concentrations will enable the detection of two additional orders of magnitude of probe concentration. This methodology is not limited to two primary probes, but may be expanded to three or more. Preferably, the methods are combined with amplification methods where one part of the target is amplified to a different level that a second part of the target.

As mentioned above, in certain embodiments, two probes are employed that are present at different concentrations that detect the same target nucleic acid molecule across a broad range of concentrations. In some embodiments involving invasive cleavage reactions, each of the two primary probes contain the same analyte specific region (ASR) but have different flap regions. Each of these two flap regions, when cleaved, reports to a different FRET cassette or other reporter sequence or system. In some embodiments, the two FRET cassettes both contain the same fluorophore molecule. In this system, an increase in dynamic range is achieved without the use of multiple different fluorophores. This system, therefore, offers a cost advantage over multiple fluorophore systems. Furthermore, expansion of dynamic range with a single fluorophore allows for multiplexing with multiple fluorophores for detection of different targets in the same vessel across a broader dynamic range than was previously feasible.

In certain embodiments, the concentration of each primary probe is present at 100-fold difference relative to each other, and the concentration of the two FRET cassettes are present at equivalent concentrations. In certain embodiments, as an example, the dynamic range with each of the primary probes present individually may be 10^4-10^6 and 10^6-10^8, respectively, while the dynamic range of the assay when both are present at the requisite different concentrations may be 10^4-10^8. The dynamic range of the serial invasive cleavage assay may be further expanded by the use of further additional primary probes, each present at different concentrations. In this manner, three, four, five or more primary probes, each having the same ASR and different flaps may report to the same number of different FRET cassettes, each reporting the same color or detection format. Such a combination of primary probes enables the expansion of the dynamic range to cover 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 orders of magnitude.

The methods of the present invention are not limited by the type of target nucleic acid. For example, the target nucleic acid may include, for example, nucleic acid

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

The methods of the present invention may be combined with amplification methods (e.g., PCR) to extend the lower limit of detection down to the theoretical limit of amplification, on the order of 1 copy per reaction vessel. Using this approach, the dynamic range of nucleic acid detection assay may be, for example, from 1 to 10^7 copies using a single set of reaction conditions and probe combinations in each reaction vessel being compared.

Additionally, methods of the present invention involve differential pre-amplification of target species prior to the detection assay. In certain embodiments, the use of differential semi-nested PCR using primers of different melting temperatures will result in a mixed population of different species, each containing the target region detected by the detection assay. The species present in higher numbers in the sample after this step can be detected by the probes present at lower concentration within its dynamic range, and the species present in lower numbers in the sample can be detected by the primary probe present at higher concentration within its dynamic range, as explained above. In addition, a population of target molecules present at different concentrations can also be generated by simultaneously combining linear and exponential amplification (or other types of amplification that lead to different levels of amplification). For example, two target-derived amplicons, both containing the target region detected by the nucleic acid detection assay, would be generated by producing one with a single PCR primer (for linear amplification) and the other with two PCR primers (for exponential amplification). As above, the different concentrations of targets can be detected with multiple primary probes tailored to detect those concentrations within their dynamic range. Further, non-amplified and amplified DNA can also be simultaneously detected using the above-described combination of probes.

Differential pre-amplification may also comprise multiple similar amplifications (*e.g.*, exponential amplifications) that are performed at different efficiencies so as to

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allow expanded dynamic range. By way of example and not to be limited to any particular embodiment or mechanism, target sequences may be selected such that one region to be amplified comprises few of a selected nucleotide, while another region to be amplified comprises an abundance of the same nucleotide. Pre-amplification under conditions wherein the dNTP required to replicated the selected nucleotide is limited or omitted will favor amplification of the sequence that is largely free of the limiting nucleotide. Conditions can be selected to allow the other sequence to amplify inefficiently, *e.g.*, by mis-incorporating bases. This is but one way in which differential pre-amplification can be configured to allow.

In some embodiments, additional probes are used to further expand the dynamic range (e.g., three probes of different concentrations that each bind to the same analyte-specific region). In some embodiments, the method detects one or more probes under each of three distinct amplification conditions: e.g., one probe or probe set that detects exponentially amplified target nucleic acid; one probe or probe set that detects linearly amplified target nucleic acid; and one probe or probe set that detects unamplified target nucleic acid. Additional amplification conditions may also be used (e.g., exponential amplification using primers or other reaction conditions that provide different amplification efficiency per cycle—e.g., a first set that is 90% efficient per cycle and a second set that is 70% efficient per cycle).

Where PCR or other amplification techniques are used, it may be desirable to use buffers and other agents and reaction conditions that minimize limitations of the respective amplification techniques. For example, where PCR is used, in some embodiments, a short amplicon is used. In some embodiments, the amplicon is less than one kilobase in length, although the present invention is not limited to such amplicons. In some embodiments, where the target nucleic is RNA, the amplicon is less than 100 bases, although the present invention is not so limited.

Accordingly, in some embodiments, the present invention provides methods and compositions for performing probe hybridization assays. In some embodiments, the method utilizes a primary or first probe and preferably at least one additional probe having different hybridization characteristics with respect to a target sequence than the primary probe. In some embodiments, a single probe that provides enhanced dynamic

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range is utilized. In preferred embodiments, the compositions and methods of the present invention utilize a combination of two or more probe oligonucleotides to increase the dynamic range of detection of the amount of a target nucleic acid present in a sample. In preferred embodiments, combinations of two or more probe oligonucleotides include a mixture of probe oligonucleotides with varying degrees of hybridization to a target nucleic acid (e.g., frequency of occupation of a hybridization site). Exemplary probe oligonucleotides of the present invention are described in greater detail below.

In some embodiments, three or more probes are used (e.g., four, five, six, etc.). Two or more of the probes may be configured to hybridize to the same region of the target nucleic acid. However, one or more of the probes may be configured to hybridize to a second region of the target nucleic acid or to a different target nucleic acid. In some embodiments, the pluralities of different probes are configured to generate a detectable signal directly or indirectly. In some embodiments, the different probes use the same type of label so that the detected signal is an additive accumulation of the signal from the first and second probes. In some such embodiments, the user of the method observes the signal throughout the broader dynamic range without knowing or needing to know the contribution provided by each type or probe.

Using such systems and methods, detection of a target nucleic acid can be achieved through a very extensive dynamic range. In some embodiments, this permits detection of target nucleic acids without the need to amplify the target nucleic acid or without the need to extensively amplify the target nucleic acid. However, the systems and methods may further be employed with amplification methods, where desired. As described herein, the systems and methods of the present invention have been exemplified with a combination of polymerase chain extension amplification and invasive cleavage-based detection. Such methods experimentally demonstrated successful detection of target nucleic acids having over an eight-log difference in starting concentration. Thus, the systems and methods of the present invention are exceptionally well suited to the detection of target nucleic acids whose concentration differs dramatically from sample to sample. For example, patients infected with viruses such as HCV and HIV differ greatly the copy number of virus target nucleic acid present in sample (e.g., blood) from very low copy (as few as one copy) to very high copy (millions

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to billions of copies or more). The ability of a single detection system to simultaneously detect viral target nucleic acid throughout this range is greatly desired. The present invention provides systems and methods that find use for such detection.

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

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

The quantitation of target nucleic acids using the methods and compositions of the present invention are utilized in a variety of clinical and research applications. For example, in some embodiments, the detection assays with increased dynamic range of the present invention are utilized in the detection and quantitation of viral pathogens in human samples. The detection assays of the present invention are suitable for use with a

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variety of purified and unpurified samples including, but not limited to, urine, stool, lymph, whole blood, and serum. In preferred embodiments, the detection assays of the present invention are suitable for use in the presence of host cells.

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

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

I. Probe Oligonucleotides

In some embodiments, the present invention provides methods for altering (e.g., increasing) the dynamic range of a nucleic acid detection assay by altering probe oligonucleotides. In some embodiments, the present invention provides combinations of two or more probe oligonucleotides for use in the same detection assay. The present invention is not limited by the manner in which probes are modified to alter hybridization characteristics. Certain exemplary embodiments are provided below.

A. Mismatch Probes

In some embodiments, the present invention provides probes with one or more (preferably one) mismatch with the target sequence. The present invention is not limited to a particular mechanism. Indeed, an understanding of the mechanism is not necessary to practice the present invention. Nonetheless, it is contemplated that the presence of one or more mismatches allows the probe to bind to the target, but with a reduced affinity as compared to a corresponding probe lacking mismatches. This decreases the percent of the time that the mismatch probe occupies the target site, thus decreasing the signal generated (or increasing the signal, depending on the detection system used). The decrease in signal allows the detection assay to remain linear or accurate for quantitation at a higher target concentration.

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In some embodiments, mismatch probes are utilized in combination with completely complementary probes. The completely complementary probes occupy the target-binding site a higher percentage of the time than the mismatch probes and thus generate more signal. The higher signal allows for the detection of lower concentrations of target nucleic acid. The use of both probes increases the dynamic range of the detection assay. In particular, as described above, it increases the linearity through a broader concentration of target molecules.

Example 1 and Figures 1 and 2 demonstrate how the use of mismatch probes can increase the dynamic range of an assay. A combination of match and mismatch probes was used in an INVADER assay to detect target nucleic acids. The mismatch probe increased the dynamic range by up to 16-fold over the use of a single completely complementary probe.

B. Lower probe concentrations

In other embodiments, the present invention provides a combination of probe concentrations to increase the dynamic range of a detection assay. In some embodiments a combination of two or more probe oligonucleotides, each of which is at a different concentration, is utilized. The probes present at a lower concentration generate a lower signal and are thus suitable for detecting higher target concentrations. The probes present at a higher concentration generate a higher signal and are thus suitable for detecting a lower concentration of target nucleic acids. By utilizing two or more probes at a range of concentrations, a broader dynamic range of target concentrations can be detected.

When probes are attached to a solid surface, lower probe concentration can be achieved, in some embodiments, through the use of different densities of probes attached to particular detection zones on the solid surface. For example, a first probe detection zone has a first density of the probe and a second probe detection zone has a lower density of the probe. Detection at the two detection zones provides enhanced dynamic range. In some embodiments, both detection zones generate the same type of signal and the total signal from the solid surface is detected (e.g., in real-time) to detect the target nucleic acid through an expanded dynamic range.

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Example 1 and Figures 1 and 2 demonstrate how the use of multiple probes present at different concentrations probes can increase the dynamic range of an assay. A combination of concentrations of probes was used in an INVADER assay to detect target nucleic acids. The use of multiple concentrations of probes increased the dynamic range of the assay over the use of a single probe.

C. Charge Modified Probes

In other embodiments, the present invention utilizes charge modified probes to alter binding efficiency of probes (See e.g., US Patent 6,780,982, herein incorporated by reference in its entirety for all reasons). In some embodiments, the charge modified probes comprise "charge tags." Positively charged moieties need not always carry a positive charge. As used herein, the term "positively charged moiety" refers to a chemical structure that possesses a net positive charge under the reaction conditions of its intended use (e.g., when attached to a molecule of interest under the pH of the desired reaction conditions). Indeed, in some preferred embodiments of the present invention, the positively charged moiety does not carry a positive charge until it is introduced to the appropriate reaction conditions. This can also be thought of as "pH-dependent" and "pH-independent" positive charges. pH-dependent charges are those that possess the charge only under certain pH conditions, while pH-independent charges are those that possess a charge regardless of the pH conditions.

The positively charged moieties, or "charge tags," when attached to another entity, can be represented by the formula:

X-Y

where X is the entity (e.g., a solid support, a nucleic acid molecule, etc.) and Y is the charge tag. The charge tags can be attached to other entities through any suitable means (e.g., covalent bonds, ionic interactions, etc.) either directly or through an intermediate (e.g., through a linking group). In preferred embodiments, where X is a nucleic acid molecule, the charge tag is attached to either the 3' or 5' end of the nucleic acid molecule.

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The charge tags may contain a variety of components. For example, the charge tag Y can be represented by the formula:

Y_1-Y_2

where Y_1 comprises a chemical component that provides the positive charge to the charge tag and where Y_2 is another desired component. Y_2 may be, for example, a dye, another chemical component that provides a positive charge to the charge tag, a functional group for attachment of other molecules to the charge tag, a nucleotide, etc. Where such a structure is attached to another entity, X, either Y_1 or Y_2 may be attached to X.

X-Y_1-Y_2 or X-Y_2-Y_1.

The charge tags are not limited to two components. Charge tags may comprise any number of desired components. For example, the charge tag can be represented by the formula:

Y_1-Y_2-Y_3-Y_n (n = any positive integer).

where any of the Y_x groups comprises a chemical component that provides the positive charge to the charge tag and where the other Y groups are any other desired components. For example, in some embodiments, the present invention provides compositions of the structure:

X-Y_1-Y_2-Y_3-Y_4

where X is an entity attached to the charge tag (e.g., a solid support, a nucleic acid molecule, etc.) and where Y_1 is a dye, Y_2 is a chemical component that provides the positive charge to the charge, Y_3 is a component containing a functional group that allows the attachment of other molecules, and Y_4 is a second chemical component that provides a positive charge. The identity of each of Y_1-Y_4 can be interchanged (i.e., the present invention is not limited by the order of the components).

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The present invention is not limited by the nature of the chemical components that provides the positive charge to the charge tag. Such chemical components include, but are not limited to, amines (primary, secondary, and tertiary amines), ammoniums, and phosphoniums. The chemical components may also comprise chemical complexes that entrap or are otherwise associated with one or more positively charged metal ions.

In preferred embodiments of the present invention, charge tags are attached to nucleic acid molecules (e.g., DNA molecules). The charge tags may be synthesized directly onto a nucleic molecule or may be synthesized, for example, on a solid support or in liquid phase and then attached to a nucleic acid molecule or any other desired molecule. In some preferred embodiments of the present invention, charge tags that are attached to nucleic acid molecules comprise one or more components synthesized by H-phosphonate chemistry, by incorporation of novel phosphoramidites, or a combination of both. For example, compositions of the present invention include structures such as:

$[X]-[Y_1-Y_2-Y_3-Y_4]$

where [X] is a nucleic acid molecule and [Y . . .] is a charge tag. In some embodiments, Y_1 is a dye, Y_2 is synthesized using H-phosphonate chemistry and comprises a chemical component that provides a positive charge to the charge tag, Y_3 is a positively charged phosphoramidite, and Y_4 is a nucleotide or polynucleotide. Any of the Y components are interchangeable with one another.

As discussed above, one or more components of a charge tag can be synthesized using H-phosphonate chemistry. Production of charge tag using the methods described herein provides a convenient and flexible modular approach for the design of a wide variety of charge tags. Since its introduction, solid phase H-phosphonate chemistry (B.C. Froehler, Methods in Molecular Biology, 20:33, S. Agrawal, Ed. Humana Press; Totowa, New Jersey[1993]) has been recognized as an efficient tool in the chemical synthesis of natural, modified and labeled oligonucleotides and DNA probes. Those skilled in the art know that this approach allows for the synthesis of the oligonucleotide fragments with a fully modified phosphodiester backbone (*e.g.,* oligonucleotide phosphorothioates; Froechler [1993], *supra*) or the synthesis of oligonucleotide fragments in which only

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specific positions of the phosphodiester backbone are modified (Agrawal, *et al.*, Proc.Natl.Acad.Sci USA , 85:7079 [1988], Froehler,Tetrahedron Lett. 27:5575[1986], Froehler, *et al.*, Nucl.Acids Res. 16:4831 [1988]). The use of H-phosphonate chemistry allows for the introduction of different types of modifications into the oligonucleotide molecule (Agrawal, *et al.,* Froehler[1986], *supra*, Letsinger, *et al.*, J.Am. Chem.Soc., 110:4470 [1988], Agrawal and Zamecnik, Nucl. Acid Res. 18:5419 [1990], Handong, *et al.*, Bioconjugate Chem. 8:49 [1997], Vinogradov, *et al.*, Bioconjugate Chem. 7:3 [1995], Schultz, *et al.*, Tetrachedron Lett. 36:8407 [1995]), however the replacement of the phosphodiester linkage by the phosphoramidate linkage is one of the most frequent changes due to its effectiveness and synthetic flexibility. Froehler and Letsinger were among first to use this approach in the synthesis of modified oligonucleotides in which phosphodiester linkages were fully or partially replaced by the phosphoramidate linkages bearing positively charged groups (e.g., tertiary amino groups; Froehler [1986], Froehler, *et al.,* [1988], and Letsinger, *et al.*, *supra*).

In some embodiments of the present invention, charge tags are generated using H-phosphonate chemistry. The charge tags may be assembled on the end of a nucleic acid molecule or may be synthesized separately and attached to a nucleic acid molecule. Any suitable phosphorylating agent may be used in the synthesis of the charge tag. For example, the component to be added may contain the structure:

A-B-P

where A is a protecting group, B is any desired functional group (e.g., a functional group that provides a positive charge to the charge tag), and P is a chemical group containing phosphorous. In preferred embodiments, B comprises a chemical group that is capable of providing a positive charge to the charge tag. However, in some embodiments B is a functional group that allows post-synthetic attachment of a positively charged group to the charge tag.

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In other embodiments, positively charged phosphoramidites (PCP) and neutral phosphoramidites (NP) are utilized to introduce both positive charge and structure modulation into the synthesized charge-balanced CRE probe (See e.g., US Patent 6,780,982, herein incorporated by reference in its entirety).

Standard coupling protocol with the use of the phosphoramidite reagents (which are compatible with the chemical synthesis of oligonucleotides) is associated with the introduction, into the growing molecule, of one negative charge per each performed coupling step, due to the formation of the phosphodiester linkage.

D. Nucleic Acid Modification Agents

The present invention is not limited to the use of charge tags as modifiers of probe hybridization efficiency. Any internal (e.g., to the probe) or external agent that alters the hybridization strength of probe binding is suitable for use with the methods and compositions of the present invention.

In some embodiments, the present invention provides probes comprising intercalating agents. Intercalating agents are agents that are capable of inserting themselves between the successive bases in DNA. In some embodiments, intercalating agents alter the binding properties of nucleic acid probes.

Examples of intercalating agents are known in the art and include, but art limited to, ethidium bromide, psoralen and derivatives, acridines, proflavine, acridine orange, acriflavine, fluorcoumanin, ellipticine, daunomycin, chloroquine, distamycin D, chromomycin, homidium, mithramycin, ruthenium polypyridyls, and anthramycin.

In other embodiments, minor groove DNA binding agents are utilized to modify (e.g., increase or decrease) the hybridization efficiency of probes. Examples of minor groove binding agents include, but are not limited to, duocarmycins (See e.g., Boger, Pure & Appl. Chem., Vol. 66, No. 4, pp. 837-844, herein incorporated by reference in its entirety), netropsin, bisbenzimidazole, aromatic diamidines, lexitropsins, distamycin, and organic dications, based on unfused-aromatic systems (See e.g., US Patent 6,613,787, herein incorporated by reference in its entirety).

In still further embodiments, modified bases are utilized to alter the hybridization efficiency of probes. For example, in some embodiments, modified bases that include

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charged groups are utilized. Examples include, but are not limited to, the substitution of a "t" nucleotide with "amino-T" in a probe and other modified nucleotides.

In yet other embodiments, one or more probe nucleotides are modified by the covalent attachment of groups that alter the hybridization properties of the probe. Examples include, but are not limited to, the attachment of amino acids to nucleotides.

In yet other embodiments, probe oligonucleotides with base analogues are utilized to alter the hybridization characteristics of probes. For example, in some embodiments, nucleotides that do not form hydrogen bonds but that still participate in base stacking are utilized. Examples include but are not limited to non-polar, aromatic nucleoside analogs such as 2,4-difluorotoluene and "universal" bases such as 5-nitroindole and 3-nitropyrrole. In other embodiments, base analogs that retain hydrogen bonding ability are utilized (See e.g., US Patent application US20040106108A1 and WO 04/065550A3, each of which is herein incorporated by reference in its entirety for all purposes).

E. Probe Length

In yet other embodiments, probe length is altered in order to alter the hybridization characteristics of a probe. For example, in some embodiments, two or more probes that hybridize to the same target sequence and share the same sequence are utilized. In some embodiments, one of the probes is shorter by one, two, three, or four or more bases. It is preferred that the probes be truncated from one or both ends. Thus, the probes share sequence in all regions except the truncated 3' or 5' ends. It is contemplated that the shorter probes will anneal with decreased hybridization efficiency and will thus be useful in the detection of higher copy numbers of target sequences than the full length probe. In preferred embodiments, a combination of full length and truncated probes is utilized to give the maximum range of target concentration detection. In some embodiments, the same length is employed, but the probe is split into two or more portions connected by linkers. Such probes hybridize with different affinity depending on a variety of factors, including secondary structure of the target nucleic acid in regions in which the probes or probe fragments hybridize.

F. Secondary Structure

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In some embodiments, probes that comprise secondary structure are utilized to alter the hybridization efficiency of the probe. For example, in some embodiments, two or more probes are designed to hybridize the same target sequence. One of the probes is designed to have minimal secondary structure. Additional probes are designed that retain target sequence recognition, but that have secondary structure. It is contemplated that the probes with secondary structure will exhibit decreased hybridization properties and will thus be suitable for the detection of large copy numbers of target sequence. The combination of probes lacking and containing secondary structure serves to detect a larger dynamic range of target nucleic acids than a single probe. Likewise, probes that hybridize to regions of the target nucleic acid that differ in secondary structure may be used. For example, a probe that has 18 of 18 bases that bind to linear target nucleic acid will hybridize differently than a similar probe shifted two bases over on target nucleic acid such that the two bases on the end of the probe correspond to a region of the target nucleic acid occupied in an internal hairpin structure or other secondary structure.

G. Competitor Oligonucleotides

In yet other embodiments, additional oligonucleotides are utilized to modify hybridization efficiency of probes. For example, in some embodiments, two probes that recognize the same target sequence are designed. One of the probes further comprises additional nucleic acid sequence (e.g., at the 3' or 5' end) that does not hybridize to the target sequence. Competitor oligonucleotides are designed to hybridize to the extra region. The binding of the competitor oligonucleotide decreases the hybridization efficiency of the probe to the target. The combination of probes with and without competitor binding sequences serves to detect a larger dynamic range of target nucleic acids than a single probe.

H. Reaction Conditions

In still further embodiments, reaction conditions are modified to alter probe hybridization characteristics. For example, in some embodiments, identical probes are utilized in separate reaction vessels, chamber, or wells. One reaction vessel utilizes "standard" reaction conditions for the detection assay (e.g., those supplied by the

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manufacturer or known in the art). The other reaction vessel comprises altered reaction conditions that increase or decrease the hybridization efficiency of the probe. Examples of parameters that affect nucleic acid hybridization conditions include, but are not limited to, ionic strength, buffer composition, pH, and additives (e.g., glycerol, polyethylene glycol, proteins).

I. Stacking Oligonucleotides

In still further embodiments, adjacently hybridizing oligonucleotides are used to alter probe hybridization characteristics. When short strands of nucleic acid align contiguously along a longer strand, the hybridization of each is stabilized by the hybridization of the neighboring fragments because the base pairs can stack along the helix as though the backbone was, in fact, uninterrupted. This cooperativity of binding can give each segment a stability of interaction in excess of what would be expected for the segment hybridizing to the longer nucleic acid alone. In the event of a perturbation in the cooperative binding, *e.g.*, by a mismatch at or near the junction between the contiguous duplexes, this cooperativity can be reduced or eliminated. In some embodiments of the present invention, probes are configured to cooperate in distinct ways with one or more adjacently hybridizing oligonucleotides, so as to provide probes having different hybridization characteristics. In some embodiments, a probe comprises one or more mismatched bases at near the junction with the adjacent oligonucleotide, so as to alter or disrupt cooperativity of binding, as compared to a probe lacking the mismatches. In other embodiments, a probe comprises one or more base analogs selected to reduce stacking interactions with adjacent bases. In yet other embodiments, it is envisioned that gaps of one or more nucleotides (*e.g.*, by the use of truncated probes) are used to alter cooperativity and thus alter hybridization characteristics. The use of a combination of probes that have a range of cooperativities of binding with an adjacently hybridized oligonucleotide, and thus having a range of different hybridization stabilities on the target, serves to detect a larger dynamic range of target nucleic acids than a single probe.

J. Multiplex Assays

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In some embodiments utilizing multiple nucleic acid probes, the probes are utilized in a biplex or multiplex assay in which a plurality of probes is included in the same reaction vessel. In some embodiments, each probe in a biplex assay comprises a differently detectable label. For example, some embodiments, each probe in a set comprises a different fluorescent label that fluoresces at a different wavelength. Many known probe binding assays are suitable for use in a multiplex format. Methods for performing multiplex assays that are unique to the particular assay format are described below.

K. Others

Any other method for altering the hybridization of characteristics of a probe may be used with the present invention. Other examples include, but are not limited to: use of sequences in probes or targets that render the sequence susceptible to differential hybridization behavior in response to buffer conditions (e.g., the use of guanosine-quartets) or protein/nucleic acid interactions (e.g., by creating binding sites for nucleic acid binding proteins or enzyme that bind or alter nucleic acid sequences); use of dangling ends (e.g., for dangling-end stabilization and stacking); attachment of iron or other magnetic agents to allow concentration of the nucleic acid in a magnetic field; use of agents that titrate out a specific probe; and the like.

One may also use different labeling techniques to achieve a differential detection of signal, independent of the hybridization properties of the probe. For example, the location of labels and quenchers in a FRET detection system may be altered between first and second probes to alter the amount of signal detected from the probes. FRET signaling can also be affected by many other parameters, including, but not limited to, the use of additional chemical moieties that influence the amount of quenching and the use of secondary structure in the probes. Additional methods for altering signal detection include the use of a helper oligonucleotide that is provided at low concentration, that when bound to a target occupied by a probe of the invention, changes the wavelength or otherwise alters the detectable aspects of the probe. The concentration of the helper can be configured to only allow detection the alteration when a particular threshold level of

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probe is hybridized to target. Any method or system that permits differential detection of hybridization events may be used in the systems and methods of the present invention.

II. Detection Assays

The present invention is not limited to a particular detection assay. Any number of suitable detection assays may be utilized. In some embodiments, the present invention provides methods and compositions for the detection of DNA or RNA (e.g., viral RNA). In some embodiments, the detection assays described below are suitable for direct detection of RNA. In other embodiments, RNA is reverse transcribed (e.g., using a reverse transcriptase enzyme such as AMV or MMLV) into DNA and the detection assay is performed on the corresponding DNA. Methods for reverse transcription are known in the art. In some embodiments, a single enzyme having both reverse transcriptase and polymerase activities is used.

Exemplary assays that find use with the methods of the present invention are described below.

A. Invader Assay

In some embodiments, the methods and compositions of the present invention are used to increase the dynamic range of the INVADER assay. The INVADER assay provides means for forming a nucleic acid cleavage structure that is dependent upon the presence of a target nucleic acid and cleaving the nucleic acid cleavage structure so as to release distinctive cleavage products. 5' nuclease activity, for example, is used to cleave the target-dependent cleavage structure and the resulting cleavage products are indicative of the presence of specific target nucleic acid sequences in the sample. When two strands of nucleic acid, or oligonucleotides, both hybridize to a target nucleic acid strand such that they form an overlapping invasive cleavage structure, as described below, invasive cleavage can occur. Through the interaction of a cleavage agent (e.g., a 5' nuclease) and the upstream oligonucleotide, the cleavage agent can be made to cleave the downstream oligonucleotide at an internal site in such a way that a distinctive fragment is produced. Such embodiments have been termed the INVADER assay (Third Wave Technologies, Madison, WI) and are described in U.S. Patent Appl. Nos. 5,846,717, 5,985,557,

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5,994,069, 6,001,567, and 6,090,543, WO 97/27214, WO 98/42873, Lyamichev et al., Nat. Biotech., 17:292 (1999), Hall et al., PNAS, USA, 97:8272 (2000), each of which is herein incorporated by reference in their entirety for all purposes.

The INVADER assay detects hybridization of probes to a target by enzymatic cleavage of specific structures by structure specific enzymes (*See*, INVADER assays, Third Wave Technologies; *See e.g.*, U.S. Patent Nos. 5,846,717; 6,090,543; 6,001,567; 5,985,557; 6,090,543; 5,994,069; Lyamichev et al., Nat. Biotech., 17:292 (1999), Hall et al., PNAS, USA, 97:8272 (2000), WO97/27214 and WO98/42873, each of which is herein incorporated by reference in their entirety for all purposes).

The INVADER assay detects specific DNA and RNA sequences by using structure-specific enzymes (*e.g.* FEN endonucleases) to cleave a complex formed by the hybridization of overlapping oligonucleotide probes. Elevated temperature and an excess of one of the probes enable multiple probes to be cleaved for each target sequence present without temperature cycling. In some embodiments, these cleaved probes then direct cleavage of a second labeled probe. The secondary probe oligonucleotide can be 5'-end labeled with fluorescent that is quenched by an internal dye. Upon cleavage, the de-quenched fluorescent labeled product may be detected using a standard fluorescence plate reader.

The INVADER assay detects specific target sequences in unamplified, as well as amplified, RNA and DNA including genomic DNA. In the embodiments shown schematically in Figure 3, the INVADER assay uses two cascading steps (a primary and a secondary reaction) both to generate and then to amplify the target-specific signal. For convenience, the alleles in the following discussion are described as wild-type (WT) and mutant (MT), even though this terminology does not apply to all genetic variations or target sequences. In the primary reaction (Figure 3, panel A), the WT primary probe and the INVADER oligonucleotide hybridize in tandem to the target nucleic acid to form an overlapping structure. An unpaired "flap" is included on the 5' end of the WT primary probe. A structure-specific enzyme (*e.g.* the CLEAVASE enzyme, Third Wave Technologies) recognizes the overlap and cleaves off the unpaired flap, releasing it as a target-specific product. In the secondary reaction, this cleaved product serves as an INVADER oligonucleotide on the WT fluorescence resonance energy transfer (WT-

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FRET) probe to again create the structure recognized by the structure specific enzyme (panel A). When the two dyes on a single FRET probe are separated by cleavage (indicated by the arrow in Figure 3), a detectable fluorescent signal above background fluorescence is produced. Consequently, cleavage of this second structure results in an increase in fluorescence, indicating the presence of the WT allele (or mutant allele if the assay is configured for the mutant allele to generate the detectable signal). In some embodiments, FRET probes having different labels (*e.g.* resolvable by difference in emission or excitation wavelengths, or resolvable by time-resolved fluorescence detection) are provided for each allele or locus to be detected, such that the different alleles or loci can be detected in a single reaction. In such embodiments, the primary probe sets and the different FRET probes may be combined in a single assay, allowing comparison of the signals from each allele or locus in the same sample.

If the primary probe oligonucleotide and the target nucleotide sequence do not match perfectly at the cleavage site (*e.g.*, as with the MT primary probe and the WT target, Figure 3, panel B), the overlapped structure does not form and cleavage is suppressed. The structure specific enzyme (e.g., CLEAVASE VIII enzyme, Third Wave Technologies) used cleaves the overlapped structure more efficiently (e.g. at least 340-fold) than the non-overlapping structure, allowing excellent discrimination of the alleles.

The probes turn over without temperature cycling to produce many signals per target (*i.e.,* linear signal amplification). Similarly, each target-specific product can enable the cleavage of many FRET probes.

The primary INVADER assay reaction is directed against the target DNA or RNA being detected. The target DNA is the limiting component in the first invasive cleavage, since the INVADER and primary probe are supplied in molar excess. In the second invasive cleavage, it is the released flap that is limiting. When these two cleavage reactions are performed sequentially, the fluorescence signal from the composite reaction accumulates linearly with respect to the target DNA amount.

In certain embodiments, the INVADER assay, or other nucleotide detection assays, are performed with accessible site designed oligonucleotides and/or bridging oligonucleotides. Such methods, procedures and compositions are described in U.S. Pat.

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6,194,149, WO9850403, and WO0198537, all of which are specifically incorporated by reference in their entireties.

In certain embodiments, the target nucleic acid sequence is amplified prior to detection (e.g. such that synthetic nucleic acid is generated). In some embodiments, the target nucleic acid comprises genomic DNA. In other embodiments, the target nucleic acid comprises synthetic DNA or RNA. In some preferred embodiments, synthetic DNA within a sample is created using a purified polymerase. In some preferred embodiments, creation of synthetic DNA using a purified polymerase comprises the use of PCR. In other preferred embodiments, creation of synthetic DNA using a purified DNA polymerase, suitable for use with the methods of the present invention, comprises use of rolling circle amplification, (*e.g.*, as in U.S. Pat. Nos. 6,210,884, 6,183,960 and 6,235,502, herein incorporated by reference in their entireties). In other preferred embodiments, creation of synthetic DNA comprises copying genomic DNA by priming from a plurality of sites on a genomic DNA sample. In some embodiments, priming from a plurality of sites on a genomic DNA sample comprises using short (*e.g.*, fewer than about 8 nucleotides) oligonucleotide primers. In other embodiments, priming from a plurality of sites on a genomic DNA comprises extension of 3' ends in nicked, double-stranded genomic DNA (*i.e.*, where a 3' hydroxyl group has been made available for extension by breakage or cleavage of one strand of a double stranded region of DNA). Some examples of making synthetic DNA using a purified polymerase on nicked genomic DNAs, suitable for use with the methods and compositions of the present invention, are provided in U.S. Patent Nos. 6,117,634, issued September 12, 2000, and 6,197,557, issued March 6, 2001, and in PCT application WO 98/39485, each incorporated by reference herein in their entireties for all purposes.

In some embodiments, synthetic DNA suitable for use with the methods and compositions of the present invention is made using a purified polymerase on multiply-primed genomic or other DNA, as provided, *e.g.*, in U.S. Patent Nos. 6,291,187, and 6,323,009, and in PCT applications WO 01/88190 and WO 02/00934, each herein incorporated by reference in their entireties for all purposes. In these embodiments, amplification of DNA such as genomic DNA is accomplished using a DNA polymerase, such as the highly processive Φ 29 polymerase (as described, *e.g.*, in US Patent Nos.

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5,198,543 and 5,001,050, each herein incorporated by reference in their entireties for all purposes) in combination with exonuclease-resistant random primers, such as hexamers.

The present invention further provides assays in which the target nucleic acid is reused or recycled during multiple rounds of hybridization with oligonucleotide probes and cleavage of the probes without the need to use temperature cycling (i.e., for periodic denaturation of target nucleic acid strands) or nucleic acid synthesis (i.e., for the polymerization-based displacement of target or probe nucleic acid strands). When a cleavage reaction is run under conditions in which the probes are continuously replaced on the target strand (*e.g.* through probe-probe displacement or through an equilibrium between probe/target association and disassociation, or through a combination comprising these mechanisms, (Reynaldo et al., J. Mol. Biol. 97: 511-520 (2000)), multiple probes can hybridize to the same target, allowing multiple cleavages, and the generation of multiple cleavage products.

As described above, in some embodiments, the present invention provides methods of utilizing the INVADER assay to quantitate the amount of target nucleic present in a sample. In some embodiments, the dynamic range of INVADER assays is increased using mismatch probes, alone or in combination with completely homologous probes. It is preferred that the mismatch is not present at the site of cleavage by the cleavage enzyme. In other embodiments, dynamic range of the INVADER assay is increased by using probes of multiple concentrations. In preferred embodiments, each probe in a multiple probe INVADER assay comprises a different label, allowing the reactions to be run in the same well or tube of the reaction vessel and detected simultaneously. However, the probes may also share the same label, permitting the combined signal to be interpreted as one detection event. In some preferred embodiments, a real time assay, in which signal is measured continuously or at time intervals, is utilized. In other embodiments, a single end-point detection is taken at a desired time point. In yet other embodiment, two or more time point readings are taken.

In other embodiments, composite or split probe oligonucleotides are utilized to increase the dynamic range is utilized in the INVADER-directed cleavage assay. For example, the probe oligonucleotide may be split into two oligonucleotides that anneal in a contiguous and adjacent manner along a target oligonucleotide. The probe

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oligonucleotide is assembled from two smaller pieces: a short segment of 6-10 nts (termed the "miniprobe"), that is to be cleaved in the course of the detection reaction, and an oligonucleotide that hybridizes immediately downstream of the miniprobe (termed the "stacker"), that serves to stabilize the hybridization of the probe. To form the cleavage structure, an upstream oligonucleotide (the INVADER oligonucleotide) is provided to direct the cleavage activity to the desired region of the miniprobe. Assembly of the probe from non-linked pieces of nucleic acid (*i.e.*, the miniprobe and the stacker) allows regions of sequences to be changed without requiring the re-synthesis of the entire proven sequence, thus improving the cost and flexibility of the detection system. In addition, the use of unlinked composite oligonucleotides makes the system more stringent in its requirement of perfectly matched hybridization to achieve signal generation, allowing this to be used as a sensitive means of detecting mutations or changes in the target nucleic acid sequences. In some embodiments, two probe/stacker designs are utilized to increase the dynamic range of the assay. A first configuration, without a gap between the probe and the stacker is utilized. This configuration occupies the target site at a high frequency and serves to generate a higher signal (e.g., in the presence of a low concentration of target). A second configuration, in which a single nucleotide gap between the probe and stacker oligonucleotide is introduced, it utilized for the detection of high concentrations of target. The gapped configuration probe and stacker oligonucleotides hybridize at a lower strength and thus occupy the target site at a lower frequency. This generates a lower signal, which is useful in the detection of high amounts of target sequences.

Additional considerations for performing the INVADER assay are discussed in more detail below.

Oligonucleotide Design for the INVADER assay

In some embodiments where an oligonucleotide is designed for use in the INVADER assay to detect a target nucleic acid, the sequence(s) of interest are entered into the INVADERCREATOR program (Third Wave Technologies, Madison, WI). Sequences may be input for analysis from any number of sources, either directly into the computer hosting the INVADERCREATOR program, or via a remote computer linked through a communication network (*e.g.*, a LAN, Intranet or Internet network). The

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program designs probes for both the sense and antisense strand. Strand selection is generally based upon the ease of synthesis, minimization of secondary structure formation, and manufacturability. In some embodiments, the user chooses the strand for sequences to be designed for. In other embodiments, the software automatically selects the strand. By incorporating thermodynamic parameters for optimum probe cycling and signal generation (Allawi and SantaLucia, Biochemistry, 36:10581 [1997]), oligonucleotide probes may be designed to operate at a pre-selected assay temperature (*e.g.*, 63°C). Based on these criteria, a final probe set (*e.g.*, match and mismatch probes and an INVADER oligonucleotide) is selected.

In some embodiments, the INVADERCREATOR system is a web-based program with secure site access that contains a link to BLAST (available at the National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health website) and that can be linked to RNAstructure (Mathews *et al.*, RNA 5:1458 [1999]), a software program that incorporates mfold (Zuker, Science, 244:48 [1989]). RNAstructure tests the proposed oligonucleotide designs generated by INVADERCREATOR for potential uni- and bimolecular complex formation. INVADERCREATOR is open database connectivity (ODBC)-compliant and uses the Oracle database for export/integration. The INVADERCREATOR system was configured with Oracle to work well with UNIX systems, as most genome centers are UNIX-based.

In some embodiments, the INVADERCREATOR analysis is provided on a separate server (*e.g.*, a Sun server) so it can handle analysis of large batch jobs. For example, a customer can submit up to 2,000 SNP sequences in one email. The server passes the batch of sequences on to the INVADERCREATOR software, and, when initiated, the program designs detection assay oligonucleotide sets. In some embodiments, probe set designs are returned to the user within 24 hours of receipt of the sequences.

Each INVADER reaction includes at least two target sequence-specific, unlabeled oligonucleotides for the primary reaction: an upstream INVADER oligonucleotide and a downstream Probe oligonucleotide. The INVADER oligonucleotide is generally designed to bind stably at the reaction temperature, while the probe is designed to freely

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associate and disassociate with the target strand, with cleavage occurring only when an uncut probe hybridizes adjacent to an overlapping INVADER oligonucleotide. In some embodiments, the probe includes a 5' flap or "arm" that is not complementary to the target, and this flap is released from the probe when cleavage occurs. In some embodiments, the released flap participates as an INVADER oligonucleotide in a secondary reaction.

The following discussion provides one example of how a user interface for an INVADERCREATOR program may be configured.

The user opens a work screen, *e.g.*, by clicking on an icon on a desktop display of a computer (*e.g.*, a Windows desktop). The user enters information related to the target sequence for which an assay is to be designed. In some embodiments, the user enters a target sequence. In other embodiments, the user enters a code or number that causes retrieval of a sequence from a database. In still other embodiments, additional information may be provided, such as the user's name, an identifying number associated with a target sequence, and/or an order number. In preferred embodiments, the user indicates (*e.g.* via a check box or drop down menu) that the target nucleic acid is DNA or RNA. In other preferred embodiments, the user indicates the species from which the nucleic acid is derived. In particularly preferred embodiments, the user indicates whether the design is for monoplex (*i.e.*, one target sequence or allele per reaction) or multiplex (*i.e.*, multiple target sequences or alleles per reaction) detection. When the requisite choices and entries are complete, the user starts the analysis process. In one embodiment, the user clicks a "Go Design It" button to continue.

In some embodiments, the software validates the field entries before proceeding. In some embodiments, the software verifies that any required fields are completed with the appropriate type of information. In other embodiments, the software verifies that the input sequence meets selected requirements (*e.g.*, minimum or maximum length, DNA or RNA content). If entries in any field are not found to be valid, an error message or dialog box may appear. In preferred embodiments, the error message indicates which field is incomplete and/or incorrect. Once a sequence entry is verified, the software proceeds with the assay design.

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In some embodiments, the information supplied in the order entry fields specifies what type of design will be created. In preferred embodiments, the target sequence and multiplex check box specify which type of design to create. Design options include but are not limited to SNP assay, Multiplexed SNP assay (*e.g.*, wherein probe sets for different alleles are to be combined in a single reaction), Multiple SNP assay (*e.g.*, wherein an input sequence has multiple sites of variation for which probe sets are to be designed), and Multiple Probe Arm assays.

In some embodiments, the INVADERCREATOR software is started via a Web Order Entry (WebOE) process (*i.e.*, through an Intra/Internet browser interface) and these parameters are transferred from the WebOE via applet <param> tags, rather than entered through menus or check boxes.

In the case of Multiple SNP Designs, the user chooses two or more designs to work with. In some embodiments, this selection opens a new screen view (*e.g.*, a Multiple SNP Design Selection view). In some embodiments, the software creates designs for each locus in the target sequence, scoring each, and presents them to the user in this screen view. The user can then choose any two designs to work with. In some embodiments, the user chooses a first and second design (*e.g.*, via a menu or buttons) and clicks a "Go Design It" button to continue.

To select a probe sequence that will perform optimally at a pre-selected reaction temperature, the melting temperature (T_m) of the SNP to be detected is calculated using the nearest-neighbor model and published parameters for DNA duplex formation (Allawi and SantaLucia, Biochemistry, 36:10581 [1997]). In embodiments wherein the target strand is RNA, parameters appropriate for RNA/DNA heteroduplex formation may be used. Because the assay's salt concentrations are often different than the solution conditions in which the nearest-neighbor parameters were obtained (1M NaCl and no divalent metals), and because the presence and concentration of the enzyme influence optimal reaction temperature, an adjustment should be made to the calculated T_m to determine the optimal temperature at which to perform a reaction. One way of compensating for these factors is to vary the value provided for the salt concentration within the melting temperature calculations. This adjustment is termed a 'salt correction'. As used herein, the term "salt correction" refers to a variation made in the value provided

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for a salt concentration for the purpose of reflecting the effect on a T_m calculation for a nucleic acid duplex of a non-salt parameter or condition affecting said duplex. Variation of the values provided for the strand concentrations will also affect the outcome of these calculations. By using a value of 0.5 M NaCl (SantaLucia, Proc Natl Acad Sci U S A, 95:1460 [1998]) and strand concentrations of about 1 mM of the probe and 1 fM target, the algorithm for used for calculating probe-target melting temperature has been adapted for use in predicting optimal INVADER assay reaction temperature. For a set of 30 probes, the average deviation between optimal assay temperatures calculated by this method and those experimentally determined is about 1.5 °C.

The length of the downstream probe to a given target sequence is defined by the temperature selected for running the reaction (*e.g.*, 63°C). Starting from the position of the variant nucleotide on the target DNA (the target base that is paired to the probe nucleotide 5' of the intended cleavage site), and adding on the 3' end, an iterative procedure is used by which the length of the target-binding region of the probe is increased by one base pair at a time until a calculated optimal reaction temperature (T_m plus salt correction to compensate for enzyme effect) matching the desired reaction temperature is reached. The non-complementary arm of the probe is preferably selected to allow the secondary reaction to cycle at the same reaction temperature. The entire probe oligonucleotide is screened using programs such as mfold (Zuker, Science, 244: 48 [1989]) or Oligo 5.0 (Rychlik and Rhoads, Nucleic Acids Res, 17: 8543 [1989]) for the possible formation of dimer complexes or secondary structures that could interfere with the reaction. The same principles are also followed for INVADER oligonucleotide design. Briefly, starting from the position N on the target DNA, the 3' end of the INVADER oligonucleotide is designed to have a nucleotide not complementary to either allele suspected of being contained in the sample to be tested. The mismatch does not adversely affect cleavage (Lyamichev *et al.*, Nature Biotechnology, 17: 292 [1999]), and it can enhance probe cycling, presumably by minimizing coaxial stabilization effects between the two probes. Additional residues complementary to the target DNA starting from residue N-1 are then added in the 5' direction until the stability of the INVADER oligonucleotide-target hybrid exceeds that of the probe (and therefore the planned assay reaction temperature), generally by 15-20 °C.

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It is one aspect of the assay design that the all of the probe sequences may be selected to allow the primary and secondary reactions to occur at the same optimal temperature, so that the reaction steps can run simultaneously. In an alternative embodiment, the probes may be designed to operate at different optimal temperatures, so that the reaction steps are not simultaneously at their temperature optima.

In some embodiments, the software provides the user an opportunity to change various aspects of the design including but not limited to: probe, target and INVADER oligonucleotide temperature optima and concentrations; blocking groups; probe arms; dyes, capping groups and other adducts; individual bases of the probes and targets (*e.g.*, adding or deleting bases from the end of targets and/or probes, or changing internal bases in the INVADER and/or probe and/or target oligonucleotides). In some embodiments, changes are made by selection from a menu. In other embodiments, changes are entered into text or dialog boxes. In preferred embodiments, this option opens a new screen (*e.g.*, a Designer Worksheet view).

In some embodiments, the software provides a scoring system to indicate the quality (*e.g.*, the likelihood of performance) of the assay designs. In one embodiment, the scoring system includes a starting score of points (*e.g.*, 100 points) wherein the starting score is indicative of an ideal design, and wherein design features known or suspected to have an adverse affect on assay performance are assigned penalty values. Penalty values may vary depending on assay parameters other than the sequences, including but not limited to the type of assay for which the design is intended (*e.g.*, monoplex, multiplex) and the temperature at which the assay reaction will be performed. The following example provides an illustrative scoring criteria for use with some embodiments of the INVADER assay based on an intelligence defined by experimentation. Examples of design features that may incur score penalties include but are not limited to the following [penalty values are indicated in brackets, first number is for lower temperature assays (*e.g.*, 62-64 °C), second is for higher temperature assays (*e.g.*, 65-66 °C)]:

1. [100:100] 3' end of INVADER oligonucleotide resembles the probe arm:

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ARM SEQUENCE: ENDS IN:		PENALTY AWARDED IF INVADER	
Arm 1 (SEQ ID NO:1):	CGCGCCGAGG	5'...GAGGX or	5'...GAGGXX
Arm 2 (SEQ ID NO:2):	ATGACGTGGCAGAC	5'...CAGACX or	5'...CAGACXX
Arm 3 (SEQ ID NO:3):	ACGGACGCGGAG	5'...GGAGX or	5'...GGAGXX
Arm 4 (SEQ ID NO:4):	TCCGCGCGTCC	5'...GTCCX or	5'...GTCCXX

2. [70:70] a probe has 5-base stretch (*i.e.*, 5 of the same base in a row) containing the polymorphism;

3. [60:60] a probe has 5-base stretch adjacent to the polymorphism;

4. [50:50] a probe has 5-base stretch one base from the polymorphism;

5. [40:40] a probe has 5-base stretch two bases from the polymorphism;

6. [50:50] probe 5-base stretch is of Gs – additional penalty;

7. [100:100] a probe has 6-base stretch anywhere;

8. [90:90] a two or three base sequence repeats at least four times;

9. [100:100] a degenerate base occurs in a probe;

10. [60:90] probe hybridizing region is short (13 bases or less for designs 65-67°C; 12 bases or less for designs 62-64°C)

11. [40:90] probe hybridizing region is long (29 bases or more for designs 65-67°C, 28 bases or more for designs 62-64°C)

12. [5:5] probe hybridizing region length – per base additional penalty

13. [80:80] Ins/Del design with poor discrimination in first 3 bases after probe arm

14. [100:100] calculated INVADER oligonucleotide Tm within 7.5°C of probe target Tm (designs 65-67°C with INVADER oligonucleotide less than $\leq$ 70.5°C, designs 62-64°C with INVADER oligonucleotide $\leq$ 69.5°C

15. [20:20] calculated probes Tms differ by more than 2.0°C

16. [100:100] a probe has calculated Tm 2°C less than its target Tm

17. [10:10] target of one strand 8 bases longer than that of other strand

18. [30:30] INVADER oligonucleotide has 6-base stretch anywhere – initial penalty

19. [70:70] INVADER oligonucleotide 6-base stretch is of Gs - additional penalty

20. [15:15] probe hybridizing region is 14, 15 or 24-28 bases long (65-67°C) or 13,14 or 26,27 bases long (62-64°C)

21. [15:15] a probe has a 4-base stretch of Gs containing the polymorphism

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In particularly preferred embodiments, temperatures for each of the oligonucleotides in the designs are recomputed and scores are recomputed as changes are made. In some embodiments, score descriptions can be seen by clicking a "descriptions" button. In some embodiments, a BLAST search option is provided. In preferred embodiments, a BLAST search is done by clicking a "BLAST Design" button. In some embodiments, this action brings up a dialog box describing the BLAST process. In preferred embodiments, the BLAST search results are displayed as a highlighted design on a Designer Worksheet.

In some embodiments, a user accepts a design by clicking an "Accept" button. In other embodiments, the program approves a design without user intervention. In preferred embodiments, the program sends the approved design to a next process step (*e.g.*, into production; into a file or database). In some embodiments, the program provides a screen view (*e.g.*, an Output Page), allowing review of the final designs created and allowing notes to be attached to the design. In preferred embodiments, the user can return to the Designer Worksheet (*e.g.*, by clicking a "Go Back" button) or can save the design (*e.g.*, by clicking a "Save It" button) and continue (*e.g.*, to submit the designed oligonucleotides for production).

In some embodiments, the program provides an option to create a screen view of a design optimized for printing (*e.g.*, a text-only view) or other export (*e.g.*, an Output view). In preferred embodiments, the Output view provides a description of the design particularly suitable for printing, or for exporting into another application (*e.g.*, by copying and pasting into another application). In particularly preferred embodiments, the Output view opens in a separate window.

The present invention is not limited to the use of the INVADERCREATOR software. Indeed, a variety of software programs are contemplated and are commercially available, including, but not limited to GCG Wisconsin Package (Genetics computer Group, Madison, WI) and Vector NTI (Informax, Rockville, Maryland). Other detection assays may be used in the present invention.

Multiplex reactions

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Since its introduction in 1988 (Chamberlain, *et al.* Nucleic Acids Res., 16:11141 (1988)), multiplex PCR has become a routine means of amplifying multiple genetic loci in a single reaction. This approach has found utility in a number of research, as well as clinical, applications. Multiplex PCR has been described for use in diagnostic virology (Elnifro, *et al.* Clinical Microbiology Reviews, 13: 559 (2000)), paternity testing (Hidding and Schmitt, Forensic Sci. Int., 113: 47 (2000); Bauer *et al.*, Int. J. Legal Med. 116: 39 (2002)), preimplantation genetic diagnosis (Ouhibi, *et al.*, Curr Womens Health Rep. 1: 138 (2001)), microbial analysis in environmental and food samples (Rudi *et al.*, Int J Food Microbiology, 78: 171 (2002)), and veterinary medicine (Zarlenga and Higgins, Vet Parasitol. 101: 215 (2001)), among others. Most recently, expansion of genetic analysis to whole genome levels, particularly for single nucleotide polymorphisms, or SNPs, has created a need for highly multiplexed PCR capabilities. Comparative genome-wide association and candidate gene studies require the ability to genotype between 100,000-500,000 SNPs per individual (Kwok, Molecular Medicine Today, 5: 538-5435 (1999); Kwok, Pharmacogenomics, 1: 231 (2000); Risch and Merikangas, Science, 273: 1516 (1996)). Moreover, SNPs in coding or regulatory regions alter gene function in important ways (Cargill *et al.* Nature Genetics, 22: 231 (1999); Halushka *et al.*, Nature Genetics, 22: 239 (1999)), making these SNPs useful diagnostic tools in personalized medicine (Hagmann, Science, 285: 21 (1999); Cargill *et al.* Nature Genetics, 22: 231 (1999); Halushka *et al.*, Nature Genetics, 22: 239 (1999)). Likewise, validating the medical association of a set of SNPs previously identified for their potential clinical relevance as part of a diagnostic panel will mean testing thousands of individuals for thousands of markers at a time.

Despite its broad appeal and utility, several factors complicate multiplex PCR amplification. Chief among these is the phenomenon of PCR or amplification bias, in which certain loci are amplified to a greater extent than others. Two classes of amplification bias have been described. One, referred to as PCR drift, is ascribed to stochastic variation in such steps as primer annealing during the early stages of the reaction (Polz and Cavanaugh, Applied and Environmental Microbiology, 64: 3724 (1998)), is not reproducible, and may be more prevalent when very small amounts of target molecules are being amplified (Walsh et al., PCR Methods and Applications, 1:

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241 (1992)). The other, referred to as PCR selection, pertains to the preferential amplification of some loci based on primer characteristics, amplicon length, G-C content, and other properties of the genome (Polz, supra).

Another factor affecting the extent to which PCR reactions can be multiplexed is the inherent tendency of PCR reactions to reach a plateau phase. The plateau phase is seen in later PCR cycles and reflects the observation that amplicon generation moves from exponential to pseudo-linear accumulation and then eventually stops increasing. This effect appears to be due to non-specific interactions between the DNA polymerase and the double stranded products themselves. The molar ratio of product to enzyme in the plateau phase is typically consistent for several DNA polymerases, even when different amounts of enzyme are included in the reaction, and is approximately 30:1 product:enzyme. This effect thus limits the total amount of double-stranded product that can be generated in a PCR reaction such that the number of different loci amplified must be balanced against the total amount of each amplicon desired for subsequent analysis, e.g. by gel electrophoresis, primer extension, etc.

Because of these and other considerations, although multiplexed PCR including 50 loci has been reported (Lindblad-Toh *et al.,* Nature Genet. 4: 381 (2000)), multiplexing is typically limited to fewer than ten distinct products. However, given the need to analyze as many as 100,000 to 450,000 SNPs from a single genomic DNA sample there is a clear need for a means of expanding the multiplexing capabilities of PCR reactions.

The present invention provides methods for substantial multiplexing of PCR reactions by, for example, combining the INVADER assay with multiplex PCR amplification. The INVADER assay provides a detection step and signal amplification that allows very large numbers of targets to be detected in a multiplex reaction. As desired, hundreds to thousands to hundreds of thousands of targets may be detected in a multiplex reaction.

Direct genotyping by the INVADER assay typically uses from 5 to 100 ng of human genomic DNA per SNP, depending on detection platform. For a small number of assays, the reactions can be performed directly with genomic DNA without target pre-

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amplification, however, for highly multiplex reactions, the amount of sample DNA may become a limiting factor.

Because the INVADER assay provides from 10^6 to 10^7 fold amplification of signal, multiplexed PCR in combination with the INVADER assay would use only limited target amplification as compared to a typical PCR. Consequently, low target amplification level alleviates interference between individual reactions in the mixture and reduces the inhibition of PCR by it's the accumulation of its products, thus providing for more extensive multiplexing. Additionally, it is contemplated that low amplification levels decrease a probability of target cross-contamination and decrease the number of PCR-induced mutations.

Uneven amplification of different loci presents one of the biggest challenges in the development of multiplexed PCR. Differences in amplification factors between two loci may result in a situation where the signal generated by an INVADER reaction with a slow-amplifying locus is below the limit of detection of the assay, while the signal from a fast-amplifying locus is beyond the saturation level of the assay. This problem can be addressed in several ways. In some embodiments, the INVADER reactions can be read at different time points, *e.g.*, in real-time, thus significantly extending the dynamic range of the detection. In other embodiments, multiplex PCR can be performed under conditions that allow different loci to reach more similar levels of amplification. For example, primer concentrations can be limited, thereby allowing each locus to reach a more uniform level of amplification. In yet other embodiments, concentrations of PCR primers can be adjusted to balance amplification factors of different loci.

The present invention provides for the design and characteristics of highly multiplex PCR including hundreds to thousands of products in a single reaction. For example, the target pre-amplification provided by hundred-plex PCR reduces the amount of human genomic DNA required for INVADER-based SNP genotyping to less than 0.1 ng per assay. The specifics of highly multiplex PCR optimization and a computer program for the primer design are described in U.S. Pat. Appln. Serial Nos. 10/967,711 and 10/321,039 herein incorporated by reference in their entireties.

In addition to providing methods for highly multiplex PCR, the present invention further provides methods of conducting reverse transcription and target and signal

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amplification reactions in a single reaction vessel with no subsequent manipulations or reagent additions beyond initial reaction set-up. Such combined reactions are suitable for quantitative analysis of limiting target quantities in very short reaction times. Methods for conducting such reactions are described in U.S. Pat. Appln. Serial No. 11/266,723, herein incorporated by reference in its entirety.

B. Other Detection Assays

The present invention is not limited to detection of target sequences by INVADER assay. The methods and compositions of the present invention find use in increasing the dynamic range of any number detection assays including, but not limited to, those described below.

1. Hybridization Assays

In some embodiments, the methods and compositions of the present invention find use in increasing the dynamic range of a hybridization assay. A variety of hybridization assays using a variety of technologies for hybridization and detection are available. A description of a selection of assays is provided below.

a. Direct Detection of Hybridization

In some embodiments, hybridization of a probe to the sequence of interest is detected directly by visualizing a bound probe (*e.g.*, a Northern or Southern assay; *See e.g.*, Ausabel *et al.* (eds.), Current Protocols in Molecular Biology, John Wiley & Sons, NY [1991]). In a these assays, genomic DNA (Southern) or RNA (Northern) is isolated from a subject. The DNA or RNA is then cleaved with a series of restriction enzymes that cleave infrequently in the genome and not near any of the markers being assayed. The DNA or RNA is then separated (*e.g.*, on an agarose gel) and transferred to a membrane. A labeled (*e.g.*, by incorporating a radionucleotide) probe or probes specific for the target sequence being detected is allowed to contact the membrane under a condition or low, medium, or high stringency conditions. Unbound probe is removed and the presence of binding is detected by visualizing the labeled probe.

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b. Detection of Hybridization Using "DNA Chip" Assays

In some embodiments of the present invention, variant sequences are detected using a DNA chip (e.g., array) hybridization assay. In this assay, a series of oligonucleotide probes are affixed to a solid support. In some embodiments, the oligonucleotide probes are designed to be unique to a given target sequence. In preferred embodiments, the arrays comprise multiple probes (e.g., mismatch or different amounts of a completely complementary probe) in order to increase the dynamic range of the assay. The DNA sample of interest is contacted with the DNA "chip" and hybridization is detected.

In some embodiments, the DNA chip assay is a GeneChip (Affymetrix, Santa Clara, CA; *See e.g.*, U.S. Patent Nos. 6,045,996; 5,925,525; and 5,858,659; each of which is herein incorporated by reference) assay. The GeneChip technology uses miniaturized, high-density arrays of oligonucleotide probes affixed to a "chip." Probe arrays are manufactured by Affymetrix's light-directed chemical synthesis process, which combines solid-phase chemical synthesis with photolithographic fabrication techniques employed in the semiconductor industry. Using a series of photolithographic masks to define chip exposure sites, followed by specific chemical synthesis steps, the process constructs high-density arrays of oligonucleotides, with each probe in a predefined position in the array. Multiple probe arrays are synthesized simultaneously on a large glass wafer. The wafers are then diced, and individual probe arrays are packaged in injection-molded plastic cartridges, which protect them from the environment and serve as chambers for hybridization.

The nucleic acid to be analyzed is isolated, amplified by PCR, and labeled with a fluorescent reporter group. The labeled DNA is then incubated with the array using a fluidics station. The array is then inserted into the scanner, where patterns of hybridization are detected. The hybridization data are collected as light emitted from the fluorescent reporter groups already incorporated into the target, which is bound to the probe array. Probes that perfectly match the target generally produce stronger signals than those that have mismatches. Since the sequence and position of each probe on the array are known, by complementarity, the identity of the target nucleic acid applied to the probe array can be determined.

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In other embodiments, a DNA microchip containing electronically captured probes (Nanogen, San Diego, CA) is utilized (*See e.g.*, U.S. Patent Nos. 6,017,696; 6,068,818; and 6,051,380; each of which are herein incorporated by reference). Through the use of microelectronics, Nanogen's technology enables the active movement and concentration of charged molecules to and from designated test sites on its semiconductor microchip. DNA capture probes unique to a given SNP or mutation are electronically placed at, or "addressed" to, specific sites on the microchip. Since DNA has a strong negative charge, it can be electronically moved to an area of positive charge.

First, a test site or a row of test sites on the microchip is electronically activated with a positive charge. Next, a solution containing the DNA probes is introduced onto the microchip. The negatively charged probes rapidly move to the positively charged sites, where they concentrate and are chemically bound to a site on the microchip. The microchip is then washed and another solution of distinct DNA probes is added until the array of specifically bound DNA probes is complete.

A test sample is then analyzed for the presence of target DNA molecules by determining which of the DNA capture probes hybridize, with complementary DNA in the test sample (*e.g.*, a PCR amplified gene of interest). An electronic charge is also used to move and concentrate target molecules to one or more test sites on the microchip. The electronic concentration of sample DNA at each test site promotes rapid hybridization of sample DNA with complementary capture probes (hybridization may occur in minutes). To remove any unbound or nonspecifically bound DNA from each site, the polarity or charge of the site is reversed to negative, thereby forcing any unbound or nonspecifically bound DNA back into solution away from the capture probes. A laser-based fluorescence scanner is used to detect binding,

In still further embodiments, an array technology based upon the segregation of fluids on a flat surface (chip) by differences in surface tension (ProtoGene, Palo Alto, CA) is utilized (*See e.g.*, U.S. Patent Nos. 6,001,311; 5,985,551; and 5,474,796; each of which is herein incorporated by reference). Protogene's technology is based on the fact that fluids can be segregated on a flat surface by differences in surface tension that have been imparted by chemical coatings. Once so segregated, oligonucleotide probes are synthesized directly on the chip by ink-jet printing of reagents. The array with its

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reaction sites defined by surface tension is mounted on a X/Y translation stage under a set of four piezoelectric nozzles, one for each of the four standard DNA bases. The translation stage moves along each of the rows of the array and the appropriate reagent is delivered to each of the reaction site. For example, the A amidite is delivered only to the sites where amidite A is to be coupled during that synthesis step and so on. Common reagents and washes are delivered by flooding the entire surface and then removing them by spinning.

DNA probes unique for the target nucleic acid are affixed to the chip using Protogene's technology. The chip is then contacted with the PCR-amplified genes of interest. Following hybridization, unbound DNA is removed and hybridization is detected using any suitable method (*e.g.*, by fluorescence de-quenching of an incorporated fluorescent group).

In yet other embodiments, a "bead array" is used for the detection of polymorphisms (Illumina, San Diego, CA; *See e.g.*, PCT Publications WO 99/67641 and WO 00/39587, each of which is herein incorporated by reference). Illumina uses a BEAD ARRAY technology that combines fiber optic bundles and beads that self-assemble into an array. Each fiber optic bundle contains thousands to millions of individual fibers depending on the diameter of the bundle. The beads are coated with an oligonucleotide specific for the detection of a given target nuclei acid. Batches of beads are combined to form a pool specific to the array. To perform an assay, the BEAD ARRAY is contacted with a prepared subject sample (e.g., DNA). Hybridization is detected using any suitable method.

In other embodiments, the array methods described in U.S. Patents 6,410,229 and 6,344,316; each of which is incorporated by reference herein, are utilized.

c. Enzymatic Detection of Hybridization

In some embodiments, hybridization of a bound probe is detected using a TaqMan assay (PE Biosystems, Foster City, CA; *See e.g.*, U.S. Patent Nos. 5,962,233 and 5,538,848, each of which is herein incorporated by reference). The assay is performed during a PCR reaction. The TaqMan assay exploits the 5'-3' exonuclease activity of DNA polymerases such as AMPLITAQ DNA polymerase. A probe, specific for a given allele

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or mutation, is included in the PCR reaction. The probe consists of an oligonucleotide with a 5'-reporter dye (*e.g.*, a fluorescent dye) and a 3'-quencher dye. During PCR, if the probe is bound to its target, the 5'-3' nucleolytic activity of the AMPLITAQ polymerase cleaves the probe between the reporter and the quencher dye. The separation of the reporter dye from the quencher dye results in an increase of fluorescence. The signal accumulates with each cycle of PCR and can be monitored with a fluorimeter.

In still further embodiments, polymorphisms are detected using the SNP-IT primer extension assay (Orchid Biosciences, Princeton, NJ; *See e.g.*, U.S. Patent Nos. 5,952,174 and 5,919,626, each of which is herein incorporated by reference). In this assay, SNPs are identified by using a specially synthesized DNA primer and a DNA polymerase to selectively extend the DNA chain by one base at the suspected SNP location. DNA in the region of interest is amplified and denatured. Polymerase reactions are then performed using miniaturized systems called microfluidics. Detection is accomplished by adding a label to the nucleotide suspected of being at the SNP or mutation location. Incorporation of the label into the DNA can be detected by any suitable method (*e.g.*, if the nucleotide contains a biotin label, detection is via a fluorescently labelled antibody specific for biotin).

In yet other embodiments, the methods and compositions of the present invention are utilized with the method described in U.S. Patent 6,528,254 (herein incorporated by reference in its entirety). The method comprises generating a cleavage structure using a primer and a nucleic acid polymerase and cleaving the cleavage structure with a FEN endonuclease.

2. Ligation assays

In other embodiments, a ligase based detection assay is utilized with the methods and compositions of the present invention. For example, in some embodiments, the method described in U.S. Patents 5,521,065 and 5,514,543 (each of which is herein incorporated by reference in its entirety) is utilized. Briefly, the method involves reacting a mixture of single-stranded nucleic acid fragments with a first probe which is complementary to a first region of the target sequence, and with a second probe which is complementary to a second region of the target sequence, where the first and second

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target regions are contiguous with one another, under hybridization conditions in which the two probes become stably hybridized to their associated target regions. Following hybridization, any of the first and second probes hybridized to contiguous first and second target regions are ligated, and the sample is tested for the presence of expected probe ligation product. The presence of ligated product indicates that the target sequence is present in the sample. In some embodiments, the ligation reaction is performed concurrent with a nucleic acid amplification reaction (See e.g., US Patents 6,130,073 and 5,912,148, each of which is herein incorporated by reference in its entirety).

C. Microarrays and Solid Supports

In some embodiments, the present invention provides microarrays. Microarrays may be utilized with any of the detection assays described herein. The below discussion describes microarrays in the context of INVADER and TAQMAN assays. However, one skilled in the art recognizes that microarrays may be adapted for use with any number of detection assays.

Microarrays may comprise assay reagents and/or targets attached to or located on or near a solid surface (i.e. a microarray spot is formed) such that a detection assay may be performed on the solid surface. In some preferred embodiments, the microarray spots are generated to possess specific and defined chemical and physical characteristics. In other embodiments, the microarray may comprise a plurality of reaction chambers (e.g., capillaries), for conducting detection assays. In some such embodiments, nucleic acids or other detection assay components are attached to the surface of the reaction chamber. In other embodiments, detection assay components are all in the liquid phase or dried down in the reaction chamber.

As used herein, the term "microarray-spot" refers to the discreet area formed on a solid surface, in a layer of non-aqueous liquid in a microwell, or in a reaction chamber containing a population of detection assay reagents. A microarray-spot may be formed, for example, on a solid substrate (e.g. glass, TEFLON) or in a layer of non-aqeous liquid or other material that is on a solid surface, when a reagent sample comprising detection assay reagents is applied to the solid surface (or film on a solid surface) by a transfer means (*e.g.* pin spotting tool, inkject printer, etc.). In preferred embodiments, the solid

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substrate (e.g. modified as described below) contains microwells and the microarray-spots are applied in the microwells. In other embodiments, the solid support serves as a platform on which microwells are printed/created and the necessary reagents are introduced to these microwells and the subsequent reaction(s) take place entirely in solution. Creation of a microwell on a solid support may be accomplished in a number of ways, including; surface tension, and etching of hydrophilic pockets (e.g. as described in patent publications assigned to Protogene Corp.). For example, the surface of a support may be coated with a hydrophobic layer, and a chemical component, that etches the hydrophobic layer, is then printed on to the support in small volumes (e.g., to generate local changes in the physical or chemical properties of the hydrophobic layer). The printing results in an array of hydrophilic microwells. An array of printed hydrophobic or hydrophilic towers may be employed to create micorarrays. A surface of a slide may be coated with a hydrophobic layer, and then a solution is printed on the support that creates a hydrophilic layer on top of the hydrophobic surface. The printing results in an array of hydrophilic towers. Mechanical microwells may be created using physical barriers, +/- chemical barriers. For example, microgrids such as gold grids may be immobilized on a support, or microwells may be drilled into the support (e.g. as demonstrated by BML). Also, a microarray may be printed on the support using hydrophilic ink such as TEFLON. Such arrays are commercially available through Precision Lab Products, LLC, Middleton, WI. In yet another variant, data of customer preferences with respect to the format of the detection assay array are stored on a database used with components of the invention. This information can be used to automatically configure products for a particular customer based upon minimal identification information for a customer, e.g. name, account number or password. In some embodiments, the desired reactions components (e.g., target nucleic acids or detection assay components) are spotted or delivered into wells and then taken up into small reaction chambers such as capillaries. The reaction then occurs within the reaction chamber.

Many types of methods may be used for printing of desired reagents into microarrays (e.g. microarray spots printed into microwells). In some embodiments, a pin tool is used to load the array (e.g. generate a microarray spot) mechanically (see, e.g.,

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Shalon, Genome Methods, 6:639 [1996], herein incorporated by reference). In other embodiments, ink jet technology is used to print oligonucleotides onto a solid surface (*e.g.*, O'Donnelly-Maloney *et al.*, Genetic Analysis:Biomolecular Engineering, 13:151 [1996], herein incorporated by reference) in order to create one or more micorarray spots in a well.

Examples of desired reagents for printing into/onto solid supports (e.g. with microwell arrays) include, but are not limited to, molecular reagents, such as INVADER reaction reagents, designed to perform a nucleic acid detection assay (e.g., an array of SNP detection assays could be printed in the wells); and target nucleic acid, such as human genomic DNA (hgDNA), resulting in an array of different samples. Also, desired reagents may be simultaneously supplied with the etching/coating reagent or printed into/onto the microwells/towers subsequent to the etching process. For arrays created with mechanical barriers the desired reagents are, for example, printed into the resulting wells. In some embodiments, the desired reagents may need to be printed in a solution that sufficiently coats the microwell and creates a hydrophilic, reaction friendly, environment such as a high protein solution (e.g. BSA, non-fat dry milk). In certain embodiments, the desired reagents may also need to be printed in a solution that creates a "coating" over the reagents that immobilizes the reagents, this could be accomplished with the addition of a high molecular weight carbohydrate such as FICOLL or dextran. In some embodiments, the coating is oil.

Application of the target solution to the microarray (or reaction reagents if the target has been printed down or taken up in a reaction chamber) may be accomplished in a number of ways. For example, the solid support may be dipped into a solution containing the target, or by putting the support in a chamber with at least two openings then feeding the target solution into one of the openings and then pulling the solution across the surface with a vacuum or allowing it to flow across the surface via capillary action. Examples of devices useful for performing such methods include, but are not limited to, TECAN – GenePaint system, and AutoGenomics AutoGene System. In yet another embodiment spotters commercially avialable from Virtek Corp. are used to spot various detection assays onto plates, slides and the like.

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In some embodiments, solutions (e.g. reaction reagents or target solutions) are dragged, rolled, or squeegeed across the surface of the support. One type of device useful for this type of application is a framed holder that holds the support. At one end of the holder is a roller/squeegee or something similar that would have a channel for loading of the target solution in front of it. The process of moving the roller/squeegee across the surface applies the target solution to the microwells. At the end opposite end of the holder is a reservoir that would capture the unused target solution (thus allowing for reuse on another array if desired). Behind the roller/squeegee is an evaporation barrier (e.g., mineral oil, optically clear adhesive tape etc.) and it is applied as the roller/squeegee move across the surface.

The application of a target solution to microwell or reaction chamber arrays results in the deposition of the solution at each of the microwell or reaction chamber locations. The chemical and/or mechanical barriers would maintain the integrity of the array and prevent cross-contamination of reagents from element to element. In some preferred embodiments, materials in the microwells or reaction chambers are dried. In some such embodiments, the reagents are rehydrated by the target solution (or detection assay component solution) resulting in an ultra-low volume reaction mix. In some embodiments, the microarray reactions are covered with mineral oil or some other suitable evaporation barrier or humidity chamber to allow high temperature incubation. The signal generated may be detected directly through the applied evaporation barrier using a fluorescence microscope, array reader or standard fluorescence plate reader.

Advantages of the use of a microwell-microarray, for running INVADER assays (e.g. dried down INVADER assay components in each well) include, but are not limited to: the ability to use the INVADER Biplex format for a nucleic acid detection assay; sufficient sensitivity to detect hgDNA directly, the ability to use "universal" FRET cassettes; no attachment chemistry needed (which means already existing off the shelf reagents could be used to print the microarrays), no need to fractionate hgDNA to account for surface effect on hybridization, low mass of hgDNA needed to make tens of thousands of calls, low volume need (e.g. a 100μm microwell would have a volume of 0.28nl, and at 10^4 microwells per array a volume of 2.8μl would fill all wells), a solution of 333ng/μl hgDNA would result in ~100 copies per microwell (this is 33X more

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concentrated than the use of 100ng ngDNA in a 20µl reaction), thus 2.8µl x 333ng/µl = 670 ng hgDNA for 10^4 calls or 0.07 ng per call. It is appreciated that other detection assays can also be presented in this format.

1. Generating and Using Microarray-Spots With Non-Aqueous Liquids

In certain preferred embodiments, the present invention provides methods for generating microarray spots in wells by applying a detection assay reagent solution to a well containing non-aqueous liquid. In other preferred embodiments, the present invention provides methods of contacting a microarray-spot with a test sample solution (e.g. comprising target nucleic acids) by shooting the test sample solution through a layer of non-aqueous liquid covering the microarray spot. In certain embodiments, the solid supports are coated with sol-gel films (described below in more detail).

In some embodiments, the present invention provides methods comprising; a) providing; i) a solid support comprising a well, ii) a non-aqueous liquid, and iii) a detection reagent solution; and b) adding the non-aqueous liquid to the well, and c) adding the detection reagent solution to the well through the non-aqueous liquid under conditions such that at least one microarray-spot is formed in the well. In other embodiments, the methods further comprise step d) contacting the at least one microarray-spot with a test sample solution. In additional embodiments, the contacting comprises propelling the test sample solution through the non-aqueous liquid in the well.

In particular embodiments, the non-aqueous liquid is oil. In other embodiments, the solid support comprises a plurality of wells, and the method is performed with the plurality of wells. In further embodiments, at least two microarray-spots are formed simultaneously (e.g. in at least two of the plurality of wells).

In some embodiments, the test sample solution comprises a target nucleic acid molecule. In preferred embodiments, the target solution comprises less than 800 copies of a target nucleic acid molecule, or less than 400 copies of a target nucleic acid molecule or less than 200 copies of a target nucleic acid molecule. In particular embodiments, the contacting the microarray-spot with the test sample solution identifies the presence or absence of a polymorphism, or other desired particular sequence to be detected, in the

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target nucleic acid molecule. In some embodiments, wells are coated with a sol-gel coating (e.g. prior to microarray-spot formation).

In other embodiments, the detection reagent solution comprises components configured for use with a detection assay selected from; TAQMAN assay, or an INVADER assay, a polymerase chain reaction assay, a rolling circle extension assay, a sequencing assay, a hybridization assay employing a probe complementary to the polymorphism, a bead array assay, a primer extension assay, an enzyme mismatch cleavage assay, a branched hybridization assay, a NASBA assay, a molecular beacon assay, a cycling probe assay, a ligase chain reaction assay, and a sandwich hybridization assay. In preferred embodiments, the detection reagent solution comprises INVADER oligonucleotides, and 5' probe oligonucleotides.

In additional embodiments, the contacting is performed with a SYNQUAD nanovolume pipetting system, or other fluid transfer system or device. In preferred embodiments, the commercially available CARTESIAN SYNQUAD nanovolume pipetting system is employed. Similar devices may also be employed, including those described in U.S. Pats. 6,063,339 and U.S. 6,258,103, both of which are specifically incorporated by reference, as well as PCT applications: WO0157254; WO0049959; WO0001798; and WO9942804; all of which are specifically incorporated by reference.

In particular embodiments, at least 2 microarray-spots are formed in the well (or at least 3 or 4 or 5 microarray-sports are formed in each well). In multi-well formats, employing multiple microarray-spots multiplies the number of reactions that can be performed on a single solid support (e.g. if 4 microarray-spots are formed in each of the 1536 wells in an a 1536 well plate, then 6144 microarray-spots would be available for performing detection reactions). In further embodiments, the present invention provides a solid support with a well (or wells) formed by the methods described above.

In some embodiments, the present invention provides methods comprising; a) providing; i) a solid support comprising a microarray-spot, ii) a non-aqueous liquid; and iii) a test sample solution; and b) covering the microarray-spot with a layer of the non-aqueous liquid, and c) contacting the microarray-spot with the test sample solution through the layer of non-aqueous liquid. In other embodiments, the test sample solution comprises a target nucleic acid molecule. In further embodiments, the contacting

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identifies the presence or absence of at least one polymorphism in the target nucleic acid molecule. In preferred embodiments, the test sample solution comprises a target nucleic acid molecule. In preferred embodiments, the target solution comprises less than 800 copies of a target nucleic acid molecule, or less than 400 copies of a target nucleic acid molecule or less than 200 copies of a target nucleic acid molecule.

In certain embodiments, the microarray-spot comprises components configured for use with a detection assay selected from; TAQMAN assay, or an INVADER assay, a polymerase chain reaction assay, a rolling circle extension assay, a sequencing assay, a hybridization assay employing a probe complementary to the polymorphism, a bead array assay, a primer extension assay, an enzyme mismatch cleavage assay, a branched hybridization assay, a NASBA assay, a molecular beacon assay, a cycling probe assay, a ligase chain reaction assay, and a sandwich hybridization assay. In preferred embodiments, the microarray-spot comprises INVADER oligonucleotides, and 5' probe oligonucleotides.

