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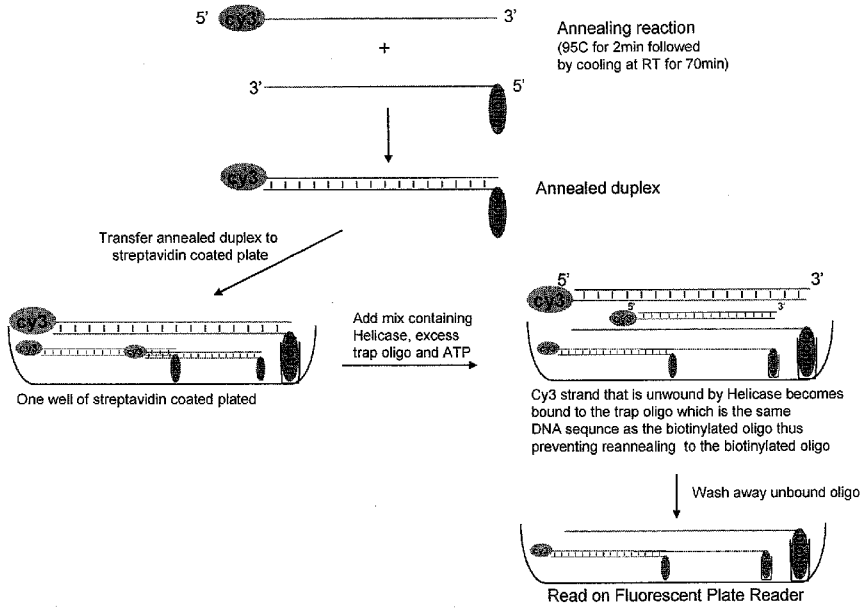
(54) Title: QUANTITATIVE HELICASE ASSAY

FIGURE 1



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(57) Abstract: Disclosed herein are methods and kits relating to detection and quantitation of helicase activity.

QUANTITATIVE HELICASE ASSAY

CROSS-REFERENCE TO RELATED PATENT APPLICATIONS

This application claims priority to U.S. Provisional Application No. 61/348,397, filed on May 26, 2010, which is hereby incorporated herein by reference in their entirety.

BACKGROUND OF THE INVENTION

Helicases are a class of enzymes that are motor proteins that move directionally along a nucleic acid phosphodiester backbone, separating two annealed nucleic acid strands (i.e. DNA, RNA, or RNA-DNA hybrid) using energy derived from ATP hydrolysis. Many cellular processes (DNA replication, transcription, translation, recombination, DNA repair, ribosome biogenesis) involve the separation of nucleic acid strands. Helicases are often utilized to separate strands of a DNA double helix or a self-annealed RNA molecule using the energy from ATP hydrolysis, a process characterized by the breaking of hydrogen bonds between annealed nucleotide bases. They move incrementally along one nucleic acid strand of the duplex with a directionality and processivity specific to each particular enzyme. There are many helicases (14 confirmed in *E. coli*, 24 in human cells) resulting from the great variety of processes in which strand separation must be catalyzed.

Studies have shown that helicases do not merely wait passively for the fork to widen, but play an active role in forcing the fork to open, thus it is an active motor unwinding its substrate.

The current technologies available that attempt to examine helicase activity include: 1) ATPase activity assays, which are quantitative but do not provide a direct method of measuring unwinding activity; and 2) radioactive helicase assays, which are not quantitative but provide a direct measurement of unwinding (or helicase activity). However, both of these assays are very laborious. What is needed in the art are efficient, uniform methods and assays for determining helicase activity.

SUMMARY OF THE INVENTION

Disclosed herein are methods of measuring helicase activity comprising providing a nucleic acid duplex comprising a first immobilizable nucleic acid strand and a labeled second strand; immobilizing the nucleic acid duplex; contacting the immobilized nucleic acid substrate duplex with a helicase to form a helicase-duplex mixture; incubating the helicase-duplex mixture with a trap oligonucleotide under conditions for helicase activity; separating

non-immobilized nucleic acids, and; detecting the label present with the immobilized nucleic acids, wherein a decrease in the amount of label present indicates helicase activity.

Also disclosed are methods for measuring helicase activity comprising: providing a nucleic acid duplex comprising a first immobilizable nucleic acid strand and a labeled second strand; immobilizing the nucleic acid duplex; contacting the immobilized nucleic acid substrate duplex with a helicase to form a helicase-duplex mixture; incubating the helicase-duplex mixture with a trap oligonucleotide under conditions for helicase activity; separating non-immobilized nucleic acids, and detecting the amount of label present with the non-immobilized nucleic acids, wherein the presence of label over background indicates helicase activity.

Further disclosed are methods of measuring helicase activity comprising: providing a nucleic acid duplex comprising a first immobilizable nucleic acid strand and a labeled second strand; contacting the immobilized nucleic acid substrate duplex with a helicase to form a helicase-duplex mixture; incubating the helicase-duplex mixture with a trap oligonucleotide under conditions for helicase activity; immobilizing the first immobilizable strand of the nucleic acid duplex, separating non-immobilized nucleic acids; and detecting the label present with the immobilized nucleic acids, wherein a decrease in the amount of label present indicates helicase activity.

Also disclosed are methods of measuring helicase activity comprising: providing a nucleic acid duplex comprising a first nucleic acid strand and a labeled second strand; contacting the nucleic acid substrate duplex of step a) with a helicase to form a helicase-duplex mixture; incubating the helicase-duplex mixture with an immobilizable trap oligonucleotide under conditions for helicase activity; immobilizing the immobilizable trap oligonucleotide; separating non-immobilized nucleic acids, and; detecting the label present with immobilized trap oligonucleotide, wherein an increase in the amount of label present indicates helicase activity.

Also disclosed are methods of measuring helicase activity comprising: providing a nucleic acid duplex comprising a first immobilizable nucleic acid strand and a labeled second strand; contacting the nucleic acid substrate duplex of step a) with a helicase to form a helicase-duplex mixture; incubating the helicase-duplex mixture with an immobilizable trap oligonucleotide under conditions for helicase activity; immobilizing the immobilizable trap oligonucleotide; separating the immobilized first immobilizable nucleic acid strand from the

immobilizable trap oligonucleotide, and; detecting the label present with the immobilized trap oligonucleotide, wherein an increase in the amount of label present indicates helicase activity.

Also disclosed are methods of measuring helicase activity comprising: providing a nucleic acid duplex comprising a first immobilizable nucleic acid strand and a labeled second strand; contacting the nucleic acid substrate duplex of step a) with a helicase to form a helicase-duplex mixture; incubating the helicase-duplex mixture with an immobilizable trap oligonucleotide under conditions for helicase activity; immobilizing the immobilizable trap oligonucleotide and the first immobilizable nucleic acid strand; separating the immobilized first immobilizable nucleic acid strand from the immobilizable trap oligonucleotide, and; detecting the label present with the immobilized first immobilizable nucleic acid strand, wherein a decrease in the amount of label present indicates helicase activity.

Further disclosed are methods for measuring helicase activity comprising: providing a nucleic acid duplex comprising a first nucleic acid strand and a second nucleic acid strand; determining the amount of label present; contacting the nucleic acid duplex with a helicase to form a helicase-duplex mixture; incubating the helicase-duplex mixture with a trap oligonucleotide under conditions for helicase activity, wherein the trap oligonucleotide comprises a fluorescent change probe portion; and detecting the label present in the mixture, wherein an increase in the amount of label present indicates helicase activity.

Further disclosed are methods for measuring helicase activity comprising: providing a nucleic acid duplex comprising a first nucleic acid strand and a labeled second nucleic acid strand; determining the amount of label present; contacting the nucleic acid duplex with a helicase to form a helicase-duplex mixture; incubating the helicase-duplex mixture with a trap oligonucleotide comprising a label quencher under conditions for helicase activity; and detecting the label present with the immobilized nucleic acids, wherein a decrease in the amount of label present indicates helicase activity.

Further disclosed are methods for measuring helicase activity comprising: providing a nucleic acid duplex comprising a first nucleic acid strand and a second nucleic acid strand, wherein the second nucleic acid strand comprises a quencher; contacting the nucleic acid duplex with a helicase to form a helicase-duplex mixture; incubating the helicase-duplex mixture with a labeled trap oligonucleotide under conditions for helicase activity; and detecting the label present with the immobilized nucleic acids, wherein a decrease in the amount of label present indicates helicase activity.

Additional advantages of the disclosed methods and compositions will be set forth in part in the description which follows, and in part will be understood from the description, or can be learned by practice of the disclosed methods and compositions. The advantages of the disclosed methods and compositions will be realized and attained by means of the elements and combinations particularly pointed out in the appended claims. It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention as claimed.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 provides a schematic representing one embodiment of the fluorescent based helicase assay of the present invention.

Figure 2 provides a schematic presenting another embodiment of the fluorescent based helicase assay of the present invention.

Figure 3 is a plot of experimental results obtained using the helicase assay. The measurements are of material bound to the streptavidin coated pate, and the decrease in fluorescence represents the loss of the complementary labeled strand due to helicase activity (unwinding of the duplex).

Figure 4 is a plot of experimental results obtained using Method 2 of the helicase assay. The results are comparable to the results in Figure 7.

Figure 5 depicts results from an experiment performance of the helicase assay at 55°C. On increasing helicase concentrations, there is a decrease in fluorescence of material bound to the plate, indicating an increase in helicase activity.

Figure 6 depicts results from an experiment performance of the helicase assay at 55°C. On increasing helicase concentrations, there is an increase in fluorescence in the supernatant, representing release of the labeled strand from the immobilized duplex.

Figure 7 shows experimental results comparing helicase activities at different temperatures using the signal: noise (S/N) ratios obtained from the pellet fraction. The greatest signal to noise ratio occurred at 65°C.

Figure 8 shows experimental results comparing helicase activities at different temperatures using the signal: noise (S/N) ratios obtained from the supernatant fraction. The greatest signal to noise ratio occurred at 60°C.

Figure 9 shows side by side comparison of the helicase assay using ATP (graph A) vs. dATP (graph B). The results show minimal difference between the two compounds as measured by the fluorescence of the pellet, which represents bound material.

Figure 10 shows side by side comparison of the helicase assay using ATP (graph A) vs. dATP (graph B). The results show minimal difference between the two compounds as measured by the fluorescence of the supernatant, which represents unbound material.

Figure 11 depicts the plot showing all the data points from the helicase assay conducted with a range of helicase concentrations and in which dATP is added individually to each well of the plate. Graph B shows the first four data points of Graph A where the linear range is in tact.

Figure 12 depicts the plot showing all the data points from the helicase assay conducted with a range of helicase concentrations and in which dATP is pre-mixed in the reaction mix. The linear range falls off quickly and proves that this would not be an effective way to add dATP to the helicase assay.

DETAILED DESCRIPTION OF THE INVENTION

The present invention comprises methods and systems directed at determining helicase activity. The disclosed method and compositions can be understood more readily by reference to the following detailed description of particular embodiments and the Example included therein and to the Figures and their previous and following description.

All patents, patent applications and publications cited herein, whether supra or infra, are hereby incorporated by reference in their entireties into this application in order to more fully describe the state of the art as known to those skilled therein as of the date of the invention described and claimed herein. It is to be understood that this invention is not limited to specific synthetic methods, or to specific recombinant biotechnology methods unless otherwise specified, or to particular reagents unless otherwise specified.

Disclosed herein are methods of directly measuring helicase activity. It is important to determine (as well as quantify) helicase activity because there are multiple methods and assays available for using helicase molecules. For example, one of skill in the art can use the disclosed methods, assays, and compositions to determine which helicase is best to use with a given assay, whether a given helicase is working optimally, as well as which reaction conditions are best for a given helicase.

For example, the methods disclosed herein can be used in conjunction with "Helicase Dependent Amplification" (HDA) to determine which helicase to use, what the optimal conditions are for a given helicase, and what optimal reaction conditions are. Helicase-Dependent Amplification (HDA) is based on the unwinding activity of a DNA helicase. HDA

uses a helicase rather than heat to separate the two strands of a DNA duplex generating single-stranded templates for the purpose of in vitro amplification of a target nucleic acid. Sequence-specific primers hybridize to the templates and are then extended by DNA polymerases to amplify the target sequence. This process repeats itself so that exponential amplification can be achieved at a single temperature.

Definitions and Nomenclature

The terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

As used in the specification and the appended claims, the singular forms "a," "an" and "the" can include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a preparation" includes mixtures of compounds, and the like. Reference to "a component" can include a single or multiple components or a mixture of components unless the context clearly dictates otherwise.

Ranges may be expressed herein as from "about" one particular value, and/or to "about" another particular value. The term "about" is used herein to mean approximately, in the region of, roughly, or around. When the term "about" is used in conjunction with a numerical range, it modifies that range by extending the boundaries above and below the numerical values set forth. In general, the term "about" is used herein to modify a numerical value above and below the stated value by a variance of 20%. When such a range is expressed, another embodiment includes from the one particular value and/or to the other particular value. Similarly, when values are expressed as approximations, by use of the antecedent "about," it will be understood that the particular value forms another embodiment. It will be further understood that the endpoints of each of the ranges are significant both in relation to the other endpoint, and independently of the other endpoint.

The word "or" as used herein means any one member of a particular list and also includes any combination of members of that list.

By "sample" is meant an animal; a tissue or organ from an animal; a cell (either within a subject, taken directly from a subject, or a cell maintained in culture or from a cultured cell line); a cell lysate (or lysate fraction) or cell extract; or a solution containing one or more molecules derived from a cell or cellular material (e.g. a polypeptide or nucleic acid), which is assayed as described herein. A sample may also be any body fluid or excretion (for example, but not limited to, blood, urine, stool, saliva, tears, bile) that contains cells or cell components.

The term "nucleic acid" refers to double stranded or single stranded DNA, RNA molecules or DNA/RNA hybrids. The phrase "nucleic acid" as used herein refers to a naturally occurring or synthetic oligonucleotide or polynucleotide, whether DNA or RNA or DNA-RNA hybrid, single-stranded or double-stranded, sense or antisense, which is capable of hybridization to a complementary nucleic acid by Watson-Crick base-pairing. Nucleic acids of the invention can also include nucleotide analogs (e.g., BrdU), and non-phosphodiester internucleoside linkages (e.g., peptide nucleic acid (PNA) or thiodiester linkages). In particular, nucleic acids can include, without limitation, DNA, RNA, cDNA, gDNA, ssDNA, dsDNA or any combination thereof. Those nucleic acids which are double stranded nucleic acid molecules may be nicked or intact. The double stranded or single stranded nucleic acid molecules may be linear or circular. The duplexes may be blunt ended or have single stranded tails. The single stranded molecules may have secondary structure in the form of hairpins or loops and stems. The nucleic acid may be isolated from a variety of sources including the environment, food, agriculture, fermentations, biological fluids such as blood, milk, cerebrospinal fluid, sputum, saliva, stool, lung aspirates, swabs of mucosal tissues or tissue samples or cells. Nucleic acid samples may obtained from cells or viruses and may include any of: chromosomal DNA, extra chromosomal DNA including plasmid DNA, recombinant DNA, DNA fragments, messenger RNA, transfer RNA, ribosomal RNA, double stranded RNA or other RNAs that occur in cells or viruses. Any of the above described nucleic acids may be subject to modification where individual nucleotides within the nucleic acid are chemically altered (for example, by methylation). Modifications may arise naturally or by *in vitro* synthesis.

The term "target nucleic acid" refers to a nucleic acid sought to be amplified, detected, or otherwise identified. For example, a "target nucleic acid" can refer to a nucleic acid strand of a nucleic acid duplex that is complementary to a trap oligonucleotide. In some aspects the target nucleic acid is *Chlamydia trachomatis* ("CT") or *Neisseria gonorrhea* ("NG") DNA or RNA.

The term "duplex" or "hybrid" refers to a nucleic acid molecule that is double stranded in whole or part. For example, a "double-stranded probe-target hybrid" refers to a nucleic acid molecule formed when an oligonucleotide probe hybridizes with a denatured target nucleic acid to form a double stranded nucleic acid molecule in the area whereby the oligonucleotide probe is specifically hybridized to the denatured target nucleic acid. A "nucleic acid duplex" refers to a nucleic acid molecule formed when two complementary

nucleic acid strands hybridize together to form a double stranded nucleic acid molecule in the region of complementarity. For example, a “nucleic acid duplex” can comprise a first immobilizable nucleic acid strand and a labeled second nucleic acid strand. The stability of a resulting hybrid or duplex can depend upon the extent of the base-pairing that occurs. The extent of base-pairing is affected by parameters such as the degree of complementarity between the probe and target molecules and the degree of stringency of the hybridization conditions. The degree of hybridization stringency is affected by parameters such as temperature, salt concentration, and the concentration of organic molecules such as formamide, and is determined by methods known to one skilled in the art.

The terms "melting," "unwinding" or "denaturing" refer to separating all or part of two complementary strands of a nucleic acid duplex or nucleic acid hybrid.

The terms "hybridization" or “hybridizes” is meant that the composition recognizes and physically interacts with another composition. For example, “hybridization” can refer to binding of an oligonucleotide primer to a region of a single-stranded nucleic acid template.

By “specifically binds” or “specifically hybridizes” is meant that the composition recognizes and physically interacts with its cognate target. For example, a primer can specifically bind to its target nucleic acid. For example, a primer specific to a sequence present in a cryptic plasmid can specifically hybridize to the cryptic plasmid and does not significantly recognize and interact with other targets or target nucleic acid sequences. The specificity of hybridization may be influenced by the length of the oligonucleotide primer, the temperature in which the hybridization reaction is performed, the ionic strength, and the pH.

By “probe,” “primer,” or “oligonucleotide” is meant a single-stranded DNA or RNA molecule of defined sequence that can base-pair to a second DNA or RNA molecule that contains a complementary sequence (the “target”). The term "primer" refers also to a single stranded nucleic acid capable of binding to a single stranded region on a target nucleic acid to facilitate polymerase dependent replication of the target nucleic acid. The stability of the resulting hybrid depends upon the extent of the base-pairing that occurs. The extent of base-pairing is affected by parameters such as the degree of complementarity between the probe and target molecules and the degree of stringency of the hybridization conditions. The degree of hybridization stringency is affected by parameters such as temperature, salt concentration, and the concentration of organic molecules such as formamide, and is determined by methods known to one skilled in the art. Probes or primers specific for target nucleic acids (for example, genes and/or mRNAs) have at least 80%-90% sequence complementarity, at least

91%-95% sequence complementarity, at least 96%-99% sequence complementarity, or at least 100% sequence complementarity to the region of the target to which they hybridize. Probes, primers, and oligonucleotides may be detectably-labeled, either radioactively, or non-radioactively, by methods well-known to those skilled in the art. Probes or oligonucleotide probes can be used for methods involving nucleic acid hybridization, such as: the described methods of forming double-stranded probe-target hybrids between an oligonucleotide probe and a denatured target nucleic acid. Primers and oligonucleotide primers can be used for methods involving nucleic acid hybridization, such as: synthesizing an extension product of an oligonucleotide primer hybridized to a target nucleic acid, which is complementary to the target nucleic acid or for amplifying a target nucleic acid in a tHDA reaction. Probes, primers and oligonucleotides can also be used for nucleic acid sequencing, reverse transcription and/or nucleic acid amplification by the polymerase chain reaction, single stranded conformational polymorphism (SSCP) analysis, restriction fragment polymorphism (RFLP) analysis, Southern hybridization, Northern hybridization, *in situ* hybridization, and electrophoretic mobility shift assay (EMSA).

By "primer set" is meant to mean at least two primers that each contain a complementary sequence to an opposite strand of the same target sequence. In a primer set, at least one of the two primers must be a "forward primer" at least one of the two primers must be a "reverse primer". A "forward primer" is a primer that is complementary to a sense strand of a target nucleic acid, wherein a "reverse primer" is a primer that is complementary to the complement of the sense strand of the target nucleic acid (also referred to as the anti-sense strand of the target nucleic acid). A primer set can be a pair of primers capable of being used in a tHDA reaction.

Similarly, by "oligonucleotide probe" is meant to mean a single-stranded DNA or RNA molecule of defined sequence that can base-pair to a second DNA or RNA molecule that contains a complementary sequence. For example, a trap oligonucleotide can be an oligonucleotide probe. In accordance with the methods described herein, one or more oligonucleotide probes can be contacted with a denatured nucleic acid sequence under conditions sufficient for the one or more polynucleotide probes to hybridize to a denatured nucleic acid duplex to form double-stranded probe-target hybrids. For example, a trap oligonucleotide can be contacted with a denatured nucleic acid duplex under conditions sufficient for the one or more trap oligonucleotides to hybridize to the denatured nucleic acid

duplex to form double-stranded trap oligo-target hybrids. In some aspects, the target nucleic acid is DNA and the oligonucleotide probes are RNA.

By “amplicon” is meant to mean pieces of DNA formed as the products of natural or artificial amplification events. For example, they can be formed via the methods described herein, tHDA, polymerase chain reactions (PCR) or ligase chain reactions (LCR), as well as by natural gene duplication.

By “specifically hybridizes” is meant that a probe, primer, or oligonucleotide recognizes and physically interacts (that is, base-pairs) with a substantially complementary nucleic acid (for example, a target nucleic acid) under high stringency conditions, and does not substantially base pair with other nucleic acids.

