSA (bovine serum albumin); CTAB (cetyltrimethylammonium bromide); HPLC (high pressure liquid chromatography); DNA (deoxyribonucleic acid); p (plasmid); µl (microliters); ml (milliliters); ng (nanograms); µg (micrograms); mg (milligrams); M (molar); mM (milliMolar);µM (microMolar); pmoles (picomoles); amoles (attomoles); zmoles (zeptomoles); nm (nanometers); kdal (kilodaltons); OD (optical density); EDTA (ethylene diamine tetra-acetic acid); FITC (fluorescein isothiocyanate) FAM (fluorescein); SDS (sodium dodecyl sulfate); NaPO4 (sodium phosphate); NP-40 (Nonidet P-40); Tris (tris(hydroxymethyl)-aminomethane); PMSF (phenylmethylsulfonylfluoride); TBE (Tris-Borate-EDTA, *i.e.*, Tris buffer titrated with boric acid rather than HCl and containing EDTA); PBS (phosphate buffered saline); PPBS (phosphate buffered saline containing 1 mM PMSF); PAGE (polyacrylamide gel electrophoresis); Tween (polyoxyethylene-sorbitan); Red or RED (REDMOND RED Dye, Epoch Biosciences, Bothell WA) Z28 (ECLIPSE Quencher, Epoch Biosciences, Bothell, WA); Promega (Promega, Corp., Madison, WI); Glen Research (Glen Research, Sterling, VA); Coriell (Coriell Cell Repositories, Camden, NJ); Third Wave Technologies (Third Wave Technologies, Madison, WI); Microsoft (Microsoft, Redmond, WA); Qiagen (Qiagen, Valencia, CA).

The following examples are provided in order to demonstrate and further illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

EXAMPLE 1

Use of Footprint Probes in an INVADER Assay During PCR

This example describes a quantitative assay composed of a combination of an INVADER assay and PCR. The INVADER assay is conducted with footprint probes that have short analyte-specific regions (*e.g.*, 11 to 12 bases). The use of such the short enzyme footprint probes of the invention allows an increased dynamic range for the combined INVADER-PCR assay. While not limited to any specific mechanism, and while not necessary to practice the invention, it is believed that the use of probes with short analyte-specific regions in an invasive cleavage assay such as the INVADER assay allows for an increased dynamic range of target detection, since the cleaved probes do not interfere with the PCR reaction. For example, it is believed that the cleaved probes, since they are short, 1) are not stably hybridized and therefore do not inhibit PCR primer extension, and 2) and do not get extended improperly as part of the PCR reaction.

In this Example, the following six targets were employed: miR-21; miR-126; miR-21; miR-155; U6 RNA; and U24 RNA. The following 10X Oligo mixes were used for each target: reverse primer at 4 uM; forward primer at 4 uM; primer-stacker at 4 uM; probe oligo at 5 uM; and FRET oligo at 2.5 uM. The following 10X reaction buffer was used for each reaction: 100 mM MOPS, pH 7.5; 75 mM $MgCl_2$; and 250 uM dNTP. A 40X enzyme mix was also used for each reaction, which was composed of: 200 ng/ul Afu FEN-1 endonuclease; 1.33 units/ul Go Taq Polymerase; 80 units/ul MMLV reverse Transcriptase; and 7 mM DTT.

The specific probes, primers, invasive oligonucleotides, and FRET cassette sequences for each target are as shown in Figure 11. Specifically, Figure 11A shows the oligonucleotide sequences for amplifying and detecting the human miR-21 target; Figure 11B shows the oligonucleotide sequences for amplifying and detecting the human miR-155 target; Figure 11C shows the oligonucleotide sequences for amplifying and detecting the human miR-126 target; Figure 11D shows the oligonucleotide sequences for amplifying and detecting the human U6 snRNA target; and Figure 11E shows the oligonucleotide sequences for amplifying and detecting the

human U24 snRNA target. In regard to Figure 11A, as an example, the identity of the sequences is as follows: probe (SEQ ID NO:1); miR-21 target sequence (SEQ ID NO:2); forward primer (SEQ ID NO:3); reverse primer/invasive oligonucleotide (SEQ ID NO:4); primer-stacker (SEQ ID NO:5); and FRET cassette sequence (SEQ ID NO:6). In regard to the primer-stacker (SEQ ID NO:5 shown in Figures 11A-C), this sequence serves to stabilize the RT-primer/microRNA hybrid. In general, without this sequence, the level of detection may drop dramatically (~100 fold). It is noted that rather than a primer-stacker, one could employ a reverse primer that loops back on itself forming a hairpin at its 5'-end.

