nanopore within a correlated range of dimensions. A payload molecule may be bound to a target analyte or a reference analyte to facilitate detection of the target analyte or reference analyte in a nanopore device. In some embodiments, the payload molecule may also be charged to act as a driver molecule. In some embodiments, the payload molecule comprises a probe binding moiety capable of specifically binding a probe molecule, which probe binds specifically to the target analyte or the reference analyte.

[0088] The term “nanopore” (or, just "pore") as used herein refers to a single nano-scale opening in a membrane that separates two volumes. The pore can be a protein channel inserted in a lipid bilayer membrane, for example, or can be engineered by drilling or etching or using a voltage-pulse method through a thin solid-state substrate, such as silicon nitride or silicon dioxide or graphene or layers of combinations of these or other materials. Geometrically, the pore has dimensions no smaller than 0.1 nm in diameter and no bigger than 1 micron in diameter; the length of the pore is governed by the membrane thickness, which can be sub-nanometer thickness, or up to 1 micron or more in thickness. For membranes thicker than a few hundred nanometers, the nanopore may be referred to as a "nano channel."

[0089] As used here, the term “nanopore instrument” or “nanopore device” refers to a device that combines one or more nanopores (in parallel or in series) with circuitry for sensing single molecule events. Each nanopore within the nanopore device, including its chambers and electrodes used to facilitate sensing with that nanopore, is referred to herein as a nanopore sensor. Specifically, nanopore instruments use a sensitive voltage-clamp amplifier to apply a specified voltage across the pore or pores while measuring the ionic current through the pore(s). When a single charged molecule such as a double-stranded DNA (dsDNA) is captured and driven through the pore by electrophoresis, the measured current shifts, indicating a capture event (*i.e.*, the translocation of a molecule through the nanopore, or the capture of a molecule in the nanopore), and the shift amount (in current amplitude) and duration of the event are used to characterize the molecule captured in the nanopore. After recording many events during an experiment, distributions of the events are analyzed to characterize the corresponding molecule according to its shift amount (*i.e.*, its current signature). In this way, nanopores provide a simple, label-free, purely electrical single-molecule method for biomolecular sensing.

[0090] As used herein, the term “electrical signal” encompasses a series of data collected on current, impedance / resistance, or voltage over time depending on configuration of the electronic circuitry. Conventionally, current is measured in a “voltage clamp” configuration;

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voltage is measured in a "current clamp" configuration, and resistance measurements can be derived in either configuration using Ohm's law V = IR. Impedance can also be generated by measured from current or voltage data collected from the nanopore device. Types of electrical signals referenced herein include current signatures and current impedance signatures, although various other electrical signals may be used to detect particles in a nanopore.

[0091] As used herein, the term "event" refers to a translocation of a detectable molecule or molecular complex through the nanopore and its associated measurement via an electrical signal, *e.g.*, change in current through the nanopore over time. It can be defined by its current, change in current from baseline open channel, duration, and/or other characteristics of detection of the molecule in the nanopore. A plurality of events with similar characteristics is indicative of a population of molecules or complexes that are identical or have similar characteristics (*e.g.*, bulk, charge).

[0092] As used herein, an "area" of an event refers to the absolute value of the duration of an event (i.e., the duration the current deviates from an open channel current signal) multiplied by the average change in current from the open channel over the duration of the event (i.e., pA*ms).

[0093] As used herein, the term "relative abundance" refers to an amount of an item relative to the total number of related items in a group. For example, in the context of a target analyte in a sample, a relative abundance of the target analyte refers to an amount of a target analyte present in a sample as compared to a reference analyte. This can be represented as a fractional abundance, e.g., the percentage of target analyte in a sample compared to the total population of target analytes and reference analytes. The relative abundance can also be represented as a ratio of, e.g., target analytes:reference analytes. In reference to an electronic signature, a relative abundance of a group of electronic signatures can refer to an amount of a first electronic signature correlated with a target analyte as compared to an amount of a second electronic signature correlated with a reference analyte. To distinguish between the actual relative abundance of a target analyte in a sample (i.e., previously measured or prepared to have a known relative abundance) and a relative abundance determined according to the methods provided herein, we often refer to the actual relative abundance as the "true relative abundance," and the relative abundance determined by the methods described herein as an "estimate of the true relative abundance."

[0094] As used herein, the term "control sample" refers to a sample containing a known relative abundance of target analyte to reference analyte. Control samples, such as reference

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control samples, target control samples, and mixed control samples are used herein to improve the accuracy of the estimate of a fractional abundance in an unknown sample. In some embodiments, control samples comprise target analytes, reference analytes, or both.

[0095] As used herein, the term "unknown sample" or an "unknown mixed sample" or a "mixed unknown sample" refers to a sample containing a relative abundance of reference analyte that is unknown. A relative abundance of a reference analyte is considered to be unknown if the relative abundance is to be determined by the method provided herein, even if some value of an estimate is already known. For some unknown samples, a quantity or concentration of a reference analyte in the sample is known.

[0096] As used herein, the term "known sample" refers to a sample containing a known relative abundance of target analyte to reference analyte, and is used to train, validate or provide an estimate of an accuracy a fractional abundance estimation model or feature of the model, such as a threshold.

Introduction / Overview

[0097] The invention provided herein, in some embodiments, is a method for determining an estimate of the true relative abundance (e.g., a fractional amount or a ratio) of a target analyte relative to a reference analyte present in a sample. This method takes advantage of a nanopore single molecule counter (i.e., a nanopore device) to detect and discriminate between target analytes and reference analytes in a sample.

[0098] The use of raw electronic event signatures correlated with target analytes and reference analytes to determine an estimate for a relative abundance of the target analyte in a sample may be inaccurate for several reasons, including false positive detection errors, false negative detection errors, and errors associated with a capture rate constant differential between target analyte and reference analyte in a mixed sample. Herein, we provide, according to some embodiments, methods to improve the accuracy of estimating the true fractional abundance of reference and target analytes in a sample. In some embodiments, these methods entail the use of control samples specifically designed to correct for one or more errors associated with electronic signal detection in a mixed sample. When the mixed sample comprises a known quantity or concentration of reference analytes, the improved estimate of relative abundance can be used to provide an improved estimate of the true quantity or concentration of target analytes in a sample.

[0099] In some embodiments, the methods provided herein improve the accuracy of an estimate of fractional abundance of a target analyte in a mixed unknown sample by

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accounting for false positive or false negative detection errors, or a capture rate constant differential between said target analyte and said reference analyte. In some embodiments, a series of controls is run to improve the accuracy of the estimate of fractional abundance, including a reference-only control to account for false positive target analyte detection errors, a target-only control to account for false negative target analyte detection errors, and one or more mixed control samples to account for a capture rate constant differential between the target analyte and the reference analyte.

[00100] In some embodiments, the capture rates between the target analyte and the reference analyte in the mixed unknown sample are relatively consistent, such that the mixed control does not need to be used to improve the estimate of the relative abundance. In some embodiments, the relative capture rates between the target analyte and the reference analyte in a mixed sample are known such that a correction term can be applied to data from a mixed unknown sample to compensate for this difference to improve the estimate of fractional abundance without running a mixed control sample. In some embodiments, data from a mixed control sample run under substantially identical nanopore conditions using the same target analyte and reference analyte species as in the mixed unknown sample is used to improve the estimate of the fractional abundance without actually running the mixed control sample as part of the method.

[00101] In some embodiments, a threshold value is determined such that a false positive value from the mixed unknown sample is negligible, and a reference-only control does not need to be used to improve the estimate of the relative abundance. In some embodiments, the false positive value from a mixed sample is known such that a correction term can be applied to data from a mixed unknown sample to compensate for a false positive error to improve the estimate of fractional abundance without running a reference-only control sample. In some embodiments, data from a reference-only control sample run under substantially identical nanopore conditions using the same reference analyte species as in the mixed unknown sample is used to improve the estimate of the fractional abundance without actually running the reference-only control as part of the method.

[00102] In some embodiments, a threshold value is determined such that a false negative value from the mixed unknown sample is negligible, and a target-only control does not need to be used to improve the estimate of the relative abundance. In some embodiments, the false negative value from a mixed sample is known such that a correction term can be applied to data from a mixed unknown sample to compensate for a false negative error to improve the estimate of fractional abundance without running a target-only control sample. In some

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embodiments, data from a target-only control sample run under substantially identical nanopore conditions using the same target analyte species as in the mixed unknown sample is used to improve the estimate of the fractional abundance without actually running the target-only control as part of the method.

Sample Uses

[00103] Determining the fractional amount of a target sequence within a nucleic acid fragment, compared to a reference nucleic acid molecule, has many applications.

[00104] In one example use case, we use the methods herein to determine the fractional amount of a transgenic sequence of a genetically modified organism (GMO) to the non-GMO reference sequence within genomic DNA, obtained from a collection of seeds for example. This determination is important for regulatory and economic reasons. The buyer and sellers of seeds with the desired trait require precise and accurate knowledge of the fraction of seeds comprising the desired trait in order for the pricing and transaction to be fair.

[00105] Therefore, in some embodiments, the methods provided herein provide %GMO content determination from aggregate seed, grain, flour, and feed presumed to contain between 1-100% GMO content. Seed developers, growers, and regulatory agencies want precise measures and the ability to resolve 10% differences (1.1-fold) in GMO content. % GMO defined as 100x (GMO event copy number) / (taxon-specific genome reference copy number).

[00106] As another example use case, we use the methods described herein to monitor the relative abundance of polynucleotide sequences comprising point mutations to non-mutant (wildtype) sequences within cell-free circulating DNA from a blood or urine sample. Relative abundance of point mutations at specific genomic loci have been correlated with cancer types and treatment outcomes. A determination of mutant relative abundance to non-mutant sequences can be used to guide diagnoses, therapies, and disease progression monitoring. Although it can take weeks for tumor imaging results to reveal a shrinking/growing mass, the methods described herein allow rapid identification of the relative abundance of mutation markers permits efficient and frequent testing (e.g., daily) by using easily accessible sample types. Critically, such technology could more effectively reveal therapy response by providing more time points of the disease dynamics, while also permitting early detection of relapse.

[00107] In some embodiments, the methods provided herein provide copy number variation determination (CNV) in hereditary cancer screening assays. Copy number variation (CNV)

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testing for hereditary cancer pre-disposition. Goal is to detecting deletions or duplications of gene regulatory elements at < 1.5-fold difference from reference. 10% differences in the copy number (1.1fold) of the BRCA1 gene for example, may warrant clinical action.

Nanopore Detection

[00108] A nanopore is formed in a solid-state silicon based substrate, and single molecule experiments are performed by applying a voltage across the pore in a buffered electrolytic solution.

[00109] Figure 1A shows a typical single-molecule event caused by a dsDNA passing through a nanopore. Events are quantitated by duration width and maximum conductance depth, max δG. The max δG is the current attenuation δI divided by applied voltage V. **Figure 1B** shows an all-event scatter plot of max δG versus duration for 1072 events of 5.6 kb dsDNA recorded in 5 minutes with a 22 nm diameter nanopore (V = 100 mV, 1 nM DNA, 1M LiCl, 10mM Tris, 1mM EDTA, pH=8.8).

[00110] Besides max δG and duration, other features of the event profile that can be quantitated are: the mean δG, the median δG, the standard deviation of the event signal, and other higher order features. Another useful feature is the absolute value of the integrated area of the event, which can be computed as the mean δG times the duration (Storm, A J, J H Chen, H W Zandbergen, and C Dekker. "Translocation of Double-Strand DNA Through a Silicon Oxide Nanopore." Physical Review E 71, no. 5 (May 2005): 051903, doi:10.1103/PhysRevE.71.051903). The integrated area, or just "area", is also known as the electric charge deficit (Fologea, Daniel, Marc Gershow, Bradley Ledden, David S McNabb, Jene A Golovchenko, and Jiali Li. "Detecting Single Stranded DNA with a Solid State Nanopore." *Nano Letters* 5, no. 10 (October 2005): 1905–9. doi:10.1021/nl051199m).

[00111] For dsDNA that is long enough to pass through the nanopore in a folded state (> 700 bp), the events can display more than one amplitude. Figure 1B is an example of this, with fully folded events displaying larger max δG values and shorter durations, and unfolded events displaying longer durations and shallower max δG values. Partially folded events display both amplitude levels within the event, starting with the deeper level and finishing with the shallower level, and having a total duration width that is in between that of unfolded and fully folded events. While the δG and duration distributions show a mixture of modes for dsDNA that can fold, the event area has a single mode distribution for dsDNA, regardless of whether or not the DNA is long enough to fold when passing through the nanopore.

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[00112] Discrimination between target analytes and reference analytes using a nanopore is based on the detection of a sufficiently different event signature upon translocation of each through the nanopore to enable reliable and sensitive detection. The differences in the average event signatures can be based on signature duration, changes in current, features within the signature, or other distinguishable features and combinations thereof. The features used are the basis for the determination of a threshold which acts as a method of identifying event signatures correlated to reference analytes and target analytes to be used for fractional abundance determination described herein.

[00113] In some embodiments, the target and reference fragments are sufficiently different length dsDNA molecules to produce different nanopore event durations.

[00114] In some embodiments, both target and reference analytes are dsDNA, and the feature that creates the distinct event types could be a difference in length of the target and reference analytes. In such embodiments, the difference in target and reference event areas, which are created by the difference in length of the target and reference analytes, are used to distinguish the target and reference event signatures (i.e., event profiles).

[00115] The event area distribution for dsDNA has a single mode. This makes area a useful event feature for classifying events as being the target type or the reference type, when the target and reference analytes are dsDNA of sufficiently different lengths. To generate sufficiently different area distributions, the lengths should be different by at least 100 bp for nanopores larger than 20 nm in diameter. For smaller nanopores 1-20 nm in diameter, e.g., formed by controlled dielectric breakdown (Yanagi, Itaru, Rena Akahori, Toshiyuki Hatano, and Ken-ichi Takeda. "Fabricating Nanopores with Diameters of Sub-1 Nm to 3 Nm Using Multilevel Pulse-Voltage Injection." Scientific Reports 4 (2014): 5000 doi:10.1038/srep05000), the dsDNA for the target and reference should be at least 20 bp different in length.

[00116] There is no apparent upper limit on how different the dsDNA lengths can be for the target and reference molecules.

[00117] **Figure 2A** shows a typical event when a 727 bp DNA goes through a 25 nm diameter solid-state nanopore at 100 mV in 1M LiCl. The event area is shown as the shaded region. Figure 2B shows how event area increases with dsDNA length. Primarily, it is event duration that is increasing while event depth remains conserved, and event area (mean depth times duration) captures this length-dependent increase since it is proportional to duration. Figure 2C shows the distribution of the log-base-10 of the area (pA*ms) of all events recorded for each DNA length shown, run sequentially on the same nanopore. The

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distribution of log-base-10 of event areas is approximately normal (Gaussian). As the DNA increases in length, the mean of the distribution increases.

[00118] To create target-sequencing comprising dsDNA and reference-sequence comprising dsDNA, with the two dsDNA lengths at least 300 bp in length, at most 100,000 bp in length. In some embodiments, the target and reference dsDNA analytes have a difference in length of at least 10 bp, 20bp, 30 bp, 40 bp, 50 bp, 60 bp, 70bp, 80 bp, 90 bp 100 bp, 150 bp, 200 bp, or 300 bp. In general, an increased difference in length between the target and reference dsDNA analytes facilitates a greater sensitivity and specificity of determination of event signatures correlated with the target and reference analytes, when discriminating by size, which improves the estimation of the relative abundance in the sample.

[00119] In some embodiments, specifying the properties of polynucleotide fragments excised from genomic DNA (gDNA) is a portion of the workflow for fractional abundance determination. These fragment specifications can include, e.g., their sequences, lengths, and secondary structures. In some embodiments, the fragment specifications enhance the capture and detection of specific sequences by the nanopore device.

[00120] In some embodiments, the target and reference fragments are bound to different payload molecules, such that the target/payload and reference payload molecules produce sufficiently different nanopore event signatures. In some embodiments, the different event signatures are a combination of event duration, event maximum depth, event mean depth, and/or other event properties.

[00121] In some embodiments, the target and reference analytes are discriminated by sequence specific payloads that, when each molecule or complex type (target-payload, reference-payload) passes through the pore, a unique nanopore event signature is generated. Methods for using probes bound to payloads that bind to each molecule type to facilitate discrimination are described in International Publication No. WO/2015/171169, "Target Detection with a Nanopore," International Publication No. WO/2014/182634, "A Method of Biological Target Detection Using a Nanopore and a Fusion Protein Binding Agent," International Publication No. WO/2016/049657, "Target Sequence Detection by Nanopore Sensing of Synthetic Probes," International Publication No. WO/2016/126746, "Nanopore Detection of Target Polynucleotides from Sample Background," and International Publication No. WO/2017/173392, "Nanopore Discrimination of Target Polynucleotides from Sample Background by Fragmentation and Payload Binding," each of which are incorporated by reference herein in its entirety.

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[00122] In some embodiments, target and/or reference analytes are dsDNA, with unique payload-bound PNAs invading each dsDNA type (target and reference) to create the two macromolecule types to be detected with the nanopore. In some embodiments, target and/or reference analytes are single-stranded nucleic acid (ssNA), including DNA or RNA. A payload-bound complementary nucleic acid (e.g., LNA) hybridizes to a region on the ssNA and one or more flanking primers hybridize to the other regions of the ssNA, to create a double-stranded molecule with payload bound, and the payloads are unique for the target and the reference in order to create the unique target and reference event profiles.

Fractional Abundance Framework

[00123] In some embodiments, the fractional abundance framework involves: 1) designing and applying biochemistry methods to convert sample material into the nanopore sensing formats, for both target analyte and reference types; 2) applying a specific nanopore experiment protocol; and 3) applying analytical methods to generate a quantitative estimate for the relative abundance of target to reference analytes. This section is focused on part 1 of the framework.

Sample Preparation for Nanopore Detection

[00124] A molecule comprising the target sequence (termed the "target analyte" or "target molecule") and a molecule comprising the reference sequence (termed a "reference analyte" or "reference molecule") may be physically similar: for example target and reference molecules may be of similar molecular weights, or polynucleotide lengths, and may differ by only single nucleotides. The goal of the biochemistry methods is to render target and reference molecules without bias to produce distinct "target" or "reference" event profiles upon translocation through the nanopore. In this way, the target:reference mixture measured on the nanopore is representative of the target:reference concentration ratio in the sample.

[00125] In some uses cases it may be advantageous to add polynucleotide sequence to target, reference, or both molecules to generate distinct event profiles. For example, the majority of DNA fragments obtained from the cell-free circulating DNA fraction of blood or urine are uniformly short 150-200bp in length. Adding polynucleotide sequences by common methods including PCR, ligation, and direct oligonucleotide hybridization allows flexibility to maximize nanopore event distinction. In other cases, hybridization of chemically modified oligonucleotide probes carrying covalently bound polymer payloads are used to alter target or

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reference analyte charge and molecular weight without affecting polynucleotide length. In all cases, the goal is distinct event profiles per target and reference molecule groups.

[00126] There are use cases, including the GMO example (fractional amount of soy seeds containing the GMO target sequence), where there is sufficient starting material that enrichment strategies can be used without needed PCR prior to nanopore sensing. There are other cases, including liquid biopsy, where PCR is required as part of enrichments, since a blood or urine sample could contain < 10 target sequences per mL of fluid. The proposed method is agnostic to the sample prep requirements, including sample collection, purification and concentration of the target and reference. The nanopore measurement and subsequent fractional abundance quantitation can be implemented provided the target and reference are sufficiently enriched (>10 pM) compared to background (< 1 pM), and provided the target and reference analytes produce electrical event signatures that can be distinguished from one another and from background, where present.

[00127] In some embodiments, target or reference analytes include polynucleotide sequences (including double and single stranded DNAs, RNAs, and synthetic polynucleotides) 20nt-100,000nt in length. In some embodiments, the polynucleotide comprising the target sequence is derived from organismal gDNA including from plants, humans, animals, insects, bacteria or viruses. In some embodiments, target polynucleotide sequences are derived from exogenous, non-genomic sequences including double or single-stranded RNA or DNA from sources including plasmid, BAC, linear sequence-verified gene blocks, expression cassettes.

[00128] In some embodiments, we provide an enrichment specific to the fractional abundance (e.g., copy number variation) detection by the nanopore device. In some embodiments, we use site-directed fragmentation methods to prepare the sample for detection by the nanopore. In some embodiments, the methods of detection provided herein include upstream fragmentation of polynucleotides fragmentation of nucleic acid samples, for example, gDNA to sizes 20-100,000nt or base pairs in length In some embodiments, the nucleic acid is fragmented sequence-specifically using restriction enzymes, or by using site-directed nucleases including Cas9/sgRNA, TALENS, zinc finger proteins / nucleases, or another fragmentation method known in the art.

[00129] In some embodiments, target or reference analyte enrichment is performed using positive and negative size selection to retain, discard, and elute target fragment sizes. For example, low ratio of SPRI beads:DNA (0.6) in the presence of PEGs to retain and discard high molecular weight polynucleotide species (for example >8,000bp DNA), followed by

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SPRI beads:DNA (1.5:1) to bind, wash and elute fragment sizes (2000-8000bp for example). In some embodiments, target or reference nucleic acids can undergo nucleic acid amplification to facilitate detection in a nanopore.

Nanopore Detection

[00130] The fractional abundance framework involves: 1) designing and applying biochemistry methods to convert sample material into the nanopore sensing formats, for both target analyte and reference types; 2) applying a specific nanopore experiment protocol; and 3) applying mathematical methods to generate a quantitative estimate for the fractional amount of target to reference (target:reference) analytes. This section is focused on part 2, experiment protocol.

[00131] Described herein are iterations of samples to be run in a nanopore to provide an improved estimate of the true relative abundance of target analytes in a mixed unknown sample. In some embodiments, the target analyte and the reference analyte are prepared to ensure reliable discrimination between each species using a nanopore sensor. In some embodiments, the characteristics of a fragment comprising a target sequence (i.e., the "target fragment") and the characteristics of a fragment comprising the reference sequence (i.e., the "reference fragment") are chosen such that the two fragments produce nanopore event signatures that can be differentiated by one or more signal properties.

[00132] In some embodiments, one or more control mixtures (i.e., control samples) is used to calibrate the estimate of the fractional amount of target to reference in an unknown mixture. In some embodiments, the calibration compensates for difference in nanopore capture efficiency between the target and the reference molecule types.

[00133] In some embodiments, an unknown mixture of target and reference analytes is measured on the nanopore, and the fraction abundance of target to reference is mathematically quantitated. In some embodiments, more than one unknown mixture of target and reference molecule types, derived from the same sample, is measured sequentially on the same nanopore. In some embodiments, more than one unknown mixture of target and reference molecule types, derived from the same sample, is measured in parallel on different nanopores.

[00134] In some embodiments, one or more controls, including 100% target alone, 100% reference alone, and known mixtures of target and reference molecules, are measured on the nanopore, prior to and/or after the unknown mixtures.

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[00135] In some embodiments, the experiment protocol involves sequentially running one or more controls on the nanopore, before or after, or before and after, running the unknown mixture on the nanopore. The controls can be made of 100% target analytes, or 100% reference analytes, and these are termed "isolated controls." The controls can also be any known mixture of target and reference analytes, referred to as "mixture controls" or "control mixtures." The control mixture could be a 1:1 ratio of target:reference analytes, or any other ratio of target:reference analytes from 0.01:1 to 100:1, or any ratio less than 0.01:1 (e.g., 0.001:1) or any ratio greater than 100:1 (e.g., 1000:1) of target:reference analytes. One or more controls can be run more than once. The controls (isolated and mixtures) and unknown mixture can be run in any order sequentially on the same nanopore. In between controls and unknown samples, the fluidic channel (i.e., chamber) from which the nanopore captures molecules is flushed.

[00136] In some embodiments, no controls are run, and only the unknown mixture is run, and compared to a reference table established by running controls in separate prior experiments, i.e., the controls are not run at the point of use.

[00137] In some embodiments, one or more fluidically isolated channels and nanopore sensors are measuring controls in parallel with a one or more fluidically isolated channels and nanopore sensors measuring unknowns. More than one nanopore could have access to each fluidic channel. In parallelized implementations, no flushing may be necessary, since each pore sees only one reagent set, i.e., a control (isolated or mixture) or an unknown (from a set of 1 or more unknowns).

[00138] In some embodiments, the ratio of the reference analyte to the target analyte in the control mixture concentration is near the anticipated ratio of reference analyte to target analyte in the unknown sample, although this may not be known ahead of time.

[00139] Any number of unknown mixtures can be sequentially run on the same nanopore, flushing out the prior unknown before each new unknown is added for measurement. This requires that the unknown mixtures are comprised of the same target and reference analyte types, though their ratios cane be the same or different in the different unknowns.

[00140] Each recording period should be long enough to detect at least 100 events for each reagent type, and performance improves as more events are recorded, where the improvement is significant when more than 500 events are recorded, and very significant when more than 1000 events are recorded. The recording period for each reagent set can be the same or different. An adaptive scheme can stop recording dynamically when the target number of molecules is detected. We have previously established a method for determining

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the number of molecules needed to achieve a desired level of confidence (e.g., 95%, 98%, 99%, 99.9%, etc.) that can be applied to any reagent set (control or unknown) in the presented workflow (SI Section 10.2, Morin, Trevor J, Tyler Shropshire, Xu Liu, Kyle Briggs, Cindy Huynh, Vincent Tabard-Cossa, Hongyun Wang, and William B Dunbar. "Nanopore-Based Target Sequence Detection." Edited by Meni Wanunu. PloS One 11, no. 5 (May 5, 2016): e0154426–21. doi:10.1371/journal.pone.0154426).