By “high stringency conditions” is meant conditions that allow hybridization comparable with that resulting from the use of a DNA probe of at least 40 nucleotides in length, in a buffer containing 0.5 M NaHPO4, pH 7.2, 7% SDS, 1 mM EDTA, and 1% BSA (Fraction V), at a temperature of 65°C, or a buffer containing 48% formamide, 4.8X SSC, 0.2 M Tris-Cl, pH 7.6, 1X Denhardt’s solution, 10% dextran sulfate, and 0.1% SDS, at a temperature of 42oC. Other conditions for high stringency hybridization, such as for PCR, Northern, Southern, or in situ hybridization, DNA sequencing, etc., are well-known by those skilled in the art of molecular biology. (See, for example, F. Ausubel et al., Current Protocols in Molecular Biology, John Wiley & Sons, New York, NY, 1998).

The term "accessory protein," refers to any protein capable of stimulating helicase activity. For example, *E. coli* MutL protein is an accessory protein (Yamaguchi et al. J. Biol. Chem. 273:9197 9201 (1998); Mechanic et al., J. Biol. Chem. 275:38337 38346 (2000)) for enhancing UvrD helicase melting activity. In embodiments of the method, accessory proteins can be used with selected helicases. In alternative embodiments, unwinding of nucleic acids may be achieved by helicases in the absence of accessory proteins.

In certain embodiments a "cofactor" may be used. A “cofactor” refers to small-molecule agents that are required for the helicase unwinding activity. Helicase cofactors include nucleoside triphosphate (NTP) and deoxynucleoside triphosphate (dNTP) and magnesium (or other divalent cations). For example, ATP (adenosine triphosphate) may be used as a cofactor for UvrD helicase at a concentration in the range of 0.1 100 mM and preferably in the range of 1 to 10 mM (for example 3 mM). Similarly, dTTP (deoxythymidine triphosphate) may be used as a cofactor for T7 Gp4B helicase in the range of 1 10 mM (for example 3 mM).

The term "HDA" refers to Helicase Dependent Amplification which is an *in vitro* method for amplifying nucleic acids by using a helicase preparation for unwinding a double stranded nucleic acid to generate templates for primer hybridization and subsequent primer-extension. This process utilizes two oligonucleotide primers, each hybridizing to the 3'-end of either the sense strand containing the target sequence or the anti-sense strand containing the reverse-complementary target sequence. The HDA reaction is a general method for helicase-dependent nucleic acid amplification.

"Thermophilic Helicase Dependent Amplification" or "tHDA" refers to an isothermal amplification technology that utilizes helicase to unwind double-stranded DNA, removing the need for thermocycling. tHDA is a true isothermal DNA amplification method and has a simple reaction scheme, similar to PCR. Basic tHDA is described in U.S. Patent No. 7,282,328 (Kong et al.) and is hereby incorporated by reference in its entirety.

The term "isothermal amplification" refers to amplification which occurs at a single temperature. This does not include the single brief time period (less than 15 minutes) at the initiation of amplification which may be conducted at the same temperature as the amplification procedure or at a higher temperature.

The term "helicase preparation" refers to a mixture of reagents that when combined with a DNA polymerase, a nucleic acid template, four deoxynucleotide triphosphates, and oligonucleotide primers are capable of achieving isothermal, specific nucleic acid amplification *in vitro*.

The term "trap oligonucleotide" refers to a single-stranded DNA or RNA molecule of defined sequence that can base-pair to a second DNA or RNA molecule that contains a complementary sequence. In accordance with the methods described herein, one or more trap oligonucleotides can be contacted with a denatured nucleic acid sequence under conditions sufficient for the one or more trap oligonucleotides to hybridize to the denatured target nucleic acid form double-stranded trap oligonucleotide-target hybrids. In some aspects, the trap oligonucleotide is designed to be complementary to the second nucleic acid sequence of a nucleic acid duplex. In some aspects, the trap oligonucleotide is designed to have the same sequence as the first nucleic acid sequence of a nucleic acid duplex.

The term "helicase" refers here to any enzyme capable of unwinding a double stranded nucleic acid enzymatically. For example, helicases are enzymes that are found in all organisms and in all processes that involve nucleic acid such as replication, recombination, repair, transcription, translation and RNA splicing. (Kornberg and Baker, DNA Replication,

W. H. Freeman and Company (2nd ed. (1992)), especially chapter 11). An example of a helicase that can be used with the methods and kits described herein is Tte-UvrD helicase. Further examples of helicases are given below.

The term "detection label" or "label" refers to any molecule that can be associated with a nucleic acid, directly or indirectly, and which results in a measurable, detectable signal, either directly or indirectly. Further examples of detection labels are given below.

Materials

Disclosed are materials, compositions, and components that can be used for, can be used in conjunction with, can be used in preparation for, or are products of the disclosed method and compositions. These and other materials are disclosed herein, and it is understood that when combinations, subsets, interactions, groups, etc. of these materials are disclosed that while specific reference of each various individual and collective combinations and permutation of these compounds may not be explicitly disclosed, each is specifically contemplated and described herein. For example, if an oligonucleotide probe is disclosed and discussed and a number of modifications that can be made to a number of molecules including the oligonucleotide probe are discussed, each and every combination and permutation of the oligonucleotide probe and the modifications that are possible are specifically contemplated unless specifically indicated to the contrary. Thus, if a class of molecules A, B, and C are disclosed as well as a class of molecules D, E, and F and an example of a combination molecule, A-D is disclosed, then even if each is not individually recited, each is individually and collectively contemplated. Thus, is this example, each of the combinations A-E, A-F, B-D, B-E, B-F, C-D, C-E, and C-F are specifically contemplated and should be considered disclosed from disclosure of A, B, and C; D, E, and F; and the example combination A-D. Likewise, any subset or combination of these is also specifically contemplated and disclosed. Thus, for example, the sub-group of A-E, B-F, and C-E are specifically contemplated and should be considered disclosed from disclosure of A, B, and C; D, E, and F; and the example combination A-D. This concept applies to all aspects of this disclosure including, but not limited to, steps in methods of making and using the disclosed compositions. Thus, if there are a variety of additional steps that can be performed it is understood that each of these additional steps can be performed with any specific embodiment or combination of embodiments of the disclosed methods, and that each such combination is specifically contemplated and should be considered disclosed.

A. Trap Oligonucleotides

A "trap oligonucleotide" refers to a single-stranded DNA or RNA molecule of defined sequence that can base-pair to a second DNA or RNA molecule that contains a complementary sequence (such as a single strand from the nucleic acid duplex that was separated by helicase activity). In accordance with the present invention, one or more trap oligonucleotides can be contacted with a denatured nucleic acid under conditions sufficient for the one or more trap oligonucleotides to hybridize to the denatured target nucleic acid (e.g. a second nucleic acid strand of a nucleic acid duplex) to form double-stranded trap-target hybrids. In one aspect, the target nucleic acid is DNA and the trap oligonucleotide is RNA.

In some aspects, one or more trap oligonucleotides are used (i.e. more than one probe). In one aspect, the trap oligonucleotides can be present in excess as compared to the nucleic acid duplex or in excess of the first nucleic acid strand of the nucleic acid duplex or in excess of the second nucleic acid strand of the nucleic acid duplex. One or more trap oligonucleotides can be specific for one or more nucleic acids (e.g. a second nucleic acid strand of a nucleic acid duplex).

In some aspects a trap oligonucleotide mixture comprising multiple sets of oligonucleotides can be used to simultaneously hybridize to a mixture of desired target nucleic acids. Furthermore, multiple trap oligonucleotides can be used to hybridize to different regions of the same target sequence.

The trap oligonucleotides described herein allow for sensitive detection of one or more target nucleic acid sequence (e.g. a second nucleic acid strand of a nucleic acid duplex), while also achieving excellent specificity against even very similar related target nucleic acid sequences.

One method of determining the one or more trap oligonucleotides can be found in U.S. Patent Application No. 12/426,076, which is specifically incorporated by reference in its entirety and especially for its teaching of oligonucleotide probes and methods of using and identifying the same. For example, depending on the target nucleic acid of interest, and the corresponding non-target nucleic acids, the one or more trap oligonucleotides can be prepared to have lengths sufficient to provide target-specific hybridization to the sought after target nucleic acid sequence.

For example, the one or more trap oligonucleotides can each have a length of at least about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 150, 200,

250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000 nucleotides, or any value between.

The one or more trap oligonucleotides can each have a length of at least about 15 nucleotides, illustratively, about 15 to about 1000, about 20 to about 800, about 30 to about 400, about 40 to about 200, about 50 to about 100, about 20 to about 60, about 20 to about 40, about 20 to about 20 and about 25 to about 30 nucleotides. In some aspects, the one or more trap oligonucleotides each have a length of about 25 to about 50 nucleotides. In some aspects, the probes have a length of 25 nucleotides. In some aspects, all of the probes in a set will have the same length, such as 25 nucleotides, and will have very similar melting temperatures to allow hybridization of all of the probes in the set under the same hybridization conditions.

The one or more trap oligonucleotides can each have a length of at least about 5 nucleotides, illustratively, about 5 to about 50, about 5 to about 100, about 5 to about 150, about 5 to about 200, about 5 to about 250, about 5 to about 300, about 5 to about 350, about 5 to about 400, about 5 to about 450, about 5 to about 500, about 5 to about 550, about 5 to about 600, about 5 to about 650, about 5 to about 700, about 5 to about 750, about 5 to about 800, about 5 to about 850, about 5 to about 900, about 5 to about 950, or about 5 to about 1000. In some aspects, all of the probes in a set will have the same length, such as 5 nucleotides, and will have very similar melting temperatures to allow hybridization of all of the probes in the set under the same hybridization conditions.

The one or more trap oligonucleotides can each have a length of at least about 5 nucleotides, illustratively, about 15 to about 50, about 15 to about 100, about 15 to about 150, about 15 to about 200, about 15 to about 250, about 15 to about 300, about 15 to about 350, about 15 to about 400, about 15 to about 450, about 15 to about 500, about 15 to about 550, about 15 to about 600, about 15 to about 650, about 15 to about 700, about 15 to about 750, about 15 to about 800, about 15 to about 850, about 15 to about 900, about 15 to about 950, or about 15 to about 1000. In some aspects, all of the probes in a set will have the same length, such as 15 nucleotides, and will have very similar melting temperatures to allow hybridization of all of the probes in the set under the same hybridization conditions.

The one or more trap oligonucleotides can each have a length of at least about 5 nucleotides, illustratively, about 50 to about 50, about 50 to about 100, about 50 to about 150, about 50 to about 200, about 50 to about 250, about 50 to about 300, about 50 to about 350, about 50 to about 400, about 50 to about 450, about 50 to about 500, about 50 to about 550,

about 50 to about 600, about 50 to about 650, about 50 to about 700, about 50 to about 750, about 50 to about 800, about 50 to about 850, about 50 to about 900, about 50 to about 950, or about 50 to about 1000. In some aspects, all of the probes in a set will have the same length, such as 50 nucleotides, and will have very similar melting temperatures to allow hybridization of all of the probes in the set under the same hybridization conditions.

Bioinformatics tools can also be employed to design the one or more trap oligonucleotides. For example, Oligoarray 2.0, a software program that designs specific oligonucleotides can be utilized. Oligoarray 2.0 is described by Rouillard et al. Nucleic Acids Research, 31: 3057-3062 (2003), which is incorporated herein by reference. Oligoarray 2.0 is a program which combines the functionality of BLAST (Basic Local Alignment Search Tool) and Mfold (Genetics Computer Group, Madison, Wis.). BLAST, which implements the statistical matching theory by Karlin and Altschul (Proc. Natl. Acad. Sci. USA 87:2264 (1990); Proc. Natl. Acad. Sci. USA 90:5873 (1993), is a widely used program for rapidly detecting nucleotide sequences that match a given query sequence One of ordinary skill in the art can provide a database of sequences, which are to be checked against, for example presence or absence of CT or NG. The target sequence of interest, e.g. the outer membrane protein gene for CT, can then be BLASTed against that database to search for any regions of identity. Melting temperature (Tm) and % GC can then be computed for one or more polynucleotide probes of a specified length and compared to the parameters, after which secondary structure also can be examined. Once all parameters of interest are satisfied, cross hybridization can be checked with the Mfold package, using the similarity determined by BLAST. The various programs can be adapted to determine the one or more polynucleotide probes meeting the desired specificity requirements. For example, the parameters of the program can be set to prepare polynucleotides of 25 nt length, Tm range of 55-95°C, a GC range of 35-65%, and no secondary structure or cross-hybridization at 55°C or below.

Trap oligonucleotides can also be immobilizable. For example, disclosed herein are immobilizable trap oligonucleotides. Immobilizable trap oligonucleotides can be immobilized as described elsewhere herein where methods and compositions regarding immobilization of nucleic acids and oligonucleotides are described.

Trap oligonucleotides can also be labeled. Suitable labels are described elsewhere herein where detection labels are described. For example, disclosed herein are trap oligonucleotides that comprise one or more detection labels.

Also disclosed herein are trap oligonucleotides that comprise a fluorescent change molecule or a fluorescent change probe portion. A "fluorescent change probe portion" of a nucleic acid strand (e.g. a trap oligonucleotide) is a part of or can comprise the entire length of the nucleic acid strand, so long as the fluorescent change probe portion does not interfere with the ability of the nucleic acid strand to hybridize to its complement. For example, disclosed herein are trap oligonucleotides that comprise molecular beacons, Amplifluors, FRET probes, cleavable FRET probes, TaqMan probes, scorpion primers, fluorescent triplex oligos including but not limited to triplex molecular beacons or triplex FRET probes, fluorescent water-soluble conjugated polymers, PNA probes, and QPNA probes. DxS' Scorpion Primers as described in U.S. Patent No. 7,445,900; Whitcombe, et al, 1999, Nature Biotech 17, 804-807;.Thelwell, et al. (2000), Nucleic Acid Research 29, 3752 – 3761; Solinas, et al. (2001), Nucleic Acid Research 29, 1-9. The disclosed trap oligonucleotides that comprise a fluorescent change probe portion can be used to hybridize to a target nucleic acid strand (e.g. a second nucleic acid strand of a nucleic acid duplex) such that when the trap oligonucleotide hybridizes to the target nucleic acid strand, the detection label present in the fluorescent change probe portion becomes activated, and thereby generates a detectable signal. Examples of fluorescent change probes and primer are described elsewhere herein.

Also disclosed herein are trap oligonucleotides that comprise a detectable label or a quencher. For example, the trap oligonucleotides can comprise a detectable label that can be quenched by a quencher present on a complementary nucleic acid strand. A trap oligonucleotide can also comprise a quencher that is capable of quenching a detectable label present on a complementary nucleic acid strand.

B. Nucleic Acid Duplex/Target Nucleic Acid Duplex

A "nucleic acid duplex" or a "target nucleic acid duplex" refers to a double stranded nucleic acid, comprising, in part a first nucleic acid strand and a second nucleic acid strand. For example, a "target nucleic acid duplex" can refer to a double stranded nucleic acid, comprising, in part a target nucleic acid sequence (e.g. second nucleic acid strand) and a complement of a target nucleic acid sequence (e.g. first nucleic acid strand). A target nucleic acid duplex can be created by synthesizing an extension product of an oligonucleotide primer which is complementary to the target nucleic acid to which the oligonucleotide primer is hybridized, by means of a DNA polymerase.

In some aspects, the target nucleic acid duplex is separated by helicase activity. When this occurs, two separate nucleic acid strands are provided.

In some aspects, one of the two strands of a nucleic acid duplex can be immobilized, while the other is not. In some aspects, neither strand is immobilized.

In some aspects, one of the two strands of a nucleic acid duplex can be labeled with a detectable label, while the other is not. In some aspects, neither strand is labeled.

In some aspects, one of the two strands of a nucleic acid duplex can be hybridized to a quencher, while the other is not. In some aspects, neither strand is hybridized to a quencher.

One of skill in the art will recognize the various ways in which the target nucleic acid duplex can be separated, labeled, immobilized, and separated.

C. Helicase Preparations

In the methods described herein, the helicase can be provided in a "helicase preparation." A "helicase preparation" must at least comprise a helicase. A "helicase preparation" may also comprise one or more other compositions that enhance helicase activity, including, but not limited to an energy source as a nucleotide triphosphate (NTP) or deoxynucleotide triphosphate (dNTP), a single strand DNA binding protein (SSB), salt, reagents to modify pH, other chemical reagents, such as denaturation reagents including urea and dimethyl-sulfoxide (DMSO), and other cofactors.

For example, a helicase preparation can include a helicase, an energy source such as a nucleotide triphosphate (NTP) or deoxynucleotide triphosphate (dNTP), and a single strand DNA binding protein (SSB). One or more additional reagents may also be included in the helicase preparation, where these are selected from the following: one or more additional helicases, an accessory protein, small molecules, chemical reagents and a buffer. Where a thermostable helicase is utilized in a helicase preparation, the presence of a single stranded binding protein is optional. Examples of various helicases that can be used with the methods and assays disclosed herein are given below.

Single-stranded DNA Binding Proteins

Some helicases show improved activity in the presence of single-strand binding proteins (SSB). In these circumstances, the choice of SSB is generally not limited to a specific protein. Examples of single strand binding proteins are T4 gene 32 protein, *E. coli* SSB, T7 gp2.5 SSB, phage phi29 SSB (Kornberg and Baker, supra (1992)) and truncated forms of the aforementioned. SSBs can be used with the methods and assays disclosed herein, but are not required.

Other Chemical Reagents

In addition to salt and pH, other chemical reagents, such as denaturation reagents including urea and dimethyl-sulfoxide (DMSO) can be added to partially denature or de-stabilize the duplex DNA. Again, while these components can be added to the methods and assays disclosed herein, they are not required. These other chemical reagents can also be part of the helicase preparation. Denaturation can be compared in different concentrations of denaturation reagents with or without SSB protein. In this way, chemical compounds can be identified which increase helicase efficiency and/or substitute for SSB in single-strand (ss) DNA stabilization. Most of the biomacromolecules such as nucleic acids and proteins are designed to function and/or form their native structures in a living cell at much high concentrations than in vitro experimental conditions. Polyethylene glycol (PEG) has been used to create an artificial molecular crowding condition by excluding water and creating electrostatic interaction with solute polycations (Miyoshi, et al., Biochemistry 41:15017 15024 (2002)). When PEG (7.5%) is added to a DNA ligation reaction, the reaction time is reduced to 5 min (Quick Ligation Kit, New England Biolabs, Inc. (Beverly, Mass.)). PEG has also been added into helicase unwinding assays to increase the efficiency of the reaction (Dong, et al., Proc. Natl. Acad. Sci. USA 93:14456 14461 (1996)). PEG or other molecular crowding reagents may increase the effective concentrations of enzymes and nucleic acids and thus reduce the reaction time and amount of protein concentration needed for the reaction.

Cofactors

ATP or TTP is a common energy source for highly processive helicases. On average one ATP molecule is consumed by a DNA helicases to unwind 1 to 4 base pairs (Kornberg and Baker, supra (1992)). In some aspects of the described methods, a UvrD-based helicase system had an optimal initial ATP concentration of 3 mM. To amplify a longer target, more ATP may be consumed as compared to a shorter target. In these circumstances, it may be desirable to include a pyruvate kinase-based ATP regenerating system for use with the helicase (Harmon and Kowalczykowski, Journal of Biological Chemistry 276:232 243 (2001)).

Helicases

The term "helicase" refers here to any enzyme capable of unwinding a double stranded nucleic acid enzymatically. For example, helicases are enzymes that are found in all organisms and in all processes that involve nucleic acid such as replication, recombination, repair, transcription, translation and RNA splicing. (Kornberg and Baker, DNA Replication,

W. H. Freeman and Company (2nd ed. (1992)), especially chapter 11). Any helicase that translocates along DNA or RNA in a 5' to 3' direction or in the opposite 3' to 5' direction may be used in present embodiments of the invention. This includes helicases obtained from prokaryotes, viruses, archaea, and eukaryotes or recombinant forms of naturally occurring enzymes as well as analogues or derivatives having the specified activity. Examples of naturally occurring DNA helicases, described by Kornberg and Baker in chapter 11 of their book, DNA Replication, W. H. Freeman and Company (2nd ed. (1992)), include E. coli helicase I, II, III, & IV, Rep, DnaB, PriA, PcrA, T4 Gp41helicase, T4 Dda helicase, T7 Gp4 helicases, SV40 Large T antigen, yeast RAD. Additional helicases that may be useful include RecQ helicase (Harmon and Kowalczykowski, J. Biol. Chem. 276:232 243 (2001)), thermostable UvrD helicases from T. tengcongensis and T. thermophilus (Collins and McCarthy, Extremophiles. 7:35 41. (2003)), thermostable DnaB helicase from T. aquaticus (Kaplan and Steitz, J. Biol. Chem. 274:6889 6897 (1999)), and MCM helicase from archaeal and eukaryotic organisms ((Grainge et al., Nucleic Acids Res. 31:4888 4898 (2003)).

Examples of helicases for use in present embodiments may also be found at the following web address: http://blocks.fhcrc.org (Get Blocks by Keyword: helicase). This site lists 49 Herpes helicases, 224 DnaB helicases, 250 UvrD-helicases and UvrD/Rep helicases, 276 DEAH_ATP-dependent helicases, 147 Papillom_E1 Papillomavirus helicase E1 protein, 608 Viral helicase1 Viral (superfamily 1) RNA helicases and 556 DEAD_ATP-dependent helicases. Examples of helicases that generally replicate in a 5' to 3' direction are T7 Gp4 helicase, DnaB helicase and Rho helicase, while examples of helicases that replicate in the 3'-5' direction include UvrD helicase, PcrA, Rep, NS3 RNA helicase of HCV.