In some embodiments, the solid support comprises a well, and the microarray-spot is located in the well. In certain embodiments, the non-aqueous liquid is oil. In other embodiments, the solid support comprises a plurality of wells, and the method is performed with the plurality of wells. In particular embodiments, at least two microarray-spots are formed simultaneously. In some embodiments, at least 2 microarray-spots are formed in the well (or at least 3 or 4 or 5 microarray-sports are formed in each well). In multi-well formats, employing multiple microarray-spots multiplies the number of reactions that can be performed on a single solid support (e.g. if 4 microarray-spots are formed in each of the 1536 wells in an a 1536 well plate, then 6144 microarray-spots would be available for performing detection reactions; if etched 3072 well plates are used, additional spots may be formed). In further embodiments, the present invention provides a solid support with a well (or wells) formed by the methods described above.

In some embodiments, the contacting comprises propelling the test sample solution through the non-aqueous liquid in the well. In other embodiments, the non-aqueous liquid is mineral oil. In additional embodiments, the non-aqueous liquid is selected from mineral oil, a seed oil, and an oil derived from petroleum.

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In additional embodiments, the contacting is performed with a SYNQUAD nanovolume pipetting system, or other fluid transfer system or device. In preferred embodiments, the commercially available CARTESIAN SYNQUAD nanovolume pipetting system is employed. Similar devices may also be employed, including those described in U.S. Pats. 6,063,339 and U.S. 6,258,103, both of which are specifically incorporated by reference, as well as PCT applications: WO0157254; WO0049959; WO0001798; and WO9942804; all of which are specifically incorporated by reference.

In some embodiments, the present invention provides systems comprising; a) a nonvolume pipetting system (e.g., SYNQUAD), and b) a solid support comprising a microarray-spot, wherein the microarray spot is covering with a layer of a non-aqueous liquid. In other embodiments, the system further comprises a test sample solution.

D. Formats for Assays on a Solid Support

In some embodiments, detection assays are performed on a solid support. The below discussion describes assays on a solid support in the context of the INVADER assay. However, one skilled in the relevant arts recognizes that the methods described herein can be adapted for use with any nucleic acid detection assay (e.g., the detection assays described herein).

The present invention is not limited to a particular configuration of the INVADER assay. Any number of suitable configurations of the component oligonucleotides may be utilized. For example, in some embodiments of the present invention, the probe oligonucleotide is bound to a solid support and the INVADER oligonucleotide and genomic DNA (or RNA) target are provided in solution. In other embodiments of the present invention, the INVADER oligonucleotide is bound to the support and the probe and target are in solution. In yet other embodiments, both the probe and INVADER oligonucleotides are bound to the solid support. In further embodiments, the target nucleic acid is bound directly or indirectly (*e.g.,* through hybridization to a bound oligonucleotide that is not part of a cleavage structure) to a solid support, and either or both of the probe and INVADER oligonucleotides are provided either in solution, or bound to a support. In still further embodiments, a primary INVADER assay reaction is carried out in solution and one or more components of a secondary reaction are bound to

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a solid support. In yet other embodiments, all of the components necessary for an INVADER assay reaction, including cleavage agents, are bound to a solid support.

The present invention is not limited to the configurations described herein. Indeed, one skilled in the art recognizes that any number of additional configurations may be utilized. Any configuration that supports a detectable invasive cleavage reaction may be utilized. Additional configurations are identified using any suitable method, including, but not limited to, those disclosed herein.

1. Probe Oligonucleotide Bound

In some embodiments, the probe oligonucleotide is bound to a solid support. In some embodiments, the probe is a labeled Signal Probe oligonucleotide. The signal probe is cleaved to release a signal molecule indicative of the presence of a given target molecule. In some embodiments, the signal molecule is a fluorescence donor in an energy transfer reaction (*e.g.*, FRET), whose emission increases in response to separation from a quenching fluorescence acceptor. In other embodiments, the signal molecule is a fluorescent moiety that is detected only upon its release into solution. It yet other embodiments, the signal molecule is a fluorescently labeled small molecule that is separated from the full length Signal Probe by carrying a distinct charge.

In some embodiments, a system is designed in which no separation steps are required to visualize the signal generated by the reaction. In some embodiments, this is accomplished in the FRET system in which the fluorescence donor remains affixed to the solid support following cleavage of the signal probe. This design has several complexities that stem from the nature of the FRET reaction. The quenching in the FRET signal molecule is only 97-99% efficient (*i.e.* not all of the energy emitted by the donor will be absorbed by the quencher). To detect the fluorescence of the unquenched donor above the background of the uncleaved probes, it is necessary to cleave 1-3% of the probe molecules. Assuming that in a 100μm X 100μm area, there are ~10^8 probes bound, then ~10^6 should be cleaved to generate a signal detectable above the inherent background generated by those probes. Probe cycling in an INVADER assay reaction on a single target molecule can generate approximately 1000-2000 cleaved probe molecules per hour (assuming a turnover rate of 15-30 events/target/min). Roughly 1000 target

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molecules are required to generate this level of cleaved Signal Probes. Assuming a reaction volume of 1 nL, the necessary target concentration becomes 1pM, well within the range of the maximum that can be manipulated (*e.g.*, 0.5-2.5 pM). At less than maximal probe densities, it would nonetheless be necessary to deliver at least 10-20 target molecules (*i.e.* a 10-20 fM solution) to each reaction area to ensure a statistical likelihood that each will contain target. The same target concentration considerations apply to other, non-FRET alternatives, for example, release of a single fluorescent group into solution, with or without a quenching fluorophore and release of a positively charged signal molecule even though <1% cleavage would be detectable with these other methods. Accordingly, in some embodiments, dilute solutions are used in conjunction with longer reaction times (*e.g.* a 100fM solution could be applied and the reactions run for 10-24 hours).

2. INVADER Oligonucleotide Bound

In some embodiment of the present invention, the INVADER oligonucleotide is bound to the solid support and the probe oligonucleotide is free in solution. In this emobodiment, there are no restrictions on the length of the INVADER oligonucleotide-target duplex, since the INVADER oligonucleotide does not need to cycle on and off the target, as does the signal probe. Thus, in some embodiments where the INVADER oligonucleotide is bound to a solid support, the INVADER oligonucleotide is used as a "capture" oligonucleotide to concentrate target molecules from solution onto the solid phase through continuous application of sample to the solid support. For example, by applying 1 ml of a 1 mg/ml target solution, it is possible to bind 10^6-10^8 target molecules in a 100μM X 100μM area. Moreover, because the INVADER oligonucleotide-target interaction is designed to be stable, in some embodiments, the support is washed to remove unbound target and unwanted sample impurities prior to applying the signal probes, enzyme, etc., to ensure even lower background levels. In other embodiments, a capture oligonucleotide complementary to a distinct region in the proximity of the locus being investigated is utilized.

Several possibilities exist for separation of cleaved from uncleaved signal probe reactions where INVADER oligonucleotides are bound the solid support and signal probe

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olignucleotides are free in solution. In preferred embodiments, a labeling strategy is utilized that makes it possible to chemically differentiate cleaved from uncleaved probe since both full length and cleaved probes are in solution. For example, in some embodiments (*e.g.*, FRET signal probe), full length probe is quenched but the cleavage product generates fluorescent signal. In other embodiment (*e.g*, CRE), the full length probe is negatively charged but the cleaved probe is positively charged.

However, in some preferred embodiments, CRE separation is utilized. First, the cleaved signal probes generated by the CRE approach are actively captured on a negatively charged electrode. This capture results in partitioning from uncleaved molecules as well as concentration of the labeled, cleaved probes by as much as an order of magnitude. Second, the use of an electric field to capture the cleaved probe eliminates the need to micromachine tiny wells to prevent diffusion of the cleaved probes.

3. Both Probe and INVADER Oligonucleotide Bound

In some embodiments of the present invention, both a probe and an INVADER oligonucleotide are bound to a solid support. In preferred embodiments, probe and INVADER oligonucleotides are placed in close proximity on the same solid support such that a target nucleic acid may bind both the probe and INVADER oligonucleotides. In some embodiments, the oligonucleotides are attached via spacer molecules in order to improve their accessibility and decrease interactions between oligonucleotides.

In some preferred embodiments, a single INVADER oligonucleotide is configured to allow it to contact and initiate multiple cleavage reactions. For example, in some embodiments, one INVADER oligonucleotide is surrounded on a solid support by multiple Signal Probe oligonucleotides. A target nucleic acid binds to an INVADER and a probe oligonucleotide. The Signal Probe is cleaved (generating signal) and released, leaving the target bound to the INVADER oligonucleotide. This target:INVADER oligonucleotide complex is then able to contact another Signal Probe and promote another cleavage event. In this manner, the signal generated from one target and one INVADER oligonucleotide is amplified.

In other embodiments, the probe and INVADER oligoucleotides are combined in one molecule. The connection between the probe and INVADER portions of the single

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molecule may be nucleic acid, or may be a non-nucleic acid linker (*e.g.*, a carbon linker, a peptide chain).

4. Secondary Reaction Bound

In some embodiments, a primary INVADER assay reaction is performed in solution and a secondary reaction is performed on a solid support. Cleaved probes from the primary INVADER assay reaction are contacted with a solid support containing one or more components of a cleavage structure, including but not limited to a secondary target nucleic acid, a secondary probe or a secondary INVADER oligonucleotide. In a preferred embodiment, the component is a one-piece secondary oligonucleotide, or cassette, comprising both a secondary target portion and a secondary probe portion. In a particularly preferred embodiment, the cassette is labeled to allow detection of cleavage of the cassette by a FRET. The secondary signal oligonucleotide may be labeled using any suitable method including, but not limited to, those disclosed herein. It will be appreciated that any of the embodiments described above for configuring an INVADER assay reaction on a support may be used in configuring a secondary or subsequent INVADER assay reaction on a support.

5. Target Bound

In some embodiments of the present invention, the target nucleic acid (e.g, genomic DNA) is bound to the solid support. In some embodiments, the INVADER and Probe oligonucleotides are free in solution. In other embodiments, both the target nucleic acid, the INVADER oligonucleotide, and the Probe (e.g, Signal Probe) oligonucleotides are bound. In yet other embodiments, a secondary oligonucleotide (e.g, a FRET oligonucleotide) is included in the reaction. In some embodiments, the FRET oligonucleotide is free in solution. In other embodiments, the FRET oligonucleotide is bound to the solid support.

6. Enzyme Bound

In some embodiments, the cleavage means (*e.g.*, enzyme) is bound to a solid support. In some embodiments, the target nucleic acid, probe oligonucleotide, and

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INVADER oligonucleotide are provided in solution. In other embodiments, one or more of the nucleic acids is bound to the solid support. Any suitable method may be used for the attachment of a cleavage enzyme to a solid support, including, but not limited to, covalent attachment to a support (*See e.g.*, Chernukhin and Klenova, Anal. Biochem., 280:178 [2000]), biotinylation of the enzyme and attachment via avidin (*See e.g.*, Suter *et al.*, Immunol. Lett. 13:313 [1986]), and attachment via antibodies (*See e.g.*, Bilkova *et al.*, J. Chromatogr. A, 852:141 [1999]).

7. Spacers

In some embodiments of the present invention, oligonucleotides are attached to a solid support via a spacer or linker molecule. The present invention is not limited to any one mechanism. Indeed, an understanding of the mechanism is not necessary to practice the present invention. Nonetheless, it is contemplated that spacer molecules enhance INVADER assay reactions by improving the accessibility of oligonucleotides and decreasing interactions between oligonucleotides. The use of linkers, which can be incorporated during oligonucleotide synthesis, has been shown to increase hybridization efficiency relative to capture oligonucleotides that contain no linkers (Guo *et al.*, Nucleic Acids Res., 22:5456 [1994]; Maskos and Southern, Nucleic Acids Res., 20:1679 [1992]; Shchepinov *et al.*, Nucleic Acids Research 25:1155 [1997]).

Spacer molecules may be comprised of any suitable material. Preferred materials are those that are stable under reaction conditions utilized and non-reactive with the components of the INVADER assay. Suitable materials include, but are not limited to, carbon chains (*e.g.*, including but not limited to C_{18}), poly nucleotides (*e.g.*, including, but not limited to, polyI, polyT, polyG, polyC, and polyA), and polyglycols (*e.g.*, hexaethylene glycol).

Spacer molecules may be of any length. Accordingly in some embodiments, multiple spacer molecules are attached end to end to achieve the desired length spacer. For example, in some embodiments, multiple C_{18} or hexaethylene glycol spacers (*e.g.*, including, but not limited to, 5, 10, or 20 spacer molecules) are combined. The optimum spacer length is dependent on the particular application and solid support used. To determine the appropriate length, different lengths are selected (e.g, 5, 10, or 20 C_{18} or

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hexaethylene glycol spacers molecules) and reactions are performed as described herein to determine which spacer gives the most efficient reaction.

8. Solid Supports

The present invention is not limited to any one solid support. In some embodiments, reactions are performed on microtiter plates (*e.g.*, polystyrene plates containing either containing 96 or 384 wells). For example, in some embodiments, streptavidin (SA) coated 96-well or 384-well microtiter plates (Boehringer Mannheim Biochemicals, Indianapolis, IN) are used as solid supports. In such embodiments, signal can be measured using standard fluorescent, chemiluminescent or colorimetric microtiter plate readers.

In some embodiments, INVADER assay reactions are carried out on particles or beads. The particles can be made of any suitable material, including, but not limited to, latex. In some embodiments, columns containing a particle matrix suitable for attachment of oligonucleotides are used. In a some embodiments, reactions are performed in minicolumns (*e.g.* DARAS, Tepnel, Cheshire, England). The columns contain microbeads to which oligonucleotides are covalently bound and subsequently used as capture probes or in enzymatic reactions. The use of minicolumns allows approximation of the bound oligonucleotide concentrations that will be attainable in a miniaturized chip format. Oligonucleotide binding is limited by the capacity of the support (*i.e.* $\sim 10^{12}/cm^2$). Thus, bound oligonucleotide concentration can only be increased by increasing the surface area to volume ratio of the reaction vessel. For example, one well of a 96-well microtiter plate, with a surface area of $\sim 1 cm^2$ and a volume of 400µl has a maximal bound oligonucleotide concentration of ~25 nM. On the other hand, a 100µm X 100µm X 100µM volume in a microchip has a surface area of 10^4 μm^2 and a volume of 1 nL, resulting in a bound oligonucleotide concentration of 0.2µM. Similar increased surface area: volume ratios can be obtained by using microbeads. Given a binding capacity of $\geq 10^{14}$ oligonucleotides in a 30µl volume, these beads allow bound oligonucleotide concentrations of 0.2-10µM, *i.e.* comparable to those anticipated for microchips.

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In some embodiments, INVADER reaction are carried out on a HydroGel (Packard Instrument Company, Meriden, CT) support. HydroGel is porous 3D hydrophilic polymer matrix. The matrix consists of a film of polyacrylamide polymerized onto a microscope slide. A coupling moiety is co-polymerized into the matrix that permits the immobilization of aminated oligonucleotide molecules by reductive amination. Covalent attachment by amine groups permits the immobilization of nucleic acid probes at specific attachment points (usually their ends), and the hydrogel provides a 3D matrix approximating a bulk solution phase, avoiding a solid/solution phase interface.

In other embodiments, INVADER reactions are conducted on a solid support using a BEADARRAY (Illumina, San Diego, CA) technology. The technology combines fiber optic bundles and beads that self-assemble into an array. Each fiber optic bundle contains thousands to millions of individual fibers depending on the diameter of the bundle. Sensors are affixed to each beads in a given batch. The particular molecules on a bead define that bead's function as a sensor. To form an array, fiber optic bundles are dipped into pools of coated beads. The coated beads are drawn into the wells, one bead per well, on the end of each fiber in the bundle.

The present invention is not limited to the solid supports described above. Indeed, a variety of other solid supports are contemplated including, but not limited to, glass microscope slides, glass wafers, gold, silicon, microchips, and other plastic, metal, ceramic, or biological surfaces.

9. Surface Coating and Attachment Chemistries

In some embodiments of the present invention, solid supports are coated with a material to aid in the attachment of oligonucleotides. The present invention is not limited to any one surface coating. Indeed, a variety of coatings are contemplated including, but not limited to, those described below.

In some embodiments, solid support INVADER assay reactions are carried out on solid supports coated with gold. The gold can be attached to any suitable solid support including, but not limited to, microparticles, microbeads, microscope slides, and microtiter plates. In some embodiments, the gold is functionalized with thiol-reactive maleimide moieties that can be reacted with thiol modified DNA (See e.g., Frutos et al.,

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Nuc. Acid. Res., 25:4748 [1997]; Frey and Corn, Analytical Chem, 68:3187 [1996]; Jordan *et al.*, Analytical Chem, 694939 [1997]; and U.S. Patent No: 5,472,881; herein incorporated by reference).

In other embodiments, solid support INVADER assay reactions are carried out on supports coated with silicon. The silicon can be attached to any suitable support, including, but not limited to, those described above and in the illustrative examples provided below.

Additionally, in some embodiments, solid supports are coated with a molecule (*e.g.*, a protein) to aid in the attachment of nucleic acids. The present invention is not limited to any particular surface coating. Any suitable material may be utilized including, but not limited to, proteins such as streptavidin. Thus, in some embodiments, oligonucleotides are attached to solid supports via terminal biotin or NH_2-mediated linkages included during oligonucleotide synthesis. INVADER oligonucleotides are attached to the support at their 5' ends and Signal Probes are attached at their 3' ends. In some embodiment, oligonucleotides are attached via a linker proximal to the attachment point. In a preferred embodiment, attachment is via a 40 atom linker with a low negative charge density as described in (Shchepinov *et al.*, Nucleic Acids Research 25: 1155 [1997]).

In other embodiments, oligonucleotides are attached to solid support via antigen:antibody interaction. For Example, in some embodiments, an antigen (*e.g.*, protein A or Protein G) is attached to a solid support and IgG is attached to oligonucleotides. In other embodiments, IgG is attached to a solid support and an antigen (*e.g.*, Protein A or Protein G) is attached to oligonucleotides.

10. Addressing of Oligonucleotides

In some embodiments, oligonucleotides are targeted to specific sites on the solid support. As noted above, when multiple oligonucleotides are bound to the solid support, the oligonucleotides may be synthesized directly on the surface using any number of methods known in the art (*e.g.*, including but not limited to methods described in PCT publications WO 95/11995, WO 99/42813 and WO 02/04597, and U.S Patent Nos. 5,424,186; 5,744,305; and 6,375,903, each incorporated by reference herein).

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Any number of techniques for the addressing of oligonucleotides may be utilized. For example, in some embodiments, solid support surfaces are electrically polarized at one given site in order to attract a particular DNA molecule (e.g, Nanogen, CA). In other embodiments, a pin tool may be used to load the array mechanically (Shalon, Genome Methods, 6:639 [1996]. In other embodiments, ink jet technology is used to print oligonucleotides onto an active surface (*e.g.*, O'Donnelly-Maloney *et al.*, Genetic Analysis:Biomolecular Engineering, 13:151 [1996]).

In some preferred embodiments utilizing gold surfaces, the gold surfaces are further modified to create addressable DNA arrays by photopatterning self-assembled monolayers to form hydrophilic and hydrophobic regions. Alkanethiol chemistry is utilized to create self-assembled monolayers (Nuzzo et al., JACS, 105:4481 [1983]). DNA is placed on the hydrophilic regions by using an automated robotic device (e.g., a pin-loading tool).

E. Reaction vessels

The detection assays of the present invention may be performed using any suitable reaction vessel. As used herein, the term "reaction vessel" refers to a system in which a reaction may be conducted, including but not limited to test tubes, wells, microwells (*e.g.*, wells in microtitre assay plates such as, 96-well, 384-well and 1536-well assay plates), capillary tubes, ends of fibers such as optical fibers, microfluidic devices such as fluidic chips, cartridges and cards (including but not limited to those described, *e.g.*, in US Patent No. 6,126,899, to Woudenberg, *et al.*, U.S. Patent Nos. 6,627,159, 6,720,187, and 6,734,401 to Bedingham, *et al.*, U.S. Patent Nos. 6,319,469 and 6,709,869 to Mian, *et al.*, U.S. Patent Nos. 5,587,128 and 6,660,517 to Wilding, *et al.*), or a test site on any surface (including but not limited to a glass, plastic or silicon surface, a bead, a microchip, or an non-solid surface, such as a gel or a dendrimer).

In some preferred embodiments, reactions are conducted using a 3M microfluidic card (3M, St. Paul, MN). The 3M card has 8 loading ports, each of which is configured to supply liquid reagent to 48 individual reaction chambers upon centrifugation of the card. The reaction chambers contain pre-dispensed and dried assay reaction components

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for detection of target nucleic acids. These reagents are dissolved when they come in contact with the liquid reagents upon centrifugation of the card.

EXPERIMENTAL

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

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

EXAMPLE 1

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Extension of Dynamic Range of Target Detection by Variation of Probe Concentration

The following example describes the use of variation of probe to extend the dynamic range of detection of an analyte. In this experimental example, the RT-INVADER+PCR method was used to detect target RNA from a sample. RNA was first reverse transcribed into cDNA, this cDNA was amplified by PCR, and this amplified DNA was detected by INVADER assay.

In this experiment, a probe containing a perfectly matched analyte specific region coupled to a FAM label arm (1968-24-03) was used at the standard INVADER assay concentration of 0.67 uM, and combined with another probe with a perfectly matched analyte-specific region coupled to a RED label arm (1978-13-02) at a 20X diluted concentration of 0.03 uM. The remaining reaction conditions were as follows: Forward primer (1931-48-05) at 1 uM, reverse primer / INVADER oligonucleotide (1931-48-01) at 1.067 uM, FRET probes at 0.33 uM, MOPS buffer at 10 mM, MgCl2 at 7.5 mM, dNTPs at 25 uM, MMLV RT at 75 units, Taq polymerase at 0.5 units, CLEAVASE enzyme at 100 ng, and the balance of water. The temperature cycling conditions of the reactions was 42°C for 30 min; 95°C for 7.5 min; 26 cycles of 95°C for 45 sec, 58°C for 30 sec, and 72°C for 2 min; 99°C for 10 min, and 63°C for 30 min. RNA template was supplied at pre-determined final concentrations of 0, 156, 313, 625, 1,250, 2,500, and 5,000 copies.

The results of the reactions were as follows: By the combined use of the perfectly matched probes being run at 1X and 20X diluted concentration, the dynamic range was extended from saturation at 625 copies of the target to saturation at 2,500 copies, an increase of 4-fold (see Figure 1).

The results of this experiment demonstrate that dynamic range can be extended by the use probes bearing different detection label arms run at different concentrations simultaneously.

EXAMPLE 2

Extension of Dynamic Range of Target Detection by Use of Mismatched Probes

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The following example describes the use of mismatch-containing probes to extend the dynamic range of detection of an analyte. In this experimental example, the RT-INVADER+PCR method was used to detect target RNA from a sample. RNA was first reverse transcribed into cDNA, this cDNA was amplified by PCR, and this amplified DNA was detected by INVADER assay.

In this experiment, a probe containing a perfectly matched analyte specific region coupled to a FAM label arm (1968-24-03) was used at the standard INVADER assay concentration of 0.67 uM, and combined with a probe containing a single mismatch in the analyte specific region coupled to a RED label arm (1978-13-01) at 0.67 uM. The remaining conditions of the reactions were as follows: Forward primer (1931-48-05) at 1 uM, reverse primer / invader oligonucleotide (1931-48-01) at 1.067 uM, FRET probes at 0.33 uM, MOPS buffer at 10 mM, MgCl2 at 7.5 mM, dNTPs at 25 uM, MMLV RT at 75 units, Taq polymerase at 0.5 units, CLEAVASE enzyme at 100 ng, and the balance of water. The temperature cycling conditions of the reactions was 42°C for 30 min; 95°C for 7.5 min; 26 cycles of 95°C for 45 sec, 58°C for 30 sec, and 72°C for 2 min; 99°C for 10 min, and 63°C for 30 min. RNA template was supplied at pre-determined final concentrations of 0, 156, 313, 625, 1,250, 2,500, and 5,000 copies.

The results of the reactions were as follows: By the combined use of the matched and mismatched probes, the dynamic range was extended from saturation at 625 copies to saturation at 5,000 copies, an increase of 8-fold (see Figure 1).

The results of this experiment demonstrate that dynamic range can be extended by the use probes bearing different detection label arms run at different concentrations simultaneously.

EXAMPLE 3

Extension of Dynamic Range of Target Detection by Variation of Probe Concentration

The following example describes the use of variation of probe to extend the dynamic range of detection of an analyte. In this experimental example, the RT-INVADER+PCR method was used to detect a second target RNA from a sample. RNA

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was first reverse transcribed into cDNA, this cDNA was amplified by PCR, and this amplified DNA was detected by INVADER assay.

In this experiment, a probe containing a perfectly matched analyte specific region coupled to a FAM label arm (1909-92-01) was used at the standard INVADER assay concentration of 0.67 uM, and combined with another probe with a perfectly matched analyte-specific region coupled to a RED label arm (1909-62-01) at a 20X diluted concentration of 0.03 uM. The remaining reaction conditions were as follows: Forward primer (1909-72-02) at 1 uM, reverse primer (1909-90-06) at 1 uM, invader oligonucleotide (1909-92-02) at 0.067 uM, FRET probes at 0.33 uM, MOPS buffer at 10 mM, MgCl2 at 7.5 mM, dNTPs at 25 uM, MMLV RT at 75 units, Taq polymerase at 0.5 units, CLEAVASE enzyme at 100 ng, and the balance of water. The temperature cycling conditions of the reactions was 42°C for 30 min; 95°C for 7.5 min; 26 cycles of 95°C for 45 sec, 65°C for 30 sec, and 72°C for 2 min; 99°C for 10 min, and 63°C for 30 min. RNA template was supplied at pre-determined final concentrations of 0, 156, 313, 625, 1,250, 2,500, and 5,000 copies.

The results of the reactions were as follows: By the combined use of the perfectly matched probes being run at 1X and 20X diluted concentration, the dynamic range was extended from saturation at 625 copies of the target to saturation at 2,500-10,000 copies, an increase of 4-16 fold (see Figure 2).

The results of this experiment demonstrate that dynamic range can be extended by the use probes bearing different detection label arms run at different concentrations simultaneously.

EXAMPLE 4

Extension of Dynamic Range of Target Detection by Use of Mismatched Probes

The following example describes the use of mismatch-containing probes to extend the dynamic range of detection of an analyte. In this experimental example, the RT-INVADER+PCR method was used to detect target RNA from a sample. RNA was first reverse transcribed into cDNA, this cDNA was amplified by PCR, and this amplified DNA was detected by INVADER assay.

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In this experiment, a probe containing a perfectly matched analyte specific region coupled to a FAM label arm (1909-92-01) was used at the standard INVADER assay concentration of 0.67 uM, and combined with another probe with an analyte-specific region containing a single mismatch coupled to a RED label arm (1909-62-02) used at 0.67 uM. The remaining reaction conditions were as follows: Forward primer (1909-72-02) at 1 uM, reverse primer (1909-90-06) at 1 uM, invader oligonucleotide (1909-92-02) at 0.067 uM, FRET probes at 0.33 uM, MOPS buffer at 10 mM, MgCl2 at 7.5 mM, dNTPs at 25 uM, MMLV RT at 75 units, Taq polymerase at 0.5 units, CLEAVASE enzyme at 100 ng, and the balance of water. The temperature cycling conditions of the reactions was 42°C for 30 min; 95°C for 7.5 min; 26 cycles of 95°C for 45 sec, 65°C for 30 sec, and 72°C for 2 min; 99°C for 10 min, and 63°C for 30 min. RNA template was supplied at pre-determined final concentrations of 0, 156, 313, 625, 1,250, 2,500, and 5,000 copies.

The results of the reactions were as follows: By the combined use of the matched and mismatched probes, the dynamic range was extended from saturation at 625 copies to saturation at 2,500-10,000 copies, an increase of 4-16 fold (see Figure 2).

The results of this experiment demonstrate that dynamic range can be extended by the use probes bearing different detection label arms run at different concentrations simultaneously.

EXAMPLE 5

Extension of Dynamic Range of Target Detection by Two Primary Probe Concentrations and a Single Dye Read-out

The following example describes the use of two probes at different concentrations that each contributes to extend the dynamic range of detection of an analyte using a single dye for detection. In this example, a different FRET cassette was provided to accumulate signal from each probe, but the FRET cassettes reported using the same dye.

Methods

Oligonucleotides were prepared and mixed as shown in Table 1.

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Table 1

Mix 1

Design	Concentration	Sequence	
2232-11-01	.004467 uM	5'-CGCGCCGAGGGGGTTTCGCAGCG-hex	SEQ ID NO: 10
2232-51-01_a4	.67 uM	5'-AGGCCACGGACGGGGGTTTCGCAGCG-hex	SEQ ID NO: 11
2232-11-02	.5 uM	5'-CGGTGCTTTCGGAACTGCTCAACAAGTG	SEQ ID NO: 12
2054-41-02	.5 uM	5'-GGGTCTTGACACTCGCGCAT	SEQ ID NO: 13
23-211	.125 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACGTCCGTGGCCT-hex	SEQ ID NO: 14
23-205	.125 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACCTCGGCGCG-hex	SEQ ID NO: 15

Mix 2

Design	Concentration	Sequence	
2232-11-01	.004467 uM	5'-CGCGCCGAGGGGGTTTCGCAGCG-hex	SEQ ID NO: 10
2232-11-02	.5 uM	5'-CGGTGCTTTCGGAACTGCTCAACAAGTG	SEQ ID NO: 12
2054-41-02	.5 uM	5'-GGGTCTTGACACTCGCGCAT	SEQ ID NO: 13
23-211	.125 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACGTCCGTGGCCT-hex	SEQ ID NO: 14
23-205	.125 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACCTCGGCGCG-hex	SEQ ID NO: 15

Mix 3

Design	Concentration	Sequence	
2232-51-01_a4	.67 uM	5'-AGGCCACGGACGGGGGTTTCGCAGCG-hex	SEQ ID NO: 11
2232-11-02	.5 uM	5'-CGGTGCTTTCGGAACTGCTCAACAAGTG	SEQ ID NO: 12
2054-41-02	.5 uM	5'-GGGTCTTGACACTCGCGCAT	SEQ ID NO: 13
23-211	.125 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACGTCCGTGGCCT-hex	SEQ ID NO: 14
23-205	.125 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACCTCGGCGCG-hex	SEQ ID NO: 15

X = Z28 Phosphoramidite
2 = Z35 (Red Dye) Phosphoramidite
hex = hexanediol

For each reaction, 333.34 ng/rxn Cleavase® VIII and 3.34 Units/rxn native Taq DNA polymerase (Promega) were combined in 5 µl of Cleavase enzyme dilution buffer (0.02 M Tris pH 8.0, 0.05 M KCl, 0.5% Tween 20, 0.5% Nonidet P40, 50% glycerol, and 100 µg/mL BSA in water). For each reaction, 10 to 10,000,000 copies of a plasmid target DNA containing the CMV sequence 5'-GCGCGTCTCGGTGCTTTCGGAACTGCTCAACAAGTGGGTTTCGCAGCGCCGTG CCGTGCGCGAATGCATGCGCGAGTGTCAAGACCC-3' (SEQ ID NO:6) was diluted into a final volume of 15 µl of a solution containing 20 ng/µl of tRNA. Each 50 µl reaction contained 5 µl of the enzyme mixture, 15 µl of the target DNA mixture and the indicated combination of oligonucleotides in buffer containing 10 mM MOPS, 7.5 mM MgCl2, and 25 µM dNTPs. The reactions were incubated as follows: 20 cycles of 95°C

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for 15 sec and 72°C for 45 sec.; 99°C for 10 min; then 63°C for 30 min. The fluorescent signal produced in each reaction vessel was quantitated on a Tecan Genios FL fluorescence plate reader. The results are shown in Figure 4. Both Probes = Mix 1, 1x Probe = Mix 3, 1/150x Probe = Mix 2.

Results

As shown in Figure 4, in this experiment the InRange™ assay expanded the dynamic range of a single reaction mixture by up to three orders of magnitude (1,000-fold) to six total orders of magnitude, as compared to the two or three orders of magnitude range achieved using either probe concentration individually.

EXAMPLE 6

Expansion of Serial Invasive Cleavage Assay Dynamic Range with InRange™ Assay Using Three Primary Probe Concentrations

The following example describes the use of three probes at different concentrations that each contributes to extend the dynamic range of detection of an analyte using a single dye for detection. In this example, a different FRET cassette was provided to accumulate signal from each probe, but the FRET cassettes reported using the same dye.

Methods

Oligonucleotides were prepared and mixed as shown in Table 2.

Table 2

Mix 1

Design	Concentration	Sequence	
2232-11-02	.5 uM	5'-CGGTGCTTTCGGAACTGCTCAACAAGTG	SEQ ID NO: 12
2054-41-02	.5 uM	5'-GGGTCTTGACACTCGCGCAT	SEQ ID NO: 13
23-211	.125 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACGTCCGTGGCCT-hex	SEQ ID NO: 14
23-205	.125 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACCTCGGCGCG-hex	SEQ ID NO: 15
23-394	.25 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACTCCGCGTCCGT-hex	SEQ ID NO: 16
2232-11-01	.67 uM	5'-CGCGCCGAGGGGGTTTCGCAGCG-hex	SEQ ID NO: 10

Mix 2

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Design	Concentration	Sequence	
2232-11-02	.5 uM	5'-CGGTGCTTTCGGAACTGCTCAACAAGTG	SEQ ID NO: 12
2054-41-02	.5 uM	5'-GGGTCTTGACACTCGCGCAT	SEQ ID NO: 13
23-211	.125 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACGTCCGTGGCCT-hex	SEQ ID NO: 14
23-205	.125 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACCTCGGCGCG-hex	SEQ ID NO: 15
23-394	.25 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACTCCGCGTCCGT-hex	SEQ ID NO: 16
2241-45-01_a3	.0054 uM	5'-ACGGACGCGGAGGGGTTTCGCAGCG-hex	SEQ ID NO: 17

Mix 3

Design	Concentration	Sequence	
2232-11-02	.5 uM	5'-CGGTGCTTTCGGAACTGCTCAACAAGTG	SEQ ID NO: 12
2054-41-02	.5 uM	5'-GGGTCTTGACACTCGCGCAT	SEQ ID NO: 13
23-211	.125 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACGTCCGTGGCCT-hex	SEQ ID NO: 14
23-205	.125 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACCTCGGCGCG-hex	SEQ ID NO: 15
23-394	.25 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACTCCGCGTCCGT-hex	SEQ ID NO: 16
2232-51-01_a4	.00067 uM	5'-AGGCCACGGACGGGGTTTCGCAGCG-hex	SEQ ID NO: 11

Mix 4

Design	Concentration	Sequence	
2232-11-02	.5 uM	5'-CGGTGCTTTCGGAACTGCTCAACAAGTG	SEQ ID NO: 12
2054-41-02	.5 uM	5'-GGGTCTTGACACTCGCGCAT	SEQ ID NO: 13
23-211	.125 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACGTCCGTGGCCT-hex	SEQ ID NO: 14
23-205	.125 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACCTCGGCGCG-hex	SEQ ID NO: 15
23-394	.25 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACTCCGCGTCCGT-hex	SEQ ID NO: 16
2232-11-01	.67 uM	5'-CGCGCCGAGGGGGTTTCGCAGCG-hex	SEQ ID NO: 10
2241-45-01_a3	.0054 uM	5'-ACGGACGCGGAGGGGTTTCGCAGCG-hex	SEQ ID NO: 17

Mix 5

Design	Concentration	Sequence	
2232-11-02	.5 uM	5'-CGGTGCTTTCGGAACTGCTCAACAAGTG	SEQ ID NO: 12
2054-41-02	.5 uM	5'-GGGTCTTGACACTCGCGCAT	SEQ ID NO: 13
23-211	.125 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACGTCCGTGGCCT-hex	SEQ ID NO: 14
23-205	.125 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACCTCGGCGCG-hex	SEQ ID NO: 15
23-394	.25 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACTCCGCGTCCGT-hex	SEQ ID NO: 16
2232-11-01	.67 uM	5'-CGCGCCGAGGGGGTTTCGCAGCG-hex	SEQ ID NO: 10
2232-51-01_a4	.00067 uM	5'-AGGCCACGGACGGGGTTTCGCAGCG-hex	SEQ ID NO: 11

Mix 6

Design	Concentration	Sequence	
2232-11-02	.5 uM	5'-CGGTGCTTTCGGAACTGCTCAACAAGTG	SEQ ID NO: 12
2054-41-02	.5 uM	5'-GGGTCTTGACACTCGCGCAT	SEQ ID NO: 13
23-211	.125 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACGTCCGTGGCCT-hex	SEQ ID NO: 14
23-205	.125 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACCTCGGCGCG-hex	SEQ ID NO: 15
23-394	.25 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACTCCGCGTCCGT-hex	SEQ ID NO: 16
2241-45-01_a3	.0054 uM	5'-ACGGACGCGGAGGGGTTTCGCAGCG-hex	SEQ ID NO: 17
2232-51-01_a4	.00067 uM	5'-AGGCCACGGACGGGGTTTCGCAGCG-hex	SEQ ID NO: 11

Mix 7

Design	Concentration	Sequence	

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2232-11-02	.5 uM	5'-CGGTGCTTTCGGAACTGCTCAACAAGTG	SEQ ID NO: 12
2054-41-02	.5 uM	5'-GGGTCTTGACACTCGCGCAT	SEQ ID NO: 13
23-211	.125 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACGTCCGTGGCCT-hex	SEQ ID NO: 14
23-205	.125 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACCTCGGCGCG-hex	SEQ ID NO: 15
23-394	.25 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACTCCGCGTCCGT-hex	SEQ ID NO: 16
2232-11-01	.67 uM	5'-CGCGCCGAGGGGGTTTCGCAGCG-hex	SEQ ID NO: 10
2241-45-01_a3	.0054 uM	5'-ACGGACGCGGAGGGGTTTCGCAGCG-hex	SEQ ID NO: 17
2232-51-01_a4	.00067 uM	5'-AGGCCACGGACGGGGTTTCGCAGCG-hex	SEQ ID NO: 11

X = Z28 Phosphoramidite
2 = Z35 (Red Dye) Phosphoramidite
hex = hexanediol

For each reaction, 333.34 ng/rxn Cleavase® VIII and 3.34 Units/rxn native Taq DNA polymerase (Promega) were combined in 5 µl of Cleavase enzyme dilution buffer (0.02 M Tris pH 8.0, 0.05 M KCl, 0.5% Tween 20, 0.5% Nonidet P40, 50% glycerol, and 100 µg/mL BSA in water). For each reaction, 10 to 10,000,000 copies of a plasmid target DNA containing the CMV sequence 5'-GCGCGTCTCGGTGCTTTCGGAACTGCTCAACAAGTGGGTTTCGCAGCGCCGTG CCGTGCGCGAATGCATGCGCGAGTGTCAAGACCC-3' (SEQ ID NO:6) was diluted into a final volume of 15 µl of a solution containing 20 ng/µl of tRNA. Each 50 µl reaction contained 5 µl of the enzyme mixture, 15 µl of the target DNA mixture and the indicated combination of oligonucleotides in buffer containing 10 mM MOPS, 7.5 mM MgCl2, and 25 µM dNTPs. The reactions were incubated as follows: 20 cycles of 95°C for 15 sec and 72°C for 45 sec.; 99°C for 10 min; then 63°C for 30 min. The fluorescent signal produced in each reaction vessel was quantitated on a Tecan Genios FL fluorescence plate reader. The results are shown in Figure 5. 1x Probe = Mix 1, 1/125x Probe = Mix 2, 1/1000x Probe = Mix 3, 3 Probe Mix = Mix 7. The remaining mixes (not shown) are various combinations of 2 probes to examine contributory effect.

Results

As shown in Figure 5, in this experiment the InRange™ assay improved the dynamic range and data quality when three probe concentrations were used together, as compared to the contribution made by any single probe concentration, or combination of two probes. As shown by the lines depicting the signal resulting from each probe

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combination individually, the independent contribution of each of these experimental conditions can readily be observed.

EXAMPLE 7

Optimization of Serial Invasive Cleavage Assay Through Alteration of Incubation Time of Invasive Cleavage Reaction.

The following example describes optimization of the multiple probe system of the present invention through alteration of the length of time of incubation of the invasive cleavage reaction. In this example, a different FRET cassette was provided to accumulate signal from each probe, but the FRET cassettes reported using the same dye.

Methods

Oligonucleotides were prepared and mixed as shown in Table 3.

Table 3

Mixes: 3 Probe and new 3 Probe

Design	Concentration	Sequence	
2232-11-02	.5 uM	5'-CGGTGCTTTCGGAACTGCTCAACAAGTG	SEQ ID NO: 12
23-211	.25 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACGTCCGTGGCCT-hex	SEQ ID NO: 14
23-205	.25 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACCTCGGCGCG-hex	SEQ ID NO: 15
23-394	.25 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACTCCGCGTCCGT-hex	SEQ ID NO: 16
2232-11-01	.0067 uM	5'-CGCGCCGAGGGGGTTTCGCAGCG-hex	SEQ ID NO: 10
2241-45-01_a3	.00067 uM	5'-ACGGACGCGGAGGGGTTTCGCAGCG-hex	SEQ ID NO: 17
2232-51-01_a4	.67 uM	5'-AGGCCACGGACGGGGTTTCGCAGCG-hex	SEQ ID NO: 11

Mix: 1/1000x Probe

Design	Concentration	Sequence	
2232-11-02	.5 uM	5'-CGGTGCTTTCGGAACTGCTCAACAAGTG	SEQ ID NO: 12
23-211	.25 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACGTCCGTGGCCT-hex	SEQ ID NO: 14
23-205	.25 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACCTCGGCGCG-hex	SEQ ID NO: 15
23-394	.25 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACTCCGCGTCCGT-hex	SEQ ID NO: 16
2241-45-01_a3	.00067 uM	5'-ACGGACGCGGAGGGGTTTCGCAGCG-hex	SEQ ID NO: 17

Mix 1/100x Probe

Design	Concentration	Sequence	
2232-11-02	.5 uM	5'-CGGTGCTTTCGGAACTGCTCAACAAGTG	SEQ ID NO: 12
23-211	.25 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACGTCCGTGGCCT-hex	SEQ ID NO: 14
23-205	.25 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACCTCGGCGCG-hex	SEQ ID NO: 15
23-394	.25 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACTCCGCGTCCGT-hex	SEQ ID NO: 16

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2232-11-01	.0067 uM	5'-CGCGCCGAGGGGGTTTCGCAGCG-hex	SEQ ID NO: 10

Mix 1x Probe

Design	Concentration	Sequence	
2232-11-02	.5 uM	5'-CGGTGCTTTCGGAACTGCTCAACAAGTG	SEQ ID NO: 12
23-211	.25 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACGTCCGTGGCCT-hex	SEQ ID NO: 14
23-205	.25 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACCTCGGCGCG-hex	SEQ ID NO: 15
23-394	.25 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACTCCGCGTCCGT-hex	SEQ ID NO: 16
2232-51-01_a4	.67 uM	5'-AGGCCACGGACGGGGTTTCGCAGCG-hex	SEQ ID NO: 11

X = Z28 Phosphoramidite
2 = Z35 (Red Dye) Phosphoramidite
hex = hexanediol

For each reaction, 100 ng/rxn Cleavase® VIII and 3.34 Units/rxn native Taq DNA polymerase (Promega) were combined in 5 µl of Cleavase enzyme dilution buffer (0.02 M Tris pH 8.0, 0.05 M KCl, 0.5% Tween 20, 0.5% Nonidet P40, 50% glycerol, and 100 µg/mL BSA in water). For each reaction, 100 to 1,000,000,000 copies of a plasmid target DNA containing the CMV sequence 5'-GCGCGTCTCGGTGCTTTCGGAACTGCTCAACAAGTGGGTTTCGCAGCGCCGTG CCGTGCGCGAATGCATGCGCGAGTGTCAAGACCC-3' (SEQ ID NO:6) was diluted into a final volume of 15 µl of a solution containing 20 ng/µl of tRNA. Each 50 µl reaction contained 5 µl of the enzyme mixture, 15 µl of the target DNA mixture and the indicated combination of oligonucleotides in buffer containing 10 mM MOPS, 7.5 mM MgCl2, and 25 µM dNTPs. The reactions were incubated at 63°C for times ranging from 30 min to 8 hours. The fluorescent signal produced in each reaction vessel was quantitated on a Tecan Genios FL fluorescence plate reader. The results are shown in Figure 6. All data shown pertains to the 3-Probe mix with readings at different time points. The other 3 mixes (not shown) were tested to examine individual probe contribution at specific probe concentrations.

Results

As shown in Figure 6, in this experiment the mixed probe assay performance was optimized through alteration of the length of time of incubation of the invasive cleavage reaction. Shorter incubation times in the range of 30 minutes led to a higher overall lower limit of detection, with no increase in overall dynamic range. By contrast, longer

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incubation times of 8 hours led to a lower limit of detection but also a lower upper limit of detection. In this experiment, an incubation time of 4 hours resulted in the best overall dynamic range, detecting a range of target concentrations from 10,000 to 1,000,000,000 copies per reaction.

EXAMPLE 8

Simultaneous Detection of Two Distinct Targets in a Single Reaction Vessel Across a Broad Dynamic Range

The following example describes the detection of two different human herpesviruses, CMV and EBV, across over six orders of magnitude of dynamic range, in the same reaction vessel. For each virus, a different FRET cassette was provided to accumulate signal from each of the different probes, but each virus-specific FRET cassettes reported using the same dye. A different dye was used for each virus' collection of FRET cassettes.

Methods

Oligonucleotides were prepared and mixed as shown in Table 4.

Table 4

Mix 1

Design	Concentration	Sequence	
2259-24-06	.67 uM	5'-CGCGAGGCCGGCGCACCGAAGC-hex	SEQ ID NO: 18
2054-42-01	.5 uM	5'-CGGCGTGACYCACCGCTTTG	SEQ ID NO: 19
2054-42-02	.5 uM	5'-GCAGTTCCGAAAGCACCGARACG	SEQ ID NO: 20
23-210	.25 uM	5'-1TCTXAAGCCGGTTTTCCGGCTGAGACTCCGCGTCCGT-hex	SEQ ID NO: 21
23-425	.25 uM	5'-1TCTXAAGCCGGTTTTCCGGCTGAGACGGCCTCGCG-hex	SEQ ID NO: 22
2250-25-01	.5 uM	5'-ATTGGGGSAATGRCGACCGTCACT	SEQ ID NO: 23
2250-25-02	.5 uM	5'-CCGTCATTCCCGTCGTGTTGC	SEQ ID NO: 24
23-211	.25 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACGTCCGTGGCCT-hex	SEQ ID NO: 25
23-205	.25 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACCTCGGCGCG-hex	SEQ ID NO: 26

Mix 2

Design	Concentration	Sequence	
2054-42-03_a4	.0067 uM	5'-CGCGCCGAGGGCGCACCGAAGC-hex	SEQ ID NO: 27
2054-42-01	.5 uM	5'-CGGCGTGACYCACCGCTTTG	SEQ ID NO: 19
2054-42-02	.5 uM	5'-GCAGTTCCGAAAGCACCGARACG	SEQ ID NO: 20
23-210	.25 uM	5'-1TCTXAAGCCGGTTTTCCGGCTGAGACTCCGCGTCCGT-hex	SEQ ID NO: 21
23-425	.25 uM	5'-1TCTXAAGCCGGTTTTCCGGCTGAGACGGCCTCGCG-hex	SEQ ID NO: 22

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2250-25-01	.5 uM	5'-ATTGGGGSAATGRCGACCGTCACT	SEQ ID NO: 23
2250-25-02	.5 uM	5'-CCGTCATTCCCGTCGTGTTGC	SEQ ID NO: 24
23-211	.25 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACGTCCGTGGCCT-hex	SEQ ID NO: 25
23-205	.25 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACCTCGGCGCG-hex	SEQ ID NO: 26

Mix 3

Design	Concentration	Sequence	
2250-25-03_a3	.67 uM	5'-ACGGACGCGGAGTCGGACTATCAACCACT-hex	SEQ ID NO: 28
2054-42-01	.5 uM	5'-CGGCGTGACYCACCGCTTTG	SEQ ID NO: 19
2054-42-02	.5 uM	5'-GCAGTTCCGAAAGCACCGARACG	SEQ ID NO: 20
23-210	.25 uM	5'-1TCTXAAGCCGGTTTTCCGGCTGAGACTCCGCGTCCGT-hex	SEQ ID NO: 21
23-425	.25 uM	5'-1TCTXAAGCCGGTTTTCCGGCTGAGACGGCCTCGCG-hex	SEQ ID NO: 22
2250-25-01	.5 uM	5'-ATTGGGGSAATGRCGACCGTCACT	SEQ ID NO: 23
2250-25-02	.5 uM	5'-CCGTCATTCCCGTCGTGTTGC	SEQ ID NO: 24
23-211	.25 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACGTCCGTGGCCT-hex	SEQ ID NO: 25
23-205	.25 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACCTCGGCGCG-hex	SEQ ID NO: 26

Mix 4

Design	Concentration	Sequence	
2250-25-03_a1	.0067 uM	5'-CGCGCCGAGGTCGGACTATCAACCACT-hex	SEQ ID NO: 29
2054-42-01	.5 uM	5'-CGGCGTGACYCACCGCTTTG	SEQ ID NO: 19
2054-42-02	.5 uM	5'-GCAGTTCCGAAAGCACCGARACG	SEQ ID NO: 20
23-210	.25 uM	5'-1TCTXAAGCCGGTTTTCCGGCTGAGACTCCGCGTCCGT-hex	SEQ ID NO: 21
23-425	.25 uM	5'-1TCTXAAGCCGGTTTTCCGGCTGAGACGGCCTCGCG-hex	SEQ ID NO: 22
2250-25-01	.5 uM	5'-ATTGGGGSAATGRCGACCGTCACT	SEQ ID NO: 23
2250-25-02	.5 uM	5'-CCGTCATTCCCGTCGTGTTGC	SEQ ID NO: 24
23-211	.25 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACGTCCGTGGCCT-hex	SEQ ID NO: 25
23-205	.25 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACCTCGGCGCG-hex	SEQ ID NO: 26

5

Mix 5

Design	Concentration	Sequence	
2259-24-06	.67 uM	5'-CGCGAGGCCGGCGCACCGAAGC-hex	SEQ ID NO: 18
2054-42-03_a4	.0067 uM	5'-CGCGCCGAGGGCGCACCGAAGC-hex	SEQ ID NO: 27
2054-42-01	.5 uM	5'-CGGCGTGACYCACCGCTTTG	SEQ ID NO: 19
2054-42-02	.5 uM	5'-GCAGTTCCGAAAGCACCGARACG	SEQ ID NO: 20
23-210	.25 uM	5'-1TCTXAAGCCGGTTTTCCGGCTGAGACTCCGCGTCCGT-hex	SEQ ID NO: 21
23-425	.25 uM	5'-1TCTXAAGCCGGTTTTCCGGCTGAGACGGCCTCGCG-hex	SEQ ID NO: 22
2250-25-01	.5 uM	5'-ATTGGGGSAATGRCGACCGTCACT	SEQ ID NO: 23
2250-25-02	.5 uM	5'-CCGTCATTCCCGTCGTGTTGC	SEQ ID NO: 24
23-211	.25 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACGTCCGTGGCCT-hex	SEQ ID NO: 25
23-205	.25 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACCTCGGCGCG-hex	SEQ ID NO: 26

Mix 6

Design	Concentration	Sequence	
2250-25-03_a3	.67 uM	5'-ACGGACGCGGAGTCGGACTATCAACCACT-hex	SEQ ID NO: 28
2250-25-03_a1	.0067 uM	5'-CGCGCCGAGGTCGGACTATCAACCACT-hex	SEQ ID NO: 29
2054-42-01	.5 uM	5'-CGGCGTGACYCACCGCTTTG	SEQ ID NO: 19
2054-42-02	.5 uM	5'-GCAGTTCCGAAAGCACCGARACG	SEQ ID NO: 20
23-210	.25 uM	5'-1TCTXAAGCCGGTTTTCCGGCTGAGACTCCGCGTCCGT-hex	SEQ ID NO: 21

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23-425	.25 uM	5'-1TCTXAAGCCGGTTTTCCGGCTGAGACGGCCTCGCG-hex	SEQ ID NO: 22
2250-25-01	.5 uM	5'-ATTGGGGSAATGRCGACCGTCACT	SEQ ID NO: 23
2250-25-02	.5 uM	5'-CCGTCATTCCCGTCGTGTTGC	SEQ ID NO: 24
23-211	.25 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACGTCCGTGGCCT-hex	SEQ ID NO: 25
23-205	.25 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACCTCGGCGCG-hex	SEQ ID NO: 26

Mix 7

Design	Concentration	Sequence	
2259-24-06	.67 uM	5'-CGCGAGGCCGGCGCACCGAAGC-hex	SEQ ID NO: 18
2054-42-03_a4	.0067 uM	5'-CGCGCCGAGGGCGCACCGAAGC-hex	SEQ ID NO: 27
2250-25-03_a3	.67 uM	5'-ACGGACGCGGAGTCGGACTATCAACCACT-hex	SEQ ID NO: 28
2250-25-03_a1	.0067 uM	5'-CGCGCCGAGGTCGGACTATCAACCACT-hex	SEQ ID NO: 29
2054-42-01	.5 uM	5'-CGGCGTGACYCACCGCTTTG	SEQ ID NO: 19
2054-42-02	.5 uM	5'-GCAGTTCCGAAAGCACCGARACG	SEQ ID NO: 20
23-210	.25 uM	5'-1TCTXAAGCCGGTTTTCCGGCTGAGACTCCGCGTCCGT-hex	SEQ ID NO: 21
23-425	.25 uM	5'-1TCTXAAGCCGGTTTTCCGGCTGAGACGGCCTCGCG-hex	SEQ ID NO: 22
2250-25-01	.5 uM	5'-ATTGGGGSAATGRCGACCGTCACT	SEQ ID NO: 23
2250-25-02	.5 uM	5'-CCGTCATTCCCGTCGTGTTGC	SEQ ID NO: 24
23-211	.25 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACGTCCGTGGCCT-hex	SEQ ID NO: 25
23-205	.25 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACCTCGGCGCG-hex	SEQ ID NO: 26

X = Z28 Phosphoramidite
1 = 6 FAM Amidite
2 = Z35 (Red Dye) Phosphoramidite
hex = hexanediol

For each reaction, 333.34 ng/rxn Cleavase® VIII and 3.34 Units/rxn native Taq DNA polymerase (Promega) were combined in 5 µl of Cleavase enzyme dilution buffer (0.02 M Tris pH 8.0, 0.05 M KCl, 0.5% Tween 20, 0.5% Nonidet P40, 50% glycerol, and 100 µg/mL BSA in water). For each reaction, approximately 4 to 1,000,000 copies of plasmid target DNA containing the CMV sequence 5'-CGGCGTGACTCACCGCTTTGTGCGTGCTTCGGTGCGCGTCTCGGTGCTTTCGGAACTGC-3' (SEQ ID NO:7)and EBV sequence 5'-ATTGGGGCAATGGCGACCGTCACTCGGACTATCAACCACTAGGAACCCAAGATCAAAGTCTGTACTTGGGATTGCAACACGACGGGAATGACGG-3' (SEQ ID NO:8) were diluted into a final volume of 15 µl of a solution containing 20 ng/µl of tRNA. Each 50 µl reaction contained 5 µl of the enzyme mixture, 15 µl of the target DNA mixture and the indicated combination of oligonucleotides in buffer containing 10 mM MOPS, 7.5 mM MgCl2, and 25 µM dNTPs. The reactions were incubated as follows: 23 cycles of 95°C for 15 sec and 72°C for 45 sec.; 99°C for 10 min; then 63°C for 30 min. The fluorescent signal produced in each reaction vessel was quantitated on a

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Tecan Genios FL fluorescence plate reader. The results are shown in Figure 7. CMV = Mix 7 (FAM dye), EBV = Mix 7 (Red dye). Data is not shown from Mixes 1 – 6, which were tested to examine individual probe contribution at specific probe concentrations for the individual target. Mix 5 is the CMV without any EBV Probes, Mix 6 is the EBV without any CMV Probes.

Results

As shown in Figure 7, in this experiment the assay of the present invention was able to accurately detect two different human herpesviruses, CMV and EBV, across over six orders of magnitude of dynamic range, in the same reaction vessel. CMV and EBV were detected in multiplex over a range from approximately 20 to 1,000,000 copies per reaction.

EXAMPLE 9

Combination of the Mixed probe Assay with Target Amplification Methods to Further Increase Dynamic Range

The following example describes combining single strand amplification (cycling primer extension for linear accumulation of single stranded product) with standard PCR (exponential accumulation of double stranded product), with detection of both products simultaneously with two sets of two probes (different concentrations) to further expand the dynamic range. As described above, a different FRET cassette was provided to accumulate signal from each probe, but the FRET cassettes reported using the same dye.

Methods

Oligonucleotides were prepared and mixed as shown in Table 5.

Table 5

Mix 1

Design	Concentration	Sequence	
2232-11-02	.5 uM	5'-CGGTGCTTTCGGAACTGCTCAACAAGTG	SEQ ID NO: 12
2054-41-02	.5 uM	5'-GGGTCTTGACACTCGCGCAT	SEQ ID NO: 13

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2241-45-01_a3	.67 uM	5'-ACGGACGCGGAGGGGTTTCGCAGCG-hex	SEQ ID NO: 17
2232-51-01_a4	.0054 uM	5'-AGGCCACGGACGGGGTTTCGCAGCG-hex	SEQ ID NO: 11
2259-24-02	.5 uM	5'-CGGCGTGACYCACCGCTTTA	SEQ ID NO: 18
23-394	.125 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACTCCGCGTCCGT-hex	SEQ ID NO: 16
23-211	.125 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACGTCCGTGGCCT-hex	SEQ ID NO: 14
23-444	.125 uM	5'-2TCTXAAGCCGGTTTTCCGGCTGAGACGGCCTCGCG-hex	SEQ ID NO: 30
23-205	.125 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACCTCGGCGCG-hex	SEQ ID NO: 15

Mix 2

Design	Concentration	Sequence	
2232-11-02	.5 uM	5'-CGGTGCTTTCGGAACTGCTCAACAAGTG	SEQ ID NO: 12
2054-41-02	.5 uM	5'-GGGTCTTGACACTCGCGCAT	SEQ ID NO: 13
2241-45-01_a3	.67 uM	5'-ACGGACGCGGAGGGGTTTCGCAGCG-hex	SEQ ID NO: 17
2232-51-01_a4	.0054 uM	5'-AGGCCACGGACGGGGTTTCGCAGCG-hex	SEQ ID NO: 11
2259-24-02	.5 uM	5'-CGGCGTGACYCACCGCTTTA	SEQ ID NO: 18
23-394	.125 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACTCCGCGTCCGT-hex	SEQ ID NO: 16
23-211	.125 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACGTCCGTGGCCT-hex	SEQ ID NO: 14
23-444	.125 uM	5'-2TCTXAAGCCGGTTTTCCGGCTGAGACGGCCTCGCG-hex	SEQ ID NO: 30
23-205	.125 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACCTCGGCGCG-hex	SEQ ID NO: 15
2259-24-01	.0054 uM	5'-CGCGAGGCCGGTGCGTGCTTCGG-hex	SEQ ID NO: 31

Mix 3

Design	Concentration	Sequence	
2232-11-02	.5 uM	5'-CGGTGCTTTCGGAACTGCTCAACAAGTG	SEQ ID NO: 12
2054-41-02	.5 uM	5'-GGGTCTTGACACTCGCGCAT	SEQ ID NO: 13
2241-45-01_a3	.67 uM	5'-ACGGACGCGGAGGGGTTTCGCAGCG-hex	SEQ ID NO: 17
2232-51-01_a4	.0054 uM	5'-AGGCCACGGACGGGGTTTCGCAGCG-hex	SEQ ID NO: 11
2259-24-02	.5 uM	5'-CGGCGTGACYCACCGCTTTA	SEQ ID NO: 18
23-394	.125 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACTCCGCGTCCGT-hex	SEQ ID NO: 16
23-211	.125 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACGTCCGTGGCCT-hex	SEQ ID NO: 14
23-444	.125 uM	5'-2TCTXAAGCCGGTTTTCCGGCTGAGACGGCCTCGCG-hex	SEQ ID NO: 30
23-205	.125 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACCTCGGCGCG-hex	SEQ ID NO: 15
2271-50-01	.67 uM	5'-CGCGCCGAGGGTGCGTGCTTCGG-hex	SEQ ID NO: 32

Mix 4

Design	Concentration	Sequence	
2232-11-02	.5 uM	5'-CGGTGCTTTCGGAACTGCTCAACAAGTG	SEQ ID NO: 12
2054-41-02	.5 uM	5'-GGGTCTTGACACTCGCGCAT	SEQ ID NO: 13
2241-45-01_a3	.67 uM	5'-ACGGACGCGGAGGGGTTTCGCAGCG-hex	SEQ ID NO: 17
2232-51-01_a4	.0054 uM	5'-AGGCCACGGACGGGGTTTCGCAGCG-hex	SEQ ID NO: 11
2259-24-02	.5 uM	5'-CGGCGTGACYCACCGCTTTA	SEQ ID NO: 18
23-394	.125 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACTCCGCGTCCGT-hex	SEQ ID NO: 16
23-211	.125 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACGTCCGTGGCCT-hex	SEQ ID NO: 14
23-444	.125 uM	5'-2TCTXAAGCCGGTTTTCCGGCTGAGACGGCCTCGCG-hex	SEQ ID NO: 30
23-205	.125 uM	5'-2TCTXTTCGGCCTTTTGGCCGAGAGACCTCGGCGCG-hex	SEQ ID NO: 15
2271-50-01	.67 uM	5'-CGCGCCGAGGGTGCGTGCTTCGG-hex	SEQ ID NO: 32
2259-24-01	.0054 uM	5'-CGCGAGGCCGGTGCGTGCTTCGG-hex	SEQ ID NO: 31

X = Z28 Phosphoramidite

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2 = Z35 (Red Dye) Phosphoramidite
hex = hexanediol

For each reaction, 333.34 ng/rxn Cleavase® VIII and 3.34 Units/rxn native Taq DNA polymerase (Promega) were combined in 5 µl of Cleavase enzyme dilution buffer (0.02 M Tris pH 8.0, 0.05 M KCl, 0.5% Tween 20, 0.5% Nonidet P40, 50% glycerol, and 100 µg/mL BSA in water). For each reaction, approximately 10 to 10,000,000,000 copies of plasmid target DNA containing the CMV sequence 5'-GCGCGTCTCGGTGCTTTCGGAACTGCTCAACAAGTGGGTTTCGCAGCGCCGTGCCGTGCGCGAATGCATGCGCGAGTGTCAAGACCC-3' (SEQ ID NO:6) and CMV sequence 5'-CGGCGTGACTCACCGCTTTGTGCGTGCTTCGGTGCGCGTCTCGGTGCTTTCGGAACTGC-3' (SEQ ID NO:7) were diluted into a final volume of 15 µl of a solution containing 20 ng/µl of tRNA. Each 50 µl reaction contained 5 µl of the enzyme mixture, 15 µl of the target DNA mixture and the indicated combination of oligonucleotides in buffer containing 10 mM MOPS, 7.5 mM MgCl2, and 25 µM dNTPs. The reactions were incubated as follows: 23 cycles of 95°C for 15 sec and 72°C for 45 sec; 99°C for 10 min; then 63°C for 30 min. The fluorescent signal produced in each reaction vessel was quantitated on a Tecan Genios FL fluorescence plate reader. The results are shown in Figure 8.

Results

As shown in Figure 8, in this experiment the assay combining different amplification procedures with use of probes at difference concentrations was able to accurately detect a broader range of target concentrations than was determined when the only the probe concentration variation was applied. Combining single strand amplification with standard PCR, and detecting both products simultaneously with two sets of two probes further broadened the dynamic range potential of the assay of the present invention to at least nine orders of magnitude (10-10,000,000,000 copies of target detected by a single reaction setup).

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EXAMPLE 10

Increased Dynamic Range of Homogeneous RT-PCR-Invader Assays.

The following example describes combining RT-PCR with the assay comprising multiple probe concentrations for detection of an RNA target over an expanded dynamic range.

Methods

Oligonucleotides were prepared and mixed as shown in Table 6.

Table 6

Mix 1

Design	Concentration	Sequence	
2178-11-08	.5 uM	5'-CCCTGCAACGCGAGTGCTGA	SEQ ID NO: 33
2178-11-09	.5 uM	5'-GTGGACCACGTACCTAGAGTGCGG	SEQ ID NO: 34
23-204	.25 uM	5'-1TCTXAAGCCGGTTTTCCGGCTGAGACGTCCGTGGCCT-hex	SEQ ID NO: 35
23-210	.25 uM	5'-1TCTXAAGCCGGTTTTCCGGCTGAGACTCCGCGTCCGT-hex	SEQ ID NO: 21
2178-40-01	.67 uM	5'-AGGCCACGGACGAGGCTGGTGTACGAC-hex	SEQ ID NO: 36

Mix 2

Design	Concentration	Sequence	
2178-11-08	.5 uM	5'-CCCTGCAACGCGAGTGCTGA	SEQ ID NO: 33
2178-11-09	.5 uM	5'-GTGGACCACGTACCTAGAGTGCGG	SEQ ID NO: 34
23-204	.25 uM	5'-1TCTXAAGCCGGTTTTCCGGCTGAGACGTCCGTGGCCT-hex	SEQ ID NO: 35
23-210	.25 uM	5'-1TCTXAAGCCGGTTTTCCGGCTGAGACTCCGCGTCCGT-hex	SEQ ID NO: 21
2206-31-01	.0054 uM	5'-ACGGACGCGGAGAGGCTGGTGTACGAC-hex	SEQ ID NO: 37

Mix 3

Design	Concentration	Sequence	
2178-11-08	.5 uM	5'-CCCTGCAACGCGAGTGCTGA	SEQ ID NO: 33
2178-11-09	.5 uM	5'-GTGGACCACGTACCTAGAGTGCGG	SEQ ID NO: 34
23-204	.25 uM	5'-1TCTXAAGCCGGTTTTCCGGCTGAGACGTCCGTGGCCT-hex	SEQ ID NO: 35
23-210	.25 uM	5'-1TCTXAAGCCGGTTTTCCGGCTGAGACTCCGCGTCCGT-hex	SEQ ID NO: 21
2178-40-01	.67 uM	5'-AGGCCACGGACGAGGCTGGTGTACGAC-hex	SEQ ID NO: 36
2206-31-01	.0054 uM	5'-ACGGACGCGGAGAGGCTGGTGTACGAC-hex	SEQ ID NO: 37

X = Z28 Phosphoramidite
1 = 6 FAM Amidite
hex = hexanediol

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For each reaction, 13.33 ng/μL Cleavase® VIII, .033 Units/μL native Taq DNA polymerase (Promega), and 2 Units/μL M-MLV RT (Promega) were combined in 5 μl of Cleavase enzyme dilution buffer (0.02 M Tris pH 8.0, 0.05 M KCl, 0.5% Tween 20, 0.5% Nonidet P40, 50% glycerol, and 100 μg/mL BSA in water). For each reaction, approximately 10 to 1,000,000 copies of a target RNA transcript containing the sequence 5'-CCCUGCAACGCGAGUGCUGAGGCUGGUGUACGACCCAUCGCUCGCCCGCUACCGCGACGUCCUGCCGCACUCUAGGUACGUGGUCCAC-3' (SEQ ID NO:9) were diluted into a final volume of 15 μl of a solution containing 20 ng/μl of tRNA. Each 50 μl reaction contained 5 μl of the enzyme mixture, 15 μl of the target RNA mixture and the indicated combination of oligonucleotides in buffer containing 10 mM MOPS, 7.5 . mM MgCl2, and 25 μM dNTPs. The reactions were incubated as follows:

Step	General Function	Temp C	Time	Cycles
1	Reverse Transcription	42	30 min.	1
2	Heat Kill RT	95	25 min.	
3	Denature	95	30 sec.	23
4	Anneal/Extend	72	1 min.	
5	Heat Kill DNA Polymerase	99	10 min.	1
6	Invader Assay Reaction	63	30 min.	1
7	Cool Down	10	Hold	1

The fluorescent signal produced in each reaction vessel was quantitated on a Tecan Genios FL fluorescence plate reader. The results are shown in Figure 9. 1X Probe = Mix 1, 1/125X Probe = Mix 2, InRange = Mix 3

Results

As shown in Figure 9, in this experiment the assay comprising multiple probe concentrations was able to accurately detect an RNA target over 5 orders of magnitude of target concentration in a homogeneous RT-PCR-Invader assay.

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

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CLAIMS

WE CLAIM:

1. A method for detecting a target nucleic acid, comprising:

a) amplifying a target nucleic acid at two different levels of amplification to generate amplification products;

b) hybridizing said amplification products to a first probe and second probe, wherein said first probe hybridizes to said amplification products at a different frequency than said second probe.

2. The method of Claim 1, wherein said second probe is present at a 10-fold lower concentration than said first probe.

3. The method of Claim 1, wherein said at least two probes bind to the same sequences.

4. A method for detecting a target nucleic acid in a plurality of samples over a broad dynamic range, comprising: exposing a first sample having less than 10^3 copies of target nucleic acid and a second sample having greater than 10^5 copies of target nucleic acid to a set of reagents under conditions such that said target nucleic acid in said first and second samples is detected, wherein method comprises exposing each of said first and second samples to a first probe and a second probe, wherein said second probe hybridizes to said target nucleic acids at a different frequency than said first probe.

5. The method of Claim 4, wherein said target nucleic acid in said first and second samples is quantitated.

6. The method of Claim 4, wherein said second probe is present at a 10-fold lower concentration than said first probe.

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7. The method of Claim 4, wherein said target nucleic acids are treated under two or more different amplification conditions prior to detection.

8. The method of Claim 4, wherein said method is conducted without any amplification of the target nucleic acid.

9. The method of Claim 4, wherein said target nucleic acid is amplified by linear amplification, by exponential amplification, or by linear and exponential amplification.

10. The method of Claim 4, wherein said target nucleic acid is amplified by two different levels of amplification.

11. A method for detecting a target nucleic acid, comprising: a) amplifying a target nucleic acid to generate amplification products; b) contacting said amplification products with first and second probes, wherein said second probe hybridizes to said amplification products at a different frequency that said first probe; c) cleaving said first and second probes; and d) detecting the cleavage of said first and second probes.

12. A kit comprising: a polymerase, a 5' nuclease, and two probes configured to hybridize to an analyte-specific region of a target nucleic acid, wherein the second probe hybridizes to said analyte-specific region at a different frequency than said first probe oligonucleotide, and wherein the first and second probes are configured to both directly or indirectly generate a detectable signal in the presence of the said target nucleic acid.

13. The kit of claim 12, wherein said first and second probes generate the same type of detectable signal.

14. The kit of claim 12, wherein said first and second probes comprise a label.

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15. The kit of claim 12, wherein said first and second probes each comprise a flap sequence that is complementary to a FRET cassette.

16. The kit of claim 15, wherein said flap of said first probe is identical to said flap of said second probe.

17. A method for detecting a target nucleic acid in a sample comprising;

a) contacting a sample suspected of containing a target nucleic acid with amplification reagents such that, if said target nucleic acid is present:

i) a first region of said target nucleic acid is either not amplified, or is amplified at a first level to generate a plurality of first product sequences; and

ii) a second region of said target nucleic acid is amplified at a second level to generate a plurality of second product sequences, wherein said second level of amplification is greater than said first level of amplification; and

b) incubating said sample with a plurality of first and second probe oligonucleotides, wherein:

i) said first and second probe oligonucleotides hybridize to said first region of said target nucleic acid, and said first product sequences if produced, at different frequencies, or

ii) said first and second probe oligonucleotides hybridize to said second product sequences at a different frequency; and

c) measuring hybridization of said first and second probe oligonucleotides thereby detecting said target nucleic acid in said sample.

18. The method of Claim 17, wherein said second product sequences are present at a level of at least 10-fold higher concentration after amplification than said target nucleic acid, or first product sequences if produced.

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19. The method of Claim 17, wherein said second product sequences are present at a level of at least 10,000-fold higher concentration after amplification than said target nucleic acid, or first product sequences if produced.

20. The method of Claim 17, wherein said second probe oligonucleotides are present in at least a 10-fold lower concentration than said first probe oligonucleotides.

21. The method of Claim 17, wherein said second probe oligonucleotides are present in at least a 100-fold lower concentration than said first probe oligonucleotides.

22. A method for detecting a target nucleic acid in a sample comprising;

a) contacting a sample suspected of containing a target nucleic acid with amplification reagents such that, if said target nucleic acid is present:

i) a first region of said target nucleic acid comprising a first probe hybridization site is either not amplified, or is amplified at a first level to generate plurality of first product sequences that comprise said first probe hybridization site; and

ii) a second region of said target nucleic acid is amplified at a second level to generate a plurality of second product sequences that comprise a second probe hybridization site, wherein said second level of amplification is greater than said first level of amplification; and

b) incubating said sample with a plurality of first and second probe oligonucleotides, wherein:

i) said first and second probe oligonucleotides occupy said first probe hybridization site on said first region of said target nucleic acid, and said first product sequences if produced, at different frequencies, or

ii) said first and second probe oligonucleotides occupy said second probe hybridization site on said second product sequences at a different frequency; and

c) measuring hybridization of said first and second probe oligonucleotides thereby detecting said target nucleic acid in said sample.

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23. The method of Claim 22, wherein said second product sequences are present at a level of at least 10-fold higher concentration after amplification than said target nucleic acid, or first product sequences if produced.

24. The method of Claim 22, wherein said second product sequences are present at a level of at least 10,000-fold higher concentration after amplification than said target nucleic acid, or first product sequences if produced.

25. The method of Claim 22, wherein said second probe oligonucleotides are present in at least a 10-fold lower concentration than said first probe oligonucleotides.

26. The method of Claim 22, wherein said second probe oligonucleotides are present in at least a 100-fold lower concentration than said first probe oligonucleotides.

27. The method of Claim 22, further comprising incubating said sample with a third probe oligonucleotide that occupies said first probe hybridization site on said first region of said target nucleic acid, and said first product sequences if produced, at a first frequency, and measuring the hybridization of said third probe oligonucleotide.

28. The method of Claim 27, further comprising incubating said sample with a fourth probe oligonucleotide that occupies said first probe hybridization site on said first region of said target nucleic acid, and said first product sequences if produced, at a second frequency, wherein said second frequency is different from said first frequency, and measuring the hybridization of said fourth probe oligonucleotide.

29. The method of Claim 27, further comprising incubating said sample with a third probe oligonucleotide that occupies said second probe hybridization site on said second product sequences at a first frequency, and measuring the hybridization of said third probe oligonucleotide.

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30. The method of Claim 29, further comprising incubating said sample with a fourth probe oligonucleotide that occupies said second probe hybridization site on said second product sequences at a second frequency, wherein said second frequency is different from said first frequency, and measuring the hybridization of said fourth probe oligonucleotide.

31. The method of claim 30, wherein said third probe oligonucleotides are present in at least a 10-fold lower concentration than said fourth probe oligonucleotides.

32. The method of Claim 22, wherein said first level of amplification is achieved by linear amplification, and wherein said second level is achieved is achieved with logarithmic amplification.

33. The method of Claim 22, wherein said first level of amplification is non-logarithmic and said second level of amplification is logarithmic.

34. The method of Claim 22, wherein said measuring detects the amount of the target nucleic acid in said sample.

35. The method of Claim 22, wherein said target nucleic acid is initially present in said sample in an amount between about 10^1 and about 10^8 molecules.

36. The method of Claim 22, wherein said target nucleic acid is initially present in said sample in an amount between about 10^1 and about 10^{10} molecules.

37. The method of Claim 22, wherein said method is conducted on two samples, wherein said target nucleic acid is initially present in one sample in an amount less than 10^3 and initially present in a second sample in an amount greater than 10^5.

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38. The method of Claim 22, wherein said method is conducted on two samples, wherein said target nucleic acid is initially present in one sample in an amount less than 10^1 and initially present in a second sample in an amount greater than 10^7.

39. The method of Claim 22, wherein said incubating and measuring steps are conducted in a single vessel.

40. The method of Claim 22, wherein at least one of said first and second probe oligonucleotides is unlabeled.

41. The method of Claim 22, wherein said first and second probe oligonucleotides are unlabeled.

42. The method of Claim 22, wherein said measuring hybridization of said first and said second probe oligonucleotides comprises performing a hybridization assay.

43. The method Claim 42, wherein said hybridization assay is real-time amplification.

44. The method of Claim 42, wherein said hybridization assay is TAQMAN.

45. The method of Claim 42, wherein said hybridization assay is selected from NASBA, TMA, and Microarrays.

46. The method of Claim 42, wherein said hybridization assay is an invasive cleavage assay.

47. The method of claim 46, wherein said invasive cleavage assay in an INVADER assay.

48. The method of Claim 22, wherein said target nucleic acid is micro-RNA.

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49. The method of Claim 22, where said first and second oligonucleotide probes have labels that are the same.

50. A method for genotyping a polymorphic locus in a target nucleic acid in a sample, comprising;

a) contacting a sample suspected of containing said target nucleic acid with amplification reagents such that, if said target nucleic acid is present, a region of said target nucleic acid containing said polymorphic locus is amplified to generate a plurality of amplified sequences, wherein said amplification is conducted until saturation;

b) incubating said a sample with a plurality of first probe oligonucleotides and a plurality of second probe oligonucleotides, wherein each of said first probe oligonucleotides comprises: i) a first analyte specific region configured for detecting a first allele at said polymorphic locus, and ii) a label capable of generating a detectable signal or a cleavable portion configured to cause a detectable signal to be generated, and wherein said second probe oligonucleotides comprise: i) a second analyte specific region configured for detecting a second allele at said polymorphic locus, ii) a label capable of generating a detectable signal or a cleavable portion configured to cause a detectable signal to be generated, wherein said plurality of second probe oligonucleotides are configured to occupy a probe hybridization site on said amplified sequences at a different frequency than said plurality of first probe oligonucleotides, and wherein the type of detectable signal from said first and second probe oligonucleotides is the same;

c) measuring the strength of said detectable signal generated, thereby determining the presence of said first allele, said second allele, or both said first and second alleles in said target nucleic acid.

51. The method of Claim 50, wherein said polymorphic locus is a single nucleotide polymorphism.

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52. The method of Claim 50, wherein said polymorphic locus is a repeat sequence.