The reactions were set up as follow. A premix of each miRNA target was made at 1 pM and a premix of the U6 and U24 RNA targets was made at 5 pM. Next, aliquot duplicates of 10 uL of targets at 1X concentrations and 5X dilutions were made into a 96 well plate. Next was added 10 ul of tRNA no-target control (20 ng/ul tRNA) for each oligo mix to be tested. Then, a reaction mix was added to each well, where the reaction mix was composed of the following components: 2 ul of 10X Oligo mix, 2 ul of 10X reaction buffer; 0.5 ul of 40X enzyme mix; and 5.5 ul of water. Finally, the plate was spun for 30 seconds at 1200 rpm, and was then placed in real time PCR thermocycler programmed for: 42 °C – 30 min; 95 °C – 2 min; and 40 cycles of (95 °C – 20 seconds --> 50 °C – 45 seconds --> 60 °C – 30 seconds). Data collection was set for end of each 50°C - 45 seconds step.

Data was plotted as fluorescence versus cycle number as shown in Figures 12A-E. For each target, a threshold was assigned that gave the most linear fit of copy number versus cycle threshold (see bottom panel in each figure). The results for each target are shown in the following figures: miR-155 (Figure 12A); miR-21 (Figure 12B); miR-126 (Figure 12C); U6 (Figure 12D); and U24 (Figure 12E). These data show facile and rapid detection of the target RNAs over a wide range of initial target numbers using the methods of the present invention.

EXAMPLE 2

Effect of Various Components of the PCR-Invasive Cleavage Reaction

This example describes the same type of PCR-Invasive Cleavage Reactions as Example 1, except certain components are left out, or included, to test the various components of the assay. In this Example, the following targets were employed: U6

RNA; GA-21-DNA; Factor V DNA; and Factor II DNA. 10X oligo mixer were generated for each target as follows: reverse primer at 4 uM; forward primer at 4 uM; invasive Oligo at 0.4 uM (omitted for certain conditions as indicated); probe oligo at 6.7 uM; and FRET oligo at 2.5 uM. A 10X reaction buffer was used for reaction which had the following makeup: 100 mM MOPS, pH 7.5; 75 mM $MgCl_2$; and 250 uM dNTP (omitted for certain conditions as indicated). A 40X enzyme mix was also employed, which was composed of the following components: 200 ng/ul Afu FEN-1 (omitted for certain conditions as indicated); 1.33 units/ul GoTaq Polymerase (native Taq DNA polymerase, Promega Corp.); 80 units/ul MMLV reverse Transcriptase (omitted for DNA targets); and 7 mM DTT.

The specific probes, primers, invasive oligonucleotides, and FRET cassette sequences for each target are as shown in Figure 13. Specifically, Figure 13A shows the oligonucleotide sequences for amplifying and detecting the U6 DNA target; Figure 13B shows the oligonucleotide sequences for amplifying and detecting the factor V DNA target; Figure 13C shows the oligonucleotide sequences for amplifying and detecting the factor II DNA target; Figure 13D shows the oligonucleotide sequences for amplifying and detecting the GA-21-R DNA target; and Figure 13E shows the oligonucleotide sequences for amplifying and detecting the human U6 RNA target.

The reactions were set up as follow. A premix was made at 1 nM of synthetic targets or 100 ng of genomic targets. Next, aliquot duplicates of 10 uL of targets at 1X concentrations and 5X dilutions into a 96 well plate were made. A 10 ul mix of tRNA no-target control (20 ng/ul tRNA) was also made for each oligo mix to be tested. To each well, 10 ul of reaction mixture is added to each well, where the reaction mix is composed of the following components: 2 ul of 10X Oligo mix; 2 ul of 10 reaction buffer; 0.5 ul 40X of enzyme mix; and 5.5 ul of water. Next, the plate is spun for 30 seconds at 1200 rpm, then placed in a real time PCR thermocycler programmed for: 95 °C – 2 min; and 40 cycles of (95 °C – 20 seconds --> 50 °C – 45 seconds --> 60 °C – 30 seconds). Data is collected at the end of each 50°C – 45 seconds step.