[00141] In some embodiments, an experiment protocol with a single nanopore is to run 1) 100% target for recording period T, 2) flush nanopore chamber, 3) 100% reference for recording period T, 4) flush nanopore chamber, 5) 50:50 target:reference mixture for recording period T, 6) flush nanopore chamber, 7) unknown mixture for recording period T. Recording period T can be 15 sec, 30 sec, 45 sec, 1 min, 5 min, 10 min, or any duration between 1-15 sec or between 10-60 min.

[00142] Another common experiment protocol is to run (1)-(7), followed by 8) flush nanopore chamber, 9) repeat 100% target for recording period T, 10) flush nanopore chamber, 11) repeat 100% reference for recording period T, 12) flush nanopore chamber, 13) repeat 50:50 target:reference mixture for recording period T.

[00143] Another common experiment protocol is to run (1)-(7), followed by 8) flush nanopore chamber, 9) repeat 50:50 target:reference mixture for recording period T, 10) flush nanopore chamber, 11) repeat 100% reference for recording period T, 12) flush nanopore chamber, 13) repeat 100% target for recording period T.

[00144] Still another common experiment protocol is to run 1) a target:reference control mixture ratio suspected to be approximately near to the target:reference ratio in the unknown mixture, for recording period T, 2) flush nanopore chamber, 3) unknown mixture for recording period T.

[00145] Still another common experiment protocol is to run 1) a 1:1 target:reference control mixture ratio for recording period T, 2) flush nanopore chamber, 3) unknown mixture for recording period T.

[00146] In some embodiments, an experiment protocol with a single nanopore is to run 1) 100% target for recording period T, 2) flush nanopore chamber, 3) 100% reference for recording period T, 4) flush nanopore chamber, 5) unknown mixture for recording period T.

[00147] In some embodiments, an experiment protocol with a single nanopore is to run 1) 100% target for recording period T, 3) flush nanopore chamber, 4) unknown mixture for recording period T.

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[00148] In some embodiments, an experiment protocol with a single nanopore is to run 1) 100% reference for recording period T, 2) flush nanopore chamber, 3) unknown mixture for recording period T.

[00149] In some embodiments, an experimental protocol with a single nanopore is to run only the unknown mixture for a recording period T, and to use data from a lookup table or previous data which contains error correction information derived from a 100% reference control sample, a 100% target control sample, a known target:reference control mixture, or any combination thereof, each run under substantially similar conditions to the experimental protocol for the unknown mixture, to provide at least one correction term to the data generated from the recording period T to improve an estimate of a fractional abundance of a target analyte in the unknown mixture.

[00150] Upon completion of the experiment protocol, the recorded events from the controls (if run) and the recorded events from the unknown(s) are mathematically analyzed to predict the fraction amount of target to reference in the one or more unknowns.

Fractional Abundance Estimation and Threshold Determination

[00151] The fractional abundance framework involves: 1) designing and applying biochemistry methods to convert sample material into the nanopore sensing formats, for both target analyte and reference types; 2) applying a specific nanopore experiment protocol; and 3) applying mathematical methods to generate a quantitative estimate for the fractional amount of target to reference (target:reference) analytes. This section is focused on part 3 of the framework.

[00152] In some cases, the estimated concentration ratio R=[t]/[r] of target sequence "t" to reference sequence "r" is quantitated. The percentage transgene, or GMO%, is the ratio R converted to a percentage. In some cases, the estimated fractional amount F=[t]/([t]+[r]) of target sequence to total (target plus reference sequence) is quantitated. A simple conversion between the ratio R and the fraction F exists, namely F = R/(R+1) or, equivalently, R = F/(1-F).

[00153] The fractional abundance method predicts the relative amount of target to reference, or target to total (sum of target and reference). In some embodiments, a calibrant molecule can be added to determine absolute concentration of either the target or the reference molecule. In some embodiments, a single nanopore event feature is compared between target and reference analyte types for calculating the fractional abundance. In some embodiments,

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more than one nanopore event feature is compared between target and reference analyte types for calculating the fractional abundance.

[00154] There are three methods we have describe herein to improve a threshold determination to discriminate event signatures correlated with target analytes and reference analytes and to correct for errors from the use of event signatures from a nanopore to determine fractional abundance: 1) The Q-test method, 2) the Support Vector Machine (SVM), and 3) the Expectation Maximization Algorithm for Gaussian Mixtures (EMGM) method.

[00155] The following general concepts apply to the methods. First, the true ratio of target analytes "t" to reference analytes "r" is denoted R=[t]/[r]. The true fraction of target analytes to total (target plus reference) analytes is denoted F=[t]/([t]+[r]). A simple conversion between the ratio R and the fraction F is F = R/(R+1) or, equivalently, R = F/(1-F). The true ratio of an unknown mixture is denoted R_{mix} and the true fraction of a mixture is denoted F_{mix}. The mathematical method generates estimates for F_{mix} and R_{mix}, which are denoted F^*_{mix} and R^*_{mix}. The target and reference molecule constructs are designed and created to give distinct nanopore event signatures.

The Q-test Method

[00156] The mathematical method first designs a criterion for binning all recorded events into one or two categories, namely, target positive (equivalently, reference negative) or target negative (equivalently, reference positive). The event criterion uses one or more event features. In some embodiments, a single feature is used to create a criterion for binning events. Given the criterion, every event is tagged as being either a target event or a reference event. These are termed "target-tagged" or "reference-tagged."

[00157] The fraction of target-tagged events is denoted Q, equal to the number of target-tagged events divided by the total number of events. The fraction of reference-tagged events is 1-Q. The tagged fraction Q is a function of the concentration fraction F above the nanopore, written as Q(F).

[00158] The fraction of target-tagged events in a mixture $Q(F_{mix})$ is denoted as Q_{mix}; the fraction of target-tagged events in a 100% target control $Q(1)$ is denoted as Q_{targ} ; the fraction of target-tagged events in a 100% reference control $Q(0)$ is denoted as Q_{ref} ; the fraction of target-tagged events in a target:reference control mixture is denoted as $Q_{X:Y}$ where X:Y is the ratio of the mixture of target-to-reference in the control mixtures. For fraction $z = X/(X +$

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Y), we have $Q(z) = Q_{X:Y}$. In some embodiments, the 1:1 ratio control mixture is preferred with $z = 0.5$ and the tagged fraction is written $Q_{1:1}$ or $Q_{50:50}$.

[00159] Typically, Q_{targ} is close to 1, with 1-Q_{targ} representing the false negative fraction. Typically, Q_{ref} is close to 0, with Q_{ref} representing the false positive fraction. The controls satisfy $Q_{targ} \geq Q_{X:Y} \geq Q_{ref}$. The mixture satisfies $Q_{targ} \geq Q_{mix} \geq Q_{ref}$.

[00160] In some embodiments, the target-tagged fractions from controls (Q_{targ}, Q_{ref}, $Q_{X:Y}$) are run separately and a lookup table is used to reference the values for any new assay that measures Q_{mix}. In some embodiments, the (Q_{targ}, Q_{ref}, $Q_{X:Y}$) are established at the point of use as part of the assay. In some embodiments, the (Q_{targ}, Q_{ref}) are run separately and a lookup table is used to reference their values, whereas the ($Q_{X:Y}$) value is established at the point of use as part of the assay that measures Q_{mix}.

[00161] In some embodiments, the target-tagged fractions from controls (Q_{targ}, Q_{ref}, $Q_{X:Y}$) are run more than once at the point of use, and their values are averaged for subsequent use in the formula below.

[00162] The formula for estimate F^*_{mix} for the true fractional amount F_{mix} is given by:

$$F^*_{mix} = \frac{\rho\alpha}{\rho\alpha+1} \qquad \text{(Equation 1)}$$

where $\rho = \left(\frac{Q_{mix}-Q_{ref}}{Q_{targ}-Q_{mix}}\right)$, and $\alpha = \left(\frac{Q_{targ}-Q_{X:Y}}{Q_{X:Y}-Q_{ref}}\right) \times \frac{X}{Y}$.

[00163] The formula for estimate R^*_{mix} for the true ratio R_{mix} is given by:

$$R^*_{mix} = \rho\alpha \qquad \text{(Equation 2)}$$

[00164] In examples for predicting the fractional amount of a transgene (GMO), the GMO (%) is equal to $R^*_{mix} \times 100$ (%).

[00165] The parameter ρ is an estimate for the ratio that can compensate for a false positive detection error, a false negative detection error, or both. In some embodiments, a value of Q_{ref} can be used to compensate for a false positive error. If no compensation for a false positive error is to be used, Q_{ref} can be set to 0. In some embodiments, a value of Q_{targ} can be used to compensate for a false negative error. If no compensation for a false negative error is to be used, Q_{targ} can be set to 0.

[00166] The parameter α is the ratio compensation multiplier. Analytically, the parameter α is the ratio of two capture rate constants. A capture rate constant is the nanopore event rate divided by concentration for a given molecule type. Specifically, the parameter α is the reference molecule capture rate constant divided by the target analyte capture rate constant.

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Thus, the multiplier α compensates for difference in nanopore capture and detection between the target and reference molecule types.

[00167] When the control mixture is a 1:1 ratio, $\alpha = \left(\frac{Q_{targ} - Q_{X:Y}}{Q_{X:Y} - Q_{ref}}\right)$.

[00168] When compensation for a capture rate constant differential between a target analyte and a reference analyte is not used, α is set equal to 1 in equations (1) and (2), to provide the estimates for F^*_{mix} and R^*_{mix}, respectively.

[00169] Applying equations (1) and (2) provide estimates for F^*_{mix} and R^*_{mix}, respectively. Uncertainty estimates, or error bars, for F^*_{mix} and R^*_{mix} can also be computed. Each Q for the isolated and mixture controls and for the unknown mixture has a standard error associated with it, std(Q) $= \sqrt{Q(1-Q)/N}$, where N is the total number of events. Numerically, random samples from each Q distribution can be drawn many times, to generate a distribution of values for F^*_{mix} and R^*_{mix}, by applying equations (1) and (2). Then distributions for F^*_{mix} and R^*_{mix} can then be used to compute uncertainty bounds, resulting in $F^*_{mix} \pm F^*_{sd}$ and $R^*_{mix} \pm R^*_{sd}$.

[00170] In some embodiments, the ratio or fraction of events matching or exceeding an event feature criterion is used to estimate the fractional amount of target to reference in an unknown mixture. In some embodiments, the criterion is a threshold.

[00171] Our previous work describes how a single tagging criterion is utilized to compute Q and its error bars (Morin, Trevor J, Tyler Shropshire, Xu Liu, Kyle Briggs, Cindy Huynh, Vincent Tabard-Cossa, Hongyun Wang, and William B Dunbar. "Nanopore-Based Target Sequence Detection." Edited by Meni Wanunu. PloS One 11, no. 5 (May 5, 2016): e0154426–21. doi:10.1371/journal.pone.0154426). As detailed in that work, by applying the criterion, each event j has the variable Z_j assigned to it. If event j is tagged, Z_j =1; otherwise, Z_j =0. For each reagent set (controls and unknowns), $Q = (\sum_j Z_j)/N$, where N is the total number of events. The same criterion is applied to all controls, isolated and mixtures, and all unknowns, to compute all Q values utilized in the formulas above (equations (1)-(2)).

[00172] The criterion involves one or more than one inequality equation, and can be a linear or nonlinear function of one or more event features. Each inequality equation has a threshold or range of thresholds associated with it. Thus, a criterion is fully specified by the set of inequalities and the corresponding set of thresholds.

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[00173] In some embodiments, the criterion is established for a class of target and reference molecule types, and new assays using types of molecules for that class will utilize the criterion already established.

[00174] In some embodiments, the criterion is identified from the control data gathered for any new assay. That is, the criterion is established at run time as part of the fractional abundance protocol.

[00175] In some embodiments, the set of inequalities for the criterion are established *a priori* from sets of previous experiments using comparable target and reference molecule types, while the set of thresholds for the one or more criterion inequalities are established at run time using the control data.

[00176] In some embodiments, a single event feature is utilized in establishing the criterion.

[00177] A threshold, labeled "q," is the scalar value that divides target-tagged events from non-target-tagged (i.e., reference-tagged) events based on an inequality. When more than one inequality is used in a criterion, q can represent the vector of threshold values used for the set of inequalities.

[00178] Consider the example of using two different length dsDNA for the target and the reference. Commonly, a single inequality using the event area is a viable criterion. When the target is dsDNA that is longer than the reference dsDNA, events are tagged if the area exceeds a threshold. When the target is dsDNA that is shorter than the reference dsDNA, events are tagged if the area is less than a threshold.

[00179] Different methods can be utilized to automate selection of the q-threshold value or values, where one q value is identified with each inequality in the criterion.

[00180] In some embodiments, the q-threshold is found as the value that produces a desired false positive for Q_{ref}. For example, the q-threshold could be set at the 95th percentile of Q_{ref} to produce a false positive of 5%. In that case, 95% of the reference molecule events have an area less than q. Alternatively, the SFT q-threshold is found as the value that produces a desired false negative for Q_{targ}, i.e., the q-threshold could set at the 5th percentile of Q_{targ} to produce a false negative of 5%.

[00181] In some embodiments, the SFT q-threshold is found as the solution to $\min_q\{Q_{targ}(q) - Q_{ref}(q)\}$. The threshold would the value that is corresponds to the greatest distance between Q_{targ} end Q_{ref}.

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[00182] In some embodiments, the q-threshold range is computed as the values that produce a desired false positive range for $Q_{ref.}$ For example, the q-threshold ranges could span the 95th to the 99th percentiles of $Q_{ref.}$

[00183] In some embodiments, where a q-threshold range is utilized, equations (1) and (2) produce a ranges of $F^*_{mix}(q)$ and $R^*_{mix}(q)$ values, and the average of these ranges are computed and reported as the predicted F^*_{mix} and R^*_{mix} values.

[00184] Consider the example of using two different payloads bound to the target DNA and the reference DNA. Commonly, three inequalities using the event mean conductance and event duration are a viable criterion. Specifically, for specific payload-target DNA molecule constructs, the target events create a unique subspace on a 2D event plot of mean δG vs. duration, and events are tagged when duration is greater than a threshold, and when mean δG is above one threshold and below another threshold. In this case, the tagging criterion is represented by three linear inequalities and three thresholds, using two event features (mean δG, duration).

The SVM Method

[00185] In some embodiments, machine learning is used to identify the set of features and feature criterion for tagging each event as a target analyte event or a reference analyte event. In some embodiments, support vector machines are used to classify events as target or reference analytes.

[00186] In some embodiments, developing a support vector machine workflow has the follows the steps: 1) load nanopore data, 2) select nanopore event features to differentiate events, 3) model training and testing using controls, 4) data calibration using controls, 5) prediction of unknown target:reference mixtures. In some embodiments, an already developed and reduced support vector machine workflow is implemented for automated fractional abundance predictions.

[00187] In some embodiments, machine learning tools are applied to automate the selection of the criterion, including selection of the event features, the form of the inequalities (linear and/or nonlinear) and the threshold values q used in the inequalities. In some embodiments, Support Vector Machines (SVMs), a supervised machine learning method that solves classification problems, are implemented to generate the tagging criterion. References on SVMs include: Cortes, C. & Vapnik, V. Machine Learning (1995) 20: 273; and Boser, B. E., Guyon, I. M., and Vapnik, V. N. (1992). "A training algorithm for optimal margin

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classifiers," *Proceedings of the fifth annual workshop on Computational learning theory,* each of which is incorporated by reference in its entirety.

[00188] An example of an application of the SVM method to our fractional abundance framework is provided below:

[00189] For data that is linearly separable, let $\{\mathbf{x}_1,\ldots,\mathbf{x}_n\}$ be the data set and let $y_i \in \{1,-1\}$ be the class label of $\mathbf{x}_i$, the decision boundary should classify all points by:

$$y_i(\boldsymbol{w}^T\boldsymbol{x_i} + b) \geq 1, \quad \forall i$$

[00190] To maximize the margin classifying all points, the classification problem becomes the following optimization problem:

$$\text{Minimize } \frac{1}{2}||\boldsymbol{w}||^2 \qquad \text{(Equation 3)}$$

$$\text{subject to } y_i(\boldsymbol{w}^T\boldsymbol{x}_i + b) \geq 1, \quad \forall i$$

[00191] The data points that are close to decision boundary are called support vectors.

[00192] For real-word problems, data are usually not linearly separable because of some outliers or noises. To optimize the classification, the margin was adjusted to allow a few misclassified points. Meanwhile, the misclassified cases were punished with high costs. This margin becomes a soft margin. A soft margin classification can be used by adding "slack" variables into the cost function (**Figure 3A**):

$$Minimize \ \frac{1}{2}||\boldsymbol{w}||^2 + C\sum_{i=1}^{n}\varepsilon_i$$

$$subject\ to: y_i(\boldsymbol{w}^T\boldsymbol{x}_i + b) \geq 1 - \varepsilon_i, \quad \varepsilon_i \geq 0$$

[00193] A second way to deal with linearly non-separable data is the kernel method (Boser, B. E., *et al.,* cited above). It transforms the input feature space to a higher dimension space. By doing so, the data can be linearly separable (**Figure 3B**). Denote the mapping function as $\emptyset(x)$, then the kernel function K can be written as:

$$K(\boldsymbol{x_i}, \boldsymbol{x_j}) = \emptyset(\boldsymbol{x_i})^T\emptyset(\boldsymbol{x_j}) \qquad \text{(Equation 4)}$$

[00194] There are sets of kernel function types available. The most common types are listed here:

[00195] Linear Kernel

$$K(\boldsymbol{x_i}, \boldsymbol{x_j}) = \boldsymbol{x_i}^T\boldsymbol{x_j} + \boldsymbol{c}$$

[00196] Polynomial Kernel

$$K(\boldsymbol{x_i}, \boldsymbol{x_j}) = (\boldsymbol{\alpha x_i}^T\boldsymbol{x_j} + \boldsymbol{c})^{\boldsymbol{d}}$$

[00197] Gaussian (RBF) Kernel

$$K(\boldsymbol{x_i}, \boldsymbol{x_j}) = \boldsymbol{exp}(-\boldsymbol{\gamma}\|\boldsymbol{x_i} - \boldsymbol{x_j}\|)$$

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[00198] Usually, both Kernel tricks and soft margin are used together to produce a better solution for classification problems.

[00199] Applying SVM to nanopore data for fractional abundance has the following steps: 1) load control and unknown data sets, including all events for each set; 2) feature selection; 3) model training and testing; 4) data calibration; and 5) prediction of F_{mix}^{*} and R_{mix}^{*}. In the examples provided, application of these 5 steps is demonstrated in greater detail. Equations (3) and (4), hyper-papameters grid search including Kernel types, soft margin constant, and any parameters that kernel funcition may depend on, are solved as part of applying the method. An assay based generalize model generated form SVM including common decision boundaris and common calibration ratio can be applied to unknown mixtures without requirement of control data sets.

[00200] An assay based generalized model generated from SVM including common decision boundaries and common calibration ratio can be applied to unknown mixtures without requirement of control data sets. Other data mining methods including decision tress, neural networks, Native Bayer, Logistic regress, K-nearest neighbor and boosting are also claimed as applicable methods for nanopore data.

The EMGM Method (Expectation Maximization Algorithm for Gaussian Mixtures)

[00201] In some embodiments, clustering methods are applied to create the criteria for tagging target events and reference events. Each event is tagged as a target event or a reference event. In some embodiments, the fractional abundance is the proportion of the target events relative to the sum of the target and reference events. Running controls that provide compensatory information allows adjustments that improve the estimate of the fractional abundance.

[00202] In some embodiments, the clustering method is a maximum likelihood method applied to parameterized models of the distributions of one or more event parameters. Iterative application of maximum likelihood estimation to control sets results in fitted model parameters, with one set of distributions associated with target analyte type and the other set of distributions associated with the reference analyte type. Subsequently, application of the parameterized models to unknown mixtures results in the assignment of events to either the target of the reference distribution(s), and the ratio of events assigned to the target distribution(s) to the total number of events assigned to target plus reference distribution(s) is used to generate the fractional amount estimate.

[00203] A log likelihood function is used as the metric for tracking progress in iterations of the algorithm, which recursively updates the membership assignment of each event in control

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data and improves the fit of the distributions to the data. In some embodiments, the data are modeled using mixtures of parameterized Gaussian distributions. Methods that use finite mixture models, including Gaussian mixture models, to characterize numerical data are well characterized in statistics and applied mathematics (Hand, David J., Heikki Mannila, and Padhraic Smyth. Principles of data mining. MIT press, 2001).

[00204] In some embodiments, given a Gaussian Mixture (GM) model, the method maximizes the likelihood function with respect to the parameters comprising the means and covariance of the components and the mixing coefficients. Since there is no closed-form solution for the log likelihood, the mode parameters and weights for assigning data to modes are iteratively computed using the Expectation Maximization (EM) technique (C.M. Bishop, Pattern Recognition and Machine Learning, Springer, 2006).

[00205] The method of applying an EM algorithm applied to GM models to nanopore data for the purpose of generating fraction abundance estimates is termed EMGM. Like the Q-test method, the EMGM method uses prior knowledge about one or more nanopore event signatures that can be used to distinguish the target events from the reference events.

[00206] As stated, the target population may be represented by a single distribution, or more than one distribution. Likewise, the reference population may be represented by a single distribution, or more than one distribution. The target and reference distribution(s) are established by applying the algorithm to one or more isolated controls and one or more control mixtures.

[00207] Subsequently, after the target distribution(s) are established, an event in an unknown mixture is tagged as a target event if it is associated with the modeled target distribution(s).

[00208] By example, a total of three Gaussian distributions could fit the entire data set in a 1:1 control mixture, with one mode associated with the target type and two modes associated with the reference type.

[00209] The algorithm requires only one control mixture for application of the EMGM. Subsequently, the resulting model can be applied to unknown mixtures. In some embodiments, an additional isolated reference control is used to offset the effects of false positives. Specifically, application of the EMGM models to 100% reference control established the false positive fraction, which is subtracted from the predicted fraction generated by applying the EMGM models to the unknown mixture. This subtraction can be referred to as false positive compensation (or “FP” compensation).

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Nanopore Devices

[00210] A nanopore device, as provided, includes at least a pore that forms an opening in a structure separating an interior space of the device into two volumes, and at least a sensor configured to identify objects (for example, by detecting changes in parameters indicative of objects) passing through the pore. Nanopore devices used for the methods described herein are also disclosed in PCT Publication WO/2013/012881, incorporated by reference in its entirety.

[00211] The pore(s) in the nanopore device are of a nano scale or micro scale. In one aspect, each pore has a size that allows a small or large molecule or microorganism to pass. In one aspect, each pore is at least about 1 nm in diameter. Alternatively, each pore is at least about 2 nm, 3 nm, 4 nm, 5 nm, 6 nm, 7 nm, 8 nm, 9 nm, 10 nm, 11 nm, 12 nm, 13 nm, 14 nm, 15 nm, 16 nm, 17 nm, 18 nm, 19 nm, 20 nm, 25 nm, 30 nm, 35 nm, 40 nm, 45 nm, 50 nm, 60 nm, 70 nm, 80 nm, 90 nm, or 100 nm in diameter.

[00212] In one aspect, the pore is no more than about 100 nm in diameter. Alternatively, the pore is no more than about 95 nm, 90 nm, 85 nm, 80 nm, 75 nm, 70 nm, 65 nm, 60 nm, 55 nm, 50 nm, 45 nm, 40 nm, 35 nm, 30 nm, 25 nm, 20 nm, 15 nm, or 10 nm in diameter.

[00213] In one aspect, the pore has a diameter that is between about 1 nm and about 100 nm, or alternatively between about 2 nm and about 80 nm, or between about 3 nm and about 70 nm, or between about 4 nm and about 60 nm, or between about 5 nm and about 50 nm, or between about 10 nm and about 40 nm, or between about 15 nm and about 30 nm.

[00214] In some aspects, the nanopore device further includes means to move a polymer scaffold across the pore and/or means to identify objects that pass through the pore. Further details are provided below, described in the context of a two-pore device.

[00215] Compared to a single-pore nanopore device, a two-pore device can be more easily configured to provide good control of speed and direction of the movement of the polymer scaffold across the pores.

[00216] In one embodiment, the nanopore device includes a plurality of chambers, each chamber in communication with an adjacent chamber through at least one pore. Among these pores, two pores, namely a first pore and a second pore, are placed so as to allow at least a portion of a target polynucleotide to move out of the first pore and into the second pore. Further, the device includes a sensor at each pore capable of identifying the target polynucleotide during the movement. In one aspect, the identification entails identifying individual components of the target polynucleotide. In another aspect, the identification

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entails identifying payload molecules bound to the target polynucleotide. When a single sensor is employed, the single sensor may include two electrodes placed at both ends of a pore to measure an ionic current across the pore. In another embodiment, the single sensor comprises a component other than electrodes.

[00217] In one aspect, the device includes three chambers connected through two pores. Devices with more than three chambers can be readily designed to include one or more additional chambers on either side of a three-chamber device, or between any two of the three chambers. Likewise, more than two pores can be included in the device to connect the chambers.