53. A method for detecting a target nucleic acid in a sample, comprising;

a) incubating a sample suspected of containing a target nucleic acid with a plurality of first and second probe oligonucleotides, a plurality of upstream oligonucleotides, and a cleavage agent,

wherein each of said first probe oligonucleotides comprise: i) a first analyte specific region configured to hybridize to a probe hybridization site on said target nucleic acid, and ii) a first non-analyte specific region configured to not hybridize to said target nucleic acid, wherein said first non-analyte specific region is 5' of said first analyte specific region, and

wherein each of said second probe oligonucleotides comprises i) a second analyte specific region configured to hybridize to said probe hybridization site on said target nucleic acid, and ii) a second non-analyte specific region configured to not hybridize to said target nucleic acid, wherein said second non-analyte specific region is not identical to said first non-analyte specific region, and wherein said plurality of second probe oligonucleotides are configured to occupy a said probe hybridization site on said target nucleic acid at a different frequency than said plurality of first probe oligonucleotides;

wherein said incubating is under conditions such that invasive cleavage structures are formed resulting in the cleavage of both said first and second probe oligonucleotides by said cleavage agent to generate: i) first non-target cleavage products comprising said first non-analyte specific region, and ii) second non-target cleavage products comprising said second non-analyte specific region; and

b) measuring hybridization of said first and said second probe oligonucleotides over time by detecting a signal generated by said first and second non-target cleavage products, thereby detecting said target nucleic acid.

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54. The method of Claim 53, wherein said second probe oligonucleotides are present in at least a 10-fold lower concentration than said first probe oligonucleotides.

55. The method of Claim 53, wherein said signal generated by said first and second non-target cleavage products is the same.

56. The method of Claim 53, wherein said signal generated by said first and second non-target cleavage products is different.

57. The method of Claim 53, wherein said upstream oligonucleotides comprise a 5' portion and a 3' portion, wherein said 5' portion is configured to hybridize to a region contiguous with said probe hybridization site on said target nucleic acid, and wherein said 3' portion is configured to not hybridize to said target nucleic acid.

58. The method of Claim 53, further comprising incubating said sample with first and second labeled sequences, wherein said first labeled sequence is configured to generate a first detectable signal when hybridized to said first non-target cleavage product, and wherein said second labeled sequence is configured to generate a second detectable signal when hybridized to said second non-target cleavage product.

59. The method of Claim 58, wherein said first and second detectable signals are the same.

60. The method of Claim 58, wherein said first and second labeled sequences comprise FRET cassettes.

61. The method of Claim 53, wherein said plurality of upstream oligonucleotides are generated in said sample.

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62. A method for detecting a target nucleic acid in a sample, comprising;

a) incubating a sample suspected of containing a target nucleic acid with a plurality of first and second probe oligonucleotides, a plurality of first upstream oligonucleotides, a plurality of second upstream oligonucleotides, and a cleavage agent,

wherein each of said first probe oligonucleotides comprise: i) a first analyte specific region configured to hybridize to a first probe hybridization site on said target nucleic acid, and ii) a first non-analyte specific region configured to not hybridize to said target nucleic acid, wherein said first non-analyte specific region is 5' of said first analyte specific region, and

wherein each of said second probe oligonucleotides comprises i) a second analyte specific region configured to hybridize to a second probe hybridization site on said target nucleic acid, wherein said second probe hybridization site is not the same as said first probe hybridization site, and ii) a second non-analyte specific region configured to not hybridize to said target nucleic acid, wherein said second non-analyte specific region is not identical to said first non-analyte specific region, and wherein said plurality of second probe oligonucleotides are present in at least 5-fold lower concentration than said first probe oligonucleotides;

wherein said incubating is under conditions such that invasive cleavage structures are formed resulting in the cleavage of both said first and second probe oligonucleotides by said cleavage agent to generate: i) first non-target cleavage products comprising said first non-analyte specific region, and ii) second non-target cleavage products comprising said second non-analyte specific region; and

b) measuring hybridization of said first and said second probe oligonucleotides over time by detecting a signal generated by said first and second non-target cleavage products, thereby detecting said target nucleic acid.

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63. A kit for quantitation of target nucleic acids in a sample, comprising:

a) a plurality of first probe oligonucleotides, wherein each of said first probe oligonucleotides comprises a first analyte specific region;

b) a plurality of second probe oligonucleotides, wherein each of said second probe oligonucleotides comprises a second analyte specific region, wherein said plurality of second probe oligonucleotides are configured to occupy a probe hybridization site on said target nucleic acids at a different frequency than said plurality of first probe oligonucleotides; and

c) reagents for performing an invasive cleavage assay using said pluralities of said first and second probe oligonucleotides.

64. The kit of Claim 63, wherein said first and second probe oligonucleotides are un-labeled.

65. The kit of claim 63, wherein said second probe oligonucleotides are present in at least a 5 fold lower concentration than said first probe oligonucleotides.

66. The kit of claim 63, wherein said second probe oligonucleotides are present in at least a 10 fold lower concentration than said first probe oligonucleotides.

67. A kit or composition comprising;

a) a plurality of first and second probe oligonucleotides, wherein said first probe oligonucleotides comprise a first 5' region and a first 3' region, and said second probe oligonucleotides comprises a second 5' region and a second 3' region, wherein both of said first and second probe oligonucleotides will form an invasive cleavage structure in the presence of the same upstream oligonucleotide and target sequence, and will both be cleaved by the same cleavage agent to form a first 5' region product and a second 5' region product, wherein said second 5' region product is not identical to said first 5' region product, and

b) first and second labeled sequences, wherein said first labeled sequence is configured to generate a first detectable signal when hybridized to

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said first 5' region product, and wherein said second labeled sequence is configured to generate a second detectable signal when hybridized to said second 5' region product.

68. The kit or composition of Claim 67, wherein said first 3' region and said second 3' region have the identical sequence.

69. The kit or composition of Claim 67, wherein said first 3' region and said second 3' region do not have identical sequences.

70. The kit or composition of Claim 67, wherein said second probe oligonucleotides are present in at least a 5 fold lower concentration than said first probe oligonucleotides.

71. The kit or composition of Claim 67, wherein said first and second probe oligonucleotides are un-labeled.

72. The kit or composition of Claim 67, further comprising a third probe oligonucleotide comprising a third 5' region and a third 3' region, wherein said third probe oligonucleotide will not form an invasive cleavage structure with said target and said upstream oligonucleotide that is cleavable by said cleavage agent.

73. A kit or composition comprising;

a) a plurality of first and second probe oligonucleotides, wherein said first probe oligonucleotides comprise a first 5' region and a first 3' region, and said second probe oligonucleotides comprises a second 5' region and a second 3' region, wherein both of said first and second probe oligonucleotides will form an invasive cleavage structure in the presence of the same upstream oligonucleotide and target sequence, and will both be cleaved by the same cleavage agent to form a first 5' region product and a second 5' region product, wherein said second 5'

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region product is identical to said first 5' region product, and wherein said first 3' region is not identical to said second 3' region, and

b) first labeled sequences, wherein said first labeled sequence is configured to generate a detectable signal when hybridized to said first or second 5' region product.

74. A kit comprising;

a) a first vessel comprising a plurality of first probe oligonucleotides and a plurality of second probe oligonucleotides, wherein the first and second probe oligonucleotides comprises an analyte specific region, wherein the plurality of second probe oligonucleotides are configured to occupy a probe hybridization site on a target nucleic acid at a different frequency than the plurality of first probe oligonucleotides;

b) a second vessel comprising a polymerase and a FEN enzyme, and

c) a third vessel comprising a buffer.

75. The kit of Claim 74, wherein said first and second oligonucleotides are unlabeled.

76. The kit of Claim 74, further comprising a control target sequence comprising said probe hybridization site.

77. The kit of Claim 74, wherein said first vessel further comprises primers for amplification.

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78. A method for detecting a target nucleic acid in a sample, comprising;

a) incubating a sample suspected of containing a target nucleic acid with a plurality of first probe oligonucleotides and a plurality of second probe oligonucleotides, wherein each of said first and said second probe oligonucleotides comprises an analyte specific region, wherein said plurality of second probe oligonucleotides are configured to occupy a probe hybridization site on said target nucleic acid with the same affinity as said plurality of first probe oligonucleotides, and wherein said plurality of second probe oligonucleotides are present in at least 5-fold lower concentration than said first probe oligonucleotides; and

b) measuring hybridization of said first and said second probe oligonucleotides over time, thereby detecting the amount of said target nucleic acid.

79. A method for detecting a target nucleic acid in a sample, comprising;

a) incubating a sample suspected of containing a target nucleic acid with a plurality of un-labeled first probe oligonucleotides and a plurality of un-labeled second probe oligonucleotides, wherein each of said first and said second probe oligonucleotides comprises an analyte specific region, wherein said plurality of second probe oligonucleotides are configured to occupy a probe hybridization site on said target nucleic acid at a different frequency than said plurality of first probe oligonucleotides;

b) measuring hybridization of said first and said second probe oligonucleotides over time, thereby detecting the amount of said target nucleic acid.

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80. A method for detecting an initial amount of a target nucleic acid in a sample without amplifying initial amount of said target nucleic acid, comprising;

a) incubating a sample initially containing 300 copies or less of a target nucleic acid with a plurality of first probe oligonucleotides and a plurality of second probe oligonucleotides, wherein each of said first and said second probe oligonucleotides comprises an analyte specific region, wherein said plurality of second probe oligonucleotides are configured to occupy a probe hybridization site on said target nucleic acid at a different frequency than said plurality of first probe oligonucleotides;

b) measuring hybridization of said first and said second probe oligonucleotides over time, thereby measuring the amount of said target nucleic acid, wherein said 300 copies or less of said target nucleic acid are not amplified prior to said measuring step.

81. The method of Claim 80, wherein said 300 copies or less is between 200 and 100 copies.

82. A method for detecting an amount of a target nucleic acid in a sample, comprising;

a) contacting a sample suspected of containing target nucleic acid with amplification reagents such that, if said target nucleic acid is present, a region of said target nucleic acid containing a probe hybridization site is amplified to generate a plurality of amplified sequences,

b) incubating said sample with a plurality of first probe oligonucleotides and a plurality of second probe oligonucleotides, wherein each of said first and said second probe oligonucleotides comprises an analyte specific region, wherein said plurality of second probe oligonucleotides are configured to occupy said a probe hybridization site on said amplified sequence at a different frequency than said plurality of first probe oligonucleotides;

c) measuring hybridization of said first and said second probe oligonucleotides over time, thereby measuring the amount of said target nucleic

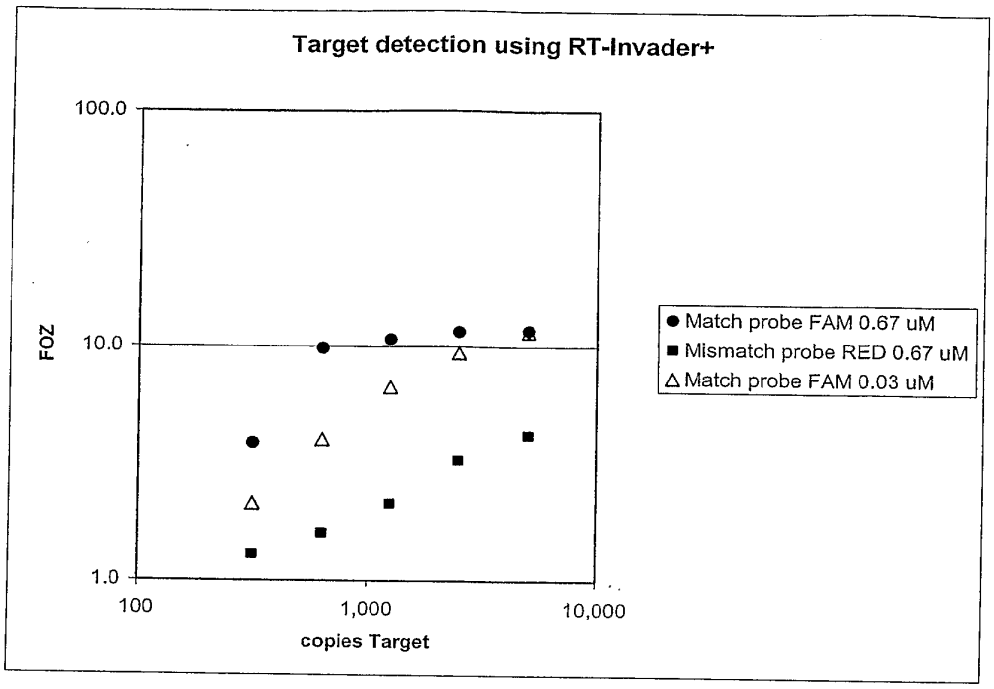
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acid, wherein said measuring is possible when said target nucleic acid is initially present in said sample in an amount between about 1 molecule and about 10^7 molecules.

5

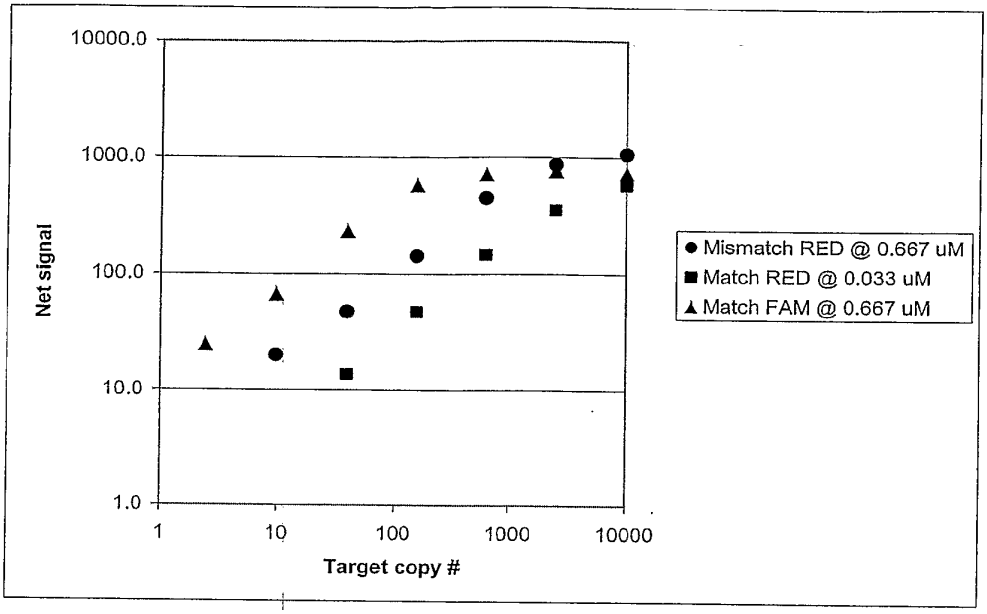
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Figure 1



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Figure 2



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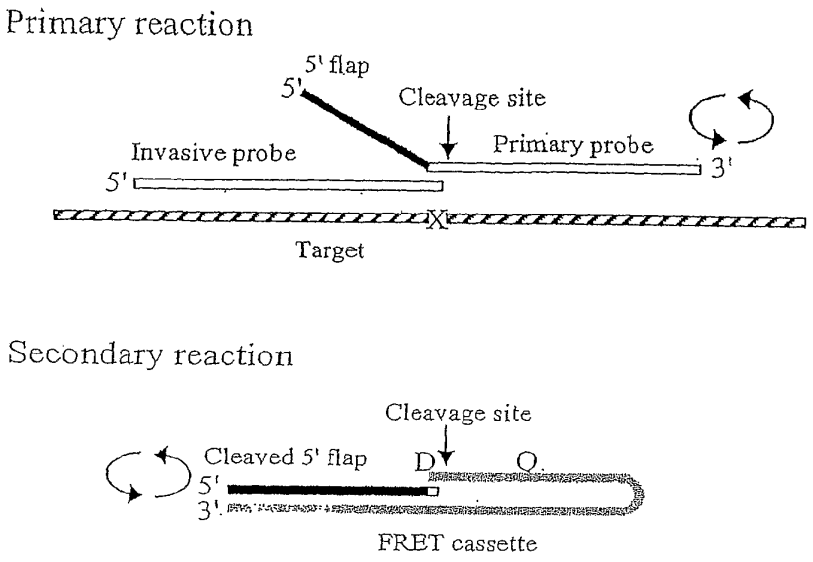
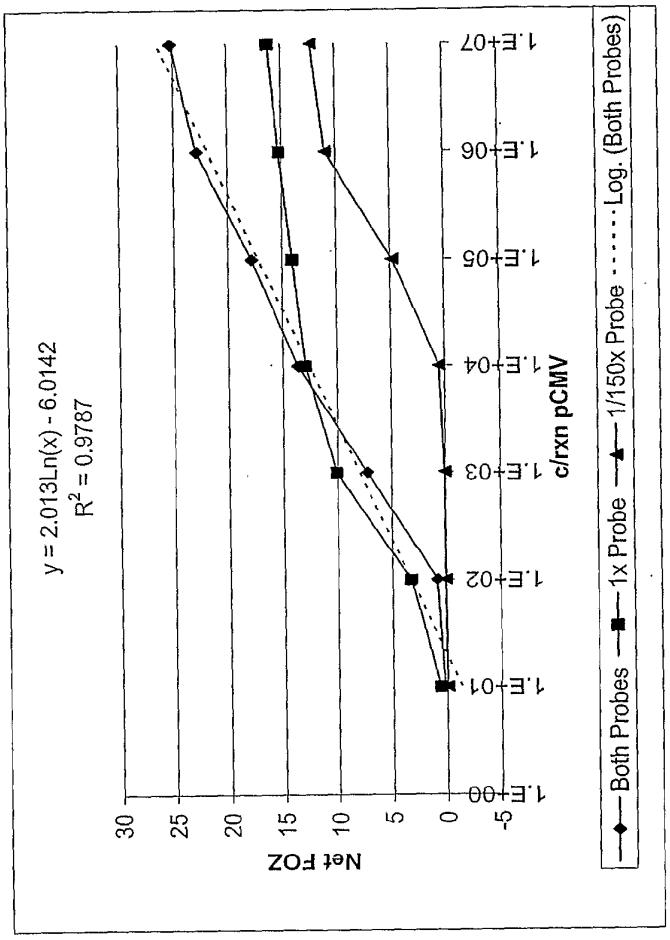


Figure 3

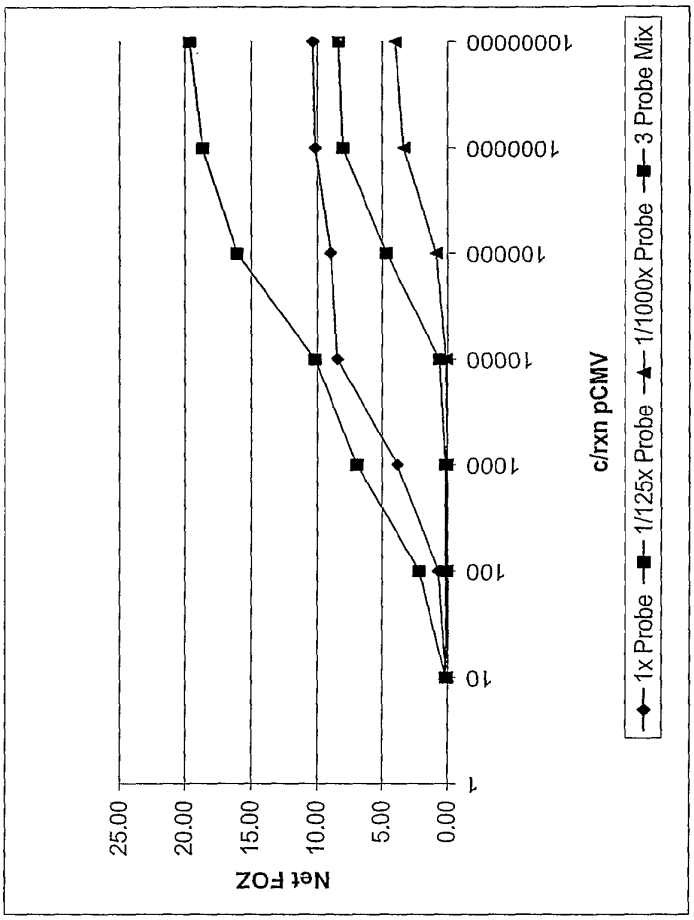
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Figure 4



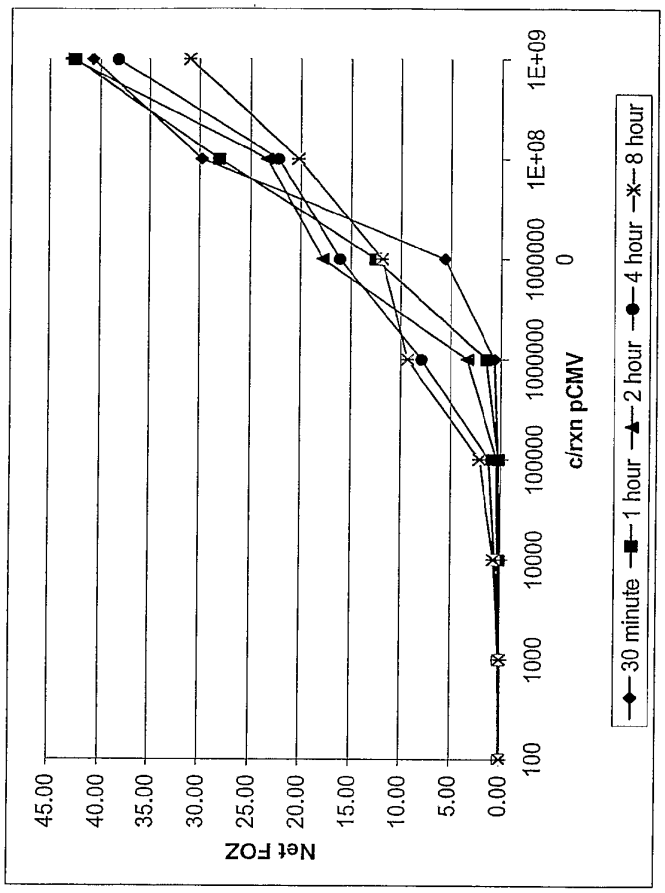
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Figure 5



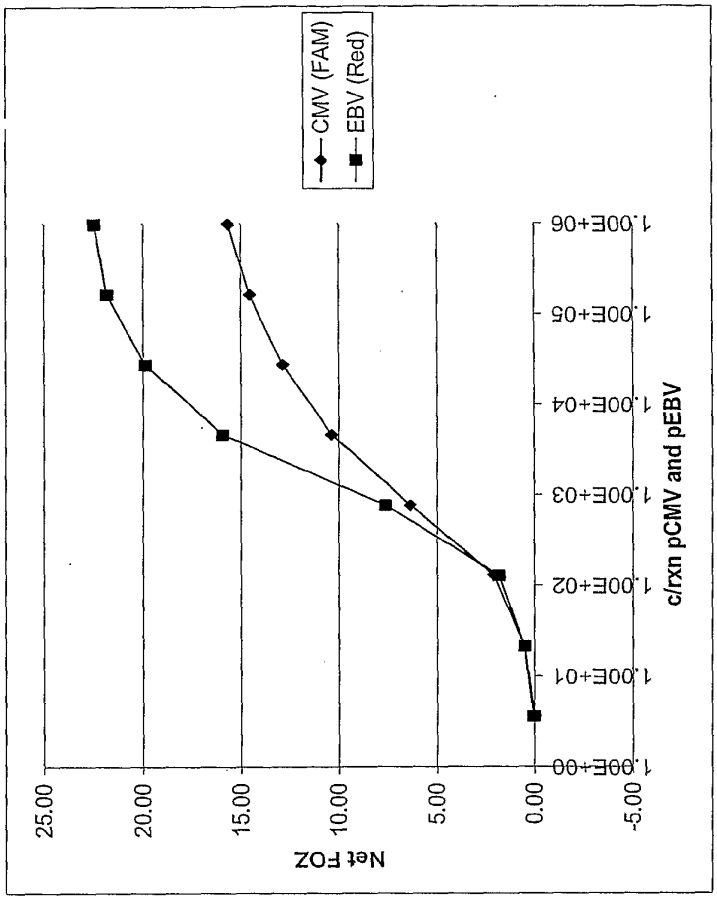
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Figure 6



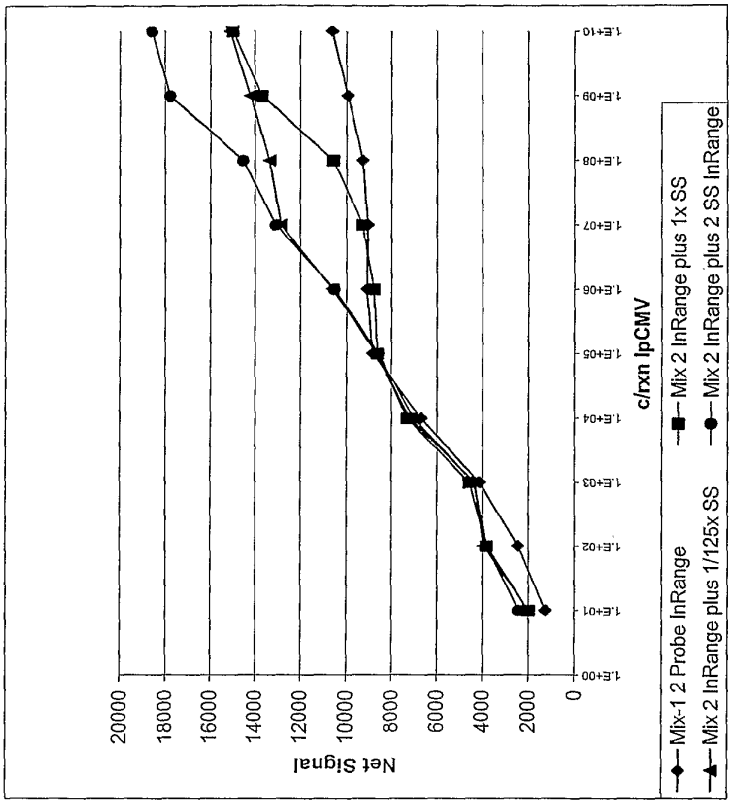
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Figure 7



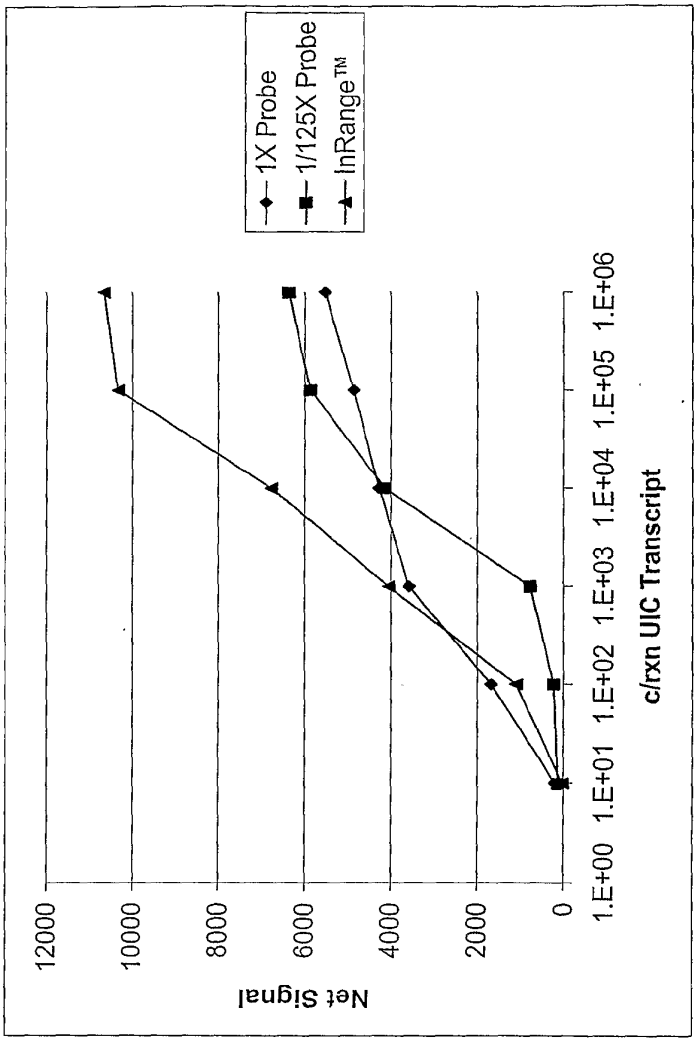
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Figure 8



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Figure 9



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(71) Applicant: **CALIFORNIA INSTITUTE OF TECHNOLOGY** [US/US]; 1200 E. California Boulevard, MC 6-32, Pasadena, CA 91125 (US).

(72) Inventors: **RAJAGOPAL, Aditya**; 221 W. Sparkleberry Avenue, Orange, CA 92865 (US). **KARTALOV, Emil P.**; 725 Second Street, Pacific Grove, CA 93950 (US).

(74) Agent: **JOHNSON, Lee** et al.; Wilson Sonsini Goodrich & Rosati, 650 Page Mill Road, Palo Alto, CA 94304-1050 (US).

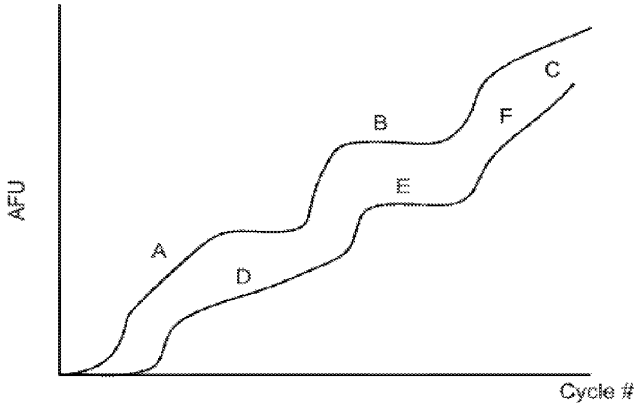
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FIG. 6



(57) Abstract: The present invention relates to methods of nucleic acid analyte detection by PCR. In particular, methods and kits for the detection of a plurality of nucleic acid analytes and the generation of kinetic signatures are provided. Further provided are methods and kits of nested PCR and PCR using limiting primers.

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NUCLEIC ACID REACTIONS AND RELATED METHODS AND COMPOSITIONS

CROSS-REFERENCE

This application claims the benefit of US Provisional Application No. 62/351,411, filed June 17, 2016, which is incorporated by reference herein in its entirety.

BACKGROUND

[0001] The polymerase chain reaction is a technique that is used for increasing the quantity of a nucleic acid target. By thermal cycling a reaction cocktail of short primer sequences, free nucleotides and DNA polymerase, a template strand can be copied. For the polymerase chain reaction (PCR), temperature plateaus are often required: a high temperature for denaturation of the amplicon of interest, a low temperature for the binding of primers to the template, and an intermediate temperature for the synthesis of a complement strand.

[0002] Specificity of the binding between the primers and the template allows for selective amplification of intended targets. For instance, a typical quantitative polymerase chain reaction is run to "saturation," wherein the amplification reaction is run to the point at which at least one of the reactants such as primers, polymerase, probe, or free nucleotides is exhausted. When fluorescent reporters such as TaqMan probes, FRET probes, or intercalating dyes are used to interpret the extent of the DNA amplification, a fluorescence curve could be generated. The sigmoidal signature of a "saturation" fluorescence curve often occurs when the reporter probe concentrations are depleted at an ever-increasing rate.

[0003] However, this reaction (and by extension, its signature fluorescence) is limited by all reactant concentrations, including the PCR primers. As an example, when a single set of primers is used in equal concentrations, and when one primer is more efficiently extended than the other, the reaction becomes rate limited. This results in a linear, as compared to an exponential, amplification of DNA.

SUMMARY OF THE INVENTION

[0004] Described herein, in some embodiments, are methods of detecting the presence or absence of a target nucleic acid analyte in a sample, the methods comprising a) performing an amplification reaction on the sample; b) measuring a signal generated during the amplification reaction and generating a kinetic signature from the measured signal; and c) comparing the generated kinetic signature to a reference signature and determining whether

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the generated kinetic signature corresponds with the reference signature, thereby detecting the presence or absence of the target nucleic acid analyte in the sample. In some embodiments, kinetic signature comprises a signal selected from an electromagnetic signal, a wavelength, and a fluorescence emission signal. In some embodiments, the kinetic signature is plotted as a curve. In some embodiments, the curve comprises at least one of an exponential region, a linear region, and a plateau region. In some embodiments, the kinetic signature comprises recorded values. In some embodiments, the values are recorded in a table. In some embodiments, the amplification reaction is a polymerase chain reaction (PCR). In some embodiments, the reference signature corresponds with a kinetic signature generated by amplification of the target nucleic acid analyte under one or more members of a set of constrained amplification parameters. In some embodiments, the set of constrained amplification parameters comprises any combination of: (i) a range of forward and/or reverse primer concentrations; (ii) a range of polymerase concentrations; (iii) polymerase type; (iv) a range of nucleotide concentrations; (v) chromophore concentration; and (vi) chromophore type; (vii) a number of thermocycles; (viii) rate of thermocycling; (ix) one or more thermocycle stage temperatures; and (x) one or more thermocycle stage time lengths; (xi) a range of target-specific molecular probes; (xii) primers associated with one or more target-specific probes; and (xiii) a concentration of one or more enzyme cofactors. In some embodiments, the amplification reaction is carried out within one or more of the constrained amplification parameters.

[0005] Described herein, in some embodiments, are methods of detecting the presence or absence of a target nucleic acid analyte in a sample, the method comprising a) performing an amplification reaction on the sample under a set of constrained amplification parameters, the set comprising any combination of one or more of (i) a range of forward and/or reverse primer concentrations; (ii) a range of polymerase concentrations; (iii) polymerase type; (iv) a range of nucleotide concentrations; (v) chromophore concentration; (vi) chromophore type; (vii) number of thermocycles; (viii) rate of thermocycling; (ix) one or more thermocycle stage temperatures; and (x) one or more thermocycler stage time lengths; (xi) a range of target-specific molecular probes; and (xii) primers associated with one or more target-specific probes; and (xiii) a concentration of one or more enzyme cofactors; b) measuring a signal generated during the amplification reaction and generating a kinetic signature from the measured signal; c) comparing the generated kinetic signature to a reference signature, the reference signature corresponding with amplification of the target nucleic acid analyte under

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the set of constrained amplification parameters; and d) determining whether the generated kinetic signature corresponds with the reference signature, thereby detecting the presence or absence of the target nucleic acid analyte in the sample. In some embodiments, the amplification reaction is carried out within one or more of the constrained amplification parameters. In some embodiments, the one or more enzyme cofactors is selected from the group consisting of magnesium, potassium, and sodium. In some embodiments, the nucleic acid analyte is from a source selected from the group consisting of an animal, a plant, a bacterium, a parasite, and a virus. In some embodiments, the animal is a human. In some embodiments, the nucleic acid analyte is selected from the group consisting of human immunodeficiency virus, herpes simplex virus, human papilloma virus, Plasmodium, Mycobacterium, dengue virus, hepatitis virus, and influenza virus. In some embodiments, the nucleic acid analyte is selected from the group consisting of human immunodeficiency virus polyprotease, human immunodeficiency virus p17, human papilloma virus E6, and human papilloma virus E7. In some embodiments, the at least one of an exponential region, a linear region, and a plateau region occurs over at least 4 cycles. In some embodiments, the at least one of an exponential region, a linear region, and a plateau region occurs over at least 8 seconds.

[0006] Described herein, in some embodiments, are methods of detecting the presence or absence of each of a plurality of target nucleic acid analytes in a sample, the method comprising a) adding a primer set, the primer set comprising at least two primer pairs, to the sample, at least one of the primer pairs comprising a limiting primer, and each primer pair defining a region of one of the target nucleic acid analytes to be amplified; performing an amplification reaction on the sample; b) measuring a signal generated during the amplification reaction; and c) making a determination as to whether each of the plurality of target nucleic acid analytes is present based on the measured signal. In some embodiments, step c) further comprises a step of generating a kinetic signature from the measured signal, and step d) further comprises a step of comparing the kinetic signature to a reference signature, the reference signature corresponding with a kinetic signature generated by an amplification of one or more of the plurality of target nucleic acid analytes with the primer set, thereby making the determination. In some embodiments, the primer set consists of three primers. In some embodiments, the three primers consist of one forward primer and two reverse primers or two forward primers and one reverse primer. In some embodiments, the primer set comprises two or more nested primers. In some embodiments, the signal is

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generated by exciting a molecule that binds an amplification product. In some embodiments, the molecule is a hybridization probe. In some embodiments, (A) the primer set comprises first and second primer pairs, the first primer pair having a first annealing temperature and the second primer pair having a second annealing temperature which is lower than the first annealing temperature, and the first primer pair defining a region of one of the target nucleic acid analytes, the second primer pair defining a region of another of the nucleic acid analytes; and (B) the amplification reaction comprises thermocycling of a set of temperature cycles comprising at least one temperature cycle having a minimum temperature which is both (i) not greater than the first annealing temperature, and (ii) greater than the second annealing temperature, the reaction further comprising at least one other temperature cycle having a minimum temperature which is not greater than the second annealing temperature. In some embodiments, the amplification reaction comprises at least one temperature cycle which has a minimum temperature not greater than the first annealing temperature and greater than the second annealing temperature, followed by at least one temperature cycle which has a minimum temperature not greater than the second annealing temperature. In some embodiments, the amplification reaction comprises at least one temperature cycle which has a minimum temperature not greater than the second annealing temperature, followed by at least one temperature cycle which has a minimum temperature which is both (x) not greater than the first annealing temperature and (y) greater than the second annealing temperature. In some embodiments, step c) further comprises a step of generating a kinetic signature from the measured signal, and step d) further comprises a step of comparing the kinetic signature to a reference signature, the reference signature corresponding to a signal generated when one or more of the plurality of target nucleic acid analytes is amplified with the primer set by a thermocycling reaction comprising the set of temperature cycles, thereby making the determination. In some embodiments, the signal comprises a kinetic signature. In some embodiments, the kinetic signature comprises a signal selected from an electromagnetic signal, a wavelength, and a fluorescence emission signal. In some embodiments, the kinetic signature is plotted as a curve. In some embodiments, the curve comprises at least one of an exponential region, a linear region, or a plateau region. In some embodiments, the kinetic signature comprises recorded values. In some embodiments, the values are recorded in a table. In some embodiments, the limiting primer is present at a concentration sufficiently lower than the concentration of a non-limiting primer to generate a characteristic signal. In some embodiments, the limiting primer is present at a concentration sufficiently lower that

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the concentration of a non-limiting primer to generate a characteristic kinetic signature. In some embodiments, the kinetic signature comprises a signal selected from an electromagnetic signal, a wavelength, and a fluorescence emission signal. In some embodiments, the kinetic signature is plotted as a curve. In some embodiments, the curve comprises at least one of an exponential region, a linear region, or a plateau region. In some embodiments, the kinetic signature comprises recorded values. In some embodiments, the values are recorded in a table. In some embodiments, the first and/or second annealing temperatures are determined by the limiting primer. In some embodiments, one or more primer of the primer pairs is linked to an antibody.

[0007] Described herein, in some embodiments, are methods of detecting the presence or absence of each of a plurality of target nucleic acid analytes in a sample, the methods comprising a) adding a primer set, the primer set comprising at least two primers, to the sample, at least one of the primer pairs comprising a limiting primer, and each primer pair defining a region of one of the target nucleic acid analytes to be amplified; b) performing a thermocycling amplification reaction on the sample, the reaction comprising a set of temperature cycles comprising at least one temperature cycle having a minimum temperature which is both (i) not greater than the first annealing temperature, and (ii) greater than the second annealing temperature, the reaction further comprising at least one other temperature cycle having a minimum temperature which is not greater than the second annealing temperature; c) measuring a signal generated during the amplification reaction; and d) making a determination as to whether each of the plurality of nucleic acid analytes is present based on the measured signal. In some embodiments, one or more primer of the primer pairs is linked to an antibody.

[0008] Described herein, in some embodiments, are methods of detecting the presence or absence of two or more of a plurality of target nucleic acid analytes in a sample, the methods comprising a) adding a primer set comprising first and second primer pairs to the sample, the first primer pair having a first annealing temperature and the second primer pair having a second annealing temperature which is lower than the first annealing temperature, and the first primer pair defining a region of one of the target nucleic acid analytes, the second primer pair defining a region of another of the nucleic acid analytes; b) performing a thermocycling amplification reaction on the sample, the reaction comprising a set of temperature cycles comprising at least one temperature cycle having a minimum temperature which is both (i) not greater than the first annealing temperature, and (ii) greater than the second annealing

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temperature, the reaction further comprising at least one other temperature cycle having a minimum temperature which is not greater than the second annealing temperature; c) measuring a signal generated during the amplification reaction; and d) making a determination as to the presence or absence of the two or more of the nucleic acid analytes based on the generated signal. In some embodiments, step c) further comprises a step of generating a kinetic signature from the measured signal, and step d) further comprises a step of comparing the kinetic signature to a reference signature, the reference signature corresponding with a signal generated when one or more of the plurality of target nucleic acid analytes is amplified with the primer set by a thermocycling reaction comprising the set of temperature cycles, thereby making the determination. In some embodiments, the first and second primer pairs comprise three primers. In some embodiments, the three primers comprise one forward primer and two reverse primers. In some embodiments, the three primers comprise two forward primers and one reverse primer. In some embodiments, the primer set comprises at least one pair of nested primers. In some embodiments, the amplification reaction comprises at least one temperature cycle which has a minimum temperature not greater than the first annealing temperature and greater than the second annealing temperature, followed by at least one temperature cycle which has a minimum temperature not greater than the second annealing temperature. In some embodiments, the amplification reaction comprises at least one temperature cycle which has a minimum temperature not greater than the second annealing temperature, followed by at least one temperature cycle which has a minimum temperature which is both (x) not greater than the first annealing temperature and (y) greater than the second annealing temperature. In some embodiments, at least one of the primer pairs comprises a limiting primer. In some embodiments, the limiting primer is present at a concentration of less than 1 μM. In some embodiments, a non-limiting primer is present at an initial concentration of at least two times greater than the limiting primer. In some embodiments, the limiting primer has a concentration-adjusted melting temperature that is equal to or greater than a non-limiting primer. In some embodiments, the signal is generated by a molecule that binds an amplification product. In some embodiments, the molecule is a hybridization probe. In some embodiments, the minimum temperature corresponds to the annealing temperature of first and second primer pairs. In some embodiments, the method comprises detecting the presence of n target nucleic acid analytes using n primer pairs, each primer pair having a different annealing temperature from that of the other primer pairs, and the set of temperature cycles

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comprising a set of minimum temperatures that are interspersed between the different annealing temperatures.. In some embodiments, n is 3. In some embodiments, n is 4. In some embodiments, n is 5. In some embodiments, n is 6. In some embodiments, n is 7. In some embodiments, one or more primer of a primer pair is linked to an antibody.

[0009] Described herein, in some embodiments, are methods of detecting the presence or absence of each of a plurality of target nucleic acid analytes in a sample, the methods comprising a) adding a primer set comprising first and second primer pairs to the sample, the first primer pair having a first annealing temperature and the second primer pair having a second annealing temperature which is lower than the first annealing temperature, and the first primer pair defining a region of one of the target nucleic acid analytes, the second primer pair defining a region of another of the nucleic acid analytes; b) performing a thermocycling amplification reaction on the sample, the reaction comprising a set of temperature cycles comprising at least one temperature cycle having a minimum temperature which is both (i) not greater than the first annealing temperature, and (ii) greater than the second annealing temperature, the reaction further comprising at least one other temperature cycle having a minimum temperature which is not greater than the second annealing temperature; c) measuring a signal generated during the amplification reaction, thereby generating a kinetic signature from the measured signal; and d) comparing the kinetic signature to a reference signature, the reference signature corresponding with a signal generated when one or more of the plurality of target nucleic acid analytes is amplified in a sample comprising the primer set by a thermocycling reaction comprising at least one temperature cycle having a minimum temperature which is both (x) not greater than the first annealing temperature, and (y) greater than the second annealing temperature, the reaction further comprising at least one other temperature cycle having a minimum temperature which is not greater than the second annealing temperature, thereby making a determination as to the presence or absence of the at least two nucleic acid analytes.

[0010] In an aspect the present disclosure provides a method for detecting a nucleic acid analyte. The method comprises performing a nested asymmetric amplification reaction on a sample, measuring a signal generated during the nested asymmetric amplification reaction, and determining whether the analyte is present based on the signal. The nested asymmetric amplification reaction of the disclosure may comprise at least three nested primers, wherein at least one pair of primers of the at least three nested primer comprises a limiting primer and an excess primer. In some cases, the at least three nested primers bind to a single analyte. In

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some examples, the at least three nested primers may comprise at least two pairs of nested primers. In some further examples, the at least three nested primers comprise at least three pairs of nested primers. In some cases, the at least three nested primers may comprise at least two pairs of nested primers that bind to the same analyte. In some cases, the at least three pairs of nested primers may bind to the same analyte. The limiting primer may be present at an initial concentration of less than 1μM. The excess primer may be present at an initial concentration of at least 2 times greater than the limiting primer. The limiting primer may have a concentration-adjusted melting temperature that is equal to or greater than the excess primer.

[0011] The method of the disclosure may detect the presence or absence of a plurality of the analytes in a sample. In some cases, the methods of the disclosure may detect the presence or absence of at least 1 analyte in a sample. The method of the disclosure may further comprise performing a melting curve analysis.

[0012] The signal may be signal that is an electromagnetic signal. For example, the signal may be a fluorescence emission signal. The signal may comprise intensity and a frequency. An analyte may be encoded by a signal intensity at a particular wavelength. An analyte may be also be encoded by a plurality of signal intensities at a plurality of wavelengths. The plurality of wavelengths may comprise at least three wavelengths. In some examples, the analyte may be encoded by at least one additional value selected from the group consisting of a value from the signal and a value from an additional signal. The least one additional value may be selected from the group consisting of a Förster resonance energy transfer (FRET) emission intensity, a FRET emission wavelength, an electrochemical signal, a chemiluminescence wavelength, a chemiluminescence intensity, a fluorescence bleaching rate, and a chemiluminescence bleaching rate. In some cases the at least one additional value is Förster resonance energy transfer (FRET) emission intensity.

[0013] The signal may be generated by a molecule that binds an amplification product. For example the signal may be generated by a hybridization probe. The hybridization probe may comprise a fluorophore and a quencher. The amplification reaction may comprise a plurality of hybridization probes that are specific for different analytes and comprise an identical fluorophore. In some examples, the number of the hybridization probes may be greater than the number of the analytes.

[0014] The signal generated by a single analyte may be plotted as a function of a variable selected from the group consisting of time and cycle number, thereby generating a plot. The

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plot may comprise at least two of the same regions selected from the group consisting of an exponential region, a linear region, and a plateau region. In some cases, both of the exponential regions occur over at least 4 cycles. In some cases, both of the exponential regions occur over at least 8 seconds. In some cases, both of the linear regions occur over at least 4 cycles. In some cases, both of the linear regions occur over at least 8 seconds. In some cases, both of the plateau regions occur over at least 4 cycles. In some cases, both of the plateau regions occur over at least 8 seconds.

[0015] The analyte may be selected from the group consisting of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). The methods of the disclosure may further comprise reverse transcribing an RNA. The analyte may be present at less than 1,000 copies. The sample may further comprise a reference analyte. The analyte may be from a source selected from the group consisting of an animal, a plant, a bacteria, a fungus, a parasite, and a virus. The animal may be human. In some cases, the analyte is from a source selected from the group consisting of human immunodeficiency virus, herpes simplex virus, human papilloma virus, Plasmodium, Mycobacterium, dengue virus, hepatitis virus, and influenza virus. In some other examples, the analyte is selected from the group consisting of human immunodeficiency virus polyprotease, human immunodeficiency virus p17, human papilloma virus E6, and human papilloma virus E7. The analyte may also be a nucleic acid variant. The analyte may be a nucleic acid encoding a 16S RNA sequence. The sample may be selected from the group consisting of a clinical sample, a food sample, an environmental sample, a pharmaceutical sample, and a sample from a consumer product.

[0016] The method of the disclosure may further comprise transforming a signal intensity into a frequency domain, thereby generating a transformed signal. The transforming may be performed by a method selected from the group consisting of a Fourier transform, a fast Fourier transform, a discrete Fourier transform, a discrete-time Fourier transform, and a wavelet transform. The method may further comprise comparing the transformed signal to a reference signal. The comparing may be used to determine if the analyte is present or absent.

[0017] The methods of the disclosure may be useful for unambiguously determining whether one or more analyte is present or absent. The methods of the disclosure may also comprise determining the initial amount of the analyte in the sample. The method of disclosure may further comprising making a clinical decision based on whether the analyte is present or absent. The clinical decision may comprise selection of an agent for administration to a subject in need thereof. The agent may be a therapeutic agent. The therapeutic agent may be

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selected from the group consisting of an antibiotic and an antiviral. The method the disclosure may further comprise transmitting information concerning the analyte through a computer network. The transmitting may be to a physician. In some examples, at least one step of the methods of the disclosure may be performed using instructions on a non-volatile computer readable medium. The instructions may be located on a remote server or on a thermal cycler.

[0018] The disclosure also provides a method of determining an amount of nucleic acid analyte. The method comprises performing an amplification reaction on a sample, measuring a signal generated during the amplification reaction, transforming the signal into a frequency domain thereby generating a transformed signal, comparing a value from the transformed signal to a value of reference signal, and determining the amount of the analyte based on the comparing. The transforming may be performed by a method selected from the group consisting of a Fourier Transform, a fast Fourier transform, a discrete Fourier transform, a discrete-time Fourier transform, and a wavelet transform. Transforming may be performed by an eigenbasis decomposition. The eigenbasis decomposition may be of a PCR curve. The eigenbasis decomposition may be of a characteristic PCR curve. The eigenbasis decomposition may comprise one or more eigenfunctions. The one or more eigenfunctions may comprise time-shifted, scaled sigmoidal, exponential, and/or linear curves corresponding to one or more primer sets. Transforming may be performed by an eigenbasis decomposition of a characteristic PCR curve comprising eigenfunctions consisting of time-shifted, scaled sigmoidal, exponential, and linear curves corresponding to specific primer-sets within a nested PCR amplification. The methods of the disclosure detect the presence or absence of a plurality of analytes. In some examples, each of the analytes produces a unique transformed signal amongst the analytes. The comparing may comprise comparing an intensity value at a particular frequency and time. In some cases, at least about 5 intensity values are generated during each cycle of the amplification reaction. The reference signal may be in a database of reference signals. The database may comprise at least 20 reference signals. The database may comprise at least 30 reference signals. The database may comprise at least 50 reference signals. The database may comprise less than 500 reference signals. The database may comprise less than 200 reference signals. The database may comprise less than 100 reference signals.

[0019] The disclosure also provides a kit for detecting a nucleic acid analyte. The kits of the disclosure comprise at least three nested primers. At least one pair of primers may comprise a

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limiting primer and an excess primer. The kit may also comprise a real time detection agent. The kit may detect the presence or absence of a plurality of analytes. At least one of the primers may be lyophilized. In some cases each of the primers is lyophilized. The kit may further comprise reagents for the detection of an amplified analyte. The reagents for the detection of an amplified analyte may comprise a fluorophore. The reagents for the detection of an amplified analyte may comprise a hybridization probe.

[0020] In some examples the at least three nested primers in the kit may bind to a single analyte. In some cases the at least three nested primers of the kit comprise at least two pairs of nested primers. The at least three nested primers may comprise at least three pairs of nested primers. In some further examples, the at least three nested primers comprise at least two pairs of nested primers that bind to the same analyte. In some cases the at least three nested primers comprise at least three pairs of nested primers that bind to the same analyte. The limiting primer may be present at an initial concentration of less than 1 μM. The excess primer may be present at an initial concentration of at least 2 times greater than the limiting primer. The limiting primer may have a concentration-adjusted melting temperature that is equal to or greater than the excess primer.

INCORPORATION BY REFERENCE

[0021] All publications and patent applications mentioned in this specification are herein incorporated in their entireties by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

[0022] The novel features of the disclosure are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present disclosure will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the disclosure are utilized, and the accompanying drawings of which:

[0023] Fig. 1 shows a diagram illustrating the method of nested PCR.

[0024] Fig. 2 shows a nested PCR with three primer pairs (A, B, C). The targeted area of interest is shown in the box.

[0025] Fig. 3 shows the fluorescence curve of a saturated, quantitative polymerase chain reaction.

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[0026] Fig. 4 shows the fluorescence curve of a biased-primer, quantitative polymerase chain reaction.

[0027] Fig. 5 shows the fluorescence curve of a nested, quantitative polymerase chain reaction.

[0028] Fig. 6 shows the characteristic fluorescence curves (curve ABC and curve DEF) of two polymerase chain reactions.

[0029] Fig. 7 shows the characteristic fluorescence curves (curve ABC and curve DEF) of two polymerase chain reactions transformed into the frequency domain.

[0030] Fig. 8 shows a block diagram illustrating a first example architecture of a computer system that can be used in connection with example embodiments of the present disclosure.

[0031] Fig. 9 shows a diagram illustrating a computer network that can be used in connection with example embodiments of the present disclosure.

[0032] Fig. 10 shows a block diagram illustrating a second example architecture of a computer system that can be used in connection with example embodiments of the present disclosure.

DETAILED DESCRIPTION OF THE INVENTION

I. General Overview

[0033] The present disclosure relates to methods, systems, compositions and kits relating to the detection and/or quantification of one or more analytes in a sample. The methods may be particularly useful for detection of a plurality of analytes in multiplexed reactions. Multiplexed reactions can permit performance of parallel reactions on the same sample, use of the same chamber to perform multiple reactions, and the ability to extract rich information from a sample in a fast and efficient manner. The methods disclosed herein may permit performance of multiplexed assays in a single solution. These methods can be used in place of or in addition to spectrally resolved fluorescence or chemiluminescence (e.g., PCR, ELISA), spatially resolved signals (e.g., microarrays, gel electrophoresis), temporally resolved signals (e.g., capillary electrophoresis), or combinations thereof (e.g., Sanger sequencing).

[0034] Specificity of the binding between PCR primers and template allows for selective amplification of intended targets. For instance, a typical quantitative polymerase chain reaction is run to "saturation," wherein the amplification reaction is run to the point at which at least one of the reactants such as primers, polymerase, probe, or free nucleotides is

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exhausted. When fluorescent reporters such as TaqMan probes, FRET probes, or intercalating dyes are used to interpret the extent of the DNA amplification, a fluorescence curve could be generated. The sigmoidal signature of a "saturation" fluorescence curve often occurs when the reporter probe concentrations are depleted at an ever-increasing rate.

[0035] However, this reaction (and by extension, its signature fluorescence) is limited by all reactant concentrations, including the PCR primers. As an example, when a single set of primers is used in equal concentrations, and when one primer is more efficiently extended than the other, the reaction becomes rate limited. This results in a linear, as compared to an exponential, amplification of DNA.

[0036] Generally, the methods of the disclosure comprise performing an amplification reaction, measuring a signal generated during the amplification reaction and determining the amount of the analytes present in the sample based on the measured signal. The method may additionally include transforming the measured signal to a second domain and comparing the transformed signal to a reference signal. The amount of the analyte present in the sample or the initial amount of analyte present in the sample may also be obtained based on the comparison of the transformed signal to the measured signal.

[0037] A signal may be anything capable of measurement. For example, a signal may be a fluorescence signal. The amplification reaction for the methods of the disclosure may comprise semi-nested or nested PCR reactions. In these PCR reactions, at least two sets of primers are used in at least two successive reactions. In the first PCR reaction, one pair of primers is used. The products from the first PCR reactions are then used as template in a second PCR reaction, using one (semi-nested PCR reactions) or two different primers (nested PCR reactions). Figure 1 shows a diagram illustrating the method of nested PCR and Figure 2 shows a nested PCR with three primer pairs (A, B, C).

[0038] The amplification reactions of the disclosure are performed so as to generate characteristic signal curves that may be indicative of the presence and/or absence of one or more analytes. The characteristic curves may also be used to measure the amount of one or more analytes present in a sample. Such characteristic curves may be generated by varying (i) the number of primer pairs per target analyte, (ii) the primer sequences used for target analytes, and/or (iii) the ratio of the forward and the reverse primers in each primer pair.

[0039] Typically the amplification reactions of the disclosure are run such that either the forward or the reverse primer in each primer pair is rate limited. This may be done by adding each primer set such that there are different ratios of reverse and forward primers. For

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example, asymmetric PCR reactions, asymmetric nested PCR reactions, or asymmetric semi-nested PCR reactions may be used as amplification reactions in the methods of the disclosure. By choosing suitable ratios of the forward and the reverse primers, the amplification reactions of the disclosure may be tailored to generate curves that exhibit a characteristic linear amplification of the analytes. While this linear amplification may be non-ideal for efficient amplification of the target analytes, it may allow for discrimination of targets. Furthermore, tuning of primer concentration and the number of primers for a particular nested reaction allows for the engineering of a particular fluorescent signature for a given target. The fluorescent signal generated by an analyte may be plotted as a function of a variable selected from the group consisting of time and cycle number, thereby generating a plot or a characteristic curve. Preferably, the fluorescence signal is provided in AFU and is plotted against the number of cycles.

[0040] The characteristic curves generated by the methods of the disclosure may comprise one or more baseline regions, one or more exponential regions, one or more linear regions, and/or one or more plateau regions. In some cases the curves comprise at least two of the same regions selected from the group consisting of a baseline region, an exponential region, a linear region, and a plateau region. For example, the curves comprise one or more of at least two baseline regions, at least two exponential regions, at least two linear regions and/or at least two plateau regions. The amplification reactions of the disclosure may be conducted such that each target analyte in a sample generates a characteristic curve, which may indicate the presence or absence of the particular target analyte. A curve can be characterized by one or more of: (i) the number of baseline regions in a curve, (ii) the number of linear regions in a curve, (iii) the number of exponential regions in a curve, (iv) the number of plateau regions in a curve, (v) the slope of the one or more linear regions in a curve, (vi) the duration/length of one or more plateau regions in a curve, (vii) the duration/length of one or more linear regions in a curve, (viii) the duration/length of one or more exponential regions in a curve, (ix) the duration/length of one or more baseline regions in a curve, (x) the duration between two consecutive baseline regions in a curve, (xi) the duration between two consecutive exponential regions in a curve, (xii) the duration between two consecutive plateau regions in a curve, and/or (xiii) the duration between two consecutive linear regions in a curve. In some cases a curve is characterized by one or more of the preceding properties along with one or more additional properties not listed here. In some cases a curve is only characterized by one or more properties not listed here.

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[0041] Once the characteristic curves are generated, these curves may be analyzed by curve fitting algorithms. These algorithms may determine the number of baseline regions in a curve, the number of linear regions in a curve, the number of exponential regions in a curve, the number of plateau regions in a curve, the slope of the one or more linear regions in a curve, the duration/length of one or more plateau regions in the curve, the duration/length of one or more linear regions in the curve, the duration/length of one or more exponential regions in the curve, the duration/length of one or more baseline regions in the curve, the duration between two consecutive baseline regions in the curve, the duration between two consecutive exponential regions in the curve, the duration between two consecutive plateau regions in the curve, and/or the duration between two consecutive linear regions in the curve. Furthermore, if these curves are in superposition (e.g. more than one target is in a single, multiplexed reaction), each target can be uniquely identified if the curves are in the linear dynamic range of the fluorometer. For example, Figure 6 shows two characteristic curves that may be generated from a sample comprising two target analytes by the methods of the disclosure.

[0042] The methods presented in this disclosure may be useful in a variety of applications. For example, the methods may be used to generate diagnostic data wherein these methods are employed to detect the presence or absence of one or more analytes that may be indicative of a disease or a condition. The methods are carried out such that each analyte, indicative of a disease, generates a characteristic curve. Exemplary diseases that may be diagnosed by the methods of the disclosure include cancer, autoimmune disease, cardio respiratory disease, liver disease, digestive disease, and so on.

[0043] In some cases, the methods may be used to detect infection with one or more pathogens. The methods provided herein may also be used to make a diagnosis and to make a clinical decision based on that diagnosis. For example, whether a particular target analyte is present or absent in a sample from an individual may assist in selection of an agent for administration into the subject. The agent is a therapeutic agent for example, antibiotic or antiviral agent. In some cases, the methods of the disclosure may indicate the presence of a bacterial polynucleotide in a sample taken from a subject, this may lead to the treatment of the subject by administering an antibiotic to the subject. In other cases, the methods of the disclosure may indicate the presence of a viral polynucleotide in a sample taken from a subject, this may lead to a clinical decision of administering an antiviral agent to the subject.

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[0044] The methods may further be used to monitor the progress of a disease in an individual or to monitor the efficiency of a treatment method. Such methods include monitoring samples from the individual at regular time intervals and studying the changes in the characteristic curves generated by the corresponding one or more disease indicative analytes.

[0045] In other examples, the methods of the disclosure may also be used to identify genetic markers in an individual or a population of individuals. The genetic marker may be a molecular genetic marker, for example PCR based markers. Non-limiting examples of techniques that may be used for analysis of genetic markers by the methods of the disclosure include RAPD (random amplification of polymorphic DNA), SSRs (simple sequence repeats (SSR or microsatellite, or short tandem repeats)), SCAR (sequence characterized amplified region), AFLP (amplified fragment length polymorphism (AFLP)), SNP (single nucleotide polymorphism), CAPS (cleaved amplified polymorphic sequence), etc.).

II. Definitions

[0046] As used in this specification and the appended claims, the singular forms "a", "an", and "the" include plural references unless the context clearly dictates otherwise. Thus, for example, references to "the method" includes one or more methods, and/or steps of the type described herein which will become apparent to those persons skilled in the art upon reading this disclosure and so forth.

[0047] "About" as used herein when referring to a measurable value such as an amount, a temporal duration, and the like, is meant to encompass variations of ±20% or ±10%, or ±5%, or even ±1% from the specified value, as such variations are appropriate for the disclosed compositions or to perform the disclosed methods.

[0048] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which this invention belongs.

[0049] The term "annealing temperature" as used herein refers to the maximum temperature at which a nucleic acid anneals to a target nucleic acid under given conditions sufficient for extension of the primer to proceed. When used to refer to the annealing temperature of a primer pair, this term refers to the maximum temperature, under given conditions, at which each member of a primer pair anneals to its corresponding sequence of a target template or analyte to be amplified, sufficient for extension of the primer to proceed. Where, as is usual, each member of a pair binds to a separate sequence, on an opposite strand, of a target double-stranded nucleic acid, the "annealing temperature" is the temperature at which both members of the pair is bound to its complementary sequence. Where one member of the pair binds at a

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lower temperature than the other member, the annealing temperature of the pair will be that lower temperature. Thus, the term "annealing temperature" refers to the annealing temperature of the primer with a lower annealing temperature in a primer pair. The "annealing temperature" of a primer or primer pair may be below the melting temperature of the primer or primer pair. The "annealing temperature" may be lower than the "melting temperature" by any number of degrees Celsius, including, but not limited to 0.5°C, 1.0°C, 1.5°C, 2.0°C, 2.5°C, 3.0°C, 3.5°C, 4.0°C, 4.5°C, 5.0°C, 5.5°C, 6.0°C, 6.5°C, 7.0°C, 7.5°C, 8.0°C, 8.5°C, 9.0°C, 9.5°C, 10.0°C. In some embodiments, annealing temperatures recited herein are concentration-adjusted annealing temperatures.

[0050] The term "melting temperature" as used herein refers to the temperature at which half of a DNA or nucleic acid duplex becomes single-stranded. The term "primer melting temperature" refers to the temperature at which half of the primers associated with a nucleic acid analyte will dissociate.

[0051] As used herein, the term analyte encompasses a nucleic acid, a portion of a nucleic acid, an open reading frame, an exonic region, or an intronic region. The analyte may be present on a chromosome, a genome, a plasmid, or a cDNA. An analyte can be an overlapping nucleic acid sequence on the same strand of a chromosome or genome, for example.

[0052] As used herein, the term "temperature cycle" refers to the temperatures between which a sample is varied during a thermocycling amplification reaction. For example, in a standard thermocycling reaction, the sample might be repeatedly varied between an annealing temperature and a melting temperature. As used herein, each "temperature cycle" would comprise both the annealing and extension temperatures, as well as one or more temperature adjustment steps. As used herein, the term "n" refers to any positive integer.

III. Curve Signatures

[0053] Typically, the methods of the disclosure comprise plotting the fluorescent signal generated by an analyte as a function of a variable selected from the group consisting of time and cycle number, thereby generating a plot or a characteristic curve. Preferably, the fluorescence signal is provided in AFU and is plotted against cycle number. Often the AFU value may be normalized to a fixed proportional scale, for example to the scale of 0 to 100.

[0054] A typical fluorescent curve generated for a target analyte by the methods of the disclosure may comprise one or more baseline regions, plateau regions, linear regions and exponential regions. In some cases the curve comprises at least two of the same regions

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selected from the group consisting of a baseline region, an exponential region, a linear region, and a plateau region. As mentioned herein, a curve can be characterized by any one or more of the following properties: (i) the number of baseline regions in the curve, (ii) the number of linear regions in the curve, (iii) the number of exponential regions in the curve, (iv) the number of plateau regions in the curve, (v) the slope of the one or more linear regions in the curve, (vi) the duration/length of one or more plateau regions in the curve, (vii) the duration/length of one or more linear regions in the curve, (viii) the duration/length of one or more exponential region in the curve, (ix) the duration/length of one or more baseline regions in the curve, (x) the duration between two consecutive baseline regions in the curve, (xi) the duration between two consecutive exponential regions in the curve, (xii) the duration between two consecutive plateau regions in the curve, and/or (xiii) the duration between two consecutive linear regions in the curve. In some cases a curve is characterized by one or more of the preceding property along with one or more additional properties not listed here. In some cases a curve is characterized only by one or more properties not listed here.

[0055] In some cases the characteristic curves may differ in any one of the above listed properties. For example, the curves may differ in number of baseline regions; or the curves may differ in number of plateau regions; or the curves may differ in the number of exponential regions; or the curves may differ in the number of linear regions; or the curves may differ in slope of the one or more linear regions; or the curves may differ in duration/length of one or more plateau regions; or the curves may differ in duration/length of one or more linear regions; or the curves may differ in duration/length of one or more exponential regions; or the curves may differ in the duration of one or more baseline regions; or the curves may only differ in the duration between any two consecutive baseline regions of the curves; or the curves may differ in the duration between any two consecutive exponential regions of the curve; or the curves may differ in the duration between any two consecutive plateau regions of a curve; or the curves may only differ in the duration between any two consecutive linear regions of a curve. For example, a curve with one linear region may indicate the presence of first target analyte in a sample while another curve with two linear regions may indicate the presence of a second target analyte in a sample. In another example, a curve with a positive slope of the linear region may indicate the presence of first target analyte in a sample while another curve with a negative slope in the linear region may indicate the presence of a second target analyte in a sample.

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[0056] In some cases the curves will differ in any two of the above listed properties. For example the curves may differ in number of plateau regions and the number of linear regions; or the curves may differ by the slope of one or more linear regions and the number of plateau regions; or the curves differ in the length/duration of one or more linear regions and the length/duration of one or more plateau region.

[0057] In other cases the curves will differ in any 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 of the above listed properties. In some cases the curves will differ in all 13 of these properties. In some cases the curves will differ in a combination of these properties plus one or more additional properties not listed here.

[0058] The number of baseline regions, plateaus, exponential regions, and the number of linear regions in a curve, indicative of a particular target analyte, may be varied by varying the number of nested primers used for a target analyte. If a single primer pair is used for a target analyte, the resulting curve will typically have a single baseline region, a single plateau, a single exponential region and a single linear region. As the number of nested primer pairs is increased to 2, 3, 4, etc. the number of baseline regions, plateaus, exponential regions, and the number of linear regions may increase correspondingly.

[0059] The characteristic curves may comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more baseline regions. For example the curve may comprise 15, 20, 25 or even more baseline regions. The characteristic curves may comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more plateau regions. For example the curve may comprise 15, 20, 25 or even more plateau regions. The characteristic curves may comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more exponential regions. For example the curve may comprise 15, 20, 25 or even more exponential regions. The characteristic curves may comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more linear regions. For example the curve may comprise 15, 20, 25 or even more linear regions.

[0060] In some case the characteristic curves will comprise equal number of baseline regions, plateau regions, exponential regions, and linear regions. For example, a characteristic curve may comprise 1 baseline region, 1 exponential region, 1 linear region and 1 plateau region. Similarly a characteristic curve may comprise 2 baseline regions, 2 exponential regions, 2 linear regions and 2 plateau regions. Alternatively characteristic curve may comprise 3 baseline regions, 3 exponential region, 3 linear regions and 3 plateau regions. A characteristic curve may also comprise 4, 5, 6, 7, 8, 9, 10 or more of each baseline regions, exponential regions, linear regions and plateau regions. For example the curve may comprise 15, 20, 25

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or even more of each baseline regions, exponential regions, linear regions and plateau regions.

[0061] In some cases the characteristic curves will comprise different numbers of baseline regions, plateau regions, exponential regions, and linear regions. In some cases the characteristic curves will comprise equal number of baseline regions, plateau regions, and exponential regions but a different number of linear regions. In some cases the characteristic curves will comprise equal number of baseline regions, exponential regions, and linear regions but a different number of plateau regions. In some cases the characteristic curves will comprise equal number of baseline regions, plateau regions and linear regions but a different number of exponential regions. In some cases the characteristic curves will comprise equal numbers of plateau regions, exponential regions, and linear regions, but a different number of baseline regions.

[0062] The curves may vary in the length or the duration of one or more plateau regions. The length/duration of a plateau region in a curve can for example be at least 2 cycles, for example at least 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, or 100 cycles. In some cases, the length or the duration of a plateau region will be at most 100 cycles, for example at most 50, 45, 40, 35, 30, 25, 20, 15, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 cycle. In some cases the length/duration of a plateau region in a curve is calculated in the unit of time. In such cases, the length/duration of a plateau region in a curve can for example be at least 30 seconds, for example at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100 minutes. In some cases, the length or the duration of a plateau region will be at most 100 minutes, for example at most 95, 90, 85, 80, 75, 70, 65, 60, 55, 50, 45, 40, 35, 30, 25, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 minute. Further multiple plateau regions of a curve may all have same or different length/duration.

[0063] Similarly, the curves may also vary in the length or duration of the linear regions. The length/duration of a linear region in a curve can for example be at least 2 cycles, for example at least 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, or 100 cycles. In some cases, the length or the duration of a linear region will be at most 100 cycles, for example at most 50, 45, 40, 35, 30, 25, 20, 15, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 cycle. In some cases the length/duration of a linear region in a curve is calculated in the unit of time. In such cases, the length/duration of a linear region in a curve can for example be at least 30 seconds, for example at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 40, 45, 50, 55, 60, 65, 70,

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75, 80, 85, 90, 95 or 100 minutes. In some cases, the length or the duration of a linear region will be at most 100 minutes, for example at most 95, 90, 85, 80, 75, 70, 65, 60, 55, 50, 45, 40, 35, 30, 25, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 minute. Multiple linear regions of a curve may all have same or different length/duration.

[0064] The curves may vary in the length or the duration of one or more baseline regions. The length/duration of a baseline region in a curve can for example be at least 2 cycles, for example at least 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, or 100 cycles. In some cases, the length or the duration of a baseline region will be at most 100 cycles, for example at most 50, 45, 40, 35, 30, 25, 20, 15, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 cycle. In some cases the length/duration of a baseline region in a curve is calculated in the unit of time. In such cases, the length/duration of a baseline region in a curve can for example be at least 30 seconds, for example at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100 minutes. In some cases, the length or the duration of a baseline region will be at most 100 minutes, for example at most 95, 90, 85, 80, 75, 70, 65, 60, 55, 50, 45, 40, 35, 30, 25, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 minute. Further multiple baseline regions of a curve may all have same or different length/duration.

[0065] The curves may vary in the length or the duration of one or more exponential regions. The length/duration of an exponential region in a curve can for example be at least 2 cycles, for example at least 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, or 100 cycles. In some cases, the length or the duration of an exponential region will be at most 100 cycles, for example at most 50, 45, 40, 35, 30, 25, 20, 15, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 cycle. In some cases the length/duration of an exponential region in a curve is calculated in the unit of time. In such cases, the length/duration of an exponential region in a curve can for example be at least 30 seconds, for example at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100 minutes. In some cases, the length or the duration of an exponential region will be at most 100 minutes, for example at most 95, 90, 85, 80, 75, 70, 65, 60, 55, 50, 45, 40, 35, 30, 25, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 minute. Further multiple exponential regions of a curve may all have same or different length/duration.

[0066] The slope (or gradient) of a linear region in a curve may be varied by varying the ratio of the corresponding forward and reverse primers. The slope of one or more linear regions of the curves may have any value. Different linear regions of a curve may have same or

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different values. In some case some of the multiple linear regions a curve have same values. In other cases multiple linear regions will all have different values. In some case a few of the multiple linear regions will have same value while the other will have different values. The slope may be steep (for example, a steep incline or steep decline) or shallow (for example, a shallow incline or a shallow decline). The value of the slope of the linear regions of the curves may be either positive or negative. In some cases the slope is positive with a numerical value in the range of 0-0.01, 0-0.1, 0-1, 0-2, 1-3, 0-4, 0-5, 0-6, 0-7, 0-8, 0-9, 0-10, 0-20, 0-30, 0-40, 0-50, 0-60, 0-70, 0-80, 0-90, or 0-100. In some cases the slope is positive with a numerical value greater than 100, 200, 300, 400, 500, 600, 700, 800, 900 or 1000. In some cases a slope is negative with a numerical value in the range of 0-0.01, 0-0.1, 0-1, 0-2, 1-3, 0-4, 0-5, 0-6, 0-7, 0-8, 0-9, 0-10, 0-20, 0-30, 0-40, 0-50, 0-60, 0-70, 0-80, 0-90, or 0-100. In some cases the slope is negative with a numerical value greater than 100, 200, 300, 400, 500, 600, 700, 800, 900 or 1000.

[0067] The curves may vary in the duration between two consecutive exponential regions. In some cases two consecutive exponential regions of a curve are separated by at least 1 cycles, for example at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, or 10,000 cycles. In some cases, the occurrence of two consecutive exponential regions of a curve are separated by at most 10,000 cycles, for example at most 900, 800, 700, 600, 500, 400, 300, 200, 100, 90, 80, 70, 60, 50, 45, 40, 35, 30, 25, 20, 15, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 cycle. In some cases two consecutive exponential regions of a curve are separated by at least 1 cycles, for example at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100 minutes. In some cases two consecutive exponential regions of a curve are separated by at most 100 minutes, for example at most 95, 90, 85, 80, 75, 70, 65, 60, 55, 50, 45, 40, 35, 30, 25, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 minute. Further, if a curve comprises more than two exponential regions, each pair of consecutive exponential regions may be separated by a same or a different number of cycles.

[0068] The curves may vary in the duration between two consecutive baseline regions. In some cases two consecutive baseline regions of a curve are separated by at least 1 cycles, for example at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, or 10,000 cycles. In some cases, the occurrence of two consecutive baseline regions of a curve are separated by at most 10,000 cycles, for example at most 900, 800, 700, 600, 500, 400, 300, 200, 100, 90, 80, 70, 60, 50, 45, 40, 35,

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30, 25, 20, 15, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 cycle. In some cases two consecutive baseline regions of a curve are separated by at least 30 seconds, for example at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100 minutes. In some cases two consecutive baseline regions of a curve are separated by at most 100 minutes, for example at most 95, 90, 85, 80, 75, 70, 65, 60, 55, 50, 45, 40, 35, 30, 25, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 minute. Further, if a curve comprises more than two baseline regions, each pair of consecutive baseline regions may be separated by a same or a different number of cycles.

[0069] The curves may vary in the duration between two consecutive plateau regions. In some cases two consecutive plateau regions of a curve are separated by at least 1 cycles, for example at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, or 10,000 cycles. In some cases, the occurrence of two consecutive plateau regions of a curve are separated by at most 10,000 cycles, for example at most 900, 800, 700, 600, 500, 400, 300, 200, 100, 90, 80, 70, 60, 50, 45, 40, 35, 30, 25, 20, 15, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 cycle. In some cases two consecutive plateau regions of a curve are separated by at least 30 seconds, for example at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100 minutes. In some cases two consecutive plateau regions of a curve are separated by at most 100 minutes, for example at most 95, 90, 85, 80, 75, 70, 65, 60, 55, 50, 45, 40, 35, 30, 25, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 minute. Further, if a curve comprises more than two plateau regions, each pair of consecutive plateau regions may be separated by a same or a different number of cycles.

[0070] The curves may vary in the duration between two consecutive linear regions. In some cases two consecutive linear regions of a curve are separated by at least 1 cycles, for example at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, or 10,000 cycles. In some cases, the occurrence of two consecutive linear regions of a curve are separated by at most 10,000 cycles, for example at most 900, 800, 700, 600, 500, 400, 300, 200, 100, 90, 80, 70, 60, 50, 45, 40, 35, 30, 25, 20, 15, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 cycle. In some cases two consecutive linear regions of a curve are separated by at least 30 seconds, for example at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100 minutes. In some cases two consecutive linear regions of a curve are separated by at most 100 minutes, for example at most 95, 90, 85, 80, 75, 70, 65, 60, 55, 50, 45, 40, 35, 30, 25, 20, 19, 18, 17,

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16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 minute. Further, if a curve comprises more than two linear regions, each pair of consecutive linear regions may be separated by a same or a different number of cycles.

[0071] When the methods of the present disclosure are used with multiplexed PCR assay, the curves obtained may be a combination/addition of the component curves. This disclosure also provides methods of interpreting combined curves. For example, combination of linear regions of two or more curves will result in a linear region wherein the resulting slope is the sum of the individual slopes. This may allow two or more reactions to be coded and decoded by their slopes. Similarly, linear region of one curve and exponential region of another curve may combine to a signature that may neither be purely linear nor purely exponential. Such combination may be decoded by a mathematical fit to the experimental data to tell that both targets may be present and amplifying. Further, combining a plateau region of a curve with a linear region of another curve may produce a result that may bear uniquely decodable information about both, e.g. the slope may identify the latter, while the magnitude of the signal at the starting cycle of the combined region may help identify the plateau.

IV. Methods

[0072] The disclosure provides methods and materials for rapidly and simultaneously performing genotypic analysis for many genetic markers of an individual. The methods comprise obtaining a nucleic acid sample from the individual to be genotyped, subjecting the sample to the amplification methods disclosed herein to amplify segments containing the genetic markers to be examined, plotting the signal measured during the amplification reaction to generate characteristic curves, and determining the presence or absence of genetic markers in the sample based on the characteristic curves obtained. The amplification reactions may be conducted by contacting the sample with one or more nested primer pairs (for example, allele-specific primers). The number of nested primer pairs, the primer sequences and their concentrations may be adjusted so that each genetic marker may generate a characteristic signal curve. Accordingly, every genetic marker present in the nucleic acid sample of the individual will generate a characteristic curve. These methods may also be repeated for a large group of individuals to obtain a genetic profile of genetic markers in a population of individuals.