Helicases use the energy of nucleoside triphosphate (for example ATP) hydrolysis to break the hydrogen bonds that hold the strands together in duplex DNA and RNA (Kornberg and Baker, DNA Replication, W. H. Freeman and Company (1992), especially chapter 11). Helicases are involved in every aspect of nucleic acid metabolism in the cell such as DNA replication, DNA repair and recombination, transcription, and RNA processing. This widespread usage may be reflected by the large numbers of helicases found in all living organisms.

Helicases have been classified according to a number of different characteristics. For example, a feature of different helicases is their oligomeric structure including helicases with single or multimeric structures. For example, one family of helicases is characterized by hexameric structures while another family consists of monomeric or dimeric helicases.

Another characteristic of helicases is the occurrence of conserved motifs. All helicases have the classical Walker A and B motifs, associated with ATP-binding and Mg^{2+}-binding (reviewed in Caruthers and McKay. Curr. Opin. Struct. Biol. 12:123 133 (2002), Soultanas and Wigley. Trends Biochem. Sci. 26:47 54 (2001)). Helicases have been classified into several superfamilies (Gorbalenya and Koonin. Curr. Opin. Struct. Biol. 3:419 429 (1993)) according to the number of helicase signature motifs and differences in the consensus sequences for motifs. Superfamilies 1 and 2 have seven characteristic helicase signature motifs and include helicases from archaea, eubacteria, eukaryotes and viruses, with helicases unwinding duplex DNA or RNA in either 3' to 5' direction or 5' to 3' direction. Examples of superfamily 1 helicases include the E. coli UvrD helicase, the T. tengcongensis UvrD helicase, and the B subunit of RecBCD. Superfamily 3 has three motifs and superfamily 4 has five motifs. Examples of superfamily 4 helicases include the T7 Gp4 helicase and DnaB helicases. A new family different from those canonical helicases is the AAA^+ family (the extended family of ATPase associated with various cellular activities).

A third type of classification relates to the unwinding directionality of helicases i.e. whether the helicase unwinds the nucleic acid duplex in a 5'-3' direction (such as T7 Gp4 helicase) or in a 3'-5' direction (such UvrD helicase) based on the strand on which the helicase binds and travels.

A fourth type of classification relates to whether a helicase preferably unwinds blunt ended nucleic acid duplexes or duplexes with forks or single stranded tails. Blunt-ended nucleic acid duplexes may not be required in the first cycle of helicase-dependent amplification but are desirable in subsequent cycles of amplification because along with the progress of the amplification reaction the blunt-ended target fragment becomes the dominant species. These blunt-ended target nucleic acids form template substrates for subsequent rounds of amplification.

In general, the temperature for suitable denaturation may occur over a range of temperatures, for example 20°C to 75°C. For example, temperature may be selected according to which helicase is selected for the melting process. Tests to determine optimum temperatures of a selected helicase can be determined by routine experimentation by varying the temperature of the reaction mixture and comparing products.

Denaturation of nucleic acid hybrids or duplexes can be accelerated by using a thermostable helicase preparation under incubation conditions that include higher temperature for example in a range of 45°C to 75. °C.

In certain aspects, it may be desirable to utilize a plurality of different helicase enzymes. For example, a helicase that has low processivity but is able to melt blunt-ended DNA may be combined with a second helicase that has great processivity but recognizes single-stranded tails at the border of duplex region for the initiation of unwinding. In this example, the first helicase initially separates the blunt ends of a long nucleic acid duplex generating 5' and 3' single-stranded tails and then dissociates from that substrate due to its limited processivity. This partially unwound substrate is subsequently recognized by the second helicase that then continues the unwinding process with superior processivity. In this way, a long target in a nucleic acid duplex may be unwound by the use of a helicase preparation containing a plurality of helicases.

D. Detection Labels

To aid in detection and quantitation of helicase activity, detection labels can be utilized. Detection labels can be directly incorporated into trap oligonucleotides, into one or both of the nucleic acid duplex nucleic acid strands. As used herein, a "detection label" is any molecule that can be associated with an oligonucleotide (e.g. trap oligonucleotides or one or both of the nucleic acid duplex nucleic acid strands) directly or indirectly, and which results in a measurable, detectable signal, either directly or indirectly. Many such labels for incorporation into nucleic acids or coupling to nucleic acids are known to those of skill in the art. Examples of detection labels suitable for use in the disclosed method are radioactive isotopes, fluorescent molecules, phosphorescent molecules, enzymes, antibodies, and ligands. Fluorescent labels, especially in the context of fluorescent change probes and primers, are useful for real-time detection of amplification.

Examples of suitable fluorescent labels include fluorescein isothiocyanate (FITC), 5,6-carboxymethyl fluorescein, Texas red, nitrobenz-2-oxa-1,3-diazol-4-yl (NBD), coumarin, dansyl chloride, rhodamine, amino-methyl coumarin (AMCA), Eosin, Erythrosin, BODIPY®, Cascade Blue®, Oregon Green®, pyrene, lissamine, xanthenes, acridines, oxazines, phycoerythrin, macrocyclic chelates of lanthanide ions such as quantum dyeTM, fluorescent energy transfer dyes, such as thiazole orange-ethidium heterodimer, and the cyanine dyes Cy3, Cy3.5, Cy5, Cy5.5 and Cy7. Examples of other specific fluorescent labels include 3-Hydroxypyrene 5,8,10-Tri Sulfonic acid, 5-Hydroxy Tryptamine (5-HT), Acid Fuchsin, Alizarin Complexon, Alizarin Red, Allophycocyanin, Aminocoumarin, Anthroyl Stearate, Astrazon Brilliant Red 4G, Astrazon Orange R, Astrazon Red 6B, Astrazon Yellow 7 GLL, Atabrine, Auramine, Aurophosphine, Aurophosphine G, BAO 9

(Bisaminophenyloxadiazole), BCECF, Berberine Sulphate, Bisbenzamide, Blancophor FFG Solution, Blancophor SV, Bodipy F1, Brilliant Sulphoflavin FF, Calcien Blue, Calcium Green, Calcofluor RW Solution, Calcofluor White, Calcophor White ABT Solution, Calcophor White Standard Solution, Carbostyryl, Cascade Yellow, Catecholamine, Chinacrine, Coriphosphine O, Coumarin-Phalloidin, CY3.1 8, CY5.1 8, CY7, Dans (1-Dimethyl Amino Naphaline 5 Sulphonic Acid), Dansa (Diamino Naphtyl Sulphonic Acid), Dansyl NH-CH3, Diamino Phenyl Oxydiazole (DAO), Dimethylamino-5-Sulphonic acid, Dipyrrometheneboron Difluoride, Diphenyl Brilliant Flavine 7GFF, Dopamine, Erythrosin ITC, Euchrysin, FIF (Formaldehyde Induced Fluorescence), Flazo Orange, Fluo 3, Fluorescamine, Fura-2, Genacryl Brilliant Red B, Genacryl Brilliant Yellow 10GF, Genacryl Pink 3G, Genacryl Yellow 5GF, Gloxalic Acid, Granular Blue, Haematoporphyrin, Indo-1, Intrawhite Cf Liquid, Leucophor PAF, Leucophor SF, Leucophor WS, Lissamine Rhodamine B200 (RD200), Lucifer Yellow CH, Lucifer Yellow VS, Magdala Red, Marina Blue, Maxilon Brilliant Flavin 10 GFF, Maxilon Brilliant Flavin 8 GFF, MPS (Methyl Green Pyronine Stilbene), Mithramycin, NBD Amine, Nitrobenzoxadidole, Noradrenaline, Nuclear Fast Red, Nuclear Yellow, Nylosan Brilliant Flavin E8G, Oxadiazole, Pacific Blue, Pararosaniline (Feulgen), Phorwite AR Solution, Phorwite BKL, Phorwite Rev, Phorwite RPA, Phosphine 3R, Phthalocyanine, Phycoerythrin R, Polyazaindacene Pontochrome Blue Black, Porphyrin, Primuline, Procion Yellow, Pyronine, Pyronine B, Pyrozal Brilliant Flavin 7GF, Quinacrine Mustard, Rhodamine 123, Rhodamine 5 GLD, Rhodamine 6G, Rhodamine B, Rhodamine B 200, Rhodamine B Extra, Rhodamine BB, Rhodamine BG, Rhodamine WT, Serotonin, Sevron Brilliant Red 2B, Sevron Brilliant Red 4G, Sevron Brilliant Red B, Sevron Orange, Sevron Yellow L, SITS (Primuline), SITS (Stilbene Isothiosulphonic acid), Stilbene, Snarf 1, sulpho Rhodamine B Can C, Sulpho Rhodamine G Extra, Tetracycline, Thiazine Red R, Thioflavin S, Thioflavin TCN, Thioflavin 5, Thiolyte, Thiozol Orange, Tinopol CBS, True Blue, Ultralite, Uranine B, Uvitex SFC, Xylene Orange, and XRITC.

Examples of fluorescent labels include fluorescein (5-carboxyfluorescein-N-hydroxysuccinimide ester), rhodamine (5,6-tetramethyl rhodamine), and the cyanine dyes Cy3, Cy3.5, Cy5, Cy5.5 and Cy7. The absorption and emission maxima, respectively, for these fluors are: FITC (490 nm; 520 nm), Cy3 (554 nm; 568 nm), Cy3.5 (581 nm; 588 nm), Cy5 (652 nm: 672 nm), Cy5.5 (682 nm; 703 nm) and Cy7 (755 nm; 778 nm), thus allowing their simultaneous detection. Other examples of fluorescein dyes include 6-carboxyfluorescein (6-FAM), 2',4',1,4,-tetrachlorofluorescein (TET), 2',4',5',7',1,4-

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), and 2'-chloro-7'-phenyl-1,4-dichloro-6-carboxyfluorescein (VIC). Fluorescent labels can be obtained from a variety of commercial sources, including Amersham Pharmacia Biotech, Piscataway, NJ; Molecular Probes, Eugene, OR; and Research Organics, Cleveland, Ohio.

Additional labels of interest include those that provide for signal only when the oligonucleotide with which they are associated is specifically bound to a target molecule (e.g. one of he nucleic acid strands of a nucleic acid duplex), where such labels include: "molecular beacons" as described in Tyagi & Kramer, Nature Biotechnology (1996) 14:303 and EP 0 070 685 B1. Other labels of interest include those described in U.S. Pat. No. 5,563,037 and PCT Applications WO 97/17471 and WO 97/17076.

Labeled nucleotides are another form of detection label since they can be directly incorporated into nucleic acid. Examples of detection labels that can be incorporated into nucleic acids include nucleotide analogs such as BrdUrd (5-bromodeoxyuridine, Hoy and Schimke, Mutation Research 290:217-230 (1993)), aminoallyldeoxyuridine (Henegariu et al., Nature Biotechnology 18:345-348 (2000)), 5-methylcytosine (Sano et al., Biochim. Biophys. Acta 951:157-165 (1988)), bromouridine (Wansick et al., J. Cell Biology 122:283-293 (1993)) and nucleotides modified with biotin (Langer et al., Proc. Natl. Acad. Sci. USA 78:6633 (1981)) or with suitable haptens such as digoxygenin (Kerkhof, Anal. Biochem. 205:359-364 (1992)). Suitable fluorescence-labeled nucleotides are Fluorescein-isothiocyanate-dUTP, Cyanine-3-dUTP and Cyanine-5-dUTP (Yu et al., Nucleic Acids Res., 22:3226-3232 (1994)). A preferred nucleotide analog detection label for DNA is BrdUrd (bromodeoxyuridine, BrdUrd, BrdU, BUdR, Sigma-Aldrich Co). Other preferred nucleotide analogs for incorporation of detection label into DNA are AA-dUTP (aminoallyl-deoxyuridine triphosphate, Sigma-Aldrich Co.), and 5-methyl-dCTP (Roche Molecular Biochemicals). A preferred nucleotide analog for incorporation of detection label into RNA is biotin-16-UTP (biotin-16-uridine-5'-triphosphate, Roche Molecular Biochemicals). Fluorescein, Cy3, and Cy5 can be linked to dUTP for direct labeling. Cy3.5 and Cy7 are available as avidin or anti-digoxygenin conjugates for secondary detection of biotin- or digoxygenin-labeled probes.

Detection labels that are incorporated into nucleic acid, such as biotin, can be subsequently detected using sensitive methods well-known in the art. For example, biotin can be detected using streptavidin-alkaline phosphatase conjugate (Tropix, Inc.), which is

bound to the biotin and subsequently detected by chemiluminescence of suitable substrates (for example, chemiluminescent substrate CSPD: disodium, 3-(4-methoxyspiro-[1,2,-dioxetane-3-2'-(5'-chloro)tricyclo [3.3.1.13,7]decane]-4-yl) phenyl phosphate; Tropix, Inc.). Labels can also be enzymes, such as alkaline phosphatase, soybean peroxidase, horseradish peroxidase and polymerases, that can be detected, for example, with chemical signal amplification or by using a substrate to the enzyme which produces light (for example, a chemiluminescent 1,2-dioxetane substrate) or fluorescent signal. Labels can also be the disclosed reagent compositions.

Molecules that combine two or more of these detection labels are also considered detection labels. Any of the known detection labels can be used with the disclosed probes, tags, and method to label and detect target nucleic acid using the disclosed method. Methods for detecting and measuring signals generated by detection labels are also known to those of skill in the art. For example, radioactive isotopes can be detected by scintillation counting or direct visualization; fluorescent molecules can be detected with fluorescent spectrophotometers; phosphorescent molecules can be detected with a spectrophotometer or directly visualized with a camera; enzymes can be detected by detection or visualization of the product of a reaction catalyzed by the enzyme; antibodies can be detected by detecting a secondary detection label coupled to the antibody. As used herein, detection molecules are molecules which interact with nucleic acid and to which one or more detection labels are coupled.

Fluorescent Change Molecules

Fluorescent change molecules refer to all nucleic acid labels that involve a change in fluorescence intensity or wavelength based on a change in the form or conformation of the nucleic acid to be detected, assayed or replicated. Examples of fluorescent change molecules include molecular beacons, Amplifluors, FRET probes, cleavable FRET probes, TaqMan probes, scorpion primers, fluorescent triplex oligos including but not limited to triplex molecular beacons or triplex FRET probes, fluorescent water-soluble conjugated polymers, PNA probes, and QPNA probes. DxS' Scorpion Primers as described in U.S. Patent No. 7,445,900; Whitcombe, et al, 1999, Nature Biotech 17, 804-807;.Thelwell, et al. (2000), Nucleic Acid Research 29, 3752 – 3761; Solinas, et al. (2001), Nucleic Acid Research 29, 1-9, all of which are hereby incorporated by reference for their teaching of Scorpion primers, can also be used.

Fluorescent change molecules can be classified according to their structure and/or function. Fluorescent change probes include hairpin quenched probes, cleavage quenched probes, cleavage activated probes, and fluorescent activated probes. The use of several types of fluorescent change probes and primers are reviewed in Schweitzer and Kingsmore, Curr. Opin. Biotech. 12:21-27 (2001). Hall et al., Proc. Natl. Acad. Sci. USA 97:8272-8277 (2000), describe the use of fluorescent change probes with Invader assays.

Hairpin quenched probes are probes that when not bound to a target sequence form a hairpin structure (and, typically, a loop) that brings a fluorescent label and a quenching moiety into proximity such that fluorescence from the label is quenched. When the probe binds to a target sequence, the stem is disrupted, the quenching moiety is no longer in proximity to the fluorescent label and fluorescence increases. Examples of hairpin quenched probes are molecular beacons, fluorescent triplex oligos, triplex molecular beacons, triplex FRET probes, and QPNA probes.

Cleavage activated probes are probes where fluorescence is increased by cleavage of the probe. Cleavage activated probes can include a fluorescent label and a quenching moiety in proximity such that fluorescence from the label is quenched. When the probe is clipped or digested (typically by the 5'-3' exonuclease activity of a polymerase during amplification), the quenching moiety is no longer in proximity to the fluorescent label and fluorescence increases. TaqMan probes (Holland et al., Proc. Natl. Acad. Sci. USA 88:7276-7280 (1991)) are an example of cleavage activated probes.

Modified TaqMan Probes

Also described herein are modified TaqMan probes. TaqMan probes are fluorescent change probes that involve a change in fluorescence intensity or wavelength based on a change in the form or conformation of the probe or primer and nucleic acid to be detected, assayed or replicated. For example, described herein are modified TaqMan probes that are comprised of a sequence that is complementary to a target sequence (e.g. one of the two nucleic acid strands of a nucleic acid duplex) and additionally have a short tail at either the 3' or 5'- end of the modified TaqMan probe complementary to the 5' or 3'- end modified TaqMan probe, respectively. The short tail can assist in forming a stem-loop structure when the modified TaqMan probe is not hybridized to a target nucleic acid. The non-tail portion of the modified TaqMan probe is complementary to the target nucleic acid and is capable of hybridizing to a target nucleic acid. In some aspects, the short tail of the modified TaqMan probe can be complementary or non-complementary to the target.

The modified TaqMan probes can be used as a detection label in the methods described herein. The modified TaqMan probes are an improvement of molecular beacons and existing TaqMan probes as they are easier to open than a molecular beacon and the modified TaqMan probes quench more predictably and efficiently than existing TaqMan probes.

Cleavage quenched probes can also be used in the methods described herein. Cleavage quenched probes are probes where fluorescence is decreased or altered by cleavage of the probe. Cleavage quenched probes can include an acceptor fluorescent label and a donor moiety such that, when the acceptor and donor are in proximity, fluorescence resonance energy transfer from the donor to the acceptor causes the acceptor to fluoresce. The probes are thus fluorescent, for example, when hybridized to a target sequence. When the probe is clipped or digested, the donor moiety is no longer in proximity to the acceptor fluorescent label and fluorescence from the acceptor decreases. If the donor moiety is itself a fluorescent label, it can release energy as fluorescence (typically at a different wavelength than the fluorescence of the acceptor) when not in proximity to an acceptor. The overall effect would then be a reduction of acceptor fluorescence and an increase in donor fluorescence. Donor fluorescence in the case of cleavage quenched probes is equivalent to fluorescence generated by cleavage activated probes with the acceptor being the quenching moiety and the donor being the fluorescent label. Cleavable FRET (fluorescence resonance energy transfer) probes are an example of cleavage quenched probes.

Fluorescent activated probes are probes or pairs of probes where fluorescence is increased or altered by hybridization of the probe to a target sequence. Fluorescent activated probes can include an acceptor fluorescent label and a donor moiety such that, when the acceptor and donor are in proximity (when the probes are hybridized to a target sequence), fluorescence resonance energy transfer from the donor to the acceptor causes the acceptor to fluoresce. Fluorescent activated probes are typically pairs of probes designed to hybridize to adjacent sequences such that the acceptor and donor are brought into proximity. Fluorescent activated probes can also be single probes containing both a donor and acceptor where, when the probe is not hybridized to a target sequence, the donor and acceptor are not in proximity but where the donor and acceptor are brought into proximity when the probe hybridized to a target sequence. This can be accomplished, for example, by placing the donor and acceptor on opposite ends a the probe and placing target complement sequences at each end of the probe where the target complement sequences are complementary to adjacent sequences in a

target sequence. If the donor moiety of a fluorescent activated probe is itself a fluorescent label, it can release energy as fluorescence (typically at a different wavelength than the fluorescence of the acceptor) when not in proximity to an acceptor (that is, when the probes are not hybridized to the target sequence). When the probes hybridize to a target sequence, the overall effect would then be a reduction of donor fluorescence and an increase in acceptor fluorescence. FRET probes are an example of fluorescent activated probes.

E. Solid Supports

Solid supports are solid-state substrates or supports with which nucleic acids (or other components used in, or produced by, the disclosed method) can be associated. The nucleic acids described herein can be associated with solid supports directly of indirectly to immobilize nucleic acids and/or oligonucleotides. For example, one or more of the nucleic acid strands of a nucleic acid duplex and/or a trap oligonucleotides can be associated with solid supports directly or indirectly. Oligonucleotides can be bound to the surface of a solid support or associated with oligonucleotide probes immobilized on solid supports. An array detector is a solid support to which multiple oligonucleotide probes can be coupled in an array, grid, or other organized pattern. Target arrays are arrays of target nucleic acids attached to solid supports. Oligonucleotide probe arrays are arrays of oligonucleotide probes attached to a solid support.

Solid-state substrates for use in solid supports can include any solid material with which components can be associated, directly or indirectly. This includes materials such as acrylamide, agarose, cellulose, nitrocellulose, glass, gold, polystyrene, polyethylene vinyl acetate, polypropylene, polymethacrylate, polyethylene, polyethylene oxide, polysilicates, polycarbonates, teflon, fluorocarbons, nylon, silicon rubber, polyanhydrides, polyglycolic acid, polylactic acid, polyorthoesters, functionalized silane, polypropylfumerate, collagen, glycosaminoglycans, polyamino acids or magnets. Solid-state substrates can have any useful form including thin film, membrane, bottles, dishes, fibers, woven fibers, shaped polymers, particles, beads, microparticles, or a combination. Solid-state substrates and solid supports can be porous or non-porous. A chip is a rectangular or square small piece of material. A useful form for a solid-state substrate is a microtiter dish. In some embodiments, a multiwell glass slide can be employed.

An array can include a plurality of components (such as target nucleic acids, target samples, detection labels, trap oligonucleotides) immobilized at identified or predefined locations on the solid support. Each predefined location on the solid support generally has

one type of component (that is, all the components at that location are the same). Alternatively, multiple types of components can be immobilized in the same predefined location on a solid support. Each location will have multiple copies of the given components. The spatial separation of different components on the solid support allows separate detection and identification of nucleic acids. Although useful, it is not required that the solid support be a single unit or structure. Sets of components can be distributed over any number of solid supports. For example, at one extreme, each component can be immobilized in a separate reaction tube or container, or on separate beads or microparticles.