[00218] In one aspect, there can be two or more pores between two adjacent chambers, to allow multiple polymer scaffolds to move from one chamber to the next simultaneously. Such a multi-pore design can enhance throughput of target polynucleotide analysis in the device. For multiplexing, one chamber could have a one type of target polynucleotide, and another chamber could have another target polynucleotide type.

[00219] In some aspects, the device further includes means to move a target polynucleotide from one chamber to another. In one aspect, the movement results in loading the target polynucleotide (e.g., the amplification product or amplicon comprising the target sequence) across both the first pore and the second pore at the same time. In another aspect, the means further enables the movement of the target polynucleotide, through both pores, in the same direction.

[00220] For instance, in a three-chamber two-pore device (a "two-pore" device), each of the chambers can contain an electrode for connecting to a power supply so that a separate voltage can be applied across each of the pores between the chambers.

[00221] In accordance with one embodiment of the present disclosure, provided is a device comprising an upper chamber, a middle chamber and a lower chamber, wherein the upper chamber is in communication with the middle chamber through a first pore, and the middle chamber is in communication with the lower chamber through a second pore. Such a device may have any of the dimensions or other characteristics previously disclosed in U.S. Publ. No. 2013-0233709, entitled Dual- Pore Device, which is herein incorporated by reference in its entirety.

[00222] In one aspect, each pore is at least about 1 nm in diameter. Alternatively, each pore is at least about 2 nm, 3 nm, 4 nm, 5nm, 6 nm, 7 nm, 8 nm, 9 nm, 10 nm, 11 nm, 12 nm, 13 nm, 14 nm, 15 nm, 16 nm, 17 nm, 18 nm, 19 nm, 20 nm, 25 nm, 30 nm, 35 nm, 40 nm, 45 nm, 50 nm, 60 nm, 70 nm, 80 nm, 90 nm, or 100 nm in diameter.

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[00223] In one aspect, each pore is no more than about 100 nm in diameter. Alternatively, the pore is no more than about 95 nm, 90 nm, 85 nm, 80 nm, 75 nm, 70 nm, 65 nm, 60 nm, 55 nm, 50 nm, 45 nm, 40 nm, 35 nm, 30 nm, 25 nm, 20 nm, 15 nm, or 10 nm in diameter.

[00224] In one aspect, the pore has a diameter that is between about 1 nm and about 100 nm, or alternatively between about 2 nm and about 80 nm, or between about 3 nm and about 70 nm, or between about 4 nm and about 60 nm, or between about 5 nm and about 50 nm, or between about 10 nm and about 40 nm, or between about 15 nm and about 30 nm.

[00225] In some aspects, the pore has a substantially round shape. "Substantially round", as used here, refers to a shape that is at least about 80 or 90% in the form of a cylinder. In some embodiments, the pore is square, rectangular, triangular, oval, or hexangular in shape.

[00226] In one aspect, the pore has a depth that is between about 1 nm and about 10,000 nm, or alternatively, between about 2 nm and about 9,000 nm, or between about 3 nm and about 8,000 nm, etc.

[00227] In some aspects, the nanopore extends through a membrane. For example, the pore may be a protein channel inserted in a lipid bilayer membrane or it may be engineered by drilling, etching, or otherwise forming the pore through a solid-state substrate such as silicon dioxide, silicon nitride, grapheme, or layers formed of combinations of these or other materials. Nanopores are sized to permit passage through the pore of the scaffold:fusion:payload, or the product of this molecule following enzyme activity. In other embodiments, temporary blockage of the pore may be desirable for discrimination of molecule types.

[00228] In some aspects, the length or depth of the nanopore is sufficiently large so as to form a channel connecting two otherwise separate volumes. In some such aspects, the depth of each pore is greater than 100 nm, 200 nm, 300 nm, 400 nm, 500 nm, 600 nm, 700 nm, 800 nm, or 900 nm. In some aspects, the depth of each pore is no more than 2000 nm or 1000 nm.

[00229] In one aspect, the pores are spaced apart at a distance that is between about 10 nm and about 1000 nm. In some aspects, the distance between the pores is greater than 1000 nm, 2000 nm, 3000 nm, 4000 nm, 5000 nm, 6000 nm, 7000 nm, 8000 nm, or 9000 nm. In some aspects, the pores are spaced no more than 30000 nm, 20000 nm, or 10000 nm apart. In one aspect, the distance is at least about 10 nm, or alternatively, at least about 20 nm, 30 nm, 40 nm, 50 nm, 60 nm, 70 nm, 80 nm, 90 nm, 100 nm, 150 nm, 200 nm, 250 nm, or 300 nm. In another aspect, the distance is no more than about 1000 nm, 900 nm, 800 nm, 700 nm, 600 nm, 500 nm, 400 nm, 300 nm, 250 nm, 200 nm, 150 nm, or 100 nm.

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[00230] In yet another aspect, the distance between the pores is between about 20 nm and about 800 nm, between about 30 nm and about 700 nm, between about 40 nm and about 500 nm, or between about 50 nm and about 300 nm.

[00231] The two pores can be arranged in any position so long as they allow fluid communication between the chambers and have the prescribed size and distance between them. In one aspect, the pores are placed so that there is no direct blockage between them. Still, in one aspect, the pores are substantially coaxial.

[00232] In one aspect, the device has electrodes in the chambers connected to one or more power supplies. In some aspects, the power supply includes a voltage-clamp or a patch-clamp, which can supply a voltage across each pore and measure the current through each pore independently. In this respect, the power supply and the electrode configuration can set the middle chamber to a common ground for both power supplies. In one aspect, the power supply or supplies are configured to apply a first voltage V_1 between the upper chamber (Chamber A) and the middle chamber (Chamber B), and a second voltage V_2 between the middle chamber and the lower chamber (Chamber C).

[00233] In some aspects, the first voltage V_1 and the second voltage V_2 are independently adjustable. In one aspect, the middle chamber is adjusted to be a ground relative to the two voltages. In one aspect, the middle chamber comprises a medium for providing conductance between each of the pores and the electrode in the middle chamber. In one aspect, the middle chamber includes a medium for providing a resistance between each of the pores and the electrode in the middle chamber. Keeping such a resistance sufficiently small relative to the nanopore resistances is useful for decoupling the two voltages and currents across the pores, which is helpful for the independent adjustment of the voltages.

[00234] Adjustment of the voltages can be used to control the movement of charged particles in the chambers. For instance, when both voltages are set in the same polarity, a properly charged particle can be moved from the upper chamber to the middle chamber and to the lower chamber, or the other way around, sequentially. In some aspects, when the two voltages are set to opposite polarity, a charged particle can be moved from either the upper or the lower chamber to the middle chamber and kept there.

[00235] The adjustment of the voltages in the device can be particularly useful for controlling the movement of a large molecule, such as a charged polymer scaffold, that is long enough to cross both pores at the same time. In such an aspect, the direction and the speed of the movement of the molecule can be controlled by the relative magnitude and polarity of the voltages as described below.

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[00236] The device can contain materials suitable for holding liquid samples, in particular, biological samples, and/or materials suitable for nanofabrication. In one aspect, such materials include dielectric materials such as, but not limited to, silicon, silicon nitride, silicon dioxide, graphene, carbon nanotubes, TiO_2, HfO_2, Al_2O_3, or other metallic layers, or any combination of these materials. In some aspects, for example, a single sheet of graphene membrane of about 0.3 nm thick can be used as the pore- bearing membrane.

[00237] Devices that are microfluidic and that house two-pore microfluidic chip implementations can be made by a variety of means and methods. For a microfluidic chip comprised of two parallel membranes, both membranes can be simultaneously drilled by a single beam to form two concentric pores, though using different beams on each side of the membranes is also possible in concert with any suitable alignment technique. In general terms, the housing ensures sealed separation of Chambers A-C.

[00238] In one aspect, the device includes a microfluidic chip (labeled as "Dual-pore chip") is comprised of two parallel membranes connected by spacers. Each membrane contains a pore drilled by a single beam through the center of the membrane. Further, the device preferably has a Teflon® housing or polycarbonate housing for the chip. The housing ensures sealed separation of Chambers A-C and provides minimal access resistance for the electrode to ensure that each voltage is applied principally across each pore.

[00239] More specifically, the pore-bearing membranes can be made with transmission electron microscopy (TEM) grids with a 5-100 nm thick silicon, silicon nitride, or silicon dioxide windows. Spacers can be used to separate the membranes, using an insulator, such as SU-8, photoresist, PECVD oxide, ALD oxide, ALD alumina, or an evaporated metal material, such as Ag, Au, or Pt, and occupying a small volume within the otherwise aqueous portion of Chamber B between the membranes. A holder is seated in an aqueous bath that is comprised of the largest volumetric fraction of Chamber B. Chambers A and C are accessible by larger diameter channels (for low access resistance) that lead to the membrane seals.

[00240] A focused electron or ion beam can be used to drill pores through the membranes, naturally aligning them. The pores can also be sculpted (shrunk) to smaller sizes by applying a correct beam focusing to each layer. Any single nanopore drilling method can also be used to drill the pair of pores in the two membranes, with consideration to the drill depth possible for a given method and the thickness of the membranes. Predrilling a micro-pore to a prescribed depth and then a nanopore through the remainder of the membranes is also possible to further refine the membrane thickness.

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[00241] By virtue of the voltages present at the pores of the device, charged molecules can be moved through the pores between chambers. Speed and direction of the movement can be controlled by the magnitude and polarity of the voltages. Further, because each of the two voltages can be independently adjusted, the direction and speed of the movement of a charged molecule can be finely controlled in each chamber.

[00242] One example concerns a target polynucleotide, having a length that is longer than the combined distance that includes the depth of both pores plus the distance between the two pores. For example, a 1000 by dsDNA is about 340 nm in length, and would be substantially longer than the 40 nm spanned by two 10 nm-deep pores separated by 20 nm. In a first step, the polynucleotide is loaded into either the upper or the lower chamber. By virtue of its negative charge under a physiological condition at a pH of about 7.4, the polynucleotide can be moved across a pore on which a voltage is applied. Therefore, in a second step, two voltages, in the same polarity and at the same or similar magnitudes, are applied to the pores to move the polynucleotide across both pores sequentially.

[00243] At about the time when the polynucleotide reaches the second pore, one or both of the voltages can be changed. Since the distance between the two pores is selected to be shorter than the length of the polynucleotide, when the polynucleotide reaches the second pore, it is also in the first pore. A prompt change of polarity of the voltage at the first pore, therefore, will generate a force that pulls the polynucleotide away from the second pore.

[00244] Assuming that the two pores have identical voltage-force influence and $|V_1| = |V_2| + \delta V$, the value $\delta V > 0$ (or < 0) can be adjusted for tunable motion in the $|V_1|$ (or V_2) direction. In practice, although the voltage-induced force at each pore will not be identical with $V_1 = V_2$, calibration experiments can identify the appropriate bias voltage that will result in equal pulling forces for a given two-pore chip; and variations around that bias voltage can then be used for directional control.

[00245] If, at this point, the magnitude of the voltage-induced force at the first pore is less than that of the voltage-induced force at the second pore, then the polynucleotide will continue crossing both pores towards the second pore, but at a lower speed. In this respect, it is readily appreciated that the speed and direction of the movement of the polynucleotide can be controlled by the polarities and magnitudes of both voltages. As will be further described below, such a fine control of movement has broad applications. For quantitating target polynucleotides, the utility of two-pore device implementations is that during controlled delivery and sensing, the target polynucleotide or payload-bound target polynucleotide can be repeatedly measured, to add confidence to the detection result.

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[00246] Accordingly, in one aspect, provided is a method for controlling the movement of a charged polymer scaffold through a nanopore device. The method comprises (a) loading a sample comprising a target polynucleotide (e.g., a target polynucleotide amplicon) in one of the upper chamber, middle chamber or lower chamber of the device of any of the above embodiments, wherein the device is connected to one or more power supplies for providing a first voltage between the upper chamber and the middle chamber, and a second voltage between the middle chamber and the lower chamber; (b) setting an initial first voltage and an initial second voltage so that the target polynucleotide moves between the chambers, thereby locating the polymer scaffold across both the first and second pores; and (c) adjusting the first voltage and the second voltage so that both voltages generate force to pull the charged target polynucleotide away from the middle chamber (voltage-competition mode), wherein the two voltages are different in magnitude, under controlled conditions, so that the target polynucleotide scaffold moves across both pores in either direction and in a controlled manner.

[00247] In one aspect, the sample containing the target polynucleotide is loaded into the upper chamber and the initial first voltage is set to pull the target polynucleotide from the upper chamber to the middle chamber and the initial second voltage is set to pull the target polynucleotide from the middle chamber to the lower chamber. Likewise, the sample can be initially loaded into the lower chamber, and the target polynucleotide can be pulled to the middle and the upper chambers.

[00248] In another aspect, the sample containing the target polynucleotide is loaded into the middle chamber; the initial first voltage is set to pull the charged polymer scaffold from the middle chamber to the upper chamber; and the initial second voltage is set to pull the target polynucleotide from the middle chamber to the lower chamber.

[00249] In one aspect, real-time or on-line adjustments to the first voltage and the second voltage at step (c) are performed by active control or feedback control using dedicated hardware and software, at clock rates up to hundreds of megahertz. Automated control of the first or second or both voltages is based on feedback of the first or second or both ionic current measurements.

Sensors

[00250] As discussed above, in various aspects, the nanopore device further includes one or more sensors to carry out the detection of the target polynucleotide.

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[00251] The sensors used in the device can be any sensor suitable for identifying a target polynucleotide amplicon bound or unbound to a payload molecule. For instance, a sensor can be configured to identify the target polynucleotide by measuring a current, a voltage, a pH value, an optical feature, or residence time associated with the polymer. In other aspects, the sensor may be configured to identify one or more individual components of the target polynucleotide or one or more components bound or attached to the target polynucleotide. The sensor may be formed of any component configured to detect a change in a measurable parameter where the change is indicative of the target polynucleotide, a component of the target polynucleotide, or preferably, a component bound or attached to the target polynucleotide. In one aspect, the sensor includes a pair of electrodes placed at two sides of a pore to measure an ionic current across the pore when a molecule or other entity, in particular a target polynucleotide, moves through the pore. In certain aspects, the ionic current across the pore changes measurably when a target polynucleotide segment passing through the pore is bound to a payload molecule. Such changes in current may vary in predictable, measurable ways corresponding with, for example, the presence, absence, and/or size of the target polynucleotide molecule present.

[00252] In a preferred embodiment, the sensor comprises electrodes that apply voltage and are used to measure current across the nanopore. Translocations of molecules through the nanopore provides electrical impedance (Z) which affects current through the nanopore according to Ohm's Law, V= IZ, where V is voltage applied, I is current through the nanopore, and Z is impedance. Inversely, the conductance G = 1/Z are monitored to signal and quantitate nanopore events. The result when a molecule translocates through a nanopore in an electrical field (e.g., under an applied voltage) is a current signature that may be correlated to the molecule passing through the nanopore upon further analysis of the current signal.

[00253] When residence time measurements from the current signature are used, the size of the component can be correlated to the specific component based on the length of time it takes to pass through the sensing device.

[00254] In one embodiment, a sensor is provided in the nanopore device that measures an optical feature of the polymer, a component (or unit) of the polymer, or a component bound or attached to the polymer. One example of such measurement includes the identification of an absorption band unique to a particular unit by infrared (or ultraviolet) spectroscopy.

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[00255] In some embodiments, the sensor is an electric sensor. In some embodiments, the sensor detects a fluorescent signature. A radiation source at the outlet of the pore can be used to detect that signature.

Equivalents and Scope

[00256] Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments in accordance with the invention described herein. The scope of the present invention is not intended to be limited to the above Description, but rather is as set forth in the appended claims.

[00257] In the claims, articles such as "a," "an," and "the" may mean one or more than one unless indicated to the contrary or otherwise evident from the context. Claims or descriptions that include "or" between one or more members of a group are considered satisfied if one, more than one, or all of the group members are present in, employed in, or otherwise relevant to a given product or process unless indicated to the contrary or otherwise evident from the context. The invention includes embodiments in which exactly one member of the group is present in, employed in, or otherwise relevant to a given product or process. The invention includes embodiments in which more than one, or all of the group members are present in, employed in, or otherwise relevant to a given product or process.

[00258] It is also noted that the term "comprising" is intended to be open and permits but does not require the inclusion of additional elements or steps. When the term "comprising" is used herein, the term "consisting of" is thus also encompassed and disclosed.

[00259] Where ranges are given, endpoints are included. Furthermore, it is to be understood that unless otherwise indicated or otherwise evident from the context and understanding of one of ordinary skill in the art, values that are expressed as ranges can assume any specific value or subrange within the stated ranges in different embodiments of the invention, to the tenth of the unit of the lower limit of the range, unless the context clearly dictates otherwise.

[00260] All cited sources, for example, references, publications, databases, database entries, and art cited herein, are incorporated into this application by reference, even if not expressly stated in the citation. In case of conflicting statements of a cited source and the instant application, the statement in the instant application shall control.

[00261] Section and table headings are not intended to be limiting.

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EXAMPLES

[00262] Below are examples of specific embodiments for carrying out the present invention. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way. Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperatures, etc.), but some experimental error and deviation should, of course, be allowed for.

[00263] The practice of the present invention will employ, unless otherwise indicated, conventional methods of protein chemistry, biochemistry, recombinant DNA techniques and pharmacology, within the skill of the art. Such techniques are explained fully in the literature. See, e.g., T.E. Creighton, Proteins: Structures and Molecular Properties (W.H. Freeman and Company, 1993); A.L. Lehninger, Biochemistry (Worth Publishers, Inc., current addition); Sambrook, et al., Molecular Cloning: A Laboratory Manual (2nd Edition, 1989); Methods In Enzymology (S. Colowick and N. Kaplan eds., Academic Press, Inc.); Remington's Pharmaceutical Sciences, 18th Edition (Easton, Pennsylvania: Mack Publishing Company, 1990); Carey and Sundberg Advanced Organic Chemistry 3rd Ed. (Plenum Press) Vols A and B(1992).

Example 1 – Q-test based FA Using Different Length dsDNA for Target and Reference

[00264] This example presents results from the application of the fractional abundance (FA) framework to data in which a transgenic (GMO) target sequence is within a 788 bp target dsDNA (i.e,. a target analyte) and the reference sequence (lectin housekeeping gene) is within a 466 bp reference dsDNA (i.e., a reference analyte). Quantitation of the fractional amount of transgene target in a sample is achieved below, first by applying the *Q*-test method with a single feature criterion based on event area and using equations (1) and (2), and second by applying the SVM method and using equations (3) and (4).

[00265] The 466 bp reference DNA and 788 bp target transgenic DNA fragments were generated by PCR from mixtures of conventional and transgene-containing genomic DNA samples using sequence specific oligonucleotide primers. PCR products were purified and concentrated using standard silica membrane columns. Precise fractional mixtures of the two amplicons were prepared from large volumes of the individually generated amplicons, and aliquots of the fractional mixtures and single amplicons were used as standard reference materials for all assays.

[00266] First, the reference control sample containing the 466 bp reference DNA was measured in a nanopore device. Next, the target control sample containing the 788 bp

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transgenic DNA was prepared and measured in the nanopore device. The length differences between the target analyte (788 bp) and the reference analyte (466 bp) generate a unique event signature upon translocation through the nanopore that can be discriminated based on area of the event signature.

[00267] Figure 4A shows all event area histograms for two isolated control runs, one for the 466 bp reference DNA and one for the 788 bp target transgenic DNA. Also shown is an area histogram from a 3:10 target:reference control mixture. Figure 4B shows the control mixtures (Q_{targ}, Q_{ref}) and the known mixture (Q_{mix}) trends as a function of the area criterion threshold q, where $Q_{mix} = Q_{3:10}$. Figure 4C shows and how the fractional amount parameter $\rho(q)$ appears graphically at a q value. The q = 5 pA*ms threshold (vertical dashed line) corresponds to a false positive of 0.05 (i.e., Q_{ref} = 0.05) and a false negative of 0.1 (i.e., Q_{targ} = 0.9).

[00268] Application of equation (2) to generate R^*_{mix} as the predicted GMO (%) is done here using the control mixtures, to test the accuracy and precision of the method of using reference only and target only controls to generate an estimate of fractional abundance for the known mixture. Equation (2) was first applied to known mixtures. Since no control mixture sample was used to generate, $Q_{X:Y}$), the estimate was generated without using compensation for a capture rate constant differential between a target analyte and a reference analyte (i.e., setting $\alpha = 1$) to validate the model. Figure 5A shows a plot of the predicted GMO (%) (R^*_{mix}) vs. true GMO (%), and for comparison 10% error margins above and below the zero-error line (slope = 1). These results were established by running 100% target and 100% reference (isolated) controls followed by five known mixtures, serially on a single nanopore. Table 1 reports the predicted values and error bars plotted in Figure 5A, and the total number of events detected for each mixture.

Table 1. GMO prediction results for Figure 5A data

True GMO %	Predicted GMO %	Percent Error (Pred. - True)	Total events
10 %	12.0 ± 1.07 %	2.0 ± 1.0 %	5,225
15%	16.3 ± 1.1 %	1.3 ± 1.1 %	4,267
20%	20.8 ± 1.1 %	0.75 ±1.1%	6,605
25%	29.3 ± 1.2 %	4.3 ± 1.2 %	6,647
30%	34.5 ± 1.4 %	4.4 ± 1.4 %	5,605

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[00269] A separate nanopore experiment following a similar protocol (two isolated controls, and six known mixtures) and produced the results shown in Figure 5B and Table 2.

Table 2. GMO prediction results for Figure 5B data

True GMO %	Predicted GMO %	Percent Error (Pred. – True)	Total events
5%	2.87 ± 0.7 %	-2.1 ± 0.7 %	4,783
10 %	11.1 ± 0.93 %	1.1 ± 0.9 %	4,884
15%	16 ± 1.1 %	1.0 ± 1.1%	4,326
20%	20.4 ± 1.1 %	0.35 ± 1.1 %	5,895
25%	27.1 ± 1.2 %	2.1 ± 1.2 %	6,587
33.33%	36.2 ± 1.3%	2.8 ± 1.3 %	7,862

[00270] The results from Figure 5A and Figure 5B and Tables 1 and 2 suggest that GMO% prediction accuracy within 5% is possible for discriminating two DNA lengths using a single nanopore. These results were achieved without using compensation for a capture rate constant differential between a target analyte and a reference analyte (setting $\alpha = 1$ in equation (2)). Compensation for a capture rate constant differential is expected to further improve results.

[00271] An example of when a q-threshold range is utilized, instead of a single value, is shown in Figure 6. Specifically, the q-threshold range was chosen to span the 75th to the 99th percentiles of Q_{ref}. Plotted the resulting $R^*_{mix}(q)$ trend over the q range, and the average value $mean\{R^*_{mix}(q)\} = 12.7\%$ compared to the known 15% GMO. This shows that the analysis framework provided herein can compensate for false positive and false negative errors over a range of thresholds, even when the threshold is not optimized, to provide an improved estimate of relative abundance of a target analyte in a sample.

[00272] The workflow demonstrated in this example for quantitating the abundance of a target sequence in a population did not require any amplification, purification, concentration or buffer exchange steps. This workflow is compatible with inexpensive, disposable sample prep cartridges, to allow a sample-in answer-out workflow in a miniaturized (handheld or desk top) unit.

[00273] In another set of experiments, varying GMO% samples were tested as unknowns. The protocol followed on each nanopore was: a) 100% 466 bp reference for 5 minutes, then flush; b) 100% 788 bp target for 5 minutes, then flush; c) run between 1 and 4 unknowns,

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each for 5 minutes, flushing in-between; d) run a control mixture. Area criterion was used and a q-threshold range spanning the 75th to the 99th percentiles of Q_{ref} was implemented, reporting the average $R^*_{mix}(q)$ as the predicted GMO%. In equation (2), the control mixture was used for compensation for a capture rate constant differential between a target analyte and a reference analyte. Experiments used a target:reference control mixture of 1:1, 0.75:1 or 0.35:1.

[00274] Table 3 reports prediction results from one nanopore assay for four "unknown" mixed samples (S1-S4) using a control mixture of 0.35:1 (35% GMO) for compensation. Unknown were blinded in each nanopore assay, so the percent error is not reported in the table. The table also reports the total number of events recorded in each 5 minute period.

Table 3. GMO prediction results for blinded samples S1-S4

True GMO %	Predicted GMO %	Total events
***35 %**	**35.1 ± 3.0%**	**1,175**
Sample S1	32.0 ± 2.2 %	2,039
Sample S2	10.2 ± 1.4 %	1,767
Sample S3	20.9 ± 2.4 %	1,031
Sample S4	6.2 ± 1.4 %	1,125

[00275] At total of 12 nanopore experiments were performed following the protocol stated above, and each mixed sample was tested 2-5 times, always on a different nanopore, and by a different experimenter or on a different date. Nanopore size range was 25-35 nm in diameter. A total of 11 mixed samples (S1-S11) were assayed. Table 4 reports the combined estimates, ordered from smallest to largest predicted GMO% value. The reported mean GMO% values are computed by averaging the single-nanopore predictions. The uncertainty of each mean estimate is computed from repeated random sampling of the individual estimates distributions (a Monte Carlo method). Reported is the numerically generated 95th-percentile confidence interval. The number of times each sample was tested and the true GMO% for each sample are also reported.