[0073] The disclosure also provides methods for the detection of one or more diseases in an individual. For example, the method may be used to detect specific mutations associated with the onset and progression of a disease. Non limiting examples of the diseases that may be

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detected by the methods of the disclosure include cancer, cystic fibrosis, heart diseases, and leukemia. Other diseases that may be detected by the methods of this disclosure include Achondroplasia, Adrenoleukodystrophy, X-Linked, Agammaglobulinemia, X-Linked, Alagille Syndrome, Alpha-Thalassemia X-Linked Mental Retardation Syndrome, Alzheimer Disease, Alzheimer Disease, Early-Onset Familial, Amyotrophic Lateral Sclerosis Overview, Androgen Insensitivity Syndrome, Angelman Syndrome, Ataxia Overview, Hereditary, Ataxia-Telangiectasia, Becker Muscular Dystrophy also The Dystrophinopathies), Beckwith-Wiedemann Syndrome, Beta-Thalassemia, Biotinidase Deficiency, Branchiootorenal Syndrome, BRCA1 and BRCA2 Hereditary Breast/Ovarian Cancer, Breast Cancer, CADASIL, Canavan Disease, Cancer, Charcot-Marie-Tooth Hereditary Neuropathy, Charcot-Marie-Tooth Neuropathy Type 1, Charcot-Marie-Tooth Neuropathy Type 2, Charcot-Marie-Tooth Neuropathy Type 4, Charcot-Marie-Tooth Neuropathy Type X, Cockayne Syndrome, Colon Cancer, Contractural Arachnodactyly, Congenital, Cranio synostosis Syndromes (FGFR-Related), Cystic Fibrosis, Cystinosis,, Deafness and Hereditary Hearing Loss, DRPLA (Dentatorubral-Pallidoluysian Atrophy), DiGeorge Syndrome (also 22ql 1 Deletion Syndrome), Dilated Cardiomyopathy, X-Linked, Down Syndrome (Trisomy 21), Duchenne Muscular Dystrophy (also The Dystrophinopathies), Dystonia, Early-Onset Primary (DYT1), Dystrophinopathies, The, Ehlers-Danlos Syndrome, Kyphoscoliotic Form, Ehlers-Danlos Syndrome, Vascular Type, Epidermolysis Bullosa Simplex, Exostoses, Hereditary Multiple, Facioscapulohumeral Muscular Dystrophy, Factor V Leiden Thrombophilia, Familial Adenomatous Polyposis (FAP), Familial Mediterranean Fever, Fragile X Syndrome, Friedreich Ataxia, Frontotemporal Dementia with Parkinsonism-17, Galactosemia, Gaucher Disease, Hemochromatosis, Hereditary, Hemophilia A, Hemophilia B, Hemorrhagic Telangiectasia, Hereditary 55, Hearing Loss and Deafness, Nonsyndromic, DFNA (Connexin 26), Hearing Loss and Deafness, Nonsyndromic, DFNB 1 (Connexin 26), Hereditary Spastic Paraplegia, Hermansky-Pudlak Syndrome, Hexosaminidase A Deficiency (also Tay-Sachs), Huntington Disease, Hypochondroplasia, Ichthyosis, Congenital, Autosomal Recessive, Incontinentia Pigmenti, Kennedy Disease (also Spinal and Bulbar Muscular Atrophy), Krabbe Disease, Leber Hereditary Optic Neuropathy, Lesch-Nyhan Syndrome Leukemias, Li-Fraumeni Syndrome, Limb-Girdle Muscular Dystrophy, Lipoprotein Lipase Deficiency, Familial, Lissencephaly, Marfan Syndrome, MELAS (Mitochondrial Encephalomyopathy, Lactic Acidosis, and, Stroke-Like Episodes), Monosomies, Multiple Endocrine Neoplasia Type 2, Multiple Exostoses, Hereditary

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Muscular Dystrophy, Congenital, Myotonic Dystrophy, Nephrogenic Diabetes Insipidus, Neurofibromatosis 1, Neurofibromatosis 2,, Neuropathy with Liability to Pressure Palsies, Hereditary, Niemann-Pick Disease Type C, Nijmegen Breakage Syndrome Norrie Disease, Oculocutaneous Albinism Type 1, Oculopharyngeal Muscular Dystrophy, Ovarian Cancer, Pallister-Hall Syndrome, Parkin Type of Juvenile Parkinson Disease, Pelizaeus-Merzbacher Disease, Pendred Syndrome, Peutz-Jeghers Syndrome Phenylalanine Hydroxylase Deficiency, Prader-Willi Syndrome, PROP 1-Related Combined Pituitary Hormone Deficiency (CPHD), Prostate Cancer, Retinitis Pigmentosa, Retinoblastoma, Rothmund-Thorns on Syndrome, Smith-Lemli-Opitz Syndrome, Spastic Paraplegia, Hereditary, Spinal and Bulbar Muscular Atrophy (also Kennedy Disease), Spinal Muscular Atrophy, Spinocerebellar Ataxia Type 1, Spinocerebellar Ataxia Type 2, Spinocerebellar Ataxia Type 3, Spinocerebellar Ataxia Type 6, Spinocerebellar Ataxia Type 7, Stickler Syndrome (Hereditary Arthroophthalmopathy), Tay-Sachs (also GM2 Gangliosidoses), Trisomies, Tuberous Sclerosis Complex, Usher Syndrome Type I, Usher Syndrome Type II, Velocardiofacial Syndrome (also 22ql 1 Deletion Syndrome), Von Hippel-Lindau Syndrome, Williams Syndrome, Wilson Disease, X-Linked Adreno leukodystrophy, X-Linked Agammaglobulinemia, X-Linked Dilated Cardiomyopathy (also The Dystrophinopathies), and X-Linked Hypotonic Facies Mental Retardation Syndrome. Methods, provided herein can also be applied to detect mutations during the course of therapeutic intervention in the treatment of disease that can decide the course and efficacy of a given treatment.

[0074] The methods may further be used to measure the efficacy of a treatment. In such cases samples from a patient will be monitored over a course of time for changes in the characteristic curves. Similarly, the methods described herein may be used to monitor the course of a disease.

[0075] The methods disclosed herein may be used with subjects at risk for developing a disease or disorder, subjects who may or may not have already been diagnosed with a disease or disorder and subjects undergoing treatment and/or therapies for a disease or disorder. The methods of the present disclosure can also be used to monitor or select a treatment regimen for a subject who has a disease or disorder.

[0076] The disclosure also provides methods for detecting the presence of one or more pathogens. In some cases, the methods may be used to determine the presence of one or more viruses, bacteria, or fungi. For example, the methods may be used for detection of DNA viruses, RNA viruses, retroviruses. In some cases the methods may be used to detect/quantify

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dsDNA viruses, ssDNA viruses, dsRNA viruses, (+)ssRNA viruses, (-)ssRNA viruses, ssRNA-RT viruses or dsDNA-RT viruses. The method may be used to detect/quantify gram-positive (for example, gram-positive cocci and gram-positive bacilli) and gram-negative (for example, gram-negative cocci and gram-negative bacilli). These methods may further include extracting cellular components of the target pathogens such as genomic DNA, ribosomal RNA, transfer RNA, or messenger RNA. In these methods, the presence of each pathogen in a sample will be indicated by the corresponding characteristic curve. Non limiting examples of pathogens that may be detected by the methods of the disclosure include HIV-1, hepatitis B and C, human papillomavirus, chlamydia trachomatis, neisseria gonorrhoeae, cytomegalovirus, influenza, salmonella typhi, west nile virus, adenovirus, actinomyces, and mycobacterium tuberculosis. The methods of the disclosure also enable the identification of GMO (genetic modified organisms).

[0077] The methods of the disclosure would also be useful in tracking the presence and/or progression of mutations in patients and related model organisms. In some cases the patients may be cancer patients and the methods of the disclosure may be used to obtain information about the presence or absence of drug-resistant mutations. Such information may be of clinical meaning and utility, for e.g. a KRAS mutation may indicate colorectal cancer, and a T790M mutation of EGFR may indicate lung cancer. Similarly, the methods of the invention may be used to track genetic information in a model organism or a population of model organisms as they are exposed to different experimental drugs in pharmaceutical research. In some cases the model organism may be a mouse or a rat.

[0078] The methods of the disclosure may also be employed in genetic retroactive studies of archival tissue cross-referenced with patient histories and outcomes and implemented medication regimens. Such studies may generate new insights both in epidemiology and in fundamental understanding of diseases, for example cancer. The methods disclosed herein further provide for a means of multiplexed expression analysis in biological samples, e.g. blood, saliva, cerebrospinal fluid, etc., which can have both fundamental and clinical utilities.

V. Amplification Reactions

A. Polymerase Chain Reaction (PCR)

[0079] Amplification refers to any process by which the copy number of a target sequence is increased. Methods for amplification of target polynucleotides are known in the art, and include without limitation, methods based on PCR. Conditions favorable to the amplification of target sequences by PCR are known in the art, can be optimized at a variety of steps in the

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process, and depend on characteristics of elements in the reaction, such as target type, target concentration, sequence length to be amplified, sequence of the target and/or one or more primers, primer length, primer concentration, polymerase used, reaction volume, ratio of one or more elements to one or more other elements, and others, some or all of which can be altered.

[0080] In general, PCR involves the steps of denaturation of the target to be amplified (if double stranded), hybridization of one or more primers to the target, and extension of the primers by a DNA polymerase, with the steps repeated (or "cycled") in order to amplify the target sequence. Steps in this process can be optimized for various outcomes, such as to enhance yield, decrease the formation of spurious products, and/or increase or decrease specificity of primer annealing.

[0081] Methods of optimization are well known in the art and include adjustments to the type or amount of elements in the amplification reaction and/or to the conditions of a given step in the process, such as temperature at a particular step, duration of a particular step, and/or number of cycles. In some embodiments, an amplification reaction comprises at least 5, 10, 15, 20, 25, 30, 35, 50, or more cycles. In some embodiments, an amplification reaction comprises no more than 5, 10, 15, 20, 25, 35, 50, or more cycles. Cycles can contain any number of steps, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more steps. Steps can comprise any temperature or gradient of temperatures, suitable for achieving the purpose of the given step, including but not limited to, 3' end extension (e.g. adaptor fill-in), primer annealing, primer extension, and strand denaturation. Steps can be of any duration, including but not limited to about, less than about, or more than about 1, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 70, 80, 90, 100, 120, 180, 240, 300, 360, 420, 480, 540, 600, or more seconds, including indefinitely until manually interrupted. Cycles of any number comprising different steps can be combined in any order. In some embodiments, different cycles comprising different steps are combined such that the total number of cycles in the combination is about, less that about, or more than about 5, 10, 15, 20, 25, 30, 35, 50, or more cycles. In some embodiments, amplification is performed following the fill-in reaction. Amplification can be performed before or after pooling of target polynucleotides from independent samples.

(i) Nested PCR reactions

[0082] In the methods of the present disclosure PCR reaction may be carried out in a traditional fashion where one set of specific primers is chosen for a signal nucleic acid target. However, strategies may also be used to increase PCR target yield. For example the

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amplification reaction may conducted under nested PCR conditions, wherein at least two pairs of primers are used, that is, a pair of outer primers and a pair of inner primers. Each primer pair comprises a forward primer and a reverse primer. Conventional nested PCR procedures utilize two sequential amplification processes. Specifically, the two sequential amplification processes include a first amplification process comprising at least one amplification step for amplifying an extended target sequence, and a second, subsequent amplification process comprising at least one amplification step for amplifying an internal sequence from the product of the first amplification process, wherein the internal sequence may or may not overlap one of the ends of the extended sequence. Each amplification step of the first amplification process employs an outer primer set typically comprising a pair of outer primers. Similarly, each amplification step of the second amplification process employs an inner primer set typically comprising a pair of inner primers. Figure 1 shows a diagram illustrating the methods of nested PCR reactions and Figure 2 shows a nested PCR with three primer pairs (A, B, C). The targeted area on interest is shown in the box. As used herein, nested PCR reactions are also meant to include semi-nested or hemi-nested PCR reactions wherein at least one of the primers used in the second amplification process is same as in the first amplification process.

[0083] The nested PCR methods of the present disclosure may be performed using plurality of primers, for example at least 3 nested primers, at least 4 nested primers, at least 5 nested primers, at least 6 nested primers, at least 7 nested primers, at least 8 nested primers, at least 9 nested primers, at least 10 nested primers, at least 11 nested primers, at least 12 nested primers, at least 13 nested primers, at least 14 nested primers, or at least 15 nested primers. In some example the methods may employ more than 15 nested primers for example 20, 25, 30, 35 or even more nested primers. In some embodiments the nested PCR methods of the present disclosure may be performed using at most 35 nested primers, 30 nested primers, 25 nested primers, 20 nested primers, 15 nested primers, 14 nested primers, 13 nested primers, 12 nested primers, 11 nested primers, 10 nested primers, 9 nested primers, 8 nested primers, 7 nested primers, 6 nested primers, 5 nested primers, 4 nested primers, 3 nested primers or 2 nested primers. In some example the methods may 35, 30, 25, 20, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, or 2 nested primers.

[0084] The plurality of primers may comprise at least 2 pairs of nested primers, at least 3 pairs of nested primers, at least 4 pairs of nested primers, at least 5 pairs of nested primes, at least 6 pairs of nested primers, at least 7 pairs of nested primers, at least 8 pairs of nested

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primers, at least 9 pairs of nested primers, at least 10 pairs of nested primers, at least 11 pairs of nested primers, at least 12 pairs of nested primers, at least 13 pairs of nested primers, at least 14 pairs of nested primers, at least 15 pairs of nested primers, at least 16 pairs of nested primers, at least 17 pairs of nested primers, at least 18 pairs of nested primers, at least 19 pairs of nested primers or at least 20 pairs of nested primers. In some embodiments the plurality of primers may comprise at most 20 pairs of nested primers, 19 pairs of nested primers, 18 pairs of nested primers, 17 pairs of nested primes, 16 pairs of nested primers, 15 pairs of nested primers, 14 pairs of nested primers, 13 pairs of nested primers, 12 pairs of nested primers, 11 pairs of nested primers, 10 pairs of nested primers, 9 pairs of nested primers, 8 pairs of nested primers, 7 pairs of nested primers, 6 pairs of nested primers, 5 pairs of nested primers, 4 pairs of nested primers, 3 pairs of nested primers, or 2 pairs of nested primers.

[0085] For instance, as shown in Figure 2, three nested primer pairs A, B, and C may be used. In this case, all three primers are effective at amplifying the targeted area of interest. By choosing primer sequences carefully for binding energy, they may exhibit different melt temperatures. This will allow the reaction kinetics for each primer to be tuned as a function of temperature.

[0086] By choosing the temperatures of the nested primer pairs, and by controlling the annealing temperature of the PCR amplification, the fluorescence signature of the amplification reaction may be tuned to generate multiple plateaus. For instance, as shown in Figure 5, the three nested primer pairs from Figure 2 may generate a unique fluorescence signature. Because the concentrations of primer sets A, B, and C may be carefully selected at the beginning of the reaction, the concentration of the nucleic acid target at plateaus A, B, and C may also be deterministic. This Information may be used to select at which concentration of targets the linear PCR regions should be run.

(ii) Asymmetric PCR reactions

[0087] The nested PCR reactions of the present disclosure may further be run in such a way that either the forward or reverse primer in the nested primer pairs may be rate limited (asymmetric PCR conditions). This may be done by adding each primer set such that there are different ratios of reverse and forward primers. In some cases this may be done by titrating each primer set such that there are different ratios of reverse and forward primers. Accordingly, it may be possible to generate curves that exhibit a characteristic linear amplification of DNA. While this is linear amplification may be non-ideal for the efficient

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amplification of DNA, it may be suitable for the careful discrimination of DNA targets. Figure 3 shows the fluorescence curve for a saturated, quantitative PCR reaction and Figure 4 shows the fluorescence curve of an asymmetric (biased primer) quantitative PCR reaction. Furthermore, tuning of primer concentrations and the number of primers for a particular nested reaction may allow for the engineering of particular fluorescent signatures for a given target.

[0088] In certain embodiments, the starting molar concentration of the limiting primer is less than the starting molar concentration of the excess primer. The ratio of the starting concentrations of the excess primer and the limiting primer may be at least 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 11:1, 12:1, 13:1, 14:1, 15:1, 16:1, 17:1, 18:1, 19:1, 20:1, 25:1, 30:1, 35:1, 40:1, 45:1, 50:1, 55:1, 60:1, 65:1, 70:1, 75:1, 80:1, 85:1, 90:1, 95:1, 100:1, 150:1, 200:1, 250:1, 300:1, 350:1, 400:1, 450:1, 500:1, 550:1, 600:1, 650:1, 700:1, 750:1, 800:1, 850:1, 900:1, 950:1, 1000:1 or more.

[0089] In certain embodiments, the starting molar concentration of the limiting primer is less than the starting molar concentration of the excess primer. The ratio of the starting concentrations of the excess primer and the limiting primer may be at most 1000:1, 950:1, 900:1, 850:1, 800:1, 750:1, 700:1, 650:1, 600:1, 550:1, 500:1, 450:1, 400:1, 350:1, 300:1, 250:1, 200:1, 150:1, 100:1, 95:1, 90:1, 85:1, 80:1, 75:1, 70:1, 65:1, 60:1, 55:1, 50:1, 45:1, 40:1, 35:1, 30:1, 25:1, 20:1, 19:1, 18:1, 17:1, 16:1, 15:1, 14:1, 13:1, 12:1, 11:1, 10:1, 9:1, 8:1, 7:1, 6:1, 5:1, 4:1, 3:1, 2:1, 1:1 or less.

[0090] In certain embodiments, the forward primer comprises a limiting primer and the reverse primer comprises an excess primer, wherein the excess primer is added to the combined sample at a concentration at least 2 times, 3 times, 4 times, 5 times, 6 times, 7 times , 8 times , 9 times , 10 times , 11 times , 12 times , 13 times , 14 times , 15 times , 16 times , 17 times , 18 times , 19 times , 20 times , 21 times , 22 times, 23 times, 24 times, 25 times, 30 times, 35 times, 40 times, 45 times, 50 times, 55 times, 60 times, 65 times, 70 times, 75 times, 80 times, 85 times, 90 times, 95 times, 100 times, 150 times, 200 times, 250 times, 300 times, 350 times, 400 times, 450 times, 500 times, 550 times, 600 times, 650 times, 700 times, 750 times, 800 times, 850 times, 900 times, 950 times, or 1000 times that of the limiting primer, and wherein the amplification conditions comprise asymmetric PCR conditions.

[0091] In certain embodiments, the forward primer comprises a limiting primer and the reverse primer comprises an excess primer, wherein the excess primer is added to the

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combined sample at a concentration at most 1000 times, 900 times, 950 times, 850 times, 800 times, 750 times, 700 times, 650 times, 600 times, 550 times, 500 times, 450 times, 400 times, 350 times, 300 times, 250 times, 200 times, 150 times, 100 times, 95 times, 90 times, 85 times, 80 times, 75 times, 70 times, 65 times, 60 times, 55 times, 50 times, 45 times, 40 times, 35 times, 30 times, 25 times, 24 times, 23 times, 22 times, 21 times, 20 times, 19 times, 18 times, 17 times, 16 times, 15 times, 14 times, 13 times, 12 times, 11 times, 10 times, 9 times, 8 times, 7 times, 6 times, 5 times, 4 times, 3 times, or 2 times that of the limiting primer, and wherein the amplification conditions comprise asymmetric PCR conditions.

[0092] In some embodiments, the forward primer comprises an excess primer and the reverse primer comprises a limiting primer, wherein the excess primer is added to the combined sample at a concentration at least 2 times, 3 times, 4 times, 5 times, 6 times, 7 times , 8 times , 9 times , 10 times , 11 times , 12 times , 13 times , 14 times , 15 times , 16 times , 17 times , 18 times , 19 times , 20 times , 21 times , 22 times, 23 times, 24 times, 25 times, 30 times, 35 times, 40 times, 45 times, 50 times, 55 times, 60 times, 65 times, 70 times, 75 times, 80 times, 85 times, 90 times, 95 times, 100 times, 150 times, 200 times, 250 times, 300 times, 350 times, 400 times, 450 times, 500 times, 550 times, 600 times, 650 times, 700 times, 750 times, 800 times, 850 times, 900 times, 950 times, or 1000 times that of the limiting primer, and wherein the amplification conditions comprise asymmetric PCR conditions.

[0093] In certain embodiments, the forward primer comprises an excess primer and the reverse primer comprises a limiting primer, wherein the excess primer is added to the combined sample at a concentration at most 1000 times, 900 times, 950 times, 850 times, 800 times, 750 times, 700 times, 650 times, 600 times, 550 times, 500 times, 450 times, 400 times, 350 times, 300 times, 250 times, 200 times, 150 times, 100 times, 95 times, 90 times, 85 times, 80 times, 75 times, 70 times, 65 times, 60 times, 55 times, 50 times, 45 times, 40 times, 35 times, 30 times, 25 times, 24 times, 23 times, 22 times, 21 times , 20 times, 19 times, 18 times, 17 times, 16 times, 15 times, 14 times, 13 times, 12 times, 11 times, 10 times, 9 times, 8 times, 7 times, 6 times, 5 times, 4 times, 3 times, or 2 times that of the limiting primer, and wherein the amplification conditions comprise asymmetric PCR conditions.

[0094] Primer length and sequence may be adjusted or modified, such that the concentration-adjusted melting temperature of the limiting primer at the start of the reaction is greater than or equal to the concentration-adjusted melting point of the excess primer. In some

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embodiments, the difference in the concentration-adjusted melting temperature of the limiting and the excess primer may be at least 0 °C , 0.5 °C, 1 °C, 1.5 °C, 2 °C, 2.5 °C, 3 °C, 3.5 °C, 4 °C, 4.5 °C, 5 °C, 5.5 °C, 6 °C, 6.5 °C, 7 °C, 7.5 °C, 8 °C, 8.5 °C, 9 °C, 9.5 °C, 10 °C or more. In some embodiments, the difference in the concentration-adjusted melting temperature of the limiting and the excess primer may be at most 10 °C , 9.5 °C, 9 °C, 8.5 °C, 8 °C, 7.5 °C, 7 °C, 6.5 °C, 6 °C, 5.5 °C, 5 °C, 4.5 °C, 4 °C, 3.5 °C, 3 °C, 2.5 °C, 2 °C, 1.5 °C, 1.0 °C, 0.5 °C, 0 °C or less.

[0095] Amplifications and assays according to embodiments of the present disclosure may be performed with initial reaction mixtures having ranges of concentrations of target molecules and primers. The methods may be used to assay samples containing less than 100 copies, 1000 copies, 10,000 copies, 20,000 copies, 30,000 copies, 40,000 copies, 50,000 copies, 60,000 copies, 70,000 copies, 80,000 copies, 90,000 copies, 100,000 copies, 200,000 copies, 300,000 copies, 400,000 copies, 500,000 copies, 600,000 copies, 70,000 copies, 800,000 copies, 900,000 copies or 1000,000 copies of the target analyte.

[0096] The methods may be used to assay samples containing more than 1000,000 copies, 900,000 copies, 800,000 copies, 700,000 copies, 600,000 copies, 500,000 copies, 400,000 copies, 300,000 copies, 200,000 copies, 100,000 copies, 90,000 copies, 80,000 copies, 70,000 copies, 60,000 copies, 50,000 copies, 40,000 copies, 30,000 copies, 20,000 copies, 10,000 copies, 1,000 copies or 100 copies of the target analyte.

[0097] The methods may be employed to assay sample comprising 0-100 copies, 0-1000 copies, 0-10,000 copies, 0-20,000 copies, 0-30,000 copies, 0-40,000 copies, 0-50,000 copies, 0-60,000 copies, 0-70,000 copies, 0-80,000 copies, 0-90,000 copies, 0-100,000 copies, 0-200,000 copies, 0-300,000 copies, 0-400,000 copies, 0-500,000 copies, 0-600,000 copies, 0-70,000 copies, 0-800,000 copies, 0-900,000 copies or 0-1000,000 copies of the target analyte.

[0098] The concentration of the limiting primer may range from a few to a several hundred nanomolars (nM). For example, the concentration of the limiting primer may be in the range of 1-50 nM, 1-100 nM, 1-150 nM, 1-200 nM, 1-250 nM, 1-300 nM, 1-350 nM, 1-400 nM, 1-450 nM, 1-500 nM, 50-100 nM, 50-150 nM, 50-200 nM, 50-250 nM, 50-300 nM, 50-350 nM, 50-400 nM, 50-450 nM, 50-500 nM, 100-150 nM, 100-200 nM, 100-250 nM, 100-300 nM, 100-350 nM, 100-400 nM, 100-450 nM, 100-500 nM, 150-200 nM, 150-250 nM, 150-300 nM, 150-350 nM, 150-400 nM, 150-450 nM, 150-500 nM, 200-250 nM, 200-300 nM, 200-350 nM, 200-400 nM, 200-450 nM, 200-500 nM, 250-300 nM, 250-350 nM, 250-400 nM, 250-450 nM, 250-500 nM, 300-350 nM, 300-400 nM, 300-450 nM, 300-500 nM, 350-

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400 nM, 350-450 nM, 350-500 nM, 400-450 nM, 400-500 nM, or 450-500 nM. Those of skill in the art appreciate that the concentration of the limiting primer may fall within any range bounded by any of these values.

[0099] In some examples the concentration of the limiting primer is less than 10 nM, 50 nM, 100 nM, 150 nM, 200 nM, 250 nM, 300 nM, 350 nM, 400 nM, 450 nM or 500 nM. In some examples the concentration of the limiting primer is greater than 10 nM, 50 nM, 100 nM, 150 nM, 200 nM, 250 nM, 300 nM, 350 nM, 400 nM, 450 nM or 500 nM.

[00100] The asymmetric nested PCR methods of the present disclosure include repeated thermal cycling through the steps of strand melting, primer annealing and primer extension. In a first-stage polymerase chain reaction, specific outer primers are added to the sample nucleic acid mixture, and the resulting mixture is subjected to an initial denaturation step to obtain single-stranded DNA templates. Following denaturation, the mixture is subjected to an initial annealing step, where the outer primers hybridize to opposite strands of the target nucleotide sequence. The temperature is then raised to allow for extension or replication of the specific segment of DNA across the region between the two primers by a thermostable DNA polymerase. The reaction is then thermocycled to allow for repeated denaturation, annealing, and extension, so that at each cycle, the amount of DNA representing the targeted nucleotide sequence between the two outer primers is doubled.

[00101] The first denaturation temperature may be in the range of 70-110 °C, for example in the range of 70-75°C, 75-80°C, 80-85°C, 85-90°C, 90-95 °C, 95-100 °C, 100-105 °C, or 105-110 °C. The first annealing temperature is may be in the range of in the range of 35-65 °C, for example in the range of 35-40 °C, 40-45 °C, 45-50 °C, 50-55 °C, 55-60 °C, or 60-65 °C. The first extension temperature may be in the range of in the range of 40-80 °C, for example in the range of 40-45 °C, 45-50 °C, 50-55 °C, 55-60 °C, 60-65 °C, 65-70 °C, 70-75 °C, or 75-80 °C.

[00102] At the end of the first-stage polymerase chain reaction, the resulting mixture is carried over into a second-stage polymerase chain reaction. In this second-stage reaction, the products of the first-stage reaction are combined with specific inner primers. This mixture is again subjected to initial denaturation, annealing, and extension steps, followed by thermocycling as before to allow for repeated denaturation, annealing, and extension or the targeted nucleotide sequence.

[00103] The second denaturation temperature may be in the range of 70-110 °C, for example in the range of 70-75°C, 75-80°C, 80-85°C, 85-90°C, 90-95 °C, 95-100 °C, 100-105

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°C, or 105-110 °C. The second annealing temperature may be in the range of 35-65 °C, for example in the range of 35-40 °C, 40-45 °C, 45-50 °C, 50-55 °C, 55-60 °C, or 60-65 °C. The second extension temperature may be in the range of in the range of 40-80 °C, for example in the range of 40-45 °C, 45-50 °C, 50-55 °C, 55-60 °C, 60-65 °C, 65-70 °C, 70-75 °C, or 75-80 °C.

[00104] Other PCR techniques that can be used in the methods of the provided disclosure include, e.g., AFLP (amplified fragment length polymorphism) PCR (see e.g.: Vos et al. 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Research 23: 4407-14), allele-specific PCR (see e.g., Saiki R K, Bugawan T L, Horn G T, Mullis K B, Erlich H A (1986). Analysis of enzymatically amplified beta-globin and HLA-DQ alpha DNA with allele-specific oligonucleotide probes Nature 324: 163-166), Alu PCR, assembly PCR (see e.g., Stemmer W P, Crameri A, Ha K D, Brennan T M, Heyneker H L (1995). Single-step assembly of a gene and entire plasmid from large numbers of oligodeoxyribonucleotides Gene 164: 49-53), asymmetric colony PCR, helicase dependent PCR (see e.g., Myriam Vincent, Yan Xu and Huimin Kong (2004). Helicase-dependent isothermal DNA amplification EMBO reports 5 (8): 795-800), hot start PCR, inverse PCR (see e.g., Ochman H, Gerber A S, Hartl D L. Genetics. 1988 November; 120(3):621-3), in situ PCR, intersequence-specific PCR or IS SR PCR, digital PCR, linear-after-the-exponential-PCR or Late PCR (see e.g., Pierce K E and Wangh L T (2007). Linear-after-the-exponential polymerase chain reaction and allied technologies Real-time detection strategies for rapid, reliable diagnosis from single cells Methods Mol. Med. 132: 65-85), long PCR, nested PCR, real-time PCR, duplex PCR, multiplex PCR, quantitative PCR, or single cell PCR. Further examples of PCR techniques that may be used with the methods of the disclosure include, but are not limited to, quantitative PCR, quantitative fluorescent PCR (QF-PCR), multiplex fluorescent PCR (MF-PCR), real time PCR, reverse transcriptase PCR (RT-PCR), single cell PCR, restriction fragment length polymorphism PCR (PCR-RFLP), PCR-RFLP/RT-PCR-RFLP, hot start PCR, nested PCR, in situ polonony PCR, in situ rolling circle amplification (RCA), bridge PCR, picotiter PCR and emulsion PCR. In some examples, the nested PCR reactions of the disclosure are conducted by having all primers added at the very beginning of PCR reaction and by programming the PCR machine to cycle among different sets of PCR temperatures over the course of the reaction, so that different sets of primers are activated in their respective phases only. For example, a nested PCR reaction may be carried out with three pairs of nested primers, say A, B, and C present at the

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same time. The reaction may be programmed in such a way that it runs for the first third of the cycles at hybridization temperature for the primers of sequence A, which is too high for the primers of B and C to hybridize. The second third of the cycles is then run at the temperature suitable for the primers of B but still too hot for hydridization of primer C. Finally, the final third of the cycles is run at temperature low enough for the primer pair C to hybridize. The lever of control in designing the methods of the disclosure include the sequence information and the programming of the temperatures and durations of the PCR cycles. The methods of the invention may in some cases involve varying the annealing and extension temperatures during the reaction, so that different primers get activated for extension at different times during the same PCR reaction.

[00105] In some cases, it may also be possible to program Taqman probes to activate at different times in the reaction as well. For example, the outer nesting primers may have annealing temperatures that are higher than the inner nested primers and the Taqman probe. This may mean that the Taqman probes may be protected from hydrolysis during the "pre-amplification", because while the template is being amplified, the probe is not hybridized and thus cannot be hydrolyzed. As a result no signal would be generated during the first stage of amplification, but when the inner pair of primers is engaged by lowering the annealing temperature, the probe would hybridize as well, and signal generation will be commenced. This characteristic may be used to keep the signal clean and help diagnose problems with the assay.

B. SPIA Amplification

[00106] Amplification of the analytes of interest employing a linear amplification method such as the single primer isothermal amplification (SPIA) can be used. SPIA enables generation of multiple copies of the strand specific sequence regions of interest and employs a single amplification primer, thus reducing the complexity associated with multiple oligonucleotide design and manufacturing, enables the use of a generic amplification primer, and can be linear.

[00107] Amplification by SPIA can occur under conditions permitting composite primer hybridization, primer extension by a DNA polymerase with strand displacement activity, cleavage of RNA from a RNA/DNA heteroduplex and strand displacement. In so far as the composite amplification primer hybridizes to the 3′-single-stranded portion (of the partially double stranded polynucleotide which is formed by cleaving RNA in the complex comprising a RNA/DNA partial heteroduplex) comprising, generally, the complement of at

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least a portion of the composite amplification primer sequence, composite primer hybridization may be under conditions permitting specific hybridization. In SPIA, all steps are isothermal (in the sense that thermal cycling is not required), although the temperatures for each of the steps may or may not be the same. It is understood that various other embodiments can be practiced given the general description provided above. For example, as described and exemplified herein, certain steps may be performed as temperature is changed (e.g., raised, or lowered).

[00108] Although generally only one composite amplification primer is described above, it is further understood that the SPIA amplification methods can be performed in the presence of two or more different first and/or second composite primers that randomly primer template polynucleotide. In addition, the amplification polynucleotide products of two or more separate amplification reactions conducted using two or more different first and/or second composite primers that randomly prime template polynucleotide can be combined.

[00109] The composite amplification primers are primers that are composed of RNA and DNA portions. In the amplification composite primer, both the RNA and the DNA portions are generally complementary and can hybridize to a sequence in the amplification-ready product to be copied or amplified. In some embodiments, a 3′-portion of the amplification composite primer is DNA and a 5′-portion of the composite amplification primer is RNA. The composite amplification primer is designed such that the primer is extended from the 3′-DNA portion to create a primer extension product. The 5′-RNA portion of this primer extension product in a RNA/DNA heteroduplex is susceptible to cleavage by RNase H, thus freeing a portion of the polynucleotide to the hybridization of an additional composite amplification primer. The extension of the amplification composite primer by a DNA polymerase with strand displacement activity releases the primer extension product from the original primer and creates another copy of the sequence of the polynucleotide. Repeated rounds of primer hybridization, primer extension with strand displacement DNA synthesis, and RNA cleavage create multiple copies of the strand-specific sequence of the polynucleotide.

[00110] In some embodiments, the composite amplification primer is generated in the amplification reaction mixture from a stem-loop chimeric pro-primer. The amplification reaction mixture can comprise a target partial duplex nucleic acid, for example a target partial duplex DNA, a chimeric stem-loop pro-primer, DNA polymerase with strand displacement activity, and an RNase targeting RNA in a RNA/DNA heteroduplex, for example RNase H.

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The RNA portion of the RNA/DNA heteroduplex at the stem of the chimeric stem-loop pro-primer can be cleaved by RNase H to generate, for example, a linear composite primer comprising a 3'-DNA and 5'-RNA. The linearized amplification primer can hybridize to a 3'-single stranded DNA portion (overhang) of a target partial duplex and can be extended by the DNA polymerase with strand displacement activity. The RNA portion of the hybridized primer in a heteroduplex can be cleaved by RNase H to free a portion of the primer binding site. A second linear composite amplification primer can hybridize to the freed primer binding site, and can be extended along the target DNA strand. The previously synthesized primer extension product (amplification product) can be displaced by the newly extended primer. Repeated cycles of primer hybridization, primer extension by strand displacement DNA polymerase, and cleavage of the RNA portion of the hybridized primer can generate multiple copies of a target nucleic acid.

C. Other Amplification Methods

[00111] Another method for amplification involves amplification of a single stranded polynucleotide using a single oligonucleotide primer. The single stranded polynucleotide that is to be amplified contains two non-contiguous sequences that are substantially or completely complementary to one another and, thus, are capable of hybridizing together to form a stem-loop structure. This single stranded polynucleotide already may be part of a polynucleotide analyte or may be created as the result of the presence of a polynucleotide analyte.

[00112] Another method for achieving the result of an amplification of nucleic acids is known as the ligase chain reaction (LCR). This method uses a ligase enzyme to join pairs of preformed nucleic acid probes. The probes hybridize with each complementary strand of the nucleic acid analyte, if present, and ligase is employed to bind each pair of probes together resulting in two templates that can serve in the next cycle to reiterate the particular nucleic acid sequence.

[00113] Another method for achieving nucleic acid amplification is the nucleic acid sequence based amplification (NASBA). This method is a promoter-directed, enzymatic process that induces in vitro continuous, homogeneous and isothermal amplification of a specific nucleic acid to provide RNA copies of the nucleic acid. The reagents for conducting NASBA include a first DNA primer with a 5′-tail comprising a promoter, a second DNA primer, reverse transcriptase, RNase-H, T7 RNA polymerase, NTP's and dNTP's.

[00114] Another method for amplifying a specific group of nucleic acids is the Q-beta-replicase method, which relies on the ability of Q-beta-replicase to amplify its RNA substrate

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exponentially. The reagents for conducting such an amplification include "midi-variant RNA" (amplifiable hybridization probe), NTP's, and Q-beta-replicase.

[00115] Another method for amplifying nucleic acids is known as 3SR and is similar to NASBA except that the RNase-H activity is present in the reverse transcriptase. Amplification by 3SR is an RNA specific target method whereby RNA is amplified in an isothermal process combining promoter directed RNA polymerase, reverse transcriptase and RNase H with target RNA. See for example Fahy et al. PCR Methods Appl. 1:25-33 (1991).

[00116] Another method for amplifying nucleic acids is the Transcription Mediated Amplification (TMA) used by Gen-Probe. The method is similar to NASBA in utilizing two enzymes in a self-sustained sequence replication. See U.S. Pat. No. 5,299,491 herein incorporated by reference.

[00117] Another method for amplification of nucleic acids is Strand Displacement Amplification (SDA) (Westin et al 2000, Nature Biotechnology, 18, 199-202; Walker et al 1992, Nucleic Acids Research, 20, 7, 1691-1696), which is an isothermal amplification technique based upon the ability of a restriction endonuclease such as HincII or BsoBI to nick the unmodified strand of a hemiphosphorothioate form of its recognition site, and the ability of an exonuclease deficient DNA polymerase such as Klenow exo minus polymerase, or Bst polymerase, to extend the 3′-end at the nick and displace the downstream DNA strand. Exponential amplification results from coupling sense and antisense reactions in which strands displaced from a sense reaction serve as targets for an antisense reaction and vice versa.

[00118] Another method for amplification of nucleic acids is Rolling Circle Amplification (RCA) (Lizardi et al. 1998, Nature Genetics, 19:225-232). RCA can be used to amplify single stranded molecules in the form of circles of nucleic acids. In its simplest form, RCA involves the hybridization of a single primer to a circular nucleic acid. Extension of the primer by a DNA polymerase with strand displacement activity results in the production of multiple copies of the circular nucleic acid concatenated into a single DNA strand.

[00119] In some embodiments of the disclosure, RCA is coupled with ligation. For example, a single oligonucleotide can be used both for ligation and as the circular template for RCA. This type of polynucleotide can be referred to as a "padlock probe" or a "RCA probe." For a padlock probe, both termini of the oligonucleotide contain sequences complementary to a domain within a nucleic acid sequence of interest. The first end of the padlock probe is substantially complementary to a first domain on the nucleic acid sequence

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of interest, and the second end of the padlock probe is substantially complementary to a second domain, adjacent to the first domain near the first domain. Hybridization of the oligonucleotide to the target nucleic acid results in the formation of a hybridization complex. Ligation of the ends of the padlock probe results in the formation of a modified hybridization complex containing a circular polynucleotide. In some cases, prior to ligation, a polymerase can fill in the gap by extending one end of the padlock probe. The circular polynucleotide thus formed can serve as a template for RCA that, with the addition of a polymerase, results in the formation of an amplified product nucleic acid. The methods of the disclosure described herein can produce amplified products with defined sequences on both the 5′- and 3′-ends. Such amplified products can be used as padlock probes.

[00120] Some aspects of the disclosure utilize the linear amplification of nucleic acids or polynucleotides. Linear amplification generally refers to a method that involves the formation of one or more copies of the complement of only one strand of a nucleic acid or polynucleotide molecule, usually a nucleic acid or polynucleotide analyte. Thus, the primary difference between linear amplification and exponential amplification is that in the latter process, the product serves as substrate for the formation of more product, whereas in the former process the starting sequence is the substrate for the formation of product but the product of the reaction, i.e. the replication of the starting template, is not a substrate for generation of products. In linear amplification the amount of product formed increases as a linear function of time as opposed to exponential amplification where the amount of product formed is an exponential function of time.

[00121] In some embodiments, amplification methods can be solid-phase amplification, polony amplification, colony amplification, emulsion PCR, bead RCA, surface RCA, surface SDA, etc., as will be recognized by one of skill in the art. In some embodiments, amplification methods that results in amplification of free DNA molecules in solution or tethered to a suitable matrix by only one end of the DNA molecule can be used. Methods that rely on bridge PCR, where both PCR primers are attached to a surface (see, e.g., WO 2000/018957 and Adessi et al., Nucleic Acids Research (2000): 28(20): E87) can be used. In some cases the methods of the disclosure can create a “polymerase colony technology,” or “polony.” referring to a multiplex amplification that maintains spatial clustering of identical amplicons (see Harvard Molecular Technology Group and Lipper Center for Computational Genetics website). These include, for example, in situ polonies (Mitra and Church, Nucleic Acid Research 27, e34, Dec. 15, 1999), in situ rolling circle

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amplification (RCA) (Lizardi et al., Nature Genetics 19, 225, July 1998), bridge PCR (U.S. Pat. No. 5,641,658), picotiter PCR (Leamon et al., Electrophoresis 24, 3769, November 2003), and emulsion PCR (Dressman et al., PNAS 100, 8817, Jul. 22, 2003). The methods of the disclosure provide new methods for generating and using polonies.

[00122] Other suitable amplification methods include transcription amplification, self-sustained sequence replication, selective amplification of target polynucleotide sequences, consensus sequence primed polymerase chain reaction (CP-PCR), arbitrarily primed polymerase chain reaction (AP-PCR), degenerate oligonucleotide-primed PCR (DOP-PCR) and nucleic acid based sequence amplification (NABSA). Other amplification methods that can be used herein include those described in U.S. Pat. Nos. 5,242,794; 5,494,810; 4,988,617; and 6,582,938. In some embodiments, amplification of target nucleic acids may occur on a bead. In other embodiments, amplification does not occur on a bead.

Multiplex Assays

[00123] In some embodiments, the present disclosure provides a multiplexed assay for simultaneous amplification, detection, and or/quantification of multiple target analytes in a sample. Multiplex assays or grammatical equivalents refer to the detection, analysis or amplification of more than one target analyte of interest in a single reaction mixture. In some embodiments the methods of the disclosure may be used to detect and/or quantify at least 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, 40, 50, 60, 70, 80, 90, 100, 500, 1000, 2000, 3000, or more different target analytes in a sample. In some embodiments the methods of the disclosure may be used to detect and/or quantify at most 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, 40, 50, 60, 70, 80, 90, 100, 500, 1000, 2000, 3000, or less different target analytes a sample. Accordingly, in some cases, the methods described herein may be used to simultaneously detect multiple diseases at once. In other cases the methods may be used to simultaneously detect multiple infectious agents in a sample. In some other cases the methods may be used to simultaneously detect multiple genetic markers in a sample.

[00124] In some embodiments the methods of the disclosure involve multiplexed PCR reactions. In some embodiments the methods may involve multiplexed nested PCR reactions or multiplexed asymmetric PCR reactions.

VI. Analytes

[00125] An analyte can be any molecule detectable by the methods provided herein. An analyte may be a polynucleotide, such as DNA, RNA, and any hybrid thereof, where the

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polynucleotide contains any combination of deoxyribo- and/or ribo-nucleotides. Polynucleotides may be single stranded or double stranded, as specified, or contain portions of both double stranded or single stranded sequence. Polynucleotides may contain any combination of nucleotides, including uracil, adenine, thymine, cytosine, guanine, inosine, xanthine, hypoxanthine, isocytosine, isoguanine and any nucleotide derivative thereof. As used herein, the term "nucleotide" may include nucleotides and nucleosides, as well as nucleoside and nucleotide analogs, and modified nucleotides, including both synthetic and naturally occurring species.

[00126] The analyte may comprise RNA. For example an analyte may comprise messenger RNA (mRNA), ribosomal RNA (rRNA), transfer RNA (tRNA), nuclear RNA (nRNA), small interfering RNA (siRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), small Cajal body-specific RNA (scaRNA), microRNA (miRNA), double stranded (dsRNA), ribozyme, riboswitch 16SRNA or viral RNA. In some cases the methods of the invention may further comprise reverse transcription of the RNA analyte into its DNA complement (cDNA) through the use of reverse transcriptase. Subsequently, the cDNA may be amplified and assayed by the methods of the disclosure.

[00127] The analyte may comprise DNA, or complex DNA, for example genomic DNA. Analytes comprising genomic DNA may be obtained from any source and using any methods known in the art. Genomic DNA may be obtained from naturally occurring or genetically modified organisms or from artificially or synthetically created genomes. Genomic DNA may be isolated with or without amplification. Amplification may include PCR amplification, multiple displacement amplification (MDA), rolling circle amplification and other amplification methods. Genomic DNA may also be obtained by cloning or recombinant methods, such as those involving plasmids and artificial chromosomes or other conventional methods (see Sambrook and Russell, *Molecular Cloning: A Laboratory Manual*., cited *supra*.). The analyte may also comprise cDNA. The cDNA can be generated from RNA, e.g., mRNA. The DNA can be cDNA made from a mixture of genomes of different species. The DNA can be mitochondrial DNA (mtDNA). The DNA can be cell-free DNA. The cell-free DNA can be obtained from, e.g., a serum or plasma sample. The DNA can comprise one or more chromosomes. For example, if the DNA is from a human, the DNA can comprise one or more of chromosome 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, X, or Y. The DNA can be from a linear or circular genome. The

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DNA can be plasmid DNA, cosmid DNA, bacterial artificial chromosome (BAC), or yeast artificial chromosome (YAC).

[00128] The DNA can be of a specific species, for example, human, rat, mouse, other animals, plants, bacteria, algae, viruses, and the like. The DNA can also be from a mixture of genomes of different species such as host-pathogen, bacterial populations and the like. The DNA can be from more than one individual or organism. The DNA can be double stranded or single stranded. The DNA can be part of chromatin. The DNA can be associated with histones. The methods described herein can be applied to high molecular weight DNA, such as is isolated from tissues or cell culture, for example, as well as highly degraded DNA, such as cell-free DNA from blood and urine and/or DNA extracted from formalin-fixed, paraffin-embedded tissues, for example.

[00129] An analyte may be a polynucleotide from a particular pathogen, such as a bacteria, virus, fungus, or helminth. For example, an analyte may be a polynucleotide from human immunodeficiency virus (HIV, for example human immunodeficiency virus p17), *Plasmodium*, herpes simplex virus, dengue virus, hepatitis virus, human papilloma virus (for example human papilloma virus E6 or human papilloma virus E7), influenza virus, *Mycobacterium*, Adenoviridae, Picornaviridae, Herpesviridae, Hepadnaviridae, Flaviviridae, Retroviridae, Orthomyxoviridae, Paramyxoviridae, Papovaviridae, Polyomavirus, Rhabdoviridae, Togaviridae, *Streptococcus*, *Pseudomonas*, *Shigella*, *Campylobacter*, *Salmonella*, or *Escherichia*.

[00130] An analyte may also be encoding an enzyme, a protein or a polypeptide. The analyte may be indicative of presence and/or quantity of a carbohydrate, sugar, small molecule, a drug or metabolite (e.g., anti-cancer drug, chemotherapeutic drug, anti-viral drug, antibiotic drug, or any other molecule, such as a co-factor, receptor, receptor ligand, hormone, cytokine, blood factor, antigen, steroid, or antibody.

[00131] The samples may be derived from animals, plants, bacterias, fungi , parasites or viruses. In some cases, the samples are derived from humans. The different samples from which the analytes are derived can comprise multiple samples from the same individual, samples from different individuals, or combinations thereof. In some embodiments, a sample comprises a plurality of polynucleotides from a single individual. In some embodiments, a sample comprises a plurality of polynucleotides from two or more individuals. An individual is any organism or portion thereof from which target polynucleotides can be derived, non-limiting examples of which include plants, animals, fungi, protists, monerans, viruses,

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mitochondria, and chloroplasts. Sample polynucleotides can be isolated from a subject, such as a cell sample, tissue sample, or organ sample derived there from, including, for example, cultured cell lines, biopsy, blood sample, or fluid sample containing a cell. Cells may comprise a specific cell type, such as a somatic cell, germline cell, wild-type cell, cancer or tumor cells, or diseased or infected cell. A cell may refer to a cell derived from a particular tissue or a particular locus in a target organism. A cell may comprise whole intact cells, or cell preparations. In some instances, samples may be obtained from bodily fluids which may include blood, urine, serum, lymph, saliva, mucosal secretions, perspiration, or semen. The subject may be an animal, including but not limited to, an animal such as a cow, a pig, a mouse, a rat, a chicken, a cat, a dog, etc., and is usually a mammal, such as a human.

[00132] Samples may also be obtained from manufactured products (such as cosmetics and foods), pharmaceutical products, or consumer products. In some instances, samples may be obtained from environmental samples including air, agricultural products, water, and soil. Samples can also be artificially derived, such as by chemical synthesis. In other instances samples may be the products of experimental manipulation including, recombinant cloning, polynucleotide amplification, polymerase chain reaction (PCR) amplification, purification methods (such as purification of genomic DNA or RNA), and synthesis reactions

[00133] Methods for the extraction and purification of nucleic acids are well known in the art. For example, nucleic acids can be purified by organic extraction with phenol, phenol/chloroform/isoamyl alcohol, or similar formulations, including TRIzol and TriReagent. Other non-limiting examples of extraction techniques include: (1) organic extraction followed by ethanol precipitation, e.g., using a phenol/chloroform organic reagent (Ausubel et al., 1993), with or without the use of an automated nucleic acid extractor, e.g., the Model 341 DNA Extractor available from Applied Biosystems (Foster City, Calif.); (2) stationary phase adsorption methods (U.S. Pat. No. 5,234,809; Walsh et al., 1991); and (3) salt-induced nucleic acid precipitation methods (Miller et al., (1988), such precipitation methods being typically referred to as "salting-out" methods. Another example of nucleic acid isolation and/or purification includes the use of magnetic particles to which nucleic acids can specifically or non-specifically bind, followed by isolation of the beads using a magnet, and washing and eluting the nucleic acids from the beads (see e.g. U.S. Pat. No. 5,705,628). In some embodiments, the above isolation methods may be preceded by an enzyme digestion step to help eliminate unwanted protein from the sample, e.g., digestion with proteinase K, or other like proteases. See, e.g., U.S. Pat. No. 7,001,724. If desired, RNase inhibitors may be

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added to the lysis buffer. For certain cell or sample types, it may be desirable to add a protein denaturation/digestion step to the protocol. Purification methods may be directed to isolate DNA, RNA, or both. When both DNA and RNA are isolated together during or subsequent to an extraction procedure, further steps may be employed to purify one or both separately from the other. Sub-fractions of extracted nucleic acids can also be generated, for example, purification by size, sequence, or other physical or chemical characteristic. In addition to an initial nucleic isolation step, purification of nucleic acids can be performed after any step in the methods of the disclosure, such as to remove excess or unwanted reagents, reactants, or products.

VII. Primers

[00134] According to the present disclosure, the term primer relates to a nucleic acid sequence, which is capable of hybridizing e.g. to the target nucleic acid sequence, to a nucleic acid sequence in the vicinity of the target sequence, or to a nucleic acid sequence overlapping with the target nucleic acid sequence. Alternatively, a primer may be capable of hybridizing to either the complementary sequence of the target nucleic acid sequence, to the complementary sequence of a nucleic acid sequence in the vicinity of the target nucleic acid sequence, or to the complementary sequence of a nucleic acid sequence overlapping with the target nucleic acid sequence.

[00135] The primers typically comprise oligonucleotides. The primers may comprise 5-50 nucleotides, for example 5-10 nucleotides, 5-15 nucleotides, 5-20 nucleotides, 5-25 nucleotides, 5-30 nucleotides, 5-35 nucleotides, 5-40 nucleotides, 5-45 nucleotides, 5-50 nucleotides, 10-15 nucleotides, 10-20 nucleotides, 10-25 nucleotides, 10-30 nucleotides, 10-35 nucleotides, 10-40 nucleotides, 10-45 nucleotides, 10-50 nucleotides, 15-20 nucleotides, 15-25 nucleotides, 15-30 nucleotides, 15-35 nucleotides, 15-40 nucleotides, 15-45 nucleotides, 15-50 nucleotides, 20-25 nucleotides, 20-30 nucleotides, 20-35 nucleotides, 20-35 nucleotides, 20-40 nucleotides, 20-45 nucleotides, 20-50 nucleotides, 25-30 nucleotides, 25-35 nucleotides, 25-40 nucleotides, 25-45 nucleotides, 25-50 nucleotides, 30-35 nucleotides, 30-40 nucleotides, 30-45 nucleotides, 30-50 nucleotides, 35-40 nucleotides, 35-45 nucleotides, 35-50 nucleotides, 40-45 nucleotides, 40-50 nucleotides, or 45-50 nucleotides. In some embodiments the primers comprise 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50 nucleotides. The primers may also comprise more than 50 nucleotides, for example 55, 60, 65, 70 or even more nucleotides.

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[00136] A primer can be purified from a restriction digest by conventional methods, or it can be produced synthetically. The primer is preferably single-stranded for maximum efficiency in amplification, but the primer can be double-stranded. Double-stranded primers may denatured, i.e., treated to separate the strands. One method of denaturing double stranded nucleic acids is by heating.

[00137] The methods of designing and preparing primers that are appropriate for amplification reaction are well known to the person of ordinary skill in the art. Important features when designing oligonucleotides to be used as amplification primers include, but are not limited to, an appropriate size amplification product to facilitate detection (e.g., by electrophoresis), melting temperatures for the members of a pair of primers, and the length of each primer (i.e., the primers need to be long enough to anneal with sequence-specificity and to initiate synthesis but not so long that fidelity is reduced during oligonucleotide synthesis). The length of the amplification primers depends on several factors including the nucleotide sequence identity and the temperature at which these nucleic acids are hybridized or used during in vitro nucleic acid amplification. Primers can be designed using, for example, a computer program such as OLIGO (Molecular Biology Insights, Inc., Cascade, Colo.).

[00138] In some embodiments primers may include a detectable label, although a label may also be introduced by other means (for example via a labeled probe) during the amplification assay. In the former case, the labeled primer will result in a labeled amplification product, as the labeled primers are incorporated into the products. Labeled primers are available from commercial suppliers such as CPG, Inc. (Lincoln Park, N.J.), or they can be prepared via conjugating a label to an unlabeled primer. Labeling oligonucleotides such as primers can be carried out using conventional coupling procedures. Specific coupling procedures, however, will vary depending on the reactive group or groups present on the label and/or on the oligonucleotide. Preferred labels include those moieties detectable by spectroscopic, photochemical, biochemical, immunochemical, or chemical means. Such labels include, without limitation, fluorescence, chemiluminescence, dyes, biotin, haptens, enzymes, enzyme substrates, enzyme cofactors, enzyme inhibitors, enzyme subunits, metal ions, electron-dense reagents, and radioactive isotopes (e.g., ^{32}P). The label moiety can be directly or indirectly attached to the primer.

[00139] For example, is primer is labeled at the 5′ ends with a fluorophore, the amplified target sequence will be labeled with the detectable fluorescent material, and the intensity of fluorescence emitted from the fluorescent material may be measured using a

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fluorescence spectrophotometer. Suitable fluorophores that may be used with the primers of the disclosure include those described above. For example the primers may be labeled with 6-FAM; Alexa fluor 405, 430, 488, 532, 546, 555, 568, 594, 633, 647, or 660; Cy2; Cy3; Cy3.5; Cy5; Cy5.5; Cy7; hydroxycoumarin; methoxycoumarin; aminocoumarin; fluorescein; HEX; R-phycoerythrin; rhodamine Red-X; ROX; Red 613; Texas Red; allophycocyanin; TruRed; BODIPY 630/650; BODIPY 650/665; BODIPY-FL; BODIPY-R6G; BODIPY-TMR; BODIPY-TRX; carboxyfluorescein; Cascade Blue; 6-JOE; Lissamine rhodamine B; Oregon Green 488, 500, or 514; Pacific Blue; REG; Rhodamine Green; SpectrumAqua; TAMRA; TET; and Tetramethylrhodamine.

VIII Signals

[00140] The methods presented in this disclosure involve the measurement of a signal. The signal may comprise a intensity and/or a frequency. In many of the exemplary embodiments described in this disclosure, a fluorescent signal may be used. The fluorescent signal is often described as being measured in color (i.e., wavelength) and intensity. Color may be measured by using a spectrophotometer or by using a band pass filter that only allows light of a certain wavelength to reach a photodetector. Such band pass filters are commonly used in qPCR machines. Intensity is measured by using a photo detector. In some cases each analyte is encoded by a signal intensity at a particular wavelength. In some cases, each analyte is encoded by a plurality of signal intensities at a plurality of wavelengths. For example, each analyte may be labeled with a single intensity of 1, 2, 3, 4, 5 or more wavelengths. In some cases an analyte is encoded by at least one additional value selected from the group consisting of a value from said signal and a value from an additional signal. In some cases, the one additional value is selected from the group consisting of a Förster resonance energy transfer (FRET) emission intensity, a FRET emission wavelength, an electrochemical signal, a chemiluminescence wavelength, a chemiluminescence intensity, a fluorescence bleaching rate, and a chemiluminescence bleaching rate. For example, at least one additional value is Förster resonance energy transfer (FRET) emission intensity.

[00141] Although many of the exemplary embodiments described in this disclosure are described in terms of a fluorescent signal, this is by no means limiting. The methods provided in this disclosure may be used with any signal, for example Forster resonance energy transfer (FRET) emission intensity, a FRET emission wavelength, a chemiluminescent wavelength, a chemiluminescent intensity, a electromagnetic signal, a fluorescence bleaching rate, or a chemiluminescence bleaching rate.

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[00142] Some of the methods provided in this disclosure utilize a reagent that generates a signal in the presence of an analyte. In some cases, these reagents are probes. The probes may be hybridization probes. The hybridization probes may be an oligonucleotide probe attached to a fluorophore and a quencher (e.g., a TAQMAN probe).

[00143] The methods of the disclosure may use one or more reagents or probes to detect the presence of each analyte. For example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or more reagents or probes may be used to detect the presence of an analyte. In some cases, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or more reagents or probes may be used to detect the presence of an analyte. In some cases, fewer than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or more reagents or probes may be used to detect the presence of an analyte.

[00144] As described above, oligonucleotide probes attached to a fluorophore and a quencher may be used to detect the presence of an analyte in a polynucleotide amplification assay. So long as the quencher and the fluorophore are in proximity, the quencher quenches the fluorescence emitted by the fluorophore upon excitation by a light source. The sequence of the oligonucleotide probe is designed to be complementary to a polynucleotide sequence present in an analyte, and therefore capable of hybridizing to the polynucleotide sequence present in the analyte. Hybridization of the oligonucleotide probe may be performed in a nucleic acid amplification reaction comprising primers (e.g., a polymerase chain reaction). Upon extension of the primers by a DNA polymerase, the 5' to 3' exonuclease activity of the polymerase degrades the probe, releasing the fluorophore and the quencher into the medium. The proximity between the fluorophore and the quencher is broken and the signal from the fluorophore is no longer quenched. Thus, the amount of fluorescence detected is a function of the amount of analyte present. If no analyte is present, the probe will not hybridize to an analyte, and the fluorophore and quencher will remain in close proximity. Little or no signal will be produced.

[00145] Oligonucleotide probes may have one or a plurality of fluorophores and quenchers per probe. For example, in some embodiments an oligonucleotide probe may comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more fluorophores. An oligonucleotide probe may comprise at least 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 fluorophores. An oligonucleotide probe may comprise fewer than 2, 3, 4, 5, 6, 7, 8, 9, or 10 fluorophores. An oligonucleotide probe may comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more quenchers. An oligonucleotide probe may comprise at least 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 quenchers. An oligonucleotide probe may comprise fewer than 2, 3, 4, 5, 6, 7, 8, 9, or 10 quenchers. Attachment of probes and

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quenchers to a probe may be performed in the same reaction or in serial reactions. A series of reactions may be performed to label probes with at least one fluorophore and the reaction products may be mixed to generate a mixture of probes with different fluorophores.

[00146] Suitable fluorophores for use in the present disclosure include, but are not limited to, fluorescent lanthanide complexes, including those of Europium and Terbium, fluorescein, rhodamine, tetramethylrhodamine, eosin, erythrosin, coumarin, methyl-coumarins, pyrene, Malacite green, stilbene, Lucifer Yellow, Cascade Blue, Texas Red, and others described in the 11th Edition of the Molecular Probes Handbook by Richard P. Haugland, hereby expressly incorporated by reference in its entirety.

[00147] Commercially available fluorescent nucleotide analogues readily incorporated into the labeling oligonucleotides include, for example, Cy3-dCTP, Cy3-dUTP, Cy5-dCTP, Cy5- dUTP (GE Healthcare), fluorescein-12-dUTP, tetramethylrhodamine-6-dUTP, Texas Red®-5-dUTP, Cascade Blue®-7-dUTP, BODIPY® FL-14-dUTP, BODIPY®R-14-dUTP, BODIPY® TR-14-dUTP, Rhodamine GreenTM-5-dUTP, Oregon Green® 488-5-dUTP, Texas Red®-12-dUTP, BODIPY® 630/650-14-dUTP, BODIPY® 650/665-1 4-dUTP, Alexa Fluor® 488-5-dUTP, Alexa Fluor® 532-5-dUTP, Alexa Fluor® 568-5-dUTP, Alexa Fluor® 594-5-dUTP, Alexa Fluor® 546-1 4-dUTP, fluorescein-12-UTP, tetramethylrhodamine-6-UTP, Texas Red®-5-UTP, Cascade Blue®-7-UTP, BODIPY® FL-14-UTP, BODIPY® TMR-14-UTP, BODIPY® TR-14-UTP, Rhodamine GreenTM-5-UTP, Alexa Fluor® 488-5-UTP, and Alexa Fluor® 546-1 4-UTP (Invitrogen).

[00148] Other fluorophores available for post-synthetic attachment include, inter alia, Alexa Fluor® 350, Alexa Fluor® 532, Alexa Fluor® 546, Alexa Fluor® 568, Alexa Fluor® 594, Alexa Fluor® 647, BODIPY 493/503, BODIPY FL, BODIPY R6G, BODIPY 530/550, BODIPY TMR, BODIPY 558/568, BODIPY 558/568, BODIPY 564/570, BODIPY 576/589, BODIPY 581/591, BODIPY 630/650, BODIPY 650/665, Cascade Blue, Cascade Yellow, Dansyl, lissamine rhodamine B, Marina Blue, Oregon Green 488, Oregon Green 514, Pacific Blue, rhodamine 6G, rhodamine green, rhodamine red, tetramethylrhodamine, Texas Red (available from Invitrogen), and Cy2, Cy3.5, Cy5.5, and Cy7 (GE Healthcare).

[00149] If an oligonucleotide probe comprises two or more fluorophores, these fluorophores may be arranged to allow Förster (Fluorescence) resonance energy transfer (FRET) to occur between the fluorophores. Briefly, FRET is a mechanism of energy transfer between fluorophores. Using FRET-based probes allows one excitation source to generate two different fluorescence emission signals by excitation of a single fluorophore. For

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example, the methods described herein may used paired probes, where one probe in the pair is attached to a fluorophore and a quencher and a second probe is attached to two fluorophores (in close enough proximity for FRET to occur) and a quencher. Any combination of fluorophores and quenchers that provide for FRET may be used. Such an approach doubles the number of fluorescent probes that may be used with an excitation source, because a single excitation source can be used to produce two signals: one from the probe with one fluorophore and one from the probe with two fluorophores in close enough proximity to provide for FRET. For example, using the encoding method described in Table 8, the number of unambiguously detectable analytes could be increased from 16 to 32 by pairing each of the probes in Table 8 with a corresponding FRET probe.

[00150] In one example, a first probe may be labeled with the fluorophore 5'-fluorescein amidite (5'-FAM) and the quencher black hole quencher 1 (BHQ-1). A second probe may be labeled with 5'-FAM in close proximity (e.g., attached to) to cyanine 5.5 (Cy5.5) and the quencher black hole quencher 3 (BHQ-3). Upon digestion of the probe, via the nuclease activity of the polymerase, two fluorescent signals are generated from a single excitation wavelength (e.g., 470 nm). The fluorophore from the first probe will fluoresce at about 520 nm. The fluorophores on the second probe undergo FRET. The donor fluorophore, FAM is excited by the excitation light source (e.g., 470 nm) and transfers its energy to the acceptor fluorophore, Cy5.5. The acceptor fluorophore emits at about 705 nm. These fluorophores and quenchers are merely exemplary. Any fluorophores that can undergo FRET and any quenchers that can quench fluorescence are suitable for use with the disclosure. Methods for producing FRET-based probes are described in Jothikumar et al., BioTechniques, 2009, 46(7):519-524.

[00151] Although many aspects of the disclosure are exemplified using nucleic acid-based probes, one of ordinary skill in the art will readily recognize that other forms of probes would work equally well with the disclosure described in this disclosure. For example, a binding molecule specific to an analyte could be used as a probe. Non-limiting exemplary binding molecules include an antibody recognizing an analyte, and generating a signal in the presence of an analyte.

[00152] Similarly, although many aspects of the disclosure are exemplified using fluorescent labels attached to probes, many other types of labels could also be used with the methods of the disclosure. The disclosure can be used with any label capable of generating a measurable signal.

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[00153] For multicolor imaging, signals of different wavelength can be obtained by multiple acquisitions or by simultaneous acquisition by splitting the signal, using RGB detectors or analyzing the whole spectrum (Richard Levenson, Cambridge Healthtech Institutes, Fifth Annual meeting on Advances in Assays, Molecular Labels, Signaling and Detection, May 17-18th Washington D.C.). Several spectral lines can be acquired by the use of a filter wheel or a monochrometer. Electronic tunable filters such as acoustic-optic tunable filters or liquid crystal tunable filters can be used to obtain multispectral imaging (e.g. Oleg Hait, Sergey Smirnov and Chieu D. Tran, 2001, Analytical Chemistry 73: 732-739). An alternative method to obtain a spectrum is hyperspectral imaging (Schultz et al., 2001, Cytometry 43:239-247).

Methods and apparatus for signal detection and processing of intensity data are disclosed in, for example, U.S. Pat. Nos. 5,143,854, 5,547,839, 5,578,832, 5,631,734, 5,800,992, 5,834,758; 5,856,092, 5,902,723, 5,936,324, 5,981,956, 6,025,601, 6,090,555, 6,141,096, 6,185,030, 6,201,639; 6,218,803; and 6,225,625, 7,689,022 and in WO99/47964, each of which also is hereby incorporated by reference in its entirety for all purposes. The fluorophores that may be used with the disclosure are not limited to any of the fluorophores described herein. For example, fluorophores with improved properties are continually developed, and these fluorophores could readily be used with the methods provided in this disclosure. Such improved fluorophores include quantum dots, which may emit energy at different wavelengths after being excited at a single wavelength. The advantage of using such fluorophores is that only a single excitation source is needed, but many different signals may be quantified, for example in terms of color and intensity.

IX. Other Additives

[00154] The PCR reagents may furthermore comprise additives such as a DNA polymerase (e.g. Taq polymerase), deoxynucleotide triphosphates (e.g. dATP, dCTP, dTTP, and dGTP), salts (e.g. KCl and $MgCl_2$), buffer (e.g. Tris-HCl), biopreservatives (e.g. sodium azide), PCR enhancers (e.g. Betaine, Trehalose, etc.), and inhibitors (e.g. RNAse inhibitors), detergents (e.g. Triton X-100, Triton X-114, NP-40, Tween 20, Tween 80 and similar non ionic detergents). Other additives may include 2-pyrrolidone, acetamide, N-methylpyrolidone (NMP), B-hydroxyethylpyrrolidone (HEP), propionamide, NN-dimethylacetamide (DMA), N-methylformamide (MMP), NN-dimethylformamide (DMF), formamide, N-methylacetamide (MMA), dimethyl sulfoxide (DMSO), polyethylene glycol, betaine, tetramethylammonium chloride (TMAC), 7-deaza-2'-deoxyguanosine, T4 gene 32 protein, 2-

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mercaptoethanol or glycerol. Paragraph 104 and 105 remove MIP 00187 for polymerase; 00188 for different types of PCR

X. Analysis of the characteristic curves

[00155] Once characteristic curves are generated for a set of reaction, these curves can be analyzed by curve-fitting algorithms. The curves may be fitted using, for example, linear regression, non-linear regression (for example Levenberg-Marquardt (LM) method), or may simply connect fluorescence data from adjacent cycles with a smoothed or non-smoothed line. The fitted curves may be then used to determine the slope of the amplification reaction in its linear regime. In the embodiments where two or more of these curves are in superposition (i.e. where two or more targets are present in multiplexed fashion) each target may be uniquely identified as long as the curves are in the linear dynamic range of the fluorometer. The multiplexed discrimination of multiple targets in a single reaction by means of reaction rates allows for another axis of orthogonality for PCR multiplexing.

[00156] In some cases the curves generated by the methods of the disclosure may be analyzed by taking the derivative of the signal with respect to cycle number and generating a derivative plot. In the derivative plot, theh plateaus will show up as zeros or near-zeros. The normal saturating amplification will show up as a sinusoidal-like peak between two zeros. The linear amplification will shows up as plateaus, where the magnitude will informative of the slope and will be proportional to the concentration of respective primer.

[00157] Another strategy for discriminating curves is to transform them into a transform domain thereby generating a transformed signal. For example, the signal may be transformed into a frequency domain. The transforming may be performed by any suitable method including Fourier transform, fast Fourier transform (FFT), discrete-time Fourier transform (DTFT), discrete-time Fourier transform (DTFT), short-time Fourier transform, fractional Fourier transform, chirplet transforms, or wavelet transform. In general, any eigenvalue transformation that is a conformal map can be used to extract the natural frequencies of the PCR curve. Once this is obtained, the frequency space signature (Figure 7) of each reaction can be used to resolve which targets were present. Careful design of the primers and probes will allow reaction rate information to be resolved as well, allowing for a built-in gauge of the quality or efficiency of the PCR reaction. Furthermore, this frequency space signature can be cross-correlated against a database of known target combinations to determine the most likely mix of targets in a particular reaction.

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[00158] Accordingly, in another aspect the disclosure provides a method of detecting a nucleic acid analyte in a sample. The method comprises performing an amplification reaction on a sample, measuring a signal generated during said amplification reaction, transforming the signal into a transform domain thereby generating a transformed signal, comparing the transformed signal to a reference signal, and based on the comparison determining whether the analyte is present or absent.

[00159] The reference signal may be a signal in a database of reference signals. The database may comprise at least 1, 5, 10, 50, 100, 200, 300, 400, 500 or more signals. In some cases, the database comprises at most 500, 400, 300, 200, 100, 50, 10, 5 or 1 signal.

XI. Kits

[00160] In one aspect, the disclosure provides kits containing any one or more of the elements disclosed in the above methods and compositions. In some embodiments, a kit comprises a composition of the disclosure, in one or more containers. The kits may further comprise additional agents, such as those described above, for use according to the methods of the disclosure. The kit elements may be provided in any suitable container, including but not limited to test tubes, vials, flasks, bottles, ampules, syringes, or the like.

[00161] An exemplary kit of the disclosure may comprise at least three nested primers, at least one pair of primers comprising a limiting primer and an excess primer, and a real time detection agent. The kits may further comprise one or more PCR reagents, for example salts, deoxynucleotide triphosphates, buffers, detergents, PCR inhibitors, PCR enhancers and nucleic acid polymerases. The kit may also comprise additives such as 2-mercaptoethanol, 2-pyrrolidone, acetamide, N-methylpyrolidone (NMP), B-hydroxyethylpyrrolidone (HEP), propionamide, NN-dimethylacetamide (DMA), N-methylformamide (MMP), NN-dimethylformamide (DMF), formamide, N-methylacetamide (MMA), dimethyl sulfoxide (DMSO), polyethylene glycol, betaine, tetramethylammonium chloride (TMAC), 7-deaza-2'-deoxyguanosine, T4 gene 32 protein, or glycerol. A kit may additionally include instructions for employing the kit components as well the use of any other reagent not included in the kit. Instructions can include variations that can be implemented.

[00162] The containers of the kits can generally include at least one vial, test tube, flask, bottle, syringe or other containers, into which a component may be placed, and preferably, suitably aliquoted. Where there is more than one component in the kit, the kit also can generally contain a second, third or other additional container into which the additional

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components can be separately placed. However, various combinations of components can be comprised in a container.

[00163] When the components of the kit are provided in one or more liquid solutions, the liquid solution can be an aqueous solution. However, the components of the kit can be provided as dried powder(s). Agents may be provided in aliquots for single-use or as stocks from which multiple uses, such as in a number of reaction, may be obtained. When reagents and/or components are provided as a dry powder, the powder can be reconstituted by the addition of a suitable solvent. Elements of the kit can further be provided, without limitation, in any suitable amounts and/or using any of the combinations (such as in the same kit or same container) described above or any other suitable combination known in the art.

[00164] The agents may be provided in a form that may be directly used in the methods of the disclosure, or in a form that requires preparation prior to use, such as in the reconstitution of lyophilized agents. For example, primers in kits provided herein may be in a lyophilized or non-lyophilized state. In some embodiments at least one primer provided in the kits is lyophilized. In some cases more than one, for example two, three, four, five, six or even more of the primers provided in a kit are in lyophilized state. In some examples, all of the primers contained in the kits of the present disclosure are lyophilized.

[00165] In certain embodiments, the concentration of the excess primer in the kits is at least 2 times, 3 times, 4 times, 5 times, 6 times, 7 times , 8 times , 9 times , 10 times , 11 times , 12 times , 13 times , 14 times , 15 times , 16 times , 17 times , 18 times , 19 times , 20 times , 21 times , 22 times, 23 times, 24 times, 25 times, 30 times, 35 times, 40 times, 45 times, 50 times, 55 times, 60 times, 65 times, 70 times, 75 times, 80 times, 85 times, 90 times, 95 times, 100 times, 150 times, 200 times, 250 times, 300 times, 350 times, 400 times, 450 times, 500 times, 550 times, 600 times, 650 times, 700 times, 750 times, 800 times, 850 times, 900 times, 950 times, or 1000 times that of the limiting primer.

[00166] In certain embodiments, the concentration of the excess primer in the kits is at most 1000 times, 900 times, 950 times, 850 times, 800 times, 750 times, 700 times, 650 times, 600 times, 550 times, 500 times, 450 times, 400 times, 350 times, 300 times, 250 times, 200 times, 150 times, 100 times, 95 times, 90 times, 85 times, 80 times, 75 times, 70 times, 65 times, 60 times, 55 times, 50 times, 45 times, 40 times, 35 times, 30 times, 25 times, 24 times, 23 times, 22 times, 21 times , 20 times, 19 times, 18 times, 17 times, 16 times, 15 times, 14 times, 13 times, 12 times, 11 times, 10 times, 9 times, 8 times, 7 times, 6 times, 5 times, 4 times, 3 times, or 2 times that of the limiting primer

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[00167] The length and sequence of the primes in the kits may be adjusted or modified such that the concentration-adjusted melting temperature of the limiting Primer is greater than or equal to the concentration-adjusted melting point of the excess primer. For example, the difference in the concentration-adjusted melting temperature of the limiting and the excess primer may be at least 0 °C , 1 °C, 1.5 °C, 2 °C, 2.5 °C , 3 °C, 3.5 °C , 4 °C, 4.5 °C , 5 °C, 5.5 °C, 6 °C, 6.5 °C, 7 °C, 7.5 °C, 8 °C, 8.5 °C, 9 °C, 9.5 °C, 10 °C or more. In some embodiments, the difference in the concentration-adjusted melting temperature of the limiting and the excess primer may be at least 10 °C , 9.5 °C, 9 °C, 8.5 °C , 8 °C, 7.5 °C , 7 °C, 6.5 °C , 6 °C, 5.5 °C, 5 °C, 4.5 °C, 4 °C, 3.5 °C, 3 °C, 2.5 °C, 2 °C, 1.5 °C, 1 °C, 0 °C or less.

[00168] The initial concentration of the limiting primers in the kits may be less than 500 nM. For example the initial concentration of the limiting primer may be less than 10 nM, 50 nM, 100 nM, 150 nM, 200 nM, 250 nM, 300 nM, 350 nM, 400 nM, 450 nM or 500 nM. In some embodiments the initial concentration of the limiting primers in the kits may be more than 500 nM. For example the initial concentration of the limiting primer may be more than 500 nM, 450 nM, 400 nM, 350 nM, 300 nM, 250 nM, 200 nM, 150 nM, 100 nM, 50 nM or 10 nM.

[00169] The at least one real time detection agent in the kits provided herein may be any reagent that generates a signal in the presence of an analyte. In some cases, these reagents are probes. The probes may be hybridization probes. The hybridization probes may be an oligonucleotide probe attached to a fluorophore and a quencher (e.g., a TAQMAN probe).

[00170] The kits may be for detection of pathogens, such as bacteria, virus, fungus, or helminth. For example, the kits provided herein may be used for detection of human immunodeficiency virus, *Plasmodium*, herpes simplex virus, dengue virus, hepatitis virus, human papilloma virus, influenza virus, *Mycobacterium*, Adenoviridae, Picornaviridae, Herpesviridae, Hepadnaviridae, Flaviviridae, Retroviridae, Orthomyxoviridae, Paramyxoviridae, Papovaviridae, Polyomavirus, Rhabdoviridae, Togaviridae, *Streptococcus*, *Pseudomonas*, *Shigella*, *Campylobacter*, *Salmonella*, *Neisseria gonorrhoeae,* or *Escherichia*.

XII. Analytical Techniques and Instrumentation

[00171] The methods provided herein are suitable for use with a variety of detection methods. For example, the methods may be applied using an analytical technique that measures the wavelength and intensity of a fluorescent signal. This may be accomplished by measuring the intensity of a signal across a spectrum of wavelengths, or by using band pass

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filters that restrict the passage of certain wavelengths of light, thereby allowing only light of certain wavelengths to reach a photodetector. Many real-time PCR and quantitative PCR instruments comprise an excitation light source and band pass filters that enable the detection of fluorescent signals in four colors (e.g., blue, green, yellow, and red). Therefore, the methods of the disclosure can be readily applied using instruments widely used in the art.

[00172] The methods are also compatible with end point PCR and digital PCR methods. Digital PCR methods (e.g., DROPLET DIGITAL (BIORAD) and DYNAMIC ARRAY (FLUIDIGM)) produce highly sensitive quantification of polynucleotide copy numbers. The methods provided herein can be easily integrated into these systems to drastically expand their throughput by allowing multiplexing in a droplet or a dynamic array.

[00173] In some cases, instruments may be modified or constructed, for example, to provide additional excitation light sources, at multiple wavelength, and/or to provide additional band pass filters or a capability of determining a complete spectrum. Including additional excitation sources would allow for the excitation of a larger variety of fluorophores. Including additional band pass filters, or modifying or constructing an instrument capable of determining an entire spectrum allows detection of a wider variety of emissions from fluorophores. These techniques can be used to increase the number of fluorophores that can be used with the methods described in this disclosure and, accordingly, to increase the number of analytes that be simultaneously detected.