Methods for immobilization of oligonucleotides to solid-state substrates are well established. Oligonucleotides, including trap oligonucleotides, can be coupled to substrates using established coupling methods. For example, suitable attachment methods are described by Pease et al., Proc. Natl. Acad. Sci. USA 91(11):5022-5026 (1994), and Khrapko et al., Mol Biol (Mosk) (USSR) 25:718-730 (1991). A method for immobilization of 3'-amine oligonucleotides on casein-coated slides is described by Stimpson et al., Proc. Natl. Acad. Sci. USA 92:6379-6383 (1995). A useful method of attaching oligonucleotides to solid-state substrates is described by Guo et al., Nucleic Acids Res. 22:5456-5465 (1994).

Methods for immobilizing oligonucleotides to solid-state substrates are well established. Immobilization can be accomplished by attachment, for example, to aminated surfaces, carboxylated surfaces or hydroxylated surfaces using standard immobilization chemistries. Examples of attachment agents are cyanogen bromide, succinimide, aldehydes, tosyl chloride, avidin-biotin, photocrosslinkable agents, epoxides and maleimides. Another example of an attachment agent is glutaraldehyde. These and other attachment agents, as well as methods for their use in attachment, are described in Protein immobilization: fundamentals and applications, Richard F. Taylor, ed. (M. Dekker, New York, 1991), Johnstone and Thorpe, Immunochemistry In Practice (Blackwell Scientific Publications, Oxford, England, 1987) pages 209-216 and 241-242, and Immobilized Affinity Ligands, Craig T. Hermanson et al., eds. (Academic Press, New York, 1992).

Each of the components immobilized on the solid support can be located in a different predefined region of the solid support. The different locations can be different reaction chambers. Each of the different predefined regions can be physically separated from each other of the different regions. The distance between the different predefined regions of the solid support can be either fixed or variable. For example, in an array, each of the components can be arranged at fixed distances from each other, while components associated

with beads will not be in a fixed spatial relationship. In particular, the use of multiple solid support units (for example, multiple beads) will result in variable distances.

Components can be associated or immobilized on a solid support at any density. Components can be immobilized to the solid support at a density exceeding 400 different components per cubic centimeter. Arrays of components can have any number of components. For example, an array can have at least 1,000 different components immobilized on the solid support, at least 10,000 different components immobilized on the solid support, at least 100,000 different components immobilized on the solid support, or at least 1,000,000 different components immobilized on the solid support.

E. Solid-State Detectors

Solid-state detectors are solid supports to which oligonucleotides, target nucleic acids, trap oligonucleotides, one or more nucleic acids strands of a nucleic acid duplex have been coupled. A preferred form of solid-state detector is an array detector. An array detector is a solid-state detector to which multiple different oligonucleotides or nucleic acids have been coupled in an array, grid, or other organized pattern.

Solid-state substrates for use in solid-state detectors can include any solid material to which oligonucleotides can be coupled. This includes materials such as acrylamide, agarose, cellulose, nitrocellulose, glass, gold, polystyrene, polyethylene vinyl acetate, polypropylene, polymethacrylate, polyethylene, polyethylene oxide, polysilicates, polycarbonates, teflon, fluorocarbons, nylon, silicon rubber, polyanhydrides, polyglycolic acid, polylactic acid, polyorthoesters, functionalized silane, polypropylfumerate, collagen, glycosaminoglycans, and polyamino acids. Solid-state substrates can have any useful form including thin film, membrane, bottles, dishes, fibers, woven fibers, shaped polymers, particles, beads, microparticles, or a combination. Solid-state substrates and solid supports can be porous or non-porous. A chip is a rectangular or square small piece of material. Preferred forms for solid-state substrates are thin films, beads, or chips. A useful form for a solid-state substrate is a microtiter dish. In some embodiments, a multiwell glass slide can be employed.

Capture oligonucleotides immobilized on a solid-state substrate allow capture of double-stranded probe-target hybrids or their amplification targets on a solid-state detector. Such capture provides a convenient means of washing away reaction components that might interfere with subsequent method steps. By attaching different capture oligonucleotides to different regions of a solid-state detector, different products can be captured at different, and therefore diagnostic, locations on the solid-state detector. For example, in a multiplex assay,

oligonucleotides specific for numerous different target nucleic acids (each representing a different target nucleic acid sequence amplified via a different set of primers) can be immobilized in an array, each in a different location. Capture and detection will occur only at those array locations corresponding to amplified nucleic acids for which the corresponding target nucleic acid sequences were present in a sample.

F. Oligonucleotide Synthesis

Trap oligonucleotides or any other oligonucleotides can be synthesized using established oligonucleotide synthesis methods. Methods to produce or synthesize oligonucleotides are well known. Such methods can range from standard enzymatic digestion followed by nucleotide fragment isolation (see for example, Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Edition (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989) Chapters 5, 6) to purely synthetic methods, for example, by the cyanoethyl phosphoramidite method. Solid phase chemical synthesis of DNA fragments is routinely performed using protected nucleoside cyanoethyl phosphoramidites (S. L. Beaucage et al. (1981) Tetrahedron Lett. 22:1859). In this approach, the 3'-hydroxyl group of an initial 5'-protected nucleoside is first covalently attached to the polymer support (R. C. Pless et al. (1975) Nucleic Acids Res. 2:773 (1975)). Synthesis of the oligonucleotide then proceeds by deprotection of the 5'-hydroxyl group of the attached nucleoside, followed by coupling of an incoming nucleoside-3'-phosphoramidite to the deprotected hydroxyl group (M. D. Matteucci et a. (1981) J. Am. Chem. Soc. 103:3185). The resulting phosphite triester is finally oxidized to a phosphorotriester to complete the internucleotide bond (R. L. Letsinger et al. (1976) J. Am. Chem. Soc. 9:3655). Alternatively, the synthesis of phosphorothioate linkages can be carried out by sulfurization of the phosphite triester. Several chemicals can be used to perform this reaction, among them 3H-1,2-benzodithiole-3-one, 1,1-dioxide (R.P. Iyer, W. Egan, J.B. Regan, and S.L. Beaucage, J. Am. Chem. Soc., 1990, 112, 1253-1254). The steps of deprotection, coupling and oxidation are repeated until an oligonucleotide of the desired length and sequence is obtained. Other methods exist to generate oligonucleotides such as the H-phosphonate method (Hall et al, (1957) J. Chem. Soc., 3291-3296) or the phosphotriester method as described by Ikuta et al., Ann. Rev. Biochem. 53:323-356 (1984), (phosphotriester and phosphite-triester methods), and Narang et al., Methods Enzymol., 65:610-620 (1980), (phosphotriester method). Protein nucleic acid molecules can be made using known methods such as those described by Nielsen

et al., Bioconjug. Chem. 5:3-7 (1994). Other forms of oligonucleotide synthesis are described in U.S. Patent No. 6,294,664 and U.S. Patent No. 6,291,669.

The nucleotide sequence of an oligonucleotide is generally determined by the sequential order in which subunits of subunit blocks are added to the oligonucleotide chain during synthesis. Each round of addition can involve a different, specific nucleotide precursor, or a mixture of one or more different nucleotide precursors. In general, degenerate or random positions in an oligonucleotide can be produced by using a mixture of nucleotide precursors representing the range of nucleotides that can be present at that position. Thus, precursors for A and T can be included in the reaction for a particular position in an oligonucleotide if that position is to be degenerate for A and T. Precursors for all four nucleotides can be included for a fully degenerate or random position. Completely random oligonucleotides can be made by including all four nucleotide precursors in every round of synthesis. Degenerate oligonucleotides can also be made having different proportions of different nucleotides. Such oligonucleotides can be made, for example, by using different nucleotide precursors, in the desired proportions, in the reaction.

Many of the oligonucleotides described herein are designed to be complementary to certain portions of other oligonucleotides or nucleic acids such that stable hybrids can be formed between them. The stability of these hybrids can be calculated using known methods such as those described in Lesnick and Freier, Biochemistry 34:10807-10815 (1995), McGraw et al., Biotechniques 8:674-678 (1990), and Rychlik et al., Nucleic Acids Res. 18:6409-6412 (1990).

So long as their relevant function is maintained, oligonucleotides can be made up of or include modified nucleotides (nucleotide analogs). Many modified nucleotides are known and can be used in oligonucleotides. A nucleotide analog is a nucleotide which contains some type of modification to either the base, sugar, or phosphate moieties. Modifications to the base moiety would include natural and synthetic modifications of A, C, G, and T/U as well as different purine or pyrimidine bases, such as uracil-5-yl, hypoxanthin-9-yl (I), and 2-aminoadenin-9-yl. A modified base includes but is not limited to 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other

8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Additional base modifications can be found for example in U.S. Pat. No. 3,687,808, Englisch et al., Angewandte Chemie, International Edition, 1991, 30, 613, and Sanghvi, Y. S., Chapter 15, Antisense Research and Applications, pages 289-302, Crooke, S. T. and Lebleu, B. ed., CRC Press, 1993. Certain nucleotide analogs, such as 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine can increase the stability of duplex formation. Other modified bases are those that function as universal bases. Universal bases include 3-nitropyrrole and 5-nitroindole. Universal bases substitute for the normal bases but have no bias in base pairing. That is, universal bases can base pair with any other base. Base modifications often can be combined with for example a sugar modification, such as 2'-O-methoxyethyl, to achieve unique properties such as increased duplex stability. There are numerous United States patents such as 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; and 5,681,941, which detail and describe a range of base modifications. Each of these patents is herein incorporated by reference in its entirety, and specifically for their description of base modifications, their synthesis, their use, and their incorporation into oligonucleotides and nucleic acids.

G. Kits

The materials described above as well as other materials can be packaged together in any suitable combination as a kit useful for performing, or aiding in the performance of, the disclosed method. It is useful if the kit components in a given kit are designed and adapted for use together in the disclosed method. For example, disclosed is a kit comprising a nucleic acid duplex comprising two strands of nucleic acid where the first strand is immobilizable and the second strand is detectably labeled, a trap oligonucleotide, an immobilization substrate, wash buffers and a helicase. The kits also can contain, for example, nucleotides, buffers, helicase, accessory proteins, topoisomerases, or a combination.

H. Mixtures

Disclosed are mixtures formed by preparing the disclosed composition or performing or preparing to perform the disclosed methods. Whenever the method involves mixing or bringing into contact compositions or components or reagents, performing the method creates

a number of different mixtures. For example, if the method includes 3 mixing steps, after each one of these steps a unique mixture is formed if the steps are performed separately. In addition, a mixture is formed at the completion of all of the steps regardless of how the steps were performed. The present disclosure contemplates these mixtures, obtained by the performance of the disclosed methods as well as mixtures containing any disclosed reagent, composition, or component, for example, disclosed herein.

I. Systems

Disclosed are systems useful for performing, or aiding in the performance of, the disclosed method. Also disclosed are systems for producing reagent compositions. Systems generally comprise combinations of articles of manufacture such as structures, machines, devices, and the like, and compositions, compounds, materials, and the like. Such combinations that are disclosed or that are apparent from the disclosure are contemplated. For example, disclosed and contemplated are systems comprising solid supports and reagent compositions.

J. Data Structures and Computer Control

Disclosed are data structures used in, generated by, or generated from, the disclosed method. Data structures generally are any form of data, information, and/or objects collected, organized, stored, and/or embodied in a composition or medium. A target fingerprint stored in electronic form, such as in RAM or on a storage disk, is a type of data structure.

The disclosed method, or any part thereof or preparation therefore, can be controlled, managed, or otherwise assisted by computer control. Such computer control can be accomplished by a computer controlled process or method, can use and/or generate data structures, and can use a computer program. Such computer control, computer controlled processes, data structures, and computer programs are contemplated and should be understood to be disclosed herein.

K. Uses

The disclosed compositions and methods are applicable to numerous areas including, but not limited to, detection and/or analysis of target nucleic acids, disease detection, protein detection, nucleic acid mapping, mutation detection, gene discovery, gene mapping, determination of helicase activity, and agricultural research. Particularly useful are assays to measure or determine helicase activity. For example, one of skill in the art can use the disclosed methods, assays, and compositions to determine which helicase is best to use with a given assay, whether a given helicase is working optimally, as well as which reaction

conditions are best for a given helicase. Other uses include, for example, detection of target nucleic acids in samples, mutation detection; detection of sexually transmitted diseases such as Chlamydia trachomatis (CT) and Neisseria gonorrhoeae (NG).

Methods

Disclosed herein are methods of measuring helicase activity comprising providing a nucleic acid duplex comprising a first immobilizable nucleic acid strand and a labeled second strand; immobilizing the nucleic acid duplex; contacting the immobilized nucleic acid substrate duplex with a helicase to form a helicase-duplex mixture; incubating the helicase-duplex mixture with a trap oligonucleotide under conditions for helicase activity; separating non-immobilized nucleic acids, and; detecting the label present with the immobilized nucleic acids, wherein a decrease in the amount of label present indicates helicase activity.

In one example, the amount of label present can be determined after the nucleic acid duplex is provided or after it is immobilized. The amount of label present can be determined again after incubation with the trap oligonucleotide and the washing step. This allows for a differential measurement in the amount of label present. A decrease in the amount of label indicates that there is helicase activity.

The trap oligonucleotide can be complementary to the first or second strand of the nucleic acid duplex, and they can form a duplex referred to as the trap-target duplex, for example. As described herein, by "complementary" is meant that the trap oligonucleotide recognizes and physically interacts (that is, base-pairs) with a substantially complementary nucleic acid (for example, the second strand of the nucleic acid duplex) under high stringency conditions, and does not substantially base pair with other nucleic acids.

A "decrease in the amount of label" means there is less label present after contact with the trap oligonucleotide and the washing step than before. The decrease can be 1, 2, 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, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99,or 100%. This can indicate the amount of labeled nucleic acid removed during the washing step.

In one example, a duplex can form between the trap oligonucleotide and the labeled second strand. When this occurs, the trap oligonucleotide can be captured and the amount of label present with the trapped oligonucleotide can be determined as well. Alternatively, if the

duplex formed between the trap oligonucleotide and the labeled second strand is washed away, the amount of label present in the wash can be determined as well.

The amount of helicase activity present can be quantified by the amount of label present. One of skill in the art can readily determine this by correlating activity with label. For example, a 5% decrease in label could indicate that the helicase is performing at 5%. In other words, if there are 100 strands of target present, a 5% decrease could indicate that that 95 out of 100 of the strands were not separated by helicase. One of skill in the art will further appreciate that background and unwashed label can be accounted for as well in quantifying helicase activity. No decrease in label could indicate that there is no helicase activity, while a 100% decrease in detected label (i.e., no label detected above background) could indicate that the helicase is 100% functional. Of course, to determine the specific percentage of activity can be affected by a number of factors including substrate concentration, enzyme concentration, etc. For example, if the substrate is in excess with relation to the enzyme, the exact percentage of enzyme activity could be higher than the amount of fluorescence due to the differing concentrations.

Any of the steps in the method outlined above can be carried out simultaneously or within a homogenous assay. For example, the steps of providing nucleic acid duplex, immobilizing the duplex, and contacting it with a helicase can all be preformed simultaneously.

Also disclosed is a method for measuring helicase activity comprising: providing a nucleic acid duplex comprising a first immobilizable nucleic acid strand and a labeled second strand; immobilizing the nucleic acid duplex; contacting the immobilized nucleic acid substrate duplex with a helicase to form a helicase-duplex mixture; incubating the helicase-duplex mixture with a trap oligonucleotide under conditions for helicase activity; separating non-immobilized nucleic acids, and detecting the amount of label present with the non-immobilized nucleic acids, wherein the presence of label over background indicates helicase activity.

In one example, the amount of label present can be determined after the nucleic acid duplex is provided. It can be determined again after incubation with the trap oligonucleotide and the washing step. This allows for a differential measurement in the amount of label present. The presence of any label over background can indicate that there is helicase activity.

Further disclosed is a method of measuring helicase activity comprising: providing a nucleic acid duplex comprising a first immobilizable nucleic acid strand and a labeled second

strand; contacting the immobilized nucleic acid substrate duplex with a helicase to form a helicase-duplex mixture; incubating the helicase-duplex mixture with a trap oligonucleotide under conditions for helicase activity; immobilizing the first immobilizable strand of the nucleic acid duplex, separating non-immobilized nucleic acids, and; detecting the label present with the immobilized nucleic acids, wherein a decrease in the amount of label present indicates helicase activity.

In this example, the immobilizable strand of the nucleic acid duplex is immobilized after the helicase-duplex mixture has been incubated with the trap oligonucleotide. One of skill in the art will appreciate that the immobilizable nucleic acid can be immobilized at any point during the method.

Also disclosed are methods of measuring helicase activity comprising: providing a nucleic acid duplex comprising a first nucleic acid strand and a labeled second strand; contacting the nucleic acid substrate duplex of step a) with a helicase to form a helicase-duplex mixture; incubating the helicase-duplex mixture with an immobilizable trap oligonucleotide under conditions for helicase activity; immobilizing the immobilizable trap oligonucleotide; separating non-immobilized nucleic acids, and; detecting the label present with immobilized trap oligonucleotide, wherein an increase in the amount of label present indicates helicase activity.

Again, one of skill in the art will appreciate that the trap oligonucleotide can be immobilized at any point during the method described. It can happen before, during, or after the trap oligonucleotide forms a duplex with the labeled second strand of the nucleic acid duplex.

Also disclosed are methods of measuring helicase activity comprising: providing a nucleic acid duplex comprising a first immobilizable nucleic acid strand and a labeled second strand; contacting the nucleic acid substrate duplex of step a) with a helicase to form a helicase-duplex mixture; incubating the helicase-duplex mixture with an immobilizable trap oligonucleotide under conditions for helicase activity; immobilizing the immobilizable trap oligonucleotide; separating the immobilized first immobilizable nucleic acid strand from the immobilizable trap oligonucleotide, and; detecting the label present with the immobilized trap oligonucleotide, wherein an increase in the amount of label present indicates helicase activity.

Also disclosed are methods of measuring helicase activity comprising: providing a nucleic acid duplex comprising a first immobilizable nucleic acid strand and a labeled second strand; contacting the nucleic acid substrate duplex of step a) with a helicase to form a

helicase-duplex mixture; incubating the helicase-duplex mixture with an immobilizable trap oligonucleotide under conditions for helicase activity; immobilizing the immobilizable trap oligonucleotide; separating the immobilized first immobilizable nucleic acid strand from the immobilizable trap oligonucleotide, and; detecting the label present with immobilized first immobilizable nucleic acid strand, wherein a decrease in the amount of label present indicates helicase activity.

Further disclosed are methods for measuring helicase activity comprising: providing a nucleic acid duplex comprising a first nucleic acid strand and a second nucleic acid strand; determining the amount of label present; contacting the nucleic acid duplex with a helicase to form a helicase-duplex mixture; incubating the helicase-duplex mixture with a trap oligonucleotide under conditions for helicase activity, wherein the trap oligonucleotide comprises a fluorescent change probe portion; and detecting the label present in the mixture, wherein an increase in the amount of label present indicates helicase activity. The above method can be conducted in an entirely closed, or homogenous, assay.

The fluorescent change probe portion of the trap oligonucleotide acts as a fluorescent activated probes. The fluorescent activated probe portion is a portion of the trap oligonucleotide where fluorescence is increased or altered by hybridization of the trap oligonucleotide to an oligonucleotide that comprises a complementary sequence to the second strand of the nucleic acid duplex. The fluorescent activated probe portion can include an acceptor fluorescent label and a donor moiety such that, when the acceptor and donor are in proximity (when the probes are hybridized to a target sequence), fluorescence resonance energy transfer from the donor to the acceptor causes the acceptor to fluoresce. The fluorescent activated probe portion can be designed to hybridize to adjacent sequences such that the acceptor and donor are brought into proximity. The fluorescent activated probe portion can also be single oligonucleotide containing both a donor and acceptor where, when the trap oligonucleotide is not hybridized to a complementary sequence, the donor and acceptor are not in proximity but where the donor and acceptor are brought into proximity when the trap oligonucleotide hybridizes to a complementary sequence. This can be accomplished, for example, by placing the donor and acceptor on opposite ends of the trap oligonucleotide and placing target complement sequences at each end of the probe where the target complement sequences are complementary to adjacent sequences in a complementary sequence. If the donor moiety of a fluorescent activated probe is itself a fluorescent label, it can release energy as fluorescence (typically at a different wavelength than the fluorescence

of the acceptor) when not in proximity to an acceptor (that is, when the probes are not hybridized to the target sequence). When the trap oligonucleotide hybridizes to a complementary sequence, the overall effect would then be a reduction of donor fluorescence and an increase in acceptor fluorescence. FRET probes are an example of fluorescent activated probes.

EXAMPLES

EXAMPLE 1

Generally, some of the methods described herein comprise an annealing step to create a duplex substrate, binding of the substrate to the solid support, addition of helicase as trap oligo, washing, and detection. The following is an exemplary protocol employed in a fluorescent-based helicase assay.

Annealing of duplex oligos:

1) Dissolve each of the labeled complementary oligo in ThermoPol Buffer (20mM, Tris-HCl, pH 8.8, 10mM KCl, 10mM $(NH_4)_2SO_4$, 2mM $MgSO_4$, 0.1% Triton-X100).
2) Combine 75nM of pHDAbio and 50nM of pHDAcy3 in a 1.5ml microfuge tube and incubate at 95°C for 2 min on a heating block. Remove the tube from the heating block and cool down to room temperature by placing tube in rack on bench (approximately 70mins). Place the tube in ice until further use. The reaction volume for each annealing reaction was 30ul.