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Table 4. Combined GMO% prediction (mean ± 2 sigma) for samples S1-S11

Sample No.	No. Tests	Mean GMO %	True GMO %	Percent Error (Pred. – True)
S4	2	7.3 ± 2.9%	5%	+2.3%
S2	2	10.2 ± 2.1%	10%	+0.2%
S9	2	14.9 ± 2.4%	15%	-0.1%
S3	2	21.2 ± 3.1%	20%	+1.2%
S7	4	24.9 ± 2.3%	25%	-0.1%
S1	2	33.8 ± 4.1%	35%	-1.2%
S8	4	41.3 ± 2.9%	42.5%	-1.2%
S5	4	67.0 ± 4.2%	67.5%	-0.5%
S11	3	76.1 ± 6.8%	75%	+1.1%
S10	5	88.9 ± 3.8%	90%	-1.1%
S6	2	104 ± 7.1%	100%	+4%

[00276] The results from Table 4 show that our method can predict a fractional abundance of a target analyte (e.g., GMO%) with high accuracy. Within the range 10-90% GMO, accuracy is within 2% by combining single-nanopore estimates. Between 5-10% and at 100% GMO, where prediction errors could be expected to increase by approaching saturation limits, combining two nanopore estimates resulted in <5% error. In general, the use of compensation for a capture rate constant differential between a target analyte and a reference analyte improves accuracy compared to no compensation for the capture rate constant differential (Tables 1-2). For the entire GMO% prediction range, more nanopore estimates will greater improve accuracy and precision. Arrayed nanopores, each measuring from a common pool, can also reduce uncertainty further, by eliminating the person-to-person and day-to-day, and reagent set-to-set variations that were present as part of this study.

Example 2 – SVM-based FA Using Different Length dsDNA for Target and Reference

[00277] The same nanopore data recorded and analyzed in Example 1 was re-analyzed here using the SVM method presented previously (equations (3)-(4)).

[00278] The isolated control sets were first used for initial feature selection. The initial selection aims to remove highly correlated features, which can cause multicollinearity problems for certain classification methods. The seven identified features were: (i) $\log_{10}$(dwell), or just "dwell", the base-10 logarithm of event duration; (ii) maxAmp: the max

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δG; (iii) sdAmpSub: standard deviation of the event signal, removing rise and fall time; (iv) medAmp: the median δG; (v) LFNmean: mean of the noise power of the event below 50 Hz; (vi) LFNmedian: median of the noise power of the event below 50 Hz; (vii) Area: the same event area used in Example 1.

[00279] Further feature extraction was performed to reduce data dimensions. The purpose of this step is to balance computation time and classification accuracy. Two algorithms have been implemented: 1) Univariate feature selection method. The ANOVA F-value was computed between each feature and label of the event. A threshold was set manually to select a portion of features that have highest F scores. 2) Recursive feature elimination (RFE). The estimator (such as SVM) is trained on the initial set of features and the importance of each feature is obtained. Least important features will be excluded from current set of features. This procedure is recursively repeated until desired number of feature set is reached.

[00280] For the example 1 data, univariate feature selection method was employed. The threshold of percentage of features was manually set to 60%. The four optimal features chosen by algorithm were: (i) dwell, (ii) sdAmpSub, (iii), medAmp, (iv) Area.

[00281] The next step in the method is model training and testing. All events collectively in the isolated controls were randomly sorted into a training dataset and a testing dataset using a 7:3 split. An SVM was trained based on the training dataset with hyper-parameters search algorithm to find the optimal parameters to perform classification. The hyper-parameters tested in grid algorithm are: the kernel type (linear, rbf), regularization parameter (C) and kernel coefficient (gamma). Area Under the Curve of ROC curve (roc_auc) was used to evaluate the performance of each hyper-parameters combination. The model having highest roc_auc scores was used for the down-stream data processing. For the best parameter combination, the average precision and recall of each class from the testing data were calculated. The model with optimal parameters was then trained by training dataset and tested on testing dataset. Prediction of accuracy on testing data set was generated and is shown in Figure 7. The accuracy across the entire set remained above 97.5%.

[00282] The next step in the method was data calibration. Calibration can be achieved by applying the model in step 3 to the control mixture data, which generates a correction ratio. The correction ratio is then multiplied by each predicted amount for a unknown mixture. This is equivalent to multiplying by the parameter α in equations (1) and (2). The value for the parameter α is generated by the applying the model to the control mixture in the SVM

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method, whereas (1) and (2) involve direct calculation of α from the control data sets Q values.

[00283] Table 5 shows a comparison of GMO% predictions between the Q-test method and the SVM-based method.

Table 5. Comparing single nanopore GMO% predictions, Q-test vs. SVM

Sample #	True GMO %	Q-test GMO%	SVM GMO%
1.	35%	28.5%	34.5%
2.	75%	80.6%	89.9%
3.	20%	20.9%	23.4%
4.	100%	101.1%	102%
5.	10%	7.2%	11.6%
6.	50%	55.3%	51.79%
7.	75%	78.8%	70.6%
8.	35%	32.3%	34.88%
9.	75%	82.0%	81.37%
10.	10%	10.13%	14%
11.	15%	17.17%	19.7%
12.	20%	20.9%	22.1%
13.	87.5%	77.8%	77.2%
14.	42.5%	42.2%	43.8%
15.	75%	73.6%	72%
16.	35%	40.9%	39.5%
17.	25%	28.7%	18.3%
18.	62.5%	70%	69.3%
19.	42.5%	40.1%	43.81%
20.	87.5%	92%	89.5%
21.	62.5%	63.7%	62.7%
22.	42.5%	40.2%	40.37%

[00284] Samples were divided into: a) SVM prediction was more accurate (1,5,6,8,9,16,19,20,21), b) Q-test prediction was more accurate (3,4,7,10,11,12,14,15,17), and c) the methods were equivalent in accuracy (2,3,18,22). For these 22 samples, the

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performance of the two methods overall was roughly equivalent, each outperforming the other in 9/22 cases.

[00285] The value of the SVM method is that it can be automated to apply to dataset which, a priori, may not have a definite criterion that can be applied, a requirement for the Q-test method. On the other hand, the Q-test method is computationally simpler, and is likely preferred for fractional abundance applications that can utilize well-characterized criterion in the Q-test format.

Example 3 – Q-test based FA using short DNA (74 bp reference, 94 bp target transgene) with unique payloads

[00286] In the context of GMO% prediction applications, this example shows that two comparable lengths can be used for the target and reference dsDNA, where discrimination in nanopore event signature is achieved by using two distinct sequence-specific payloads.

[00287] Methods: Using validated qPCR primer sets (publicly available from the European Union Reference Laboratory for GM Food and Feed) we amplified both 94bp transgene-specific and 74bp taxon-specific fragments from mixtures of conventional and transgene-containing genomic DNA samples. Prior to nanopore detection, these amplicons were hybridized (method described in Data Storage patent #5520281-v2-29517, 5/16/2016) with sequence-specific oligonucleotide probes covalently linked to PEG polymer probes (see International Publication No. WO/2016/187159, "Methods and Compositions for Target Detection in a Nanopore Using a Labelled Polymer Scaffold," incorporated herein by reference in its entirety. Specifically, the transgene-targeting probe was linked to a 4-arm 40kDa PEG and the reference-targeting probe was linked to an 8-arm 40kDa PEG.

[00288] As a representative example of an all event scatter plot, Figure 8 shows an event plot for two molecule types that were run as isolated controls sequentially on the same pore. First, a sample containing a 96 bp DNA/probe-payload complex was prepared and measured in a nanopore device. The complex is a model for a fragment comprising the target sequence and bound with a probe-payload. The probe-payload was a PNA-PEG with a 4-arm PEG structure. Next, the fragment comprising the reference sequence was designed to generate a unique event signature upon translocation through the nanopore with which fractional abundance calculations could be achieved. The reference molecule is a 74bp DNA with PNA-PEG bound, where the PEG has an 8-arm structure. The key is that the reference/probe-payload molecule generates a unique event subpopulation that is distinct from the

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target/probe-payload molecule, and both are distinct from any background events when present.

[00289] The protocol followed on each nanopore was: a) 100% 74 bp/payload-2 reference for 5 minutes, then flush; b) 100% p4 bp/payload-1 target for 5 minutes, then flush; c) run between 1 and 4 unknowns, each for 5 minutes, flushing in-between; d) run a control mixture. Area criterion was used and a q-threshold range spanning the 75th to the 99th percentiles of Q_{ref} was implemented, reporting the average $R^{*}_{mix}(q)$ as the predicted GMO%. In equation (2), the 1:1 control mixture was used for compensation for a capture rate constant differential between a target analyte and a reference analyte.

[00290] A set of nanopore experiments were performed following the protocol stated above, and each mixed sample was tested 2-4 times, always on a different nanopore, and by a different experimenter or on a different date. Nanopore size range was 25-35 nm in diameter. A total of 6 mixed samples (Sp1-Sp6) were assayed. Table 6 reports the combined estimates, ordered from smallest to largest predicted GMO% value. The reported mean GMO% values are computed by averaging the single-nanopore predictions. The uncertainty of each mean estimate is computed and reported as 95th-percentile confidence interval. The number of times each sample was tested and the true GMO% for each sample are also reported.

Table 6. Combined GMO% predictions using distinct payloads to discriminate target/reference

Sample No.	No. Tests	Mean GMO %	True GMO %	Percent Error (Pred. – True)
Sp1	2	14.8 ± 1.8%	10%	+4.8%
Sp2	2	16.8 ± 1.9%	15%	+1.8%
Sp3	4	20.5 ± 1.5%	20%	+0.5%
Sp4	2	22.3 ± 2.0%	25%	-2.7%
Sp5	4	28.3 ± 1.7%	30%	-1.7%
Sp6	2	45.8 ± 3.5%	40%	+5.8%

[00291] Prediction performance with the two payloads appears to be not quite as good as when using dsDNA length discrimination (Examples 1, 2). In any case, accuracy is better than 6% in all cases, and can be further improved by having more nanopores measuring the pool of molecules in parallel, and combining the resulting estimates.

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Example 4 – Q-test and SVM methods for FA of KRAS G12D SNP compared to wild-type using short DNA (89 bp) and two unique payloads

[00292] We designed primers to amplify short (58bp, 70bp, or 89bp) fragments of the human KRAS gene from highly fragmented, cell-free, circulating DNAs. (cfDNA primer sequences were designed to anneal on either side of KRAS G12D SNP sequence (CosmicID 521). Amplicons were generated from the cell-free circulating DNA fraction obtained from blood plasma and subject to hybridization with oligonucleotide probes targeting both wildtype and mutant KRAS alleles and covalently linked to PEG polymer payloads: probes that target the KRAS wt alleles (c.35G) were linked to either 40kDa 8-arm or 80kDa 2-branch PEG polymers and probes targeting the G12D (c.35G->A) allele were linked to a 40kDa 3-branch PEG polymers.

[00293] **Figure 9A** shows a representative event plot of mean δG vs. duration for the 100% target analyte control sample (blue closed circles) and the 100% reference molecule control sample (black open squares) overlaid. The target analyte was 89bp DNA with G12D-bound probe linked to a 3-branch PEG (denoted G12D-3bPEG). The reference molecule was 89bp DNA with wild-type (c.35G)-bound probe linked to an 8-arm PEG (denoted WT-8armPEG). The two controls were run sequentially using a 35 nm diameter nanopore at 215 mV (1.0 M LiCl 10mM tris 1mM EDTA). Visually, the plot suggests a criterion based on three inequalities for tagging target events:

$$duration \geq q_1$$
$$mean\ \partial G \geq q_2$$
$$mean\ \partial G \leq q_3$$

[00294] The thresholds $q_1 = 1$ msec, $q_2 = 0.4$ nS and $q_3 = 0.65$ nS create the target tagging box (dashed line) also shown in Figure 9A. Using the criterion of the three inequalities with stated thresholds, the isolated controls produce $Q_{ref} = 0.006$ and $Q_{targ} = 0.795$. An equimolar concentration of target-payload and reference-payload molecules resulted in $Q_{1:1} = 0.274$, used as the control mixture. Two subsequent unknown samples, A and B, registered $Q_A = 0.066$ and $Q_B = 0.041$. The two samples are overlaid on the two isolated controls in an event plot, shown in Figure 9B. Visually, sample A shows higher G12D content than sample B, though both are positive compared to the 0.6% false positive rate of the 100% WT control. After applying equation (1) and using the control mixture for compensation, the predicted fractions of G12D mutant to wild type are $F_A^* = 11.1 \pm 0.9\%$ and $F_B^* = 6.0 \pm 0.7\%$ for samples A and B, respectively.

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[00295] Table 7 shows the results for samples A and B in rows 1 and 2. Also shown are the results for all patient samples tested. A total of 5 different patient samples were assayed. Samples C and C2 were subsamples from the same patient sample; likewise for sample D, D2 and E, E2. Different subsamples taken from the same patient sample were, in all three cases considered, within 2% of one another. This is despite different people running each nanopore experiment on a different nanopore, and in two cases on a different day. This suggests a reproducible workflow and quantitative fractional abundance method.

Table 7. Predicted G12D mutant fraction in blood samples using Q-test method

Nanopore ID, diameter	Sample Label	Estimated G12D Fraction %	Total Events
NP1, 35 nm	A	11 ± 0.89%	1,494
NP1, 35 nm	B	6.9 ± 0.75	1,508
NP2, 30 nm	C	7 ± 0.9%	1,488
NP3, 33 nm	D	5.9 ± 0.5%	2,503
NP4, 33 nm	C2	5.3 ± 0.9%	1,188
NP5, 38 nm	D2	6.5 ± 1.0%	1,741
NP6, 23 nm	E	30 ± 0.9%	2,455
NP7, 32 nm	E2	28 ± 0.9%	3,299

[00296] The true amount of G12D is unknown for these samples. Samples were collected from patients several weeks after the onset of cancer treatment (chemotherapy), and after each patients DNA was sequence and found to be positive for the G12D mutation. Non positive control samples from a control patient were also assayed, and the predicted fraction of G12D was 2% or less, suggesting a total workflow false positive of 2%. Further optimization in the workflow can reduce the limit of detection further.

[00297] The SVM method was applied for comparison. Using one representative experiment (nanopore NP4 in Table 1), the data was processed using the steps described for applying the SVM method. An event scatter plot of median δG vs. $\log_{10}$(duration) is shown in Figure 10 for the 100% reference control and the 100% target control overlaid. Also plotted is the SVM-identified decision boundary. The predicted G12D fraction in sample C2 is reported in Table 8 for both the Q-test and SVM methods. The two methods are within 5% of each other.

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Table 8. Predicted G12D fraction using the Q-test and SVM to determine an optimized threshold (q).

Nanopore ID	Sample Label	Q-test G12D Frac. (%)	SVM G12D Frac. (%)
NP4	C2	5.3 %	10.4%

Example 5: EMGM for FA of KRAS G12D SNP compared to wild-type using short DNA (89 bp) and two unique payloads

[00298] Application of Expectation Maximization Algorithm for Gaussian Mixtures (EMGM) to a representative data set is described. The target and reference are the mutant KRASG12D SNP and wild-type sequences within payload-bound dsDNA fragments, as described in Example 4. In a representative workflow, only a 1:1 control mixture was measured and only one 100% reference control was measured, followed by the unknown mixture.

[00299] Step 1: log of dwell time (log(dwell)) and median amplitude (medAmp) of 50% target & 50% reference mixture sample was used as input data for the EMGM algorithm (Figure 11). The initially identified expected region of the target, the mutant KRASG12D SNP, is marked as the rectangular region in the plot, using previously established knowledge about this assay. The prior knowledge was established by testing the 100% target control in comparable conditions (same buffer) in separate experiments. The box is not used for tagging. Rather, after the EMGM is applied to the control mixture, any events associated with a Gaussian mixture within the box are tagged as target events.

[00300] Step 2: Based on the population, a 3-Gaussian mixture model was used to train the model. This model predicted the mutant (target) region in one cluster (diamond). The other 2 clusters (star and square) correspond to wild-type (Figure 12). We observe that some events within the initial target domain box (Figure 11) are associated with the reference modes by the EMGM algorithm. This is different than the Q-test method, where the box itself defines the population of events that are tagged as targets vs. reference.

[00301] Step 3: The model was applied on 100% wild-type (reference) sample. The ratio number of events in the mutant (target) region over the total number of events establishes the false positive fraction (Figure 13), which can be used to improve the fractional abundance estimate.

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[00302] Step 4: The model was used to predict unknown mixtures. The ratio number of events in mutant region over the total number of events was used a predictor of the percentage of mutant molecules in unknown mixture (Figure 14).

[00303] As a test of performance enhancement by false positive compensation, the false positive fraction from step 3 was subtracted from computed fraction in step 4 as a correction. The results of applying EMGM to multiple mixtures within a set of nanopore experiments are reported in Table 9. Mixtures were blinded until EMGM results were assembled, and then the results were compared to the true G12D fractional abundance values.

Table 9. Predicted G12D fraction comparing the EMGM without and with false positive (FP) compensation

Nanopore ID	Known G12D Frac. (%)	EMGM G12D Frac. (%)	EMGM + FP-comp G12D Frac. (%)
NP-a	50%	50.7 %	43.2%
	20%	28.1%	20.5%
	10%	13.3%	5.7%
NP-b	50%	56.4%	47.3%
	20%	31.5%	22.4%
	10%	19.9%	10.8%
NP-c	50%	50.8%	--
	20%	19.7%	--
	10%	5.3%	--

[00304] In the case of NP-a, performance was enhanced only in the 20% case by using false positive compensation. For NP-b, performance was enhanced in all cases. False positive compensation was not tested for NP-c, although performance was already good, particularly for the 50% and 20% estimates.

[00305] In summary, only a control mixture is needed to apply the EMGM method, prior to applying the EMGM models to an unknown mixture for fractional abundance estimation.

OTHER EMBODIMENTS

[00306] It is to be understood that the words which have been used are words of description rather than limitation, and that changes may be made within the purview of the appended claims without departing from the true scope and spirit of the invention in its broader aspects.

[00307] While the present invention has been described at some length and with some particularity with respect to the several described embodiments, it is not intended that it

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should be limited to any such particulars or embodiments or any particular embodiment, but it is to be construed with references to the appended claims so as to provide the broadest possible interpretation of such claims in view of the prior art and, therefore, to effectively encompass the intended scope of the invention.

[00308] All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, section headings, the materials, methods, and examples are illustrative only and not intended to be limiting.

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CLAIMS

What is claimed is:

1. A method of a determining an improved estimate of a true relative abundance of a target analyte in a mixed unknown sample using a nanopore device, comprising

    applying a voltage across a nanopore in a nanopore device to generate a detectable electronic signature and to induce translocation of charged analytes through said nanopore separately for each of:

        a control sample comprising a known relative abundance of target analytes to reference analytes, and

        a mixed unknown sample comprising said target analytes and said reference analytes, wherein the relative abundance of said target analytes in said sample is to be determined;

    generating a plurality of event signatures generated by translocation of said target analytes or said reference analytes through said nanopore for each sample;

    identifying a quantity of first event signatures associated with said target analyte and a quantity of second event signatures associated with said reference analyte from said plurality of event signatures to determine a detected relative abundance of first and second event signatures for each sample; and

    adjusting a detected relative abundance of said first and second event signatures in said mixed unknown sample using the detected relative abundance of said first and second event signatures in said control sample to correct for an error in the detected relative abundance, thereby determining an improved estimate of the true relative abundance of said target analyte in said mixed unknown sample.

2. The method of claim 1, wherein said control sample is a target control sample comprising said target analytes, but not said reference analytes.

3. The method of claim 1, wherein said control sample is a reference control sample comprising said reference analytes, but not said target analytes.

4. The method of claim 3, further comprising applying a voltage to a nanopore device to induce translocation of charged analytes through a nanopore sensor for a target control sample comprising said target analytes, but not said reference analytes.

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5. The method of any one of claims 1-4, wherein said adjustment of said detected relative abundance of said first and second event signatures in said unknown sample comprises using the detected relative abundance of said first and second event signatures in said target control sample and in said reference control sample to correct for said error in the detected relative abundance.

6. The method of any one of claims 1-5, wherein said error comprises a false positive or a false negative detection error of said target analyte.

7. The method of any one of claims 1-6, further comprising applying a voltage to a nanopore device to induce translocation of charged analytes through a nanopore sensor for a mixed control sample comprising said target analytes and said reference analytes, wherein the relative abundance of said target analytes and said reference analytes is known.

8. The method of claim 7, wherein said adjustment of said detected relative abundance of said first and second event signatures in said unknown sample comprises using the detected relative abundance of said first and second event signatures in said target control sample, said reference control sample, and said mixed control sample to correct for said error in the detected relative abundance.

9. The method of any one of claims 1-8, wherein said error comprises a false positive target analyte detection error, a false negative target analyte detection error, a capture rate constant differential between said target analyte and said reference analyte, or any combination thereof.

10. The method of claim 1, wherein said control sample is a mixed control sample comprising said target analytes and said reference analytes, wherein the relative abundance of said target analytes and said reference analytes is known.

11. The method of claim 10, wherein said error comprises a capture rate constant differential between said target analyte and said reference analyte.

12. The method of any one of claims 7-11, wherein said mixed control sample comprises a relative abundance of said target analytes to said reference analytes that differs by no more than a factor of 1.2, a factor of 1.5, a factor of 2, a factor of 5, or a factor of 10 relative to said mixed unknown sample.

13. The method of any one of claims 1-12, wherein said estimate of the true relative abundance is an estimate of the true ratio of said target analyte to said reference analyte in

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said mixed unknown sample.

14. The method of claim 13, wherein said estimate (R^*_{mix}) of the true ratio is determined by $R^*_{mix} = \rho\alpha$, wherein the parameter ρ is an estimate for the ratio that can compensate for a false positive detection error, a false negative detection error, or both, and wherein the parameter α can be used to compensate for a capture rate constant differential between said target analyte and said reference analyte.

15. The method of claim 14, wherein α is an estimate of the ratio of the reference analyte capture rate divided by the target analyte capture rate.

16. The method of any one of claims 1-12, wherein said estimate of the true relative abundance is an estimate of the true fraction of said target analytes in a population of said reference analytes and said target analytes in said mixed unknown sample.

17. The method of claim 16, wherein said estimate of the true fraction (F^*_{mix}) is determined by $F^*_{mix} = \frac{\rho\alpha}{\rho\alpha+1}$, wherein the parameter ρ is an estimate for the ratio that can compensate for a false positive detection error, a false negative detection error, or both, and wherein the parameter α can be used to compensate for a capture rate constant differential between said target analyte and said reference analyte.

18. The method of claim 17, wherein α is an estimate of the ratio of the reference analyte capture rate divided by the target analyte capture rate

19. The method of claim 14 or 17, wherein $\rho = \left(\frac{Q_{mix}-Q_{ref}}{Q_{targ}-Q_{mix}}\right)$, and $\alpha = \left(\frac{Q_{targ}-Q_{X:Y}}{Q_{X:Y}-Q_{ref}}\right) \times \frac{X}{Y}$.

20. The method of claim 19, wherein Q_{targ} is the fraction of said first event signature observed in said target control sample if said control sample is used, or $Q_{targ} = 1$ if no target control sample is used.

21. The method of claim 19 or 20, wherein Q_{ref} is the fraction of said first event signature observed in said reference control sample if said reference control sample is used, or $Q_{ref} = 0$ if no reference control sample is used.

22. The method of any one of claims 19-21, wherein $Q_{X:Y}$ is the fraction of said first event signature observed in said mixed control sample and wherein $\frac{X}{Y}$ is the known ratio of target analytes (X) to reference analytes (Y) in the mixed control sample if said control sample is used, or $\alpha = 1$ if no mixed control sample is used.

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23. The method of any one of claims 19-22, wherein Q_{mix} is the fraction of said first event signature observed in said mixed unknown sample.

24. The method of any of the above claims, wherein said unknown or control sample is prepared by nucleic acid amplification.

25. The method of any one of claims 1-23, wherein said unknown or control sample is not prepared by nucleic acid amplification.

26. The method of any of the above claims, wherein said sample is purified to substantially consist of reference and target molecules.

27. The method of any one of claims 1-25, wherein said sample is not purified.

28. The method of any of the above claims, wherein the quantity or concentration of said reference analytes in said mixed unknown sample are known.

29. The method of claim 28, further comprising determining an estimate of the absolute quantity or concentration of said target analytes in said mixed unknown sample using said estimate of the true relative abundance of said target analytes to said reference analytes in said mixed unknown sample and said known quantity or concentration of said reference analytes in said mixed unknown sample.

30. The method of any of the above claims, wherein said quantity of first event signatures associated with said target analyte and said quantity of second event signatures associated with said reference analyte are identified according to a defined threshold.

31. The method of claim 30, further comprising optimizing said threshold to increase accuracy of detection of said reference analytes and/or said target analytes using a Q-test, a support vector machine, or an expectation maximization algorithm.

32. The method of claim 31, wherein said support vector machine is trained using electronic signatures from control samples comprising known quantities of target analytes and reference analytes.

33. The method of claim 30, wherein said defined threshold is a function of one or more features of an event signature selected from the group consisting of: an event duration, a maximum δG, a median δG, an average δG, a standard deviation of the event signature, a mean or median of the noise power of the event below 50 Hz, a unique pattern in said event signature, an area of an event, or any combination thereof.