Data was plotted as fluorescence versus cycle number as shown in Figures 14-17. The results for the Factor II target are shown in Figures 14A-D. Figures 14A-B show the results of this Example with both the invasive oligonucleotide and the FEN-1 enzyme present, while Figures 14C-D show the results with the invasive

oligonucleotide and FEN-1 enzyme not present. The target level of each well for Factor II is shown in Table 1 below. "Cleavase" as used here refers to Afu FEN-1 endonuclease.

Table 1

Factor II: Target Level Per Well

	Well #				Target level (g)
	1	13	25	37	1.00E-06
	2	14	26	38	1.00E-07
	3	15	27	39	1.00E-08
	4	16	28	40	1.00E-09
	5	17	29	41	1.00E-10
	6	18	30	42	1.00E-11
	7	19	31	43	1.00E-12
	8	20	32	44	1.00E-13
	9	21	33	45	1.00E-14
	10	22	34	46	1.00E-15
	11	23	35	47	1.00E-16
	12	24	36	48	0.00E+00
Cleavase	+	+	-	-	
Invasive Oligo	+	+	-	-	
					Target is 10X diluted PCR amplicon of FII DNA 35 PCR cycles
					~ 1 ug

The results for the Factor V target are shown in Figures 15A-F. Figures 15A-B show the results of this Example with all the reactions components present. Figures 15C-D show the results with the invasive oligonucleotide and FEN-1 enzyme not present. Figures 15E-F show the same results as Figures 15C-D, except the y-axis maximum is 10,000 rather than 60,000.

The results for the corn trans gene GA-21-R target are shown in Figures 16A-D. Figures 16A-B show the results of this Example with both the invasive oligonucleotide and the FEN-1 enzyme present, while Figures 16C-D show the results with the invasive oligonucleotide and FEN-1 enzyme not present. The target level of each well for GA-21-R is shown in Table 2 below.

Table 2

GA-21-R Target Number Per Well

Afu FEN-1	Invasive Oligo		1	2	3	4	5	6	7	8	9	10	11	12
-	-	A	6.00E+10	6.00E+09	6.00E+08	6.00E+07	6.00E+06	6.00E+05	6.00E+04	6.00E+03	6.00E+02	6.00E+01	6.00E+00	0
-	-	B	6.00E+10	6.00E+09	6.00E+08	6.00E+07	6.00E+06	6.00E+05	6.00E+04	6.00E+03	6.00E+02	6.00E+01	6.00E+00	0
+	+	C	6.00E+10	6.00E+09	6.00E+08	6.00E+07	6.00E+06	6.00E+05	6.00E+04	6.00E+03	6.00E+02	6.00E+01	6.00E+00	0
+	+	D	6.00E+10	6.00E+09	6.00E+08	6.00E+07	6.00E+06	6.00E+05	6.00E+04	6.00E+03	6.00E+02	6.00E+01	6.00E+00	0
			Target copies/rxn											

The results for the U6 target are shown in Figures 17A-D. Figures 17A-B show the results of this Example with both the invasive oligonucleotide and the FEN-1 enzyme present, while Figures 17C-D show the results with the invasive oligonucleotide and FEN-1 enzyme not present. The target level of each well for U6 is shown in Table 3 below. "Cleavase" as used here refers to Afu FEN-1 endonuclease.