Attaching annealed mix to streptavidin coated plate

3) Prewash the streptavidin coated plates with 200ul wash buffer (25mM Tris-HCl, pH 7.4, 0.05% Tween-20, 150mM NaCl).
4) Add 30ul of wash buffer to each 30ul of the annealing reaction from step 2. Add the entire mix (60ul) to the streptavidin coated plate.
5) Incubate the plate at RT for 30 min with shaking.

Addition of Helicase Mix

6) Remove the liquid and add different concentrations of Tte-UvrD Helicase and capture oligo(pHDAC) at a concentration of 800nM in a total reaction volume of 50ul in ThermoPol Buffer (20mM, Tris-HCl, pH 8.8, 10mM KCl, 10mM $(NH_4)_2SO_4$, 2mM $MgSO_4$, 0.1% Triton-X100). Pre-incubate at 65°C for 2 min.
7) Initiate the reaction by adding ATP to 3mM and continue incubation at 65°C for 10 min.

8) Remove the liquid and wash the streptavidin coated plate three times with streptavidin wash buffer. Add 50ul water to each well.

9) Incubate the plate at RT for 60 min with shaking.

10) Read the plate in a fluorescent plate reader with excitation of 535nm and emission of 590nm.

The concentration of Tte-UvrD Helicase used was 150ng/uL, the MW of protein is 82.66kDa, and 150ng/uL of UvrD corresponds to approximately 1.8uM. The concentration of substrate used was approximately 50nM. The assay used between 5-100nM of Helicase for the reaction in hopes of obtaining a linear plot. The different concentration points used were 0nM, 5nM, 10nM, 25nM, 50nM, 100nM. Optionally, instead of water TE buffer may be used (10mM Tris, 1mM EDTA, brought to pH of approximately 7.5 to 8.0 with hydrochloric acid).

Table 1 shows a table of the reagents and materials used in an exemplary Fluorescent based Helicase Assay.

Table 1: Reagents and Materials for the Fluorescent based DNA Helicase Assay.

Reagents and Equipment	**Company**	**Catalog Number**
100mM ATP	Amersham	27-2056-01
100mM dATP	Amersham	27-2050-03
pHDAcy3	IDT	N/A
pHDAbio	IDT	N/A
pHDAC	IDT	N/A
Streptavidin Wash Buffer	N/A	N/A
ThermoPol Buffer	N/A	N/A
Reacti-Bind Streptavidin HBC coated 96-well	Pierce	15503

black plates		
Tte-UvrD Helicase	BioHelix	N/A
Tecan Fluorescent plate Reader	GeniosPro	S/N 05668
Hybrid Capture Heating Blocks	Digene	

Table 2 shows a table of the oligos used in the Helicase Assay. pHDAcy3 has a cy3 fluorophore at is 5' end and is complementary to pHDAbio, which has a biotin label at its 5' end. pHDAC, the trap oligo, consists of the same sequence as pHDAbio without the 5' biotin and therefore is complementary to pHDAcy3.

Table 2: The sequences of the oligonucleotides used in the Helicase Assay.

Name	Sequence	SEQ. ID. NO.:
pHDAcy3	5' Cy3-AAT TGT TTC CAA ATG CAC TGG CCG TCG TTT TAC	1
pHDAbio	5' Bio-GTA AAA CGA CGG CCA GTG CAT TTG GAA ACA ATT	2
pHDAC	5' GTA AAA CGA CGG CCA GTG CAT TTG GAA ACA ATT	3

Figure 3 is a plot of the data obtained with this protocol. This reaction is dependent upon ATP as removal of ATP results in no activity seen. Also the trap oligo plays some role in ensuring that the unwound cy3 strand remains sequestered and does not re-associate with the biotinylated strand. The concentrations of Helicase used in this assay were outside of the linear range and thus the assay falls off very quickly.

Table 4 shows the data obtained using the above protocol. Because the assay measures the material bound to the streptavidin coated bioplate, one expects a decrease in fluorescence to represent the loss or unwinding of the complementary cy3 strand to the biotinylated strand.

Table 3: Constituency of the Buffers used in the Fluorescent based Helicase Assay.

ThermoPol Buffer (Lot #022607NA)	Streptavidin Wash Buffer (Lot #120506NA)
20mM Tris-HCl, pH 8.8	25 mM Tris-HCl, pH 7.4
10mMKCl	0.05% Tween-20
10mM $(NH_4)_2SO_4$	150mM NaCl
2mM $MgSO_4$	
0.1% Triton-X100	

Table 4: Data obtained using Method 1 of the Helicase Assay.

Data Analysis of Method 1

Material	Fluorescence	MFI	Corrected MFI	Std. Dev	%CV
0nM Helicase	34103	32362	30062	1554	5
	31870				
	31114				
5nM Helicase	16451	17090	14790	694	4
	17829				
	16990				
10nM Helicase	8630	8707	6407	976	11
	9719				
	7771				
25nM Helicase	4452	4858	2558	381	8
	5208				
	4913				
50nM Helicase	3825	3884	1584	144	4
	4049				
	3779				
100nM Helicase	3843	3815	1515	65	2
	3862				

	3741				
100nM, NoATP	43303	39766	37466	3214	8
	37024				
	38970				
100nM, No pHDAC	6537	6987	4687	542	8
	7589				
	6835				
Oligo+Buffer	46572	39014	36714	7351	19
	38582				
	31889				
No Oligo+buffer	2282	2300	0	19	1
	2319				
	2299				

EXAMPLE 2

The following is another protocol employed in the Fluorescent-based Helicase Assay. In this method, the Helicase Mix is added to the annealed duplex oligo solution in a regular 96 well plate. After the Helicase reaction has proceed for 10 min at 65°C, the mix is then transferred to the straptavidin coated bioplate.

Annealing of duplex oligos:

1) Dissolve each of the labeled complementary oligo in ThermoPol Buffer (20mM, Tris-HCl, pH 8.8, 10mM KCl, 10mM $(NH_4)_2SO_4$, 2mM $MgSO_4$, 0.1% Triton-X100).

2) Combine 75nM of pHDAbio and 50nM of pHDAcy3 in a 1.5ml microfuge tube and incubate at 95°C for 2 min on a heating block. Remove the tube from the heating block and cool down to room temperature by placing tube in rack on bench (approximately 70mins). Place the tube in ice until further use. The reaction volume for each annealing reaction was 30ul.

Addition of Helicase Mix

3) Mix the annealed oligos with 30ul of differing amounts of Tte-UvrD Helicase and trap DNA at a concentration of 800nM in ThermoPol Buffer in a regular 96 well plate, incubate this mix at 65°C for 2 min.

4) Initiate the reaction by adding ATP to 3mM and continue incubation for 10 min at 65°C.

Attaching reaction mix to streptavidin coated plate

5) Prewash the streptavidin coated plates with 200ul wash buffer (25mM Tris-HCl, pH 7.4, 0.05% Tween-20, 150mM NaCl).

6) Add 60ul of wash buffer to each of the wells of the regular 96 well plate. Transfer the entire mix to the streptavidin coated plate.

7) Incubate the plate at RT for 30 min with shaking.

8) Remove the liquid and wash the well three times with wash buffer. Add 50ul water to each well.

9) Incubate the plate at RT for 60 min with shaking.

10) Read the plate in a fluorescent plate reader with excitation of 535nm and emission of 590nm.

Figure 9 is a plot of the data obtained from using Helicase Assay Method 2. Note that the results are again outside of the linear range using these concentrations of Tte-UvrD Helicase. A comparison of Method 1 vs. Method 2 shows that they are very comparable in the data produced. Method 1 is less time-consuming than Method 2 and involves less sample transfer.

Table 5 presents data obtained using Method 2 and the results seen here coincide with the data seen in Method 1. ATP and the capture oligo play similar roles to that seen in Method 1. The pHDAC trap oligo has more dramatic effect on preventing re-annealing.

Table 5: Data obtained using Method 2, the results seen here coincide greatly with the data seen in Method 1. ATP and the capture oligo play similar roles to that seen in Method 1. The pHDAC trap oligo has more dramatic effect on preventing re-annealing.

Table 5.

Data Analysis of Method 2 (material left on plate, pellet)

Material	Fluores.	Avg. Fluores.	Corrected MFI	Std. Dev	%CV
0nM	5234	5366	5130	142	3
	5516				
	5347				
5nM Helicase	1075	1050	1034	137	13
	902				
	1173				
10nM Helicase	940	918	682	31	3
	931				

	882				
25nM Helicase	913	890	654	20	2
	875				
	881				
50nM Helicase	840	910	675	102	11
	863				
	1027				
100nM Helicase	1180	1085	850	125	11
	1132				
	944				
100nM, NoATP	6362	6487	6252	270	4
	6302				
	6796				
100nM, No pHDAC	6074	6939	6704	1052	15
	6632				
	8110				
Oligo+Buffer	7583	6277	6042	1145	18
	5806				
	5443				
No oligo+buffer	236	235	0	2	1
	233				
	237				

EXAMPLE 3

Previous work using ATPase assays to assess the activity of Tte-UvrD helicase showed that Tte-UvrD Helicase achieved maximal activity at 55°C and lost 30% of its ATPase activity after 90 minutes of continuous incubation at 65°C. The following protocol was utilized to assess the activity of the helicase at 55°C.

Annealing of duplex oligos:

1) Dissolve each of the labeled complementary oligo in ThermoPol Buffer (20mM, Tris-HCl, pH 8.8, 10mM KCl, 10mM $(NH_4)_2SO_4$, 2mM $MgSO_4$, 0.1% Triton-X100).
2) Combine 75nM of pHDAbio dissolved oligo with 50nM of pHDAcy3 dissolved oligo in a 1.5mL microfuge tube and incubate at 95°C for 2 min on a heating block. Remove the tube from the heating block and cool down to room temperature by placing tube in rack on bench (approximately 70-90min). Place the tube in ice until

further use. Keep the reaction tube containing cy3 in the dark as much as possible. The reaction volume for each annealing reaction was 30ul.

Attaching annealed mix to streptavidin coated plate

3) Prewash the streptavidin coated plates with 200ul wash buffer (25mM Tris-HCl, pH 7.4, 0.05% Tween-20, 150mM NaCl).
4) Add 30ul of wash buffer to each 30ul of annealed oligo mix. Add the entire mix (60ul total) to each well of the streptavidin coated plate.
5) Incubate the plate at RT for 30min with shaking.

Addition of Helicase Mix

6) Remove the liquid and wash the well three times with wash buffer. Add different concentrations of Tte-UvrD Helicase and capture oligo (pHDAC) at a concentration of 800nM in a total reaction volume of 50ul in ThermoPol Buffer (20mM, Tris-HCl, pH 8.8, 10mM KCl, 10mM $(NH_4)_2SO_4$, 2mM $MgSO_4$, 0.1% Triton-X100). Pre-incubate at 55°C for 2 min.
7) Initiate the reaction by adding ATP to 3mM and continue incubation at 55°C for 10 min.
8) Transfer liquid to a regular U bottom hybridization plate until further use, make sure to keep the plate in the dark as much as possible.
9) Wash the streptavidin coated plate three times with streptavidin wash buffer. Add 50ul water to each well.
10) Transfer the liquid from the U bottom hybridization plate to unused wells on the streptavidin coated plate (this represents the released cy3- strand from the helicase assay).
11) Incubate the plate at RT for 60 min with shaking.
12) Read the plate in a fluorescent plate reader with excitation of 535nm and emission of 590nm.

The concentration of Tte-UvrD Helicase used was between 2.5-20nM, in the hopes of obtaining a linear plot. The different Helicase concentrations used include 0nM, 2.5nM, 5nM, 10nM, 15nM, 20nM.

Table 6 shows data obtained from the Helicase assay performed at 55°C. These values represent the material that remained bound to the streptavidin coated bioplate (pellet).

Table 6: Data obtained from performing the Helicase Assay at 55°C.

Helicase Assay (pellet) 030507NA

Material	Fluorescence	MFI	Corrected MFI	Std. Dev.	%CV
0nM Helicase	28317	28698	26849	448	2
	28585				
	29191				
2.5nM Helicase	23976	25797	23948	1854	7
	27682				
	25732				
5nM Helicase	23653	24983	23134	1329	5
	24985				
	26311				
10nM Helicase	25816	24689	22840	1279	5
	23298				
	24952				
15nM Helicase	26175	24137	22288	1768	7
	23004				
	23233				
20nM Helicase	21896	23325	21476	1668	7
	25157				
	22921				
20nM, NoATP	33326	31272	29423	2322	7
	31737				
	28753				
20nM, No pHDAC	27632	26863	25014	676	3
	26593				
	26364				
Oligo+Buffer	24053	30651	28802	5722	19
	33647				
	34253				
0nM, no pHDAC	28726	29854	28005	1152	4
	31029				
	29806				
No Oligo+buffer	1845	1849	0	71	4

	1921				
	1780				
1/10 reaction mix	5954	5558	3709	430	8
	5101				
	5619				
1/100 reaction mix	2170	2219	370	45	2
	2228				
	2258				

Figure 5 is a plot of this data and represents the material bound to the plate (pellet). On increasing Helicase concentrations, there is a decrease in fluorescence units representing the loss of pHDAcy3 from pHDAbio and an increase in Helicase activity.

Table 7: Data depicting material released from the plate (supernatant).

Helicase AssaySupernatant (030507NA)

Material	Fluorescence	MFI	Std. Dev.	% CV	Corrected MFI	bkgd corrected MFI
0nM Helicase	24472	19756	4827	24	16741	1879
	19970					
	14825					
2.5nM Helicase	30445	29047	4405	15	26032	11170
	24113					
	32583					
5nM Helicase	22657	26521	4923	19	23506	8644
	24841					
	32064					
10nM Helicase	33379	34957	4976	14	31942	17080
	30961					
	40530					
15nM Helicase	26244	30069	5038	17	27054	12192
	35778					
	28185					
20nM Helicase	25006	38769	12020	31	35754	20892
	44095					
	47206					

20nM, NoATP	14110	16862	3243	19	13847	N/A
	20438					
	16038					
20nM, No pHDAC	39672	32289	8918	28	29274	14412
	22381					
	34813					
Oligo+Buffer	19463	13769	5626	41	10754	N/A
	13632					
	8213					
0nM, no pHDAC	16310	17877	1713	10	14862	0
	17615					
	19705					
No Oligo+buffer	2419	3015	1495	50	0	
	1909					
	4716					
1/10 annealing mix	14446	15008	576	4	11993	
	15597					
	14981					
1/100 annealing mix	3120	3196	135	4	181	
	3116					
	3352					
Unbound cy3	21680	24662	6810	28	21647	
	19852					
	32454					

Figure 13 is a plot of the experimental results showing an increase in fluorescence units representing the release of pHDAcy3 in the supernatant and an increase in Helicase activity with increasing concentatrations of Helicase. Performing the Helicase Assay at 55°C did not show the increase in Helicase activity as has been previously reported. The range of enzyme concentration used may have been too low.

EXAMPLE 4

Experiments were conducted to find the optimal temperature for Tte-UvrD Helicase Activity using the Helicase Assay. The assay was performed as outlined below in Example 6 with the exception that only two concentration points were used in the Helicase Assay, 10nM

and 20nM and the temperatures ranged from 55°C to 65°C. The different temperature points used were 55°C, 57°C, 60°C, 62°C, and 65°C.

Table 8 represents a compilation of four different tables representing the results from two different temperature points, 55°C and 57°C.

Table 8A: Helicase Assay
pellet 030607, 55C

Material	RFU	MFI	Corrected MFI	Std. Dev.	% CV	S/N ratios
0nM Helicase	21591	19567	18593	1784	9	1
	18887					
	18222					
10nM Helicase	13682	13810	12836	209	2	1.45
	14051					
	13696					
20nM Helicase	11741	12726	11752	1007	8	1.6
	12685					
	13753					
0nM, no pHDAC	18510	18247	17273	422	2	N/A
	18470					
	17760					
20nM, NoATP	18636	19315	18341	760	4	N/A
	19172					
	20136					
Oligo+Buffer	24487	21427	20453	2650	12	N/A
	19928					
	19867					
No Oligo+buffer	971	974	0	6	1	N/A
	981					
	969					

Table 8B: Helicase Assay
pellet 030607, 57C

Material	RFU	MFI	Corrected MFI	Std. Dev.	% CV	S/N ratios
0nM Helicase	23872	21388	20206	2153	10	1
	20218					
	20073					
10nM	16395	16491	15309	207	1	1.32

Helicase	16728					
	16349					
20nM Helicase	13561	13735	12553	188	1	1.6
	13710					
	13934					
Oligo+Buffer	20842	21268	20086	404	2	N/A
	21645					
	21318					
20nM, NoATP	20550	20157	18975	387	2	N/A
	20146					
	19776					
0nM, no pHDAC	19292	20359	19177	972	5	N/A
	20591					
	21194					
No Oligo+buffer	1164	1182	0	17	1	N/A
	1197					
	1184					

Table 8C: Helicase Assay supernatant

030607, 55C

Material	RFU	MFI	Corrected MFI	Std. Dev.	% CV	Corrected MFI	S/N ratios
0nM Helicase	15278	14548	12007	769	5	4876	1
	14621						
	13746						
10nM Helicase	35861	33684	31143	2986	9	24012	4.9
	30280						
	34911						
20nM Helicase	21808	33880	31339	10520	31	24208	5
	38744						
	41088						
0nM, no pHDAC	5799	9672	7131	3623	37	0	N/A
	12977						
	10241						
20nM, NoATP	12312	14374	11833	2011	14	4702	N/A
	14480						

	16329						
Oligo+Buffer	15228	8586	6045	5803	68	N/A	N/A
	6033						
	4498						
No Oligo+Buffer	913	2541	0	1412	56	N/A	N/A
	3265						
	3444						

Table 8D: Helicase Assay supernatant
030607, 57C

Material	RFU	MFI	Corrected MFI	Std. Dev.	% CV	Adjusted MFI	S/N ratios
0nM Helicase	19455	16817	15647	2321	14	1791	1
	15908						
	15087						
10nM Helicase	24948	24147	22977	816	3	9121	5.1
	24177						
	23317						
20nM Helicase	39752	34240	33070	4833	14	19214	11
	32242						
	30726						
Oligo+Buffer	4835	8138	6968	3732	46	N/A	N/A
	12186						
	7393						
20nM, NoATP	10363	10980	9810	1775	16	N/A	N/A
	12982						
	9596						
0nM, no pHDAC	17445	15026	13856	2736	18	0	N/A
	12057						
	15576						
No Oligo+buffer	1195	1170	0	22	2	N/A	N/A
	1152						
	1163						

Table 9 represents a compilation of six different tables representing the results from three different temperature points, 60°C and 62°C and 65°C. Plotting all the S/N ratios that were obtained at different temperatures and two different concentrations of Tte-UvrD, the Helicase activities at different temperatures was compared.