XIII. Systems and Software

[00174] The methods of this disclosure may also be performed as part of a system. For example, one or more steps of the methods described herein may be performed by software contained on a computer readable medium. The software may be used to convert measurements made on an instrument (e.g., a PCR machine) into the presence or absence of an analyte. The software may be used to calculate the slopes of the linear region of the generated characteristic curves. The software may also be used to transform the measured signal into a transform domain and to thereby generate a transformed signal. The software may compare the transformed signal to a reference signal and based on the comparison determine the amount of analyte present in the sample. In some cases the determined amount of the analyte in the sample may be used to conclude whether a said analyte is present or absent in the sample.

[00175] In one example, the software is embedded in the instrument making the measurement. In another example, the software is installed on a computer attached to the

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instrument making the measurement. In another example, the software is in the "cloud" -- i.e., on a computer that another computer may communicate with through a network. Each of these elements may be connected to a controller that communicates with each of the elements and coordinates their actions. For example, a controller may initiate the measurement of the signal and generation of characteristic curves. The controller can execute software to transform the signal into a transform domain, thereby generating a transformed signal. The controller may also initiate the comparison of the measured signal or the transformed signal to a reference signal.

[00176] Alternatively, the controller may operate an oligonucleotide synthesis instrument that can synthesize the appropriate probes. After analysis of the sample on the instrument (which may also be automated), the controller can process the results of the analysis using the reference signal values. The controller may then provide these results to a user.

[00177] Figure 8 is a block diagram illustrating an example architecture of a computer system **100** that can be used in connection with example embodiments of the present disclosure. As depicted in Figure 8, the example computer system can include a processor **102** for processing instructions. Non-limiting examples of processors include: Intel Xeon™ processor, AMD Opteron™ processor, Samsung 32-bit RISC ARM 1176JZ(F)-S v1.0™ processor, ARM Cortex-A8 Samsung S5PC100™ processor, ARM Cortex-A8 Apple A4™ processor, Marvell PXA 930™ processor, or a functionally-equivalent processor. Multiple threads of execution can be used for parallel processing. In some embodiments, multiple processors or processors with multiple cores can also be used, whether in a single computer system, in a cluster, or distributed across systems over a network comprising a plurality of computers, cell phones, and/or personal data assistant devices.

[00178] As illustrated in Figure 8, a high speed cache **104** can be connected to, or incorporated in, the processor **102** to provide a high speed memory for instructions or data that have been recently, or are frequently, used by processor **102**. The processor **102** is connected to a north bridge **106** by a processor bus **108**. The north bridge **106** is connected to random access memory (RAM) **110** by a memory bus **112** and manages access to the RAM **110** by the processor **102**. The north bridge **106** is also connected to a south bridge **114** by a chipset bus **116**. The south bridge **114** is, in turn, connected to a peripheral bus **118**. The peripheral bus can be, for example, PCI, PCI-X, PCI Express, or other peripheral bus. The north bridge and south bridge are often referred to as a processor chipset and manage data

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transfer between the processor, RAM, and peripheral components on the peripheral bus **118**. In some alternative architectures, the functionality of the north bridge can be incorporated into the processor instead of using a separate north bridge chip.

[00179] In some embodiments, system **100** can include an accelerator card **122** attached to the peripheral bus **118**. The accelerator can include field programmable gate arrays (FPGAs) or other hardware for accelerating certain processing. For example, an accelerator can be used for adaptive data restructuring or to evaluate algebraic expressions used in extended set processing.

[00180] Software and data are stored in external storage **124** and can be loaded into RAM **110** and/or cache **104** for use by the processor. The system **100** includes an operating system for managing system resources; non-limiting examples of operating systems include: Linux, Windows™, MACOS™, BlackBerry OS™, iOS™, and other functionally-equivalent operating systems, as well as application software running on top of the operating system for managing data storage and optimization in accordance with example embodiments of the present disclosure.

[00181] In this example, system **100** also includes network interface cards (NICs) **120** and **121** connected to the peripheral bus for providing network interfaces to external storage, such as Network Attached Storage (NAS) and other computer systems that can be used for distributed parallel processing.

[00182] Figure 9 is a diagram showing a network **200** with a plurality of computer systems **202a**, and **202b**, a plurality of cell phones and personal data assistants **202c**, and Network Attached Storage (NAS) **204a**, and **204b**. In example embodiments, systems **202a**, **202b**, and **202c** can manage data storage and optimize data access for data stored in Network Attached Storage (NAS) **204a** and **204b**. A mathematical model can be used for the data and be evaluated using distributed parallel processing across computer systems **202a**, and **202b**, and cell phone and personal data assistant systems **202c**. Computer systems **202a**, and **202b**, and cell phone and personal data assistant systems **202c** can also provide parallel processing for adaptive data restructuring of the data stored in Network Attached Storage (NAS) **204a** and **204b**. Figure 9 illustrates an example only, and a wide variety of other computer architectures and systems can be used in conjunction with the various embodiments of the present disclosure. For example, a blade server can be used to provide parallel processing. Processor blades can be connected through a back plane to provide parallel processing.

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Storage can also be connected to the back plane or as Network Attached Storage (NAS) through a separate network interface.

[00183] In some example embodiments, processors can maintain separate memory spaces and transmit data through network interfaces, back plane or other connectors for parallel processing by other processors. In other embodiments, some or all of the processors can use a shared virtual address memory space.

[00184] Figure 10 is a block diagram of a multiprocessor computer system **300** using a shared virtual address memory space in accordance with an example embodiment. The system includes a plurality of processors **302a-f** that can access a shared memory subsystem **304**. The system incorporates a plurality of programmable hardware memory algorithm processors (MAPs) **306a-f** in the memory subsystem **304**. Each MAP **306a-f** can comprise a memory **308a-f** and one or more field programmable gate arrays (FPGAs) **310a-f**. The MAP provides a configurable functional unit and particular algorithms or portions of algorithms can be provided to the FPGAs **310a-f** for processing in close coordination with a respective processor. For example, the MAPs can be used to evaluate algebraic expressions regarding the data model and to perform adaptive data restructuring in example embodiments. In this example, each MAP is globally accessible by all of the processors for these purposes. In one configuration, each MAP can use Direct Memory Access (DMA) to access an associated memory **308a-f**, allowing it to execute tasks independently of, and asynchronously from, the respective microprocessor **302a-f**. In this configuration, a MAP can feed results directly to another MAP for pipelining and parallel execution of algorithms.

[00185] The above computer architectures and systems are examples only, and a wide variety of other computer, cell phone, and personal data assistant architectures and systems can be used in connection with example embodiments, including systems using any combination of general processors, co-processors, FPGAs and other programmable logic devices, system on chips (SOCs), application specific integrated circuits (ASICs), and other processing and logic elements. In some embodiments, all or part of the computer system can be implemented in software or hardware. Any variety of data storage media can be used in connection with example embodiments, including random access memory, hard drives, flash memory, tape drives, disk arrays, Network Attached Storage (NAS) and other local or distributed data storage devices and systems.

[00186] In example embodiments, the computer system can be implemented using software modules executing on any of the above or other computer architectures and systems.

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In other embodiments, the functions of the system can be implemented partially or completely in firmware, programmable logic devices such as field programmable gate arrays (FPGAs) as referenced in Figure 10, system on chips (SOCs), application specific integrated circuits (ASICs), or other processing and logic elements. For example, the Set Processor and Optimizer can be implemented with hardware acceleration through the use of a hardware accelerator card, such as accelerator card **122** illustrated in Figure 8.

XIV. Services

[00187] The methods provided herein may also be performed as a service. For example, a service provider may obtain the identity of a series of analytes that a customer wishes to analyze. The service provider may then encode each analyte to be detected by any of the methods described herein and provide appropriate reagents to the customer for the assay. The customer may perform the assay and provide the results to the service provider for decoding. The service provider may then provide the decoded results to the customer. The customer may also encode analytes, generate probes, and/or decode results by interacting with software installed locally (at the customer's location) or remotely (e.g., on a server reachable through a network). Exemplary customers include clinical laboratories, physicians, manufacturers, and the like.

EXAMPLES

Example 1: General Materials & Methods

1. DNA Sequences, Primers, and Probes

[00188] Synthesis of the oligonucleotides for the following examples is done by INTEGRATED DNA TECHNOLOGIES (Coralville, Iowa). HSV-1 and HSV-2 sequences from the Los Alamos National Laboratory reference sequence are selected for the exemplary study.

[00189] The 198-mer HSV-2 oligonucleotide is reconstituted with TE buffer at a concentration of 1.7 μM. Forward and reverse primers A for the HSV-2 oligonucleotide are reconstituted with TE buffer at a concentration of 100 μM and 1 μM respectively. Forward and reverse primers B for the HSV-2 oligonucleotide are reconstituted with TE buffer at a concentration of 40 μM and 1 μM respectively. Forward and reverse primers C for the HSV-2 oligonucleotide are reconstituted with TE buffer at a concentration of 3 μM and 1 μM respectively. A TAQMAN sense probe ("HSV-2_probe1") is synthesized with a 6-FAM fluorophore at the 5' end and a BHQ-1 quencher at the 3' end.

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[00190] The 199-mer HSV-1 oligonucleotide is reconstituted with TE buffer at a concentration of 1.5 μM. Forward and reverse primers D for the HSV-1 oligonucleotide are reconstituted with TE buffer at a concentration of 1 μM and 100 μM respectively. Forward and reverse primers E for the HSV-1 oligonucleotide are reconstituted with TE buffer at a concentration of 1 μM and 40 μM respectively. Forward and reverse primers F for the HSV-1 oligonucleotide are reconstituted with TE buffer at a concentration of 1 μM and 3 μM respectively. A TAQMAN sense probe ("HSV-1_probe1") is synthesized with a 6-FAM fluorophore at the 5' end and a BHQ-1 quencher at the 3' end. An additional TAQMAN sense probe ("HSV-1_probe2") is synthesized with Cy3 at the 5' end and BHQ-2 at the 3' end.

2. Polymerase Chain Reactions

[00191] All PCR reactions are performed in a ROCHE 480 LIGHTCYCLER instrument. Forty-five thermal cycles are performed, with a 60 second hot-start at 95°C. The cycling conditions for denaturation, annealing, and extension are 45 seconds, 50 seconds, and 60 seconds at 95°C, 65°C, and 70°C, respectively. Each experiment is run in quintuplicate, with a reaction volume of 15 μL. Fluorescence measurements are obtained in the following channels after every annealing step: 483 nm to 533 nm (FAM), 523 nm to 568 nm (Cy3), 558 nm to 610 nm (ROX), and 615 nm to 670 nm (Cy5).

Example 2: Detection of Polynucleotide Markers from Pathogens

[00192] This example describes the detection of polynucleotides from HSV-1 and HSV-2.

1. PCR Reaction Components

[00193] Three PCR reactions are assembled, using the reagents described in Table 1.

Table 1. Reagents for the three PCR reactions.

Reaction No.	Type of Reagent	Reagent	Concentration	Volume
1		UltraPure Water	-	34 μL
		Taq 5x Master Mix	-	42 μL
	Template	HSV-2 Template	17 nM	2 μL
	Primers	HSV-2 FWD Primer A	100 nM	2 μL
		HSV-2 RVS Primer A	1 nM	2 μL

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		HSV-2FWD Primer B	40 nM	2 µL
		HSV-2 RVS Primer B	1 nM	2 µL
		HSV-2 FWD Primer C	3 nM	2 µL
		HSV-2 RVS Primer C	1 nM	2 µL
	Probe	HSV-2 Probe 1	1 nM	2 µL
2		UltraPure Water	-	34 µL
		Taq 5x Master Mix	-	42 µL
	Template	HSV-1 Template	17 nM	2 µL
	Primers	HSV-1 FWD Primer D	1 nM	2 µL
		HSV-1 RVS Primer D	100 nM	2 µL
		HSV-1 FWD Primer E	1 nM	2 µL
		HSV-1 RVS Primer E	40 nM	2 µL
		HSV-1 FWD Primer F	1 nM	2 µL
		HSV-1 RVS Primer F	3 nM	2 µL
	Probe	HSV-1 Probe 1	1 nM	2 µL
3		UltraPure Water	-	34 µL
		Taq 5x Master Mix	-	42 µL
	Template	HSV-2 Template	17 nM	2 µL
		HSV-1 Template	17 nM	2 µL
	Primers	HSV-2 FWD Primer A	100 nM	2 µL
		HSV-2 RVS Primer A	1 nM	2 µL
		HSV-2 FWD Primer B	40 nM	2 µL
		HSV-2 RVS Primer B	1 nM	2 µL
		HSV-2 FWD Primer C	3 nM	2 µL
		HSV-2 RVS Primer C	1 nM	2 µL
		HSV-1 FWD Primer D	1 nM	2 µL
		HSV-1 RVS Primer D	100 nM	2 µL
		HSV-1 FWD Primer E	1 nM	2 µL
		HSV-1 RVS Primer E	40 nM	2 µL
		HSV-1 FWD Primer F	1 nM	2 µL
		HSV-1 RVS Primer F	3 nM	2 µL
	Probe	HSV-2 Probe 1	1 nM	2 µL

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		HSV-1 Probe 1	1 nM	2 μL

[00194] The signal generated from PCR reactions 1-3 is measured in arbitrary fluorescence units (AFU) and plotted as a function of number of cycles. A typical curve generated by a uniplex PCR reaction (for example reaction 1 and 2) is shown in Figure 5.

[00195] A typical curve generated from a multiplex PCR reaction of the disclosure (for example, from reaction 3 where two targets are present in a single multiplexed reaction) is shown in Figure 6. The curves generated are analyzed by curve fitting algorithms. These algorithms calculate the slope of the reaction in its linear regime. Each target present in these multiplexed reactions can be uniquely identified as long as the curves are in the linear dynamic range of the fluorometer.

Example 3: Generation of characteristic curves

[00196] Figure 5 shows three consecutive plateaus separated by normal amplification regions. Such plot may be generated by the different reactions depending on the starting reagents.

(i) Three independent sequences, A, B, and C are present at the same time and addressed by standard PCR with a single intercalator dye and symmetric primers optimized for maximal exponential performance but designed in such a way that they hybridize at different temperatures. The reaction is programmed in such a way that it runs for the first third of the cycles at hybridization temperature for the primers of sequence A, which is too high for the primers of B and C to hybridize. The second third of the cycles is then run at the temperature suitable for the primers of B to hybridize but still too hot for the primers of C. Finally, the final third of the cycles are run at temperature low enough for C to hybridize. As there is normal amplification in the first third of the cycles, the conclusion is that A is present. As there is normal amplification in the second third of the cycles, the conclusion is that B is present. As there is normal amplification in the final third of the cycles, the conclusion is that C is present. Thus this curve decodes to positive results for A, B, and C under this setup. If any of the plateaus were missing, we would conclude that the respective sequences were missing.

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(ii) A multiplex PCR reactions contains a nested set of two pairs of symmetric primers A and B for sequence #1 and a normal set of primer C for sequence #2. The outer primers of sequence #1 A have the highest melting temperature, followed by the inner primers sequence #1 B, followed by the primer C of sequence #2. The PCR program runs the first third at hybridization temperature that allows only the outer set of primers to sequence #1 to hybridize, leading to normal symmetric exponential amplification for the outer set of sequence #1. The reaction saturates by the end of the first third of the cycles. Then the program lowers the hybridization temperature so that the inner pair hybridizes but it is still too hot for the primers of sequence #2. Thus the second third sees normal symmetric exponential amplification of the inner primers. Finally, in the end third of the cycles, the temperature is low enough for sequence #2 to amplify symmetrically and exponentially and saturate by the end of the reaction. Looking at the curve and the reagent setup, it is concluded that both sequences are present.

(iii) Three pairs of nested primers A, B, and C are used for the same sequence. A, B, and C in the graph indicate the correct workings of the assay and the successful detection of the sequence. The use of three pairs of nested primers as opposed to just one primer pair may provide increased reliability. For example, if a single pair of primer is used, and the primers misprime on another sequence, e.g. in genomic DNA, amplification would still happen and the intercalator dye would report it, resulting in a false positive. This may not be possible when nested primer pairs are used because not all plateaus will be present. For example, if the sequence is not present but the outermost pair misprimes, A will be seen as normal, but B and C will not happen, so the results would be negative.

[00197] Figure 6 can be interpreted in very similar terms as described above for Figure 5, except that the amplification is linear asymmetric, so the respective coding is by asymmetrically chosen primer pairs. The difference between A and D is the concentration of the limiting primer, which can be used as a coding method. For example, if the curve looks like DEF instead of ABC, then a different set of target sequences has been detected using the same multiplexed assay.

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Example 4: Discrimination of curves by basis transformation

The qPCR signals generated from a 3-target multiplexed qPCR assay similar to the one in Example 2 are transformed using a basis transformation to obtain frequency space signatures for the reactions. These frequency space signatures may be used to resolve which targets are present. In this example, Target 1 has a unique signature that is reproducible across qPCR reaction replicates. The target is identified by a specific (height, width) tuple that is specific only to Target 1. For this multiplexed reaction chemistry, a single peak signature of (0.096, 14) identifies Target 1. Furthermore, Target 2 has a distinct signature from Target 1. When Target 2 is present with this qPCR reaction chemistry, the kinetic signature observed is a dual peak curve with (height, width) tuples of (0.01, 7) and (0.1, 10). Furthermore, when Targets 1 and 2 are present in a qPCR reaction with this multiplex assay, the generated kinetic signature is unique and highly reproducible. In this example, the signature is dual peaked with (height, width) tuples of (0.018, 7) and (0.09, 12). With this example assay set, we demonstrate the proof-of-principle of unambiguous discrimination of targets using basis-transformed qPCR signals. Another typical frequency space signature obtained from the multiplexed PCR reactions of the disclosure is shown in Figure 7.

Example 5: Detection of one or more nucleic acid analytes

[00198] This example illustrates a method for the detection of one or more of a nucleic acid analyte in a sample.

[00199] In one aspect, the presence or absence of a target nucleic acid analyte in a sample is detected. An amplification reaction is performed on the sample and a signal generated during the amplification reaction is measured. A kinetic signature is generated from the measured signal, which is compared to a reference signature to determine whether the generated kinetic signature corresponds to the reference signature. Comparison of the generated kinetic signature to the reference signature allows for detection of the presence or absence of the target nucleic acid analyte in the sample. For example, a nucleic acid analyte is determined to be present in a sample if the generated kinetic signature corresponds to the reference signature. Conversely, a nucleic acid analyte is determined to be absent if the generated kinetic signature does not correspond to the reference signature.

[00200] In another aspect, the presence or absence of each of a plurality of target nucleic acid analytes in a sample is detected. A primer set is added to the sample that comprises at least two primer pairs. At least one of the primer pairs comprises a limiting primer, and each primer pair defines a region of one of the target nucleic acid analytes to be

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amplified. An amplification reaction is performed on the sample and a signal generated during the amplification reaction is measured. The measured signal is used to determine the presence or absence of the each of the plurality of target nucleic acid analytes. In some aspects, the concentration of a limiting primer may be sufficiently lower relative to other primers in the amplification reaction so as to affect the generated signal. For example, where the generated signal is plotted as an amplification curve, the concentration of the limiting primer may be sufficiently lower such that the generated amplification curve is not sigmoidal.

[00201] In yet another aspect, the presence or absence of two or more of a plurality of target nucleic acids in a sample is detected. A first and second primer pair is added to the sample, each primer pair defining a region of a nucleic acid analyte and having a first annealing temperature and a second annealing temperature, respectively. A thermocycling amplification reaction is performed on the sample. The thermocycling amplification reaction comprises a set of temperature cycles. At least one temperature cycle is performed that has a minimum temperature, *i.e.* an annealing temperature, that is not greater than the annealing temperature of the first primer pair that corresponds to the first annealing temperature, but is greater than the annealing temperature of the second primer pair, *i.e.* the second annealing temperature. At least one other temperature cycle is performed that has a minimum temperature, *i.e.* an annealing temperature, that is not greater than the annealing temperature of the second primer pair, *i.e.* the second annealing temperature. A signal generated during the amplification reaction is measured, and the measured signal is used to determine the presence or absence of each of the two or more of a plurality of target nucleic acid analytes.

[00202] Unless the context requires otherwise, throughout the present specification and claims, the word "comprise" and variations thereof, such as, "comprises" and "comprising," which is used interchangeably with “including,” “containing,” or “characterized by,” is inclusive or open-ended language and does not exclude additional, unrecited elements or method steps. The phrase “consisting of” excludes any element, step, or ingredient not specified in the claim. The phrase “consisting essentially of” limits the scope of a claim to the specified materials or steps and those that do not materially affect the basic and novel characteristics of the claimed invention. The present disclosure contemplates embodiments of the invention compositions and methods corresponding to the scope of each of these phrases. Thus, a composition or method comprising recited elements or steps contemplates particular embodiments in which the composition or method consists essentially of or consists of those elements or steps.

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[00203] Reference throughout this specification to "one embodiment" or "an embodiment" or "an aspect" means that a particular feature, structure or characteristic described in connection with the embodiment is included in at least one embodiment of the present invention. Thus, the appearances of the phrases "in one embodiment" or "in an embodiment" in various places throughout this specification are not necessarily all referring to the same embodiment. Furthermore, the particular features, structures, or characteristics may be combined in any suitable manner in one or more embodiments.

[00204] The various embodiments described above can be combined to provide further embodiments. These and other changes can be made to the embodiments in light of the above-detailed description. In general, in the following claims, the terms used should not be construed to limit the claims to the specific embodiments disclosed in the specification and the claims, but should be construed to include all possible embodiments along with the full scope of equivalents to which such claims are entitled. Accordingly, the claims are not limited by the disclosure.

[00205] While preferred embodiments of the present invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein may be employed in practicing the invention. It is intended that the following claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.

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CLAIMS

WHAT IS CLAIMED IS:

1. A method of detecting the presence or absence of a target nucleic acid analyte in a sample, the method comprising:
    a) performing an amplification reaction on the sample;
    b) measuring a signal generated during the amplification reaction and generating a kinetic signature from the measured signal; and
    c) comparing the generated kinetic signature to a reference signature and determining whether the generated kinetic signature corresponds with the reference signature, thereby detecting the presence or absence of the target nucleic acid analyte in the sample.
2. The method of claim 1, wherein the kinetic signature comprises a signal selected from an electromagnetic signal, a wavelength, and a fluorescence emission signal.
3. The method of claim 1, wherein the kinetic signature is plotted as a curve.
4. The method of claim 1, wherein the reference signature corresponds with a kinetic signature generated by amplification of the target nucleic acid analyte under one or more members of a set of constrained amplification parameters, the set of constrained amplification parameters comprising any combination of: (i) a range of forward and/or reverse primer concentrations; (ii) a range of polymerase concentrations; (iii) polymerase type; (iv) a range of nucleotide concentrations; (v) chromophore concentration; and (vi) chromophore type; (vii) a number of thermocycles; (viii) rate of thermocycling; (ix) one or more thermocycle stage temperatures; and (x) one or more thermocycle stage time lengths; (xi) a range of target-specific molecular probes; (xii) primers associated with one or more target-specific probes; and (xiii) a concentration of one or more enzyme cofactors.
5. The method of claim 4, wherein the amplification reaction is carried out within one or more of the constrained amplification parameters.
6. The method of claim 3, wherein the at least one of an exponential region, a linear region, and a plateau region of the curve occurs over at least 4 thermocycles or over at least 8 thermocycles.

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7. A method of detecting the presence or absence of each of a plurality of target nucleic acid analytes in a sample, the method comprising:
    a) adding a primer set, the primer set comprising at least two primer pairs, to the sample, at least one of the primer pairs comprising a limiting primer, and each primer pair defining a region of one of the target nucleic acid analytes to be amplified;
    b) performing an amplification reaction on the sample;
    c) measuring a signal generated during the amplification reaction; and
    d) making a determination as to whether each of the plurality of target nucleic acid analytes is present based on the measured signal.
8. The method of claim 7, wherein step c) further comprises a step of generating a kinetic signature from the measured signal, and wherein step d) further comprises a step of
comparing the kinetic signature to a reference signature, the reference signature corresponding with a kinetic signature generated by an amplification of one or more of the plurality of target nucleic acid analytes with the primer set, thereby making the determination.
9. The method of claim 7, wherein the primer set comprises one forward primer and two reverse primers or two forward primers and one reverse primer.
10. The method of claim 7, wherein the primer set comprises two or more nested primers.
11. The method of claim 7, wherein the signal is generated by exciting a molecule that binds an amplification product.
12. The method of claim 7, wherein
    (A) the primer set comprises first and second primer pairs, the first primer pair having a first annealing temperature and the second primer pair having a second annealing temperature which is lower than the first annealing temperature, and the first primer pair defining a region of one of the target nucleic acid analytes, the second primer pair defining a region of another of the nucleic acid analytes;
    and wherein
    (B) the amplification reaction comprises thermocycling of a set of temperature cycles comprising at least one temperature cycle having a

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minimum temperature which is both (i) not greater than the first annealing temperature, and (ii) greater than the second annealing temperature, the reaction further comprising at least one other temperature cycle having a minimum temperature which is not greater than the second annealing temperature.

13. The method of claim 12, wherein the amplification reaction comprises at least one temperature cycle which has a minimum temperature not greater than the first annealing temperature and greater than the second annealing temperature, followed by at least one temperature cycle which has a minimum temperature not greater than the second annealing temperature.
14. The method of claim 12, wherein the amplification reaction comprises at least one temperature cycle which has a minimum temperature not greater than the second annealing temperature, followed by at least one temperature cycle which has a minimum temperature which is both (x) not greater than the first annealing temperature and (y) greater than the second annealing temperature.
15. The method of claim 7, wherein step c) further comprises a step of generating a kinetic signature from the measured signal, and wherein step d) further comprises a step of comparing the kinetic signature to a reference signature, the reference signature corresponding to a signal generated when one or more of the plurality of target nucleic acid analytes is amplified with the primer set by a thermocycling reaction comprising the set of temperature cycles, thereby making the determination.
16. The method of claim 12, wherein the limiting primer is present at a concentration sufficiently lower that the concentration of a non-limiting primer to generate a characteristic kinetic signature.
17. The method of claim 12, wherein the first and/or second annealing temperatures are determined by the limiting primer.
18. A method of detecting the presence or absence of two or more of a plurality of target nucleic acid analytes in a sample, the method comprising:
    a) adding a primer set comprising first and second primer pairs to the sample, the first primer pair having a first annealing temperature and the second primer pair having a second annealing temperature which is lower than the first annealing temperature, and the first primer pair defining a region of

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one of the target nucleic acid analytes, the second primer pair defining a region of another of the nucleic acid analytes;

b) performing a thermocycling amplification reaction on the sample, the reaction comprising a set of temperature cycles comprising at least one temperature cycle having a minimum temperature which is both (i) not greater than the first annealing temperature, and (ii) greater than the second annealing temperature, the reaction further comprising at least one other temperature cycle having a minimum temperature which is not greater than the second annealing temperature;

c) measuring a signal generated during the amplification reaction; and

d) making a determination as to the presence or absence of the two or more of the nucleic acid analytes based on the generated signal.

19. The method of claim 18, wherein step c) further comprises a step of generating a kinetic signature from the measured signal, and wherein step d) further comprises a step of

comparing the kinetic signature to a reference signature, the reference signature corresponding with a signal generated when one or more of the plurality of target nucleic acid analytes is amplified with the primer set by a thermocycling reaction comprising the set of temperature cycles, thereby making the determination.

20. The method of claim 18, wherein the first and second primer pairs comprise one forward primer and two reverse primers.

21. The method of claim 18, wherein the primer set comprises at least one pair of nested primers.

22. The method of claim 18, wherein the amplification reaction comprises at least one temperature cycle which has a minimum temperature not greater than the first annealing temperature and greater than the second annealing temperature, followed by at least one temperature cycle which has a minimum temperature not greater than the second annealing temperature.

23. The method of claim 18, wherein the amplification reaction comprises at least one temperature cycle which has a minimum temperature not greater than the second annealing temperature, followed by at least one temperature cycle which has a minimum temperature which is both (x) not greater than the first annealing temperature and (y) greater than the second annealing temperature.

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24. The method of claim 18, wherein at least one of the primer pairs has a limiting primer, the limiting primer having a concentration-adjusted melting temperature that is equal to or greater than a non-limiting primer.
25. The method of claim 18, wherein the method comprises detecting the presence of n target nucleic acid analytes using n primer pairs and wherein the minimum temperature of each temperature cycle is lower than the minimum temperature of the previous temperature cycle.
26. The method of claim 18, wherein one or more primer of a primer pair is linked to an antibody.
27. A method of detecting a nucleic acid analyte, comprising:
    a. performing a nested asymmetric amplification reaction on a sample, wherein said nested asymmetric amplification reaction comprises:
    at least three nested primers, wherein at least one pair of primers of said at least three nested primer comprises a limiting primer and an excess primer;
    b. measuring a signal generated during said nested asymmetric amplification reaction; and
    c. determining whether said analyte is present based on said signal.
28. The method of claim 27, wherein said at least three nested primers bind to a single analyte.
29. The method of claim 27, wherein said limiting primer has a concentration-adjusted melting temperature that is equal to or greater than said excess primer.
30. The method of claim 27, wherein said analyte is encoded by at least one additional value selected from the group consisting of a value from said signal and a value from an additional signal.
31. The method of claim 27, wherein said at least one additional value is selected from the group consisting of a Förster resonance energy transfer (FRET) emission intensity, a FRET emission wavelength, an electrochemical signal, a chemiluminescence wavelength, a chemiluminescence intensity, a fluorescence bleaching rate, and a chemiluminescence bleaching rate, wherein said at least one additional value is Förster resonance energy transfer (FRET) emission intensity.
32. A method of determining an amount of nucleic acid analyte, comprising:

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a. performing an amplification reaction on a sample;
b. measuring a signal generated during said amplification reaction;
c. transforming said signal into a frequency domain, thereby generating a transformed signal;
d. comparing a value from said transformed signal to a value of reference signal; and
e. determining said amount of said analyte based on said comparing.

33. The method of claim 32, wherein said transforming is performed by a method selected from the group consisting of a Fourier Transform, a fast Fourier transform, a discrete Fourier transform, a discrete-time Fourier transform, and a wavelet transform, wherein said method detects the presence or absence of a plurality of analytes, and wherein said reference signal is in a database of reference signals.

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FIG. 1

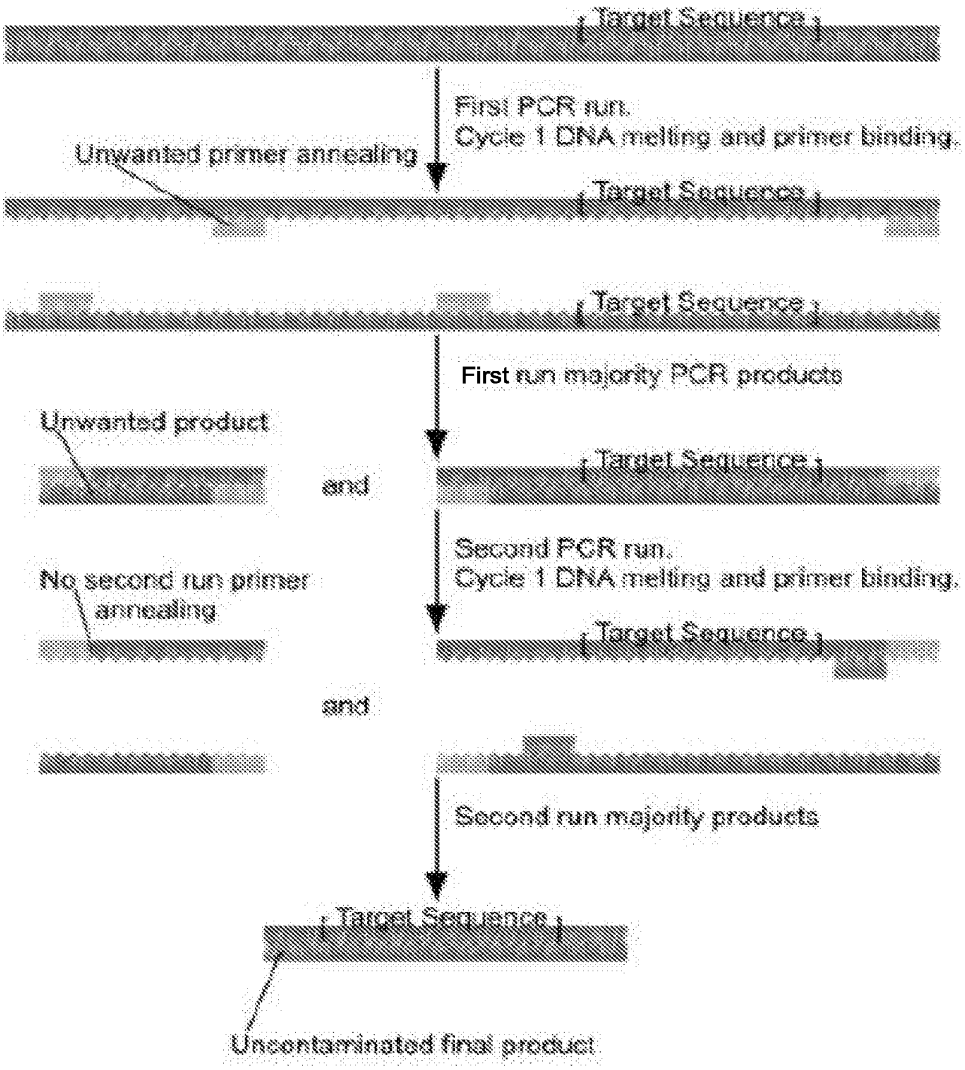

Target Sequence
First PCR run.
Cycle 1 DNA melting and primer binding.
Unwanted primer annealing
Target Sequence
Target Sequence
First run majority PCR products
Unwanted product
and
Target Sequence
Second PCR run.
Cycle 1 DNA melting and primer binding.
No second run primer annealing
Target Sequence
and
Second run majority products
Target Sequence
Uncontaminated final product

FIG. 2

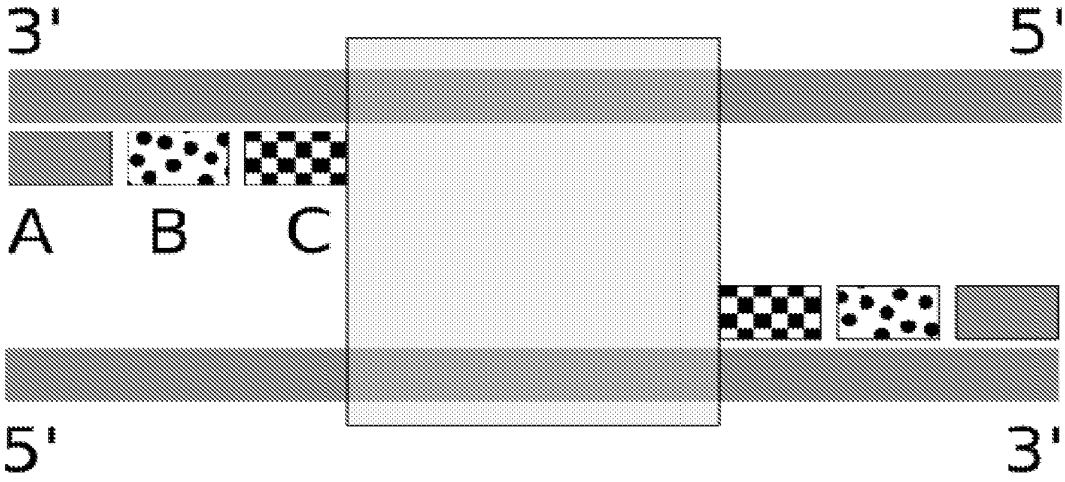


FIG. 3

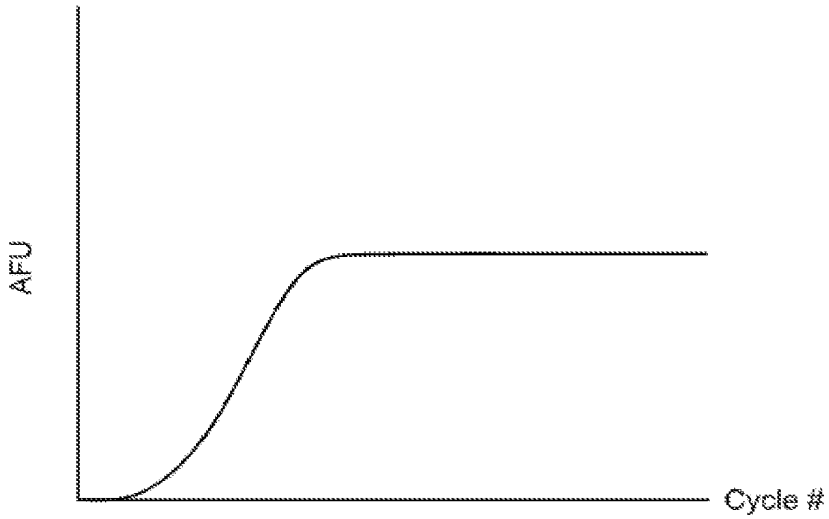


FIG. 4

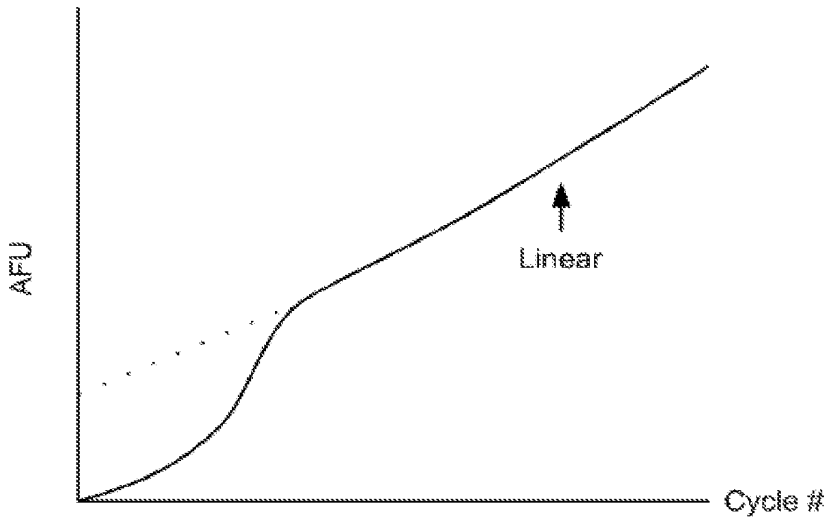


FIG. 5

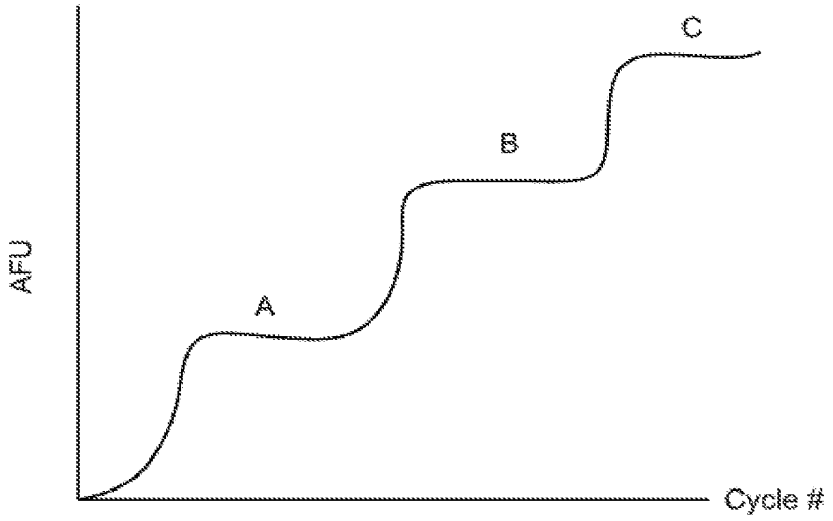


FIG. 6

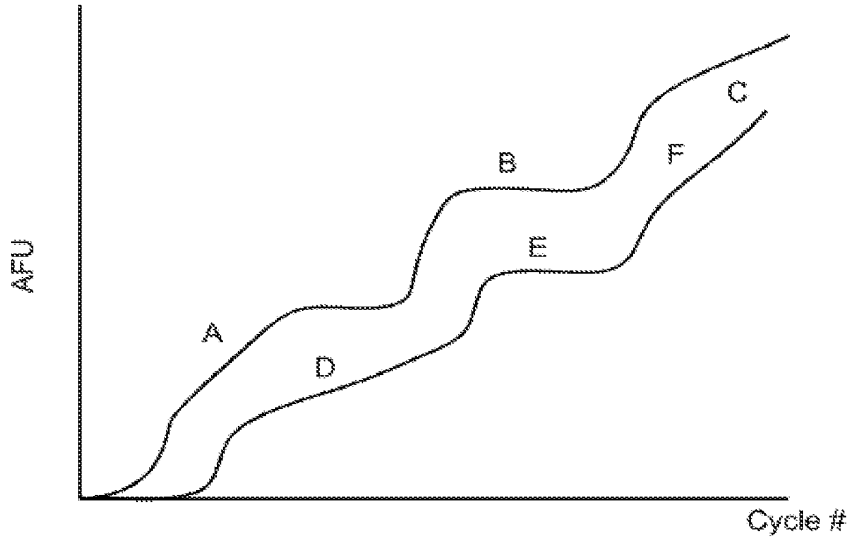


FIG. 7

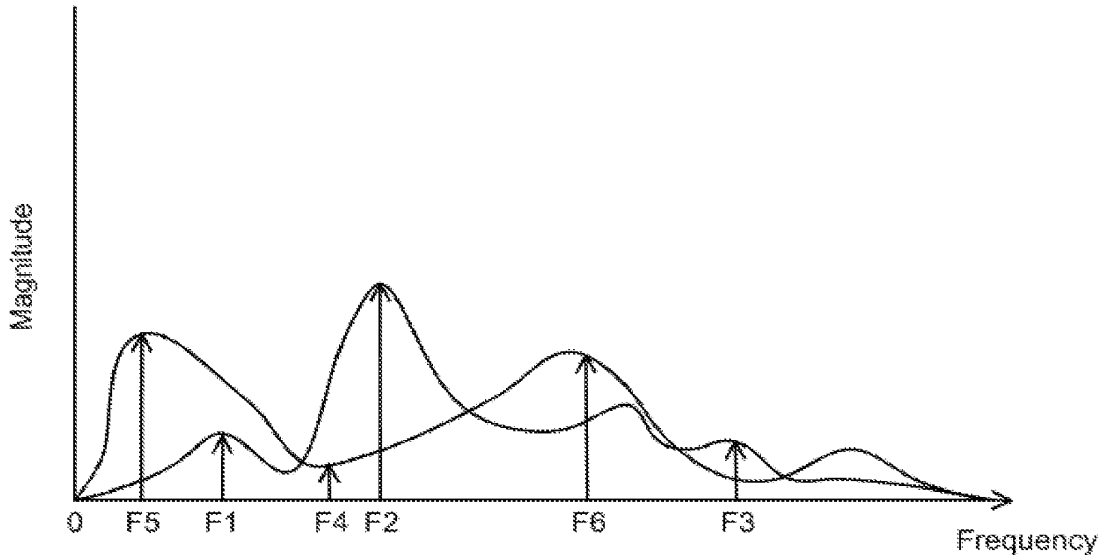


FIG. 8

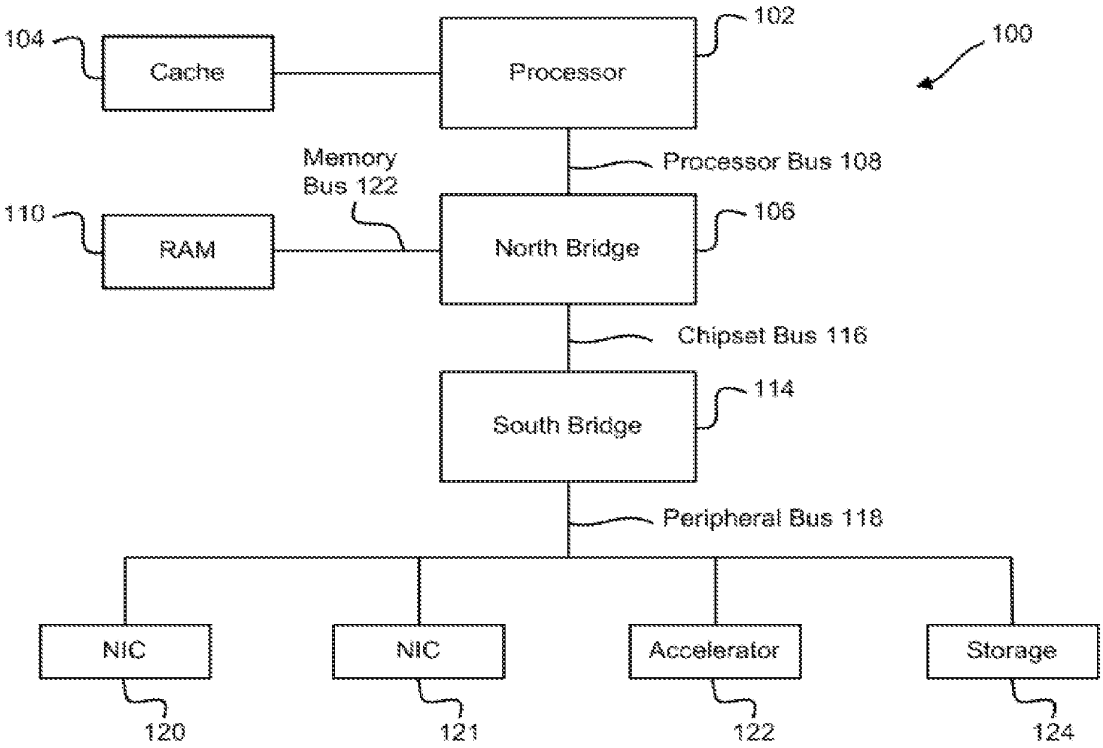

104
Cache
Processor
102
100
Memory
Bus 122
Processor Bus 108
110
RAM
North Bridge
106
Chipset Bus 116
South Bridge
114
Peripheral Bus 118
NIC
NIC
Accelerator
Storage
120
121
122
124

FIG. 9

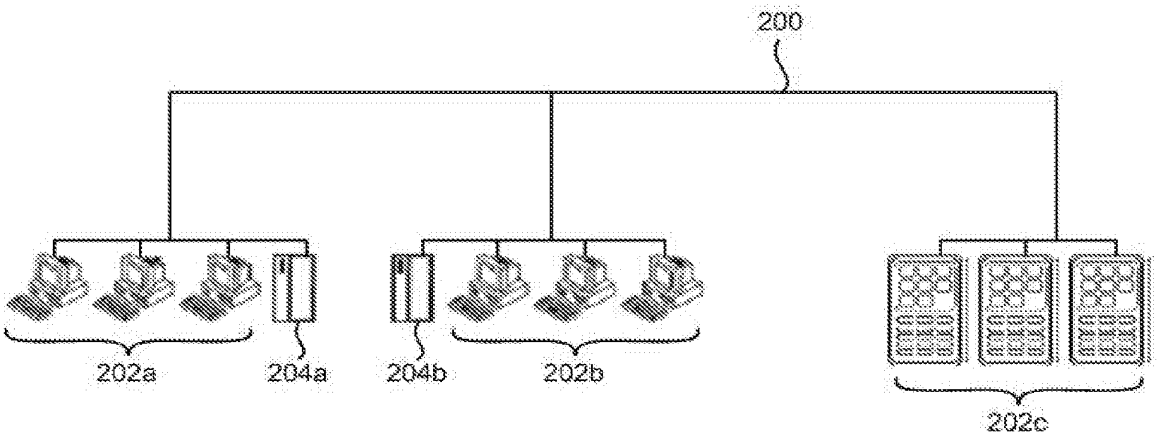
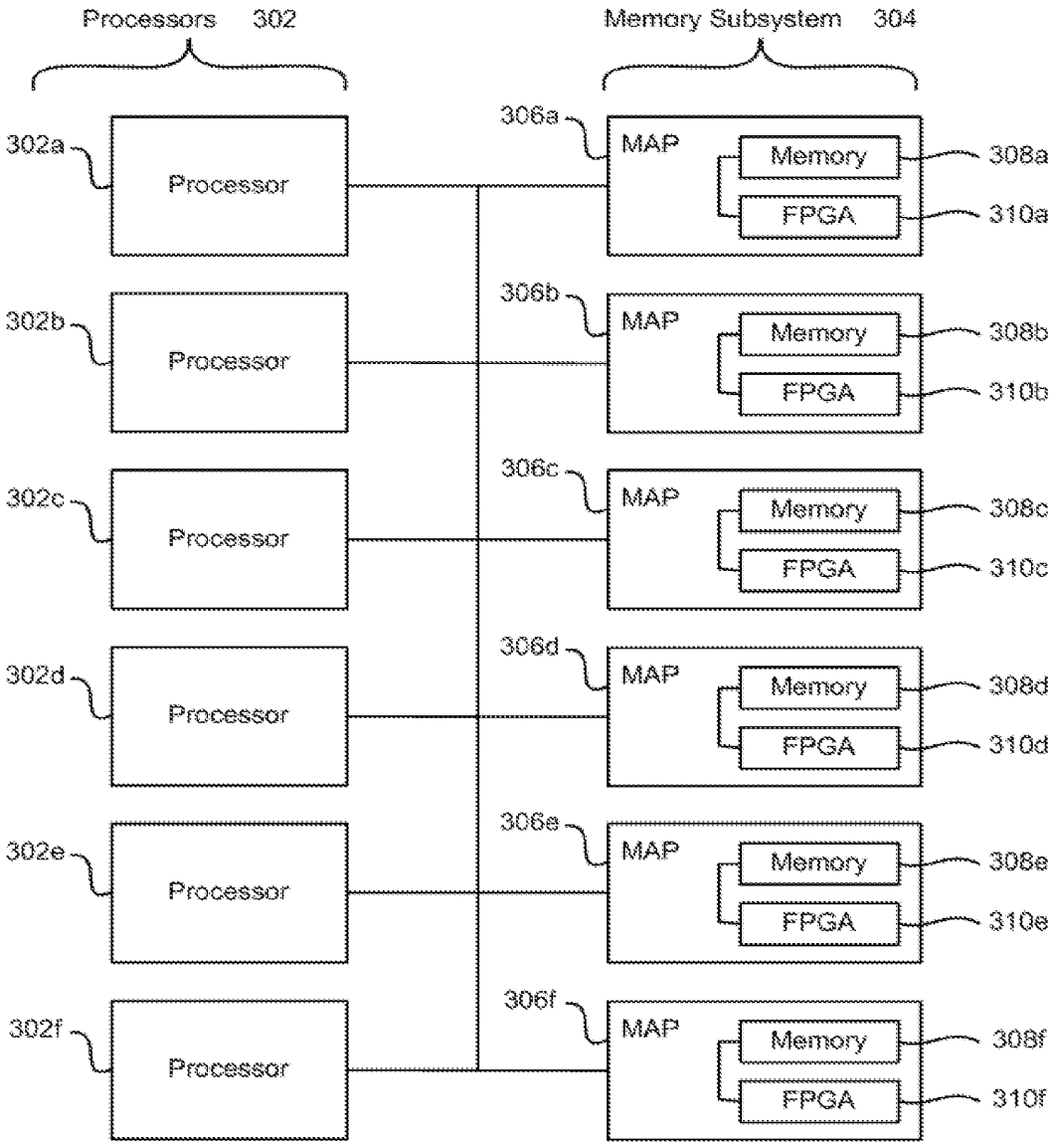


FIG. 10


Processors 302
Memory Subsystem 304
302a
Processor
306a
MAP
Memory
FPGA
308a
310a
302b
Processor
306b
MAP
Memory
FPGA
308b
310b
302c
Processor
306c
MAP
Memory
FPGA
308c
310c
302d
Processor
306d
MAP
Memory
FPGA
308d
310d
302e
Processor
306e
MAP
Memory
FPGA
308e
310e
302f
Processor
306f
MAP
Memory
FPGA
308f
310f

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 17/37682

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12Q 1/68, C12P 19/34 (2017.01)
CPC - C12Q 1/6844, C12N 15/10

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)
See Search History Document

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2007/0072211 A1 (NEWTON et al.,) 29 March 2007 (29.03.2007) Claim 1; Claim 3; para [0007]	1-6

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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

Date of the actual completion of the international search	Date of mailing of the international search report
22 August 2017	16 NOV 2017
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 17/37682

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

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

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

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

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

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

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

----------Please see continuation in first extra sheet----------

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

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

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

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-6

Remark on Protest

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

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

☐ No protest accompanied the payment of additional search fees.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 17/37682

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

Group I: Claims 1-6, drawn to a method of detecting an analyte comprising generating a kinetic signature from a signal generated during an amplification reaction .

Group II: Claims 7-17, 27-31, drawn to methods of detecting target analytes comprising performing an amplification reaction by using a primer set that comprises at least one limiting primer.

Group III: Claims 18-26, drawn to a method of detecting an analyte comprising performing a amplification on a sample by using a primer set that comprises primer pairs with different annealing temperatures.

Group IV: Claims 32-33, drawn to a method of determining an amount of nucleic acid analyte, comprising generating transformed signal by transforming a signal generated during an amplification reaction into a frequency domain.

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

Special Technical Features

Group I requires generating a kinetic signature from a signal generated during an amplification reaction, not required by Groups II, III, and IV.

Group II requires the detection of target analytes by using a set of primers comprising at least one limiting primers, not required by Groups I, III, and IV.

Group III requires the detection of target analytes by using a set of primers by using a primer set that comprises primer pairs with different annealing temperatures, not required by Groups I, II, and IV.

Group IV requires generating transformed signal by transforming a signal generated during an amplification reaction into a frequency domain, not required by Groups I, II, and III.

Common Technical Features

The feature shared by Groups I, II, III, and IV is a method of detecting a nucleic acid analyte in a sample, the method comprising:
performing an amplification reaction on the sample;
measuring a signal generated during the amplification reaction; and
detecting the presence or absence of the target nucleic acid analyte in the sample.

Another feature shared by Groups I and IV is the method step of: comparing a test value to a reference value and determining whether the test value corresponds with the reference value.

However, these shared technical features do not represent a contribution over prior art, because the shared technical features are taught by US 2007/0072211 A1 to Newton et al., (hereinafter 'Newton').

Newton teaches a method of detecting the presence or absence of a target nucleic acid analyte in a sample (para [0011] "the invention comprises methods for identifying one or more nucleic acid targets in a sample"), the method comprising:
performing an amplification reaction on the sample (para [0011] "Such methods comprise: performing asymmetric kinetic PCR in a reaction mixture containing one or more labeled 5'-nuclease probes and one or more labeled hybridization probes");
measuring a signal generated during the amplification reaction (para [0011] "monitoring one or more growth curves from the kinetic PCR"); and
detecting the presence or absence of the target nucleic acid analyte in the sample (para [0011] "thus, identifying the nucleic acid targets in the sample").

Newton further teaches the method step of: comparing a test value to a reference value and determining whether the test value corresponds with the reference value (para [0011] "correlating the melting curve or annealing curve, thus produced, to standard melting or annealing curves of completely or partially complementary probes of known nucleic acid targets or the same probe tested against a known sample, thus, identifying the nucleic acid targets in the sample").

As the technical features were known in the art at the time of the invention, they cannot be considered special technical features that would otherwise unify the groups.

-----Please see continuation in next extra sheet----------

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International application No.
PCT/US 17/37682

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

Another feature shared by Group II and III is the detection of target analytes by using a set of primers.

However, this shared technical feature does not represent a contribution over prior art, because the shared technical feature is taught by US 2010/0233686 A1 to Higuchi et al., (hereinafter 'Higuchi').

Higuchi teaches detection of target analytes by using a set of primers (para [0012] "The present invention is a method for amplification, detection and quantification of one or more target nucleic acids in a single sample container comprising the steps of contacting a sample, suspected of containing one or more target nucleic acids, with at least one set of oligonucleotides, each oligonucleotide within the set labeled with the same one or more reporter moieties"; para [0042] "amplification of several target nucleic acid sequences in the same reaction vessel. Each tube contains one or more sets of probes, each set labeled with the same fluorescent reporter. However, within the set, each probe is characterized by a unique melting temperature with its target nucleic acid. For the maximum multiplex ability, several sets of probes are present in the same reaction vessel"; para [0057] "In some embodiments, the present invention involves asymmetric PCR. In an asymmetric PCR mixture, one of the amplification primers is present in greater amount than the other primer. The primers are referred to as "excess primer" and "limiting primer" respectively. The nucleic acid strands resulting from the extension of these primers are referred to as "excess strand" and "limiting strand" respectively").

As the technical feature was known in the art at the time of the invention, it cannot be considered a special technical feature that would otherwise unify the groups.

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

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Electronic Acknowledgement Receipt

EFS ID:	33469114
Application Number:	15914356
International Application Number:	
Confirmation Number:	2457
Title of Invention:	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS
First Named Inventor/Applicant Name:	Emil P. Kartalov
Customer Number:	21971
Filer:	J Kyle Bruner/Mary Kelly (KBRU)
Filer Authorized By:	J Kyle Bruner
Attorney Docket Number:	38075-716.302
Receipt Date:	14-AUG-2018
Filing Date:	
Time Stamp:	20:25:09
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		IDS38075-716-302-08-14-2018.pdf	321879 6709798c7e6450a9b1111b1701a33e41d6d5a0db	yes	8

	Multipart Description/PDF files in .zip description				
	Document Description	**Start**	**End**		
	Information Disclosure Statement (IDS) Form (SB08)	5	8		
	Transmittal Letter	1	4		
Warnings:					
Information:					
2	Non Patent Literature	EVANS-DIGITAL-PCR-244.PDF	171553 bca5ac0ecabe273a601a7076790008e2fd44c8dd	no	4
Warnings:					
Information:					
3	Non Patent Literature	HUDECOVA-MATERNAL-PLASMA-2014.PDF	663513 be93886c3bebaa9c77d8627b7f3ac2c4527573e6	no	7
Warnings:					
Information:					
4	Non Patent Literature	NOA-06-14-2018-US14-451876.PDF	444780 b30a597a0ea6db36c0703e4d13c0808bf7eb126b	no	9
Warnings:					
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5	Non Patent Literature	NOA-07-03-2018-US15-623974.PDF	414231 60e9f1be8e573b0577a363de899235f8485ab869	no	7
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Information:					
6	Non Patent Literature	NOA-07-11-2018-US14-451876.PDF	86245 0f9c84316b264f4f8edafb44c900cc196431f135	no	3
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7	Non Patent Literature	OA-US15677772-07JUNE2018.PDF	745251 9209597effc71cd116eb6d711279d4f8cf85a4ba	no	13
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8	Non Patent Literature	OA-US15677772-08FEB2018.PDF	641523 92966a8f860fe4e3959dc91fe89f98611127f21f	no	16
Warnings:					
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9	Non Patent Literature	OA-US15892245-11MAY2018.PDF	313299 92a7a636096d24fe2b0001610f9a055692f52222	no	6
Warnings:					
Information:					
10	Foreign Reference	WO2006079049A2.PDF	8053093 cb4fee77564e10a5e559753aac779a172f694156	no	153
Warnings:					
Information:					
11	Foreign Reference	WO2017218777A1.PDF	4736844 1ccfea4063edf028c786314c1951a11f93bf38e3	no	88
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Information:					
12	Non Patent Literature	YANG-2008.PDF	927540 7409c35c38cbc98b1b0c7ea34db29e7ce168c5e0	no	13
Warnings:					
Information:					
13	Non Patent Literature	ZHANG-2003.PDF	374637 2acabc011a144a8ba98f87da41a575d3746a8af2	no	8
Warnings:					
Information:					
		Total Files Size (in bytes):	17894388		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

RAW SEQUENCE LISTING

Loaded by SCORE, no errors detected.

Application Serial Number: 15914356
Source: OPAP
Date Processed by SCORE: 10/18/2018

ENTERED

SEQUENCE LISTING

<110> KARTALOV, EMIL P.
RAJAGOPAL, ADITYA
SCHERER, AXEL

<120> SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

<130> 38075-716.301

<140> US 15/914,356
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<150> US 14/451,876
<151> 2014-08-05

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<213> Human immunodeficiency virus 1

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120

tcagatactc atagaaatct gtggacataa agctataggt acagtattag taggacctac
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acctgtcaac ataattgg
198

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<211> 26
<212> DNA

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California Institute of Technology - Ex. 2003 Page 956

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primer

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<213> Human immunodeficiency virus 1

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24

<210> 8
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probe

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California Institute of Technology - Ex. 2003 Page 958

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IPR2024-01178
California Institute of Technology - Ex. 2003 Page 959

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IPR2024-01178
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PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 15/914,356

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY FEE($)
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	300
SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	660
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	760
TOTAL CLAIMS (37 CFR 1.16(i))	20 minus 20 =	*			OR	x 100 =	0.00
INDEPENDENT CLAIMS (37 CFR 1.16(h))	1 minus 3 =	*				x 460 =	0.00
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						0.00
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							0.00
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	1720

APPLICATION AS AMENDED - PART II

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
AMENDMENT A	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE($)	ADDITIONAL FEE($)		RATE($)	ADDITIONAL FEE($)
AMENDMENT B	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
15/914,356	03/07/2018	2877	1720	38075-716.302	20	1

CONFIRMATION NO. 2457

21971
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FILING RECEIPT

OC000000103175091

Date Mailed: 10/19/2018

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Inventor(s)

Emil P. Kartalov, Pasadena, CA;
Aditya Rajagopal, Orange, CA;
Axel Scherer, Pasadena, CA;

Applicant(s)

California Institute of Technology, Pasadena, CA;

Power of Attorney: None

Domestic Priority data as claimed by applicant

This application is a CON of 14/451,876 08/05/2014 PAT 10068051
which is a CON of 13/756,760 02/01/2013 PAT 8838394
which claims benefit of 61/703,093 09/19/2012
and claims benefit of 61/594,480 02/03/2012

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.) - None.
Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

Permission to Access Application via Priority Document Exchange: Yes

Permission to Access Search Results: Yes

Applicant may provide or rescind an authorization for access using Form PTO/SB/39 or Form PTO/SB/69 as appropriate.

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If Required, Foreign Filing License Granted: 10/18/2018

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 15/914,356**

Projected Publication Date: To Be Determined - pending completion of Corrected Papers

Non-Publication Request: No

Early Publication Request: No

Title

SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

Preliminary Class

356

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
15/914,356	03/07/2018	Emil P. Kartalov	38075-716.302

CONFIRMATION NO. 2457

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650 PAGE MILL ROAD
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FORMALITIES LETTER

OC000000103175092

Date Mailed: 10/19/2018

NOTICE TO FILE CORRECTED APPLICATION PAPERS

Filing Date Granted

An application number and filing date have been accorded to this application. The application is informal since it does not comply with the regulations for the reason(s) indicated below. Applicant is given TWO MONTHS from the date of this Notice within which to correct the informalities indicated below. Extensions of time may be obtained by filing a petition accompanied by the extension fee under the provisions of 37 CFR 1.136(a).

The required item(s) identified below must be timely submitted to avoid abandonment:

- A substitute specification in compliance with 37 CFR 1.52, 1.121(b)(3), and 1.125, is required. The substitute specification must be submitted with markings and be accompanied by a clean version (without markings) as set forth in 37 CFR 1.125(c) and a statement that the substitute specification contains no new matter (see 37 CFR 1.125(b)). The specification, claims, and/or abstract page(s) submitted is not acceptable and cannot be scanned or properly stored because:
  - The application papers (including any electronically submitted papers) are not in compliance with 37 CFR 1.52 because pages 27-29, 53-55, 62-67 contain text that is unreadable or of insufficient clarity. Application papers (including any electronically submitted papers) must be presented in a form having sufficient clarity and contrast between the background of the paper and the writing thereon to permit the Office to electronically reproduce the papers by use of digital imaging and optical character recognition. See 37 CFR 1.52(a)(1)(v).

Applicant is cautioned that correction of the above items may cause the specification and drawings page count to exceed 100 pages. If the specification and drawings exceed 100 pages, applicant will need to submit the required application size fee.

Replies must be received in the USPTO within the set time period or must include a proper Certificate of Mailing or Transmission under 37 CFR 1.8 with a mailing or transmission date within the set time period. For more information and a suggested format, see Form PTO/SB/92 and MPEP 512.

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/tle/

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SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

CROSS-REFERENCE

[0001] This application is a continuation of U.S. Patent Application No. 14/451,876, filed August 5, 2014, which is a continuation of U.S. Patent Application No. 13/756,760, filed February 1, 2013, which claims the benefit of U.S. provisional applications 61/594,480, filed February 3, 2012 and 61/703,093, filed September 19, 2012. The content of these applications is incorporated herein by reference in its entirety.

SEQUENCE LISTING

[0001.1] The instant application contains a Sequence Listing which has been submitted in ASCII format via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on August 20, 2014, is named 38075-716-301-Seqlist.txt and is 9,260 bytes in size.

STATEMENT AS TO FEDERALLY SPONSORED RESEARCH

[0001.5] This invention was made with the support of the United States government under Grant No. 1144469 awarded by NSF. The government has certain rights in the invention.

BACKGROUND

[0002] Multiplexed reactions offer significant advantages over traditional uniplex reactions, including performance of parallel reactions on the same sample, use of the same chamber to perform multiple reactions, and the ability to extract rich information from a sample in a fast and efficient manner. However, to achieve these benefits, multiplexed assays generally require complex reporting mechanisms, namely spectrally resolved fluorescence or chemiluminescence (e.g., PCR, ELISA), spatially resolved signals (e.g., microarrays, gel electrophoresis), temporally resolved signals (e.g., capillary electrophoresis), or combinations thereof (e.g., Sanger sequencing). There is a need for multiplexed reactions that can be carried out in a single solution.

SUMMARY OF THE INVENTION

[0003] This disclosure provides methods, compositions, systems, and kits for the multiplexed detection of analytes. In some cases, this disclosure provides assays that are capable of unambiguously detecting

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the presence or absence of each of at least 7 analytes, in any combination of presence or absence, in a single sample volume without immobilization, separation, mass spectrometry, or melting curve analysis. In some examples, each of the analytes is encoded as a value of one (or at least one) component of a signal.

[0004] In some examples, an assay provided in this disclosure is capable of unambiguously detecting the presence or absence of M analytes, in any combination of presence or absence, where $M = \log_2 (F + 1)$ and F is the maximum cumulative value of one component of a signal (e.g., an intensity) when all the analytes are present.

[0005] In some cases, each analyte is encoded as at least one first value in a first component of a signal (e.g., at least one intensity or range of intensities) and at least one second value in a second component of a signal (e.g., at least one wavelength or range of wavelengths). In some examples, each analyte is encoded as a first value in a first component of a signal at each of a plurality of second values in a second component of a signal (e.g., a signal intensity or range of signal intensities at each of a plurality of wavelengths or ranges of wavelengths).

[0006] In some examples, an assay provided herein is capable of unambiguously detecting the presence or absence of M analytes, in any combination of presence or absence, where $M = C * \log_2 (F + 1)$, where C is the number of the second values used to encode the analytes and F is the maximum cumulative value of the first component of the signal, for any second value, when all of the analytes are present.

[0007] In some cases, an assay provided in this disclosure is capable of unambiguously detecting the presence or absence of M analytes, in any combination of presence or absence, where $M = (P * T) + 1$, where P is the number of codes per tier in a coding scheme and T is the number of tiers. In some cases, the number of tiers $T = \log_4 (F + 1)$, and F is the maximum cumulative value of a first component of a signal, for any second value, when all of the analytes are present.

[0008] In some examples, the first value is an intensity or range of intensities. In some cases, a coding scheme comprises at least three intensities or ranges of intensities.

[0009] In some cases, the second value is a wavelength or range of wavelengths. In some examples, the coding scheme comprises at least five wavelengths or ranges of wavelengths.

[0010] In some examples, the signal is an electromagnetic signal. In some cases, the electromagnetic signal is a fluorescence emission signal. In some examples, the intensity of the fluorescence emission signal is measured at at least four wavelengths or ranges of wavelengths.

[0011] In some cases, an assay provided herein is performed with reagents that are lyophilized prior to use.

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[0012] In some cases, a detecting step is performed with reagents comprising hybridization probes. In some examples, the number of the hybridization probes is greater than the number of analytes. In some cases, the set of hybridization probes comprise one or more hybridization probes specific for different analytes and comprising an identical fluorophore or combination of fluorophores. In some examples, a sample is contacted with at least 18 of said hybridization probes. In some examples, a detecting step is performed with reagents comprising at least one pair of primers. In some cases, at least one pair of primers are capable of amplifying a region complementary to at least three of said hybridization probes.

[0013] In some examples, the signal that is measured is generated during a polymerase chain reaction. In some cases, the polymerase chain reaction is selected from the group consisting of an end-point polymerase chain reaction, a real-time polymerase chain reaction, a digital polymerase chain reaction, and combinations thereof.

[0014] In some examples, at least one cumulative measurement is performed on a solution.

[0015] In some examples, at least one analyte is encoded by at least one additional value (i.e., at least two values together), wherein the at least one additional value is selected from the group consisting of a value from at least one additional component of a signal, a value from at least one component of a different signal, and combinations thereof. For example, the at least one additional value may be selected from the group consisting of a fluorescence emission intensity, a fluorescence emission wavelength, a Förster resonance energy transfer (FRET) emission intensity, a FRET emission wavelength, an electrochemical signal, a chemiluminescence wavelength, a chemiluminescence intensity, a fluorescence bleaching rate, a chemiluminescence bleaching rate, and combinations thereof.

[0016] In some cases, a chromatogram is constructed. The chromatogram may be constructed by plotting all possible combinations of first values and second values for positive control samples for each analyte.

[0017] In some examples, at least one of the analytes comprises a polynucleotide. In some cases, the polynucleotide is from a source selected from the group consisting of an animal, a plant, a bacteria, a fungus, a parasite, and a virus. In some examples, the polynucleotide is from a source selected from the group consisting of human immunodeficiency virus, herpes simplex virus, human papilloma virus, Plasmodium, Mycobacterium, dengue virus, hepatitis virus, and influenza virus. In some cases, the polynucleotide is selected from the group consisting of human immunodeficiency virus polyprotease, human immunodeficiency virus p17, human papilloma virus E6, and human papilloma virus E7.

[0018] In some cases, a sample is selected from the group consisting of a clinical sample, a food sample, an environmental sample, a pharmaceutical sample, and a sample from a consumer product.

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[0019] In some examples, information concerning the presence or absence of an analyte is transmitted through a computer network.

[0020] In some cases, information concerning the presence or absence of an analyte is provided to a physician. In some examples, a clinical decision is made based on such information.

[0021] In some examples, at least one step of a method provided herein is performed using instructions on a computer readable medium. In some cases, the instructions are located on a remote server. In some examples, the instructions are located on a thermal cycler. In some cases, the instructions are located on a computer in communication with a thermal cycler.

[0022] In some cases, at least one of the analytes is a positive control analyte.

[0023] In some examples, the coding scheme is non-degenerate. In some cases, the coding scheme is designed to be non-degenerate. In some examples, the coding scheme is made non-degenerate by enumerating every legitimate result that can be obtained from the coding scheme, identifying each legitimate result that is degenerate, and eliminating at least one potential analyte code from the coding scheme to eliminate degeneracy.

[0024] In some cases, an assay provided herein is an end-point assay. In some examples an assay provided in this disclosure is ended at a threshold number of cycles set by the limit of detection of an instrument.

[0025] In certain examples, an assay provided herein is a liquid phase assay.

[0026] In some cases, an assay provided herein is quantitative.

[0027] In some cases, this disclosure provides a method of detecting the presence or absence of each analyte of a plurality of analytes, comprising: (a) encoding each of said analytes as a first value of a signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said first value, wherein said encoding is performed in a manner that eliminates degeneracy; (b) providing a sample comprising, or potentially comprising, at least one of said analytes; (c) contacting said sample with analyte-specific reagents that generate said first value, as represented in said coding scheme, when each of said analytes is present; (d) cumulatively measuring said first values of said signal within said sample, thereby providing a cumulative measurement; and (e) determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme.

[0028] In some examples, the method described in the preceding paragraph is capable of unambiguously detecting the presence or absence of M analytes, in any combination of presence or absence, where $M = \log_2 (F + 1)$ and F is the maximum cumulative value of the first values when all of the analytes are present. In some cases, the first value is an intensity or range of intensities. In some examples, the first

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value has a minimum value in the coding scheme that is selected from the group consisting of at least 1, at least 2, at least 4, at least 8, at least 16, at least 32, and at least 64. In some cases, the first value is incremented in the coding scheme by an amount equal to the cumulative maximum of said preceding first values plus one. In other cases, the first value is incremented in the coding scheme by an amount greater than the cumulative maximum of the preceding first values plus one.

[0029] In some cases, this disclosure provides a method of detecting the presence or absence of each analyte of a plurality of analytes, comprising: (a) encoding each of said analytes as at least one first value and at least one second value, wherein said first value is a value from a first component of a signal and said second value is a value from a second component of said signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said at least one first value and said at least one second value, wherein said encoding is performed in a manner that reduces or eliminates degeneracy; (b) providing a sample comprising, or potentially comprising, at least one of said analytes; (c) contacting said sample with analyte-specific reagents that generate said at least one first value and at least one said second value, as represented in said coding scheme, when each of said analytes is present; (d) cumulatively measuring said first values and said second values within said sample, thereby providing a cumulative measurement; and (e) determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme, wherein, when degeneracy is eliminated, said method is capable of unambiguously detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said reagents generates only one second value.

[0030] In some cases, each of the analytes of the method described in the preceding paragraph is encoded as a first value in a first component of the signal at each of a plurality of second values in a second component of the signal. In some cases, when degeneracy is eliminated, this method is capable of unambiguously detecting the presence or absence of each of at least seven analytes in a single volume using four second values. In some examples, when degeneracy is eliminated, the coding scheme comprises T non-degenerate tiers, wherein $T = \log_4 (F + 1)$ and F is the maximum cumulative value of the first component of the signal, for any second value, when all of the analytes are present. In some cases, when degeneracy is eliminated, the method is capable of unambiguously detecting the presence or absence of M analytes, in any combination of presence or absence, where $M = (P * T) + 1$ and P is the number of codes per tier. In some examples, when degeneracy is eliminated, the method is capable of unambiguously detecting the presence or absence of M analytes, in any combination of presence or absence, where $M = C * \log_2 (F + 1)$, C is the number of second values in the coding scheme, and F is

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the maximum cumulative value of the first component of the signal, for any second value, when all of the analytes are present.

[0031] This disclosure also provides non-degenerate coding schemes capable of unambiguously encoding the presence or absence of each of at least 7 analytes, in any combination of presence or absence, in a single sample volume without immobilization, separation, mass spectrometry, or melting curve analysis.

[0032] In some examples, a non-degenerate coding scheme of this disclosure is generated by a method comprising: (a) generating a code for each potential analyte, wherein each potential analyte is encoded by at least one value of at least one component of a signal; (b) enumerating every legitimate cumulative result for all possible combinations of presence or absence of each analyte; (c) identifying each legitimate result that is degenerate; and (d) eliminating at least one code to eliminate degeneracy. In some cases, the coding scheme may be expanded by adding additional codes that are at least one unit greater than the sum of all previous codes in the at least one component of said signal. In some cases a coding scheme is generated by a method of mathematical iteration that guarantees non-degeneracy by construction.

[0033] In some cases, this disclosure provides systems for detecting the presence or absence of each analyte of a plurality of analytes, comprising: (a) encoding each of said analytes as a first value of a signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said first value, wherein said encoding is performed in a manner that eliminates degeneracy; (b) providing a sample comprising, or potentially comprising, at least one of said analytes; (c) contacting said sample with analyte-specific reagents that generate said first value, as represented in said coding scheme, when each of said analytes is present; (d) cumulatively measuring said first values of said signal within said sample, thereby providing a cumulative measurement; and (e) determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme.

[0034] In some cases, this disclosure provides systems for detecting the presence or absence of each analyte of a plurality of analytes, comprising: (a) encoding each of said analytes as at least one first value and at least one second value, wherein said first value is a value from a first component of a signal and said second value is a value from a second component of said signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said at least one first value and said at least one second value, wherein said encoding is performed in a manner that reduces or eliminates degeneracy; (b) providing a sample comprising, or potentially comprising, at least one of said analytes; (c) contacting said sample with analyte-specific reagents that generate said at least one first

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value and said at least one second value, as represented in said coding scheme, when each of said analytes is present; (d) cumulatively measuring said first values and said second values within said sample, thereby providing a cumulative measurement; and (e) determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme, wherein, when degeneracy is eliminated, said system is capable of unambiguously detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said reagents generates only one second value.

[0035] In some cases, this disclosure provides methods of detecting the presence or absence of each analyte of a plurality of analytes for a third party, comprising: (a) obtaining the identity of each of said analytes from a party; (b) encoding each of said analytes as a first value of a signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said first value, wherein said encoding is performed in a manner that eliminates degeneracy; (c) providing said party with analyte-specific reagents that generate said first value, as represented in said coding scheme, when each of said analytes is present, said party: (i) contacting a sample comprising, or potentially comprising, at least one of said analytes with said reagents; and (ii) cumulatively measuring said first values within said sample, thereby providing a cumulative measurement; and (d) obtaining said cumulative measurement from said party; (e) determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme; and (f) providing said party with information about the presence or absence of each of said analytes.

[0036] In some cases, this disclosure provides methods of detecting the presence or absence of each analyte of a plurality of analytes for a third party, comprising: (a) obtaining the identity of each of said analytes from a party; (b) encoding each of said analytes as at least one first value and at least one second value, wherein said first value is a value from a first component of a signal and said second value is a value from a second component of said signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said at least one first value and said at least one second value, wherein said encoding is performed in a manner that reduces or eliminates degeneracy; (c) providing said party with analyte-specific reagents that generate said at least one first value and said at least one second value, as represented in said coding scheme, when each of said analytes is present, said party: (i) contacting a sample comprising, or potentially comprising, at least one of said analytes with said reagents; and (ii) cumulatively measuring said first values and said second values within said sample, thereby providing a cumulative measurement; and (d) obtaining said cumulative measurement from said party; (e) determining whether each of said analytes is present or absent based on said

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cumulative measurement and said coding scheme; and (f) providing said party with information about the presence or absence of each of said analytes.

[0037] In some cases, this disclosure provides compositions for detecting the presence or absence of each analyte of a plurality of analytes, comprising analyte-specific reagents, each reagent generating a signal comprising a first value, wherein said reagents are capable of unambiguously detecting the presence or absence of each of at least seven analytes in a single sample volume, in any combination of presence or absence, without immobilization, separation, mass spectrometry, or melting curve analysis.

[0038] In some cases, this disclosure provides compositions for detecting the presence or absence of each analyte of a plurality of analytes, comprising analyte-specific reagents, each reagent generating a signal comprising at least one first value that is a value from a first component of said signal and at least one second value that is a value from a second component of said signal, wherein said reagents are capable of detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said reagents generates only one second value.

[0039] In some cases, this disclosure provides kits for detecting the presence or absence of each analyte of a plurality of analytes, comprising analyte-specific reagents, packaging, and instructions, each reagent generating a signal comprising a first value, wherein said kit is capable of unambiguously detecting the presence or absence of each of at least seven analytes in a single sample volume, in any combination of presence or absence, without immobilization, separation, mass spectrometry, or melting curve analysis.