Table 3

U6 RNA Target Number Per Well

Cleavase	Invasive Oligo											
-	-	W1	W2	W3	W4	W5	W6	W7	W8	W9	W10	W11	W12
-	-	W13	W14	W15	W16	W17	W18	W19	W20	W21	W22	W23	W24
+	+	W25	W26	W27	W28	W29	W30	W31	W32	W33	W34	W35	W36
+	+	W37	W38	W39	W40	W41	W42	W43	W44	W45	W46	W47	W48

Cleavase	Invasive Oligo	U6 Copies per rxn	U6 Copies per rxn	U6 Copies per rxn	U6 Copies per rxn	U6 Copies per rxn	U6 Copies per rxn	U6 Copies per rxn	U6 Copies per rxn	U6 Copies per rxn	U6 Copies per rxn	U6 Copies per rxn	U6 Copies per rxn
-	-	6.00E+09	6.00E+08	6.00E+07	6.00E+06	6.00E+05	6.00E+04	6.00E+03	6.00E+02	60	6.0	0.6	-
-	-	6.00E+09	6.00E+08	6.00E+07	6.00E+06	6.00E+05	6.00E+04	6.00E+03	6.00E+02	60	6.0	0.6	-
+	+	6.00E+09	6.00E+08	6.00E+07	6.00E+06	6.00E+05	6.00E+04	6.00E+03	6.00E+02	60	6.0	0.6	-
+	+	6.00E+09	6.00E+08	6.00E+07	6.00E+06	6.00E+05	6.00E+04	6.00E+03	6.00E+02	60	6.0	0.6	-

In light of the above results, it is clear that essentially no signal is generated when the FEN-1 enzyme and the Invasive oligo are absent, even though the polymerase enzyme comprises a 5' nuclease activity.

EXAMPLE 3

Use of Short Probes in an INVADER assay Configured for SNP Detection During PCR

This Example describes the use of probes with a short analyte-specific region (12 bases in this Example) in an invasive cleavage assays conducted during PCR for genotyping. The target in this Example is Factor V. The specific probes, primers, invasive oligonucleotides, and FRET cassette sequences for detecting the SNP in Factor V is shown in Figure 5.

The wild-type and mutant reactions were set up as follows. An aliquot 10 ul of genomic DNA (1 ng/ul) or tRNA no-target control (20 ng/ul tRNA) in placed in a 96 well plate. 10 ul of reaction mixture is added to each well, where the reaction mixtures (as well as the 40X enzyme mix) is as described in Example 1. The 10X oligo mixture, which is part of the reaction mixtures, is composed of the following sequences as shown in Table 4.

Table 4

10X Oligo Mixture

	10X Q-Invader FV Oligo mix =	
	Oligo #	10X conc (uM)
	2749-70-07	4
	2749-70-06	4
	2749-70-05	0.4
	2749-70-04	5
	2749-70-03	5
	Arm 6 FAM FRET	2.5
	Arm 4 Yellow FRET	2.5

The plate was spun for 30 seconds at 1200 rpm, then placed in a real time PCR thermocycler programmed for: 95 °C – 2 min; and 50 cycles of (95 °C – 20 seconds --> 52 °C – 60 seconds). Data was collected at the end of each 52°C – 45 seconds step.

Data is plotted as fluorescence data versus cycle, as shown in Figure 6. The results for the mutant target detection are shown in Figure 6A, the results for the wild-type target detection are shown in Figure 6B, and the results for heterogenous target detection are shown in Figure 6C. For each target, a cycle threshold (Ct) that gives the least cross reactivity for each genotype is assigned. Data for detection of the same

alleles using the TAQMAN assay as described in Luderer, *et al.*, Clinical Chemistry 50, No. 4 (2004):787-788 is shown in Figures 6D-6F.

Figure 7 shows a scatter plot of the data from Luderer, *et al.* (panel A), compared to a scatter plot of FAM Ct versus Yellow Ct in the PCR +invasive cleavage using foot print probes, as described here (panel B). The genotype is determined based on the Ct dye assignment. In both panels A and B, the mutant is shown in the upper left hand corner of the plot; wild-type is shown in the bottom right hand corner of the plot. In panel A, the heterogeneous result is shown in the upper right, while in panel B, the heterogeneous result is shown in the bottom left hand corner of the plot, and the no-target controls are similarly inverted (lower left for panel A and upper right hand corner of the plot for panel B).

All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described compositions and methods of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in the relevant arts are intended to be within the scope of the following claims.