Table 9A: Helicase Assay pellet 030607, 60C

Material	RFU	MFI	Corrected MFI	Std. Dev.	% CV	S/N ratios
0nM Helicase	10791	10752	9990	35	0	1
	10726					
	10738					
10nM Helicase	6584	6597	5835	48	1	1.7
	6557					
	6650					
20nM Helicase	4443	4407	3645	478	11	2.7
	3913					
	4866					
Oligo+Buffer	12063	11923	11161	265	2	N/A
	12088					
	11617					
20nM, NoATP	11360	11390	10628	44	0	N/A
	11370					
	11441					
0nM, no pHDAC	14957	12497	11735	2140	17	N/A
	11061					
	11473					
No Oligo+buffer	756	762	0	7	1	N/A
	769					
	760					

Table 9B: Helicase Assay pellet 030607, 62C

Material	RFU	MFI	Corrected MFI	Std. Dev.	% CV	S/N ratios
0nM Helicase	18000	18380	17162	457	2	1
	18253					
	18888					
10nM Helicase	9759	8714	7496	905	10	2.3
	8180					
	8202					
20nM Helicase	3512	4013	2795	1275	32	6.1
	3065					

	5462					
Oligo+Buffer	24001	23479	22261	458	2	N/A
	23287					
	23148					
20nM, NoATP	21586	21247	20029	345	2	N/A
	21260					
	20896					
0nM, no pHDAC	16938	18766	17548	1651	9	N/A
	19214					
	20147					
No Oligo+buffer	1197	1218	0	18	1	N/A
	1227					
	1230					

Table 9C: Helicase Assay
pellet 030607, 65C

Material	RFU	MFI	Corrected MFI	Std. Dev.	% CV	S/N ratios
0nM Helicase	6113	6192	5677	205	3	1
	6039					
	6425					
10nM Helicase	1431	1592	1077	140	9	5.3
	1684					
	1661					
20nM Helicase	874	807	292	105	13	19.4
	861					
	686					
Oligo+Buffer	8601	9004	8489	587	7	N/A
	8733					
	9678					
20nM, NoATP	8674	7753	7238	815	11	N/A
	7458					
	7127					
0nM, no pHDAC	4898	5310	4795	540	10	N/A
	5922					
	5111					
No Oligo+buffer	509	515	0	6	1	N/A
	521					

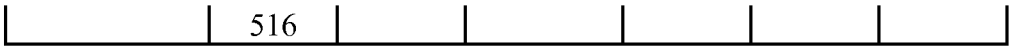


Table 9D: Helicase Assay supernatant
030607, 60C

Material	RFU	MFI	Corrected MFI	Std. Dev.	% CV	Adjusted MFI	S/N ratios
0nM Helicase	16332	15288	14473	2218	15	1075	1
	16791						
	12741						
10nM Helicase	25807	30038	29223	3972	13	15825	14.7
	30620						
	33686						
20nM Helicase	39851	42552	41737	2811	7	28339	26.4
	45462						
	42344						
Oligo+Buffer	9874	9862	9047	12	0	N/A	N/A
	9851						
	9860						
20nM, NoATP	11233	11636	10821	1653	14	N/A	N/A
	10222						
	13454						
0nM, no pHDAC	12895	14213	13398	1355	10	0	N/A
	15603						
	14142						
No Oligo+buffer	830	815	0	88	11	N/A	N/A
	895						
	721						

Table 9E: Helicase Assay supernatant
030607, 62C

Material	RFU	MFI	Corrected MFI	Std. Dev.	% CV	Adjusted MFI	S/N ratios
0nM Helicase	33218	30439	29051	2479	8	3616	1
	28456						
	29642						
10nM Helicase	41752	41734	40346	894	2	14911	4.1
	40831						
	42619						
20nM Helicase	44277	47156	45768	2558	5	20333	5.6

	49167						
	48025						
Oligo+Buffer	20326	28211	26823	6996	25	1388	N/A
	33674						
	30632						
20nM, NoATP	9503	11809	10421	3396	29	N/A	N/A
	10216						
	15709						
0nM, no pHDAC	27169	32054	30666	4344	14	5231	N/A
	35484						
	33510						
No Oligo+buffer	1372	1388	0	36	3	N/A	N/A
	1363						
	1430						

Table 9F: Helicase Assay supernatant
030607, 65C

Materials	RFU	MFI	Corrected MFI	Std. Dev.	% CV	Adjusted MFI	S/N ratios
0nM Helicase	17798	13711	13169	5560	41	4514	1
	7380						
	15955						
10nM Helicase	26027	32896	32354	6400	19	23699	5.25
	38690						
	33971						
20nM Helicase	34835	36908	36366	5589	15	27711	6.14
	32652						
	43237						
Oligo+Buffer	10170	8264	7722	2208	27	N/A	N/A
	8778						
	5844						
20nM, NoATP	11397	10188	9646	1333	13	991	N/A
	8759						
	10408						
0nM, no pHDAC	19951	9197	8655	9582	104	0	N/A
	6073						
	1567						
No Oligo+buffer	484	542	0	62	11	N/A	N/A

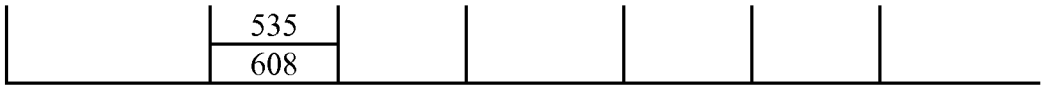


Figure 7 depicts a comparison of the Tte-UvrD Helicase activities at different temperatures using the S/N values obtained from the pellet. Figure 8 depicts a comparison of the Tte-UvrD Helicase activities at different temperatures using the S/N values obtained from the supernatant. Looking at the values obtained for the pellet, the temperature point that shows the greatest signal to noise ratio is 65°C; however looking at the results from the supernatant, the greatest signal to noise ratio is 60°C.

EXAMPLE 5

In this experiment, a direct comparison between ATP and dATP in the Helicase Assay was performed. The Assay was again carried out as in Example 4 but in some instances 3 mM dATP was added instead of 3 mM ATP and half the supernatant was added to the streptavidin coated plate instead of the entire mix.

Table 10 depicts data obtained from analyzing the pellet fraction and performing a comparison of ATP vs. dATP (in **BOLD**).

Helicase Assay 030907, 60C ATP, vs dATP (red)

Material	RFU	MFI	Corrected MFI	Std. Dev.	%CV
0nM Helicase	3621	4228	4019	538	13
	4415				
	4648				
2.5nM Helicase	4195	4171	3962	205	5
	4363				
	3955				
5nM Helicase	3384	3407	3198	34	1
	3390				
	3446				
10nM Helicase	2943	3027	2818	78	3
	3042				
	3097				
15nM Helicase	2361	2368	2159	50	2
	2422				
	2322				
20nM Helicase	1102	1567	1358	403	26

	1788				
	1811				
20nM, NoATP	4858	4408	4199	771	17
	4849				
	3518				
0nM, No pHDAC	4716	4486	4277	211	5
	4440				
	4301				
Oligo+Buffer	4846	4953	4744	129	3
	4918				
	5096				
No Oligo+buffer	212	209	0	3	1
	206				
	208				
2.5nM Helicase	**3889**	**3950**	**3741**	**127**	**3**
	4096				
	3864				
5nM Helicase	**3231**	**3014**	**2805**	**596**	**20**
	2340				
	3470				
10nM Helicase	**2805**	**2721**	**2512**	**90**	**3**
	2626				
	2733				
15nM Helicase	**2497**	**2530**	**2321**	**129**	**5**
	2672				
	2420				
20nM Helicase	**1009**	**1568**	**1359**	**524**	**33**
	1648				
	2047				

Table 11 (A) and (B) give a side by side comparison of the different values obtained in the Helicase Assay using ATP vs. dATP. Looking at both plots, the results for ATP and dATP were very comparable and there were minimal differences seen between the two. The Table depicts data obtained from analyzing the supernatant fraction and performing a comparison of ATP vs. dATP (in **BOLD**).

Helicase Assay 030907, 60C ATP vs dATP(bold), supernatant

Material	RFU	MFI	Corrected MFI	Std. Dev	% CV	Adjusted MFI
0nM Helicase	405	453	354	44	10	156
	462					
	491					
2.5nM Helicase	552	556	457	106	19	259
	664					
	453					
5nM Helicase	431	404	305	62	15	107
	447					
	333					
10nM Helicase	1158	844	745	383	45	547
	958					
	417					
15nM Helicase	1397	1466	1367	208	14	1169
	1699					
	1301					
20nM Helicase	455	890	791	693	78	593
	526					
	1690					
20nM, NoATP	232	288	189	57	20	N/A
	288					
	345					
0nM, No pHDAC	355	297	198	53	18	0
	284					
	251					
Oligo+Buffer	209	245	146	33	14	N/A
	253					
	274					
No Oligo+buffer	101	99	0	3	3	N/A
	96					
	101					
2.5nM Helicase	**406**	**663**	**564**	**228**	**34**	**366**
	840					
	744					

5nM Helicase	**1060** **743** **706**	**836**	**737**	**195**	**23**	**539**
10nM Helicase	**813** **1143** **468**	**808**	**709**	**338**	**42**	**511**
15nM Helicase	**630** **627** **699**	**652**	**553**	**41**	**6**	**355**
20nM Helicase	**651** **601** **656**	**636**	**537**	**30**	**5**	**339**

The table represents the plot of the supernatants from the Helicase assay using ATP vs. dATP, respectively. A direct comparison of using ATP or dATP in the Helicase assay(pellet) shows that the two compounds are very comparable. Looking at the supernatant, the results were very variable and this does not seem to be a very efficient way at looking at the Helicase activity. The variation in the results seen for the supernatant could stem from the fact that the samples were being transferred twice, once to a regular 96-well plate and then again to the streptavidin coated plate. This could have resulted in loss of samples during transfer. Additionally, the supernatant consists of a mix of different added reagents (e.g. Helicase ATP, trap oligo), and some of the constituents from these reagents may lead to the variability seen. Because of the increased variability seen using the supernatant, the pellet values were used when assessing the Helicase assay.

EXAMPLE 6

Since dATP/ATP is usually added after the reaction mixture has been at the desired temperature for two minutes, the effect on the Helicase Assay was tested of premixing dATP in the reaction mix and allowing the reaction to proceed at 65°C.

Annealing of duplex oligos:

1) Dissolve each of the labeled complementary oligo in ThermoPol Buffer (20mM, Tris-HCl, pH 8.8, 10mM KCl, 10mM $(NH_4)_2SO_4$, 2mM $MgSO_4$, 0.1% Triton-X100).

2) Combine 75nM of pHDAbio dissolved oligo with 50nM of pHDAcy3 dissolved oligo in a 1.5mL microfuge tube and incubate at 95°C for 2 min on a heating block. Remove the tube from the heating block and cool down to room temperature by placing tube in rack on bench (approximately 70-90 min). Place the tube in ice until further use. The reaction volume for each annealing reaction was 30ul.

Attaching annealed mix to streptavidin coated plate

3) Prewash the streptavidin coated plates with 200ul wash buffer (25mM Tris-HCl, pH 7.4, 0.05% Tween-20, 150mM NaCl).
4) Add 30ul of wash buffer to each 30ul of annealing mix. Add the entire mix (60ul total) to each well of the streptavidin coated plate.
5) Incubate the plate at RT for 30 min with shaking.

Addition of Helicase Mix

6) Remove the liquid and wash the well three times with wash buffer. Add different concentrations of Tte-UvrD Helicase and capture oligo(pHDAC) at a concentration of 400nM in a total reaction volume of 50ul in ThermoPol Buffer (20mM, Tris-HCl, pH 8.8, 10mM KCl, 10mM $(NH_4)_2SO_4$, 2mM $MgSO_4$, 0.1% Triton-X100). Pre-incubate at 65°C for 2 min.
7) Initiate the reaction by adding dATP to 3mM and continue incubation at 65°C for 10 min.
8) Remove the liquid and wash the streptavidin coated plate containing bound material (pellet) three times with streptavidin wash buffer. Add 50ul water to each well.
9) Incubate the plate with the pellet at RT for 60 min with shaking.
10) Read the plates in a fluorescent plate reader with excitation of 535nm and emission of 590nm.

Alternatively after step 5:

Addition of Helicase Mix

6) Remove the liquid and wash the well three times with wash buffer. Add different concentrations of Tte-UvrD Helicase, capture oligo(pHDAC) at a concentration of 400nM and dATP at a concentration of 3mM in a total reaction volume of 50ul in ThermoPol Buffer (20mM, Tris-HCl, pH 8.8, 10mM KCl, 10mM $(NH_4)_2SO_4$, 2mM $MgSO_4$, 0.1% Triton-X100).
7) Incubate the plate at 65°C for 10 min.

8) Remove the liquid and wash the streptavidin coated plate containing bound material (pellet) three times with streptavidin wash buffer. Add 50ul water to each well.
9) Incubate the plate with the pellet at RT for 60 min with shaking.
10) Read the plates in a fluorescent plate reader with excitation of 535nm and emission of 590nm.

The capture oligo concentration used in this experiment was 400nM instead of 800nM, and the concentration of Helicase used ranged between 1.25 to 10nM. The different concentration points used were 1.25nM, 2.5nM, 5nM, 7.5nM and 10nM. In order to aid in the addition of Helicase to the reaction mix, a freshly made diluted stock of Helicase (25ng/ul) was made in ThermoPol Buffer from the original 150ng/ul stock of Tte-UvrD Helicase.

Table 12 provides a table of data obtained using original method of dATP addition, where dATP is added individually to each well. Figure 11 (A) depicts the plot showing all the data points from the assay ranging from 0nM to 10nM and from the graph, one can see that the linear range of this assay starts breaking down around 7.5nM at 65°C. Figure 11 (B) depicts the plot showing the first four data points where the linear range is still in tact.

Helicase Assay 031607, 65C, original, pellet

Material	RFU	MFI	Corrected MFI	Std. Dev.	%CV
0nM Helicase	32614 34041 35461	34039	31284	1424	4
1.25nM Helicase	25646 23626 25869	25047	22292	1236	5
2.5nM Helicase	21519 21378 24243	22380	19625	1615	7
5nM Helicase	14196 13717 15303	14405	11650	813	6
7.5nM Helicase	13628 13687 13943	13753	10998	167	1
10nM Helicase	8480 9102	8600	5845	454	5

	8217				
0nM, no pHDAC	38529 39944 35565	38013	35258	2235	6
10nM, NoATP	43419 44153 42421	43331	40576	869	2
Oligo+Buffer	45742 45889 44274	45302	42547	893	2
No Oligo+buffer	2773 2714 2779	2755	0	36	1

Table 13 provides a table of data obtained from premixing dATP to the reaction mixture. Figure 12 presents the plot of this data showing data points obtained from pre-mixing dATP in the reaction mix. Note that the linear range of the graph breaks down almost instantaneously. The helicase reaction is allowed to proceed immediately, and so this would not be an effective way to add dATP to the Helicase Assay.

Helicase Assay 031607, 65C, alternate, pellet

Material	RFU	MFI	Corrected MFI	Std. Dev.	%CV
0nM Helicase	35839 37531 38870	37413	34155	1519	4
1.25nM Helicase	26527 17385 17697	20536	17278	5190	25
2.5nM Helicase	13064 18477 19345	16962	13704	3404	20
5nM Helicase	13213 14517 14824	14185	10927	855	6
7.5nM Helicase	11877 10488 9912	10759	7501	1010	9
10nM	9146	9961	6703	706	7

Helicase					
	10377				
	10361				
0nM, no pHDAC	37313	34686	31428	2535	7
	34492				
	32254				
10nM, NoATP	42416	41681	38423	829	2
	41845				
	40782				
Oligo+Buffer	45346	46342	43084	877	2
	47001				
	46679				
No Oligo+buffer	3296	3258	0	35	1
	3228				
	3251				

Comparison of adding the dATP individually after the reaction has been pre-heated to 65°C vs. adding the dATP in the reaction mix from the beginning shows that using pre-mixed dATP is not an efficient way to add dATP to the Helicase assay as the linear range breaks down instantly.

EXAMPLE 7

The following experiment was conducted to apply the Helicase Assay to assign Unit Definition for Tte-UvrD Helicase. The purity of BioHelix Tte-UvrD was assessed to be about 95% pure (information obtained from BioHelix using gel analysis of the purified protein).

Table 14.

Material	RFU	MFI	Corrected MFI	Std. Dev	%CV	Conc of pHHDAcy3
1/100 annealing mix	5101	5151	2370	46	1	0.025pmoles
	5159					
	5192					
Buffer	2744	2780	0	37	1	N/A
	2817					
	2780					

Table 14 summarizes data obtained from the experiment. The annealing Mix from Step 1 in the Helicase Assay, containing a known amount of pHDAcy3 was diluted 100 fold in water.

50ul of this mix was added directly to a well in the streptavidin coated plate and read on the fluorescent plate reader. To convert from nM to moles a conversion tool from was used; use the MW of Tte-UvrD Helicase as 82.66kDa.

Using the values above, at 0nM Helicase, a reading of 31284 would correspond to approximately 0.33pmoles of pHDACy3 bound.

Using the Unit definition of Tte-UvrD Helicase as: The release of 0.1 pmoles of Cy3 labeled oligonucleotide from its complementary strand for 10 min at 65°C, then 0.1pmoles of pHDAcy3 would be equivalent to 9480 MFI, the release of 0.1 pmoles would be equivalent to 31284-9480=21804 MFI.

Using the equation above, a value of 2.03nM of Helicase was obtained from the data shown in Table 7.

Using the value of 2.03nM of Helicase, this would be equivalent to 0.182ng/ul; since the reaction volume used was 50ul, this would then result in 8.4ng of Tte-UvrD Helicase.

Specific activity of Tte-UvrD Helicase would be 1U/8.4 ng which would be equivalent to 119000U/mg, thus the specific activity of Tte-UvrD Helicase at 65°C is 119000U/mg.

Using similar methods of calculations at 55°C and 60°C, specific activities of Tte-UvrD Helicase were also obtained. The specific activity of Tte-UvrD Helicase at 55°C is 13,750U/mg. The specific activity of Tte-UvrD at 60°C was calculated under two conditions, using ATP and using dATP. The specific activity of Tte-UvrD Helicase using ATP was 27,500U/mg, while the specific activity of Tte-UvrD Helicase using dATP was 31,000U/mg. This indicates that ATP and dATP were comparable for use in the Helicase assay with dATP giving slightly better results in the Helicase activity.

The greatest specific activity was seen using Tte-UvrD at 65°C suggesting that this is the optimal temperature for helicase activity.

CLAIMS

What is claimed is:

1. A method for measuring helicase activity comprising:
    a. providing a nucleic acid duplex comprising a first immobilizable nucleic acid strand and a labeled second strand;
    b. immobilizing the nucleic acid duplex;
    c. contacting the immobilized nucleic acid substrate duplex with a helicase to form a helicase-duplex mixture;
    d. incubating the helicase-duplex mixture with a trap oligonucleotide under conditions for helicase activity;
    e. separating non-immobilized nucleic acids; and
    f. detecting the label present with the immobilized nucleic acids, wherein a decrease in the amount of label present indicates helicase activity.
2. The method of claim 1, further comprising detecting the amount of label present after step a) or step b).
3. The method of claim 2, further comprising comparing the amount of label present in step a) or b) with the amount of label present in step f).
4. The method of claim 1, wherein in step d), a duplex can form between the trap oligonucleotide and the labeled second strand.
5. The method of claim 1, further comprising quantifying the amount of helicase activity by comparing the amount of label present after step a) or b) to the amount of label present in step f).
6. The method of claim 1, wherein the helicase is Tte-UvrD helicase.
7. The method of claim 1, wherein the trap oligonucleotide is complementary to the labeled second strand, and they hybridize to each other.
8. The method of claim 1, wherein the trap oligonucleotide is present in excess.
9. The method of claim 1, wherein ATP, dATP, UTP, CTP, dCTP, GTP or dTTP is added during step c).
10. The method of claim 1, wherein steps (a), (b), (c), and/or (d) are carried out simultaneously.
11. The method of claim 1, wherein amplification is isothermal.
12. A method for measuring helicase activity comprising:

a. providing a nucleic acid duplex comprising a first immobilizable nucleic acid strand and a labeled second strand;
b. immobilizing the nucleic acid duplex;
c. contacting the immobilized nucleic acid substrate duplex with a helicase to form a helicase-duplex mixture;
d. incubating the helicase-duplex mixture with a trap oligonucleotide under conditions for helicase activity;
e. separating non-immobilized nucleic acids; and
f. detecting the amount of label present with the non-immobilized nucleic acids, wherein the presence of label over background indicates helicase activity.

13. A method of measuring helicase activity comprising:
a. providing a nucleic acid duplex comprising a first immobilizable nucleic acid strand and a labeled second strand;
b. contacting the immobilized nucleic acid substrate duplex with a helicase to form a helicase-duplex mixture;
c. incubating the helicase-duplex mixture with a trap oligonucleotide under conditions for helicase activity;
d. immobilizing the first immobilizable strand of the nucleic acid duplex,
e. separating non-immobilized nucleic acids, and;
f. detecting the label present with the immobilized nucleic acids, wherein a decrease in the amount of label present indicates helicase activity.

14. A method of measuring helicase activity comprising:
a. providing a nucleic acid duplex comprising a first nucleic acid strand and a labeled second strand;
b. contacting the nucleic acid substrate duplex of step a) with a helicase to form a helicase-duplex mixture;
c. incubating the helicase-duplex mixture with an immobilizable trap oligonucleotide under conditions for helicase activity;
d. immobilizing the immobilizable trap oligonucleotide;
e. separating non-immobilized nucleic acids, and;
f. detecting the label present with immobilized trap oligonucleotide, wherein a decrease in the amount of label present indicates helicase activity.

15. The method of claim 12, wherein an immobilizable trap oligonucleotide-labeled second strand duplex forms in step c).
16. A method for measuring helicase activity comprising:
    a. providing a nucleic acid duplex comprising a first immobilizable nucleic acid strand and a labeled second strand;
    b. determining the amount of label present;
    c. immobilizing the nucleic acid duplex;
    d. contacting the immobilized nucleic acid substrate duplex with a helicase to form a helicase-duplex mixture;
    e. incubating the helicase-duplex mixture with a trap oligonucleotide comprising a quencher under conditions for helicase activity; and
    f. detecting the label present with the immobilized nucleic acids, wherein a decrease in the amount of label present indicates helicase activity.
17. The method of claim 16, wherein steps (a) through (f) are conducted in a homogenous assay.
18. A kit comprising a nucleic acid duplex comprising two strands of nucleic acid where the first strand is immobilizable and the labeled second strand is detectably labeled, a trap oligonucleotide, an immobilization substrate, wash buffers and a helicase.