[0040] In some examples, this disclosure provides kits for detecting the presence or absence of each analyte of a plurality of analytes, comprising analyte-specific reagents, packaging, and instructions, each reagent generating a signal comprising at least one first value that is a value from a first component of said signal and at least one second value that is a value from a second component of said signal, wherein said kit is capable of detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said reagents generates only one second value.

[0041] In some cases, this disclosure provides kits for detecting the presence or absence of each analyte of a plurality of analytes, comprising a kit body (601), clamping slots arranged in the kit body for placing bottles, a bottle comprising probes for the detection of an analyte (602), a bottle comprising primers for amplification (603), and a bottle comprising reagents for amplification (604), wherein said kit is capable of unambiguously detecting the presence or absence of each of at least seven analytes in a single sample volume, in any combination of presence or absence, without immobilization, separation, mass spectrometry, or melting curve analysis.

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[0042] In some examples, this disclosure provides kits for detecting the presence or absence of each analyte of a plurality of analytes, a kit body (601), clamping slots arranged in the kit body for placing bottles, a bottle comprising probes for the detection of an analyte (602), a bottle comprising primers for amplification (603), and a bottle comprising reagents for amplification (604), each probe generating a signal comprising at least one first value that is a value from a first component of said signal and at least one second value that is a value from a second component of said signal, wherein said kit is capable of detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said probes generates only one second value.

INCORPORATION BY REFERENCE

[0043] All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

[0044] The novel features of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

[0045] Fig. 1 shows a comparison between a traditional encoding method of detecting four analytes with four colors and an encoding method of the invention able to detect 16 or more sequences with four colors. Colors are indicated by B, G, Y, and R, which indicate blue, green, yellow, and red, respectively.

[0046] Fig. 2 shows a schematic representation of detection of six analytes (including control) with four colors. The analytes are detected using hybridization probes attached to a fluorophore (B, G, Y, R; blue, green, yellow, and red, respectively) and a quencher (oval with "X"). The control sequence and the five other sequences (Dengue Fever = dengue virus; Tuberculosis = *Mycobacterium tuberculosis*; P17 = HIV p17; Malaria = *Plasmodium falciparum;* and Herpes = herpes simplex virus 2) are all detected using a probe labeled with a blue fluorophore. The non-control analytes are each detected using 1-3 additional probes. For example, the dengue virus analyte is detected using three additional probes with green,

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yellow, and red fluorophores; the herpes simplex virus 2 analyte is detected using one additional probe with a red fluorophore; and so on.

[0047] Fig. 3 shows chromatograms of experimental results obtained as described in Example 2.

[0048] Fig. 4 shows an exemplary embodiment of the invention in which a computer is used to perform one or more steps of the methods provided herein.

[0049] Fig. 5 shows a schematic of two different methods of detecting wavelength and intensity of a light signal, such as a fluorescence emission signal.

[0050] Fig. 6 shows a schematic of exemplary kits.

[0051] Fig. 7 shows chromatograms of experimental results obtained as described in Example 3.

[0052] Fig. 8 shows a schematic of a legitimate result (top) and an illegitimate result (bottom).

DETAILED DESCRIPTION OF THE INVENTION

[0053] Fluorescence detection has been a preferred technique for multiplexed assays because of several desirable features, including compatibility with biochemical assays, the relatively small size of fluorescent labels, simple means of conjugation to molecules of interest, affordability, low toxicity, stability, robustness, detectability with inexpensive optics, and an ability to be combined with spatial arrays. However, standard fluorophores have wide emission spectra. Therefore, in order to avoid spectral overlap (i.e., to preserve spectral resolution) only a relatively small number of colors (e.g., 4 to 6) are typically used simultaneously in multiplexed fluorescent assays.

[0054] The traditional encoding method for multiplexed fluorescent assays has been to encode each analyte with a single color; i.e., M=N, where M is the number of analytes that can be detected and N is the number of spectrally resolved fluorescent probes. Whenever higher factors of multiplexing are required (i.e., M>N), fluorescence is generally combined with other techniques, such as aliquoting, spatial arraying, or sequential processing. These additional processing steps are labor-intensive and frequently require relatively expensive and complex optical and mechanical systems (e.g., spectrometers, mechanized microscopy stages, microfluidics, droplet generators, scanners, and the like). Such systems are often impractical to deploy in certain settings, particularly point-of care and low-resource settings. Thus, there is a significant need for multiplexed encoding and decoding methods that can provide an inexpensive means of multiplexing while avoiding the use of expensive additional processing steps.

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[0055] This disclosure provides methods, systems, compositions, and kits for the detection of multiple analytes in a sample. Analytes are detected based on the encoding, analysis, and decoding methods presented herein. In some examples, each analyte to be detected is encoded as a value of a signal (e.g., intensity), where the values are assigned so that the results of the assay unambiguously indicate the presence or absence of the analytes being assayed. In other examples, each analyte to be detected is encoded as a value in each of at least two components of a signal (e.g., intensity and wavelength). The at least two components of a signal may be orthogonal. Similarly, as described more fully elsewhere in this disclosure, multiple orthogonal signals may be used, such as a combination of a fluorescent signal and an electrochemical signal. The analyte may be any suitable analyte, such as a polynucleotide, a protein, a small molecule, a lipid, a carbohydrate, or mixtures thereof. The signal may be any suitable signal such as an electromagnetic signal, a light signal, a fluorescence emission signal, an electrochemical signal, a chemiluminescent signal, and combinations thereof. The at least two components of the signal may be any suitable two components, such as an amplitude and a frequency or an intensity and a wavelength.

[0056] After encoding of the analytes, a sample is provided wherein the sample comprises or may comprise at least one of the encoded analytes. The sample is contacted with an analyte-specific reagent or reagents that generate a particular signal, as specified for each analyte in the coding scheme, in the presence of an analyte. A reagent may be any suitable reagent that is capable of generating such a signal in the presence of its corresponding analyte, for example, an oligonucleotide probe attached to a fluorophore and a quencher (e.g., a TAQMAN probe). If the reagent is an oligonucleotide probe attached to a fluorophore and a quencher, a nucleic acid amplification may be performed to generate the signal.

[0057] After addition of the reagent(s), the signal is quantified. In some cases, this quantification is performed by measuring one component of the signal (e.g., fluorescence intensity) and determining the presence and absence of certain analytes based on the values used to encode the presence of each analyte and the cumulative value of the signal.

[0058] In some cases, at least two components of a signal (e.g., intensity and wavelength) are cumulatively measured for the sample. This measurement can be performed, for example, by measuring the intensity at a particular wavelength or the intensity within a particular range of wavelengths. The presence or absence of an analyte may then be determined based on the values of each of the at least two components of the signal and the values used to encode the presence of the analyte (i.e., those values in the coding scheme).

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[0059] The encoding may be performed in a manner that reduces or eliminates the number of possible degenerate (e.g., ambiguous) results that can be obtained by the method. As described elsewhere in this specification, the full coding capability of a particular coding scheme may be enumerated, and certain potential analyte codes may be eliminated from the coding scheme to reduce or eliminate degeneracy. Similarly, a coding scheme may be designed to be non-degenerate, so that a reduction or elimination of degeneracy is not necessary. A decoding matrix may be constructed to translate cumulative signal measurements (e.g., intensities or intensities at particular wavelengths) into the presence or absence of certain analytes, corresponding to the constituent signals of the cumulative signal measurement.

I. Definitions

[0060] The terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

[0061] As used herein, the singular forms "a," "an," and "the" are intended to include the plural forms as well, unless the context clearly indicates otherwise. Furthermore, to the extent that the terms "including," "includes," "having," "has," "with," "such as," or variants thereof, are used in either the specification and/or the claims, such terms are not limiting and are intended to be inclusive in a manner similar to the term "comprising".

[0062] The term "about," as used herein, generally refers to a range that is 15% greater than or less than a stated numerical value within the context of the particular usage. For example, "about 10" would include a range from 8.5 to 11.5.

[0063] The terms "dimension" and "component," as used herein when referring to a signal, generally refer to an aspect of the signal that may be quantified. For example, if a signal is generated by a fluorescent molecule, it may be quantified in terms of its wavelength (e.g., a first dimension or component) and its intensity (e.g., a second dimension or component).

[0064] The term "decoding," as used herein, generally refers to a method of determining which analytes are present based on the cumulative signal and a coding scheme or decoding matrix that enables the conversion of a cumulative signal to information concerning the presence or absence of one or more analytes.

[0065] The term "encoding," as used herein, generally refers to the process of representing an analyte using a code comprising values of a signal, such as intensity, or values in each of at least two components of a signal or signals, such as wavelength and intensity.

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[0066] The terms "oligonucleotide probe attached to a fluorophore and a quencher" and "TAQMAN probe" generally refer to hydrolysis probes used to detect the presence of an analyte in a polynucleotide amplification assay. These probes comprise an oligonucleotide probe attached to a fluorophore and a quencher. So long as the quencher and the fluorophore are in proximity, the quencher quenches the fluorescence emitted by the fluorophore upon excitation by a light source. The sequence of the oligonucleotide probe is designed to be complementary to a polynucleotide sequence present in an analyte, and therefore capable of hybridizing to the polynucleotide sequence present in the analyte. Hybridization of the oligonucleotide probe is performed in a nucleic acid amplification reaction comprising primers (e.g., a polymerase chain reaction). Upon extension of the primers by a DNA polymerase, the 5' to 3' exonuclease activity of the polymerase degrades the probe, releasing the fluorophore and the quencher into the medium. The proximity between the fluorophore and the quencher is broken and the signal from the fluorophore is no longer quenched. Thus, the amount of fluorescence detected is a function of the amount of analyte present. If no analyte is present, the probe will not hybridize to an analyte, and the fluorophore and quencher will remain in close proximity. Little or no signal will be produced.

[0067] The term "orthogonal," as used herein, generally refers to at least two components of a signal (e.g., wavelength and intensity), or at least two different signals (e.g., fluorescence emission and electrochemical signal), that can be varied independently or approximately independently. For example, wavelength and intensity are considered orthogonal or approximately orthogonal when fluorescent molecules are used. Among other factors, the wavelength of fluorescence emission will depend on the composition of the fluorescent molecule and the intensity of the fluorescence will depend on the amount of molecule present. Although wavelength and intensity are examples of two components of a signal that can be varied approximately independently, the methods described herein are not limited to components that can be varied independently or approximately independently. Components of a signal, or signals, that vary non-independently may also be used, so long as the components or signals are characterized well enough to enable encoding, measurement, and decoding. For example, if the variance in one component or signal affects the variance in another component or signal, the two components or signals may still be used so long as the relationship between the variances is understood.

[0068] The terms "polynucleotide," "oligonucleotide," or "nucleic acid," as used herein, are used herein to refer to biological molecules comprising a plurality of nucleotides. Exemplary polynucleotides include deoxyribonucleic acids, ribonucleic acids, and synthetic analogues thereof, including peptide nucleic acids.

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[0069] The term "probe," as used herein, generally refers to a reagent capable of generating a signal in the presence of a particular analyte. A probe generally has at least two portions: a portion capable of specifically recognizing an analyte, or a portion thereof, and a portion capable of generating a signal in the presence of an analyte, or a portion thereof. A probe may be an oligonucleotide probe attached to a fluorophore and a quencher, as described above and elsewhere in this disclosure. A probe may also be any reagent that generates a signal in the presence of an analyte, such as an antibody that detects an analyte, with a fluorescent label that emits or is quenched upon binding of the antibody to an analyte. Any suitable probe may be used with the methods presented in this disclosure, so long as the probe generates a quantifiable signal in the presence of an analyte. For example, the analyte-specific portion of a probe may be coupled to an enzyme that, in the presence of an analyte, converts an uncharged substrate into a charged product, thereby increasing the electrical conductivity in the medium over time. In this case, different analytes may be encoded by coupling the analyte-specific portion of the probe (e.g., hybridization probe or antibody) to an enzyme at different ratios. The resulting rate of increased conductivity in the medium will be cumulative for all analytes present in the medium. Encoding analytes according to the methods provided herein enables conversion of the conductivity measurements into unambiguous (i.e., non-degenerate) results providing information about the presence or absence of particular analytes. Similarly, a probe may comprise an enzyme producing a chemiluminescent product from a substrate. The amount of chemiluminescence may then be used to encode the presence of particular analytes.

II. Encoding and Decoding Methods

A. Traditional Fluorescent Encoding and Decoding Method

[0070] A commonly used method of determining the presence of an analyte uses four spectrally resolved fluorescent molecules to indicate the presence or absence of four analytes. An example of this method is presented on the left-hand side of **Fig. 1**. The left-hand side of **Fig. 1** shows an encoding method where four analytes (Seq 1, Seq 2, Seq 3, and Seq 4) are each encoded by a single color (blue, green, yellow, and red, respectively). The color represents a fluorophore attached to an oligonucleotide probe that also comprises a quencher. In the system shown on the left-hand side of **Fig. 1**, there are four different oligonucleotide probes, each comprising a single fluorophore (blue, green, yellow, or red) and a quencher. The presence or absence of an analyte is determined based on the presence or absence of a signal in a particular color.

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[0071] The chart on the left-hand side of **Fig. 1** shows intensity versus color for a hypothetical sample containing two analytes: Seq 1 and Seq 3. The presence of these analytes is determined based on the measurement of a blue signal (corresponding to Seq 1) and a yellow signal (corresponding to Seq 3). The absence of Seq 2 and Seq 4 is indicated by the absence of a blue and red signal.

[0072] Table 1 shows a translation of this coding scheme into a binary format. Each analyte is encoded as a value in each of two components of a fluorescent signal: (1) color (also known as wavelength; or range of wavelengths) and (2) intensity (indicated by the numbers within the table). For example, A (e.g., Seq 1) has a color of blue and an intensity of 1; B (e.g., Seq 2) has a color of green and an intensity of 1; and so on. The intensity of the signal within each color range may be quantified as described herein, for example by measuring the intensity of the signal within a particular wavelength range determined by a band pass filter. A result of 1000 indicates that only analyte A is present; a result of 1100 indicates that analytes A and B are present; and so on.

Table 1. Traditional encoding of four analytes with four probes, each probe having a single color, and one probe per analyte.

	Blue	Green	Yellow	Red
A	1	0	0	0
B	0	1	0	0
C	0	0	1	0
D	0	0	0	1

B. Encoding Methods Using More Than One Color Per Analyte

[0073] The traditional method described above suffers from the fact that it is limited by the number of spectrally resolvable fluorophores. More specifically, the number of detectable analytes is equal to the number of spectrally resolvable fluorophores. Therefore, the number of analytes may only be increased by increasing the number of spectrally resolvable fluorophores.

[0074] This disclosure provides methods that overcome this limitation. More specifically, in some cases, by utilizing at least two components of a signal during encoding, the methods described herein may be used to detect more than one analyte per fluorophore. For example, using the method provided herein 1.5, 1.6, 1.7, 1.8, 1.9, 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, or 50 analytes may be detected per fluorophore. In some cases, the methods provided herein may be used to

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detect at least 1.5, 1.6, 1.7, 1.8, 1.9, 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, or 50 analytes may be detected per fluorophore. In some cases, the methods provided herein may be used to detect 1.5-2, 2-4, 1.5-4, 4-6, 2-6, 6-10 analytes per fluorophore.

[0075] In some cases, the methods provided in this disclosure may include the use of a control color. The control color may be attached to one or more probes binding a positive control analyte, and each analyte to be detected, in a sample. If the same sequence occurs in the positive control analyte and each analyte to be detected, a single control probe may be used. If the same sequence does not occur in the positive control analyte and each analyte to be detected, different probes may be used, but each probe may still be attached to the control color.

[0076] For example, building on the traditional methods described above, one color (e.g., blue) may be used to encode the presence of a control analyte that is always present in the sample. The control analyte may be added to the sample, or may be inherently present in the sample. The remaining colors (e.g., green, yellow, red) may be used to encode the presence of additional analytes. Table 2 shows an example of one such method.

Table 2. Encoding of four analytes, including control, with four colors and up to two colors per analyte.

	Blue	Green	Yellow	Red
Ctrl. (p)	1	0	0	0
A	1	0	0	1
B	1	0	1	0
C	1	1	0	0

[0077] In Table 2, the presence of the control (p) is indicated by a result of 1000. The presence of the control and analyte A is indicated by a result of 2001. The presence of the control and all three other analytes is indicated by a result of 4111. The control color (blue) provides an indication that the assay is functioning properly. The intensity of the blue color reports the number of analytes that are present in a test sample. Of course, any color may be used as the control color. One of skill in the art will recognize that certain practical considerations might make it preferable to use one color over another as the control color. For example, if one color is better detected in a particular optical system (or system of fluorophores), it might be practically preferable to use that color as a control color.

[0078] The coding scheme shown in Table 2 encodes each analyte using one control color and one additional color (up to 2 probes per analyte). However, as shown below, the number of analytes that can

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be encoded increases when up to 4 colors are used per analyte. Table 3 shows an exemplary coding scheme where each analyte is encoded by up to 4 colors. In the scheme shown in Table 3, the control color (blue) is generated in the presence of the control sequence and each of the other seven analytes (A-G). The other seven analytes are each encoded by the presence of one to three additional colors. The colors may be contained on different probes (e.g., oligonucleotide probes attached to a fluorophore and a quencher) or on the same probe (which can have multiple fluorophores and multiple quenchers).

Table 3. Encoding of eight analytes (including control) with four colors and up to four colors per analyte.

	Blue	Green	Yellow	Red
Ctrl. (p)	1	0	0	0
A	1	0	0	1
B	1	0	1	0
C	1	1	0	0
D	1	0	1	1
E	1	1	0	1
F	1	1	1	0
G	1	1	1	1

[0079] In Table 3, the presence of the control and all seven other analytes is indicated by a result of 8444. Three analytes (A, B, and C) are encoded by two colors. Three analytes (D, E, and F) are encoded by three colors, and one analyte (G) is encoded by four colors. The presence of the control (p) is indicated by a result of 1000. The presence of the control and analyte A is indicated by a result of 2001. The presence of the control and analyte G is indicated by a result of 2111. These results express the cumulative intensity of the signal in each color. For example, the result 2111 has a 2X signal intensity in the blue channel, while the result 1000 has only a 1X signal intensity in the blue channel.

[0080] Using Table 3, each of the possible cumulative assay results can be enumerated, in terms of color (blue, green, yellow, red) and intensity (0-8). Table 4 shows a "decoding matrix" generated by enumerating each of the possible cumulative assay results based on the encoding method presented in Table 3 and providing the corresponding decoded result of each assay in terms of the analyte(s) present in the sample. A similar decoding matrix may be generated for any coding scheme described herein, by enumerating each of the possible cumulative assay results based on the coding scheme and providing the corresponding decoded result of each assay in terms of the analyte(s) present in (or absent from) the sample. In some cases, as described below, one or more analytes may be removed from the coding scheme in order to reduce or eliminate degeneracy.

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Table 4. Decoding matrix for encoding method presented in Table 3.

Cumulative Assay Result				
Blue	**Green**	**Yellow**	**Red**	**Analyte(s) Present**
1	0	0	0	p
2	0	0	1	pA
2	0	1	0	pB
2	1	0	0	pC
2	0	1	1	pD
2	1	0	1	pE
2	1	1	0	pF
2	1	1	1	pG
3	0	1	1	pAB
3	1	0	1	pAC
3	0	1	2	pAD
3	1	0	2	pAE
3	1	1	1	pAF, pCD, pBE
3	1	1	2	pAG, pDE
3	1	1	0	pBC
3	0	2	1	pBD
3	1	2	0	pBF
3	1	2	1	pBG, pDF
3	2	0	1	pCE
3	2	1	0	pCF
3	2	1	1	pCG, pEF
3	1	2	2	pDG
3	2	1	2	pEG
3	2	2	1	pFG
4	1	1	1	pACB
4	0	2	2	pABD
4	2	2	0	pBCF
4	2	0	2	pACE
4	1	1	2	pABE, pACD
4	1	2	1	pABF, pBCD
4	2	1	1	pACF, pBCE
4	1	2	2	pADF, pABG, pBDE
4	2	1	2	pACG, pCDE, pAEF
4	2	2	1	pBCG, pBEF, pCDF
4	2	2	2	pCDG, pAFG, pBEG, pDEF
4	1	1	3	pADE
4	1	3	1	pBDF
4	3	1	1	pCEF
4	1	2	3	pADG
4	3	2	1	pCFG
4	2	1	3	pAEG

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4	2	3	1	pBFG
4	1	3	2	pBDG
4	3	1	2	pCEG
4	2	2	3	pDEG
4	3	2	2	pEFG
5	1	2	2	pABCD
5	2	1	2	pABCE
5	2	2	1	pABCF
5	2	2	2	pABCG, pABEF, pBCDE, pACDF
5	1	2	3	pABDE
5	2	1	3	pACDE
5	1	3	2	pABDF
5	2	1	3	pBCDF
5	3	1	2	pACEF
5	3	2	1	pBCEF
5	2	2	3	pABEG, pACDG, pADEF
5	2	3	2	pABFG, pBCDG, pBDEF
5	3	2	2	pACFG, pBCEG, pCDEF
5	1	3	3	pABDG
5	3	1	3	pACEG
5	3	3	1	pBCFG
5	2	3	3	pADFG, pBDEG
5	3	3	2	pBEFG, pDEFG
5	3	2	3	pCDEG, pAEFG
5	2	2	4	pADEG
5	2	4	2	pBDFG
5	4	2	2	pCEFG
5	3	3	3	pDEFG
6	2	2	3	pABCDE
6	2	3	2	pABCDF
6	3	2	2	pABCEF
6	2	3	3	pABCDG, pABDEF
6	3	2	3	pABCEG, pACDEF
6	3	3	2	pABCFG, pBCDEF
6	4	3	2	pBCEFG
6	4	2	3	pACEFG
6	3	3	3	pABEFG, pACDFG, pBCDEG
6	3	4	2	pBCDFG
6	2	4	3	pABDFG
6	3	2	4	pACDEG
6	2	3	4	pABDEG
6	4	3	3	pCDEFG
6	3	4	3	pBDEFG
6	3	3	4	pADEFG
7	4	4	3	pBCDEFG
7	4	3	4	pACDEFG

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7	3	4	4	pABDEFG
7	4	3	3	pABCEFG
7	3	4	3	pABCDFG
7	3	3	4	pABCDEG
7	3	3	3	pABCDEF
8	4	4	4	pABCDEFG

[0081] The decoding matrix provided in Table 4 is constructed using two assumptions. First, the decoding matrix assumes that the positive control (p) always produces a positive outcome. Second, the decoding matrix assumes that, within each color, the intensity is additive and scales in the same way with changing probe concentration, regardless of which probe the signal may come from. This essentially means that the signals are additive and digital. The conditions underlying these assumptions may be met by properly preparing the assay. If a fluorescent signal is used, the intensity need only be approximately additive and digital, as demonstrated in the Examples provided herein.

[0082] Table 4 allows the conversion of a cumulative measurement of intensity in four ranges of fluorescent wavelengths (i.e., signal intensity within each color range), into the corresponding analytes present in the sample. For example, a result of 4321 indicates that p, C, F, and G are present and the other analytes are not present. This result is referred to as a "legitimate" result, because it is present in the decoding matrix. By contrast, a result of 4000, while possible to measure, does not occur in the decoding matrix. More specifically, the result of 4000 cannot be achieved by adding any combination of control and analyte codes from Table 3. This result is referred to as an "illegitimate" result. An illegitimate result may indicate that the assay malfunctioned. Thus, the control (p) and the decoding matrix provide a means of verifying that the assay is functioning properly.

[0083] Table 4 is exhaustive for any combination of fluorophores generating four resolvable emission spectra (e.g., colors). The term "rank" is used to describe the number of detected analytes, including the control. In the example provided above, if the assay functions properly, the rank is equal to the value of the blue signal. For example, a rank of 8 indicates that the control and all seven other analytes (A-G) are present. A rank of 2 indicates that the control and only one analyte are present. The lowest rank is a rank of 1, which indicates that only the control is present. The number of possibilities at each rank can be enumerated. For example, continuing to refer to the encoding and decoding method described in Tables 3-4, there are 7 possibilities for rank 2. The number of possibilities at rank 3 can be calculated as a combination of 7 take 2, or $7!/(5! * 2!) = 21$ possibilities. More generally, the number of possibilities for a combination of N take K is $N!/((N-K)! * K!)$. Analogously, at ranks 4, 5, 6, 7, and 8, the number

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of possibilities is 35, 35, 21, 7, and 1, respectively. Referring to Table 4 shows that the table agrees with the theoretical prediction. Thus, Table 4 is an exhaustive decoding matrix for the encoding method provided in Table 3.

C. Reducing or Eliminating Degeneracy

[0084] The terms "degenerate" and "degeneracy," as used herein, generally describe a situation where a legitimate result is not definitive, because it can indicate more than one possibility in terms of the presence or absence of an analyte. For example, with reference to Table 4, result 5233 is degenerate because it can be decoded as either pADFG or pBDEG. Similarly, result 4222 is degenerate because it can be decoded as any of the following: pCDG, pAFG, pBEG, pDEF. By contrast, result 3110 can only indicate pBC and thus is not degenerate.

[0085] This disclosure provides methods of reducing or eliminating degeneracy, thereby increasing the confidence with which an analyte is detected. In one embodiment, degeneracy is eliminated by a method comprising (i) encoding each potential analyte to be detected as a value of a signal and, optionally, as a value in each of at least two components of a signal; (ii) enumerating every legitimate result that can be obtained from the coding scheme; (iii) identifying each legitimate result that is degenerate; and (iv) eliminating at least one potential analyte (or potential analyte code) from the coding scheme, wherein eliminating the at least one potential analyte reduces or eliminates degeneracy. For example, with reference to the coding scheme described in Table 3 and the decoding matrix described in Table 4 (enumerating every legitimate result), eliminating any two of analytes D, E, and F eliminates the degeneracy. Eliminating any one of analytes D, E, and F would not eliminate the degeneracy, but would reduce it.

[0086] With continued reference to the coding scheme described in Table 3, eliminating any two of analytes D, E, and F from the coding scheme results in a scheme where six analytes (including control) can be analyzed, with no degeneracy, using only 4 colors. By contrast, conventional methods of multiplexing would allow for only the reporting of 3 analytes and 1 control using 4 colors. Therefore, the number of analytes that can be analyzed is nearly doubled by using the methods provided herein.

[0087] **Fig. 2** shows an exemplary embodiment of the invention in which 4 colors are used to detect five analytes and a control. The analytes are nucleic acids from dengue virus ("Dengue Fever"), *Mycobacterium tuberculosis* ("Tuberculosis"), human immunodeficiency virus (HIV) p17 (P17), *Plasmodium* ("Malaria"), and herpes simplex ("Herpes"). The "colors" in this example are fluorophores attached to oligonucleotide probes that also comprise a quencher. The oligonucleotide probes will

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generally be different for different analytes, whereas the color is the same. For example, all probes designated as Probe 1 have a blue color but generally will have a different oligonucleotide sequence. Of course, probes designated as Probe 1 could also have the same oligonucleotides sequence, if the complementary sequence was present in each of the analytes. The mechanism of detection with these probes is described elsewhere in this disclosure. Probe 1 (blue) hybridizes to all six analytes, including control. Probe 2 (green) hybridizes to analytes from dengue virus, *Mycobacterium*, and HIV p17. Probe 3 (yellow) hybridizes to analytes from dengue fever and *Plasmodium*. Probe 4 hybridizes to analytes from dengue virus, *Mycobacterium*, and herpes simplex. The coding scheme defined by these probes is illustrated in Table 5. With reference to Table 3, potential analytes D (1011) and F (1110) have been eliminated from the coding scheme. Therefore, the coding scheme presented in Table 5 is non-degenerate and each legitimate result from the assay corresponds to the presence or absence of a unique combination of analytes in a sample.

Table 5. Coding scheme for detection of dengue virus, *Mycobacterium*, HIV, *Plasmodium*, and herpes simplex, as exemplified in Fig. 2.

	Blue	**Green**	**Yellow**	**Red**
Ctrl. (p)	1	0	0	0
Herpes Simplex	1	0	0	1
Plasmodium	1	0	1	0
HIV	1	1	0	0
Mycobacterium	1	1	0	1
Dengue Virus	1	1	1	1

[0088] The methods for encoding and decoding presented above, including the methods for eliminating degeneracy, are all equally applicable to encoding methods using additional colors. For example, Table 3 could be extended by including additional colors and additional intensities (described further below). The decoding matrix is then generated as described above, enumerating every legitimate result that can be obtained from the coding scheme. A decoding matrix analogous to the decoding matrix provided in Table 4 may be constructed for any coding scheme described in this disclosure. The legitimate results that are degenerate are then identified and at least one potential analyte code is eliminated from the coding scheme to reduce or eliminate degeneracy. The method of eliminating degeneracy may be

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carried out using software on a computer readable medium, or hardware configured to carry out the method (e.g., a microchip).

[0089] Although degeneracy can be reduced or eliminated by the methods described above, and elsewhere in this disclosure, this disclosure also provides coding schemes that are non-degenerate by design. For example, after elimination of degeneracy in the coding scheme described in Table 3, the coding scheme may be extended indefinitely in a non-degenerate manner where the non-degeneracy is by design (see, e.g., the coding scheme exemplified in Table 6, described more fully below). Similarly, this disclosure provides coding schemes that are completely non-degenerate by design and therefore do not require any reduction or elimination of degeneracy (see, e.g., the coding scheme exemplified in Table 8, described more fully below). Thus, the coding schemes provided in this disclosure may have reduced or eliminated degeneracy, or be non-degenerate by design.

D. Encoding Methods Using More Than One Color and More Than One Intensity

[0090] The encoding method described above (e.g., Tables 3-5) may be further extended by allowing analytes to be encoded by an intensity greater than 1. For example, each of the analytes encoded in the coding scheme provided in Table 5 is encoded by a fluorescence intensity of either 1 or 0. Allowing higher values for the signal intensity in at least one color further increases the number of analytes that can be encoded by any of the methods provided in this disclosure. In some examples, these higher intensity values may be assigned any color except for the control color, in order to maintain the analyte counting capability of the control color.

[0091] Table 6 shows the first three tiers of an exemplary coding scheme that utilizes four colors and multiple intensities. Tier 1 of Table 6 is a reproduction of Table 3, showing the encoding of seven analytes and a control with four colors. As described above, any two of potential analytes D, E, and F may be eliminated from the coding scheme in order to produce a non-degenerate coding scheme. Tier 1 of Table 6 indicates that potential analytes D (1011) and F (1110) have been eliminated from the coding scheme to eliminate degeneracy. Therefore, the coding scheme presented in Tier 1 of Table 6 is capable of determining the presence of five analytes and one control using four colors, as described above.

[0092] The coding scheme of Tier 1 of Table 6 may be expanded to a second tier (Tier 2) by allowing the intensity in any of the colors to increase. As described above, the intensity of the control color may be maintained at 1, in order to preserve the sequence counting capability of the control. Increasing the intensity of any of the remaining three colors will yield codes 100Y, 10Y0, 1Y00, 10YY, 1Y0Y, 1YY0, and 1YYY, where $Y > 1$. The minimal value of Y for a new tier of encoding is equal to the cumulative

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maximum value from the prior tier(s) plus 1. As described below, a value greater than 1 could also be used, to maximize the differences between the intensities.

[0093] Thus, in this context, the term "tier" is generally used to describe a set of codes that fully utilize the coding capability provided by a particular number of first values (e.g., intensities) and second values (e.g., colors), without degeneracy. For example, Tier 1 of Table 6 fully utilizes the coding capability provided by four colors with up to one intensity in each color, without degeneracy. As shown in Table 6, Tier 1, this results in six encoded analytes, including the control. To introduce a second tier, the minimum value of Y (described above) may be incremented to equal the cumulative maximum result from the prior tier(s) plus one (or more than one). In the example provided in Table 6, the intensity of the blue (control) color is maintained as one, to preserve the sequence counting capability in this color. Thus, Tier 2 consists of five non-degenerate encoding possibilities obtained by incrementing the intensities in the green, yellow, and red channels to equal the cumulative maximum results in each of these channels from Tier 1, plus one. All possibilities of these codes may then be enumerated for Tier 2, and codes resulting in degeneracy may be eliminated, or Tier 2 may be made non-degenerate by design, using the information used to eliminate the corresponding codes from Tier 1. Further coding capacity may then be achieved by adding a third tier, or further tiers, which are constructed according to analogous methods. A coding scheme may have an infinite number of tiers, for example 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, 60, 70, 80, 90, 100 or more tiers.

[0094] More specifically, with reference to Table 6, Tier 2, analyte H is encoded by 1004. The value of the control color is maintained as 1. The value of the red color is equal to the cumulative maximum result from the prior tier (3) plus 1, or 4. Similarly, analytes I and J are encoded by 1030 and 1400, respectively. Combinations of these codes are used to encode the remaining four analytes (K-N), as was done for analytes D-G in Tier 1. This completes Tier 2. In Tier 2, the inclusion of potential analytes K (1034) and M (1430) results in degeneracy. Therefore, these analytes have been eliminated from the coding scheme, to eliminate degeneracy.

[0095] Continuing to refer to Table 6, a third tier (Tier 3) is constructed using the same principles described above. Analyte O is encoded by 1-0-0-16. The value of the control color is still maintained as 1. The value of the red color is equal to the cumulative maximum result from the prior tiers (15) plus 1, or 16. Similarly, analytes P and Q are encoded by 1-0-9-0 and 1-16-0-0, respectively. Combinations of these codes are used to encode the remaining four analytes (R-U), as was done for analytes D-G in Tier 1 and analytes K-N in Tier 2. In Tier 3, the inclusion of potential analytes R (1-0-9-16) and T (1-16-9-0)

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results in degeneracy. Therefore, these analytes have been eliminated from the coding scheme, to eliminate degeneracy.

[0096] The three-tier coding scheme shown in Table 6 shows the encoding of 15 analytes and one control using four colors. This coding scheme may be indefinitely extended, by adding more intensities to generate additional tiers and/or adding more colors, to generate additional coding capability within the tiers. The methods of reducing or eliminating degeneracy, as described in this disclosure, may be used with the addition of each intensity and/or color, to reduce or eliminate degenerate results.

[0097] More generally, the coding scheme depicted in Table 6 is a non-degenerate, infinite extension of the coding scheme depicted in Tables 3-5. The maximum intensity of the cumulative measurement at the first tier is 6. The maximum intensity of the cumulative measurement at the second tier is 15. The maximum intensity of the cumulative measurement at the third tier is 63, and so on. Given a maximum cumulative intensity value (F), the maximum number of tiers (T) available in this coding scheme is $T = \log_4 (F + 1)$. The coding scheme depicted in Table 6 provides five non-degenerate codes per tier (P). Thus, the maximum number of codes $M = 5 * \log_4 (F + 1)$, or $M = P * T$, where P is the number of non-degenerate codes per tier and T is the number of tiers. For example, given $F = 63$, the maximum number of codes (i.e., analytes) is 15 for 5 non-degenerate codes per tier. This formula does not include the 1000 code, which is reserved for a positive control in Table 6. To include the control in the total number of analytes, one would simply add one, to provide the formula $M = (P * T) + 1$.

[0098] The methods provided in this disclosure may be used to expand this coding scheme infinitely. For example, by utilizing combinations of different intensities (i.e., first values) and colors (i.e., second values) one can encode any number of analytes (M) by varying the number of non-degenerate codes per tier (P) and the number of tiers (T). For example, the number of non-degenerate codes per tier is 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, 60, 70, 80, 90, 100, or more. Similarly, the number of tiers may 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, 60, 70, 80, 90, 100, or more.

Table 6. Encoding of 15 analytes and one control using four colors and multiple intensities.

Tier	Analyte	B	G	Y	R	Comments
1	**Ctrl. (p)**	1	0	0	0	
	A	1	0	0	1	
	B	1	0	1	0	

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	C	1	1	0	0	
	~~**D**~~	~~1~~	~~0~~	~~1~~	~~1~~	Eliminated from coding scheme to eliminate degeneracy.
	E	1	1	0	1	
	~~**F**~~	~~1~~	~~1~~	~~1~~	~~0~~	Eliminated from coding scheme to eliminate degeneracy.
	G	1	1	1	1	
Cumulative Maximum Result		*6*	*3*	*2*	*3*	
2	**H**	1	0	0	4	
	I	1	0	3	0	
	J	1	4	0	0	
	~~**K**~~	~~1~~	~~0~~	~~3~~	~~4~~	Eliminated from coding scheme to eliminate degeneracy.
	L	1	4	0	4	
	~~**M**~~	~~1~~	~~4~~	~~3~~	~~0~~	Eliminated from coding scheme to eliminate degeneracy.
	N	1	4	3	4	
Cumulative Maximum Result		*11*	*15*	*8*	*15*	
3	**O**	1	0	0	16	
	P	1	0	9	0	
	Q	1	16	0	0	
	~~**R**~~	~~1~~	~~0~~	~~9~~	~~16~~	Eliminated from coding scheme to eliminate degeneracy.
	S	1	16	0	16	
	~~**T**~~	~~1~~	~~16~~	~~9~~	~~0~~	Eliminated from coding scheme to eliminate degeneracy.
	U	1	16	9	16	
Cumulative Maximum Result						
Cumulative Maximum Result for Three Non-Degenerate Tiers		***16***	***63***	***26***	***63***	

[0099] The right-hand side of **Fig. 1** shows one exemplary embodiment of the method described above. More specifically, the right-hand side of **Fig. 1** shows an encoding method where nine or more analytes

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(Seq 1 - Seq 9, etc.) are each encoded by at least two colors, with varying intensities within each color. The color represents a fluorophore attached to an oligonucleotide probe that also comprises a quencher. The system can be designed so that each probe is labeled with single fluorophore, or each probe is labeled with more than one fluorophore. For example, the code for analyte H, in Table 6, is 1004. The intensity of 4 in the red channel may be achieved by either using an H-specific probe comprising 4 red fluorophores, or by using 4 H-specific probes each comprising a single red fluorophore. Of course, any combination of probes and fluorophores producing a result of 4 in the red channel would be equally appropriate, such as 2 probes with 2 red fluorophores each, and 1 probe with 1 red fluorophore and 1 probe with 3 red fluorophores, or simply one probe with one red fluorophore but present at 4x amount in the reaction mixture.

[00100] The coding scheme depicted on the right-hand side of **Fig. 1** may be represented as in Table 7. The result of the analysis shown on the right-hand side of **Fig. 1** is 4112, or an intensity of 4 in the blue channel, 1 in the green channel, 1 in the yellow channel, and 2 in the red channel. Using a decoding matrix constructed as described herein, this result is decoded to indicate the presence of Seq 1, Seq 3, Seq 4, and Seq 5.

Table 7. Coding scheme illustrated on the right-hand side of Fig. 1.

Tier	Analyte	B	G	Y	R	Comments
1	**Seq 1**	1	0	0	0	
	Seq 2	1	1	0	0	
	Seq 3	1	0	1	0	
	Seq 4	1	0	0	1	
	Seq 5	1	1	0	1	
	Seq 6	1	1	1	1	
	~~Eliminated Potential Seq~~	~~1~~	~~0~~	~~1~~	~~1~~	Eliminated from coding scheme to eliminate degeneracy.
	~~Eliminated Potential Seq~~	~~1~~	~~1~~	~~1~~	~~0~~	Eliminated from coding scheme to eliminate degeneracy.
Cumulative Maximum Result		*6*	*3*	*2*	*3*	
2	**Seq 7**	1	4	0	0	
	Seq 8	1	0	3	0	
	Seq 9	1	0	0	4	
Etc. - i.e. Continue as provided in Table 6.						

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E. Encoding Methods Using One Color and One Intensity Per Analyte but Different Intensities Among Analytes

[00101] In some methods provided herein, each analyte is encoded by a single color and intensity combination. For example, in a four color system, the first four analytes may be encoded by 1000, 2000, 4000, and 8000. The next four analytes may be encoded by 0100, 0200, 0400, and 0800. Analytes 9-12 and 13-16 would be assigned analogously, as shown in Table 8.

[00102] Like the encoding method described in Table 6, this coding scheme is theoretically infinite, non-degenerate by construction, and only limited by the bandwidth of the instrument used to measure the signal. However, this coding scheme enables more analytes to be quantified per unit of bandwidth than the encoding method described in Table 6. The reason is that the available multiplicity of signal is used with maximal efficiency, as each level of intensity is utilized in the coding (i.e. there are no gaps in the chromatogram; see below for description of chromatograms). Table 8 shows one embodiment of this method, illustrating four tiers of encoding based on four colors and intensities 1, 2, 4, and 8. The coding scheme is non-degenerate, and the result if all 16 analytes are present is 15-15-15-15. This encoding method is more efficient, in terms of bandwidth utilization, than the encoding methods presented above. However, this method does not have the proofreading capability of the first scheme, as all the results decode to legitimate outcomes in the absence of gaps in the chromatogram.

Table 8. Example of encoding method using one color and one intensity per analyte, but different intensities among analytes.

Tier	Analyte	B	G	Y	R
1	**A**	1	0	0	0
	B	2	0	0	0
	C	4	0	0	0
	D	8	0	0	0
2	**E**	0	1	0	0
	F	0	2	0	0
	G	0	4	0	0
	H	0	8	0	0
3	**I**	0	0	1	0
	J	0	0	2	0
	K	0	0	4	0

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	L	0	0	8	0
4	**M**	0	0	0	1
	N	0	0	0	2
	O	0	0	0	4
	P	0	0	0	8
		15	***15***	***15***	***15***

[00103] The method presented above, and exemplified in Table 8, may be extended by introducing additional colors and/or intensities. For example, 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, 60, 70, 80, 90, 100, or more colors may be used. Additional intensity levels, such as 16, 32, 64, 128, 255, 512, 1024, 2048, 4096, 8192, 16384, 32768, 65536, and so on may also be used. The method is generalizable and may be used to encode an infinite number of analytes. Each analyte is represented by a code in a single color, wherein the value of the code in that single color equal to the sum of all previous values plus one. For example, if the first code contains a 1 in a particular color, the next codes are 2, 4, 8, 16, 32, 64, 128 and so on. Other progressions are possible, but will not be as efficient in terms of usage of the bandwidth. However, one of ordinary skill in the art will recognize that less efficient use of bandwidth may be desirable when one wishes to maximize separation between values. Similarly, the value 1 could be excluded from the coding scheme, for example to maximize the difference in the intensity between the first encoded value and instrumental noise. For example, in some cases a coding scheme may begin with a value of 2, which would provide a progression of 2, 4, 8, 16, 32, 64, 128, and so on for the analyte codes, while the progression of cumulative results would be 2, 4, 6, 8, 10, 12, 14, 16, and so on. This approach generates uniform gaps in the possible cumulative results, allowing for higher tolerance to noise in comparison to an analyte code progression of 1, 2, 4, 8, 16, 32, and so on, and its corresponding cumulative result progression of 1, 2, 3, 4, 5, 6, 7, 8, and so on. In this example, the increased robustness in the measurement comes at a cost of a decrease in the number of available codes within a fixed bandwidth of multiplicity. For example, if only 63 multiplicity states can be measured reliably, the coding scheme starting with 1 offers 7 codes per color whereas the coding scheme starting with 2 offers 6 codes per color. If 4 colors are available, the former will offer 28 codes, while the latter will offer 24 codes. In summary, both the starting signal intensity and the progression (i.e., difference between intensity values) may be scaled in order to maximize the ability to distinguish

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over instrumental noise and maximize the differences between the intensity values, thereby enhancing the ability to distinguish between distinct experimental outcomes.

[00104] The coding scheme illustrated in Table 8 is non-degenerate by design. Although the coding scheme illustrated in Table 8 uses both intensity and color to encode each of the 16 analytes, each of the analytes could also be encoded by simply utilizing intensity. For example, given the 16 analytes provided in Table 8 (A-P), a single color coding scheme encoding all 16 analytes may assign the values 1, 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024, 2048, 4096, 8192, and 16384 to analytes A, B, C, D, E, F, G, H, I, J, K, L, M, N, O, and P, respectively. Such a coding scheme is non-degenerate by design and the cumulative intensity result can be unambiguously decoded to indicate the presence or absence of analytes A-P. This coding scheme is capable of detecting the presence or absence of M analytes, where $M = \log_2 (F + 1)$ and F is the maximum cumulative value of a signal (e.g., signal intensity). By adding a second component to this signal (e.g., color, as depicted in Table 8), the capacity of this coding scheme can be increased to $M = C * \log_2 (F + 1)$, where C is the number of colors used in said coding scheme.

[00105] As described throughout this specification, any suitable value may be used for C or F. For example, C may be at least about 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, 60, 70, 80, 90, 100, or more. F may be at least about 2, 4, 8, 16, 32, 64, 128, 255, 512, 1024, 2048, 4096, 8192, 16384, 32768, 65536, 131072, 262144, 524288, 1048576, and so on. As described elsewhere in this disclosure, both the initial starting value of F and the steps in its progression may also be varied.

III. Analytes

[00106] An analyte may be any suitable analyte that may be analyzed using the methods and compositions of the invention, where the analyte is capable of interacting with a reagent in order to generate a signal with at least two components that can be measured. An analyte may be naturally-occurring or synthetic. An analyte may be present in a sample obtained using any methods known in the art. In some cases, a sample may be processed before analyzing it for an analyte. The methods and compositions presented in this disclosure may be used in solution phase assays, without the need for particles (such as beads) or a solid support.

[00107] In some cases, an analyte may be a polynucleotide, such as DNA, RNA, peptide nucleic acids, and any hybrid thereof, where the polynucleotide contains any combination of deoxyribo- and/or

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ribo-nucleotides. Polynucleotides may be single stranded or double stranded, or contain portions of both double stranded or single stranded sequence. Polynucleotides may contain any combination of nucleotides or bases, including, for example, uracil, adenine, thymine, cytosine, guanine, inosine, xanthine, hypoxanthine, isocytosine, isoguanine and any nucleotide derivative thereof. As used herein, the term "nucleotide" may include nucleotides and nucleosides, as well as nucleoside and nucleotide analogs, and modified nucleotides, including both synthetic and naturally occurring species. Polynucleotides may be any suitable polynucleotide for which one or more reagents (or probes) as described herein may be produced, including but not limited to cDNA, mitochondrial DNA (mtDNA), messenger RNA (mRNA), ribosomal RNA (rRNA), transfer RNA (tRNA), nuclear RNA (nRNA), small interfering RNA (siRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), small Cajal body-specific RNA (scaRNA), microRNA (miRNA), double stranded (dsRNA), ribozyme, riboswitch or viral RNA. Polynucleotides may be contained within any suitable vector, such as a plasmid, cosmid, fragment, chromosome, or genome.

[00108] Genomic DNA may be obtained from naturally occurring or genetically modified organisms or from artificially or synthetically created genomes. Analytes comprising genomic DNA may be obtained from any source and using any methods known in the art. For example, genomic DNA may be isolated with or without amplification. Amplification may include PCR amplification, multiple displacement amplification (MDA), rolling circle amplification and other amplification methods. Genomic DNA may also be obtained by cloning or recombinant methods, such as those involving plasmids and artificial chromosomes or other conventional methods (see Sambrook and Russell, *Molecular Cloning: A Laboratory Manual*., cited *supra*.) Polynucleotides may be isolated using other methods known in the art, for example as disclosed in *Genome Analysis: A Laboratory Manual Series* (Vols. I-IV) or *Molecular Cloning: A Laboratory Manual*. If the isolated polynucleotide is an mRNA, it may be reverse transcribed into cDNA using conventional techniques, as described in Sambrook and Russell, *Molecular Cloning: A Laboratory Manual*., cited *supra*.

[00109] An analyte may be a protein, polypeptide, lipid, carbohydrate, sugar, small molecule, or any other suitable molecule that can be detected with the methods and compositions provided herein. An analyte may be an enzyme or other protein. An analyte may be a drug or metabolite (e.g., anti-cancer drug, chemotherapeutic drug, anti-viral drug, antibiotic drug, or biologic). An analyte may be any molecule, such as a co-factor, receptor, receptor ligand, hormone, cytokine, blood factor, antigen, steroid, or antibody.

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[00110] An analyte may be any molecule from any pathogen, such as a virus, bacteria, parasite, fungus, or prion (e.g., PrP^{Sc}). Examples of viruses include those from the families Adenoviridae, Flaviviridae, Hepadnaviridae, Herpesviridae, Orthomyxoviridae, Papovaviridae, Paramyxoviridae, Picornaviridae, Polyomavirus, Retroviridae, Rhabdoviridae, and Togaviridae. Specific examples of viruses include adenovirus, astrovirus, bocavirus, BK virus, coxsackievirus, cytomegalovirus, dengue virus, Ebola virus, enterovirus, Epstein-Barr virus, feline leukemia virus, hepatitis virus, hepatitis A virus, hepatitis B virus, hepatitis C virus, hepatitis D virus, hepatitis E virus, herpes simplex virus (HSV), HSV type 1, HSV type 2, human immunodeficiency virus (HIV), HIV type 1, HIV type 2, human papilloma virus (HPV), HPV type 1, HPV type 2, HPV type 3, HPV type 4, HPV type 6, HPV type 10, HPV type 11, HPV type 16, HPV type 18, HPV type 26, HPV type 27, HPV type 28, HPV type 29, HPV type 30, HPV type 31, HPV type 33, HPV type 34, HPV type 35, HPV type 39, HPV type 40, HPV type 41, HPV type 42, HPV type 43, HPV type 44, HPV type 45, HPV type 49, HPV type 51, HPV type 52, HPV type 54, HPV type 55, HPV type 56, HPV type 57, HPV type 58, HPV type 59, HPV type 68, HPV type 69, influenza virus, JC virus, Marburg virus, measles virus, mumps virus, Norwalk virus, parovirus, polio virus, rabies virus, respiratory syncytial virus, retrovirus, rhinovirus, rotavirus, Rubella virus, smallpox virus, vaccinia virus, West Nile virus, and yellow fever virus.

[00111] Examples of bacteria include those from the genuses *Bordetella, Borrelia, Brucella, Campylobacter*, *Chlamydia, Clostridium, Corynebacterium, Enterococcus, Escherichia, Francisella, Haemophilus, Helicobacter, Legionella, Leptospira, Listeria, Mycobacterium*, *Mycoplasma, Neisseria, Pseudomonas*, *Rickettsia, Salmonella*, *Shigella*, *Staphylococcus, Streptococcus*, *Treponema, Vibrio, and Yersinia.* Specific examples of bacteria include *Bordetella par apertussis, Bordetella pertussis, Borrelia burgdorferi, Brucella abortus, Brucella canis, Brucella melitensis, Brucella suis, Campylobacter jejuni, Chlamydia pneumoniae, Chlamydia psittaci, Chlamydia trachomatix, Clostridium botulinum, Clostridium difficile, Clostridium perfringens, Clostridium tetani, Corynebacterium diphtheriae, Enterococcus faecalis, Enterococcus faecium, Escherichia coli*, *Francisella tularensis, Haemophilus influenzae, Helicobacter pylori, Legionella pneumophila, Leptospira interrogans, Listeria monocytogenes, Mycobacterium leprae, Mycobacterium tuberculosis, Mycobacterium ulcerans, Mycoplasma pneumoniae, Neisseria gonorrhoeae, Neisseria meningitidis, Pseudomonas aeruginosa, Rickettsia rickettsii, Salmonella choleraesuis, Salmonella dublin, Salmonella enteritidis, Salmonella typhi, Salmonella typhimurium, Shigella sonnei, Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus saprophyticus, Streptococcus agalactiae, Streptococcus pneumoniae, Streptococcus pyogenes, Treponema pallidum, Vibrio cholerae, Yersinia pestis, and Yersinia enterocolitica.*

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[00112] Examples of parasites include those from the genuses *Acanthamoeba, Babesia, Balamuthia, Balantidium, Blasocystis, Cryptosporidium, Dientamoeba, Entamoeba, Giardia, Isospora, Leishmania, Naegleria, Pediculus, Plasmodium, Rhinosporidium, Sarcocystis, Schistosoma, Toxoplasma, Trichomonas,* and *Trypanosoma.* Specific examples of parasites include *Babesia divergens, Babesia bigemina, Babesia equi, Babesia microfti, Babesia duncani, Balamuthia mandrillaris, Balantidium coli, Dientamoeba fragilis, Entamoeba histolytica, Giardia lamblia, Isospora belli, Naegleria fowleri, Pediculus humanus, Plasmodium falciparum, Plasmodium knowlesi, Plasmodium malariae, Plasmodium ovale, Plasmodium vivax, Rhinosporidium seeberi, Sarcocystis bovihominis, Sarcocystis suihominis, Schistosoma mansoni, Toxoplasma gondii, Trichomonas vaginalis, Trypanosoma brucei,* and *Trypansoma cruzi.*

[00113] Examples of fungi include those from the genuses *Apophysomyces, Aspergillus, Blastomyces, Candida, Cladosporium, Coddidioides, Cryptococcos, Exserohilum, Fusarium, Histoplasma, Pichia, Pneumocystis, Saccharomyces, Sporothrix, Stachybotrys,* and *Trichophyton.* Specific examples of fungi include *Aspergillus fumigatus, Aspergillus flavus, Aspergillus clavatus, Blastomyces dermatitidis, Candida albicans, Coccidioides immitis, Crytptococcus neoformans, Exserohilum rostratum, Fusarium verticillioides, Histoplasma capsulatum, Pneumocystis jirovecii, Sporothrix schenckii, Stachybotrys chartarum,* and *Trichophyton mentagrophytes.*

[00114] In some cases, the methods provided in this disclosure may be used to detect any one of the analytes described above, or elsewhere in the specification. In some cases the methods provided in this disclosure may be used to detect panels of the analytes described above, or elsewhere in the specification. For example, a panel may comprise an analyte selected from the group consisting of any 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, 60, 70, 80, 90, 100 or more analytes described above or elsewhere in the specification.

[00115] An analyte may be obtained from any suitable location, including from organisms, whole cells, cell preparations and cell-free compositions from any organism, tissue, cell, or environment. Analytes may be obtained from environmental samples, biopsies, aspirates, formalin fixed embedded tissues, air, agricultural samples, soil samples, petroleum samples, water samples, or dust samples. In some instances, an analyte may be obtained from bodily fluids which may include blood, urine, feces, serum, lymph, saliva, mucosal secretions, perspiration, central nervous system fluid, vaginal fluid, or semen. Analytes may also be obtained from manufactured products, such as cosmetics, foods, personal care products, and the like. Analytes may be the products of experimental manipulation including,

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recombinant cloning, polynucleotide amplification, polymerase chain reaction (PCR) amplification, purification methods (such as purification of genomic DNA or RNA), and synthesis reactions.

[00116] More than one type of analyte may be detected in each multiplexed assay. For example, a polynucleotide, a protein, a polypeptide, a lipid, a carbohydrate, a sugar, a small molecule, or any other suitable molecule may be detected simultaneously in the same multiplexed assay with the use of suitable reagents. Any combination of analytes may be detected at the same time.

[00117] Detection of an analyte may be useful for any suitable application, including research, clinical, diagnostic, prognostic, forensic, and monitoring applications. Exemplary applications include detection of hereditary diseases, identification of genetic fingerprints, diagnosis of infectious diseases, cloning of genes, paternity testing, criminal identification, phylogeny, anti-bioterrorism, environmental surveillance, and DNA computing. For example, an analyte may be indicative of a disease or condition. An analyte may be used to make a treatment decision, or to assess the state of a disease. The presence of an analyte may indicate an infection with a particular pathogen, or any other disease, such as cancer, autoimmune disease, cardiorespiratory disease, liver disease, digestive disease, and so on. The methods provided herein may thus be used to make a diagnosis and to make a clinical decision based on that diagnosis. For example, a result that indicates the presence of a bacterial polynucleotide in a sample taken from a subject may lead to the treatment of the subject with an antibiotic.

[00118] In some cases the methods and compositions of the invention may be used to detect at least 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, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, or 1000 analytes. In some cases the methods and compositions of the invention may be used to detect 7-50, 8-40, 9-30, 10-20, 10-15, 8-12, or 7-12 analytes.

[00119] In some cases, this disclosure provides assays that are capable of unambiguously detecting the presence or absence of each of 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, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, or 1000 analytes, in any combination of presence or absence, in a single sample volume without immobilization, separation, mass spectrometry, or melting curve analysis. In some cases, this disclosure provides assays that are capable of unambiguously detecting the presence or absence of each of at least 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,

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46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, or 1000 analytes, in any combination of presence or absence, in a single sample volume without immobilization, separation, mass spectrometry, or melting curve analysis. In some cases, this disclosure provides assays that are capable of unambiguously detecting the presence or absence of less than 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, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, or 1000 analytes, in any combination of presence or absence, in a single sample volume without immobilization, separation, mass spectrometry, or melting curve analysis.

IV. Signals

[00120] The methods presented in this disclosure may be used with any quantifiable signal. In some cases, this disclosure provides coding schemes and methods to encode an infinite number of targets without degeneracy, using a single component of a signal (e.g., intensity). For example, as described above and in Example 3, a coding scheme may rely on a multiplicity of signal intensity without consideration of color. Although fluorescent probes have been used to illustrate this principle, the coding scheme is equally applicable to any other method providing a quantifiable signal, including an electrochemical signal and a chemiluminescent signal, as described elsewhere in this disclosure.

[00121] The methods presented in this disclosure may also utilize the measurement of a signal in at least two dimensions, also referred to as the measurement of at least two components of a signal. In comparison to the coding scheme described in the paragraph above, which relies on, for example, signal intensity to differentiate between analytes, utilization of at least two components of a signal (e.g., color and intensity) allows the generation of more unique codes per unit of signal intensity bandwidth. When at least two components of a signal are utilized, a cumulative measurement of the at least two components may be obtained for a single sample volume. For example in the coding scheme described in Table 5, the presence of each analyte results in a particular intensity (one component of the signal) in each of the four colors (a second component of a signal). The combination of these constituent signals leads to a cumulative signal that may be measured by measuring an intensity at each wavelength or range of wavelengths. The corresponding coding scheme or decoding matrix may then be used to convert the cumulative measurement into a determination of the presence or absence of an analyte.

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[00122] In some cases, a quantifiable signal comprises a waveform that has both a frequency (wavelength) and an amplitude (intensity). A signal may be an electromagnetic signal. An electromagnetic signal may be a sound, a radio signal, a microwave signal, an infrared signal, a visible light signal, an ultraviolet light signal, an x-ray signal, or a gamma-ray signal. In some cases, an electromagnetic signal may be a fluorescent signal, for example a fluorescence emission spectrum that may be characterized in terms of wavelength and intensity.

[00123] In certain portions of this disclosure, the signal is described and exemplified in terms of a fluorescent signal. This is not meant to be limiting, and one of ordinary skill in the art will readily recognize that the principles applicable to the measurement of a fluorescent signal are also applicable to other signals. For example, like fluorescent signals, any of the electromagnetic signals described above may also be characterized in terms of a wavelength and an intensity. The wavelength of a fluorescent signal may also be described in terms of color. The color may be determined based on measuring intensity at a particular wavelength or range of wavelengths, for example by determining a distribution of fluorescent intensity at different wavelengths and/or by utilizing a band pass filter to determine the fluorescence intensity within a particular range of wavelengths. Such band pass filters are commonly employed in a variety of laboratory instrumentation, including quantitative PCR machines. Intensity may be measured with a photodetector. A range of wavelengths may be referred to as a "channel."

[00124] In some cases, the methods provided in this disclosure may be used with any signal where the cumulative signal scales with the constituent signals of the same color, frequency, absorption band, and so on. However, the cumulative signal need not be digital or scale linearly with the number and intensity of the constituent signals. For example, if the physical principle of measurement is absorption, the cumulative attenuation is a product of constituent attenuations while the constituent concentrations are additive, due to the exponential nature of the Beer-Lambert law. The logarithm of the cumulative attenuation will then scale linearly with constituent concentrations in each absorption band (the equivalent of color, if fluorescent detection is used). The methods of the invention are therefore applicable. The methods of the invention may also be used with chemiluminescent signals and electrochemical signals.

[00125] The number of signals, and the number of dimensions or components measured, may also be expanded beyond the numbers shown in the exemplary embodiments of the invention, leading to an expansion in multiplexing capability. The exemplary embodiments provided in this disclosure utilize coding schemes constructed utilizing a fluorescent signal with one or two components measured: wavelength and/or intensity. The number of analytes that can be encoded can be increased by increasing

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the number of wavelengths and/or intensities. The number of analytes that can be encoded can also be increased by increasing the number of signals, for example by combining a fluorescent signal with an electrochemical signal or a FRET signal (fluorescence resonance energy transfer).

[00126] In some cases, more than two components of a signal may be measured. For example, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 18, 20 or more components of a signal may be measured. At least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 18, or 20 components of a signal may be measured. At least 2, but fewer than 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 18, or 20 components of a signal may be measured. In some cases, 2-3, 2-4, 2-5, 2-6, 3-5, 3-6, 3-8, or 5-10 components of a signal may be measured. These additional components may include kinetic components, such as a rate of signal decay and rate of photobleaching.

[00127] If a fluorescent signal is employed, the number of analytes that can be encoded may be further expanded by utilizing additional fluorophores. For example, 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 or more fluorophores may be used. In some cases, at least 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, or 50 fluorophores may be used. In some cases, fewer than 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, or 50 fluorophores may be used. In some cases, 4-6, 4-7, 4-8, 4-9, 4-10, 5-6, 5-7, 5-8, 5-9, 5-10, 10-15, or 10-20 fluorophores may be used.

[00128] Generally, the number of analytes that can be encoded may be further expanded by utilizing additional first values (e.g., intensities) that are values or ranges of values from a first component of a signal. For example, 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, or more first values that are values or ranges of values from a first component of a signal may be used. In some cases, at least 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 or more first values that are values or ranges of values from a first component of a signal may be used. In some cases, fewer than 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, or 50 first values that are values or ranges of values from a first component of a signal may be used. In some

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cases, 4-20, 4-6, 4-7, 4-8, 4-9, 4-10, 5-6, 5-7, 5-8, 5-9, 5-10, 10-15, or 10-20 first values that are values or ranges of values from a first component of a signal may be used.

[00129] In instances in which intensity is a component of a signal that is quantified, such as when a fluorescent signal is utilized, the presence of an analyte may be encoded using a variety of intensities or ranges of intensities. For example, a coding scheme may utilize 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, or more intensities or ranges of intensities. A coding scheme may utilize at least 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, or more intensities or ranges of intensities. A coding scheme may utilize fewer than 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, or 50 intensities or ranges of intensities. In some cases, a coding scheme may utilize 2-20, 2-3, 2-4, 2-5, 2-6, 2-7, 2-8, 2-9, 2-10, 3-4, 3-5, 3-6, 3-7, 3-8, 3-9, 3-10, 4-5, 4-6, 4-7, 4-8, 4-9, 4-10, 5-6, 5-7, 5-8, 5-9, or 5-10 intensities or ranges of intensities.

[00130] The number of analytes that can be encoded may be further expanded by utilizing additional second values (e.g., wavelengths) that are values or ranges of values from a second component of a signal. For example, 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, or more second values that are values or ranges of values from a second component of a signal may be used. In some cases, at least 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 or more second values that are values or ranges of values from a second component of a signal may be used. In some cases, fewer than 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, or 50 second values that are values or ranges of values from a second component of a signal may be used. In some cases, 4-20, 4-6, 4-7, 4-8, 4-9, 4-10, 5-6, 5-7, 5-8, 5-9, 5-10, 10-15, or 10-20 second values that are values or ranges of values from a second component of a signal may be used.

[00131] In instances in which wavelength is a component of a signal that is quantified, such as when a fluorescent signal is utilized, the presence of an analyte may be encoded using a variety of wavelengths or ranges of wavelengths. For example, 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, or more wavelengths or ranges of wavelengths may be used. In some cases, at

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least 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 or more wavelengths or ranges of wavelengths may be used. In some cases, fewer than 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, or 50 wavelengths or ranges of wavelengths may be used. In some cases, 4-20, 4-6, 4-7, 4-8, 4-9, 4-10, 5-6, 5-7, 5-8, 5-9, 5-10, 10-15, or 10-20 wavelengths or ranges of wavelengths may be used.

[00132] In some cases, when degeneracy is eliminated, the methods of the invention are capable of detecting the presence or absence of at least 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, or more analytes in a single volume when each reagent used to generate a signal in the volume generates only one second value (e.g., each reagent, emits light at only one wavelength).

[00133] In other cases, when degeneracy is eliminated, the methods of the invention are capable of detecting the presence or absence of at least 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, or more analytes in a single volume using a total of four second values in that volume (e.g., a total of four wavelengths or ranges or wavelengths, which might be implemented by using four spectrally resolvable fluorophores).

[00134] As described throughout the specification, the assay provided herein utilizes cumulative measurements on a sample. A cumulative measurement may be, for example, a single measurement of intensity values, or a measurement of intensity values at one or more wavelengths or ranges of wavelengths. A plurality of cumulative measurements may be obtained. For example, an intensity may be measured at 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, or more wavelengths or ranges of wavelengths. An intensity may be measured at at least 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, or more wavelengths or ranges of wavelengths. An intensity may be measured at less than 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, or 50 wavelengths or ranges of wavelengths.

[00135] More generally a cumulative measurement may be obtained for any quantifiable component of a signal, and for any quantifiable component of a signal at another quantifiable component

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of a signal. For example, at least a first component of a signal may be measured at 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, or more second components of a signal. At lest a first component of a signal may be measured at at least 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, or more second components of a signal. At least a first component of a signal may be measured at less than 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, or 50 second components of a signal.

[00136] As is apparent from this disclosure, each analyte to be detected can be encoded as a code utilizing any number of suitable components of a signal or any number of signals. For example, each analyte to be detected can be encoded in 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, or more components of a signal or signals. In some cases, each analyte to be detected can be encoded in at least 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 or more components of a signal or signals. In some cases, each analyte to be detected can be encoded in fewer than 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, or 50 components of a signal or signals. In some cases, 1-4, 4-20, 4-6, 4-7, 4-8, 4-9, 4-10, 5-6, 5-7, 5-8, 5-9, 5-10, 10-15, or 10-20 components of signal or signals may be used to encode each analyte. Each analyte in a coding scheme may be encoded by the same number of components of a signal, or signals, or different numbers of components of a signal, or signals.

A. Probes, Primers, Fluorophores, and Quenchers

[00137] Some of the methods provided in this disclosure utilize a reagent that generates a signal in the presence of an analyte. Any suitable reagent may be used with the invention. Generally, a reagent will have an analyte-specific component and a component that generates a signal in the presence of the analyte. In some cases, these reagents are referred to as probes. The probes may be hybridization probes. The hybridization probes may be an oligonucleotide probe attached to a fluorophore and a quencher (e.g., a TAQMAN probe).

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[00138] The methods of the invention may use one or more reagents or probes to detect the presence or absence of each analyte. For example, 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 or more or probes may be used to detect the presence or absence of each analyte. In some cases, at least 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 probes may be used to detect the presence or absence of each analyte. In some cases, fewer than 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 probes may be used to detect the presence or absence of each analyte. In some cases, the number of probes used to detect the presence or absence of each analyte is 1-10, 2-9, 2-8, 2-7, 2-6, 2-5, 2-4, 1-3, or 1-4.

[00139] In some cases, a sample is contacted with 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, or more probes to detect the presence or absence of all analytes. In some cases, a sample is contacted with at least 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 or more probes to detect the presence or absence of all analytes. In some cases, a sample is contacted with fewer than 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, or 50 probes to detect the presence or absence of all analytes. In some cases, the number of probes that a sample is contacted with to detect the presence or absence of all analytes is 4-50, 4-40, 4-30, 4-20, 5-15, 5-10, or 3-10.

[00140] As described above, oligonucleotide probes attached to a fluorophore and a quencher may be used to detect the presence of an analyte in a polynucleotide amplification assay. So long as the quencher and the fluorophore are in proximity, the quencher quenches the fluorescence emitted by the fluorophore upon excitation by a light source. The sequence of the oligonucleotide probe is designed to be complementary to a polynucleotide sequence present in an analyte, and therefore capable of hybridizing to the polynucleotide sequence present in the analyte. Hybridization of the oligonucleotide probe may be performed in a nucleic acid amplification reaction comprising primers (e.g., a polymerase chain reaction). Upon extension of the primers by a DNA polymerase, the 5' to 3' exonuclease activity of the polymerase degrades the probe, releasing the fluorophore and the quencher into the medium. The proximity between the fluorophore and the quencher is broken and the signal from the fluorophore is no longer quenched. Thus, the amount of fluorescence detected is a function of the amount of analyte

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present. If no analyte is present, the probe will not hybridize to an analyte, and the fluorophore and quencher will remain in close proximity. Little or no signal will be produced.

[00141] Oligonucleotide probes may have one or a plurality of fluorophores and quenchers per probe. For example, in some embodiments an oligonucleotide probe may comprise 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 or more fluorophores. An oligonucleotide probe may comprise at least 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, or 50 fluorophores. An oligonucleotide probe may comprise fewer than 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, or 50 fluorophores.

[00142] An oligonucleotide probe may comprise 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 or more quenchers. An oligonucleotide probe may comprise at least 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, or 50 quenchers. An oligonucleotide probe may comprise fewer than 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, or 50 quenchers.

[00143] Attachment of probes and quenchers to a probe may be performed in the same reaction or in serial reactions. A series of reactions may be performed to label probes with at least one fluorophore and the reaction products may be mixed to generate a mixture of probes with different fluorophores.

[00144] If an oligonucleotide probe comprises two or more fluorophores, these fluorophores may be arranged to allow Förster (Fluorescence) resonance energy transfer (FRET) to occur between the fluorophores. Briefly, FRET is a mechanism of energy transfer between fluorophores. Using FRET-based probes allows one excitation source to generate two different fluorescence emission signals by excitation of a single fluorophore. For example, the methods described herein may used paired probes, where one probe in the pair is attached to a fluorophore and a quencher and a second probe is attached to two fluorophores (in close enough proximity for FRET to occur) and a quencher. Any combination of fluorophores and quenchers that provide for FRET may be used. Such an approach doubles the number of fluorescent probes that may be used with an excitation source, because a single excitation source can be used to produce two signals: one from the probe with one fluorophore and one from the probe with two fluorophores in close enough proximity to provide for FRET. For example, using the encoding

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method described in Table 8, the number of unambiguously detectable analytes could be increased from 16 to 32 by pairing each of the probes in Table 8 with a corresponding FRET probe.

[00145] The primers may be specific for a particular analyte and capable of amplifying a region complementary to a probe. In some cases, the number of pairs of primers used is equivalent to the number of probes. In other cases, the number of probes used may exceed the number of primer pairs used. In still other cases, the number of primer pairs used may exceed the number of probes used. In some cases a sample to be analyzed is contacted with 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 or more pairs of primers. In some cases a sample to be analyzed is contacted with at least 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 or more pairs of primers. In some cases a sample to be analyzed is contacted with fewer than 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, or 50 pairs of primers. In some cases, the number of pairs of primers is 2-10, 3-15, 4-20, 3-10, 4-10, 5-10, 6-8, or 6-10.

[00146] The primers may amplify regions of a polynucleotide in which different numbers of hybridization probes hybridize. For example, at least one pair of primers may amplify a region complementary to at least 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, or 50 hybridization probes. In some cases, all of said pairs of primers may amplify a region complementary to at least 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, or 50 hybridization probes.

[00147] In one example, a first probe may be labeled with the fluorophore 5'-fluorescein amidite (5'-FAM) and the quencher black hole quencher 1 (BHQ-1). A second probe may be labeled with 5'-FAM in close proximity (e.g., attached to) to cyanine 5.5 (Cy5.5) and the quencher black hole quencher 3 (BHQ-3). Upon digestion of the probe, via the nuclease activity of the polymerase, two fluorescent signals are generated from a single excitation wavelength (e.g., 470 nm). The fluorophore from the first probe will fluoresce at about 520 nm. The fluorophores on the second probe undergo FRET. The donor fluorophore, FAM is excited by the excitation light source (e.g., 470 nm) and transfers its energy to the acceptor fluorophore, Cy5.5. The acceptor fluorophore emits at about 705 nm. These fluorophores and quenchers are merely exemplary. Any fluorophores that can undergo FRET and any quenchers that can

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quench fluorescence are suitable for use with the invention. Methods for producing FRET-based probes are described in Jothikumar et al., BioTechniques, 2009, 46(7):519-524.

[00148] In some examples, a single hybridization probe may be used for each analyte. In order to utilize multi-color encoding (e.g., as shown in Table 3), each probe may be labeled with a plurality of fluorophores at pre-determined ratios. The ratio may be determined so that a positive control signal provides the same intensity as other positive control signals of the same intensity, in the same color. This approach reduces the number of probes that must be synthesized and may be less expensive to deploy than methods utilizing multiple probes. Moreover, in the case of hybridization probes, it is easier to fit one hybridization probe to an analyte sequence than multiple probes.

[00149] Although many aspects of the invention are exemplified using nucleic acid-based probes, one of ordinary skill in the art will readily recognize that other forms of probes would work equally well with the invention described in this disclosure. For example, a binding molecule specific to an analyte could be used as a probe. Non-limiting exemplary binding molecules include an antibody recognizing an analyte, and generating a signal in the presence of an analyte.

[00150] In embodiments of the invention that utilize fluorescent labels, any suitable fluorescent label may be used. Exemplary fluorescent labels suitable for use with the invention include rhodamine, rhodol, fluorescein, thiofluorescein, aminofluorescein, carboxyfluorescein, chlorofluorescein, methylfluorescein, sulfofluorescein, aminorhodol, carboxyrhodol, chlororhodol, methylrhodol, sulforhodol; aminorhodamine, carboxyrhodamine, chlororhodamine, methylrhodamine, sulforhodamine, and thiorhodamine, cyanine, indocarbocyanine, oxacarbocyanine, thiacarbocyanine, merocyanine, cyanine 2, cyanine 3, cyanine 3.5, cyanine 5, cyanine 5.5, cyanine 7, oxadiazole derivatives, pyridyloxazole, nitrobenzoxadiazole, benzoxadiazole, pyrene derivatives, cascade blue, oxazine derivatives, Nile red, Nile blue, cresyl violet, oxazine 170, acridine derivatives, proflavin, acridine orange, acridine yellow, arylmethine derivatives, auramine, crystal violet, malachite green, tetrapyrrole derivatives, porphin, phtalocyanine, and bilirubin. Exemplary quenchers include black hole quenchers, such as BHQ-0, BHQ-1, BHQ-2, BHQ-3; ATTO quenchers, such as ATTO 540Q, ATTO580Q, and ATTO612Q; and the like.

[00151] The fluorophores that may be used with the invention are not limited to any of the fluorophores described herein. For example, fluorophores with improved properties are continually developed, and these fluorophores could readily be used with the methods provided in this disclosure. Such improved fluorophores include quantum dots, which may emit energy at different wavelengths after being excited at a single wavelength. The advantage of using such fluorophores is that only a

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single excitation source is needed, but many different signals may be quantified, for example in terms of color and intensity. Moreover, fluorophores with narrow emission spectra would be particularly useful with the method described herein, as such fluorophores could be included in multiplex assays with minimal or no overlap between their emission spectra, thereby offering many more "colors" and boosting significantly the overall number of coded analytes.

[00152] In some cases, one or more reagents are lyophilized. Any suitable reagent may be lyophilized. For example, probes, primers, enzymes, antibodies, or any other reagent used for detection may be lyophilized. In some cases, a sample comprising an analyte may also be lyophilized. Lyophilization may be useful, for example, when distributing reagents and/or samples in developing regions where access to cold storage is expensive, not readily available, or not reliably available. In one example, lyophilized reagents comprise any probe or analyte-specific reagent described in this disclosure or otherwise suitable for use with the invention. In another example, lyophilized reagents comprise PCR primers. In yet another example, lyophilized reagents comprise reagents suitable for performing a PCR reaction.

V. Analytical Techniques and Instrumentation

[00153] The methods provided herein are suitable for use with a variety of detection methods. For example, the methods may be applied using an analytical technique that measures the wavelength and intensity of a fluorescent signal. This may be accomplished by measuring the intensity of a signal across a spectrum of wavelengths, or by using band pass filters that restrict the passage of certain wavelengths of light, thereby allowing only light of certain wavelengths to reach a photodetector. Many real-time PCR and quantitative PCR instruments comprise an excitation light source and band pass filters that enable the detection of fluorescent signals in four colors (e.g., blue, green, yellow, and red). Therefore, the methods of the invention can be readily applied using instruments widely used in the art. Importantly, the methods and compositions provided herein may be used to detect multiple analytes by obtaining a cumulative measurement on a single solution. No separation is necessary. The invention does not require the use of beads or a solid phase. Of course, one of ordinary skill in the art would understand that the invention could be used with separation, beads, or a solid phase, if desired.

[00154] **Fig. 5** shows two examples of instrumental configurations that may be used to obtain cumulative measurements useful for the methods of the invention. With reference to **Fig. 5**, an instrument with a detector configured to detect both wavelength and intensity (e.g., a fluorometer) is shown in **501.** With reference to **501**, at least one excitation light source **502** is directed into a chamber

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503 containing analytes and reagents that generate a signal in the presence of the analytes **504.** As described elsewhere, the analyte may be a nucleic acid and the reagents generating the signal may be hybridization probes comprising a fluorophore and a quencher. If the analyte is present, an emission signal **505** is generated. In the configuration shown in **501**, the wavelength and intensity of this emission signal are measured across a spectrum by a detector capable of generating a fluorescence emission spectrum **506.**

[00155] The wavelength and intensity may also be determined using a combination of a photodetector and band pass filters. This configuration is used in several thermal cyclers known in the art. With reference to **Fig. 5**, an instrument with band pass filters and a photodetector is depicted in **507.** With reference to **507**, at least one excitation light source **508** is directed into a chamber **509** containing analytes and reagents that generate a signal in the presence of the analytes **510.** As described elsewhere, the analyte may be a nucleic acid and the reagents generating the signal may be hybridization probes comprising a fluorophore and a quencher. If the analyte is present, an emission signal **511** is generated. In the configuration shown in **507**, band pass filters **512, 513,** and **514** are used to restrict the passage of light to light within certain ranges of wavelengths. For example band pass filter **512** may restrict the passage of light to light within wavelength range 1. Band pass filter **513** may restrict the passage of light to light within wavelength range 2. Band pass filter **514** may restrict the passage of light to light within wavelength range 3. Any number of band pass filters (e.g., 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, or more) may be used with the invention. In addition, the positions of the band pass filters in **507** are not meant to be limiting. In some cases, emitted light may be passed through more than one band pass filter at any time. In other cases, the band pass filters may be configured so that all emitted light passes through only one band pass filter at a time. After passage through a band pass filter, filtered light **515, 516,** and **517** is detected by a photodetector. For example, filtered light **515** may be light within wavelength range 1, filtered light **516** may be light within wavelength range 2, and filtered light **517** may be light within wavelength range 3. The intensity of each of the filtered lights **515, 516,** and **517** may then be quantified by a photodetector **518.**

[00156] The methods described in this disclosure are compatible with a variety of amplification methods, including quantitative PCR (qPCR) methods, end point PCR methods, reverse transcriptase PCR, and digital PCR methods. Digital PCR methods (e.g., DROPLET DIGITAL (BIORAD) and DYNAMIC ARRAY (FLUIDIGM)) produce highly sensitive quantification of polynucleotide copy numbers. The methods provided herein can be easily integrated into these systems to significantly expand their throughput by allowing multiplexing in a droplet or a dynamic array.