WE CLAIM:

1. A method of analyzing a target nucleic acid, comprising: a) amplifying a target nucleic acid in the presence of a synthetic probe and a FEN-1 endonuclease under conditions such that said synthetic probe is cleaved during an amplification reaction to generate cleaved fragments; wherein said synthetic probe is a footprint probe comprising an analyte-specific portion and a non-target portion, wherein said non-target potion is substantially non-complementary to said target nucleic acid and wherein said analyte-specific portion is no more than 12 nucleotides in length, and contains up to 12 nucleotides that are complementary to said target nucleic acid; and b) detecting said cleaved fragments during said amplification reaction.

2. The method of claim 1, wherein said analyzing comprises detecting the presence of said target nucleic acid by detecting said cleaved fragments during said amplification reaction.

3. The method of claim 1, wherein said analyzing comprises identifying the presence of a polymorphism in said target nucleic acid.

4. The method of claim 1, wherein said analyzing comprises determining an identity of an organism from which the target nucleic acid is derived.

5. The method of claim 1, wherein said analyzing comprises detecting the amount of said target nucleic acid.

6. The method of claim 1, wherein said target nucleic acid is isolated from a sample.

7. The method of claim 6, wherein said sample is selected from the group consisting of a cell sample, a tissue sample, a fluid sample, a culture sample, and an environmental sample.

8. The method of claim 1, wherein said target nucleic acid is from an organism selected from the group consisting of: an animal, a plant, a bacterium, a virus, and a fungus.

9. The method of claim 1, wherein said target nucleic acid is DNA.

10. The method of claim 1, wherein said target nucleic acid is RNA.

11. The method of claim 10, wherein said method further comprises the step of reverse transcribing said RNA into DNA prior to or concurrent with said amplification.

12. The method of claim 1, wherein said amplifying comprises a polymerase chain reaction.

13. The method of claim 1, wherein said amplifying employs a polymerase.

14. The method of claim 13, wherein said polymerase is a thermostable polymerase.

15. The method of claim 13, wherein said polymerase lack 5' to 3' exonuclease activity.

16. The method of claim 1, wherein said FEN-1 endonuclease is a thermostable FEN-1 endonuclease.

17. The method of claim 1, wherein said FEN-1 endonuclease is from an archaeal species.

18. The method of claim 1, wherein said amplifying employs first and second primer oligonucleotides.

19. The method of claim 1, wherein a cleavage structure is formed before said probe is cleaved, wherein said cleavage structure is formed by association of said target nucleic acid with: a) said probe at a first region of said target nucleic acid; and b) a second oligonucleotide that associates with a second region of said target nucleic, wherein said second region is downstream of said first region.

20. The method of claim 19, wherein said second region is contiguous with said first region.

21. The method of claim 19, wherein at least one nucleotide at the 3' end of the second oligonucleotide, in said cleavage structure, overlaps with a region of hybridization between said probe and said target nucleic acid.

22. The method of claim 21, wherein the 3' terminal nucleotide of said second oligonucleotide is not complementary to said target nucleic acid, in said cleavage structure.

23. The method of claim 19, wherein said second oligonucleotide is also a primer used in said amplifying.

24. The method of claim 1, wherein said target binding portion of said probe contains no more than 11 nucleotides that are complementary to said target nucleic acid.

25. The method of claim 1, wherein said target binding portion of said probe contains no more than 10 nucleotides that are complementary to said target nucleic acid.

26. The method of claim 1, wherein said target binding portion of said probe contains no more than 9 nucleotides that are complementary to said target nucleic acid.

27. The method of claim 1, wherein said target binding portion of said probe contains no more than 8 nucleotides that are complementary to said target nucleic acid.

28. The method of claim 1, wherein said target binding portion of said probe contains no more than 7 nucleotides that are complementary to said target nucleic acid.

29. The method of claim 1, wherein said target binding portion of said probe contains no more than 6 nucleotides that are complementary to said target nucleic acid.

30. The method of claim 1, wherein said probe is unlabeled.

31. The method of claim 1, wherein said probe does not contain non-natural nucleotides.

32. The method of claim 1, wherein said non-target portion of said probe is at least 10 nucleotides in length.

33. The method of claim 1, wherein said detecting said cleaved fragments comprises associating one or more of said cleaved fragments with a synthetic detection oligonucleotide.