FIGURE 1

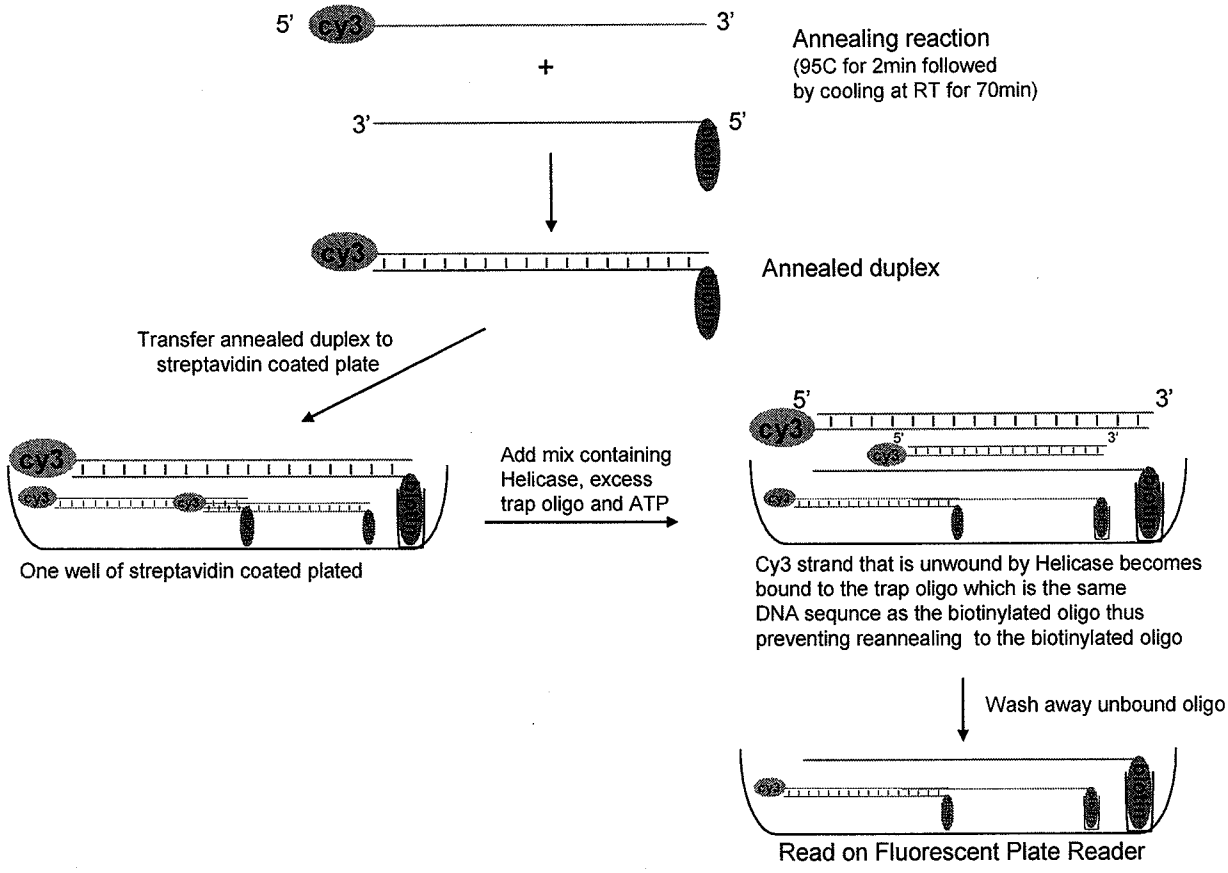

5'
cy3
3'
+
3'
5'
Annealing reaction
(95C for 2min followed
by cooling at RT for 70min)
cy3
Annealed duplex
Transfer annealed duplex to
streptavidin coated plate
cy3
cy3
One well of streptavidin coated plated
Add mix containing
Helicase, excess
trap oligo and ATP
5'
3'
cy3
5'
3'
cy3
Cy3 strand that is unwound by Helicase becomes
bound to the trap oligo which is the same
DNA sequnce as the biotinylated oligo thus
preventing reannealing to the biotinylated oligo
Wash away unbound oligo
cy3
Read on Fluorescent Plate Reader

FIGURE 2

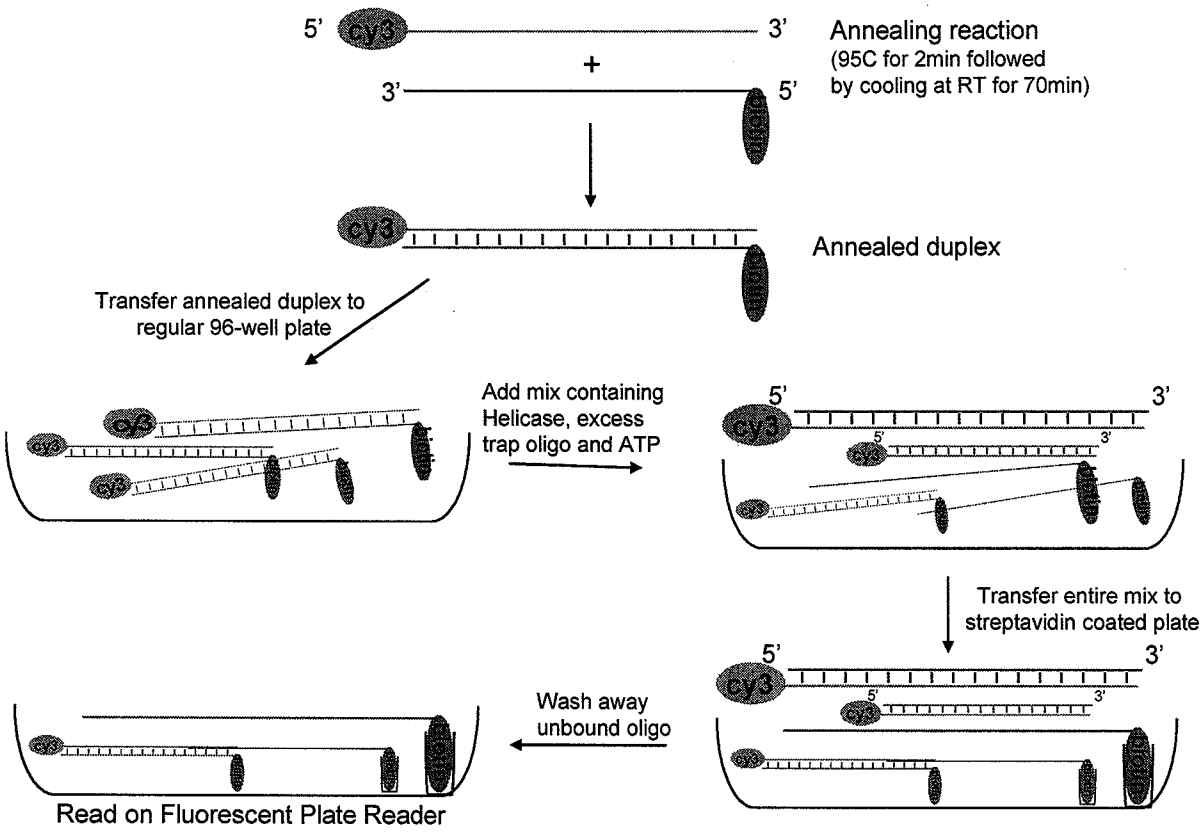

5'
cy3
3'
+
3'
5'
Annealing reaction
(95C for 2min followed
by cooling at RT for 70min)
cy3
Annealed duplex
Transfer annealed duplex to
regular 96-well plate
Add mix containing
Helicase, excess
trap oligo and ATP
5'
3'
cy3
Transfer entire mix to
streptavidin coated plate
Wash away
unbound oligo
Read on Fluorescent Plate Reader

FIGURE 3

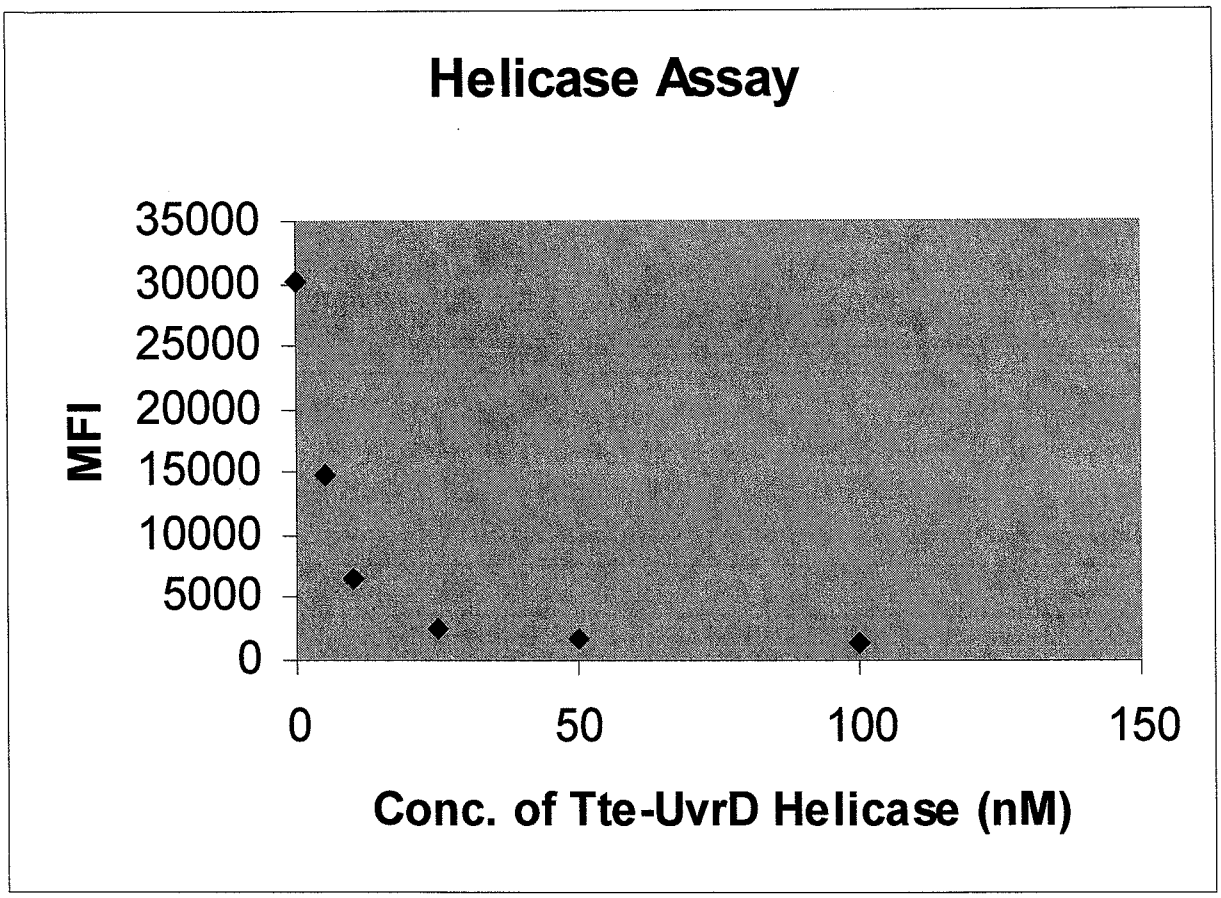

Helicase Assay
MFI
35000
30000
25000
20000
15000
10000
5000
0
0
50
100
150
Conc. of Tte-UvrD Helicase (nM)

FIGURE 4

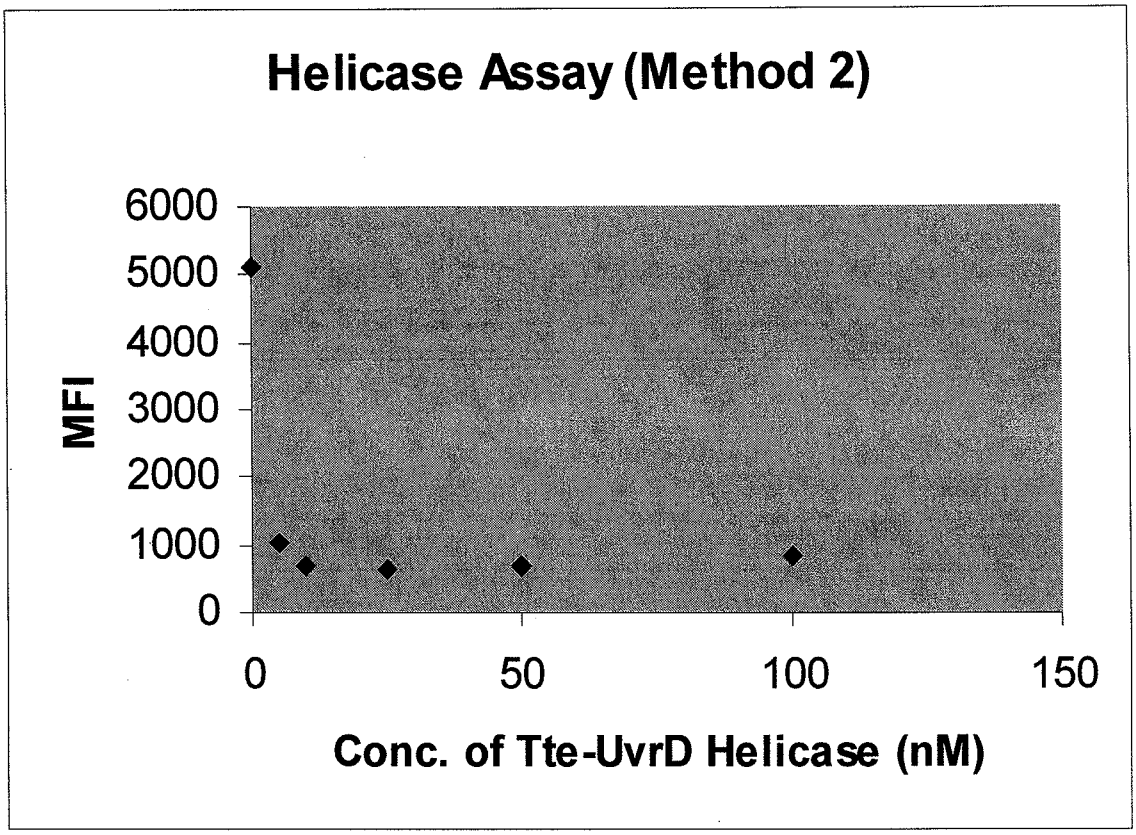

Helicase Assay (Method 2)
MFI
6000
5000
4000
3000
2000
1000
0
0
50
100
150
Conc. of Tte-UvrD Helicase (nM)

FIGURE 5

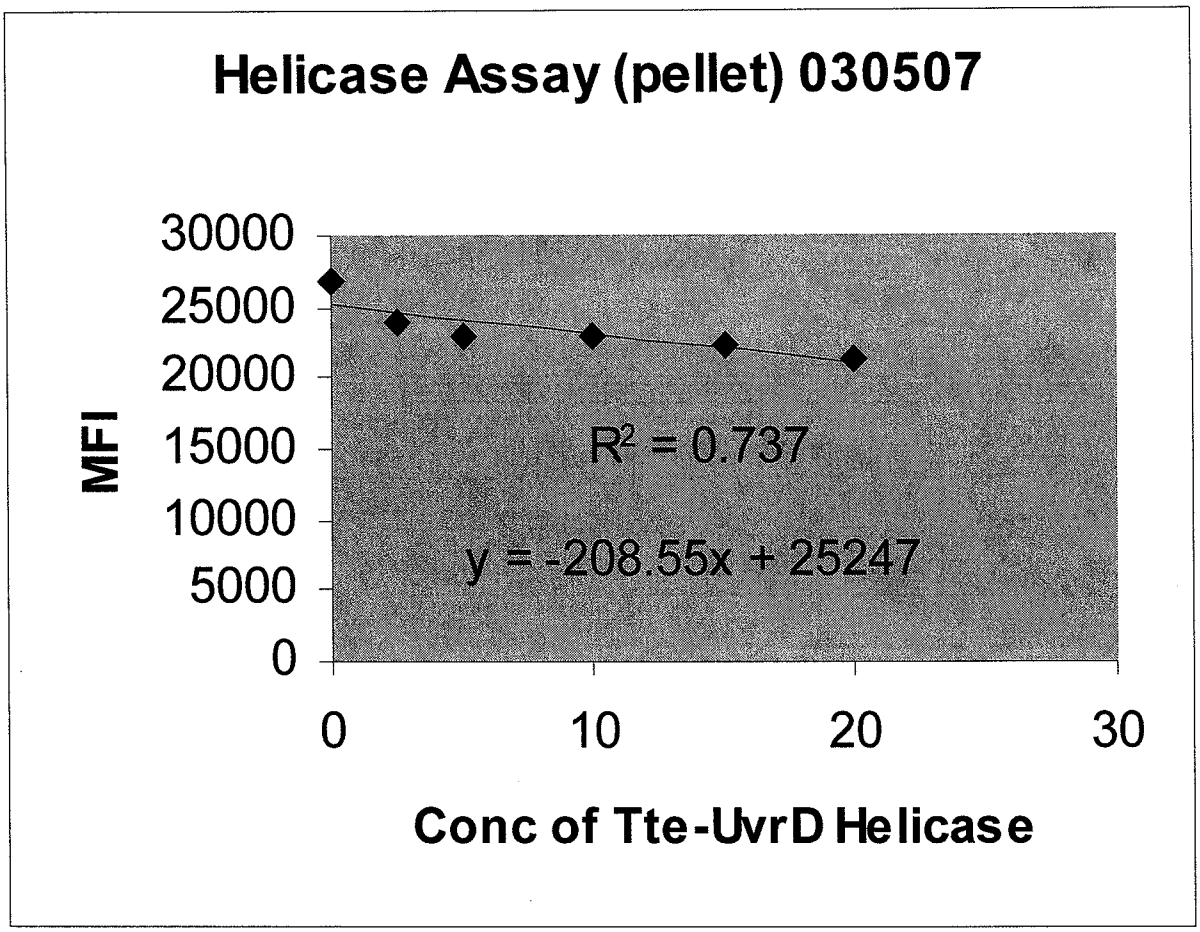

Helicase Assay (pellet) 030507
MFI
30000
25000
20000
15000
10000
5000
0
R2 = 0.737
y = -208.55x + 25247
0
10
20
30
Conc of Tte-UvrD Helicase

FIGURE 6

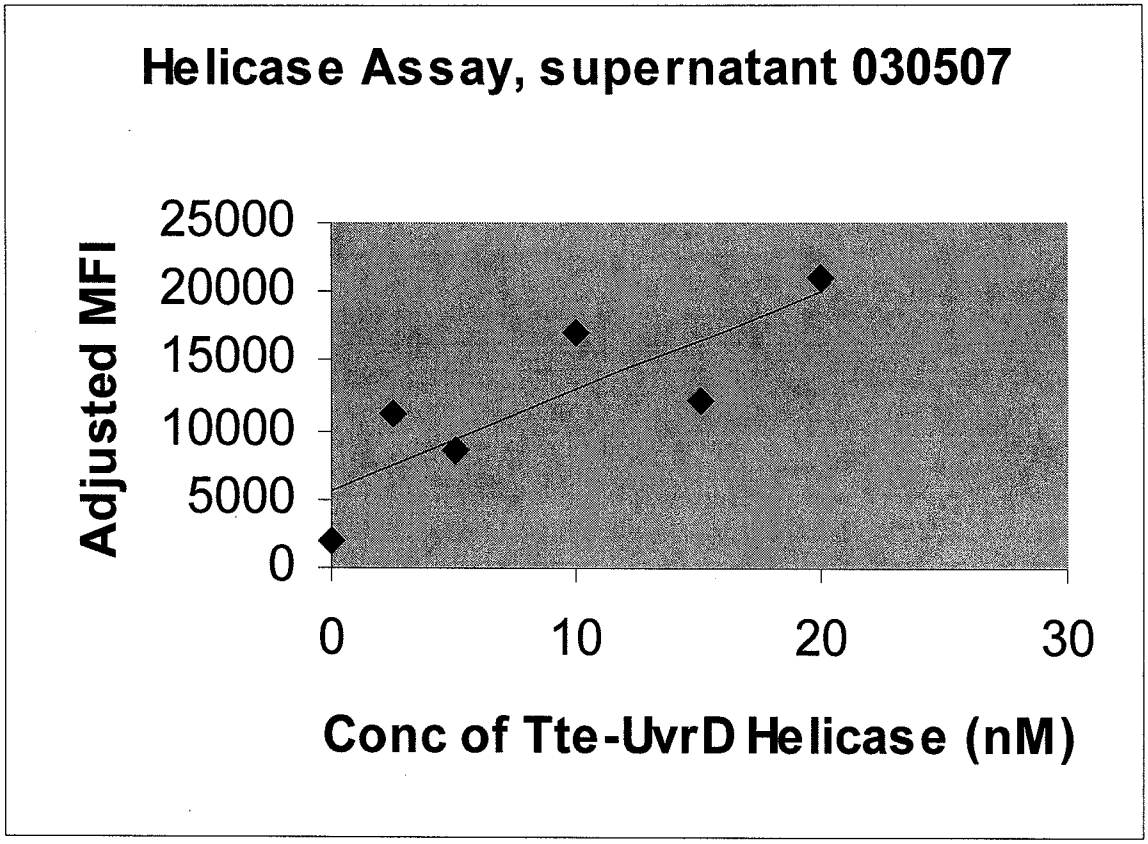

Helicase Assay, supernatant 030507
Adjusted MFI
25000
20000
15000
10000
5000
0
0
10
20
30
Conc of Tte-UvrD Helicase (nM)