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34. The method of any of the above claims, wherein said adjustment of said detected relative abundance of said first and second event signatures in said mixed unknown sample to correct for said error in the detected relative abundance is performed using a Q-test, a support vector machine, or an expectation maximization algorithm.

35. The method of any of the above claims, wherein said target analyte and said reference analyte each comprise a polynucleotide.

36. The method of 35, wherein said target analyte polynucleotide and said reference analyte polynucleotide are of different lengths.

37. The method of claim 36, wherein said lengths are different by at least 10 nucleotides, at least 20 nucleotides, at least 50 nucleotides, at least 100 nucleotides, at least 150 nucleotides or at least 200 nucleotides.

38. The method of any of the above claims, further comprising contacting said control or unknown samples with a first probe bound to a first payload, wherein said first probe is configured to bind specifically to said first analyte.

39. The method of any of the above claims, further comprising contacting said control or unknown samples with a second probe bound to a second payload, wherein said second probe is configured to bind specifically to said second analyte.

40. The method of any of the above claims, wherein said target analyte is correlated with a genetically modified organism.

41. The method of any of the above claims, wherein said target analyte comprises a marker associated with the presence or absence of cancer in a patient.

42. A method of determining a relative quantity of a target analyte in a sample comprising:

running separately in a nanopore system each of:

a first control sample comprising reference analytes and no target analytes,

a second control sample comprising target analytes and no reference analytes,

a third control sample comprising a known relative abundance of said target analytes and said reference analytes, and

an experimental sample comprising an unknown relative abundance of said target analytes and said reference analytes;

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detecting a quantity of first event signatures associated with a reference analyte and a quantity of second event signatures associated with a target analyte for each sample; and

comparing a relative abundance of said quantity of first and second event signatures from said experimental sample with a relative abundance of said quantity of first and second event signatures from each of said first control sample, said second control sample, and said third control sample to determine an estimate of the true relative abundance of said reference analyte and said target analyte in said experimental sample.

43. The method of claim 42, wherein said event signature comprises an electrical signal induced by translocation of said reference analyte through said nanopore.

44. The method of claim 42, wherein said target analyte and said reference analyte each comprise a polynucleotide.

45. The method of claim 44, wherein said reference analyte and said target analyte are discriminated by length

46. The method of claim 42, wherein said reference analyte and said target analyte are each bound to a sequence-specific probe comprising a payload to facilitate discrimination between said reference analyte and said target analyte in said nanopore device.

47. The method of claim 42, wherein said relative abundance is a fractional amount of said target analyte compared to the total population of the target analyte and the reference analyte in said sample.

48. A method of determining a relative abundance of a target analyte in an unknown sample, comprising

providing an unknown sample comprising a plurality of reference analytes and a plurality of target analytes;

loading said unknown sample into a first chamber of a nanopore device comprising a nanopore disposed between said first chamber and a second chamber;

applying a voltage across said nanopore to pass said reference analytes and said target analytes through said nanopore from said first chamber to said second chamber;

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detecting a number of first electrical signals each associated with the translocation of said reference analyte through the nanopore;

detecting a number of second electrical signals each associated with the translocation of said target analyte through the nanopore; and

converting a relative abundance of the number of detected first electrical signals and the number of detected second electrical signals to an estimate of the true relative abundance of said target analyte in said unknown sample using a reference value that accounts for at least one error associated with said electrical signal relative abundance.

49. The method of claim 48, wherein said reference value is determined from a fractional abundance of said first electrical signal determined from a mixed control sample comprising a known amount of target analytes and reference analytes.

50. The method of claim 48, wherein said reference value is determined from a fractional abundance of said first electrical signal determined from a mixed control sample comprising a known amount of target analytes and reference analytes.

51. The method of claim 48, wherein said reference value is determined from a fractional abundance of said first electrical signal determined from a mixed control sample comprising a known amount of target analytes and reference analytes.

52. The method of any one of claims 49-51, wherein said mixed control sample, said target control sample, or said refrence control sample is run in said nanopore device under conditions substantially identical to conditions in said nanopore device during said detection of said first and second electrical signals from said unknown sample.

53. The method of any one of claims 48-52, wherein said nanopore device comprises a membrane that separates an interior space of the device into a first chamber and a second chamber, wherein said membrane comprises said nanopore, wherein said first chamber and said second chamber are in fluid communication through said nanopore, and wherein said device comprises an electrode in each chamber for applying a voltage across said nanopore.

54. The method of claim 53, wherein said electrodes are configured to monitor electrical current through said nanopore.

55. The method of claim 53 or 54, wherein said electrodes are connected to a power supply.

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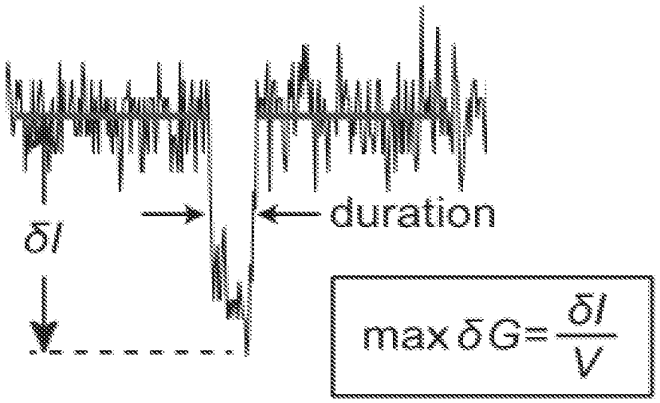


Figure 1A

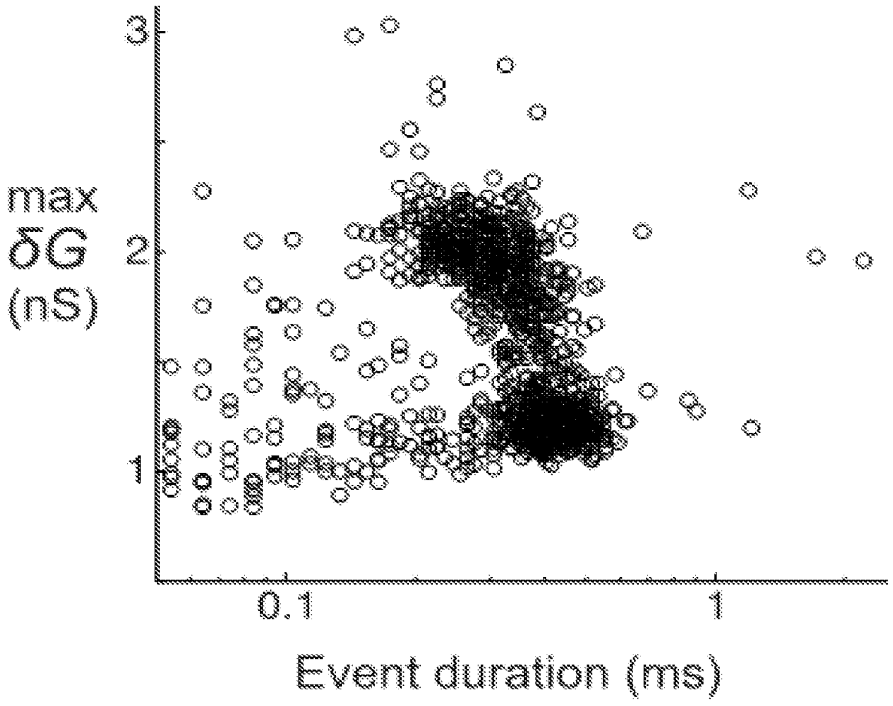


Figure 1B

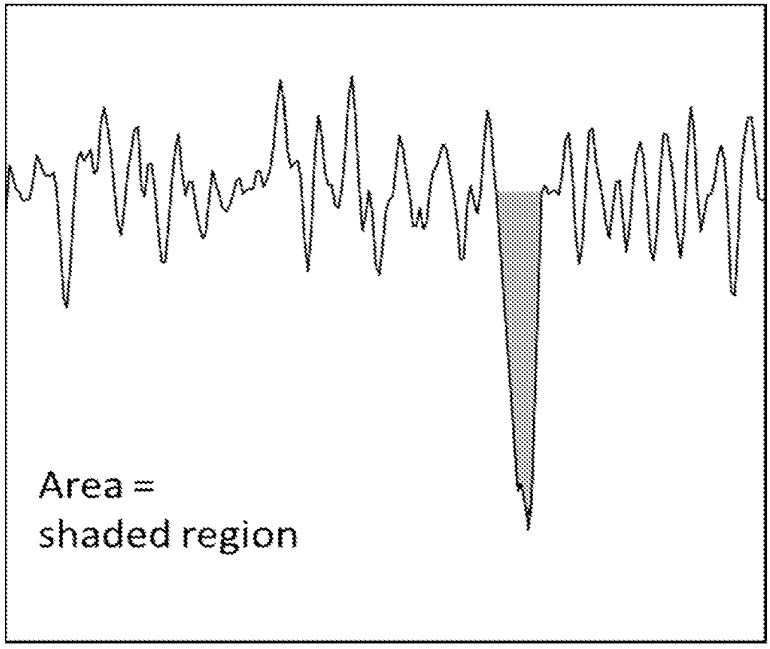


Figure 2A

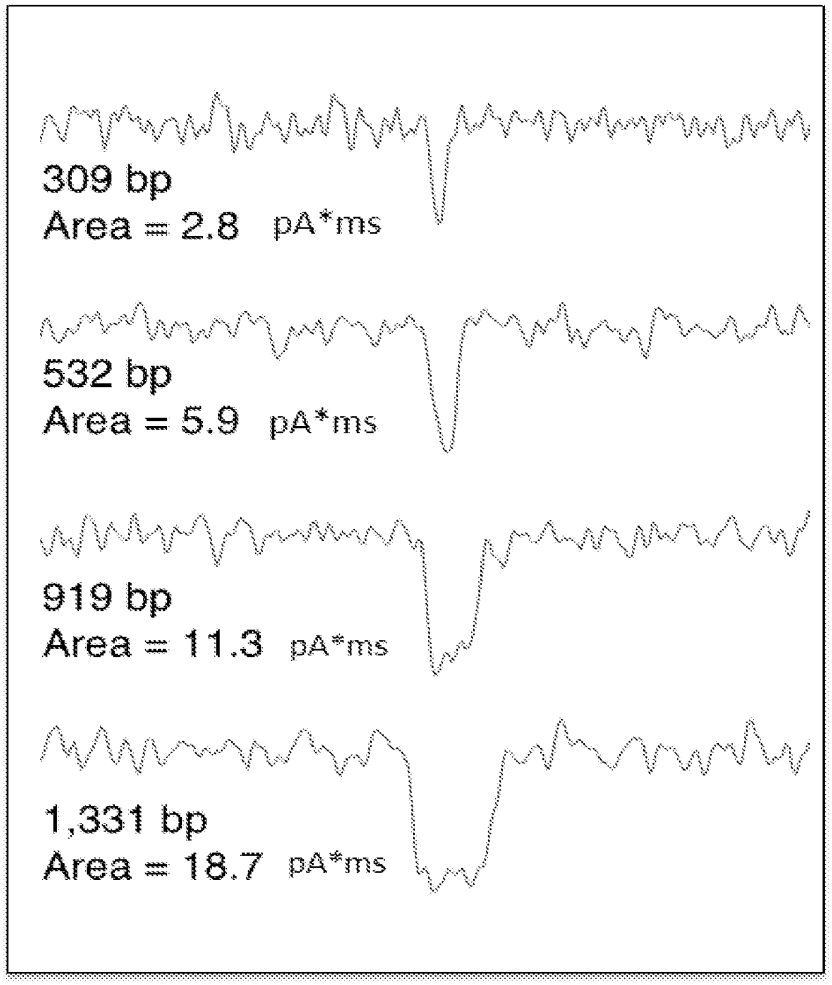

309 bp
Area = 2.8 pA*ms
532 bp
Area = 5.9 pA*ms
919 bp
Area = 11.3 pA*ms
1,331 bp
Area = 18.7 pA*ms

Figure 2B

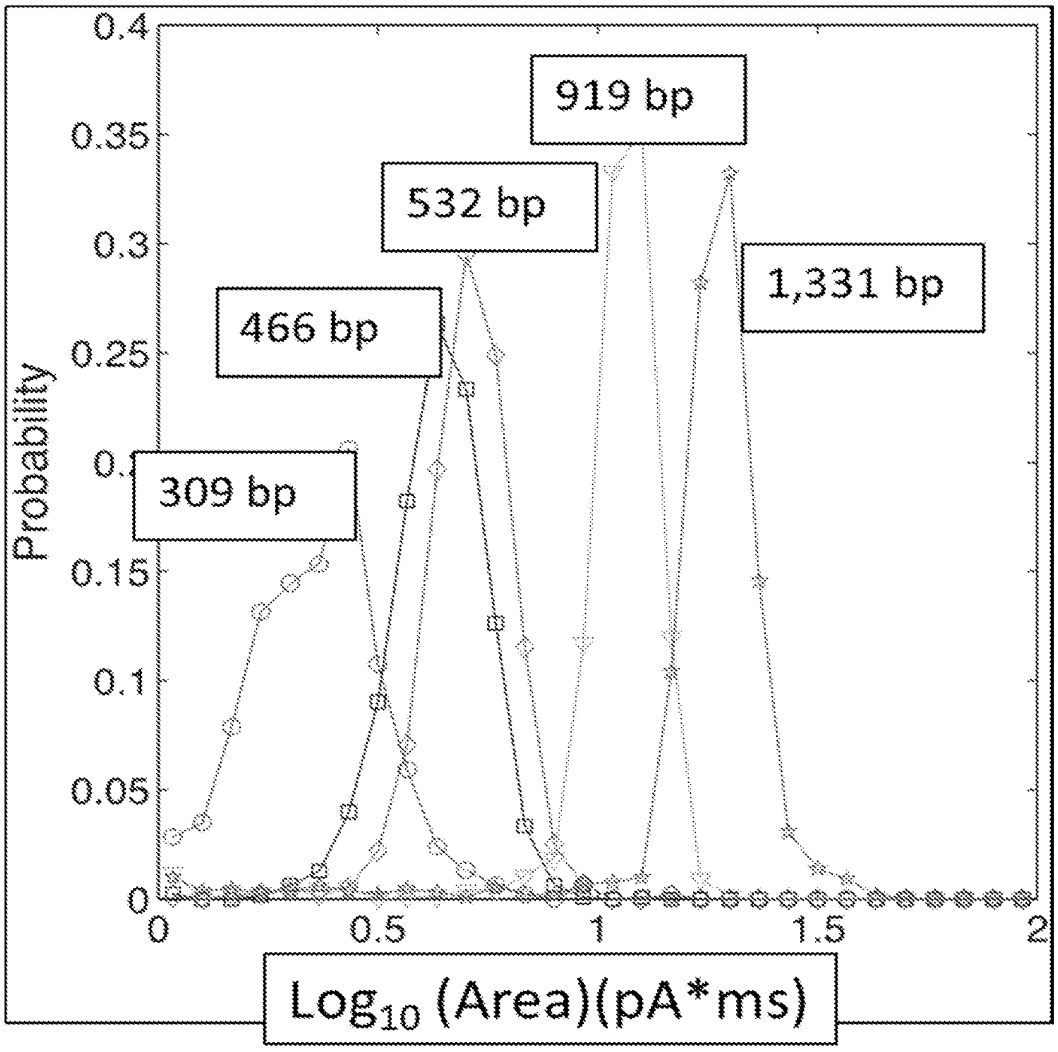

919 bp
532 bp
1,331 bp
466 bp
309 bp
Probability
0.4
0.35
0.3
0.25
0.15
0.1
0.05
0
0
0.5
1
1.5
2
Log10 (Area)(pA*ms)

Figure 2C

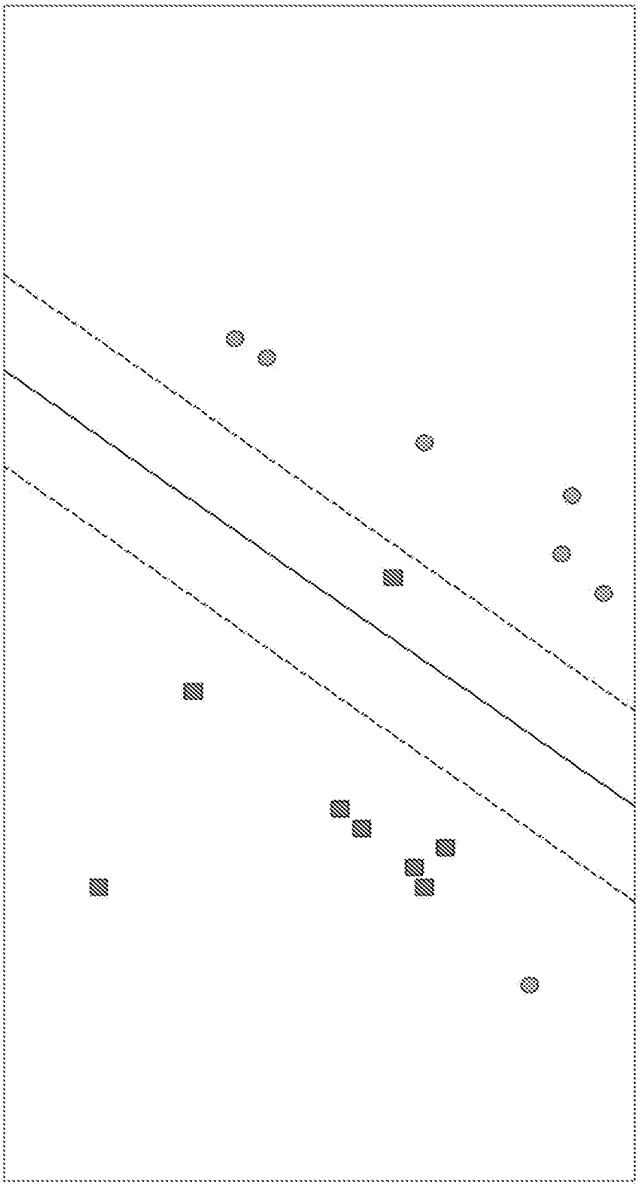


Figure 3A

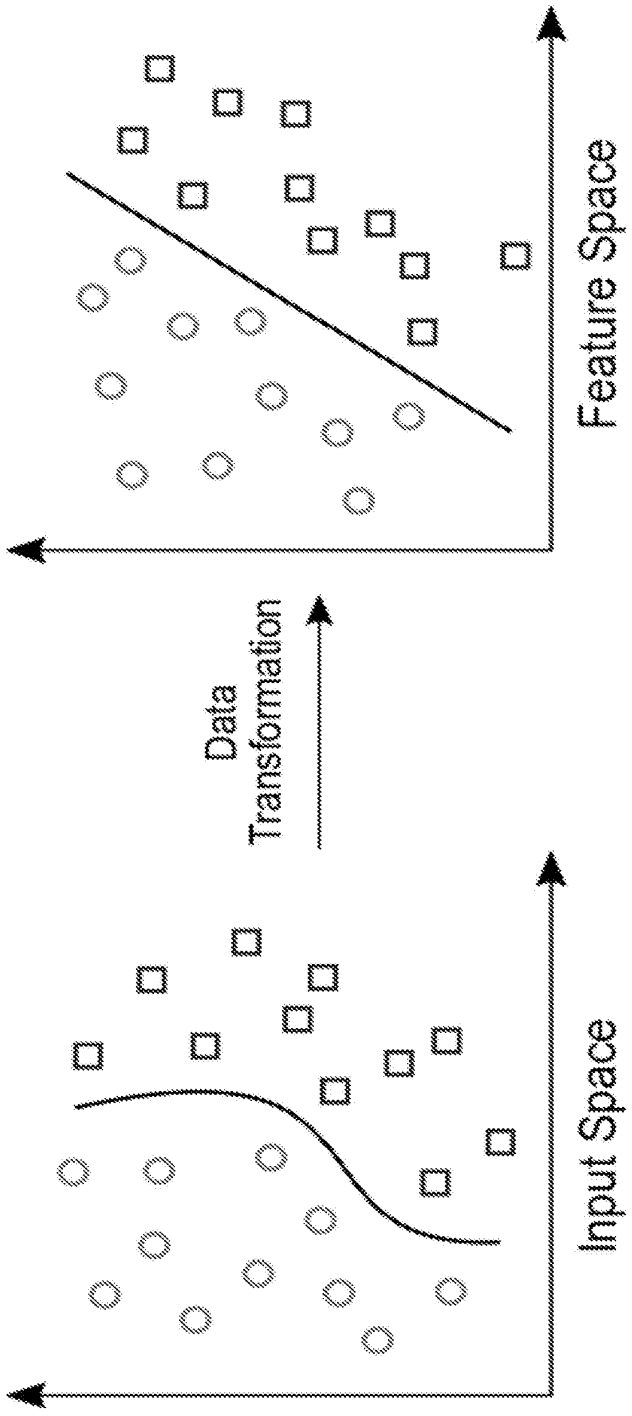


Figure 3B

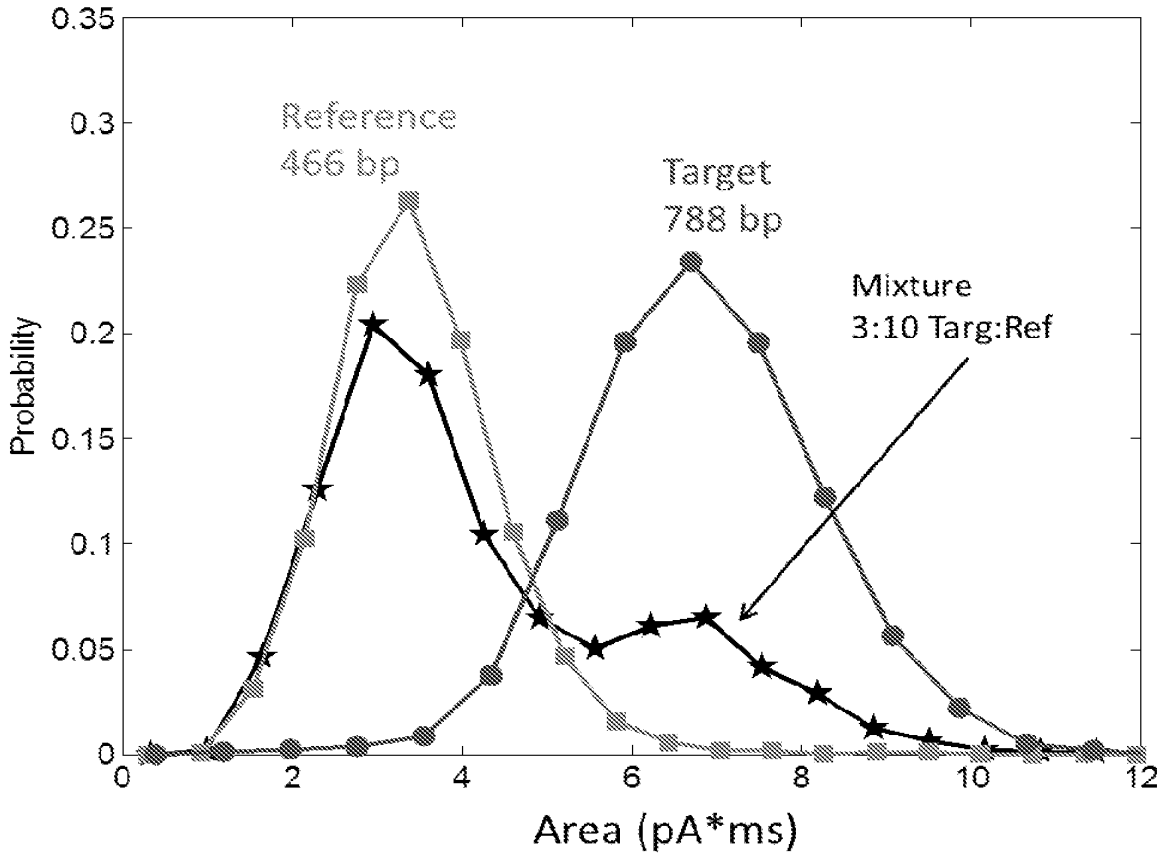

0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Probability
Reference
466 bp
Target
788 bp
Mixture
3:10 Targ:Ref
0
2
4
6
8
10
12
Area (pA*ms)