34. The method of claim 33, wherein said synthetic detection oligonucleotide has a region of self-complementarity that forms a hairpin structure.

35. The method of claim 33, wherein said synthetic detection oligonucleotide comprises a label.

36. The method of claim 35, wherein said label is a fluorescent label.

37. The method of claim 36, wherein said synthetic detection oligonucleotide further comprises a fluorescent quencher moiety.

38. The method of claim 33, wherein said cleaved fragments, when associated with said synthetic detection oligonucleotide, form a cleavage structure that is cleavable by said FEN-1 endonuclease.

39. The method of claim 38, wherein said detecting comprises cleaving said cleavage structure comprising said synthetic detection oligonucleotide to generate a detectable signal.

40. The method of claim 1, wherein a known amount of a synthetic control target nucleic acid is also analyzed.

41. The method of claim 40, wherein the analyzed synthetic control target nucleic acid is used to determine the amount of said target nucleic acid.

42. A composition comprising: a) a target nucleic acid; b) amplification primers; c) a polymerase; d) a FEN-1 endonuclease; and e) an unlabeled synthetic probe comprising an analyte-specific portion and a non-target portion, wherein said non-target potion is substantially non-complementary to said target nucleic acid and wherein said analyte-specific portion is no more than 12 nucleotides in length, and contains up to 12 nucleotides that are complementary to said target nucleic acid.

43. The composition of claim 42, wherein said composition is a reaction mixture.

44. The composition of claim 42, wherein said composition is a kit.

45. The composition of claim 44, wherein said kit comprises a plurality of containers housing one or more of the target nucleic acid, amplification primers, polymerase, FEN-1 endonuclease, and unlabeled synthetic probe.

46. The composition of claim 42, wherein said target nucleic acid is from an organism selected from the group consisting of: an animal, a plant, a bacterium, a virus, and a fungus.

47. The composition of claim 42, wherein said target nucleic is DNA.

48. The composition of claim 42, wherein said target nucleic acid RNA.

49. The composition of claim 42, further comprising a reverse transcriptase enzyme.

50. The composition of claim 42, wherein said polymerase is a thermostable polymerase.

51. The composition of claim 50, wherein said polymerase lack 5' to 3' exonuclease activity.

52. The composition of claim 42, wherein said FEN-1 endonuclease is a thermostable FEN-1 endonuclease.

53. The composition of claim 42, wherein said FEN-1 endonuclease is from an archaeal species.

54. The composition of claim 42, further comprising a second oligonucleotide configured to form a cleavage structure with said probe in the presence of said target nucleic, wherein said cleavage structure is formed by association of said target nucleic acid with: a) said probe at a first region of said target nucleic acid; and b) said second oligonucleotide that associates with a second region of said target nucleic, wherein said second region is downstream of said first region.

55. The composition of claim 54, wherein said second region is contiguous with said first region.

56. The composition of claim 54, wherein at least one nucleotide at the 3' end of the second oligonucleotide, in said cleavage structure, overlaps with a region of hybridization between said probe and said target nucleic acid.

57. The composition of claim 54, wherein the 3' terminal nucleotide of said second oligonucleotide is not complementary to said target nucleic acid, in said cleavage structure.

58. The composition of claim 42, wherein said analyte-specific portion of said probe contains no more than 11 nucleotides that are complementary to said target nucleic acid.

59. The composition of claim 42, wherein said analyte-specific portion of said probe contains no more than 10 nucleotides that are complementary to said target nucleic acid.

60. The composition of claim 42, wherein said analyte-specific portion of said probe contains no more than 9 nucleotides that are complementary to said target nucleic acid.

61. The composition of claim 42, wherein said analyte-specific portion of said probe contains no more than 8 nucleotides that are complementary to said target nucleic acid.

62. The composition of claim 42, wherein said analyte-specific portion of said probe contains no more than 7 nucleotides that are complementary to said target nucleic acid.

63. The composition of claim 42, wherein said analyte-specific portion of said probe contains no more than 6 nucleotides that are complementary to said target nucleic acid.

64. The composition of claim 42, wherein said probe is unlabeled.

65. The composition of claim 42, wherein said probe does not contain non-natural nucleotides.

66. The composition of claim 42, wherein said non-target potion portion of said probe is at least 10 nucleotides in length.