FIGURE 7

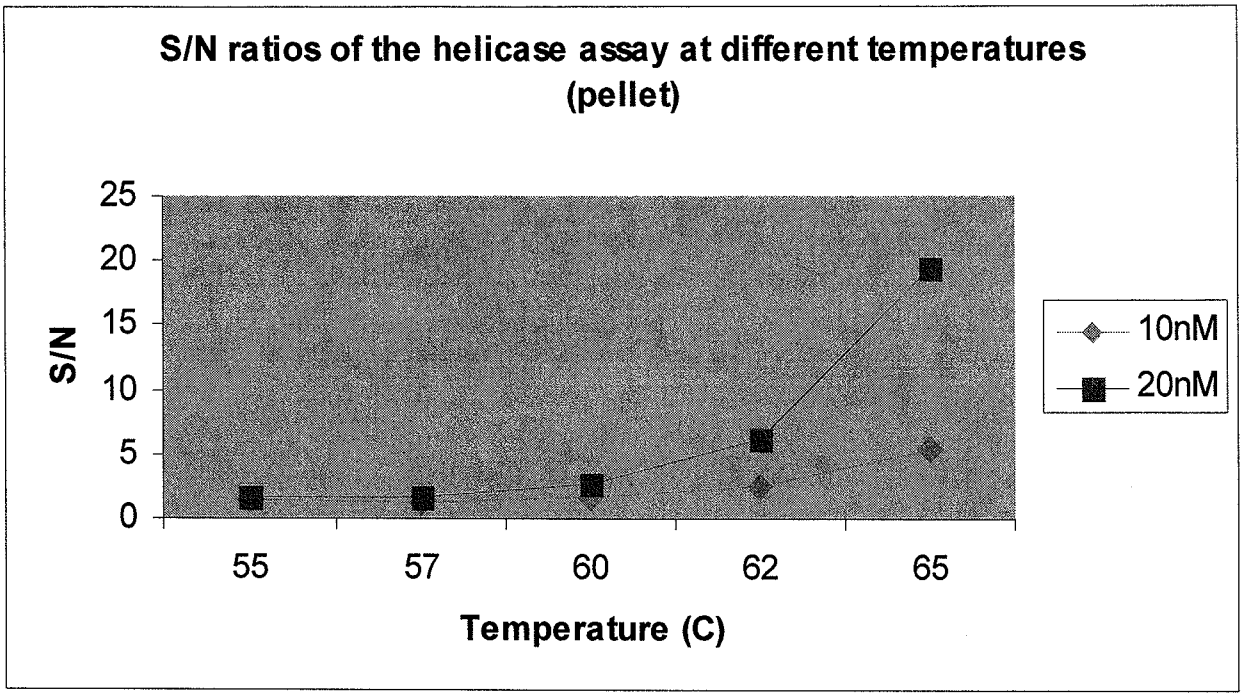

S/N ratios of the helicase assay at different temperatures (pellet)
S/N
25
20
15
10
5
0
55
57
60
62
65
Temperature (C)
10nM
20nM

FIGURE 8

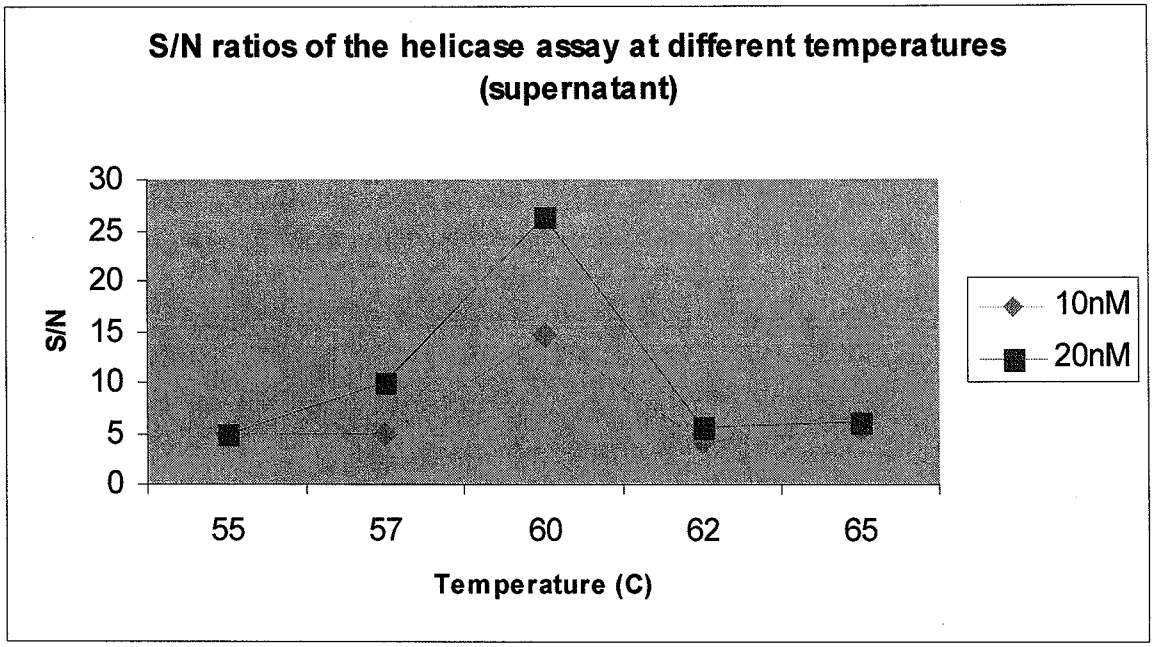
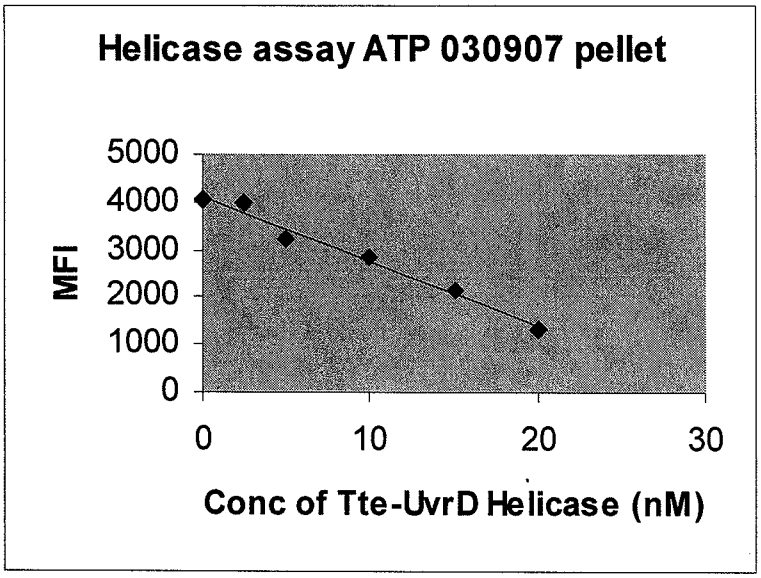

S/N ratios of the helicase assay at different temperatures (supernatant)
30
25
20
15
10
5
0
S/N
55
57
60
62
65
Temperature (C)
10nM
20nM

FIGURE 9

A


Helicase assay ATP 030907 pellet
MFI
5000
4000
3000
2000
1000
0
0
10
20
30
Conc of Tte-UvrD Helicase (nM)

B

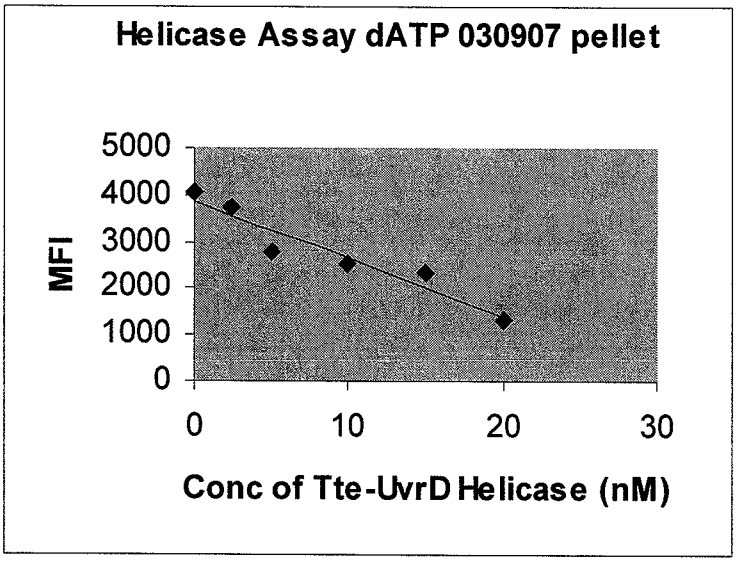
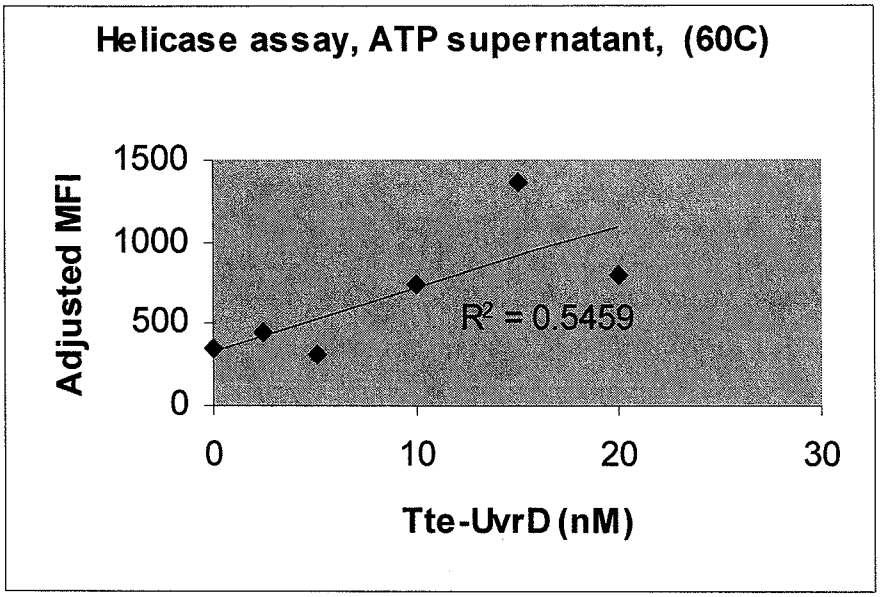

Helicase Assay dATP 030907 pellet
MFI
5000
4000
3000
2000
1000
0
0
10
20
30
Conc of Tte-UvrD Helicase (nM)

FIGURE 10

A


Helicase assay, ATP supernatant, (60C)
Adjusted MFI
1500
1000
500
0
R² = 0.5459
0
10
20
30
Tte-UvrD (nM)

B

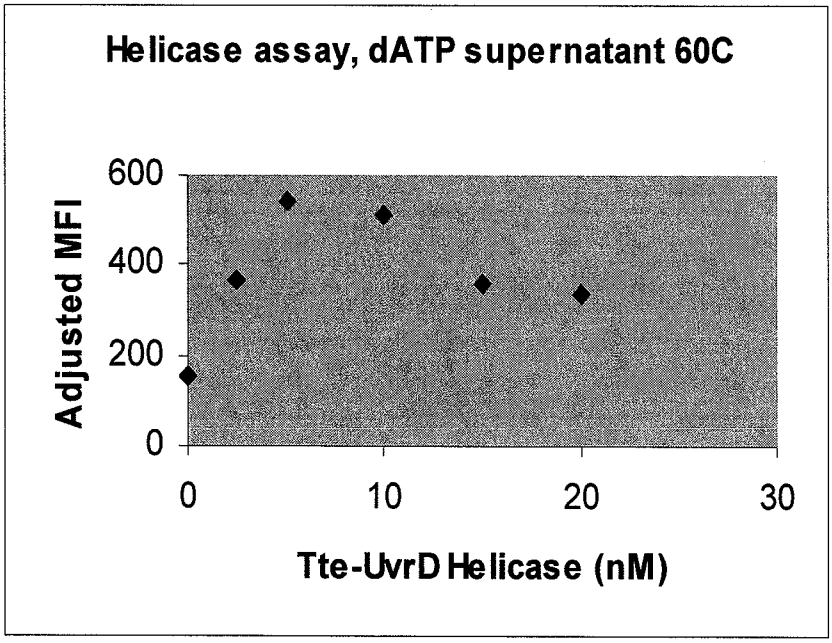
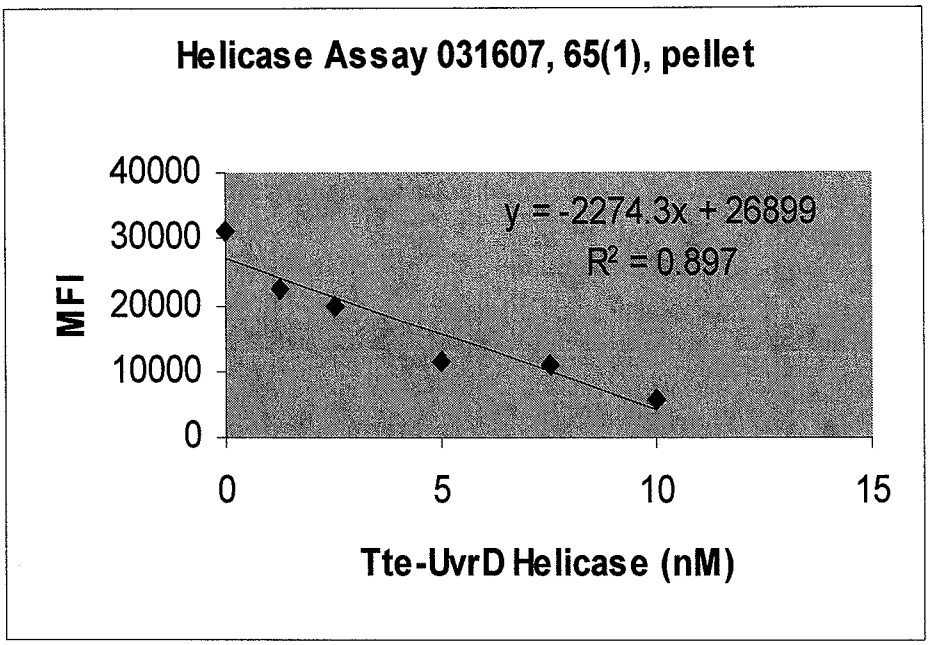

Helicase assay, dATP supernatant 60C
Adjusted MFI
600
400
200
0
0
10
20
30
Tte-UvrD Helicase (nM)

FIGURE 11

A


Helicase Assay 031607, 65(1), pellet
MFI
40000
30000
20000
10000
0
y = -2274.3x + 26899
R² = 0.897
0
5
10
15
Tte-UvrD Helicase (nM)

B

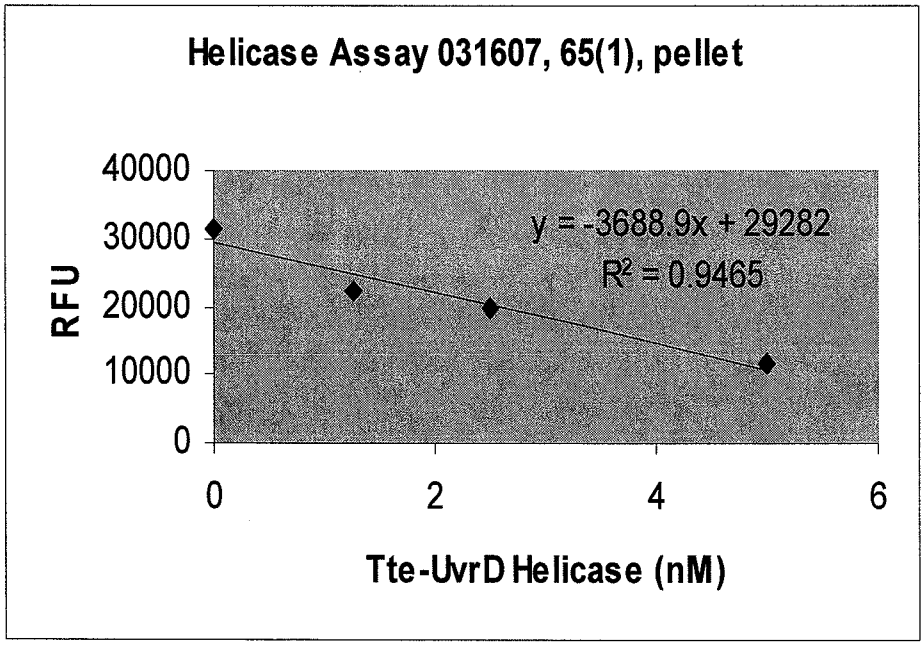
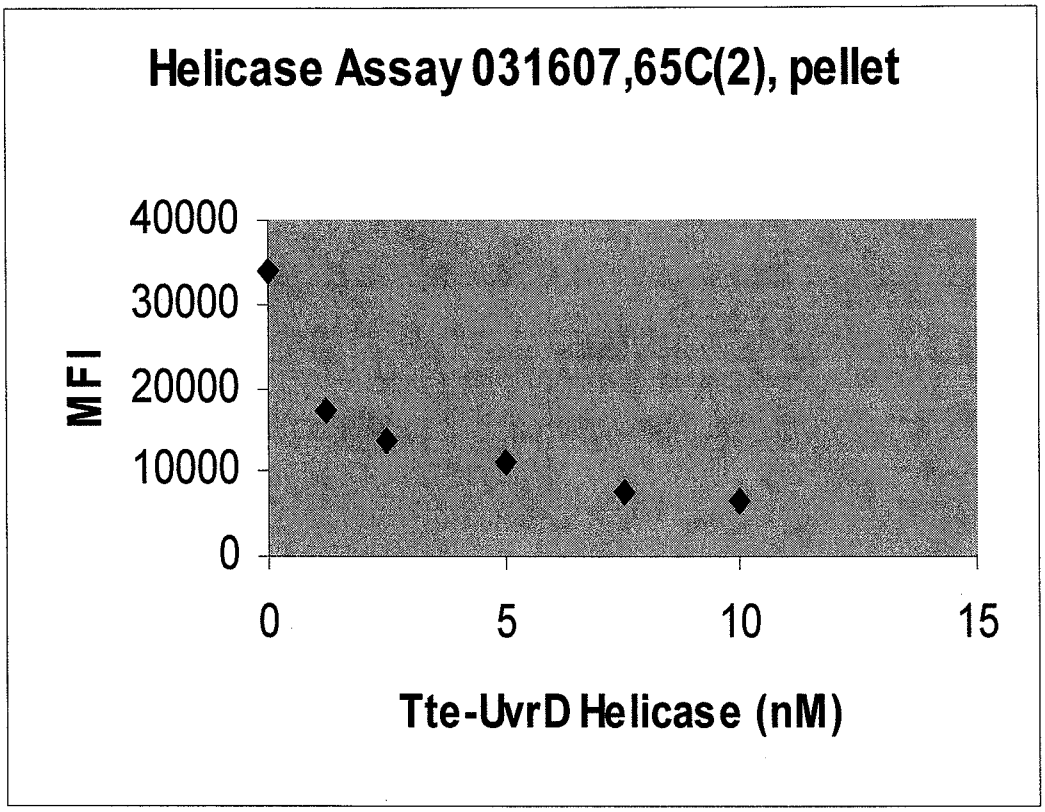

Helicase Assay 031607, 65(1), pellet
RFU
40000
30000
20000
10000
0
y = -3688.9x + 29282
R² = 0.9465
0
2
4
6
Tte-UvrD Helicase (nM)

FIGURE 12


Helicase Assay 031607,65C(2), pellet
40000
30000
20000
10000
0
MFI
0
5
10
15
Tte-UvrD Helicase (nM)

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2011/038167

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/533 C12Q1/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, COMPENDEX, EMBASE, FSTA, INSPEC, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 01/25487 A2 (AVENTIS PHARM PROD INC [US]; ZANG LITAO [US]; HARRISON RICHARD K [US]) 12 April 2001 (2001-04-12) abstract claim 5	1-18
X	WO 00/06710 A1 (TULARIK INC [US]) 10 February 2000 (2000-02-10) abstract; claim 4 Page 3, last paragraph to page 4 end of second paragraph.	1-18

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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Date of the actual completion of the international search	Date of mailing of the international search report
12 July 2011	29/07/2011

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Jacques, Patrice

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2011/038167

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HICHAM ALAOUI-ISMAILI M ET AL: "A novel high throughput screening assay for HCV NS3 helicase activity", ANTIVIRAL RESEARCH, ELSEVIER BV, NL, vol. 46, no. 3, 1 June 2000 (2000-06-01), pages 181-193, XP002234885, ISSN: 0166-3542, DOI: DOI:10.1016/S0166-3542(00)00085-1 Page 184, section 2.2figure 2	1-18
Y	JP 2008 099619 A (NAT INST OF ADV IND & TECHNOL (INCLUDING THE AUTOMATIC TRANSLATION)) 1 May 2008 (2008-05-01) claim 5	1-18
T	-& JP 2008 099619 A (AUTOMATIC ENGLISH TRANSLATED VERSION) 1 May 2008 (2008-05-01)	
Y	CRAIG BELON ET AL: "Monitoring helicase activity with molecular beacons", BIOTECHNIQUES, vol. 45, no. 4, 1 October 2008 (2008-10-01), pages 433-442, XP55002526, ISSN: 0736-6205, DOI: 10.2144/000112834 abstract	16
A	KYONO KIYOSHI ET AL: "Detection of hepatitis C virus helicase activity using the scintillation proximity assay system", ANALYTICAL BIOCHEMISTRY, ACADEMIC PRESS INC, NEW YORK, vol. 257, no. 2, 15 March 1998 (1998-03-15), pages 120-126, XP002170874, ISSN: 0003-2697, DOI: DOI:10.1006/ABIO.1998.2560 the whole document	1-18

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2011/038167

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0125487 A2	12-04-2001	AT 334227 T	15-08-2006
		AU 783653 B2	17-11-2005
		AU 7858900 A	10-05-2001
		BR 0014507 A	11-06-2002
		CA 2386642 A1	12-04-2001
		DE 60029607 T2	26-07-2007
		DK 1222311 T3	06-11-2006
		EP 1222311 A2	17-07-2002
		ES 2265989 T3	01-03-2007
		HK 1046934 A1	20-10-2006
		IL 148941 A	19-08-2007
		JP 2003511660 A	25-03-2003
		MX PA02003385 A	10-09-2004
		NO 20021585 A	04-06-2002
		NZ 518010 A	30-05-2003
		PT 1222311 E	31-10-2006
		US 2003032041 A1	13-02-2003
		US 6448004 B1	10-09-2002
		US 2002127549 A1	12-09-2002
		ZA 200202331 A	27-08-2003
WO 0006710 A1	10-02-2000	AU 5545399 A	21-02-2000
		US 6569619 B1	27-05-2003
JP 2008099619 A	01-05-2008	NONE	