Figure 4A

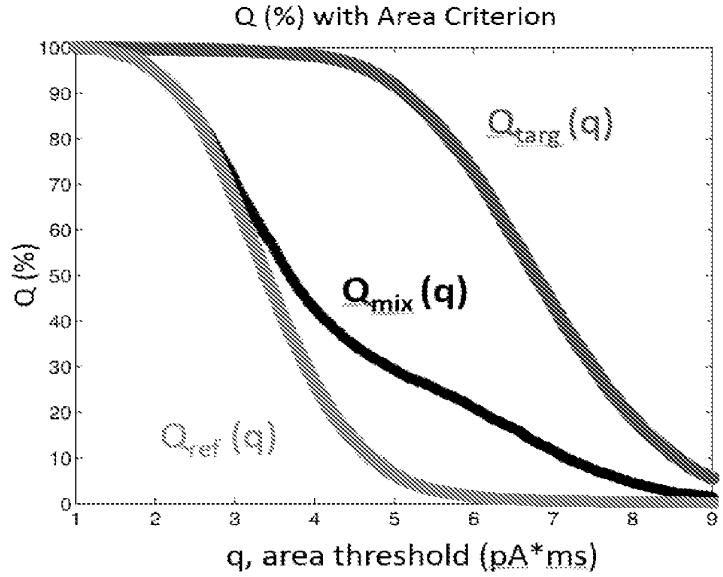


Figure 4B

$$\rho(q) = \frac{Q_{mix}(q) - Q_{ref}(q)}{Q_{targ}(q) - Q_{mix}(q)}$$

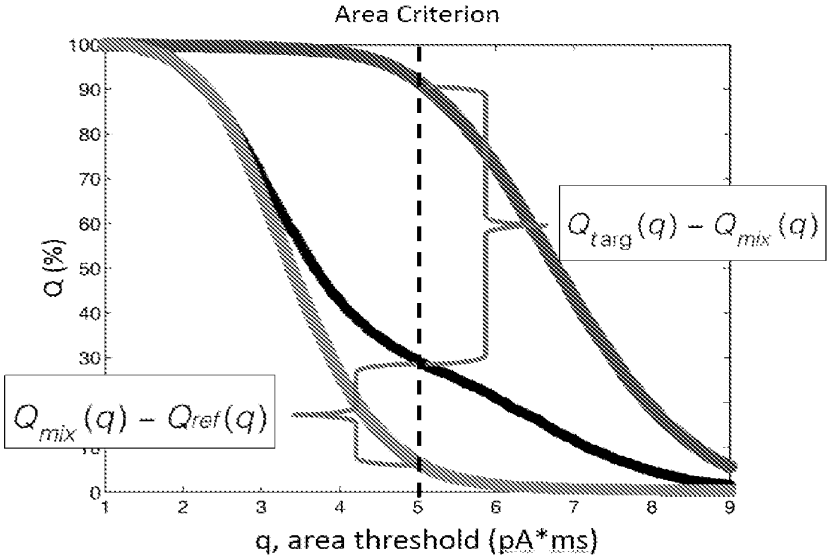


Figure 4C

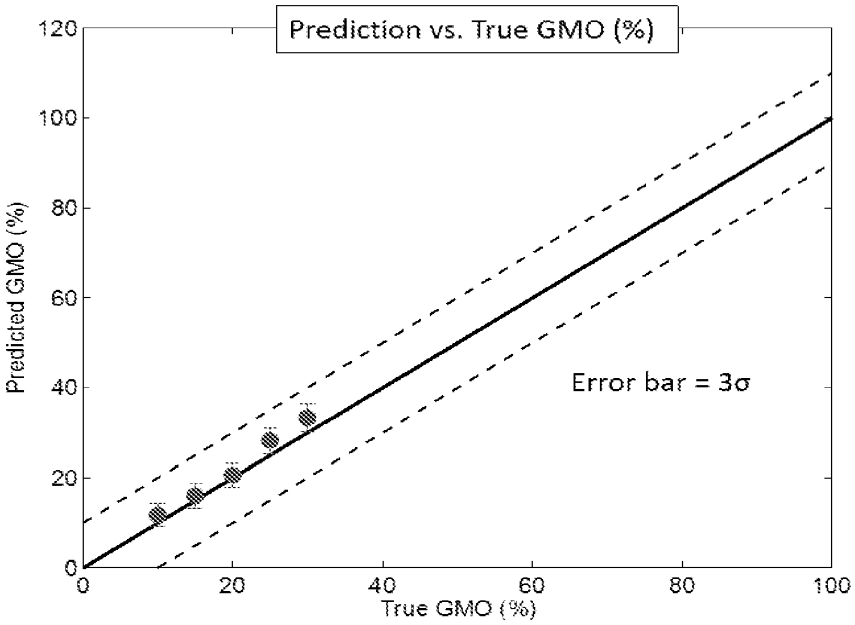


Figure 5A

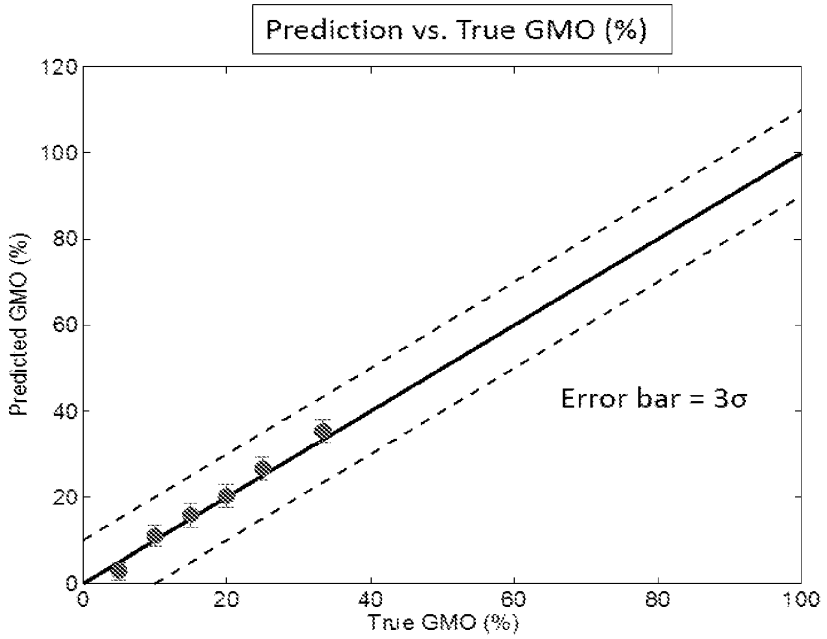


Figure 5B

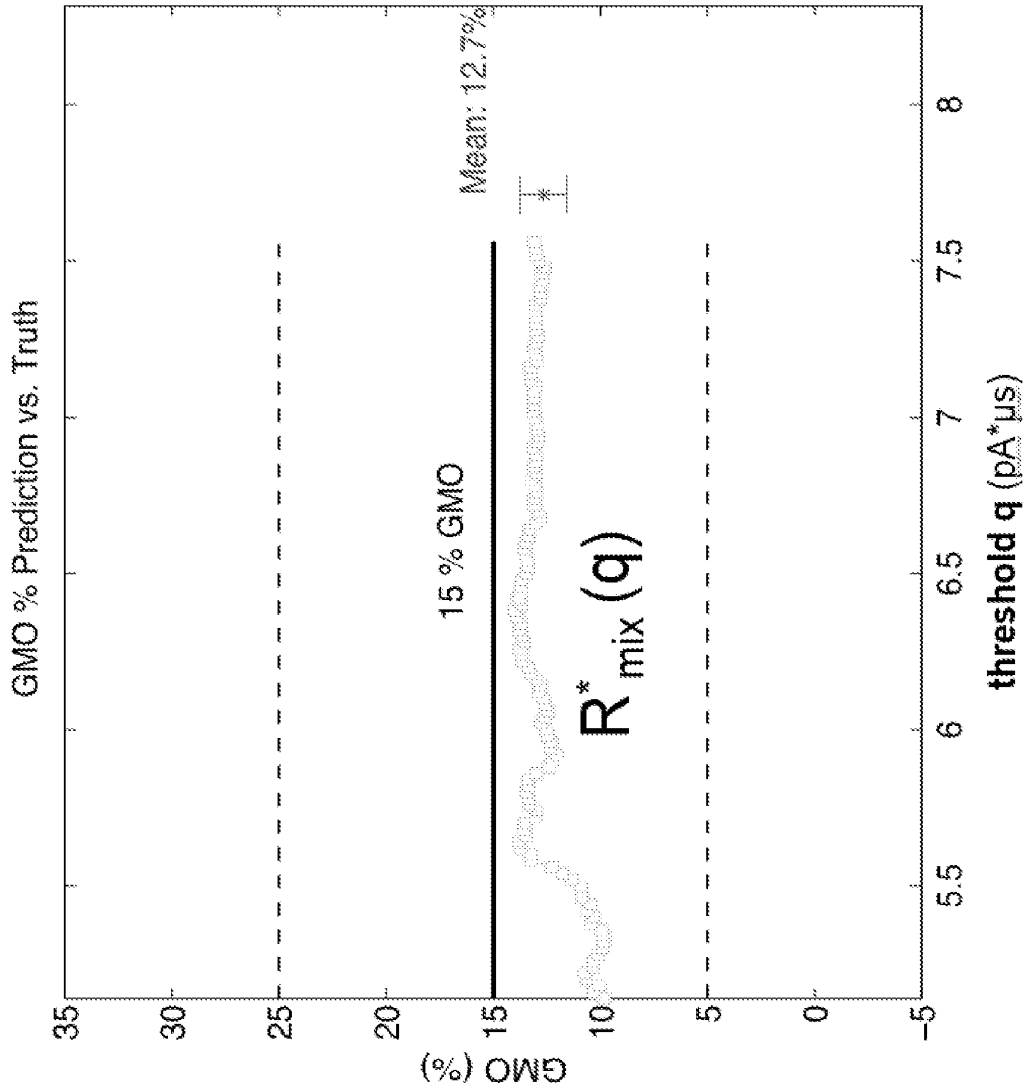

GMO % Prediction vs. Truth
Mean: 12.7%
15 % GMO
$R^*_{mix}(q)$
GMO (%)
35
30
25
20
15
10
5
0
−5
5.5
6
6.5
7
7.5
8
threshold q (pA*µs)

Figure 6

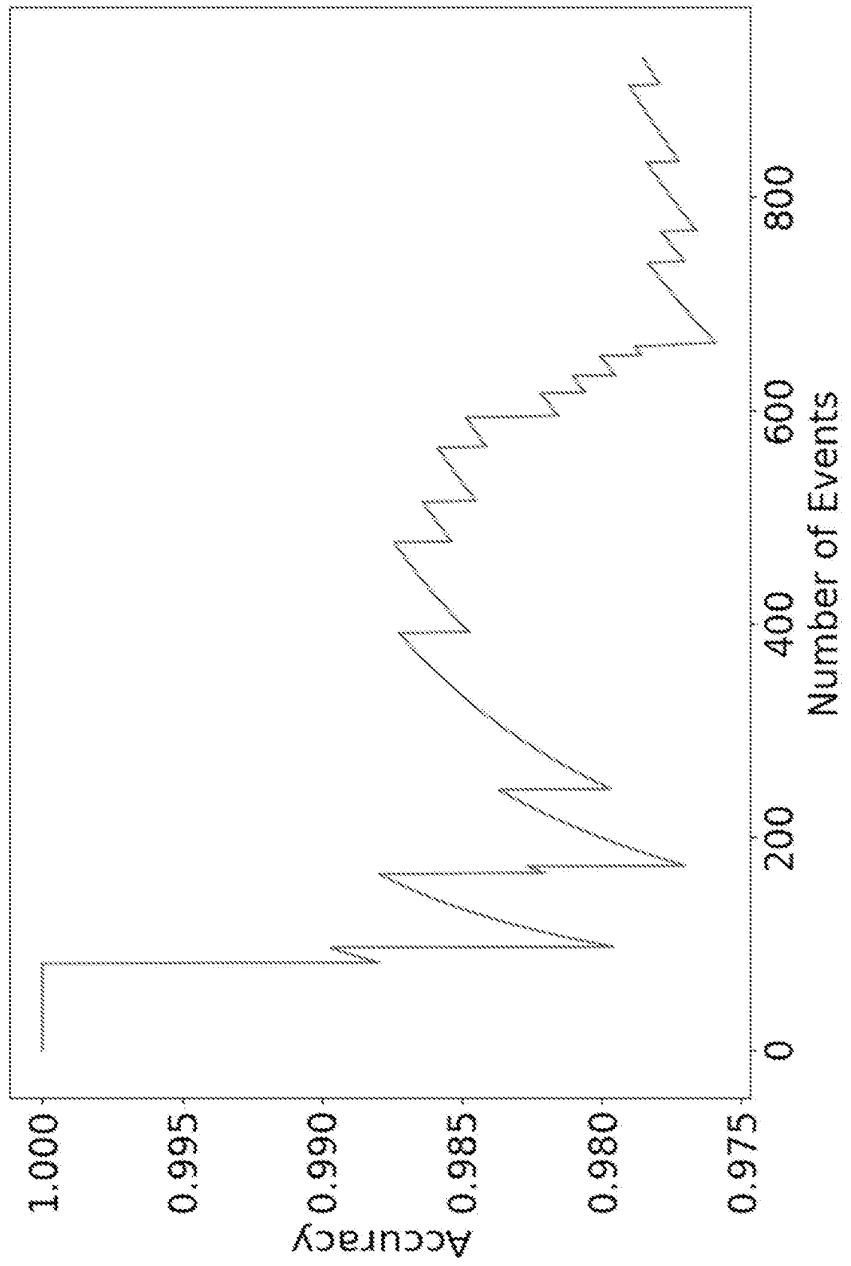

1.000
0.995
0.990
0.985
0.980
0.975
Accuracy
0
200
400
600
800
Number of Events

Figure 7

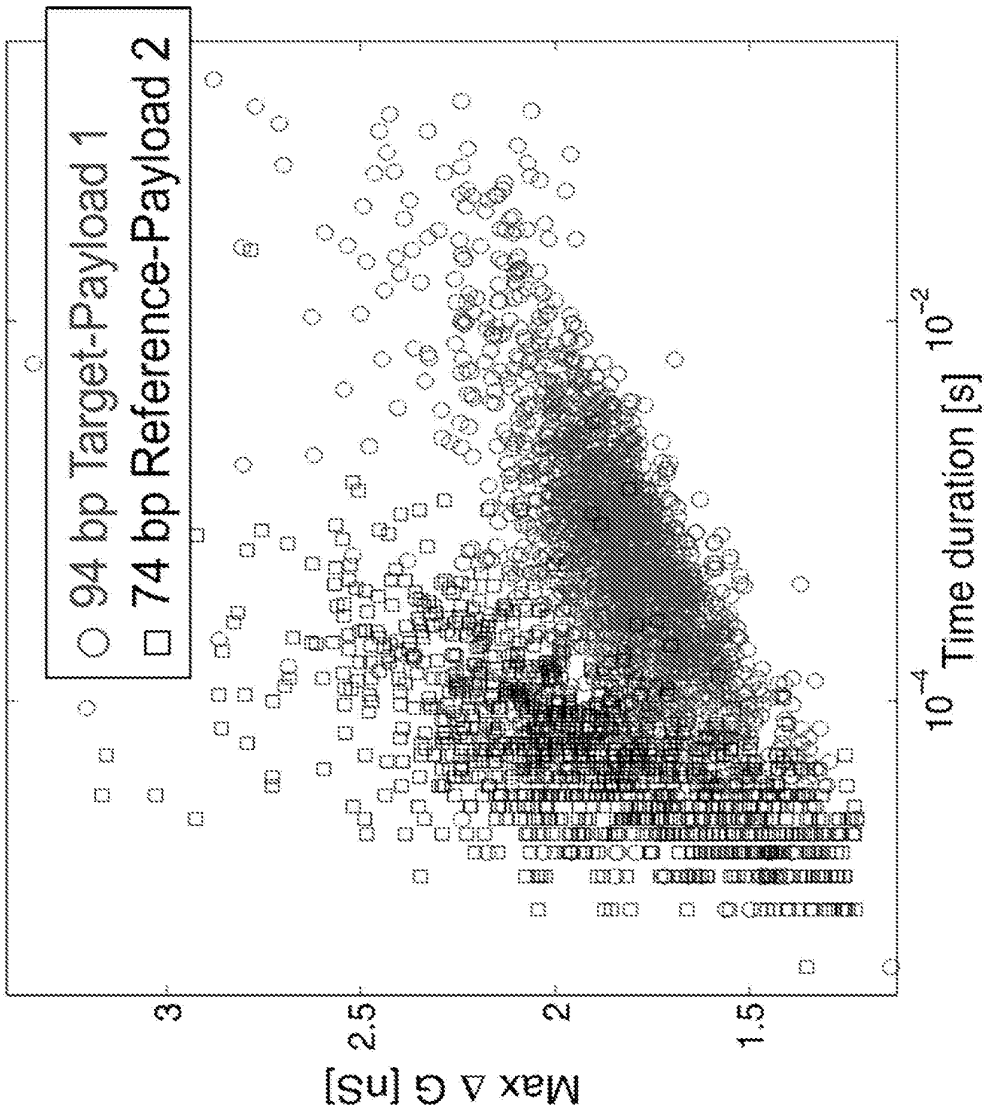

○ 94 bp Target-Payload 1
□ 74 bp Reference-Payload 2
Max Δ G [nS]
3
2.5
2
1.5
Time duration [s]
10^-4
10^-2

Figure 8

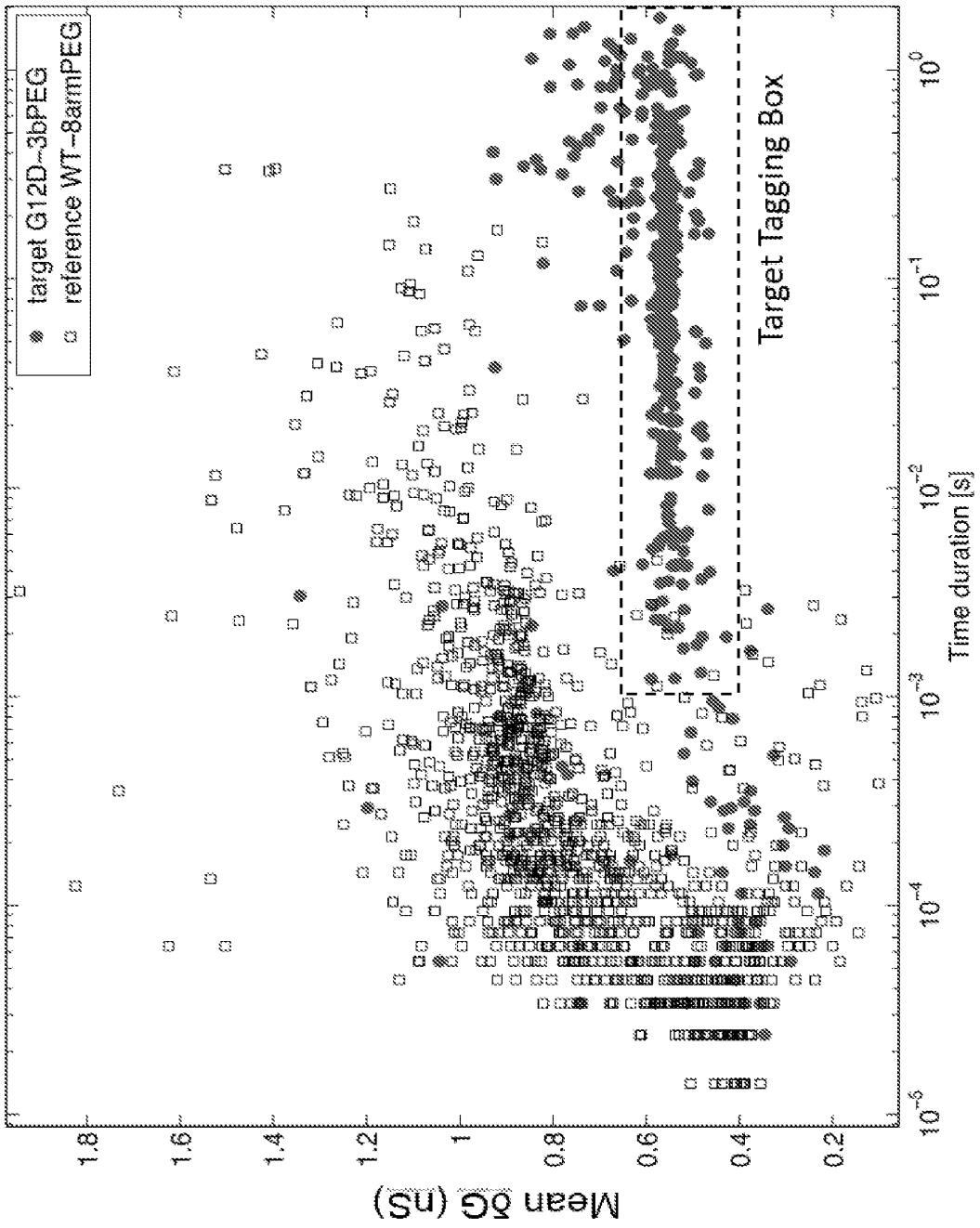

target G12D-3bPEG
reference WT-8armPEG
Target Tagging Box
Mean δG (nS)
Time duration [s]
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
10^{-5}
10^{-4}
10^{-3}
10^{-2}
10^{-1}
10^{0}

Figure 9A

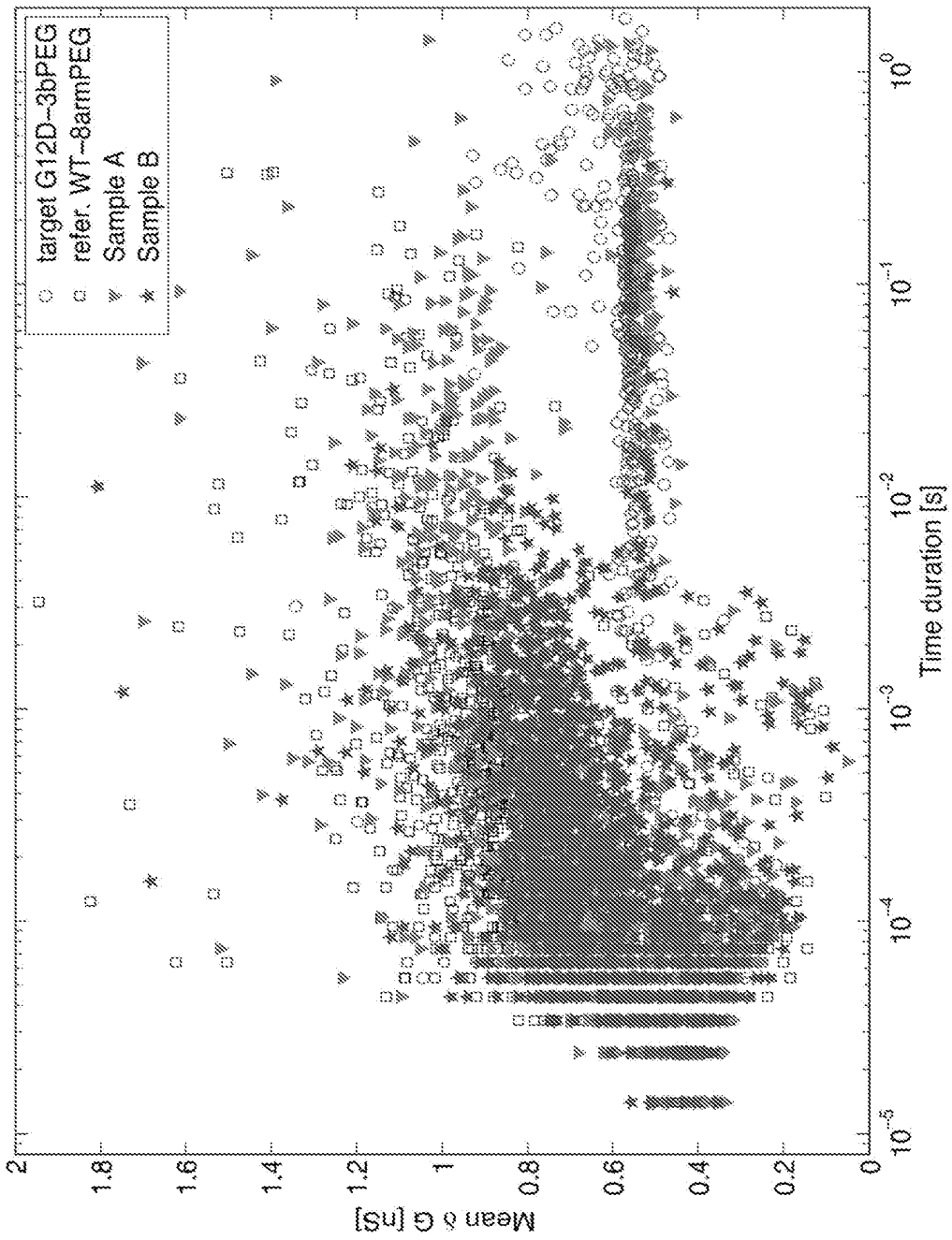

target G12D–3bPEG
refer. WT–8armPEG
Sample A
Sample B
Mean δ G [nS]
Time duration [s]
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
10^{-5}
10^{-4}
10^{-3}
10^{-2}
10^{-1}
10^{0}