67. The composition of claim 42, further comprising a synthetic detection oligonucleotide having a region complementary to said non-target potion of said probe.

68. The composition of claim 67, wherein said synthetic detection oligonucleotide has a region of self-complementarity that forms a hairpin structure.

69. The composition of claim 67, wherein said synthetic detection oligonucleotide comprises a label.

70. The composition of claim 69, wherein said label is a fluorescent label.

71. The composition of claim 70, wherein said synthetic detection oligonucleotide further comprises a fluorescent quencher moiety.

72. A system comprising the composition of claim 42.

73. The system of claim 72, further comprising a detector.

74. The system of claim 73, wherein said detector detect fluorescent signal.

75. A composition comprising: a) a target nucleic acid; b) amplification primers; c) a polymerase; d) a FEN-1 endonuclease; and e) an unlabeled synthetic probe comprising an analyte-specific portion and a non-target portion, wherein said non-target potion is substantially non-complementary to said target nucleic acid.

76. A method of analyzing a target nucleic acid comprising: providing the composition of claim 75, forming a cleavage structure between said probe and said target nucleic acid during an amplification reaction, cleaving said probe with said

FEN-1 endonuclease to generate a cleavage product containing said non-target portion of said probe, and detecting said cleavage product.

77. A method of analyzing a target nucleic acid, comprising: a) amplifying a target nucleic acid in the presence of a synthetic probe and a FEN-1 endonuclease under conditions such that said synthetic probe is cleaved during an amplification reaction to generate cleaved fragments; wherein said synthetic probe is a footprint probe for said FEN-1 endonuclease, said footprint probe comprising an analyte-specific portion and a non-target portion, wherein said non-target potion is substantially non-complementary to said target nucleic acid and wherein, if said amplification reaction is an isothermal reaction, said analyte-specific portion of said footprint probe has a calculated T_m with said target that is at least 5° C below the temperature at which said isothermal reaction is conducted, or if said amplification reaction is a thermal cycling reaction, said analyte-specific portion of said footprint probe has a calculated T_m with said target that is at least 5° C below the lowest temperature used in said thermal cycling, and wherein said footprint probe does not comprise a minor groove binder moiety; and b) detecting said cleaved fragments during said amplification reaction.

78. The method of Claim 77, wherein said analyte-specific portion of said footprint probe has a calculated T_m with said target nucleic acid that is at least 8° C below the temperature at which said isothermal reaction is conducted, or if said amplification reaction is a thermal cycling reaction, said analyte-specific portion of said footprint probe has a calculated T_m with said target nucleic acid that is at least 8° C below the lowest temperature used in said thermal cycling.

79. The method of Claim 77, wherein said analyte-specific portion of said footprint probe has a calculated T_m with said target that is at least 10° C below the temperature at which said isothermal reaction is conducted, or if said amplification reaction is a thermal cycling reaction, said analyte-specific portion of said footprint probe has a calculated T_m with said target that is at least 10° C below the lowest temperature used in said thermal cycling.

80. A method of analyzing a target nucleic acid, comprising:

a) selecting a 5' nuclease, wherein a said 5' nuclease recognizes a structure comprising a nucleic acid duplex;

b) determining a footprint duplex length for said 5' nuclease;

c) amplifying a target nucleic acid in the presence of a synthetic footprint probe and said 5' nuclease under conditions such that said synthetic probe is cleaved during a target amplification reaction to generate cleaved fragments, wherein said synthetic probe comprises an analyte-specific portion and a non-target portion, wherein said non-target potion is substantially non-complementary to said target nucleic acid and wherein said analyte-specific portion forms a probe-target duplex with said target nucleic acid that is no longer than said footprint duplex length for said 5' nuclease enzyme;

d) detecting said cleaved fragments during said amplification reaction.

81. The method of Claim 80, wherein said target amplification reaction is a polymerase chain reaction.

82. The method of Claim 80, wherein said 5' nuclease is selected from the group consisting of a natural FEN-1 endonuclease, a modified FEN-1 endonuclease, and a chimerical protein comprising at least a portion of at least one FEN-1 endonuclease.