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[00157] Similarly, the methods described in this disclosure may be applied in a real-time PCR assay. For example, real-time data may be fully recorded as the PCR runs to completion. The end-point values may then be decoded to indicate the presence or absence of analytes. The individual cycle thresholds (Ct) values may be analyzed for each color by detecting the maxima of the second derivative of the real-time curve in that color. As a particular analyte is amplified, fluorophores are released from its corresponding probes in the same ratios as the color multiplicities in the coding of that sequence. Hence, e.g. a code of 1100 will have the same Ct in blue and green, while a code of 1400 will have its green Ct precede its blue Ct by 2 cycles. This additional data enables determination of the identity and starting quantity of each analyte.

[00158] The methods described herein may also be used directly on a tissue sample. For example, a tissue sample may be obtained and photolithographic methods may be used to build wells directly onto the tissue sample. The tissue sample may be fixed prior to building the wells. The tissue sample may then be analyzed by dispensing appropriate reagents into the wells in the tissue sample. The encoding and decoding methods described in this disclosure may be used for the multiplexed detection of analytes within the wells etched into the tissue. Each well may correspond to a single cell or a few cells, providing excellent spatial resolution when analyzing different portions of a tissue for an analyte. This method may be used to detect analytes in different areas of the same tissue.

[00159] In some cases, instruments may be modified or constructed, for example, to provide additional excitation light sources, at multiple wavelength, and/or to provide additional band pass filters or a capability of determining a complete spectrum. Including additional excitation sources would allow for the excitation of a larger variety of fluorophores. Including additional band pass filters, or modifying or constructing an instrument capable of determining an entire spectrum allows detection of a wider variety of emissions from fluorophores. These techniques can be used to increase the number of fluorophores that can be used with the methods described in this disclosure and, accordingly, to increase the number of analytes that be simultaneously detected.

[00160] In some cases, the methods described in this disclosure utilize end-point PCR methods. However, in some cases a result may be obtained before the end-point. This may be advantageous when, for example, results are needed as soon as possible. For example, in one case calibration experiments are performed in which different starting concentrations of an analyte are analyzed to determine the cycle number at which the signal becomes saturated, as a function of the starting amount of the analyte. Using this data, a computer monitoring a PCR reaction in real-time can be programmed to search for saturation up to the maximal cycle number according to the limit of detection (LOD) of the

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particular system. If there is no amplification of an analyte (e.g., other than a positive control, if present), by the maximal cycle number, the result for that analyte is negative. If there is amplification of the analyte by the maximal cycle number, the result for that analyte is positive and the saturation intensity in each color may be used to decode the result using the coding scheme. In both cases, there would be no need to run the PCR reaction beyond the number of cycles set by the LOD for that instrument. Although PCR has been used to illustrate this quantitative method, one of ordinary skill in the art will readily recognize that similar principles could be applied in any catalytic system where the starting amount of an analyte limits the rate of reaction, and the rate of reaction can be measured and reported by an evolution in a signal intensity over time.

VI. Compositions and Kits

[00161] This disclosure also provides compositions and kits for use with the methods described herein. The compositions may comprise any component, reaction mixture and/or intermediate described herein, as well as any combination. For example, the disclosure provides detection reagents for use with the methods provided herein. Any suitable detection reagents may be provided, including hybridization probes labeled with a fluorophore and a quencher and primers, as described elsewhere in the specification.

[00162] In some cases, compositions comprise reagents for the detection of at least seven analytes using four fluorophores. In some cases, compositions comprise reagents for the detection of at least 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, or 50 analytes using four fluorophores. In some cases, compositions comprise reagents for the detection of at least 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, or 50 analytes using five fluorophores. In some cases, compositions comprise reagents for the detection of at least 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, or 50 analytes using six fluorophores. In some cases, compositions comprise reagents for the detection of at least 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, or 50 analytes using seven fluorophores.

[00163] In some cases the compositions comprise primers. The compositions may comprise at least 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, or 50 pairs of primers.

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[00164] The invention also provides kits for carrying out the methods of the invention. Accordingly, a variety of kits are provided in suitable packaging. The kits may be used for any one or more of the uses described herein, and, accordingly, may contain instructions for detecting the presence or absence of each analyte of a plurality of analytes. A kit may comprise a coding scheme, or a decoding matrix, to assist the user in converting a cumulative measurement to a result indicating the presence or absence of each of a plurality of analytes. A kit may be a diagnostic kit, for example, a diagnostic kit suitable for the detection of any analyte, including the analytes recited herein. A kit may contain any of the compositions provided in this disclosure, including those recited above.

[00165] **Fig. 6** shows schematics of exemplary kits of the invention. With reference to **Fig. 6**, a kit comprising a kit body **601** is shown that contains reagents for the detection of analytes (e.g., polynucleotide probes comprising a fluorophore and a quencher) **602** and primers for the amplification of a nucleic acid **603.** The kit may also contain any other reagents, such as reagents suitable for performing an amplification reaction **604.** In the embodiment depicted in **601**, the reagents for the detection of analytes and the primers are each contained in a single volume (e.g., tube or a bottle). However, these reagents may also be provided in separate volumes, as depicted the kit body shown in **605.** The kit body **605** contains seven separate volumes. Three volumes (**606, 607,** and **608**) contain reagents for the detection of analytes (or mixtures of such reagents) and three volumes (**609, 610,** and **611**) contain primers (or mixtures of primers). The kit may also contain reagents suitable for performing an amplification reaction **604.**

[00166] **Fig. 6** is provided for illustrative purposes only. Any number of reagents for the detection of analytes (e.g., probes) and any number of primers may be included in a single tube or bottle, as appropriate for the application. For example, in some embodiments, 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 or more reagents for the detection of analytes may be included in a single bottle. In other cases, 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 or more primers for amplification may be included in a single bottle or tube. In some cases, primers and probes may be provided in the same bottle or tube. For example, 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 or more probes may be provided per pair of primers. In other cases, 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 or more primers may be provided

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per pair of probes. The kits may contain 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 or more bottles or tubes. Control analytes, and their associated detection reagents and primers, may also be included in the kit. These may be included in a separate bottle or tube or, for example, included within the bottle or tube providing reagents for an amplification reaction **604**. As described elsewhere in the specification, any reagent in the kits may be lyophilized.

VII. Systems and Software

[00167] The methods of this disclosure may also be performed as part of a system. For example, one or more steps of the methods described herein may be performed by software contained on a computer readable medium. The software may be used to encode analytes, check for degeneracy, reduce or eliminate degeneracy, and/or produce a decoding matrix. The software may also be used to convert cumulative measurements made on an instrument (e.g., a PCR machine) into information concerning the presence or absence of an analyte. For example, with reference to **Fig. 4**, a computer **401** may be connected to an input device **403**, a display **402**, and a printer **404**. The computer may execute one or more of the steps of any of the methods described in this disclosure, including, for example, encoding methods **410**, analysis methods **420**, and decoding methods **430.** A computer may reduce or eliminate degeneracy, for example as part of the encoding methods **410.** Similarly, a computer may generate a coding schme that is non-degenerate by design using, for example, mathematical iteration. A computer may also be connected to a network **405**. The network may be a local network and/or the Internet. The network may be used to perform the methods of the invention on multiple computers in different locations. For example, one computer may perform the encoding, another computer may perform the measuring, and yet another computer may perform the decoding. A computer may perform any or all of these methods. The network may be used to transmit information obtained using the methods described in this disclosure throughout the world, for example across a border.

[00168] In one example, the software is embedded in the instrument making the measurement, such as a thermal cycler. In another example, the software is installed on a computer attached to the instrument making the measurement, such as a computer attached to a thermal cycler. In another example, the software is in the "cloud" -- i.e., on a computer that another computer may communicate with through a network.

[00169] Each of the elements described above may be connected to a controller that communicates with each of the elements and coordinates their actions. For example, a controller may

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initiate the encoding of a series of analytes into values of color and intensity. As described throughout this disclosure, the controller can execute software to reduce or eliminate degeneracy by removing one or more potential analyte codes from the encoding matrix. The controller may then automatically order probes corresponding to this code from a manufacturer of probes. Alternatively, the controller may operate an oligonucleotide synthesis instrument that can synthesize the appropriate probes. After analysis of the sample on the instrument (which may also be automated), the controller may process the results of the analysis using the decoding matrix. The controller may then provide these results to a user.

VIII. Services

[00170] The methods provided herein may also be performed as a service. For example, a service provider may obtain the identity of a plurality of analytes that a customer wishes to analyze. The service provider may then encode each analyte to be detected by any of the methods described herein and provide appropriate reagents to the customer for the assay. The customer may perform the assay and provide the results to the service provider for decoding. The service provider may then provide the decoded results to the customer. The customer may also encode analytes, generate probes, and/or decode results by interacting with software installed locally (at the customer's location) or remotely (e.g., on a server reachable through a network). Exemplary customers include clinical laboratories, physicians, manufacturers of food and consumer products, industrial manufacturers (e.g., petroleum companies) and the like. A customer or party may be any suitable customer or party with a need or desire to use the methods, systems, compositions, and kits of the invention.

EXAMPLES

Example 1: General Materials & Methods

1. General Reagents

[00171] TE Buffer, pH7 (LIFE TECHNOLOGIES, Carlsbad, CA); UltraPure RNAse-free Water (LIFE TECHNOLOGIES, Carlsbad, CA); Taq 5x Master Mix (FISHER SCIENTIFIC COMPANY, Tustin, CA).

2. DNA Sequences, Primers, and Probes

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[00172] Five nucleic acids from organisms of clinical relevance were chosen for the exemplary study: (1) human immunodeficiency virus 1 (HIV-1); (2) *Plasmodium falciparum* (Malaria); (3) herpes simplex virus-2 (HSV-2); (4) *Mycobacterium tuberculosis* (TB); (5) and dengue virus type 3 (dengue fever).

[00173] Two regions of diagnostic relevance from the HIV-1 genome, p17 and poly-protease, were selected from the Los Alamos National Laboratory HIV-1 reference sequence. A diagnostic sequence from the *Plasmodium falciparum* ChR7 gene was obtained from the UCSC *Plasmodium falciparum* Genome Browser. A sequence for Herpes Simplex Virus-2 was synthesized from the sequence obtained from the European Molecular Biology Library. Similarly a diagnostic sequence for the rpoB gene in *Mycobacterium tuberculosis* was synthesized from a sequence obtained from the European Molecular Biology Library. A PCR diagnostic sequence for Dengue Virus Type 3 was obtained from the National Institute of Health genetic sequence database.

[00174] Oligonucleotides were synthesized by INTEGRATED DNA TECHNOLOGIES (IDT). Probes and primer pairs were chosen for each analyte using OLIGOANALYZER, from IDT. Sense probes containing a fluorophore at the 5' end and a quencher at the 3' end were synthesized for all analytes. All oligonucleotides were lyophilized and reconstituted in TE buffer before use. Tables 9 - 14 show sequence information, target sequence, primers, and probes for each of the six analytes.

Table 9. HIV-1 polyprotease sequence information.

Sequence Information	HIV-1 Poly protease 198mer synthesized from bases 2253 – 2550
Source	HIV-1 Reference Sequence, Los Alamos National Laboratory
Target sequence (3' to 5')	GGAAGCTCTATTAGATACAGGAGCAGATGATACAGTATTAGAAGAAATGA GTTTGCCAGGAAGATGGAAACCAAAAATGATAGGGGGAATTGGAGGTTTT ATCAAAGTAAGACAGTATGATCAGATACTCATAGAAATCTGTGGACATAA AGCTATAGGTACAGTATTAGTAGGACCTACACCTGTCAACATAATTGG (SEQ ID NO: 1)
Forward Primer (3' to 5')	GGAAGCTCTATTAGATACAGGAGCAG **(SEQ ID NO: 2)**
Reverse Primer (3' to 5')	CCAATTATGTTGACAGGTGTAGGTCC **(SEQ ID NO: 3)**
Probe 1 (3' to 5')	**/56-FAM/**TGAGTTTGCCAGGAAGATGGAAACCA/3BHQ_1/ **(SEQ ID NO: 4)**

Table 10. HIV p17 sequence information.

Sequence Name	HIV-1 P17 199mer synthesized from bases 790 – 1186

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Source	HIV-1 Reference Sequence, Los Alamos National Laboratory
Target sequence (3' to 5')	CAGCTACAACCATCCCTTCAGACAGGATCAGAAGAACTTAGATCATTATA TAATACAGTAGCAACCCTCTATTGTGTGCATCAAAGGATAGAGATAAAAG ACACCAAGGAAGCTTTAGACAAGATAGAGGAAGAGCAAAACAAAAGTAAG AAAAAAGCACAGCAAGCAGCAGCTGACACAGGACACAGCAATCAGGTCA **(SEQ ID NO: 5)**
Forward Primer (3' to 5')	CAGCTACAACCATCCCTTCAGACA **(SEQ ID NO: 6)**
Reverse Primer (3' to 5')	TGACCTGATTGCTGTGTCCTGTGT **(SEQ ID NO: 7)**
Probe 1 (3' to 5')	/**56-FAM**/AGCAACCCTCTATTGTGTGCATCAAAGG/3BHQ_1 **(SEQ ID NO: 8)**
Probe 2 (3' to 5')	/**5Cy3**/AAAGCACAGCAAGCAGCAGCTGA/3BHQ_2/ **(SEQ ID NO: 9)**

Table 11. *Plasmodium* ChR7 sequence information.

Sequence Name	*Plasmodium* ChR7 199mer synthesized from bases 1139138 - 1141223
Source	UCSC Plasmodium falciparum Genome Browser Gateway
Target sequence (3' to 5')	GCCTAACATGGCTATGACGGGTAACGGGGAATTAGAGTTCGATTCCGGAG AGGGAGCCTGAGAAATAGCTACCACATCTAAGGAAGGCAGCAGGCGCGTA AATTACCCAATTCTAAAGAAGAGAGGTAGTGACAAGAAATAACAATGCAA GGCCAATTTAAAACCTTCCCAGAGTAACAATTGGAGGGCAAGTCTGGTG (SEQ ID NO: 10)
Forward Primer (3' to 5')	GCCTAACATGGCTATGACGGGTAA **(SEQ ID NO: 11)**
Reverse Primer (3' to 5')	CACCAGACTTGCCCTCCAATTGTT **(SEQ ID NO: 12)**
Probe 1 (3' to 5')	/**56-FAM**/ATTCCGGAGAGGGAGCCTGAGAAATA/3BHQ_1/ **(SEQ ID NO: 13)**
Probe 2 (3' to 5')	/**56-ROXN**/AAGGAAGGCAGCAGGCGCGTAAATTA/3BHQ_2/ **(SEQ ID NO: 14)**

Table 12. Herpes simplex virus 2 sequence information.

Sequence Name	HSV-2 193mer synthesized from HSV-2 genome
Source	EMBL Bank AJ303204
Target sequence (3' to	TCAGCCCATCCTCCTTCGGCAGTATGGAGGGTGTCGCGGCGGCGAGCCGC

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5')	CGTCCCCAAAGACGTGCGGGTCGTACACGTACACGTACCAGGGCGGCGGG CCTCCGACCCGGTACGCTCTCGTAAATGCTTCCCTGCTGGTGCCGATCTG GGACCGCGCCGCGGAGACATTCGAGTACCAGATCGAACTCGG **(SEQ ID NO: 15)**
Forward Primer (3' to 5')	TCAGCCCATCCTCCTTCGGCAGTAT **(SEQ ID NO: 16)**
Reverse Primer (3' to 5')	CCGAGTTCGATCTGGTACTCGAATGT **(SEQ ID NO: 17)**
Probe 1 (3' to 5')	**/56-FAM/**AAAGACGTGCGGGTCGTACACGTACA/3BHQ_1/ **(SEQ ID NO: 18)**
Probe 2 (3' to 5')	**/5Cy5/**TAAATGCTTCCCTGCTGGTGCCGAT/3IAbRQSp/ **(SEQ ID NO: 19)**

Table 13. *Mycobacterium tuberculosis* rpoB sequence information

Sequence Name	Mycobacterium Tuberculosis rpoB 200mer synthesized from Mycobacterium tuberculosis rpoB genome
Source	EMBL Bank GQ395623
Target sequence (3' to 5')	GAGTGCAAAGACAAGGACATGACGTACGCGGCCCCGCTGTTCGTCACGGC CGAGTTCATCAACAACAACACCGGCGAGATCAAGAGCCAGACGGTGTTCA TGGGTGACTTCCCGATGATGACCGAGAAGGGCACCTTCATCATCAACGGC ACCGAGCGCGTCGTGGTCAGCCAGCTGGTCCGCTCGCCCGGTGTGTACTT **(SEQ ID NO: 20)**
Forward Primer (3' to 5')	GAGTGCAAAGACAAGGACATGACG **(SEQ ID NO: 21)**
Reverse Primer (3' to 5')	AAGTACACACCGGGCGAGC **(SEQ ID NO: 22)**
Probe 1 (3' to 5')	**/56-FAM/**CGCTGTTCGTCACGGCCGAGTTCAT/3BHQ_1/ **(SEQ ID NO: 23)**
Probe 2 (3' to 5')	**/5Cy3/**AGATCAAGAGCCAGACGGTGTTCATG/3BHQ_2/ **(SEQ ID NO: 24)**
Probe 3 (3' to 5')	**/5Cy5/**AAGGGCACCTTCATCATCAACGGCA/3IAbRQSp/ **(SEQ ID NO: 25)**

Table 14. Dengue virus type 3 sequence information.

Sequence Name	Dengue Virus Type 3 200mer synthesized from Dengue Virus Type 3 genome
Source	GenBank M93130
Target sequence (3' to 5')	ATGCCAACTGTGATTGAGCACTTAGAAAGACTACAAAGGAAACATGGAGG AATGCTTGTGAGAAATCCACTCTCACGAAACTCCACGCACGAAATGTATT GGATATCCAATGGTACAGGCAACATCGTCTCATTCACAATGACACACAGG AGACCCACCATAGAGAAAGATGTGGATTTAGGAGCAGGAACCCGACATGT **(SEQ ID NO: 26)**
Forward Primer (3' to 5')	ATGCCAACTGTGATTGAGCACT **(SEQ ID NO: 27)**

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Reverse Primer (3' to 5')	ACATGTCGGGTTCCTGCTCCTAAA **(SEQ ID NO: 28)**
Probe 1 (3' to 5')	**/56-FAM/**ACAAAGGAAACATGGAGGAATGCTTGTGA/3BHQ_1/ **(SEQ ID NO: 29)**
Probe 2 (3' to 5')	**/5Cy3/**ACTCTCACGAAACTCCACGCACGAAA/3BHQ_2/ **(SEQ ID NO: 30)**
Probe 3 (3' to 5')	**/56-ROXN/**ACAATGACACACAGGAGACCCACCAT/3BHQ_2/ **(SEQ ID NO: 31)**
Probe 4 (3' to 5')	**/5Cy5/**GGATATCCAATGGTACAGGCAACATCGT/3IAbRQSp/ **(SEQ ID NO: 32)**

3. Polymerase Chain Reactions

[00175] All PCR reactions were performed in a ROCHE 480 LIGHTCYCLER instrument. Forty-five thermal cycles were performed, with a 60 second hot-start at 95°C. The cycling conditions for denaturation, annealing, and extension were 45 seconds, 50 seconds, and 60 seconds at 95°C, 65°C, and 70°C, respectively. Each experiment was run in quintuplicate, with a reaction volume of 15 µL. Fluorescence measurements were obtained in the following channels after every annealing step: 483 nm to 533 nm (FAM), 523 nm to 568 nm (Cy3), 558 nm to 610 nm (ROX), and 615 nm to 670 nm (Cy5). Fluorescence measurements were also obtained after the hot-start and the end of the 45 thermal cycles. The change in the fluorescence intensity between these two measurements (after hot-start and at the end of the 45 cycles) determined the end-point fluorescent signal.

Example 2: Simultaneous Detection of Polynucleotide Markers from Pathogens Based on an Encoding Method Using More Than One Color Per Analyte

[00176] This example describes the simultaneous detection of up to four polynucleotides selected from HIV (2 polynucleotides), HSV-2, *Mycobacterium*, *Plasmodium*, and dengue virus. Fourteen PCR reactions were assembled, using the reagents described in Table 15.

Table 15. Reagents for 14 PCR reactions.

Reaction No.	Type of Reagent	Reagent	Concentration	Volume
1		UltraPure Water	-	34 µL
		Taq 5x Master Mix	-	42 µL
	Template	Poly Protease Template	17 nM	2 µL
	Primers	Poly Protease FWD Primer	1 nM	2 µL
		Protease RVS Primer	1 nM	2 µL

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	Probe	Poly Protease Probe 1	1 nM	2 µL
2		UltraPure Water	-	28 µL
		Taq 5x Master Mix	-	42 µL
	Templates	Malaria Template	15 nM	2 µL
		Herpes Template	17 nM	2 µL
		TB Template	16 nM	2 µL
		Dengue Virus Template	17 nM	2 µL
	Primers	Poly Protease FWD Primer	1 nM	2 µL
		Protease RVS Primer	1 nM	2 µL
	Probe	Poly Protease Probe 1	1 nM	2 µL
3		UltraPure Water	-	32 µL
		Taq 5x Master Mix	-	42 µL
	Template	Malaria Template	15 nM	2 µL
	Primers	Malaria FWD Primer	1 µM	2 µL
		Malaria RVS Primer	1 µM	2 µL
	Probes	Malaria Probe 1	1 µM	2 µL
		Malaria Probe 2	1 µM	2 µL
4		UltraPure Water	-	26 µL
		Taq 5x Master Mix	-	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Herpes Template	17 nM	2 µL
		TB Template	16 nM	2 µL
		Dengue Virus Template	17 nM	2 µL
	Primers	Malaria FWD Primer	1 µM	2 µL
		Malaria RVS Primer	1 µM	2 µL
	Probes	Malaria Probe 1	1 µM	2 µL
		Malaria Probe 2	1 µM	2 µL
5		UltraPure Water	-	32 µL
		Taq 5x Master Mix	-	42 µL
	Template	Herpes Template	17 nM	2 µL
	Primers	Herpes FWD Primer	1 µM	2 µL
		Herpes RVS Primer	1 µM	2 µL
	Probes	Herpes Probe 1	1 µM	2 µL
		Herpes Probe 2	1 µM	2 µL
6		UltraPure Water	-	26 µL
		Taq 5x Master Mix	-	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Malaria Template	15 nM	2 µL
		TB Template	16 nM	2 µL
		Dengue Virus Template	17 nM	2 µL
	Primers	Herpes FWD Primer	1 µM	2 µL
		Herpes RVS Primer	1 µM	2 µL
	Probes	Herpes Probe 1	1 µM	2 µL
		Herpes Probe 2	1 µM	2 µL
7		UltraPure Water	-	30 µL
		Taq 5x Master Mix	-	42 µL

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	Template	TB Template	16 nM	2 µL
	Primers	TB FWD Primer	1 µM	2 µL
		TB RVS Primer	1 µM	2 µL
	Probes	TB Probe 1	1 µM	2 µL
		TB Probe 2	1 µM	2 µL
		TB Probe 3	1 µM	2 µL
8		UltraPure Water	-	30 µL
		Taq 5x Master Mix	-	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Malaria Template	15 nM	2 µL
		Herpes Template	17 nM	2 µL
		Dengue Virus Template	17 nM	2 µL
	Primers	TB FWD Primer	1 µM	2 µL
		TB RVS Primer	1 µM	2 µL
	Probes	TB Probe 1	1 µM	2 µL
		TB Probe 2	1 µM	2 µL
		TB Probe 3	1 µM	2 µL
9		UltraPure Water	-	28 µL
		Taq 5x Master Mix	-	42 µL
	Template	Dengue Virus Template	17 nM	2 µL
	Primers	Dengue Virus FWD Primer	1 µM	2 µL
		Dengue Virus RVS Primer	1 µM	2 µL
	Probes	Dengue Virus Probe 1	1 µM	2 µL
		Dengue Virus Probe 2	1 µM	2 µL
		Dengue Virus Probe 3	1 µM	2 µL
		Dengue Virus Probe 4	1 µM	2 µL
10		UltraPure Water	-	22 µL
		Taq 5x Master Mix	-	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Malaria Template	15 nM	2 µL
		Herpes Template	17 nM	2 µL
		TB Template	16 nM	2 µL
	Primers	Dengue Virus FWD Primer	1 µM	2 µL
		Dengue Virus RVS Primer	1 µM	2 µL
	Probes	Dengue Virus Probe 1	1 µM	2 µL
		Dengue Virus Probe 2	1 µM	2 µL
		Dengue Virus Probe 3	1 µM	2 µL
		Dengue Virus Probe 4	1 µM	2 µL
11		Taq 5x Master Mix	-	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Malaria Template	15 nM	2 µL
		Herpes Template	17 nM	2 µL
		Dengue Virus Template	17 nM	2 µL
	Primers	Poly Protease FWD Primer	1 µM	2 µL
		Poly Protease RVS Primer	1 µM	2 µL
		Malaria FWD Primer	1 µM	2 µL

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		Malaria RVS Primer	1 µM	2 µL
		Herpes FWD Primer	1 µM	2 µL
		Herpes RVS Primer	1 µM	2 µL
		Dengue Virus FWD Primer	1 µM	2 µL
		Dengue Virus RVS Primer	1 µM	2 µL
	Probes	Poly Protease Probe 1	1 µM	2 µL
		Malaria Probe 1	1 µM	2 µL
		Malaria Probe 2	1 µM	2 µL
		Herpes Probe 1	1 µM	2 µL
		Herpes Probe 2	1 µM	2 µL
		Dengue Virus Probe 1	1 µM	2 µL
		Dengue Virus Probe 2	1 µM	2 µL
		Dengue Virus Probe 3	1 µM	2 µL
		Dengue Virus Probe 4	1 µM	2 µL
12		UltraPure Water	-	2 µL
		Taq 5x Master Mix	-	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Malaria Template	15 nM	2 µL
		Herpes Template	17 nM	2 µL
		TB Template	16 nM	2 µL
	Primers	Poly Protease FWD Primer	1 µM	2 µL
		Poly Protease FWD Primer	1 µM	2 µL
		Malaria FWD Primer	1 µM	2 µL
		Malaria RVS Primer	1 µM	2 µL
		Herpes FWD Primer	1 µM	2 µL
		Herpes RVS Primer	1 µM	2 µL
		TB FWD Primer	1 µM	2 µL
		TB RVS Primer	1 µM	2 µL
	Probes	Poly Protease Probe 1	1 µM	2 µL
		Malaria Probe 1	1 µM	2 µL
		Malaria Probe 2	1 µM	2 µL
		Herpes Probe 1	1 µM	2 µL
		Herpes Probe 2	1 µM	2 µL
		TB Probe 1	1 µM	2 µL
		TB Probe 2	1 µM	2 µL
		TB Probe 3	1 µM	2 µL
13		UltraPure Water	-	12 µL
		Taq 5x Master Mix	-	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Herpes Template	17 nM	2 µL
		TB Template	16 nM	2 µL
	Primers	Poly Protease FWD Primer	1 µM	2 µL
		Poly Protease FWD Primer	1 µM	2 µL
		Herpes FWD Primer	1 µM	2 µL
		Herpes RVS Primer	1 µM	2 µL
		TB FWD Primer	1 µM	2 µL

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		TB RVS Primer	1 µM	2 µL
	Probes	Poly Protease Probe 1	1 µM	2 µL
		Herpes Probe 1	1 µM	2 µL
		Herpes Probe 2	1 µM	2 µL
		TB Probe 1	1 µM	2 µL
		TB Probe 2	1 µM	2 µL
		TB Probe 3	1 µM	2 µL
14		UltraPure Water	-	10 µL
		Taq 5x Master Mix	-	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Malaria Template	15 nM	2 µL
		Dengue Virus Template	17 nM	2 µL
	Primers	Poly Protease FWD Primer	1 µM	2 µL
		Poly Protease FWD Primer	1 µM	2 µL
		Malaria FWD Primer	1 µM	2 µL
		Malaria RVS Primer	1 µM	2 µL
		Dengue Virus FWD Primer	1 µM	2 µL
		Dengue Virus RVS Primer	1 µM	2 µL
	Probes	Poly Protease Probe 1	1 µM	2 µL
		Malaria Probe 1	1 µM	2 µL
		Malaria Probe 2	1 µM	2 µL
		Dengue Virus Probe 1	1 µM	2 µL
		Dengue Virus Probe 2	1 µM	2 µL
		Dengue Virus Probe 3	1 µM	2 µL
		Dengue Virus Probe 4	1 µM	2 µL

[00177] Referring to Table 15, reactions 1, 3, 5, 7, and 9 (containing HIV polyprotease, *Plasmodium*, HSV, *Mycobacterium*, and dengue analytes, respectively) were positive controls that provided baseline fluorescence intensity for each polynucleotide analytes alone, in the absence of other analytes. The change in fluorescence intensity in each color was used to provide the expected cumulative intensity level for each color in the chromatogram **(Fig. 3)**, as described more fully below. Reactions 2, 4, 6, 8, and 10 were negative controls used to determine the extent of non-specific amplification. In these reactions, the primers provided were not specific for the analytes provided.

[00178] Experiments 11, 12, 13, and 14 were multiplex detection experiments. Encoding was performed as indicated in Table 16. The measured fluorescence intensity for each assay, in each color, was plotted as black circles in the chromatogram of **Fig. 3.**

Table 16. Coding scheme for detection of pathogens.

	FAM	**Cy3**	**ROX**	**Cy5**
HIV poly-protease	1	0	0	0

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HIV p17	1	1	0	0
Plasmodium	1	0	1	0
HSV	1	0	0	1
Mycobacterium	1	1	0	1
Dengue Virus	1	1	1	1

[00179] To construct the chromatogram, the signals of the positive controls (reactions 1, 3, 5, 7, and 9) were used to assemble every possible combination of present sequences, in each color. The cumulative signals were plotted in a diagram, called a "chromatogram" (**Fig. 3**). Cumulative signals corresponding to all possible combinations of the same rank in the same color were organized into their own "band." Doing this for all four colors produced a level and band structure, against which the experimental results of each combination (reactions 11, 12, 13, and 14) could be evaluated. **Fig. 3** shows three replicates of a chromatogram constructed using the positive control samples, with experimental results 4112, 3121, and 3102 superimposed on the chromatogram. The experimental results are indicated by black dots.

[00180] The intensity value for each color in reactions 11-14 was measure in arbitrary fluorescence units (AFU), plotted on the chromatogram, and then assigned to a band based on two criteria. First, if an experimental result was within a band, then the result was assigned to the multiplicity of the band (e.g., the Cy3 value of 1 in the left-most chromatogram of **Fig. 3**). Second, if an experimental result was between two bands, a two-step analysis was employed. If the experimental result was between two legitimate results, the experimental result was assigned to the closest band. (e.g., the Cy5 value of 2 in the right-most chromatogram of **Fig. 3**). If the experimental result was between a legitimate result and an illegitimate result, the result was assigned to the band with the legitimate result (e.g., the FAM value of 3 in the middle chromatogram of **Fig. 3**). **Fig. 8** schematically shows a legitimate result of 4112 (top) indicating the presence of HIV polyprotease, *Plasmodium*, herpes simplex, and *Mycobacterium*; and an illegitimate result of 4110 (bottom), which cannot be decoded and indicates a malfunction in the assay.

[00181] Using these criteria, the output of Experiment 12 was designated 4112, which was the correct answer. The output of Experiment 14 could have been designated as 2121 or 3121, but 2121 is illegitimate, so the result must be 3121, which is the correct answer. The output of Experiment 13 could be designated as 3102 or 3101, both of which are legitimate, but the result is clearly closer to 2 than 1 in the Cy5 channel, so 3102, the correct answer, was designated as the result. These results provide experimental validation of the methods of the provided herein.

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[00182] The results indicate a trend of slightly lower combined signal than what would be expected from a simple summation of positive-control signals. This may be because the combination of signals is not perfectly linear. This may be a result of additional effects for which the chromatogram construction does not account for at present. These effects may be related to the absolute concentration of quenchers. The higher the absolute concentration of quenchers, the larger the percentage of reaction volume they quench, so that the same percentage of released fluorophores fails to contribute as much as expected to the cumulative signal. One solution to this problem is to use low concentrations of probes, so that the percentile loss in signal is negligible, regardless of how much quencher is released.

[00183] **Fig. 3** shows that each rank containing multiple combinations has a relatively wide band. The width of the band is ultimately the difference between the highest and lowest cumulative signals for the positive control samples. If all positive controls in the particular color produced exactly the same fluorescence signal, then the width of each band would be zero. Since instead those signals are somewhat different, the resulting bands are relatively wide. However, there is a simple means to tighten the bands. Instead of loading all probes at the same concentration, as was done for the experiment above, the concentration of each probe can be adjusted so that each of the resulting positive-control signals is the same as the others in the same color. This approach would significantly tighten the bands, making it easier to determine the multiplicity of a signal for a sample containing analytes.

Example 3: Simultaneous Detection of Polynucleotide Markers from Pathogens Based on an Encoding Method Using One Color Per Analyte and Different Intensities

[00184] This example describes the simultaneous detection of up to three polynucleotides selected from dengue virus, HIV p17, and HIV polyprotease. The coding scheme was as depicted in Table 11. As shown in Table 17, each analyte was encoded as a single value of fluorescence intensity in a single color. In this case, as described elsewhere in this disclosure, the coding scheme is non-degenerate by design. This is achieved by assigning an intensity value to each analyte that is equal to the cumulative intensity values for the prior analytes, plus one. Of course, as described elsewhere in the specification, the initial assigned value can be greater than one, for example to better distinguish over noise. Similarly, the values assigned to each analyte can be greater than the cumulative intensity values for the prior analytes plus one, for example, to increase the separation between the intensities and enable more simple assignment of intensities.

Table 17. Coding scheme for detection of dengue virus, HIV polyprotease, and HIV p17.

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	FAM
Dengue Virus	1
HIV p17	2
HIV polyprotease	4

[00185] The same reagents (e.g., templates, primers, and probes) used in Example 2 were used here. The fluorescence signals generated by the respective probes in positive-control end-point PCR reactions were measured and used to calculate the probe concentrations that would produce 1x, 2x, and 4x signal intensities. Experiments were then set up to detect the seven non-null combinations of analyte occurrences. Tables 18-24 provide the PCR reaction components for each of these mixtures.

Table 18. Binary FAM Experiment 1 Cocktail: HIV Polyprotease, HIV p17, and dengue virus

Reagents	**Concentration**	**Volume Added**
UltraPure Water	-	50µL
Taq 5x Master Mix	-	25µL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	4µL
P17 Template	15nM	4µL
Dengue Virus Template	17nM	4µL
Primers	**Concentration**	**Volume Added**
Poly Protease FWD Primer	1µM	4µL
Poly Protease RVS Primer	1µM	4µL
P17 FWD Primer	1µM	4µL
P17 RVS Primer	1µM	4µL
Dengue Virus FWD Primer	1µM	4µL
Dengue Virus RVS Primer	1µM	4µL
Probes	**Concentration**	**Volume Added**
Poly Protease Probe 1	800nM	4µL
P17 Probe 1	400nM	4µL
Dengue Virus Probe 1	200nM	4µL

Table 19. Binary FAM Experiment 2 Cocktail: dengue virus and HIV p17

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Reagents	**Concentration**	**Volume Added**
UltraPure Water	-	54µL
Taq 5x Master Mix	-	25µL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	-
P17 Template	15nM	4µL
Dengue Virus Template	17nM	4µL
Primers	**Concentration**	**Volume Added**
Poly Protease FWD Primer	1µM	4µL
Poly Protease RVS Primer	1µM	4µL
P17 FWD Primer	1µM	4µL
P17 RVS Primer	1µM	4µL
Dengue Virus FWD Primer	1µM	4µL
Dengue Virus RVS Primer	1µM	4µL
Probes	**Concentration**	**Volume Added**
Poly Protease Probe 1	800nM	4µL
P17 Probe 1	400nM	4µL
Dengue Virus Probe 1	200nM	4µL

Table 20. Binary FAM Experiment 3 Cocktail: dengue virus and HIV polyprotease

Reagents	**Concentration**	**Volume Added**
UltraPure Water	-	54µL
Taq 5x Master Mix	-	25µL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	4µL
P17 Template	15nM	-
Dengue Virus Template	17nM	4µL
Primers	**Concentration**	**Volume Added**
Poly Protease FWD Primer	1µM	4µL
Poly Protease RVS Primer	1µM	4µL
P17 FWD Primer	1µM	4µL

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P17 RVS Primer	1μM	4μL
Dengue Virus FWD Primer	1μM	4μL
Dengue Virus RVS Primer	1μM	4μL
Probes	**Concentration**	**Volume Added**
Poly Protease Probe 1	800nM	4μL
P17 Probe 1	400nM	4μL
Dengue Virus Probe 1	200nM	4μL

Table 21. Binary FAM Experiment 4 Cocktail: HIV polyprotease and HIV p17

Reagents	**Concentration**	**Volume Added**
UltraPure Water	-	54μL
Taq 5x Master Mix	-	25μL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	4μL
P17 Template	15nM	4μL
Dengue Virus Template	17nM	-
Primers	**Concentration**	**Volume Added**
Poly Protease FWD Primer	1μM	4μL
Poly Protease RVS Primer	1μM	4μL
P17 FWD Primer	1μM	4μL
P17 RVS Primer	1μM	4μL
Dengue Virus FWD Primer	1μM	4μL
Dengue Virus RVS Primer	1μM	4μL
Probes	**Concentration**	**Volume Added**
Poly Protease Probe 1	800nM	4μL
P17 Probe 1	400nM	4μL
Dengue Virus Probe 1	200nM	4μL

Table 22. Binary FAM Experiment 5 Cocktail: dengue virus

Reagents	**Concentration**	**Volume Added**
UltraPure Water	-	58μL

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Taq 5x Master Mix	-	25µL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	-
P17 Template	15nM	-
Dengue Virus Template	17nM	4µL
Primers	**Concentration**	**Volume Added**
Poly Protease FWD Primer	1µM	4µL
Poly Protease RVS Primer	1µM	4µL
P17 FWD Primer	1µM	4µL
P17 RVS Primer	1µM	4µL
Dengue Virus FWD Primer	1µM	4µL
Dengue Virus RVS Primer	1µM	4µL
Probes	**Concentration**	**Volume Added**
Poly Protease Probe 1	800nM	4µL
P17 Probe 1	400nM	4µL
Dengue Virus Probe 1	200nM	4µL

Table 23. Binary FAM Experiment 6 Cocktail: HIV p17

Reagents	**Concentration**	**Volume Added**
UltraPure Water	-	58µL
Taq 5x Master Mix	-	25µL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	-
P17 Template	15nM	4µL
Dengue Virus Template	17nM	-
Primers	**Concentration**	**Volume Added**
Poly Protease FWD Primer	1µM	4µL
Poly Protease RVS Primer	1µM	4µL
P17 FWD Primer	1µM	4µL
P17 RVS Primer	1µM	4µL
Dengue Virus FWD Primer	1µM	4µL

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Dengue Virus RVS Primer	1μM	4μL
Probes	**Concentration**	**Volume Added**
Poly Protease Probe 1	800nM	4μL
P17 Probe 1	400nM	4μL
Dengue Virus Probe 1	200nM	4μL

Table 24. Binary FAM Experiment 7 Cocktail: HIV polyprotease

Reagents	**Concentration**	**Volume Added**
UltraPure Water	-	58μL
Taq 5x Master Mix	-	25μL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	4μL
P17 Template	15nM	-
Dengue Virus Template	17nM	-
Primers	**Concentration**	**Volume Added**
Poly Protease FWD Primer	1μM	4μL
Poly Protease RVS Primer	1μM	4μL
P17 FWD Primer	1μM	4μL
P17 RVS Primer	1μM	4μL
Dengue Virus FWD Primer	1μM	4μL
Dengue Virus RVS Primer	1μM	4μL
Probes	**Concentration**	**Volume Added**
Poly Protease Probe 1	800nM	4μL
P17 Probe 1	400nM	4μL
Dengue Virus Probe 1	200nM	4μL

[00186] The total fluorescence signal of each experiment was plotted on a chromatogram **(Fig. 7)** that was constructed as described above. The width of the bands around each positive control measurement is the propagated uncertainty of the 1X measurement. That uncertainty was equated to the standard deviation of the fluorescence signals of the last five PCR cycles in saturation.

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[00187] **Fig. 7** shows the results of this experiment. The three right-most results show the measured intensities for dengue virus alone (DEN), HIV p17 alone (P17), and HIV polyprotease (TPP). The assigned intensities for each of these analytes were as indicated in Table 11, and **Fig. 7** confirms that the three analytes yielded the expected intensity results when alone (see DEN, P17, and TPP). At the end of the assay, essentially all of the probes have been hydrolyzed, releasing essentially all of the fluorophores, which emit a signal. A fixed amount of a fluorophore produces a fixed and predictable amount of signal. Therefore a target that is present should generally produce a fixed amount of signal, as determined by the amount of probe added to the reaction volume.

[00188] As expected, the intensity values for the combinations of analytes are equivalent to the sum of the assigned intensities. Since this coding scheme is designed to be non-degenerate, each result can be unambiguously decoded into the presence or absence of a particular analyte. For example, starting from the left side of **Fig. 7**, a result of 7 indicates the presence of all three analytes. A result of 3 indicates the presence of dengue virus and HIV p17 analytes, and the absence of HIV polyprotease. A result of 5 indicates the presence of dengue virus and HIV polyprotease, and the absence of HIV p17. Finally a result of 6 indicates the presence of HIV p17 and HIV polyprotease, and the absence of dengue virus. This coding scheme can be extended to encode additional analytes, as described elsewhere in this disclosure.

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Electronic Acknowledgement Receipt

EFS ID:	34096550
Application Number:	15914356
International Application Number:	
Confirmation Number:	2457
Title of Invention:	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS
First Named Inventor/Applicant Name:	Emil P. Kartalov
Customer Number:	21971
Filer:	J Kyle Bruner/Katherine Lopez
Filer Authorized By:	J Kyle Bruner
Attorney Docket Number:	38075-716.302
Receipt Date:	24-OCT-2018
Filing Date:	07-MAR-2018
Time Stamp:	09:54:03
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Applicant Response to Pre-Exam Formalities Notice	Caltech_38075_716_302_Signed_Response_Corrected_Appl_Papers.pdf	141504 cfdb668886f32cf84283d1559331f79fa54b5bd7	no	3

Warnings:

Information:					
2	Specification	Caltech_38075_716_302_Clean_Spec.pdf	540262 93294ac97a53fd277c7e176ea64e37f61d676dac	no	67
Warnings:					
Information:					
			Total Files Size (in bytes):	681766	

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If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Electronically filed on: October 24, 2018 PATENT

Attorney Docket No.: 38075-716.302

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventors: Emil Kartlov, *et al.*	Group Art Unit: 2877
Serial Number: 15/914,356	Examiner: Unassigned
Filing Date: March 7, 2018	**CONFIRMATION NO: 2457**
Title: SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS	

RESPONSE TO NOTICE TO FILE CORRECTED APPLICATION PAPERS

AND

PRELIMINARY AMENDMENT

Mail Stop Missing Parts
Commissioner for Patents
P.O. Box 1450
Alexandria, Virgina 22313

Dear Commissioner:

Prior to an examination on the merits of the above-identified application, please amend the application as follows:

Amendments to the Specification begin on page **2** of this paper.

Remarks begin on page **3** of this paper.

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California Institute of Technology - Ex. 2003 Page 1036

AMENDMENTS TO THE SPECIFICATION

Applicant submits herewith a Substitute Specification (clean copy only) in compliance with 37 C.F.R 1.121. The specification is amended to remove the grey background from certain cells of Tables 6-8 on pages 25-29 and Tables 9-14 on pages 52-55, and to modify the background color and text formatting for certain rows of Tables 18-24 on pages 62-66, pursuant to 37 C.F.R 1.52. Since this amendment is not able to be marked, a marked version of the Substitute Specification is not provided. No new matter has been introduced by this amendment.

REMARKS

Applicant submits herewith a Substitute Specification (clean copy only) in compliance with 37 C.F.R 1.121. The specification is amended to correct certain cells of Tables 6-8, 9-14, and 18-24. Specifically, gray or black background of certain cells has been removed to ensure that the text is readable and sufficiently clear as required by 37 C.F.R. 1.52. No new matter has been introduced by these amendments.

CONCLUSION

Applicants respectfully request that the Examiner advance the application to issuance. Should the Examiner have any questions, the Examiner is encouraged to contact the undersigned at (512) 338-5444.

The Commissioner is authorized to charge any additional fees which may be required, or credit any overpayment, to Deposit Account No. 23-2415 (Docket No. 38075-716.302).

Respectfully submitted,

WILSON SONSINI GOODRICH & ROSATI

Date: October 24, 2018

By: /Kyle Bruner/
Kyle Bruner, Reg. No. 75,716

650 Page Mill Road
Palo Alto, California 94304
Telephone No.: (512) 338-5444
Facsimile No.: (650) 493-6811
Customer No. 021971

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APPLICATION NUMBER	FILING or 371(c) DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	TOT CLAIMS	IND CLAIMS
15/914,356	03/07/2018	2877	1720	38075-716.302	20	1

CONFIRMATION NO. 2457

UPDATED FILING RECEIPT

21971
WILSON, SONSINI, GOODRICH & ROSATI
650 PAGE MILL ROAD
PALO ALTO, CA 94304-1050

OC000000105377121

Date Mailed: 10/26/2018

Receipt is acknowledged of this non-provisional patent application. The application will be taken up for examination in due course. Applicant will be notified as to the results of the examination. Any correspondence concerning the application must include the following identification information: the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please submit a written request for a Filing Receipt Correction. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections**

Inventor(s)

Emil P. Kartalov, Pasadena, CA;
Aditya Rajagopal, Orange, CA;
Axel Scherer, Pasadena, CA;

Applicant(s)

California Institute of Technology, Pasadena, CA;

Power of Attorney: None

Domestic Priority data as claimed by applicant

This application is a CON of 14/451,876 08/05/2014 PAT 10068051
which is a CON of 13/756,760 02/01/2013 PAT 8838394
which claims benefit of 61/703,093 09/19/2012
and claims benefit of 61/594,480 02/03/2012

Foreign Applications for which priority is claimed (You may be eligible to benefit from the **Patent Prosecution Highway** program at the USPTO. Please see http://www.uspto.gov for more information.) - None.
Foreign application information must be provided in an Application Data Sheet in order to constitute a claim to foreign priority. See 37 CFR 1.55 and 1.76.

Permission to Access Application via Priority Document Exchange: Yes

Permission to Access Search Results: Yes

Applicant may provide or rescind an authorization for access using Form PTO/SB/39 or Form PTO/SB/69 as appropriate.

If Required, Foreign Filing License Granted: 10/18/2018

The country code and number of your priority application, to be used for filing abroad under the Paris Convention, is **US 15/914,356**

Projected Publication Date: 01/31/2019

Non-Publication Request: No

Early Publication Request: No

Title

SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

Preliminary Class

356

Statement under 37 CFR 1.55 or 1.78 for AIA (First Inventor to File) Transition Applications: No

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Applicants may wish to consult the USPTO booklet, "General Information Concerning Patents" (specifically, the section entitled "Treaties and Foreign Patents") for more information on timeframes and deadlines for filing foreign patent applications. The guide is available either by contacting the USPTO Contact Center at 800-786-9199, or it can be viewed on the USPTO website at http://www.uspto.gov/web/offices/pac/doc/general/index.html.

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Title 35, United States Code, Section 184

Title 37, Code of Federal Regulations, 5.11 & 5.15

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PATENT APPLICATION FEE DETERMINATION RECORD

Substitute for Form PTO-875

Application or Docket Number: 15/914,356

APPLICATION AS FILED - PART I

FOR	(Column 1) NUMBER FILED	(Column 2) NUMBER EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY FEE($)
BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	300
SEARCH FEE (37 CFR 1.16(k), (i), or (m))	N/A	N/A	N/A			N/A	660
EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	760
TOTAL CLAIMS (37 CFR 1.16(i))	20 minus 20 =	*			OR	x 100 =	0.00
INDEPENDENT CLAIMS (37 CFR 1.16(h))	1 minus 3 =	*				x 460 =	0.00
APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $310 ($155 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						0.00
MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							0.00
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	1720

APPLICATION AS AMENDED - PART II

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE($)	SMALL ENTITY ADDITIONAL FEE($)	OR	OTHER THAN SMALL ENTITY RATE($)	OTHER THAN SMALL ENTITY ADDITIONAL FEE($)
AMENDMENT A	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE($)	ADDITIONAL FEE($)	OR	RATE($)	ADDITIONAL FEE($)
AMENDMENT B	Total (37 CFR 1.16(i))	*	Minus	**	=	x =		OR	x =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	x =		OR	x =	
	Application Size Fee (37 CFR 1.16(s))									
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".
The "Highest Number Previously Paid For" (Total or Independent) is the highest found in the appropriate box in column 1.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/914,356	03/07/2018	Emil P. Kartalov	38075-716.302	2457

21971 7590 11/08/2018

WILSON, SONSINI, GOODRICH & ROSATI
650 PAGE MILL ROAD
PALO ALTO, CA 94304-1050
UNITED STATES OF AMERICA

EXAMINER	
ART UNIT	PAPER NUMBER
1639	

NOTIFICATION DATE	DELIVERY MODE
11/08/2018	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

patentdocket@wsgr.com

IPR2024-01178
California Institute of Technology - Ex. 2003 Page 1043

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Doc Code: TRACK1.GRANT

Decision Granting Request for Prioritized Examination (Track I or After RCE)	Application No.: 15/914,356

1. THE REQUEST FILED March 7, 2018 IS **GRANTED.**

The above-identified application has met the requirements for prioritized examination
A. ☒ for an original nonprovisional application (Track I).
B. ☐ for an application undergoing continued examination (RCE).

2. **The above-identified application will undergo prioritized examination.** The application will be accorded special status throughout its entire course of prosecution until one of the following occurs:

A. filing a **petition for extension of time** to extend the time period for filing a reply;
B. filing an **amendment to amend the application to contain more than four independent claims, more than thirty total claims**, or a multiple dependent claim;
C. filing a **request for continued examination**;
D. filing a notice of appeal;
E. filing a request for suspension of action;
F. mailing of a notice of allowance;
G. mailing of a final Office action;
H. completion of examination as defined in 37 CFR 41.102; or
I. abandonment of the application.

Telephone inquiries with regard to this decision should be directed to Liana Walsh at 571-272-3206. In his/her absence, calls may be directed to the Office of Petitions Help Desk at 571-272-3282.

/Liana Walsh/
[*Signature*]

Petitions Lead Paralegal Specialist, Office of Petitions
(Title)

U.S. Patent and Trademark Office
PTO-2298 (Rev. 02-2012)

Office of Petitions: Decision Count Sheet

Mailing Month: 11

Application No.: 15914356

15914356

For US serial numbers: enter number only, no slashes or commas. Ex: 10123456
For PCT: enter "51+single digit of year of filing+last 5 numbers", Ex. for PCT/US05/12345, enter 51512345

Deciding Official: WALSH, LIANA

Count (1) - Palm Credit 15914356

Decision: GRANT

FINANCE WORK NEEDED
☐ Select Check Box for YES

GRANT

Decision Type: 643 - Track One request

643

Notes:

Count (2)

Decision: n/a

FINANCE WORK NEEDED
☐ Select Check Box for YES

Decision Type: NONE

Notes:

Count (3)

Decision: n/a

FINANCE WORK NEEDED
☐ Select Check Box for YES

Decision Type: NONE

Notes:

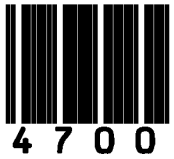
Initials of Approving Official (if required)

If more than 3 decisions, attach 2nd count sheet & mark this box ☐

Printed on: 11/5/2018

Office of Petitions Internal Document - Ver. 5.0

Office of Petitions: Routing Sheet



Application No. 15914356

This application is being forwarded to your office for further processing. A decision has been rendered on a petition filed in this application, as indicated below. For details of this decision, please see the document PET.OP.DEC filed on the same date as this document.

- [X] **GRANTED**
- [ ] **DISMISSED**
- [ ] **DENIED**

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
15/914,356	03/07/2018	Emil P. Kartalov	38075-716.302

CONFIRMATION NO. 2457

21971
WILSON, SONSINI, GOODRICH & ROSATI
650 PAGE MILL ROAD
PALO ALTO, CA 94304-1050

PUBLICATION NOTICE

OC000000105506448

Title:SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

Publication No.US-2019-0034583-A1
Publication Date:01/31/2019

NOTICE OF PUBLICATION OF APPLICATION

The above-identified application will be electronically published as a patent application publication pursuant to 37 CFR 1.211, et seq. The patent application publication number and publication date are set forth above.

The publication may be accessed through the USPTO's publically available Searchable Databases via the Internet at www.uspto.gov. The direct link to access the publication is currently http://www.uspto.gov/patft/.

The publication process established by the Office does not provide for mailing a copy of the publication to applicant. A copy of the publication may be obtained from the Office upon payment of the appropriate fee set forth in 37 CFR 1.19(a)(1). Orders for copies of patent application publications are handled by the USPTO's Public Records Division. The Public Records Division can be reached by telephone at (571) 272-3150 or (800) 972-6382, by facsimile at (571) 273-3250, by mail addressed to the United States Patent and Trademark Office, Public Records Division, Alexandria, VA 22313-1450 or via the Internet.

In addition, information on the status of the application, including the mailing date of Office actions and the dates of receipt of correspondence filed in the Office, may also be accessed via the Internet through the Patent Electronic Business Center at www.uspto.gov using the public side of the Patent Application Information and Retrieval (PAIR) system. The direct link to access this status information is currently https://portal.uspto.gov/pair/PublicPair. Prior to publication, such status information is confidential and may only be obtained by applicant using the private side of PAIR.

Further assistance in electronically accessing the publication, or about PAIR, is available by calling the Patent Electronic Business Center at 1-866-217-9197.

Office of Data Managment, Application Assistance Unit (571) 272-4000, or (571) 272-4200, or 1-888-786-0101

IPR2024-01178
California Institute of Technology - Ex. 2003 Page 1047

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/914,356	03/07/2018	Emil P. Kartalov	38075-716.302	2457

21971 7590 03/08/2019

WILSON, SONSINI, GOODRICH & ROSATI
650 PAGE MILL ROAD
PALO ALTO, CA 94304-1050

EXAMINER	
NEGIN, RUSSELL SCOTT	

ART UNIT	PAPER NUMBER
1631	

NOTIFICATION DATE	DELIVERY MODE
03/08/2019	ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

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IPR2024-01178
California Institute of Technology - Ex. 2003 Page 1048

Office Action Summary	Application No. 15/914,356	Applicant(s) Kartalov et al.	
	Examiner RUSSELL S NEGIN	Art Unit 1631	AIA (FITF) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☐ Responsive to communication(s) filed on _____.

☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2a)☐ This action is **FINAL.** 2b) ☑ This action is non-final.

3)☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

4)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims*

5) ☑ Claim(s) 1-20 is/are pending in the application.

5a) Of the above claim(s) _____ is/are withdrawn from consideration.

6) ☐ Claim(s) _____ is/are allowed.

7) ☑ Claim(s) 1-20 is/are rejected.

8) ☐ Claim(s) _____ is/are objected to.

9) ☐ Claim(s) _____ are subject to restriction and/or election requirement

* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to **PPHfeedback@uspto.gov.**

Application Papers

10)☐ The specification is objected to by the Examiner.

11)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a)☐ All b)☐ Some** c)☐ None of the:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

** See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☑ Notice of References Cited (PTO-892)

2) ☑ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b) Paper No(s)/Mail Date _____.

3) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date _____.

4) ☐ Other: _____.

DETAILED ACTION

Comments

The present application is being examined under the pre-AIA first to invent provisions. In the event the determination of the status of the application as subject to AIA 35 U.S.C. 102 and 103 (or as subject to pre-AIA 35 U.S.C. 102 and 103) is incorrect, any correction of the statutory basis for the rejection will not be considered a new ground of rejection if the prior art relied upon, and the rationale supporting the rejection, would be the same under either status.

Claims 1-20 are pending and examined in the instant Office action.

Information Disclosure Statements

The IDSs that have been filed have been considered.

Priority

The instant application is a continuation of 14/451,876, filed 8/5/2014. Application '876 is a continuation of 13/756,760, filed on 2/1/2013. Application '760 claims benefit to provisional applications 61/703,093, filed on 9/19/2012 and 61/594,480, filed 2/3/2012. The provisional applications do not disclose the independent claim limitation of partitioning the mixture into a plurality of droplets. Consequently, the benefit date for claims 1-20 is 2/1/2013.

Claim Rejections - 35 USC § 103

The following is a quotation of pre-AIA 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

This application currently names joint inventors. In considering patentability of the claims under pre-AIA 35 U.S.C. 103(a), the examiner presumes that the subject matter of the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of pre-AIA 35 U.S.C. 103(c) and potential pre-AIA 35 U.S.C. 102(e), (f) or (g) prior art under pre-AIA 35 U.S.C. 103(a).

35 U.S.C. 103 Rejection #1:

Claims 1-12 and 20 is/are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Han et al. [Nature Biotechnology, volume 19, 2001, pages 631-635; on IDS] in view of Lin et al. [WO 2012/058638 A2] in view of Vogelstein et al. [PNAS, volume 96, 1999, pages 9236-9241].

Claim 1 is drawn to a method of detecting any unique combination of presence or absence of at least five polynucleotides in a plurality of droplets. The method comprises providing a sample potentially comprising at least one of the at least five polynucleotide analytes. The method comprises forming a mixture of the sample and at least five

hybridization probes, each comprising a fluorophore selected from at most four fluorophores. The method comprises partitioning the mixture into the plurality of droplets. The method comprises exciting the fluorophores to generate signals of one or more of the at least five polynucleotide analytes is present in said plurality of droplets. The signals comprise at least one signal generated by excitement of the one or more fluorophores. The method comprises measuring the signals to generate a cumulative measurement. The cumulative measurement corresponds to the presence of a unique combination of presence or absence of the at least five polynucleotide analytes in the sample. The method does not require any step of immobilization of the at least five polynucleotide analytes or mass spectrometry.

Claim 2 is further limiting wherein each of the at least five hybridization probes is complementary to one of the at least five polynucleotide analytes.

Claims 3-6 and 8-9 provides further constraints on the numbers of analytes, probes, and fluorophores.

Claim 7 is further limiting and requires PCR.

The document of Han et al. studies quantum dot tagged microbeads for multiplexed optical coding of biomolecules [title]. Figure 5 on page 634 of Han et al. illustrates four different polynucleotide analytes labeled with four different combinations of three different fluorophores. The bottom three polynucleotide analytes are hybridized to complementary polynucleotide probes. When the sample undergo spectroscopy, the optical code on the right panel of the figure is the cumulative measurement of the combination of fluorophores for each of the four polynucleotide analytes. The technique of Han et al. does not involve immobilization or mass spectrometry.

Han et al. does not teach a mixture of at least five hybridization analytes. Han et al. does not teach partitioning the mixture into droplets or conducting PCR.

The document of Lin et al. teaches nucleic acid nanostructure barcode probes [title]. Figure 2 of Lin et al. illustrates histograms for a combinatorial number of probes having one of 27 different barcodes.

Han et al. and Lin et al. do not teach partitioning the mixture into droplets or conducting PCR.

The document of Vogelstein et al. studies digital PCR [title]. The methods section of Vogelstein et al. teaches diluting and partitioning a genome into many small droplets on many 96 well plates such that anywhere between zero to one to all of the polynucleotide analytes may be present in each droplet.

With regard to claim 10, the experiments in Han et al. are performed on DNA that is cell-free.

With regard to claims 11-12, Figure 5 of Vogelstein et al. teaches performing digital PCR on a stool sample of a subject to determine presence of cancer.

With regard to claim 20, the methods section of Vogelstein et al. teaches diluting the mixtures of polynucleotide analytes into a fixed concentration in the mixture of the plurality of droplets such that Figure 1 of Vogelstein et al. illustrates presence/absence patterns of the polynucleotide analyzes in each of the droplet wells.

It would have been obvious to someone of ordinary skill in the art at the time of the instant invention to modify the labeling of Han et al. by use of the labeling of Lin et al. wherein the motivation would have been that the barcodes of Lin et al. generate a combinatorial number of labels [Figure 2 of Lin et al.].

It would have been obvious to someone of ordinary skill in the art at the time of the instant invention to modify the labeling of Han et al. and Lin et al. by use of the digital PCR of Vogelstein wherein the motivation would have been that the partitioning into droplets facilitates identifying presence/absence patterns of polynucleotide analytes.

There would have been a reasonable expectation of success in combining Han et al., Lin et al., and Vogelstein et al. because all three studies analogously pertain to fluorophore labeling of polynucleotide analytes to determine their presence/absence patterns.

35 U.S.C. 103 Rejection #2:

Claims 13 and 17-19 is/are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Han et al. in view of Lin et al. in view of Vogelstein et al. as applied to claims 1-12 and 20 above, in further view of Empedocles et al. [US PGPUB 2002/0022273 A1; on IDS].

Claims 13 and 17-19 recite computer limitations pertaining the coding of polynucleotide analytes.

Han et al., Lin et al., and Vogelstein et al. make obvious using combinatorial hybridization and fluorophore labeling to identify presence/absence patterns of polynucleotide analytes, as discussed above.

Han et al., Lin et al., and Vogelstein et al. do not teach the computer limitations of the claims.

The document of Empedocles et al. studies differentiable spectral bar code methods and systems [title]. Figure 1 of Empedocles et al. illustrates the computer limitations of the claims.

It would have been obvious to someone of ordinary skill in the art at the time of the instant invention to modify the labeling of Han et al. and Lin et al. and the digital PCR of Vogelstein by use of the computers of Empedocles et al. wherein the motivation would have been that automation facilitates the execution of the method [Figure 1 of Empedocles et al.].

There would have been a reasonable expectation of success in combining Han et al., Lin et al., Vogelstein et al., and Empedocles et al. because all four studies analogously pertain to fluorophore labeling of polynucleotide analytes to determine their presence/absence patterns.

35 U.S.C. 103 Rejection #3:

Claims 14-16 is/are rejected under pre-AIA 35 U.S.C. 103(a) as being unpatentable over Han et al. in view of Lin et al. in view of Vogelstein et al. in view of Empedocles et al. as applied to claims 1-13 and 17-20 above, in further view of Newton et al. [US PGPUB 2007/0072211 A1; on IDS].

Claims 14-16 recite having a health care professional make a clinical decision on cancer treatment based on the polynucleotide data.

Han et al., Lin et al., Vogelstein et al., and Empedocles et al. make obvious using combinatorial hybridization and fluorophore labeling to identify presence/absence patterns of polynucleotide analytes, as discussed above. Figure 5 of Vogelstein et al. conducts digital PCR on the stool of a subject to determine the presence of cancer.

Han et al., Lin et al., Vogelstein et al., and Empedocles et al. do not teach the clinical treatment of the claims.

The document of Newton et al. studies asymmetric PCR coupled with post-PCR characterization for the identification of nucleic acids [title]. Paragraphs 111 and 119 of Newton et al. teach that a health care professional makes clinical decisions on cancer treatment based on the nucleic acid data provided.

It would have been obvious to someone of ordinary skill in the art at the time of the instant invention to modify the labeling of Han et al. and Lin et al., the digital PCR of Vogelstein by, and the computers of Empedocles et al. by use of the cancer treatment of Newton et al, wherein the motivation would have been that cancer treatment gives a physiological application to the combinatorial analysis of barcoded polynucleotide analytes [paragraphs 111 and 119 of Newton et al.].

There would have been a reasonable expectation of success in combining Han et al., Lin et al., Vogelstein et al., Empedocles et al., and Newton et al. because all five studies analogously pertain to analysis of polynucleotide analytes for biological applications.

Double Patenting

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on nonstatutory double patenting provided the reference application or patent either is shown to be commonly owned with the examined application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement. See MPEP § 717.02 for applications subject to examination under the first inventor to file provisions of the AIA as explained in MPEP § 2159. See MPEP §§ 706.02(l)(1) - 706.02(l)(3) for applications not subject to examination under the first inventor to file provisions of the AIA. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

The USPTO Internet website contains terminal disclaimer forms which may be used. Please visit www.uspto.gov/patent/patents-forms. The filing date of the application in which the form is filed determines what form (e.g., PTO/SB/25, PTO/SB/26, PTO/AIA/25, or PTO/AIA/26) should be used. A web-based eTerminal Disclaimer may be filled out completely online using web-screens. An eTerminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more information about eTerminal Disclaimers, refer to www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp.

Claims 1, 13, 14, 15, and 16 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1, 5, 6, 7, and 8, respectively, of U.S. Patent No. 10,068,051 B2 in view of Vogelstein et al.. Although the claims at issue are not identical, they are not patentably distinct from each other because both sets of claims are directed to combinatorial manner of barcoding mixtures of polynucleotide analytes without immobilization or mass spectrometry.

The claims of ‘051 do not teach partitioning the mixture into droplets.

The document of Vogelstein et al. studies digital PCR [title]. The methods section of Vogelstein et al. teaches diluting and partitioning a genome into many small droplets on many 96 well plates such that anywhere between zero to one to all of the polynucleotide analytes may be present in each droplet.

It would have been obvious to someone of ordinary skill in the art at the time of the instant invention to modify the labeling of the claims of ‘051 by use of the partitioning into droplets of Vogelstein wherein the motivation would have been that the partitioning into droplets facilitates identifying presence/absence patterns of polynucleotide

analytes. There would have been a reasonable expectation of success in combining the claims of '051 and Vogelstein et al. because both studies analogously pertain to fluorophore labeling of polynucleotide analytes to determine their presence/absence patterns.

E-mail Communications Authorization

Per updated USPTO Internet usage policies, Applicant and/or applicant's representative is encouraged to authorize the USPTO examiner to discuss any subject matter concerning the above application via Internet e-mail communications. See MPEP 502.03. To approve such communications, Applicant must provide written authorization for e-mail communication by submitting the following statement via EFS-Web (using PTO/SB/439) or Central Fax (571-273-8300):

> Recognizing that Internet communications are not secure, I hereby authorize the USPTO to communicate with the undersigned and practitioners in accordance with 37 CFR 1.33 and 37 CFR 1.34 concerning any subject matter of this application by video conferencing, instant messaging, or electronic mail. I understand that a copy of these communications will be made of record in the application file.

Written authorizations submitted to the Examiner via e-mail are **NOT** proper. Written authorizations must be submitted **via EFS-Web** (using PTO/SB/439) **or Central Fax** (571-273-8300). A paper copy of e-mail correspondence will be placed in the patent application when appropriate. E-mails from the USPTO are for the sole use of the intended recipient, and may contain information subject to the confidentiality requirement set forth in 35 USC § 122. See also MPEP 502.03.

Conclusion

No claim is allowed.

Any inquiry concerning this communication or earlier communications from the Examiner should be directed to Russell Negin, whose telephone number is (571) 272-1083. This Examiner can normally be reached from Monday through Thursday from 10:30 am to 5 pm and variable hours on Fridays.

If attempts to reach the Examiner by telephone are unsuccessful, the Examiner's Supervisor, Peter Paras, Supervisory Patent Examiner, can be reached at (571) 272-4517.

Information regarding the status of the application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only.

/RUSSELL S NEGIN/
Primary Examiner, Art Unit 1631
5 March 2019

Notice of References Cited	Application/Control No. 15/914,356	Applicant(s)/Patent Under Reexamination Kartalov et al.	
	Examiner RUSSELL S NEGIN	Art Unit 1631	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	CPC Classification	US Classification
*	A	US-10068051-B2	09-2018	Kartalov; Emil	C12Q1/68	1/1
	B					
	C					
	D					
	E					
	F					
	G					
	H					
	I					
	J					
	K					
	L					
	M					

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	CPC Classification
	N	WO 2012/058638	05-2012	WIPO	Lin et al.	
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	Vogelstein et al. Digital PCR, PNAS, volume 96, pages 9236-9241. (Year: 1999)
	V	
	W	
	X	

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Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	15/914,356	Kartalov et al.
	Examiner	Art Unit
	RUSSELL S NEGIN	1631

CPC - Searched*		
Symbol	Date	Examiner

CPC Combination Sets - Searched*		
Symbol	Date	Examiner

US Classification - Searched*			
Class	Subclass	Date	Examiner

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

Search Notes		
Search Notes	Date	Examiner
see attached search history printouts	02/24/2019	RSN
EAST prior art search	02/24/2019	RSN
EAST inventor search on all inventors	02/24/2019	RSN
PALM inventor search on all inventors	02/24/2019	RSN

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner

/RUSSELL S NEGIN/ Primary Examiner, Art Unit 1631	

IPR2024-01178
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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

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Sheet 1 of 15

Complete if Known	
Application Number	15914356
Filing Date	03-07-2018
First Named Inventor	KARTALOV; Emil P.
Art Unit	2877
Examiner Name	
Attorney Docket Number	38075-716.302

U. S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	001	US-5079351	01-07-1992	SNINSKY; John J. et al.	
	002	US-5538848	07-23-1996	LIVAK; Kenneth J. et al.	
	003	US-5618711	04-08-1997	GELFAND; David H. et al.	
	004	US-5677152	10-14-1997	BIRCH; David Edward et al.	
	005	US-5723591	03-03-1998	LIVAK; Kenneth J. et al.	
	006	US-5773258	06-30-1998	BIRCH; David Edward et al.	
	007	US-5789224	08-04-1998	GELFAND; David H. et al.	
	008	US-5804375	09-08-1998	GELFAND; David H. et al.	
	009	US-5876930	03-02-1999	LIVAK; Kenneth J. et al.	
	010	US-5928862	07-27-1999	MORRISON; Larry E.	
	011	US-5948360	09-07-1999	RAO; Prabhakar P. et al.	
	012	US-5981180	11-09-1999	CHANDLER; Van S. et al.	
	013	US-5994056	11-30-1999	HIGUCHI; Russell G.	
	014	US-6030787	02-29-2000	LIVAK; Kenneth J. et al.	
	015	US-6127155	10-03-2000	GELFAND; David H. et al.	
	016	US-6171785	01-09-2001	HIGUCHI; Russell G.	
	017	US-6258569	07-10-2001	LIVAK; Kenneth J. et al.	
	018	US-6534266	03-18-2003	SINGER; Robert H.	
	019	US-6548259	04-15-2003	WARD; David C. et al.	
	020	US-6642062	11-04-2003	KAUVAR; Lawrence M. et al.	
	021	US-7101663	09-05-2006	GODFREY; Tony E. et al.	

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Sheet 2 of 15

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Attorney Docket Number	38075-716.302

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	022	US-7141377	11-28-2006	GELFAND; David H. et al.	
	023	US-7348141	03-25-2008	FRENCH; David John et al.	
	024	US-7385043	06-10-2008	KRAMER; Fred R.	
	025	US-7410764	08-12-2008	GOCKE; Christopher D. et al.	
	026	US-7413708	08-19-2008	MAYRAND; Paul E.	
	027	US-7473767	01-06-2009	DIMITROV; Krassen	
	028	US-7575864	08-18-2009	BEDZYK; Laura A. et al.	
	029	US-7667024	02-23-2010	MAO; Fei et al.	
	030	US-7767423	08-03-2010	KOPRESKI; Michael S. et al.	
	031	US-7771949	08-10-2010	KRAMER; Fred R.	
	032	US-7919237	04-05-2011	DIMITROV; Krassen et al.	
	033	US-7919244	04-05-2011	MADEJON; Seiz Antonio et al.	
	034	US-7941279	05-10-2011	HWANG; Jenq-Neng et al.	
	035	US-8148512	04-03-2012	DIMITROV; Krassen et al.	
	036	US-9458497	10-04-2016	HASSIBI; Arjang et al.	
	037	US-20020022273	02-21-2002	EMPEDOCLES; Stephen A. et al.	
	038	US-20030148280	08-07-2003	HARRIS; Barbara J. et al.	
	039	US-20030148544	08-07-2003	NIE; Shuming et al.	
	040	US-20050064435	03-24-2005	SU; Xing et al.	
	041	US-20050164264	07-28-2005	SHIPWASH; Edward	

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Sheet 3 of 15

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	042	US-20050214753	09-29-2005	SHULTZ; John W. et al.	
	043	US-20050260640	11-24-2005	ANDERSEN; Mark R. et al.	
	044	US-20060216708	09-28-2006	VENEMA; Fokke	
	045	US-20070178485	08-02-2007	EL-DEIRY; Wafik S. et al.	
	046	US-20070231824	10-04-2007	CHEE; Mark S. et al.	
	047	US-20080050737	02-28-2008	ARIELI; Boaz et al.	
	048	US-20080069733	03-20-2008	MALTEZOS; George et al.	
	049	US-20090048785	02-19-2009	KATZIR; Nir et al.	
	050	US-20090062129	03-05-2009	MCKERNAN; Kevin et al.	
	051	US-20100015607	01-21-2010	GEISS; Gary K. et al.	
	052	US-20100047924	02-25-2010	WEBSTER; Philippa J. et al.	
	053	US-20100112710	05-06-2010	GEISS; Gary K. et al.	
	054	US-20100159447	06-24-2010	LI; Bin et al.	
	055	US-20100210472	08-19-2010	EMPEDOCLES; Stephen A. et al.	
	056	US-20100248257	09-30-2010	JACOBSEN; Kivin et al.	
	057	US-20100261026	10-14-2010	FERREE; Sean M. et al.	
	058	US-20100267064	10-21-2010	KARTALOV; Emil P. et al.	
	059	US-20100273173	10-28-2010	HIRAI; Mitsuharu et al.	
	060	US-20110104684	05-05-2011	HOOPER; Dennis G.	
	061	US-20110151459	06-23-2011	ROTHMANN; Thomas et al.	
	062	US-20110207623	08-25-2011	DIMITROV; Krassen et al.	

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Sheet 4 of 15

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Art Unit	2877
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Attorney Docket Number	38075-716.302

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	063	US-20110237459	09-29-2011	NOVA; Michael P. et al.	
	064	US-20120003646	01-05-2012	JOO; Sunghae A. et al.	
	065	US-20120045756	02-23-2012	ROTHMANN; Thomas et al.	
	066	US-20120077195	03-29-2012	LI; Qingge et al.	
	067	US-20120122704	05-17-2012	ATCHLEY; Daniel H. et al.	
	068	US-20120141995	06-07-2012	LI; Qingge et al.	
	069	US-20120171677	07-05-2012	LUDOWISE; Peter D.	
	070	US-20120184017	07-19-2012	CHATTERJEE; Deb K.	
	071	US-20120190030	07-26-2012	CHUN; Jong Yoon et al.	
	072	US-20120196283	08-02-2012	BABIEL; Reiner et al.	
	073	US-20120252017	10-04-2012	REED; Michael W. et al.	
	074	US-20120258457	10-11-2012	JAROSCH; Florian et al.	
	075	US-20130116780	05-09-2013	MILLER; Eran et al.	

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FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3]-Number[4]-Kind Code[5] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear	T[6]
	001	EP-1448581-B1	11-12-2008	INST SYSTEMS BIOLOGY [US]		☐
	002	EP-1963531-B1	09-21-2011	NANOSTRING TECHNOLOGIES INC [US], et al.		☐

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	003	WO-02056014-A2	07-18-2002	GLAXO GROUP LTD [GB], et al.		☐
	004	WO-02056014-A3	10-09-2003	GLAXO GROUP LTD [GB], et al.		☐
	005	WO-2007076128-A2	07-05-2007	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	006	WO-2007076128-A3	11-29-2007	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	007	WO-2007076129-A2	07-05-2007	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	008	WO-2007076129-A3	03-06-2008	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	009	WO-2007076132-A2	07-05-2007	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	010	WO-2007076132-A3	09-07-2007	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	011	WO-2007139766-A2	12-06-2007	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	012	WO-2007139766-A3	12-11-2008	NANOSTRING TECHNOLOGIES INC [US], et al.		☐

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	013	WO-2008124847-A2	10-16-2008	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	014	WO-2008124847-A3	02-19-2009	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	015	WO-2010019826-A1	02-18-2010	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	016	WO-2011047087-A2	04-21-2011	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	017	WO-2011047087-A3	08-18-2011	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	018	WO-2011100541-A2	08-18-2011	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	019	WO-2011100541-A3	01-05-2012	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	020	WO-2011116088-A2	09-22-2011	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	021	WO-2011116088-A3	02-02-2012	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	022	WO-2012058638-A2	05-03-2012	HARVARD COLLEGE [US], et al.		☐

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Attorney Docket Number	38075-716.302

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3]-Number[4]-Kind Code[5] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear	T[6]
	023	WO-2012058638-A3	12-20-2012	HARVARD COLLEGE [US], et al.		☐
	024	WO-2012106428-A2	08-09-2012	UNIV FLORIDA [US], et al.		☐
	025	WO-2012135340-A2	10-04-2012	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	026	WO-2012135340-A3	12-20-2012	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	027	WO-9919515-A1	04-22-1999	LUMINEX CORP [US]		☐
	028	WO-9952708-A1	10-21-1999	LUMINEX CORP [US]		☐

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Sheet 8 of 15

Complete if Known	
Application Number	15914356
Filing Date	03-07-2018
First Named Inventor	KARTALOV; Emil P.
Art Unit	2877
Examiner Name	
Attorney Docket Number	38075-716.302

NON-PATENT LITERATURE DOCUMENTS			
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	001	BEIGE, et al. Clinical evaluation of a Mycobacterium tuberculosis PCR assay. J Clin Microbiol. 1995 Jan;33(1):90-5.	☐
	002	CHAMBERLAIN, et al. Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification. Nucleic Acids Res. 1988 Dec 9;16(23):11141-56.	☐
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	005	Chromatogram, 2011, 2 pages. Dorland's illustrated medical dictionary. Retrieved online on 22 January 2014 from «http://www.credoreference.com».	☐
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	009	EL-HAJJ, et al. Detection of rifampin resistance in Mycobacterium tuberculosis in a single tube with molecular beacons. J Clin Microbiol. 2001 Nov;39(11):4131-7.	☐

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	010	EMBL-Bank: AJ303204. http://www.ebi.ac.uk/Tools/dbfetch/emblfetch?db=embl&id=AJ303204&format=default&style=default&Retrieve=Retrieve. Accessed February 2012.	☐
	011	EMBL-Bank: GQ395623. http://www.ebi.ac.uk/Tools/dbfetch/emblfetch?db=embl&id=GQ395623&format=default&style=default&Retrieve=Retrieve. Accessed February 2012.	☐
	012	FODOR, et al. Multiplexed biochemical assays with biological chips. Nature. 1993 Aug 5;364(6437):555-6.	☐
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	020	HOREJSH, et al. A molecular beacon, bead-based assay for the detection of nucleic acids by flow cytometry. Nucleic Acids Res. 2005 Jan 19;33(2):e13.	☐
	021	HUANG, et al. Identification of 8 foodborne pathogens by multicolor combinational probe coding technology in a single real-time PCR. Clin Chem. 2007 Oct;53(10):1741-8. Epub 2007 Aug 10.	☐
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	028	LEE, et al. Seven-color, homogeneous detection of six PCR products. Biotechniques. 1999 Aug;27(2):342-9.	☐
	029	LIEW, et al. Validating a custom multiplex ELISA against individual commercial immunoassays using clinical samples. Biotechniques. 2007 Mar;42(3):327-8, 330-3.	☐

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	030	LIVAK, et al. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. PCR Methods Appl. 1995 Jun;4(6):357-62.	☐
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	034	OU, et al. DNA amplification for direct detection of HIV-1 in DNA of peripheral blood mononuclear cells. Science. 1988 Jan 15;239(4837):295-7.	☐
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	039	PTAK, et al. Inhibition of human immunodeficiency virus type 1 replication in human cells by Debio-025, a novel cyclophilin binding agent. Antimicrob Agents Chemother. 2008 Apr;52(4):1302-17. doi: 10.1128/AAC.01324-07. Epub 2008 Jan 22.	☐
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	049	WANG, et al. Locked nucleic acid molecular beacons. J Am Chem Soc. 2005 Nov 16;127(45):15664-5.	☐
	050	WATERS, et al. Microchip device for cell lysis, multiplex PCR amplification, and electrophoretic sizing. Anal Chem. 1998 Jan 1;70(1):158-62.	☐
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(71) Applicant *(for all designated States except US)*: **PRESIDENT AND FELLOWS OF HARVARD COLLEGE** [US/US]; 17 Quincy Street, Cambridge, MA 02138 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **LIN, Chenxiang** [CN/US]; c/o President and Fellows of Harvard College, 17 Quincy Street, Cambridge, MA 02138 (US). **LI, Chao** [CN/US]; c/o President and Fellows of Harvard College, 17 Quincy Street, Cambridge, MA 02138 (US). **JUNGMANN, Ralf** [DE/US]; c/o President and Fellows of Harvard College, 17 Quincy Street, Cambridge, MA 02138 (US). **SHIH, William** [US/US]; c/o President and Fellows of Harvard College, 17 Quincy Street, Cambridge, MA 02138 (US). **YIN, Peng** [CN/US]; 99 Pond Avenue, #901, Brookline, MA 02445 (US).