Figure 9B

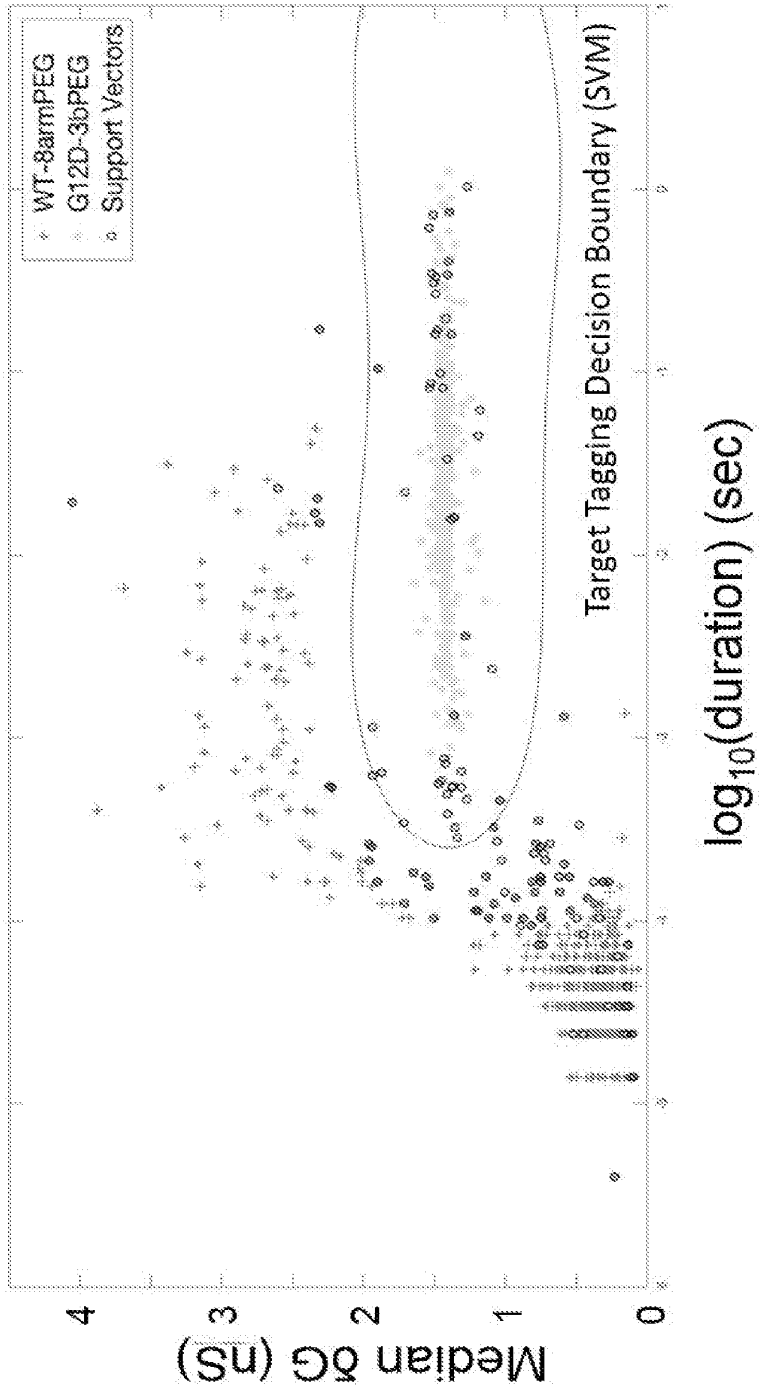

WT-8armPEG
G12D-3bPEG
Support Vectors
Target Tagging Decision Boundary (SVM)
Median δG (nS)
log10(duration) (sec)
0
1
2
3
4

Figure 10

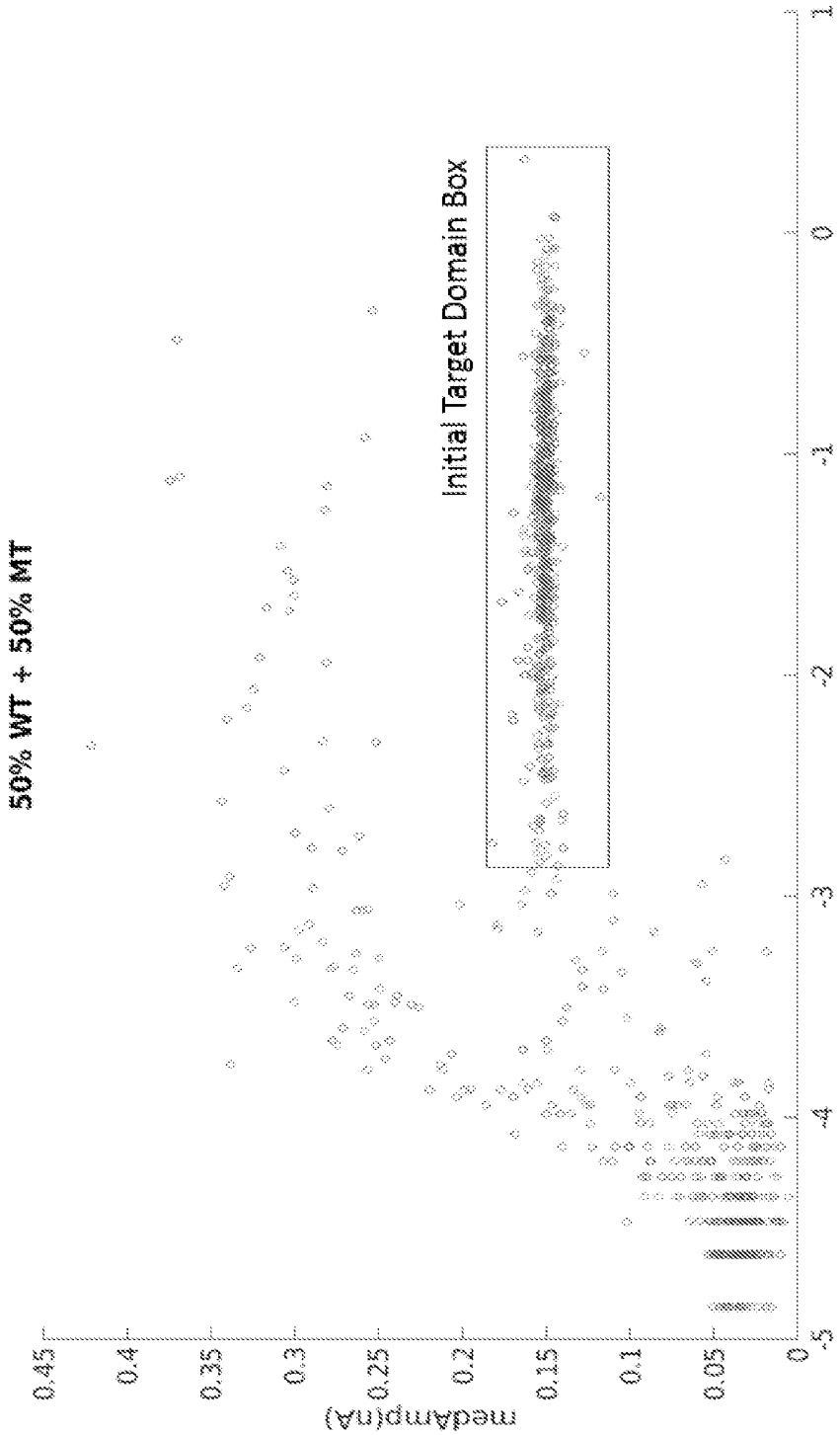

50% WT + 50% MT
Initial Target Domain Box
medAmp(nA)
log(dwell)
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
-5
-4
-3
-2
-1
0
1

Figure 11

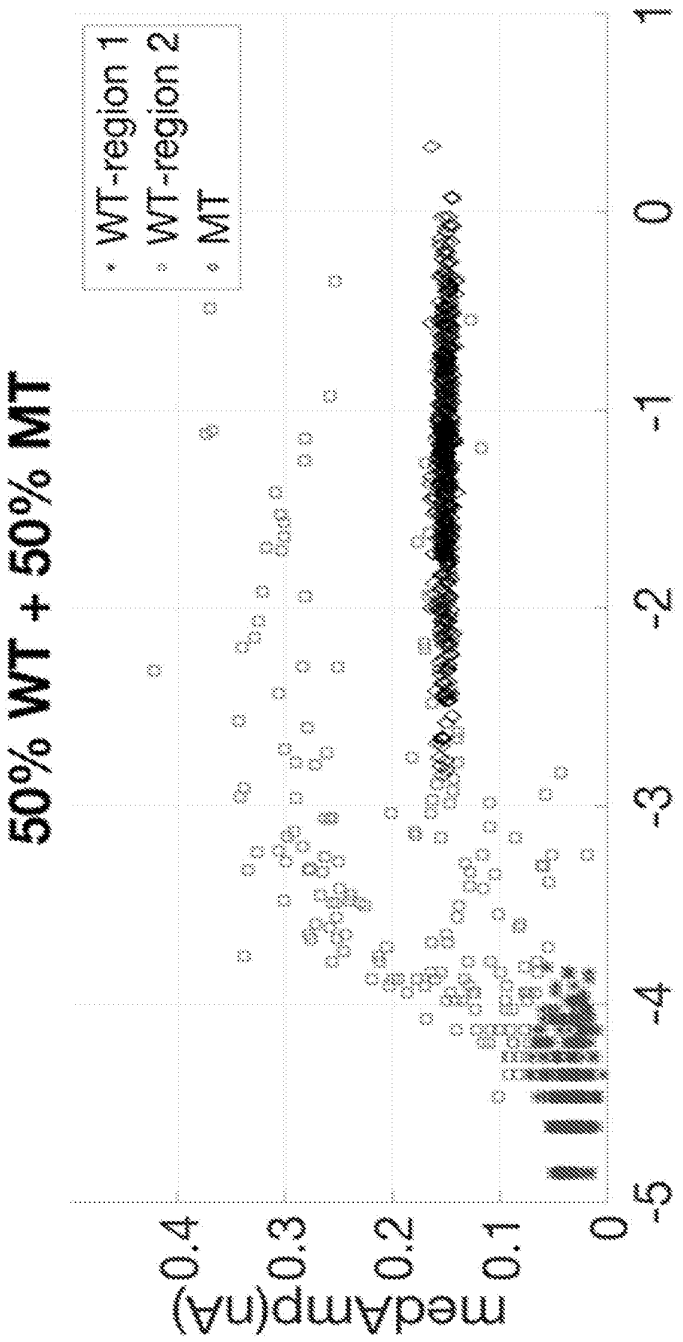

50% WT + 50% MT
WT-region 1
WT-region 2
MT
medAmp(nA)
0.4
0.3
0.2
0.1
0
-5
-4
-3
-2
-1
0
1
log(dwell)

Figure 12

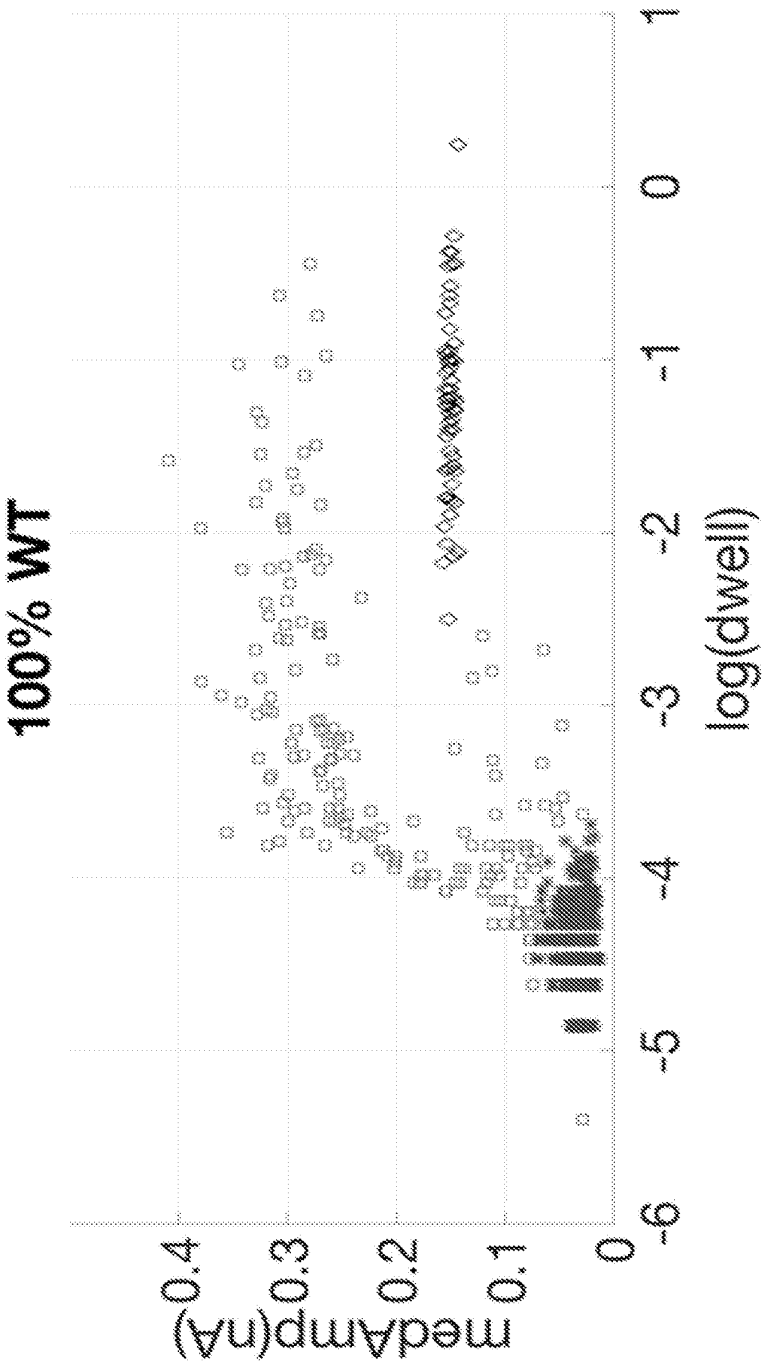


Figure 13

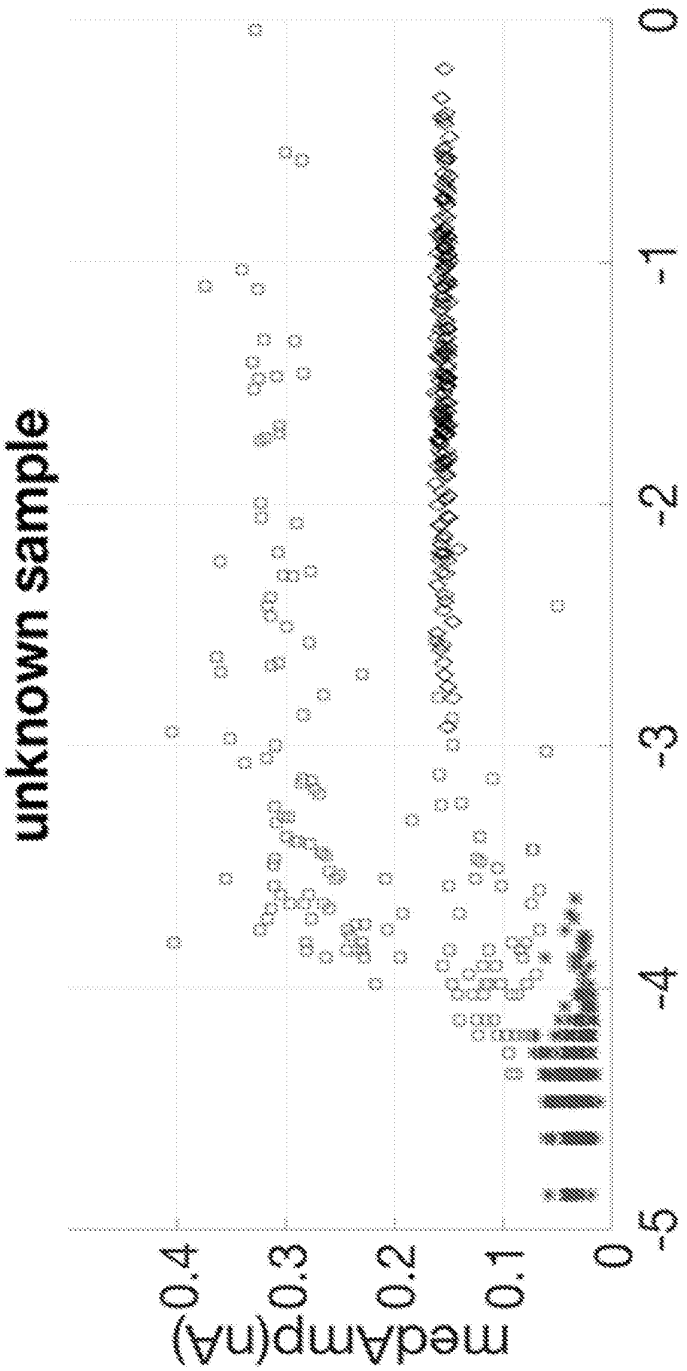


Figure 14

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2017/058159

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - G01N 27/447; G01N 33/487; G01N 33/53 (2017.01)
CPC - G01N 27/4473; G01N 33/48721; G01N 33/5302; G01N 33/5438 (2017.08)

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

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.
A	US 8,764,968 B2 (AFZALI-ARDAKANI et al) 01 July 2014 (01.07.2014) entire document	1-5, 10, 11, 42-52
A	US 2013/0260472 A1 (HOLT) 03 October 2013 (03.10.2013) entire document	1-5, 10, 11, 42-52
A	US 2016/0266089 A1 (TWO PORE GUYS INC) 15 September 2016 (15.09.2016) entire document	1-5, 10, 11, 42-52
A	WO 2015/176034 A1 (TWO PORE GUYS INC) 19 November 2015 (19.11.2015) entire document	1-5, 10, 11, 42-52
A	WO 2016/049657 A1 (TWO PORE GUYS INC) 31 March 2016 (31.03.2016) entire document	1-5, 10, 11, 42-52

☐ 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
30 November 2017	26 DEC 2017
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Authorized officer Blaine R. Copenheaver 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/US2017/058159

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.: 6-9, 12-41, 53-55
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:

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.:

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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(74) Agent: **BRUNER, Kyle**; WILSON SONSINI GOODRICH & ROSATI, 650 Page Mill Road, Palo Alto, California 94304 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:
— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*
— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

Published:
— *with international search report (Art. 21(3))*

(54) Title: MULTIPLEXED FLUOROMETRIC MEASUREMENTS WITH DROPLET PCR SYSTEMS

(57) Abstract: The present disclosure provides methods and compositions for detection of multiple nucleic acid targets in a single optical channel in a digital assay. In some cases, three or more nucleic acid targets may be detected in a single channel. Nucleic acid targets may be partitioned, amplified, used to generate signals, where each signal corresponds to a unique combination of nucleic acid targets in a partition.

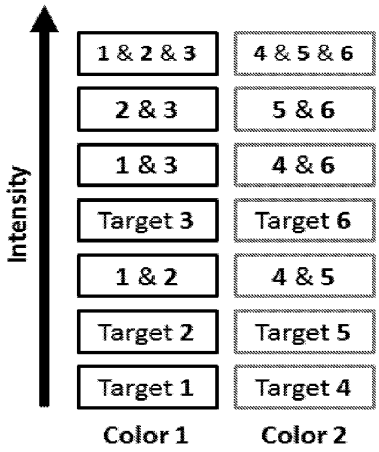


FIG. 1

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MULTIPLEXED FLUOROMETRIC MEASUREMENTS WITH DROPLET PCR SYSTEMS

CROSS-REFERENCE

[0001] This application claims the benefit of U.S. Provisional Patent Application No. 62/615,560, filed on January 10, 2018, and U.S. Provisional Patent Application No. 62/526,272, filed on June 28, 2017, which applications are incorporated herein by reference in their entirety.

BACKGROUND

[0002] Droplet Digital PCR (ddPCR) is a useful method for detection and quantification of nucleic acid targets. The use of labeled oligonucleotide probes enables specific detection of a target present in an emulsion (i.e. droplet). However, ddPCR is limited in the number of targets that can be measured in a single reaction. Current multiplex ddPCR technologies are limited to the measurement of two targets in a single reaction using two distinct color channels.

SUMMARY

[0003] Disclosed herein are methods and compositions for increasing the number of targets that can be measured in a single digital assay (e.g., digital PCR, ddPCR, etc.) by enabling the detection of multiple targets using a single optical channel. In some cases, three, four, or more targets are detected using a single optical channel. This is a key improvement over existing digital multiplexing technologies.

[0004] In some aspects, provided herein is a method for performing a digital assay, comprising: (a) partitioning into a plurality of partitions (i) three or more nucleic acid targets and (ii) a set of nucleic acid probes comprising a first nucleic acid probe, a second nucleic acid probe, and a third nucleic acid probe; (b) amplifying the three or more nucleic acid targets in the partitions; (c) generating N signals from the set of nucleic acid probes, each of which N signals corresponds to the presence of a unique combination of nucleic acid targets of the three or more nucleic acid targets in a partition of the plurality of partitions; and (d) detecting the N signals in a single optical channel; wherein, in (a), the concentration of the first nucleic acid probe is about X, the concentration of the second nucleic acid probe is at least about 2X, and the concentration of the third nucleic acid probe is at least about 4X. In some embodiments, N is 3 or more. In some embodiments, N is 4 or more. In some embodiments, the three or more nucleic acid targets are four or more nucleic acid targets. In some embodiments, the set of nucleic acid probes further comprises a fourth nucleic acid probe. In some embodiments, in (a), the concentration of the fourth nucleic acid probe is at least about 8X. In some embodiments, each of the N signals corresponds to a fluorescence intensity level. In some embodiments, the N signals are generated

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from the set of nucleic acid probes. In some embodiments, each of the set of nucleic acid probes comprises an identical fluorophore. In some embodiments, each of the set of nucleic acid probes comprises a different fluorophore. In some embodiments, the amplifying comprises polymerase chain reaction. In some embodiments, the amplifying comprises linear amplification. In some embodiments, the amplifying comprises a nucleic acid extension reaction.

[0005] In some aspects, provided herein is method for performing a digital assay, comprising: (a) partitioning into a plurality of partitions (i) three or more nucleic acid targets and (ii) a set of nucleic acid probes comprising a first nucleic acid probe, a second nucleic acid probe, and a third nucleic acid probe; (b) amplifying the three or more nucleic acid targets in the partitions; (c) generating N signals from the set of nucleic acid probes, each of which N signals corresponds to the presence of a unique combination of nucleic acid targets of the three or more nucleic acid targets in a partition of the plurality of partitions; and (d) detecting the N signals in a single optical channel; wherein, in (a), the first nucleic acid probe has an intensity level of about Y, the second nucleic acid probe has an intensity level of at least about 2Y, and the third nucleic acid probe has an intensity level of at least about 4Y. In some embodiments, the first nucleic acid probe comprises one fluorophore with an intensity level of about Y, the second nucleic acid probe comprises at least two fluorophores each with an intensity level of about Y, and the third nucleic acid probe comprises at least four fluorophores each with an intensity level of about Y. In some embodiments, the first nucleic acid probe comprises a fluorophore with an intensity level of about Y, the second nucleic acid probe comprises a fluorophore with an intensity level of at least about 2Y, and the third nucleic acid probe comprises a fluorophore with an intensity level of at least about 4Y. In some embodiments, N is 3 or more. In some embodiments, N is 4 or more. In some embodiments, the three or more nucleic acid targets are four or more nucleic acid targets. In some embodiments, the set of nucleic acid probes further comprises a fourth nucleic acid probe. In some embodiments, the fourth nucleic acid probe has an intensity level of 8X. In some embodiments, the fourth nucleic acid probe comprises a fluorophore with an intensity level of at least about 8X. In some embodiments, each of the N signals corresponds to a fluorescence intensity level. In some embodiments, the N signals are generated from the set of nucleic acid probes. In some embodiments, each of the set of nucleic acid probes comprises an identical fluorophore. In some embodiments, each of the set of nucleic acid probes comprises a different fluorophore. In some embodiments, the amplifying comprises polymerase chain reaction. In some embodiments, the amplifying comprises linear amplification. In some embodiments, the amplifying comprises a nucleic acid extension reaction.

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[0006] In some aspects, provided herein is a method for performing a digital assay, comprising: (a) partitioning three or more nucleic acid targets into a plurality of partitions; (b) amplifying the three or more nucleic acid targets in the partitions; (c) generating N signals each of which corresponds to the presence of a unique combination of nucleic acid targets of the three or more nucleic acid targets in a partition of the plurality of partitions; and (d) detecting the signals in a single optical channel. In some embodiments, N is 3 or more. In some embodiments, N is 4 or more. In some embodiments, the three or more nucleic acid targets are four or more nucleic acid targets. In some embodiments, each signal of the N signals corresponds to a fluorescence intensity level. In some embodiments, (a) further comprises partitioning a set of nucleic acid probes comprising three or more nucleic acid probes into the plurality of partitions. In some embodiments, the N signals are generated from the set of nucleic acid probes. In some embodiments, the set of nucleic acid probes each comprise one or more fluorophores. In some embodiments, the set of nucleic acid probes are provided in (a) at concentrations such that each of the N signals corresponds to the presence of a unique combination of nucleic acid targets of the three or more nucleic acid targets in the partition. In some embodiments, each nucleic acid probe of the set of nucleic acid probes comprises a different number of fluorophores, such that the signal corresponds to the presence of a unique combination of nucleic acid targets of the three or more nucleic acid targets in the partition. In some embodiments, each nucleic acid probe of the set of nucleic acid probes in (a) is provided at a different concentration. In some embodiments, the set of nucleic acid probes comprises a first nucleic acid probe, a second nucleic acid probe, and a third nucleic acid probe. In some embodiments, in (a), the concentration of the first nucleic acid probe is about X, the concentration of the second nucleic acid probe is at least about 2X, and the concentration of the third nucleic acid probe is at least about 4X. In some embodiments, the set of nucleic acid probes further comprises a fourth nucleic acid probe and wherein in (a), the concentration of the fourth nucleic acid probe is at least about 8X. In some embodiments, in (a), the first nucleic acid probe comprises one fluorophore, the second nucleic acid probe comprises at least two fluorophores, and the third nucleic acid probe comprises at least four fluorophores. In some embodiments, the set of nucleic acid probes further comprises a fourth nucleic acid probe and wherein in (a), the fourth nucleic acid probe comprises at least six fluorophores. In some embodiments, each of the set of nucleic acid probes comprises an identical fluorophore. In some embodiments, each of the set of nucleic acid probes comprises a different fluorophore. In some embodiments, the amplifying comprises polymerase chain reaction. In some embodiments, the amplifying comprises linear amplification. In some embodiments, the amplifying comprises a nucleic acid extension reaction.