(74) Agent: **TREVISAN, Maria, A.**; Wolf, Greenfield & Sacks, P.C., 600 Atlantic Avenue, Boston, MA 02210-2206 (US).

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(54) **Title**: NUCLEIC ACID NANOSTRUCTURE BARCODE PROBES

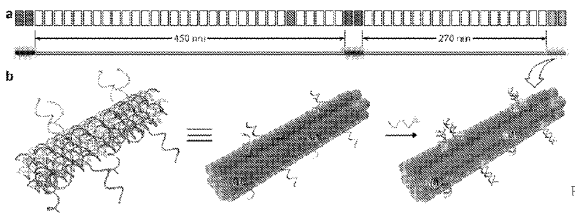


FIG.1

(57) **Abstract**: Provided herein are, inter alia, barcode probes comprised of transiently or stably fluorescently labeled nucleic acid nanostructures that are fully addressable and able to be read using standard fluorescent microscope and methods of use thereof including methods of use as detectable labels for probes.

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NUCLEIC ACID NANOSTRUCTURE BARCODE PROBES

RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application Serial No. 61/408358, filed on October 29, 2010, entitled "DNA NANOSTRUCTURE BARCODE PROBES", the entire contents of which are incorporated by reference herein.

REFERENCE TO A COMPACT DISK APPENDIX

A computer program listing is appended to this disclosure and is hereby incorporated herein by this reference. The appendix includes the file programlisting.txt which includes listings for 6ht-v5-1color-1.json and 6ht-v6-2color-1.json.

BACKGROUND OF INVENTION

A major challenge in modern biology and nanotechnology is to observe, identify and differentiate a large number of different molecular species in real time. Fluorescent microscopy is a powerful tool for non-destructively and dynamically monitoring many individual molecular events. However, the multiplexing ability of fluorescent imaging is limited by the number of spectrally non-overlapping fluorophores available. There is therefore a great need for novel addressable fluorescent probes useful in multiplex detection systems.

BRIEF SUMMARY OF INVENTION

The invention provides, inter alia, nucleic acid barcode probes that comprise a nucleic acid nanostructure comprising one or more fluorescently labeled regions that may be stably or transiently bound (and thus labeled) with fluorophore-bearing oligonucleotides. The barcode probes may further comprise a target binding moiety. The invention also provides methods of use for such barcode probes including but not limited to their use in analyte detection assays (e.g., assays for detecting and optionally quantitating one or more analytes). The nature of the fluorescent signals (e.g., the wavelength or "color", intensity, etc.) and the pattern (or orientation, or arrangement or geometry) of such signals on the barcode can also be used to identify particular analytes

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in a sample. This facilitates multiplexed assays in which a plurality of analytes are detected simultaneously (or at a minimum with a single sample or a single aliquot from a sample).

In one aspect, the invention provides a method of detecting a target comprising: contacting a sample with a nucleic acid barcode probe, and determining whether the nucleic acid barcode probe binds to one or more components in the sample, wherein binding of the nucleic acid barcode probe to one or more components of the sample indicates presence of a target in the sample, and wherein the nucleic acid barcode probe comprises a nucleic acid nanostructure comprising a target binding moiety and at least two fluorescently labeled regions.

In some embodiments, the method further comprises identifying the target based on the color and/or orientation of the fluorescently labeled regions of the nucleic acid barcode probe bound to one or more components of the sample.

In some embodiments, whether the nucleic acid barcode probe binds to one or more components in the sample comprises contacting the nucleic acid barcode probe with soluble, transiently binding fluorophore-bearing oligonucleotides.

In some embodiments, the nucleic acid barcode probe comprises stably bound fluorophore-bearing oligonucleotides.

In another aspect, the invention provides a method of detecting a target comprising contacting the target with a nucleic acid barcode probe, under conditions sufficient for the target to bind to the nucleic acid barcode probe; separating the target from material that is not bound to the target; and detecting the presence of the nucleic acid barcode probe bound to the target, wherein the nucleic acid barcode probe comprises a nucleic acid nanostructure comprising a target binding moiety and at least two fluorescently labeled regions.

In some embodiments, the method further comprises identifying the target based on the color and/or orientation of the fluorescently labeled regions of the nucleic acid barcode probe bound to the target.

In some embodiments, detecting the presence of the nucleic acid barcode probe bound to the target comprises contacting the nucleic acid barcode probe with soluble, transiently bound fluorophore-bearing oligonucleotides.

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In some embodiments, the nucleic acid barcode probe comprises stably bound fluorophore-bearing oligonucleotides.

In another aspect, the invention provides a nucleic acid barcode probe comprising a nucleic acid nanostructure, such as a DNA barcode probe comprising a DNA nanostructure, having at least two fluorescently-labeled regions. In some embodiments, the nanostructure comprises at least three fluorescently-labeled regions, and the orientation is determinable due to asymmetric spacing of the fluorescently-labeled regions. The locations of each of the fluorescently-labeled regions on the nanostructure are such that a pattern of the fluorescent labels is determinable based on the emission of visible light by the fluorescently-labeled regions. In some embodiments, the orientation of the barcode probe is determinable based on the emission of visible light by the fluorescently-labeled regions.

In some embodiments, each of the fluorescently-labeled regions has a center that is located at least 200 nm from the centers of the other fluorescently-labeled regions. In some embodiments, each of the fluorescently-labeled regions has a center that is located at least 250 nm from the centers of the other fluorescently-labeled regions. In some embodiments, each of the fluorescently-labeled regions has a center that is located at least about 25 nm, at least about 50 nm, or at least about 75 nm, or at least about 100 nm, or at least 150 nm from the centers of the other fluorescently-labeled regions.

In some embodiments, the nanostructure comprises a scaffold strand and plurality of staple strands. In some embodiments, the scaffold strand has a sequence derived from M13 bacteriophage. In some embodiments, the fluorescently-labeled regions of the DNA nanostructure comprise fluorophore-labeled staple strands. In some embodiments, the fluorophore-labeled staple strand is a transiently binding fluorophore-labeled staple strand. In some embodiments, the fluorophore-labeled staple strand is transiently binding at room temperature. In some embodiments, the fluorophore-labeled staple strand is 7-12 nucleotides in length. In some embodiments, the fluorophore-labeled staple strand is about 9 nucleotides in length. In some embodiments, the fluorophore-labeled staple strand is a stably binding fluorophore-labeled staple strand. In some embodiments, the fluorophore-labeled staple strand is a stably binding fluorophore-labeled staple strand at room temperature. In some embodiments, the

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fluorophore-labeled staple strand is at least 18 nucleotides in length, including in some embodiments 18-25 nucleotides in length.

In some embodiments, the fluorophore-labeled staple strands are directly labeled. In some embodiments, the fluorophore-labeled staple strands are indirectly labeled.

In some embodiments, the fluorophore-labeled staple strands have a staple domain hybridized to a scaffold strand and a handle domain hybridized to a fluorophore-labeled oligonucleotide. In some embodiments, the handle domain is 7-12 nucleotides in length. In some embodiments, the handle domain is about 9 nucleotides in length. In some embodiments, the handle domain is at least 18 nucleotides in length. In some embodiments, the handle domain is 18-25 nucleotides in length.

In some embodiments, the nanostructure is a nanotube. In some embodiments, the nanostructure is prepared using a DNA origami method. In some embodiments, the nanostructure comprises a single-stranded DNA tile. In some embodiments, the nanostructure comprises DNA hairpins.

In some embodiments, the barcode probe further comprises a target binding moiety. In some embodiments, the target binding moiety is a single-stranded nucleic acid complementary to a nucleic acid target. In some embodiments, the target binding moiety is an antibody. In some embodiments, the target binding moiety is a protein or peptide.

In some embodiments, wherein the nanostructure, once assembled, is sufficiently immutable in a resting state that the pattern of the fluorescent regions can be detected using light microscopy in that resting state. In some embodiments, the resting state comprises absence of an applied electric field.

In another aspect, the invention provides a composition comprising a plurality of any of the foregoing nucleic acid nanostructure barcode probes. In some embodiments, some or all members of the plurality are different from other members in the plurality. The members may differ from each other based on the target binding moiety and the signal and orientation (arrangements) of the barcode. In some embodiments, the plurality is equal to or less than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 40, 50, 100, 200 or more. In some instances, the plurality is equal to or less than 216.

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In another aspect, the invention provides a method of identifying a target nucleic acid comprising contacting the target nucleic acid with any of the foregoing barcode probes under conditions such that the target nucleic acid binds to the barcode probe; separating the target nucleic acid from material that is not bound to the target nucleic acid; and detecting the presence of the barcode probe bound to the target nucleic acid.

In another aspect, the invention provides a method of identifying a target protein or cell comprising contacting the target protein or cell with any of the foregoing barcode probes under conditions such that the target protein or cell binds to the barcode probe; separating the target protein or cell from material that is not bound to the target protein or cell; and detecting the presence of the barcode probe bound to the target protein or cell.

In another aspect, the invention provides a method of identifying a target protein or cell comprising contacting the target protein or cell with any of the foregoing barcode probes under conditions such that the target protein or cell binds to the barcode probe; separating the target protein or cell from material that is not bound to the target protein or cell; and detecting the presence of the barcode probe bound to the target protein or cell.

In another aspect, the invention provides a method of detecting a target comprising contacting a sample with any of the foregoing nucleic acid barcode probes, and determining whether the nucleic acid barcode probe binds to one or more components in the sample, wherein binding of the nucleic acid barcode probe to one or more components in the sample indicates presence of a target in the sample.

In some embodiments, the method further comprises removing components in the sample that are not bound to the nucleic acid barcode probe following the contacting step.

In some embodiments, the target is a nucleic acid, a protein, a peptide, or a cell, or a combination thereof.

In some embodiments, the presence of the barcode probe is detected by fluorescent microscopy.

In some embodiments, the presence of the barcode probe is detected without exposing the DNA barcode probe to an electric field.

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In some embodiments, each probe comprises a nucleic acid nanostructure having at least two fluorescently-labeled regions and a target binding moiety, wherein the locations of each of the fluorescently-labeled regions on the nanostructure are such that a pattern of the fluorescent labels of the barcode probe and the identity of the target binding moiety is determinable based on the emission of visible light by the fluorescently-labeled regions; and each of the fluorescently-labeled regions has a center that is located at least 200 nm from the centers of the other fluorescently-labeled regions.

In another aspect, the invention provides a population of nucleic acid barcode probes such as DNA barcode probes, wherein each probe comprises a nucleic acid nanostructure such as a DNA nanostructure having at least two fluorescently-labeled regions and a target binding moiety, wherein the locations of each of the fluorescently-labeled regions on the nucleic acid nanostructure are such that a pattern of the fluorescent labels of the nucleic acid barcode probe and the identity of the target binding moiety is determinable based on the emission of visible light by the fluorescently-labeled regions.

In some embodiments, each of the fluorescently-labeled regions has a center that is located at least about 25 nm, at least about 50 nm, at least about 100 nm, at least about 200 nm, or at least about 250 nm from the centers of the other fluorescently-labeled regions.

These and other aspects and embodiments of the invention will be described in greater detail herein.

BRIEF DESCRIPTION OF DRAWINGS

The patent or application file contains at least one Figure executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee. Publication of the application includes gray-scale renderings of the Figures. Color versions of these Figures are available upon request.

FIG. 1. Design of the DNA nanotube based barcode. (A) Two schematic drawings of the Blue--Red-Green (BRG, "--" and "-" denotes larger and smaller inter-zone distance in the barcode, respectively) barcode with a segment diagram on the top and a 3D view at the bottom. The main-body of the barcode is a DNA nanotube formed from by dimerizing two origami monomers, each consisting of 28 segments with 42-bp

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(13.6 nm) length. The grey segment in the middle represents the junction where the two monomers are joined together through cross-hybridization between their scaffolds and staples. Three 84-bp zones of the nanotube are fluorescently labeled (shown as blue, red and green segments) to produce the BRG barcode with an inter-zone distance of 450-nm between the first two zones and 270-nm between the last two. Note that each zone is only labeled with one fluorophore species. The resulting barcodes are thus referred as single-labeled-zone barcodes. (B) 3D cartoons showing the details of one fluorescently labeled zone. Left: a scaffold-plus-staple model of such an 84-bp zone before labeling. Each of the twelve 63-base-long staples (shown in rainbow colors) contains two parts: the 42-base region at the 5′-end weaves through three double-helices to fold the scaffold (shown in black) into a six-helix bundle nanotube; and the 21-base extension at the 3′-end protrudes out for fluorescent labeling. Middle: an identical but simplified model to emphasize the six-helix bundle structure (each helix shown as a semi-transparent grey cylinder) and the positioning of the twelve 21-base staple extensions (each shown as a light-grey curl). Right: Cartoon representation of a "green" 84-bp zone. The labeling is achieved by hybridizing the Cy3 (shown the glowing green spheres at the 3′-ends) modified strands to the staple extensions.

FIG. 2. Single-labeled-zone fluorescent barcodes. (A) Superimposed TIRF microscopy images of five barcode species (top) and the statistics from manual counting (bottom). From left to right are the BBB, BRG, BRR, GRG and RGB barcodes with a representative image on top of the corresponding bar-graph. Each bar-graph is generated based on the manual sorting and counting of the objects found in a 50 x 50 μm^2 image (~40 barcodes, the exact sample size N is noted beside the corresponding bar-graph). (B) A representative image of the equimolar mixture of 27 barcode species. (C) Statistics obtained by analyzing twenty-seven 50 x 50 μm^2 images of the 27 barcode mixture (~1,500 barcodes in total). Left: barcode counts of the 27 species (average count of 55 with a standard deviation of 9). A representative TIRF image (1.4 x 0.7 μm^2) of each barcode type is placed underneath the corresponding bar. Right: sorting result of the observed objects shown as a pie-chart. Color scheme used for the bar-graphs and the pie-chart (unrelated to the pseudo-colors of the fluorophores): blue: correct barcodes (qualified barcode with expected identity); green: incorrect barcodes (qualified barcode

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with unexpected identity); red: monomer nanotubes (one spot or two connecting spots); purple: barcodes with wrong geometry (i.e., bending angle <120°); and orange: barcodes containing at least one spot with two colors. In the 27-barcode pool, correct vs. incorrect barcodes were not distinguishable because all barcode types are expected. As a result the bars and pie representing the qualified barcodes in (C) are shown in blue. Scale bars: 5 μm.

FIG. 3. Representative TIRF and software reconstructed images of BRG and GRG nano-barcodes. (A) TIRF image of BRG nano-barcodes. (B) Software constructed image of (A), inset showing calculation of barcode bending angle. (C) TIRF image of BRG nano-barcodes. (D) Software constructed image of (C). (E) Theoretical designed distances between each region of BRG (upper) and GRG (lower) nano-barcodes. Scale bar: 10 pixels or 0.714 μm.

FIG. 4. Schematic illustration and result of a 21-mer oligonucleotide detection using nano-barcodes. (A) A GRG barcode with a capture probe is biotinylated as a result of the target binding and a nude BRG barcode stays untouched. Note that the drawing is not to scale. (B) TIRF images of nano-barcodes immobilized on streptavidin coated glass cover-slip. Left: with target; Right: without target. (C) Statistical detection result showing the average barcode counts per 71.4 x 71.4 μm^2 area. Error bars represent the standard deviation.

FIG. 5. Dual-labeled-zone fluorescent barcodes. (A) Typical TIRF microscopy images of five selected barcode species, shown both in separate channels and after superimposing. Scale bar: 5 μm. (B) Statistics obtained by analyzing two 50 x 50 μm^2 images of each barcode species (~85 barcodes, the exact sample size N is noted beside the corresponding bar-graph). The barcode types are noted under the x-axis of the diagram. Color scheme (unrelated to the pseudo-colors of the fluorophores): blue: correct barcodes (correct geometry and color identity); green: incorrect barcodes (correct geometry but incorrect color identity); red: monomer nanotubes (one spot or two connecting spots); and purple: barcodes with wrong geometry (i.e., bending angle <120°). (C) Computer-aided barcode counting results of the 72-barcode pool (N=2,617) and the 216-barcode pool (N=7,243) plotted as bar-graphs with descending barcode counts from left to right (data not shown). A computer-generated reference barcode image is placed underneath the corresponding bar. (D) A table containing one

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representative TIRF image (1.4×0.7 μm^2) for each of the 216 dual-labeled-zone barcode species.

FIG. 6. Super-resolution fluorescent barcodes. (A) Scheme of DNA-PAINT used for super-resolution barcode imaging. The 400 nm nanotube consists of 4 binding zones spaced by ~114 nm. Each zone can be decorated with the desired combination of "docking" sequences for red, green or blue imager strands. The orthogonal imager strands bind transiently to their respective "docking" sites on the nanotube, creating the necessary "blinking" for super-resolution reconstruction (B) Top: Segment diagram (similar to the one used in FIG. 1) of the DNA nanotube monomers used for creating five barcodes for super-resolution imaging. Bottom: super-resolution images of the five barcodes shown in each channel separately and as an overlay of all channels. Scale bar: 100 nm. (C) Super-resolution image showing all five barcodes in one mixture. Scale bar: 500 nm.

FIG. 7. Tagging yeast cells with the GRG barcodes as in situ imaging probes. (A) Cartoon illustrating the tagging mechanism. The biotinylated barcodes are anchored on the yeast cell through streptavidin molecules bound to biotinylated polyclonal antibodies coated on the yeast surface. Only two of the ten biotinylated staples on the barcode are shown here for clarity. (B) Overlaid microscope images (acquired in bright field and TIRF) of the yeast cells treated with the barcodes. Top: Yeast cells treated as illustrated in (A). Bottom: Negative control: yeast cells treated with non-biotinylated barcodes. Scale bars: 5 μm.

FIG. 8. Fluorescent barcode with non-linear geometry. (A) A schematic of three identical ~400-nm long DNA nanotubes are linked to the outer edge of a DNA ring with diameter of ~60 nm through the hybridization between staple extensions. The ring and the end of the tube are labeled by Cy3 (green) and Cy5 (red), respectively. (B) A representative TIRF microscope image of the barcode shown in (a). Scale bar: 5 μm.

DETAILED DESCRIPTION OF INVENTION

Provided herein are nucleic acid (e.g., DNA) nanostructure barcode probes that are fully addressable and able to be read using standard fluorescent microscope.

Previously described molecular barcodes are constructed using one of two strategies: (1) By combining multiple spectrally non-overlapping fluorophores in a

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controlled molar ratio to generate a mixed color signature; or (2) by separating fluorophores beyond the diffraction limit (~200 nm) and arranging them in a specific geometric pattern. The first category of barcode has been made, for example, from dendrimeric DNA (*see, e.g.,* Li *et al., Nat. Biotechnol.* 23:885-889 (2005)), self-assembled 2D DNA arrays (*see, e.g.,* Lin *et al., Nano Lett.* 7:507-512 (2007)) and quantum dots (*see, e.g.,* Han *et al., Nat. Biotechnol.* 19:631-635 (2001)). The successful construction and decoding of such barcodes heavily depends on both the fluorescent labeling efficiency and the imaging instrument's ability to precisely detect different fluorescent intensity levels.

In contrast, barcodes constructed according to the second strategy are more robust, and can be constructed and detected even when the labeling efficiency and imaging conditions are sub-optimal. Furthermore, when such methods are used, the ability to multiplex increases exponentially with each additional fluorescent spot that is incorporated. Examples of barcodes that fall in this category include rare-earth doped glass microfibers (~100 μm long) (*see, e.g.,* Dejneka *et al., Proc. Nat. Acad. Sci. USA* 100:389-393 (2003)) and double-stranded linear DNA with one strand fluorescently tagged at specific locations (2–5 μm long) (*see, e.g.,* Xiao *et al., Nat. Methods* 6:199-201 (2009) and Geiss *et al., Nat. Biotechnol.* 26:317-325 (2008)). However, existing methods using this strategy are limited both in the addressability of the fluorescent spots and because, in the absence of specialized equipment, the labeled barcodes will fold into a conformation that brings the fluorescent spots within the diffraction limit of visible light, rendering the barcode unreadable using a fluorescent microscope.

The instant inventors recognized that the problems associated with existing molecular barcode strategies could be addressed by using nucleic acid nanostructures such as DNA nanostructures to create novel molecular barcode probes. Nucleic acids such as DNA may be folded into predetermined one-, two- or three-dimensional nanostructures using a variety of techniques, such as DNA origami (Rothemund US-2007/0117109 A1), single-stranded tiles (Yin et al., "Programming DNA Tube Circumferences," *Science* (2008): 321: 824-826), DNA hairpins (Yin et al. US-2009/0011956 A1; Yin et al., "Programming biomolecular self-assembly pathways," *Nature* (2008) 451:318-323), and others.

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In general, the DNA origami process involves the folding of one or more long, "scaffold" DNA strands into a particular shape using a plurality of rationally designed "staple" DNA strands. The sequences of the staple strands are designed such that they hybridize to particular portions of the scaffold strands and, in doing so, force the scaffold strands into a particular shape. Methods useful in the making of DNA origami structures can be found, for example, in U.S. Pat. App. Pub. Nos. 2007/0117109, 2008/0287668, 2010/0069621 and 2010/0216978, each of which is incorporated by reference in its entirety. Staple design can be facilitated using, for example, caDNAno software, available on the internet at the cadnano website.

In certain embodiments, a DNA nanostructure barcode probe may include a DNA nanostructure made of one or more scaffold strands held in a specific shape by rationally designed staple strands. The sequence listing provides the nucleotide sequence of a scaffold strand, as SEQ ID NO:1, that may be used to construct an exemplary DNA origami barcode probe. The sequence listing also provides nucleotide sequences of staple strands, as SEQ ID NOs: 13-361, that may be used to construct an exemplary DNA origami barcode probe, together with the scaffold strand represented by SEQ ID NO:1. As will be discussed in greater detail herein, SEQ ID NOs: 13-186 represent nucleotide sequences of staple strands used to form a front monomer and SEQ ID NOs: 187-361 represent nucleotide sequences of staple strands used to form a rear monomer in an exemplary DNA origami barcode probe. As used herein, the term "DNA nanostructures" is used for convenience and it is to be understood that the invention contemplates nucleic acid nanostructures generally. The nanostructures of the invention may be linear (e.g., nanorods) or non-linear (e.g., star-shaped, triangular, etc.).

The DNA nanostructure has at least two non-overlapping, fluorescently-labeled regions. In certain embodiments, the DNA nanostructure barcode probe has at least 3, 4, 5, 6, 7, 8, 9, 10, 11 or 12 non-overlapping, fluorescently-labeled regions.

The fluorophore pattern of the probe is determined using light microscopy. In some embodiments, each of the fluorescently labeled regions can be visibly distinguished using a fluorescent light microscope. In some of these embodiments, the center of each fluorescently labeled region is separated from the centers of other fluorescently labeled regions by a distance greater than the visible light diffraction limit of visible light (*i.e.* at least 200 nm). In certain embodiments, therefore, each

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fluorescently labeled region is at least 200 nm from any other fluorescently labeled region. In some embodiments, each fluorescently labeled region is at least 250 nm from any other fluorescently labeled region.

In some embodiments, even higher resolution is achievable and the fluorescently labeled regions may be spaced apart from each other at even shorter distances (i.e., a distance of less than 200 nm). Accordingly, in some embodiments, the distance between the fluorescently labeled regions may be equal to or about 175 nm, 150 nm, 125 nm, 100 nm, 75 nm, 50 nm, 25 nm, or smaller distances. When the fluorescently labeled regions are spaced apart at distances that are less than the diffraction limit of visible light, the barcoded probes are imaged using super-resolution techniques such as Point Accumulation for Imaging in Nanoscale Topography (PAINT, DNA-PAINT), Stimulated Emission Depletion Microscopy (STED), Reversible Saturable Optical Fluorescence Transitions (RESOLFT), Stochastic Optical Reconstruction Microscopy (STORM, dSTORM), Photoactivated Localization Microscopy (PALM), Blink Microscopy (BM), and any other form of super-resolution microscopy.

It is to be understood that the term "fluorescently-labeled region" embraces regions that are stably labeled and those that are transiently labeled with fluorophore-bearing moieties such as fluorophore-bearing oligonucleotides that are complementary to a handle domain or docking strand (or sequence), as described in greater detail herein. In some instances, the barcode probes of the invention are provided with such fluorophore-bearing oligonucleotides bound thereto, and therefore these probes would be fluorescent. In other instances, the barcode probes of the invention are provided without such fluorophore-bearing oligonucleotides bound thereto, and therefore these probes may not be fluorescent until they are bound to a fluorophore-bearing oligonucleotide. In these latter instances, a probe may be provided together with a plurality of fluorophore-bearing oligonucleotides that are specific for (e.g., typically, complementary to) the handle domains or docking strands of the probe. The probes and the oligonucleotides may be provided in a kit, optionally with each in a separate container within the kit.

The positions of the fluorescently labeled regions can be selected such that the orientation of the DNA nanostructure barcode may be determinable through visualization of the barcode on a fluorescent microscope (typically by some form of asymmetry in the

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arrangement of the labeled regions or by some arrangement convention, such a particular color or pattern reserved to indicate an end of the barcode). In some instances, orientation information is unnecessary and the barcode pattern need not be absolutely unique. Thus, barcodes with even just two labels may be used, and the presence and/or intensity of various labels provides the information required.

In some instances, the fluorescently labeled regions are arranged in a linear manner. An examples is shown in, for example, FIG. 1. The fluorescently labeled regions may however be arranged in a non-linear manner as well. An example is shown in FIG. 8. Other non-linear arrangements will be apparent to those of ordinary skill in the art and are embraced by the invention. In some instances, a non-linear orientation (or arrangement or geometry) of the fluorescently labeled regions is used to detect and/or identify analytes. In some instances, the signal (including for example the color or color combination) of non-linearly arranged fluorescently labeled regions is used to detect and/or identify analytes. In still other instances, orientation and signal are used together to detect and/or identify analytes. Thus, it is to be understood that the nanostructure may be linear or non-linear and/or the arrangement of the fluorescently labeled regions may be linear or non-linear.

The DNA nanostructure, once assembled, is sufficiently immutable in a resting state (i.e., in the absence of external influence from e.g. an electrical field) that the spatial pattern of the fluorescent regions can be detected using light microscopy in that resting state. Portions of the nanostructure may be flexible, such as unlabeled portions. In some embodiments, a probe may include a (relatively) rigid portion carrying label and an unlabeled flexible portion. in some embodiments, a probe may have two rigid labeled regions connected by a deformable linker.

In certain embodiments, the DNA nanostructure of the DNA barcode probe can be of any one-, two- or three dimensional shape that is able to support at least three fluorescently labeled regions at least 200 nm apart from each other. As stated herein, however, the invention is not so limited as nanostructures having fluorescently labeled regions spaced at distances of less than 200 nm from each other are also contemplated.

In certain embodiments, the DNA nanostructure is a nanotube. In some embodiments, the nanotube is at least 200, 300, 400, 500, 600, 700 or 800 nm long. In certain embodiments, the DNA nanostructure is a six helix bundle dimer nanotube.

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Description of DNA nanotube design (via DNA origami) can be found in, for example, Douglas *et al., Proc. Natl. Acad. Sci. USA* 104:6644-6648 (2006) and U.S. Pat. App. Pub. No. 2010/0216978, each of which is incorporated by reference in its entirety.

In some embodiments, the DNA barcode probes are designed such that the orientation of the DNA barcode probe is determinable based on the emission of visible light by the fluorescently-labeled regions. Any property of the barcode can be used to render the orientation of the DNA barcode can be determinable. For example, if the DNA barcode probe has a substantially one-dimensional (linear) structure, such as a nanotube, the fluorescently labeled regions can be positioned asymmetrically along the DNA nanostructure. The orientation of a substantially one-dimensional (linear) DNA barcode probe can also be made determinable by asymmetrically using a particular fluorophore or combination of fluorophores to label one side of the DNA nanostructure consistently. If the DNA nanostructure is two- or three-dimensional, the orientation of the DNA barcode probe can also be rendered determinable based, for example, on an asymmetry of the shape of the structure itself.

In certain embodiments, the fluorescently labeled regions of the DNA barcode probe are generated by labeling specific staples of the DNA nanostructure with a fluorescent moiety. The staples can be directly labeled or indirectly labeled. As used herein, the term "directly labeled" refers to a nucleic acid that is covalently bonded to a detectable moiety. In contrast, the term "indirectly labeled" refers to a nucleic acid that is attached to a detectable moiety through one or more non-covalent interactions.

In certain embodiments, certain staples of the DNA nanostructure are directly labeled with a fluorescent moiety. In such embodiments, the staple can be, for example, synthesized with a particular fluorescent moiety attached, or covalently bonded to a fluorescent moiety prior to its incorporation into the DNA nanostructure.

Any combination of fluorescent moieties may be used in a single barcode, provided the fluorescent signal from each labeled region is detectable. Preferably, the combination of fluorescent moieties is chosen so that there is no energy transfer between the fluorescent moieties (i.e., the fluorophore combination used on a single barcode probe does not contain pairs of fluorophores that act together as a donor-acceptor pair).

In some embodiments, certain staples of the DNA nanostructure are indirectly labeled with a fluorescent moiety. In such embodiments, such staples can be synthesized

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to have at least two domains, a staple domain and a handle domain. The staple domain is a region (or nucleotide sequence) of the staple that hybridizes to the scaffold strand to contribute to the formation and stability of the DNA nanostructure. The handle domain contains additional nucleic acid sequence that is not necessary for the creation of the DNA nanostructure. Before, during or after the formation of the DNA nanostructure, the handle sequences are available to be hybridized by oligonucleotides having a complementary DNA sequence. Thus, such staples can be indirectly labeled by hybridizing the handle domain to another nucleic acid that has a nucleic acid sequence complementary to the handle and that is itself either directly or indirectly labeled with a fluorescent moiety.

In some embodiments, each fluorescent region of the DNA barcode probe includes at least one fluorescently labeled staple. In certain embodiments, the fluorescently labeled region may include a plurality of fluorescently labeled staples. For example, in certain embodiments, each fluorescently labeled region may include at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or more fluorescently labeled staples. In regions designed to have multiple staples labeled, all the staples may be labeled by a single type of fluorophore, or the staples may be labeled by a combination of fluorophore types. For example, in certain embodiments, a fluorescently labeled region is designed to be labeled by multiple types of fluorophores present at a predetermined ratio. An example of this latter type of labeling, referred to as dual-labeling, is demonstrated in the Examples. In dual-labeling, a single region is labeled with two fluorophores in a predetermined ratio, to yield a fluorescent signal that is distinguishable from either of the contributing fluorophores. In this way, the variety of barcode probes is increased.

In still other embodiments, the barcode probe may comprise one or more regions that are transiently labeled with fluorophore-bearing oligonucleotide strands. These embodiments may be used when the fluorescently labeled regions are within 200 nm of each other. In some embodiments, all the regions contributing to the barcode will be designed to be transiently labeled. Transient labeling of a region is achieved by using a nanostructure comprised of staple strands with handle domains that are shorter in length than those used for more permanent binding of a fluorescently labeled oligonucleotide. As an example, permanent (or stable) binding of fluorophore-bearing oligonucleotides to a handle domain of a staple strand can be achieved using

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oligonucleotides and handle domains that are about 21 nucleotides in length. When shorter oligonucleotides and handle domains are used, the strength of binding between the two is reduced and accordingly they are more likely to dissociate than are longer strands. At room temperature, oligonucleotides and handle domains that are about 9 nucleotides in length associate with each other only transiently. As will be understood in the art, at higher temperatures, the length of the oligonucleotide and handle domain will typically be increased in order to achieve the same association/dissociation kinetics.

Accordingly, the invention contemplates handle domains that are directly or indirectly labeled with fluorophores as well as those that are transiently labeled with fluorophores. The length of the handle domain (and similarly its complementary oligonucleotide) will depend upon the nature of the binding (i.e., whether it is intended to be permanent or transient binding), and the reaction conditions such as but not limited to temperature, salt concentration, and the like. Such lengths may range, without limitation, from about 5 nucleotides to 30 nucleotides, or from about 7 nucleotides to about 25 nucleotides, or from about 9 nucleotides to about 21 nucleotides.

In some embodiments, the DNA barcode probe may further include a target binding moiety that acts as a binding partner for the target (or analyte) of interest. As used herein, the term "binding" refers to an association between at least two molecules due to, for example, electrostatic, hydrophobic, ionic and/or hydrogen-bond interactions under physiological conditions. Such a target binding moiety can be, for example, without limitation, a nucleic acid such as an oligonucleotide, a protein or peptide such as an antibody or antibody fragment, a carbohydrate or a polysaccharide, or a small molecule. The target binding moiety and the target to which it binds can also be viewed in a receptor and ligand relationship. In certain embodiments, the DNA barcode probe may include multiple target capture moieties, which may be identical or different.

The target capture moiety can be attached to the DNA nanostructure of the DNA barcode probe using any method known in the art. For example, the target capture moiety can be covalently bonded to a staple strand or a scaffold strand. The capture moiety can also be indirectly attached to either a staple strand or the scaffold strand by, for example, hybridizing to a handle domain of a staple strand, as described above.

In certain embodiments, the DNA barcode probe may include a target binding moiety that is an oligonucleotide or other nucleic acid capable of hybridizing to a target

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nucleic acid. In general, an oligonucleotide is capable of hybridizing to a target nucleic acid if it includes a nucleic acid sequence that is substantially complementary a sequence of the target nucleic acid. In certain embodiments, the oligonucleotide may include a sequence that is perfectly complementary to a target (*i.e.* that is able to base pair at every nucleotide with the target sequence). In some embodiments, the oligonucleotide is less than perfectly complementary to the target but is still able to hybridize to a target nucleic acid under certain conditions. Thus, in certain embodiments the sequence of the oligonucleotide is at least 10%, 20%, 30%, 40%, 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90% or 95% complementary to a sequence of the target nucleic acid. In certain embodiments, the DNA barcode probe may include a target binding moiety that is an aptamer.

In certain embodiments, the DNA barcode probe may include a target binding moiety that is an antibody. As used herein, the term "antibody" includes full-length antibodies and any antigen binding fragment (*i.e.*, "antigen-binding portion") or single chain thereof. The term "antibody" includes, but is not limited to, a glycoprotein comprising at least two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds, or an antigen binding portion thereof. Antibodies may be polyclonal or monoclonal; xenogeneic, allogeneic, or syngeneic; or modified forms thereof (*e.g.*, humanized, chimeric).

As used herein, the phrase "antigen-binding portion" of an antibody, refers to one or more fragments of an antibody that retain the ability to specifically bind to an antigen. The antigen-binding function of an antibody can be performed by fragments of a full-length antibody. Examples of binding fragments encompassed within the term "antigen-binding portion" of an antibody include (i) a Fab fragment, a monovalent fragment consisting of the V_H, V_L, CL and CH1 domains; (ii) a $F(ab')_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the V_H and CH1 domains; (iv) a Fv fragment consisting of the V_H and V_L domains of a single arm of an antibody, (v) a dAb fragment (Ward *et al.*, (1989) Nature 341:544 546), which consists of a V_H domain; and (vi) an isolated complementarity determining region (CDR) or (vii) a combination of two or more isolated CDRs which may optionally be joined by a synthetic linker. Furthermore, although the two domains of the Fv fragment, V_H and V_L, are coded for by separate

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genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the V_H and V_L regions pair to form monovalent molecules (known as single chain Fv (scFv); see *e.g.*, Bird *et al.* (1988) Science 242:423 426; and Huston *et al.* (1988) Proc. Natl. Acad. Sci. USA 85:5879 5883). Such single chain antibodies are also intended to be encompassed within the term "antigen-binding portion" of an antibody. These antibody fragments are obtained using conventional techniques known to those with skill in the art, and the fragments are screened for utility in the same manner as are intact antibodies.

In certain embodiments the target binding moiety is a protein or peptide. For example, a protein or peptide can be used to bind the DNA barcode probe to the protein's ligand. DNA barcodes having protein or peptide target binding moieties can also be used, for example, to identify antibodies or receptors such as but not limited to cell surface receptors such as but not limited to T cell receptors and B cell receptors and intracellular receptors such as but not limited to hormone receptors that are able to bind to an epitope present on the protein or peptide.

In certain embodiments, the target binding moiety is a small molecule. DNA barcode probes having small molecule target binding moieties can, for example, be used to identify molecular or cellular targets of small molecules of interest. Furthermore, libraries of small molecules, where each small molecule is individually tagged with a DNA barcode, can be used in small molecule library screens to identify small molecules that specifically bind to a target of interest.

DNA barcode probes may be provided as a population of distinct species. The number of distinct barcode probes in the population is limited only by the multiplexing capability of the particular barcodes in the population. In certain embodiments, the population contains at least 10, 50, 100, 500, 1000, 2000, 3000, 4000, 5000, 10^4, 50000, 10^5, 10^5, 10^6, 10^7, 10^8, 10^9, 10^{10}, 10^{11} distinct barcode probes. The population may contain 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, or more distinct species. The population may contain less than or equal to about 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 or more distinct species. In some embodiments, the population comprises 216 distinct barcode probes. The barcode probes described herein are sufficiently adaptable and expandable to permit creation of such large-scale populations of distinguishable species.

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Methods of Use

The DNA nanostructure barcode probes can be used, inter alia, in any assay in which existing molecular barcode technologies are used. For example, the DNA barcode probes can be used according to the methods described in U.S. Pat. App. Pub. No. 2010/0015607, the content of which is incorporated by reference in its entirety.

Typically assays include detection assays including diagnostic assays, prognostic assays, patient monitoring assays, screening assays, biowarfare assays, forensic analysis assays, prenatal genomic diagnostic assays, and the like. The assay may be an in vitro assay or an in vivo assay. The sample being analyzed may be a biological sample, such as blood, sputum, lymph, mucous, stool, urine and the like. The sample may be an environmental sample such as a water sample, an air sample, a food sample, and the like. The assay may be carried out with one or more components of the binding reaction immobilized. Thus, the targets or the barcodes may be immobilized. The assay may be carried out with one or more components of the binding reaction non-immobilized. The assays may involve detection of a number of targets in a sample, essentially at the same time, in view of the multiplexing potential offered by the barcode probes of the invention. As an example, an assay may be used to detect a particular cell type (e.g., based on a specific cell surface receptor) and a particular genetic mutation in that particular cell type. In this way, an end user may be able to determine how many cells of a particular type carry the mutation of interest, as an example.

In certain embodiments, a method of identifying a target nucleic acid may include contacting the target nucleic acid with a DNA barcode having an oligonucleotide target capture moiety that includes a nucleic acid sequence capable of hybridizing to a sequence of the target nucleic acid. In certain embodiments, the target nucleic acid is contacted with a DNA barcode probe under conditions such that the target nucleic acid binds to the DNA barcode probe. In some embodiments, the target nucleic acid is then separated from at least some material that is not bound to the target nucleic acid. In certain embodiments the presence of the target nucleic acid is then determined by detecting the presence of the DNA barcode probe bound to the target nucleic acid. The detection of the DNA barcode probe can be accomplished by any appropriate method known in the art, including through fluorescent microscopy.

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In some embodiments, the above-described method may further include immobilizing a capture probe containing a nucleic acid sequence complementary to a second target nucleic acid sequence to a solid support. In some embodiments the target nucleic acid is contacted with both the capture probe and the DNA barcode probe. The target nucleic acid can be contacted by the capture probe first, the DNA barcode probe first, or by both probes simultaneously. The capture probe can be immobilized on the solid support before hybridizing to the target nucleic acid, after hybridizing to the target nucleic acid, or simultaneously with hybridizing to the target nucleic acid. In such embodiments, the presence of the target nucleic acid will cause the formation of a tertiary complex that includes the capture probe, the target nucleic acid and the DNA barcode probe, immobilized to a solid support. As described above, the presence of the target nucleic acid is then determined by detecting the presence of the DNA barcode probe by any appropriate method known in the art, including through fluorescent microscopy

In certain embodiments, a method of identifying a target protein or cell may include contacting the target protein or cell with a DNA barcode probe described herein. In certain embodiments, the DNA probe has a protein, peptide or antibody target capture moiety. In some embodiments, the DNA barcode probe is contacted to a target cell or protein under conditions such that the target cell or protein binds to the DNA barcode probe. In some embodiments, the method further includes the step of separating the target protein or cell from at least some material that is not bound to the target protein or cell. In certain embodiments, the method further includes identifying a target protein or cell by detecting the presence of the DNA barcode probe bound to the target protein or cell. In some embodiments, the target protein is an antibody or receptor such as but not limited to a T cell receptor.

It will be appreciated that for some of the aspects and embodiments of the invention, it is preferable for the barcode probe and/or target to remain stationary during image acquisition, particularly if a super-resolution approach is being used and/or if the imaging technique requires superimposition of separately acquired images.

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Super-Resolution Imaging

As discussed herein, in some aspects of the invention, the barcode probes comprise fluorescently labeled regions that are spaced at distances less than 200 nm (i.e., the diffraction limit of visible light). In some of these instances, the probe is contacted to a sample and once bound to one or more components of that sample it is exposed to soluble oligonucleotides each of which is specific for one of the regions and is labeled with fluorophores. Such soluble, transiently binding oligonucleotides may be referred to herein as "imager" strands. In some instances, the unbound components in the sample are removed prior to the addition of the fluorophore-bearing oligonucleotides. The presence of the barcode probe (and its identity based on its barcode) is then determined by imaging the remaining sample components using a series of single time lapsed images or a time lapsed movie in order to detect the binding of a fluorophore-bearing oligonucleotide to its complement on the probe. The complement on the probe may be referred to herein as a "docking" strand. The conditions are set such that at any given time only a single fluorophore-bearing oligonucleotide is bound to the barcode probe. If only a single fluorophore is bound at any given time, its color identity can be determined even if the neighboring regions are less than 200 nm away (since there would be no fluorophores bound to the neighboring regions at the same time). In this way, one is able to position adjacent "fluorescently labeled" regions at distances of less than 200 nm, and thereby generate an even greater diversity of barcodes. It is to be understood that in these embodiments, the fluorescently labeled regions are only transiently fluorescent and that the barcodes are only fluorescent when bound by imager strands.

In some embodiments, the imager and/or docking strands are about 5 to about 18 nucleotides in length, or about 6 to about 15 nucleotides in length, or about 7 to about 12 nucleotides in length. In some embodiments, the length is about 8, 9, 10, 11 or 12 nucleotides.

The art is familiar with other localization-based super-resolution fluorescence techniques including, for example, the STORM technique as described in U.S. Patent No. 7838302, the disclosure of which relating to STORM is incorporated by reference herein. The invention contemplates labeling of the nanostructure barcode probes in any manner sufficient for any super-resolution technique.

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In other aspects of the invention, the nanostructure barcode probes of the invention may be used to calibrate and/or confirm calibration of fluorescence microscopy equipment, including standard or conventional fluorescence microscopes, confocal microscopes, super-resolution fluorescence microscopes, and the like. Accordingly, nanostructure barcode probes may be used as calibration standards (or tools). Of particular interest in these instances are the barcode probes having "fluorescently-labeled" regions that are spaced apart at less than the diffraction limit of visible light (i.e., less than about 200 nm). When used in this manner, the probes may or may not have a target binding moiety. As an example, they may comprise a target binding moiety such as biotin or streptavidin that facilitates their immobilization for imaging purposes.

EXAMPLES

Example 1: Creation of a DNA Barcode

DNA origami barcode probes were constructed by fluorescently labeling three separate regions of an 800-nm six-helix bundle dimer nanotube (FIG. 1). Each of the approximately 28 nm long fluorescently labeled regions was modified with twelve oligonucleotides attached to one of the three fluorophores (rhodamine-green, Cy3 or Cy5). The DNA origami barcode probe was designed to be asymmetric by placing the first two labeled regions further apart (approximately 460 nm apart) than the last two (approximately 270 nm apart). It is to be understood that other arrangements, including symmetric arrangements, are also contemplated by the invention.

The main structure of the DNA origami barcode probe is a DNA six-helix bundle nanotube dimer designed using the caDNAno software available at the cadnano website. The sequence of the scaffold strand used for each monomer is provided as SEQ ID NO:1. The sequences of the staples used in the construction of the DNA origami barcode are provided as SEQ ID NOs: 13-361. Certain selected staples were elongated (after rational design) to include 21-nt single stranded overhangs (handles) for fluorescent labeling. Table 1 provides the handle and anti-handle sequences used.

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Table 1: DNA sequences used for fluorescently labeling nano-barcode.

Name	Sequence (SEQ. ID. NO.)
Handle 1	5'-TTCCTCTACCACCTACATCAC-3' (SEQ ID NO:2)
Handle 2	5'-TAACATTCCTAACTTCTCATA-3' (SEQ ID NO:3)
"Blue" anti-handle 1	5'-GTGATGTAGGTGGTAGAGGAA/rhodamine green/-3' (SEQ ID NO:4)
"Green" anti-handle 1	5'-GTGATGTAGGTGGTAGAGGAATTT/Cy3/-3' (SEQ ID NO:5)
"Red" anti-handle 1	5'-GTGATGTAGGTGGTAGAGGAA/Cy5/-3' (SEQ ID NO:6)
"Blue" anti-handle 2	5'-TATGAGAAGTTAGGAATGTTA/Alexa Fluor 488/-3' (SEQ ID NO:7)
"Green" anti-handle 2	5'-TATGAGAAGTTAGGAATGTTA/Cy3/-3' (SEQ ID NO:8)
"Red" anti-handle 2	5'-TATGAGAAGTTAGGAATGTTA/Cy5/-3' (SEQ ID NO:9)

Accordingly, of the staple strands provided in the Sequence Listing, SEQ ID NOs: 16, 17, 70-73, 126-129, 182 and 183 are labeled "blue"; SEQ ID NOs: 195, 196, 239, 240, 251, 252, 295, 296, 307, 308, 351 and 352 are labeled "red"; and SEQ ID NOs: 216-219, 272-275 and 328-331 are labeled "green".

caDNAno JSON files showing the arrangement of the front and rear monomers of the probe are provided in the computer program listing appendix as "6ht-v5-1color-1.json" and "6ht-v6-2color-1.json" respectively.

To assemble the DNA origami barcode probe, the front and rear monomers were each assembled and fluorescently labeled in a separate test tube and then mixed together to form the dimer. The assembly of each monomer is accomplished in one-pot reaction by mixing 100 nM scaffold strand derived from M13 bacteriophage (termed p7308) (SEQ ID NO:1) with a pool of oligonucleotide staple strands (SEQ ID NOs: 13-186 for the front monomer and 187-361 for the rear monomer; 600 nM of each; reverse-phase cartridge purified, Bioneer Inc.) in folding buffer containing 5 mM Tris, 1 mM EDTA, 20 mM $MgCl_2$, 50 mM NaCl (pH 8) and subjecting the mixture to a thermal-annealing ramp that cooled from 80°C to 60°C over the course of 80 minutes and then cooled from 60°C to 24°C over 15 hours. Excessive staples were removed by polyethylene glycol (PEG) fractionation before each monomer was incubated with anti-handles carrying appropriate fluorophores at 1:1.2 molar ratio for labeling. For

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dimerization, a stoichiometric amount of the fluorescently labeled front and rear monomers were mixed and incubated a 37°C for 2 hours. The final product was purified by agarose gel (non-denaturing, 1.0%) electrophoresis. The purified nano-barcode can be stored at -20 °C for at least 3 months.

Although this example uses a barcode made of two monomers, a DNA barcode probe could include 3, 4, 5, or more monomers. The platform is therefore scalable to any desired level, and can provide any desired number of distinct probes.

Example 2: Detection of a Singly-Labeled DNA Barcode Using Fluorescent Microscopy

Two of the monomer nanotubes were folded and fluorescently labeled separately and combined together as described above to yield the nano-barcode. The final product was purified by agarose gel electrophoresis and directly deposited on a glass slide for imaging using a total internal reflection fluorescence microscope (TIRFM). In brief, 5 μL of the purified nano-barcode (~ 20 pM) was deposited on a glass slide, sandwiched by a coverslip (No. 1.5, 18 x 18 mm^2, ~0.17 mm thick) and let sit for 5 minutes before being imaged on a Leica DM16000B TIRFM. The samples were imaged sequentially through three channels, each assigned a false color (blue, green and red). For the blue channel, the 488 nm laser beam was reflected by a dichroic mirror (430/505/575/670) and shined on the sample through a 100x objective (HCX PL APO 100x/1.47 oil CORR TIRF, Leica). The emission light was collected through the same objective, filtered through the same dichroic mirror and an external emission filter (525/36) and integrated for 800 ms on an EM-CCD camera (Hamamatsu C9100-02). For the green and red channels, similar configurations were used except for the excitation laser (561 and 635 nm, respectively), emission filter (605/52 and 705/72, respectively) and exposure time (500 and 700 ms, respectively). The imaging process was automatically controlled by a Leica LAS software. The images were processed by *Image J* and a custom written software for decoding.

The following false-colors were assigned to the fluorescently labeled regions: Alexa Fluor 488/rhodamine green (blue), Cy3 (green) and Cy5 (red). TIRFM images resolved the DNA origami barcode as a strip of three distinct bright spots. Notably, the distance between blue and green spots is farther than that between green and red showing the asymmetry of the barcode (data not shown)

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A DNA origami barcode system having three fluorescently labeled regions, each of which is labeled by one of three distinct fluorophores can generate 3^3 (27) distinct DNA origami barcode probes. All 27 possible DNA origami barcode probes were assembled separately and purified together by agarose gel electrophoresis.

Five distinct barcodes from the 27 members in the barcode family were randomly chosen for quality control experiments. The barcodes were assembled and purified separately and imaged under the same experimental conditions. Two distinct features of the barcode were clearly visible from the TIRF images (FIG. 2A, top panel): first, each fluorescently labeled zone on a barcode was resolved as a single-color spot and each complete barcode consisted of three of such spots; Second, two of the neighboring spots were separated by a small gap while the other 7 two neighbors sat closely together. Therefore one can visually recognize and decode those geometrically encoded barcodes based on the color identity of the spots and their relative spatial positions, even without the aid of any specialized decoding software. Using a custom-written software that localizes the center of each spot on the BRG barcodes, we measured the average center-to-center distance between the neighboring spots to be 433±53 nm (mean±s.d., N=70; larger distance) and 264±52 nm (mean±s.d., N=70; smaller distance), confirming the correct formation of the barcodes. These experimentally measured distances were slightly smaller than the designed values (478 nm and 298 nm). We attribute this discrepancy to random thermal bending of the nanotubes (persistence length of ~1–2 μm), which has been observed previously by others[23,28] and confirmed by us (data not shown) using transmission electron microscopy (TEM). It is important to note that unlike some other geometrically encoded barcoding systems (e.g., NanoString nCounter[12]), there was no molecular combing step involved in the sample preparation. The separation between the fluorescent spots was exclusively created by the inherently rigid structure of the six-helix bundle nanotube. It is also notable that the spot intensities were not perfectly uniform across the whole image, which can be explained by factors such as the uneven illumination of the sample stage and differences in labeling efficiency. Nevertheless, the TIRF images proved that the barcodes were successfully assembled and can be resolved unambiguously. We then manually investigated TIRF images with an area of 50×50 μm^2 for the selected five

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barcodes (FIG. 2A, bottom panel). The objects found within the images were first sorted into qualified (i.e., three single-color spots arranged in a nearly linear and asymmetric fashion as designed) and unqualified (i.e., all other objects) barcodes. The qualified 8 barcodes were further categorized into correct and incorrect (false-positive) barcodes based on the fluorescent signatures of the composing spots to reflect whether the barcode was the expected type. The unqualified barcodes were further sorted into (1) monomer nanotubes (single spot or two "kissing" spots), (2) barcode with "wrong" geometry (i.e., extreme bending), and (3) barcode containing at least one spot with multiple colors. Our statistics revealed that more than 70% of the visible objects were qualified barcodes, which we further determined to be exclusively the expected type (i.e., zero false-positive out of 188 qualified barcodes observed). The unqualified barcodes arose likely from folding defects, sample damage during handling and overlapping nanotubes on the surface, which can be largely reduced by optimizing the sample preparation and imaging protocol.

Typically, a number of different barcode species coexist in one pool. Thus it is important to examine the robustness of our system by mixing different types of barcode together. In an initial test, we synthesized BRG and RGB barcodes separately, mixed them together at equal molar ratio and co-purified them via gel electrophoresis. The TIRF analysis of the purified mixture confirmed the 1:1 stoichiometry of the two barcodes and the overall assembly success rate (qualified barcode/all objects) of ~80%, suggesting that both barcodes maintained their integrity in the mixing and co-purification process. In addition, over 98% of the qualified barcodes fell into one of the two expected types (BRG and RGB). The 2% false-positive rate was due to an unexpected barcode namely BGB, which could be attributed to a rare occasion when the front monomer of the BRG barcode lay in proximity of the rear monomer of the RGB barcode. This can be eliminated using a more stringent purification condition to minimize the amount of leftover monomers.

We next challenged the system by imaging a pool of all 27 members of the barcode family in which all species were mixed at equimolar amount. The TIRF images (FIG. 2B) showed that all types of barcodes were resolved. Statistics by sampling twenty-seven 50×50 μm^2 images (~1,500 barcodes in total) revealed an average count of 55 per barcode type with a standard deviation of 9 (FIG. 2C), fitting

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well with the expected stoichiometry considering the pipetting and sampling error. The distribution of observed objects over the four categories (note that here correct *vs.* incorrect barcodes were not distinguishable as all 27 types were included) was consistent with the values measured from the single-type barcode samples. The above observations suggest that the sub-micrometer-long DNA nanotube represents a reliable platform to construct geometrically encoded barcodes with built-in structural rigidity.

Software could be used to recognize and decode the image of different types of DNA origami barcode probes automatically. Typically, such software has three basic steps: recognition, filtering and decoding. In the first step, the fluorescent spots are identified. For each fluorescent channel (blue, red or green), the software identifies fluorescent spots based on their intensity and size, then compares them to automatically determined thresholds using overall image intensity and pre-defined spot sizes. The fluorescent spots below those thresholds are removed along with general background noise. After processing all three channels, a barcode image is constructed by merging spots identified in three channels. An example of such a barcode image is provided in FIG. 3. In the second step, the distances (calculated from (x,y) coordinates of fluorescent spots) between each region of DNA origami barcode probes and the barcode bending angle (based on the center spot) are calculated. The software then filters DNA origami barcode probes and removes those with region distances that disagreed with the theoretical region distances or had a barcode bending angle smaller than a currently arbitrarily set threshold angle of 140 degrees. As an example, the BRG barcodes were filtered using three distance rules: (1) The distance between blue and green spot cannot be greater than 15 pixels (theoretical distance was 11 pixels); (2) The distance between red and blue spot cannot be greater than 9 pixels (theoretical distance was 6.8 pixels); and (3) The distance between red and green spot cannot be greater than 6 pixels (theoretical distance was 4.2 pixels). The BRG barcodes that had a bending angle (at the red spot) smaller than 140 degrees were removed. In the last step, after all barcodes are identified and filtered, the software uses different criteria (based on theoretical distance and color) to sort barcodes into specific types. Therefore, multiple types of DNA barcode probes can be decoded based on how they were originally designed. Besides the final constructed DNA barcode probe image, the software can output statistical information

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regarding the (*x,y*) coordinates of each DNA barcode probe fluorescent spot, distances between adjacent spots, barcode bending angle, and barcode types.

The software described above was used to detect BRG and GRG DNA origami barcode probes. As shown in FIG. 3, the BRG and GRG DNA origami barcode probes were clearly identified by the automated decoding software. The software used a number of filters to select the valid DNA origami barcode probes. As a result, 88% and 85% of BRG and GRG nano-barcodes were counted. The software generated statistical information on BRG and GRG barcodes (Table 2) that showed that the measured distances between each region on those DNA origami barcode probes were in good agreement with the theoretical region distances as designed (FIG. 3E).

Table 2

Barcode type	Barcode identified	Barcode discarded	Barcode bending angle α (degree)	Distance 1 (pixel)	Distance 2 (pixel)
BRG	70	9	165.11±10.08	6.18±0.76	3.77±0.74
GRG	81	15	161.51±11.73	6.12±0.95	3.95±0.98

Example 3: Detection of an Oligonucleotide Target Using a DNA Barcode Probe

A 42-nt DNA oligonucleotide target was detected using a DNA origami barcode probe. Two different purified barcodes Green, Red, Green (GRG) with the 21-nt oligonucleotide target capture moiety specific for a 42-nt oligonucleotide target (Target 1, *see* Table 3 for sequences) and Blue, Red, Green (BRG) without a target capture moiety, were mixed at 1:1 ratio and diluted to 20 pM in folding buffer. Biotin probe specific for Target 1 (0.2 µL of 100 nM) and Target 1 (0.4 µL of 10 nM) was added to 100 µL of the barcode mixture. In a separate test tube containing 100 µL of the barcode mixture, 0.2 µL of 100 nM biotin probe for Target 1 and 0.4 µL of 1x folding buffer was added as a negative control. The above samples were both incubated at 37 °C overnight before being run through separate channels of a micro-fluidic cell with a streptavidin coated glass cover-slip (Xenopore) substrate. Both channels were then washed with 40 µL of washing buffer (5 mM Tris-HCl pH 8.0, 500 mM NaCl) and imaged on a Leica TIRFM as described above. Ten images were taken in each channel

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at random locations and the total number of DNA origami barcode probes was counted manually.

Table 3: DNA sequences used in oligonucleotide target detection

Name	Sequence (SEQ ID NO:)
Target oligo	5'-GAATCGGTCACAGTACAACCGCGCCGTAGGGCTG-ATCAAAGC-3' (SEQ ID NO:10)
Biotin probe	5'-/Biotin/ GCTTTGATCAGCCCTACGGCG-3' (SEQ ID NO:11)
Capture probe	5'-CGGTTGTACTGTGACCGATTC-3' (SEQ ID NO:12)

In the presence of the target oligonucleotide, the biotin probe hybridized to one 21 nt sequence of the target oligonucleotide, while the GRG barcode hybridized to a second 21 nt sequence of the target oligonucleotide. The BRG probe, however, lacking a target capture moiety specific for the target oligonucleotide does not hybridize to the target and therefore does not become bound by the biotin probe. (FIG. 4A). As a consequence, when the detection reaction mixture was run through the streptavidin-coated glass surface, the GRG barcode binds to the surface, while BRG barcode is washed away. TIRF images and statistical analysis supported the specificity of the detection (FIGs. 4B and C). In the presence of 40 pM oligonucleotide target, there were ~20 times more GRG barcodes than BRG barcodes bound to the surface. In the absence of the target, both barcodes did not show significant surface binding.

Example 4: Dual-Labeled Barcode Probes

In order to enhance multiplexing capability even further, the sequence of six staple extensions per zone was changed so that instead of using twelve identical fluorescent oligonucleotides for labeling, a combination of up to two fluorophores was used to create more unique fluorescence signatures (pseudo-colors) for each zone. Six pseudo-colors (B, R, G, BG, BR, and GR) were generated by this "dual-labeling" strategy using three spectrally differentiable fluorophores. Consequently, the total number of distinct barcodes was raised from 27 to 6^3 =216, which represented an order of magnitude increase in the multiplexing capability.

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Similar to the single-labeled-zone barcode family, 5 members from the dual-labeled-zone barcode family were chosen for quality control purpose. The barcodes can be visually decoded either solely from the superimposed image or by examining all different channels simultaneously. For example, as shown in the first column of FIG. 5A, the barcode "BG--GR-BR" ("--" and "-" denotes larger and smaller inter-zone distance in the barcode, respectively) exhibited two spots each in the blue, green and red channels but with descending gaps between them, matching its design. In the superimposed image, the barcodes were seen as Cyan--Yellow-Pink, an expected consequence of color mixing caused by the dual-labeling strategy. In a similar fashion, we further verified the correct formation of the other four selected barcodes (FIG. 5A). Although the final pseudo-color from the dual-labeled zones was not always uniform (e.g., some yellow spots were green-tinted while the others were red-tinted) due to the inconsistent labeling efficiency and minor sample displacement during imaging, the fluorescence signature of any given spot could be identified by checking the raw images acquired from the three imaging channels. We manually analyzed two 50 x 50 μm^2 images of each dual-labeled-zone barcode and plotted the statistical data in FIG. 5B. Here, objects were sorted into qualified barcodes and unqualified barcodes based on their geometry and the qualified ones were further categorized as correct and incorrect. 75–95% of the objects were qualified barcodes, among which 80–90% were the correct type (percentage varies depending on the exact type of barcode). Compared to the single-labeled-zone barcode family, the percentage of qualified barcodes remained the same, while the false positive rate increased significantly from zero to 10–20%. This observed increased false positive rate is consistent with the expected decreased robustness of the dual-labeling strategy (as compared to single-labeling). In one design, a dual-labeled zone carried 6 staple extensions for each fluorophore species, only half as many as in a single-labeled zone.

As a result, the dual-labeled-zone barcode consisted of dimmer spots that were more susceptible to damages such as photo bleaching. In this sense, a single-labeled-zone barcode can be thought of as a redundantly encoded dual-labeled-zone barcode. For instance, a single-labeled-zone barcode can still be recognized as the correct type when six fluorophores were missing from each zone, a scenario in which the dual-labeled-zone barcode could be disqualified or categorized as incorrect. In the latter case, it will increase the false positive rate. In principle, the false positive rate can be decreased by

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increasing the copy number of each fluorophore species in a dual-labeled zone. The five barcodes we examined have each of their zones labeled with two distinct fluorophore species, making them likely among the most error-prone members of the dual-labeled-zone barcode family. Therefore, we would expect a smaller false-positive rate on average from the whole family.

We further tested the dual-labeled-zone barcoding system by imaging a mixture containing 72 barcode species that were individually assembled and co-purified. Custom MATLAB scripts were used to assist the decoding process in two steps. In step one, a three-channel (red, green, blue) TIRF image containing barcodes was pre-processed to remove background and thresholded so that only pixels containing qualified barcodes remained. The resulting three-channel binary image was merged to generate a single-channel binary image. Next, the software identified the location and orientation of geometrically legitimate barcodes based on their shape in the binary image. In step two, for each barcode located in step one, the corresponding region of the three-channel image was compared against a library of all possible reference barcodes. The observed barcode was assigned the identity of the reference barcode with the highest correlation. The fully automated decoding process (unsupervised mode) ended after the above two steps. In an optional supervised mode, the software presented the user with the observed barcode and its most likely identity for approval. Comparison between supervised and unsupervised decoding results confirmed >80% agreement between the computer and the user. The computer-aided (supervised mode) analysis of thirty-six 64 x 64 μm^2 three-channel images registered ~2,600 qualified barcodes that belonged to 116 different species (FIG. 5C, top panel). The expected 72 species constituted ~98% of the total barcode population with an average barcode count of 36 per species and a standard deviation of 8. In contrast, the unexpected species averaged only ~1.4 barcodes per species (maximum 4 counts).

Finally, we analyzed a mixture containing all the 216 members of the dual-labeled-zone barcode. Sixty 64 x 64 μm^2 images of this mixture were processed by the decoding software in the unsupervised mode. The fully automated analysis registered ~34 barcode counts per species (~7,200 barcodes total) with a standard deviation of 17 (FIG. 5C, bottom panel). The relatively large standard deviation could be attributed to

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the decoding error in the fully automated data analysis. Our study demonstrated that 216 barcode species were successfully constructed and resolved (FIG. 5D).

Example 5: Super-Resolution Barcode Probes

Barcodes with higher spatial information density were also generated using geometrically encoded super-resolution barcodes with fluorescent features spaced by ~100-nm.

As a feasibility demonstration of the latter approach, we applied DNA-PAINT[31], a recently developed super-resolution fluorescence technique, to image the barcodes. Over the last years, several techniques have been developed that allow imaging beyond the diffraction limit using far-field fluorescence microscopy[40-44]. In most super-resolution implementations, fluorophores are switched between fluorescence ON- and OFF-states, so that individual molecules can be localized consecutively. In methods relying on targeted readout schemes such as in Stimulated Emission Depletion Microscopy[45] (STED) or other Reversible Saturable Optical Fluorescence Transitions[40] (RESOLFT) techniques, fluorescence emission is actively confined to an area below the diffraction limit. Switching of fluorescent molecules can also be carried out stochastically such as in (direct) Stochastic Optical Reconstruction Microscopy[46,47] (STORM, dSTORM), Photoactivated Localization Microscopy[48] (PALM) and Blink Microscopy[49] (BM) where most fluorescent molecules are "prepared" in a dark state and only stochastically switched on to emit fluorescence. In Point Accumulation for Imaging in Nanoscale Topography[50] (PAINT), fluorescence switching is obtained by targeting a surface with fluorescent molecules. In all stochastic approaches, fluorescence from single molecules is localized[51,52] in a diffraction-limited area to yield super-resolved images. DNA-PAINT uses transient binding of fluorescently labeled oligonucleotides (imager strands) to complementary "docking" strands on DNA nanostructures to obtain switching between a fluorescence ON- and OFF-state, which is necessary for localization-based super-resolution microscopy (cf. FIG. 6A). By adjusting the length of the imager/docking strand duplex and the concentration of imager strands in solution, fluorescence ON- and OFF-times can be tuned independently.

For this study, we extended the DNA-PAINT technique to three-color imaging using orthogonal imager strand sequences coupled to three spectrally distinct dyes

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(Atto488 for blue, Cy3b for green and Atto655 for red excitation). To demonstrate the feasibility of the three-color super-resolution barcode system, we designed a DNA nanotube monomer with 4 binding zones in a symmetric arrangement. The neighboring zones were separated by ~114 nm (i.e., well below the diffraction limit). Each binding zone consists of 18 staple strands, which can be extended to display three groups of orthogonal sequences (six per group) for the red, green or blue imager strands to bind. As a proof-of-principle experiment, we designed five different barcodes (FIG. 6A and top panel of B). The bottom panel of FIG. 6B shows the super-resolution reconstruction of the five barcodes for each channel separately as well as an overlay of all channels. FIG. 6C shows a larger area containing all five barcodes. The unique pattern of the barcodes in all three channels can be resolved. Some barcodes moved during the sequential imaging of all three color channels, but were still resolvable. Imaging could be improved by alternating excitation and faster image acquisition to prevent this effect. The transient, repetitive binding of imager strands to docking sequences on the nanotube not only creates the necessary "blinking" behavior for localization but also makes the imaging protocol more robust, as DNA-PAINT is not prone to photo-bleaching or incorrectly labeled strands. With the microscope setup we used, DNA-PAINT provides a resolution of ~46 nm (FWHM of a Gaussian fit to the reconstructed PSF) in the red, ~25 nm in the green and ~29 nm in the blue channel. The lower resolution in the red imaging channel is a result from weaker laser excitation power. When using a higher power TIRF system, a resolution of 24 nm, similar to the green and blue channel can be obtained. The obtainable resolution and imaging specificity suggests that 6 positions on one nanotube monomer could be robustly resolved while keeping the geometrical asymmetry of the barcode, which would lead to $6^7 = 279{,}936$ possible different barcodes. Furthermore the modularity of the nanotube design enables the customized reengineering of barcodes with inter-zone distances tailored to the resolving power of the used microscope, thus making it applicable for a wide range of microscope setups.

Example 6: In Situ Labeling

In a proof-of-principle experiment, the GRG barcode was used to tag wild-type *Candida albicans* yeast. The yeast cells were first mixed with a biotinylated polyclonal antibody specific to *C. albicans*, then coated with a layer of streptavidin, and finally

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incubated with biotinylated GRG barcodes (FIG. 7A). TIRF microscopy revealed the barcodes attached to the bottom surface of the yeast cells (FIG. 7B, top panel). While some of the nanotubes landed awkwardly on the uneven cell walls of the yeast cells, a number of GRG barcodes can be clearly visualized. In contrast, no barcode tagging was observed when non-biotinylated antibodies or barcodes were used to treat the yeasts (FIG. 7B, bottom panel), suggesting that little to no non-specific interaction existed between the barcode and the cell surface.

Example 7: Non-Linear Labeled Nanostructures

DNA nanostructures with non-linear geometry could be assembled to generate more sophisticated barcodes. FIG. 8 shows an example where three ~400 nm DNA tubes were linked to the outer edge of a ~60 nm DNA ring through hybridization between the staple extensions (FIG. 8A, inset). Fluorescently labeling the ring and the far end of the nanotubes generated a three-point-star-like structure clearly resolvable under fluorescence microscopy. TIRF microscopy and TEM studies (FIG. 8B) revealed that about 50% of successfully folded barcodes featured three nanotubes surrounding the ring with roughly 120° angle between each other as designed, while many other barcodes had significantly biased angles between neighboring nanotubes due to the semi-flexible double-stranded DNA linker between the ring and the nanotubes. It is conceivable that using similar design to connect three identical "satellite" linear barcodes to a central hub (here the three satellite barcodes may share the hub as a common fluorescently labeled zone), one can construct barcodes with triplicated encoding redundancy that feature outstanding reliability. In addition, more rigid linkers between the ring and the protrusions (e.g., multi-helix DNA with strand crossovers) could be employed to enforce better-defined barcode geometry.

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EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents of the specific embodiments of the subject matter described herein. Such equivalents are intended to be encompassed by the following claims.

All publications, patents, and patent applications cited in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

What is claimed is:

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29],[0,428,0,430],[0,429,0,431],[0,430,0,432],[0,431,0,433],[0,432,0,434],[0,433,0,435],[0,434,0,436],[0,435,0,437],[0,436,0,438],[0,437,0,439],[0,438,0,440],[0,439,0,441],[0,440,0,442],[0,441,0,443],[0,442,0,444],[0,443,0,445],[0,444,0,446],[0,445,0,447],[0,446,0,448],[0,447,0,449],[0,448,0,450],[0,449,0,451],[0,450,0,452],[0,451,0,453],[0,452,0,454],[0,453,0,455],[0,454,0,456],[0,455,0,457],[0,456,0,458],[0,457,0,459],[0,458,0,460],[0,459,0,461],[0,460,0,462],[0,461,0,463],[0,462,0,464],[0,463,0,465],[0,464,0,466],[0,465,0,467],[0,466,0,468],[0,467,0,469],[0,468,0,470],[0,469,0,471],[0,470,0,472],[0,471,0,473],[0,472,0,474],[0,473,0,475],[0,474,0,476],[0,475,0,477],[0,476,0,478],[0,477,0,479],[0,478,0,480],[0,479,0,481],[0,480,0,482],[0,481,0,483],[0,482,0,484],[0,483,0,485],[0,484,0,486],[0,485,0,487],[0,486,0,488],[0,487,0,489],[0,488,0,490],[0,489,0,491],[0,490,0,492],[0,491,0,493],[0,492,0,494],[0,493,0,495],[0,494,0,496],[0,495,0,497],[0,496,0,498],[0,497,0,499],[0,498,0,500],[0,499,0,501],[0,500,0,502],[0,501,0,503],[0,502,0,504],[0,503,0,505],[0,504,0,506],[0,505,0,507],[0,506,0,508],[0,507,0,509],[0,508,0,510],[0,509,0,511],[0,510,0,512],[0,511,0,513],[0,512,0,514],[0,513,0,515],[0,514,0,516],[0,515,0,517],[0,516,0,518],[0,517,0,519],[0,518,0,520],[0,519,0,521],[0,520,0,522],[0,521,0,523],[0,522,0,524],[0,523,0,525],[0,524,0,526],[0,525,0,527],[0,526,0,528],[0,527,0,529],[0,528,0,530],[0,529,0,531],[0,530,0,532],[0,531,0,533],[0,532,0,534],[0,533,0,535],[0,534,0,536],[0,535,0,537],[0,536,0,538],[0,537,0,539],[0,538,0,540],[0,539,0,541],[0,540,0,542],[0,541,0,543],[0,542,0,544],[0,543,0,545],[0,544,0,546],[0,545,0,547],[0,546,0,548],[0,547,0,549],[0,548,0,550],[0,549,0,551],[0,550,0,552],[0,551,0,553],[0,552,0,554],[0,553,0,555],[0,554,0,556],[0,555,0,557],[0,556,0,558],[0,557,0,559],[0,558,0,560],[0,559,0,561],[0,560,0,562],[0,561,0,563],[0,562,0,564],[0,563,0,565],[0,564,0,566],[0,565,0,567],[0,566,0,568],[0,567,0,569],[0,568,0,570],[0,569,0,571],[0,570,0,572],[0,571,0,573],[0,572,0,574],[0,573,0,575],[0,574,0,576],[0,575,0,577],[0,576,0,578],[0,577,0,579],[0,578,0,580],[0,579,0,581],[0,580,0,582],[0,581,0,583],[0,582,0,584],[0,583,0,585],[0,584,0,586],[0,585,0,587],[0,586,0,588],[0,587,0,589],[0,588,0,590],[0,589,0,591],[0,590,0,592],[0,591,0,593],[0,592,0,594],[0,593,0,595],[0,594,0,596],[0,595,0,597],[0,596,0,598],[0,597,0,599],[0,598,0,600],[0,599,0,601],[0,600,0,602],[0,601,0,603],[0,602,0,604],[0,603,0,605],[0,604,0,606],[0,605,0,607],[0,606,0,608],[0,607,0,609],[0,608,0,610],[0,609,0,611],[0,610,0,612],[0,611,0,613],[0,612,0,614],[0,613,0,615],[0,614,0,616],[0,615,0,617],[0,616,0,618],[0,617,0,619],[0,618,0,620],[0,619,0,621],[0,620,0,622],[0,621,0,623],[0,622,0,624],[0,623,0,625],[0,624,0,626],[0,625,0,627],[0,626,0,628],[0,627,0,629],[0,628,0,630],[0,629,0,631],[0,630,0,632],[0,631,0,633],[0,632,0,634],[0,633,0,635],[0,634,0,636],[0,635,0,637],[0,636,0,638],[0,637,0,639],[0,638,0,640],[0,639,0,641],[0,640,0,642],[0,641,0,643],[0,642,0,644],[0,643,0,645],[0,644,0,646],[0,645,0,647],[0,646,0,648],[0,647,0,649],[0,648,0,650],[0,649,0,651],[0,650,0,652],[0,651,0,653],[0,652,0,654],[0,653,0,655],[0,654,0,656],[0,655,0,657],[0,656,0,658],[0,657,0,659],[0,658,0,660],[0,659,0,661],[0,660,0,662],[0,661,0,663],[0,662,0,664],[0,663,0,665],[0,664,0,666],[0,665,0,667],[0,666,0,668],[0,667,0,669],[0,668,0,670],[0,669,0,671],[0,670,0,672],[0,671,0,673],[0,672,0,674],[0,673,0,675],[0,674,0,676],[0,675,0,677],[0,676,0,678],[0,677,0,679],[0,678,0,680],[0,679,0,681],[0,680,0,682],[0,681,0,683],[0,682,0,684],[0,683,0,685],[0,684,0,686],[0,685,0,687],[0,686,0,688],[0,687,0,689],[0,688,0,690],[0,689,0,691],[0,690,0,692],[0,691,0,693],[0,692,0,694],[0,693,0,695],[0,694,0,696],[0,695,0,697],[0,696,0,698],[0,697,0,699],[0,698,0,700],[0,699,0,701],[0,700,0,702],[0,701,0,703],[0,702,0,704],[0,703,0,705],[0,704,0,706],[0,705,0,707],[0,706,0,708],[0,707,0,709],[0,708,0,710],[0,709,0,711],[0,710,0,712],[0,711,0,

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1,-1,-1,-1],[-1,-1,-1,-1],[-1,-1,-1,-1],[-1,-1,-1,-1],[-1,-1,-1,-1],[-1,-
1,-1,-1],[-1,-1,-1,-1],[-1,-1,-1,-1],[-1,-1,-1,-1],[-1,-1,-1,-1],[-1,-1,-
1,-1],[-1,-1,-1,-1],[-1,-1,-1,-1],[-1,-1,-1,-1],[-1,-1,-1,-1],[-1,-1,-1,-
1],[-1,-1,-1,-1],[-1,-1,-1,-1],[-1,-1,-1,-1],[-1,-1,-1,-1],[-1,-1,-1,-1],[-
1,-1,-1,-1],[-1,-1,-1,-1],[-1,-1,-1,-1],[-1,-1,-1,-1],[-1,-1,-1,-1],[-1,-
1,-1,-1],[-1,-1,-1,-1],[-1,-1,-1,-1],[-1,-1,-1,-1],[-1,-1,-1,-1],[-1,-1,-
1,-1],[-1,-1,-1,-1],[-1,-1,-1,-1],[-1,-1,-1,-1],[-1,-1,-1,-1],[-1,-1,-1,-
1],[-1,-1,-1,-1],[-1,-1,-1,-1],[-1,-1,-1,-1],[-1,-1,-1,-1],[-1,-1,-1,-1],[-
1,-1,-1,-1],[-1,-1,-1,-1],[-1,-1,-1,-1],[-1,-1,-1,-1],[-1,-1,-1,-1],[-1,-
1,-1,-1],[-1,-1,-1,-1],[-1,-1,-1,-1],[-1,-1,-1,-1],[-1,-1,-1,-1],[-1,-1,-
1,-1],[-1,-1,-1,-1],[-1,-1,-1,-1],[-1,-1,-1,-1],[-1,-1,-1,-1],[-1,-1,-1,-