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[0007] In some aspects, provided herein is a method of performing a digital assay, comprising: (a) amplifying at least three nucleic acid targets from a sample in a plurality of droplets comprising a set of nucleic acid probes comprising three or more nucleic acid probes labeled with fluorophores capable of being detected in a single optical channel, wherein each nucleic acid probe is complementary to a target of the at least three nucleic acid targets; (b) detecting N signals from the plurality of droplets if one or more of the at least three nucleic acid targets is present, wherein each of the N signals corresponds to a unique combination of one or more of the at least three nucleic acid targets present in the droplet; and (c) determining, from the N signals, the presence or absence of each of the at least three nucleic acid targets in the sample. In some embodiments, N is 3 or more. In some embodiments, N is 4 or more. In some embodiments, the three or more nucleic acid targets are four or more nucleic acid targets. In some embodiments, each of the N signals corresponds to a fluorescence intensity level. In some embodiments, the N signals are generated from the set of nucleic acid probes. In some embodiments, the set of nucleic acid probes are provided in (a) at concentrations such that the signal corresponds to the presence of a unique combination of nucleic acid targets of the three or more nucleic acid targets in the partition. In some embodiments, each nucleic acid probe of the set of nucleic acid probes comprises a different number of fluorophores, such that the signal corresponds to the presence of a unique combination of nucleic acid targets of the three or more nucleic acid targets in the partition. In some embodiments, the set of nucleic acid probes comprise a first nucleic acid probe, a second nucleic acid probe, and a third nucleic acid probe. In some embodiments, in (a), the concentration of the first nucleic acid probe is about X, the concentration of the second nucleic acid probe is at least about 2X, and the concentration of the third nucleic acid probe is at least about 4X. In some embodiments, the set of nucleic acid probes further comprise a fourth nucleic acid probe and wherein in (a), the concentration of the fourth nucleic acid probe is at least about 8X. In some embodiments, the first nucleic acid probe comprises a fluorophore with an intensity level of about X, the second nucleic acid probe comprises a fluorophore with an intensity level of at least about 2X, and the third nucleic acid probe comprises a fluorophore with an intensity level of at least about 4X. In some embodiments, the set of nucleic acid probes further comprises a fourth nucleic acid probe and wherein in (a), the fourth nucleic acid probe comprises a fluorophore with an intensity level of at least about 8X. In some embodiments, the set of nucleic acid probes each comprise one or more fluorophores. In some embodiments, the fluorophores are identical fluorophores. In some embodiments, the fluorophores are different fluorophores. In some embodiments, the amplifying comprises polymerase chain reaction. In some embodiments, the amplifying comprises linear

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amplification. In some embodiments, the amplifying comprises a nucleic acid extension reaction.

BRIEF DESCRIPTION OF THE DRAWINGS

[0008] 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:

[0009] FIG. 1 shows a schematic representation of example signal readouts from a multiplex digital assay of the present disclosure where each signal corresponds to a target or combination thereof.

[0010] FIG. 2 shows a schematic representation of example signal readouts from a multiplex digital assay of the present disclosure where each signal corresponds to a single target.

[0011] FIG. 3 shows the results of the experiment described in Example 1, demonstrating the generation of signals which uniquely correspond to each combination of nucleic acid targets which signals are detected in a single optical channel.

[0012] FIG. 4 shows the results of the experiment described in Example 2, illustrating the detection of signals in a single optical channel generated from a nucleic acid target in a droplet digital PCR reaction at various nucleic acid probe concentrations.

[0013] FIG. 5 shows the results of the experiment described in Example 4, illustrating the detection of six targets across two color channels in a single digital PCR reaction.

DETAILED DESCRIPTION

[0014] Polymerase Chain Reaction (PCR) is a method of exponential amplification of specific target nucleic acid in a reaction mix with a nucleic acid polymerase and primers. Primers are short single stranded oligonucleotides which are complementary to the 3' sequences of the positive and negative strand of the target sequence. The reaction mix is cycled in repeated heating and cooling steps. The heating cycle denatures or splits a double stranded nucleic acid target into single stranded templates. In the cooling cycle, the primers bind to complementary sequence on the template. After the template is primed the nucleic acid polymerase creates a copy of the original template. Repeated cycling exponentially amplifies the target 2 fold with each cycle leading to approximately a billion-fold increase of the target sequence in 30 cycles (Saiki et al 1988).

[0015] Digital PCR is a process of partitioning a sample containing one or more targets into a plurality of partitions (e.g., wells, droplets, etc.), performing a PCR reaction in each partition,

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and recording the fluorescence generated by, for example, a target-specific reporter probe. This is generally performed on a digital PCR instrument that measures the fluorescence from each partition in an optical channel through one or more excitation/emission filter sets.

[0016] Frequently, the target-specific nucleic acid probe is a short oligonucleotide complementary to one strand of the amplified target. The probe lacks a 3' hydroxyl and therefore is not extendable by the DNA polymerase. TaqMan (ThermoFisher Scientific) chemistry is a common reporter probe method used for multiplex Real-Time PCR (Holland et al. 1991). The TaqMan oligonucleotide probe is covalently modified with a fluorophore and a quenching tag (i.e., quencher). In this configuration the fluorescence generated by the fluorophore is quenched and is not detected by the real time PCR instrument. When the target of interest is present, the probe oligonucleotide base pairs with the amplified target. While bound, it is digested by the 5' to 3' exonuclease activity of the Taq polymerase thereby physically separating the fluorophore from the quencher and liberating signal for detection by the real time PCR instrument.

[0017] Current methods that use a unique probe for each target of interest limit the number of targets that can be measured in a single digital assay. There is a need for methods which allow for detection of multiple (e.g., three or more) targets in a single optical channel, thereby increasing the number of targets that can be measured in a single digital PCR reaction. Disclosed herein are methods and compositions for detecting three or more nucleic acid targets using digital PCR in a single optical channel.

Overview

[0018] The present disclosure provides methods for detecting more than one nucleic acid target with digital PCR in a single optical channel. In some cases, the methods of the present disclosure are used for detecting two, three, four, or more nucleic acid targets with digital PCR in a single optical channel.

[0019] In some cases, detection of multiple nucleic acid targets in a single optical channel can be achieved by providing multiple nucleic acid probes, each specific for one of multiple targets, where the probes are labeled with the same color signal tag but are provided at varying concentrations, thereby identifying each target with a unique signal readout (e.g., signal intensity value). For example, in order to detect three nucleic acid targets with digital PCR in a single optical channel, a first nucleic acid probe may be provided at a concentration of X, a second nucleic acid probe may be provided at a concentration of at least about 2X, and a third nucleic acid probe may be provided at a concentration of at least about 4X, thereby generating signals in a digital PCR reaction where each signal uniquely corresponds to the presence of each target or combination thereof in a partition.

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[0020] In one example, a sample may be suspected of comprising one or more of three nucleic targets, corresponding with influenza A virus (InfA), influenza B virus (InfB), and respiratory syncytial virus B (RsvB). Unique nucleic acid primers and probes complementary to each of the three targets are provided for amplification and detection of the targets in a digital PCR reaction. Each primer set is provided at about the same concentration. Each nucleic acid probe comprises a signal tag (e.g., fluorophore) with about equal emission wavelengths, but is added to the sample at distinct concentrations to enable unique identification of each target. A probe complementary to InfA is added at a lowest concentration (e.g., X), a probe complementary to InfB is added at a moderate concentration (e.g., at least 2X), and a probe complementary to RsvB is added at a highest concentration (e.g., at least 4X). Standard digital PCR techniques are used to separate, amplify, and detect the targets, with the assumption that only one oligonucleotide molecule per target is present per droplet. The use of distinct concentrations for the oligonucleotide probes allows for the presence or absence of each target to be detected based on a given signal intensity. In this case, a signal at the lowest intensity indicates the presence of InfA, a signal of a moderate intensity indicates the presence of InfB, and a signal of the greatest intensity indicates the presence of RsvB. By this method, all three targets can be identified in a single reaction using only a single color channel. Further, through use of precise concentrations of each probe, signals corresponding with any combination of presence or absence of the three targets can be discerned. For example, where a signal intensity of 50x indicates the presence or InfA, a signal intensity of 100x indicates the presence of InfB, and a signal intensity of 200x indicates the presence of RsvB, any combination of one, two, or three targets will result in a unique signal intensity (0x = no target present, 50x = InfA only, 100x = InfB only, 150x = InfA + InfB, 200x = RsvB only, 250x = InfA + RsvB, 300x = InfB + RsvB, 350x InfA + InfB + RsvB). In this way, it is possible to unambiguously resolve the presence or absence of the three targets, in any combination of presence or absence.

[0021] In some cases, detection of more than one nucleic acid target using a single optical channel can be achieved by providing oligonucleotide probes, each specific for one of multiple targets, where each probe is labeled with one or more copies of a luminescent signal tag, such that the probes have varying intensity levels, thereby identifying each target with a unique signal readout (e.g., signal intensity value). For example, in order to detect three nucleic acid targets with digital PCR in a single optical channel, a first nucleic acid probe having an intensity level of X may be provided, a second nucleic acid probe having an intensity level of 2X may be provided, and a third nucleic acid probe having an intensity level of 4X may be provided, thereby generating signals in a digital PCR reaction where each signal uniquely corresponds to the presence of each target and combination thereof in a partition.

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[0022] In one example, a sample may be suspected of comprising three targets, InfA, InfB, and RsvB. Unique nucleic acid primers and probes complementary to each of the three targets are provided for amplification and detection of the targets in a digital PCR reaction. Each oligonucleotide probe comprises a signal tag (e.g., fluorophore) with a different intensity level to uniquely identify each target. A probe complementary to InfA comprises a luminescent signal tag with an intensity level of X, a probe complementary to InfB comprises a luminescent signal tag with an intensity level of at least about 2X, and a probe complementary to RsvB comprises a luminescent signal tag with an intensity level of at least about 4X. The use of signal tags with varying intensity levels allows for the presence or absence of each target to be detected based on a given intensity. In this case, a signal at 1x intensity indicates the presence of InfA, a signal at 2x intensity indicates the presence of InfB, and a signal at 4x intensity indicates the presence of RsvB. Similarly, a signal intensity of 3x indicates the presence of InfA + InfB, an intensity of 5x indicates the presence of InfA + RsvB, an intensity of 6x indicates the presence of InfB + RsvB, and an intensity of 7x indicates the presence of InfA, InfB, and RsvB. By this method, the presence or absence of all three targets, in any combination, can be identified in a single reaction using only a single optical channel.

[0023] In addition to the use of varying intensities within a single color channel to identify multiple targets, multiple color channels can be combined to further increase the number of detectable targets in a single reaction. This can be achieved by providing probes specific to a given target which comprise different signal tags at varying concentrations and/or with varying intensity levels.

[0024] By combining the detection of more than one target within a single color channel with the use of multiple color channels, the amount of targets that can be detected in a single reaction can be increased significantly. In general, the number of targets that can be detected in a single digital PCR reaction can be equal to m*n, where m is the number of distinct intensity levels within a color channel and n is the number of color channels available. For example, where three targets can be detected within a given color channel, and where two color channels are used, nine unique targets can be identified in a single reaction. The ability to measure many targets using only a small number of color channels is a substantial improvement over existing multiplex ddPCR technologies.

[0025] FIG. 1 shows a schematic representation of the signal readouts which can be used with the methods of the present disclosure to detect large numbers of nucleic acid targets in a single digital PCR reaction, where each partition contains a single target or combination of targets. Each intensity level within each color may represent a unique target or target combination, as indicated.

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[0026] FIG. 2 shows a schematic representation of the signal readouts which can be used with the methods of the present disclosure to detect large numbers of nucleic acid targets in a single digital PCR reaction, where each partition comprises at most one nucleic acid target. Each intensity level within each color may represent a unique target, as indicated.

[0027] In some cases, each target or combination thereof is assigned a specific fluorescence signature (color combination and color intensity) by titrating the fluorogenic probes in specific ratios associated with that target. The probe and primer concentrations are chosen to be significantly higher than the target concentration, to mitigate for statistical sample partitioning variation (e.g. Poisson distribution of reagents amongst partitions).

[0028] In cases where each partition comprises at most one nucleic acid target, each partition may be measured individually, and its fluorescence signature used to identify the target present in that partition. Table 1 shows reagent concentrations for an example digital assay which can be used to accomplish the following signal output for multiple targets:

- 1x signal level in channel 1 and a 0x signal level in channel 2 identifies Target A
- 2x signal level in channel 1 and a 0x signal level in channel 2 identifies Target B
- 0x signal level in channel 1 and a 1x signal level in channel 2 identifies Target C
- 1x signal level in channel 1 and a 1x signal level in channel 2 identifies Target D

Table 1

	Probe Color 1	Probe Color 2	Forward Primer	Reverse Primer
Target A	300nM	0nM	1200nM	1200nM
Target B	600nM	0nM	1200nM	1200nM
Target C	0nM	300nM	1200nM	1200nM
Target D	300nM	300nM	1200nM	1200nM

Digital Assays

[0029] In some aspects, the present disclosure provides methods for performing a digital assay. A method for performing a digital assay may comprise partitioning a plurality of nucleic acid targets and a plurality of nucleic acid probes into a plurality of partitions. In some cases, two, three, four, five, or more nucleic acid targets may be partitioned into a plurality of partitions together with two, three, four, five, six, seven, eight, nine, ten, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more nucleic acid probes. Following partitioning, the nucleic acid targets may be amplified in the partitions, for example, by polymerase chain reaction (PCR). Next, N signals may be generated from the nucleic acid probes. Each signal of the N signals may correspond to the presence of a unique combination of nucleic acid targets in a partition. Following signal generation, the N signals may be detected in a single optical channel. The signals may be detected using, for example, fluorescence detection in a single color channel.

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[0030] A method for performing a digital assay may comprise amplifying nucleic acid targets from a sample in a plurality of partitions comprising nucleic acid probes complementary to the nucleic acid targets. Each nucleic acid probe may be labeled with a fluorophore. The fluorophores may be capable of being detected in a single optical channel. For example, the fluorophores may each comprise similar emission wavelength spectra, such that they can be detected in a single optical channel. Following partitioning, N signals may be detected from the plurality of partitions if one or more of the nucleic acid targets is present. Each of the N signals may correspond to a unique combination of one or more of the nucleic acid targets present in a partition. From the N signals, the presence or absence of each of the nucleic acid targets in the sample may be determined.

[0031] In some cases, the concentrations of each of the nucleic acid probes provided in the disclosed methods are such that each signal generated from the nucleic acid probes uniquely identifies a specific combination of nucleic acid targets in a partition. For example, where three nucleic acid probes are provided, a first nucleic acid probe may be provided at a concentration of about X, a second nucleic acid probe may be provided at a concentration of at least about 2X, and a third nucleic acid probe may be provided at a concentration of at least about 4X, such that a signal generated in a partition corresponds to a unique combination of nucleic acid targets in the partition. Where four nucleic acid probes are provided, a first nucleic acid probe may be provided at a concentration of about X, a second nucleic acid probe may be provided at a concentration of at least about 2X, a third nucleic acid probe may be provided at a concentration of at least about 4X, and a fourth nucleic acid probe may be provided at a concentration of at least about 8X, such that a signal generated in a partition corresponds to a unique combination of nucleic acid targets in the partition. Various combinations of nucleic acid probe concentrations may be used in the disclosed methods, as described elsewhere herein.

[0032] In some cases, the intensity levels of each of the nucleic acid probes provided in the disclosed methods are such that the signals generated from the nucleic acid probes uniquely identify a specific combination of nucleic acid targets in a partition. For example, where three nucleic acid probes are provided, a first nucleic acid probe may have an intensity level of about Y, a second nucleic acid probe may have an intensity level of at least about 2Y, and a third nucleic acid probe may have an intensity level of at least about 4Y. Where four nucleic acid probes are provided, a first nucleic acid probe may have an intensity level of about Y, a second nucleic acid probe may have an intensity level of at least about 2Y, and a third nucleic acid probe may have an intensity level of at least about 4Y, and a fourth nucleic acid probe may have an intensity level of at least about 8Y. Each nucleic acid probe may comprise one fluorophore having various intensity levels, such that each nucleic acid probe has a different intensity level.

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Alternatively or in addition, each nucleic acid probe may comprise multiple fluorophores, each having about the same intensity level, such that each nucleic acid probe has a different intensity level.

Amplification

[0033] Amplification of nucleic acid targets may be performed using a suitable amplification method. Amplification may comprise linear amplification. Amplification may comprise polymerase chain reaction. Amplification may comprise a nucleic acid extension reaction. Amplification may be performed before or after partitioning. In some cases, amplification of nucleic acid targets is performed in a plurality of partitions. For example, nucleic acid targets may be partitioned into a plurality of droplets, and amplification performed within each droplet, thereby generating a signal if at least one nucleic acid target is present in a droplet.

[0034] Amplification conditions may be modified such that the signals generated in a digital assay uniquely identify each combination of targets. Amplification conditions may comprise thermal cycling conditions, including temperature and length in time of each thermal cycle. The use of particular amplification conditions may serve to modify the signal intensity of each signal, thereby enabling each signal to correspond to a unique combination of nucleic acid targets in a partition.

Signals

[0035] A signal may be a fluorescent signal. A signal may correspond to a fluorescence intensity level. Each signal measured in the methods of the present disclosure may have a distinct fluorescence intensity value, thereby corresponding to the presence of a unique combination of nucleic acid targets in a partition. A signal may be generated by one or more nucleic acid probes. The number of signals generated in a digital assay may correspond to the number of nucleic acid probes that are partitioned, the number of nucleic acid targets that are partitioned, and, in some cases, the partitioning conditions. For example, where three nucleic acid targets and three nucleic acid probes are partitioned such that each partition may comprise one, two, or all three nucleic acid targets, seven signals may be generated, where each signal corresponds to the presence of a unique combination of the three nucleic acid targets in the partition. N may be a number of signals detected in a single optical channel in a digital assay of the present disclosure. N may be at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 25, 30, 40, 50 or more. N may be at most 50, 40, 30, 24, 20, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, or 2. N may be 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 25, 30, 40, or 50. As will be recognized and is described elsewhere herein, sets of signals may be generated in multiple different optical channels, where each set of signals is detected in a single optical channel, thereby significantly increasing the number of nucleic acid targets that can be measured in a single digital PCR reaction. In some

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cases, two sets of signals are detected in a single reaction. Each set of signals detected in a reaction may comprise the same number of signals, or different numbers of signals.

Partitioning

[0036] Methods of the present disclosure may comprise partitioning nucleic acid targets, nucleic acid probes, and, in some cases, additional reagents into a plurality of partitions. A partition may be a droplet. A partition may be an emulsion. A partition may be a well. A partition may be a microwell. Partitioning may be performed using a microfluidic device. In some cases, partitioning is performed using a droplet generator. Partitioning may comprise dividing a mixture (e.g., a mixture comprising nucleic acid targets, and nucleic acid probes) into water-in-oil droplets. A droplet may comprise one or more nucleic acid targets. A droplet may comprise a single nucleic acid target. A droplet may comprise two or more nucleic acid targets. A droplet may comprise no nucleic acid targets.

Nucleic acid probes

[0037] Any number of different nucleic acid probes may be partitioned together with nucleic acid targets in the disclosed methods. In some cases, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 30, 40, 50, or more different nucleic acid probes are partitioned into a plurality of partitions. Each probe may be specific for (e.g., capable of binding to) a given nucleic acid target in a sample. For example, three nucleic acid probes may be provided comprising a first nucleic acid probe, a second nucleic acid probe, and a third nucleic acid probe, where the first nucleic acid probe is specific for a first nucleic acid target, the second nucleic acid probe is specific for a second nucleic acid target, and a third nucleic acid probe is specific for a third nucleic acid target. Each nucleic acid probe may comprise a signal tag with about equal emission wavelengths. In some cases, each nucleic acid probe comprises an identical fluorophore. In some cases, each nucleic acid probe comprises a different fluorophore, where each fluorophore is capable of being detected in a single optical channel.

[0038] Each nucleic acid probe of a plurality of nucleic acid probes may be provided at a specific concentration, such that each signal generated from the nucleic acid probes in a partition corresponds to a unique combination of target nucleic acids. Where each signal of N signals corresponds to a unique combination of target nucleic acids, the signals may be described as "non-degenerate". In one aspect, a first nucleic acid probe is provided at a concentration of about X. Where a first nucleic acid probe is provided at a concentration of about X, additional nucleic acid probes may be provided at a specific concentration greater than X, thereby enabling the generation of non-degenerate signals.

[0039] In some cases, a second nucleic acid probe is provided at a concentration of at least about 2X, about 3X, about 4X, about 5X, about 6X, about 7X, about 8X, or more. In some cases, a

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second nucleic acid probe is provided at a concentration of at most about 8X, about 7X, about 6X, about 5X, about 4X, about 3X, or about 2X. In some cases, a second nucleic acid probe is provided at a concentration of about 2X, about 3X, about 4X, about 5X, about 6X, about 7X, or about 8X.

[0040] In some cases, a third nucleic acid probe is provided at a concentration of at least about 4X, about 5X, about 6X, about 7X, about 8X, about 9X, about 10X, about 11X, about 12X or more. In some cases, a third nucleic acid probe is provided at a concentration of at most about 12X, about 11X, about 10X, about 9X, about 8X, about 7X, about 6X, about 5X, or about 4X. In some cases, a third nucleic acid probe is provided at a concentration of about 4X, about 5X, about 6X, about 7X, about 8X, about 9X, about 10X, about 11X, or about 12X.

[0041] In some cases, a fourth nucleic acid probe is provided at a concentration of at least about 8X, about 9X, about 10X, about 11X, about 12X, about 13X, about 14X, about 15X, about 20X, or more. In some cases, a fourth nucleic acid probe is provided at a concentration of at most about 20X, about 15X, about 14X, about 13X, about 12X, about 11X, about 10X, about 9X, or about 8X. In some cases, a fourth nucleic acid probe is provided at a concentration of about 8X, about 9X, about 10X, about 11X, about 12X, about 13X, about 14X, about 15X, or about 20X.

[0042] Each nucleic acid probe may be provided at a concentration that is distinct from all other nucleic acid probes. Any combination of nucleic acid probe concentrations which is capable of producing non-degenerate signals (i.e., signals which each uniquely identify a specific combination of nucleic acid targets in a partition) may be used. In one example, a first nucleic acid probe may be provided at a concentration of about X, a second nucleic acid probe may be provided at a concentration of about 3X, and a third nucleic acid probe may be provided at a concentration of about 8X, thereby generating non-degenerate signals in a plurality of partitions corresponding to the presence of a unique combination of nucleic acid targets.

[0043] X may be a concentration of a nucleic acid probe provided in the disclosed methods. In some cases, X is at least 50 nM, 100 nM, 150 nM, 200 nM, 250 nM, 300 nM, 350 nM, 400 nM, 450 nM, 500 nM, or greater. In some cases, X is at most 500 nM, 450 nM, 400 nM, 350 nM, 300 nM, 250 nM, 200 nM, 150 nM, 100 nM, or 50 nM. X may be any concentration of a nucleic acid probe which is capable of being partitioned.

[0044] Each nucleic acid probe of a plurality of nucleic acid probes may be labeled with one or more copies of a luminescent signal tag, such that the probes have varying intensity levels, thereby enabling the generation of signals which uniquely identify each combination of nucleic acid targets in a partition. In one aspect, a first nucleic acid probe has an intensity level of about Y. Where a first nucleic acid probe has an intensity level of about Y, additional nucleic acid probes may have an intensity level greater than Y, thereby enabling the generation of non-

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degenerate signals. A first nucleic acid may comprise a fluorophore with an intensity level of about Y.

[0045] In some cases, a second nucleic acid probe has an intensity level of at least about 2Y, about 3Y, about 4Y, about 5Y, about 6Y, about 7Y, about 8Y, or more. In some cases, a second nucleic acid probe has an intensity level of at most about 8Y, about 7Y, about 6Y, about 5Y, about 4Y, about 3Y, or about 2Y. In some cases, a second nucleic acid probe has an intensity level of about 2Y, about 3Y, about 4Y, about 5Y, about 6Y, about 7Y, or about 8Y. A second nucleic acid may comprise a single luminescent signal tag (e.g., fluorophore) with an intensity level of at least about 2Y. Alternatively or in addition, a second nucleic acid may comprise two or more fluorophores each with an intensity level of about Y.

[0046] In some cases, a third nucleic acid probe has an intensity level of at least about 4Y, about 5Y, about 6Y, about 7Y, about 8Y, about 9Y, about 10Y, about 11Y, about 12Y or more. In some cases, a third nucleic acid probe has an intensity level of at most about 12Y, about 11Y, about 10Y, about 9Y, about 8Y, about 7Y, about 6Y, about 5Y, or about 4Y. In some cases, a third nucleic acid probe has an intensity level of about 4Y, about 5Y, about 6Y, about 7Y, about 8Y, about 9Y, about 10Y, about 11Y, or about 12Y. A third nucleic acid may comprise a single luminescent signal tag (e.g., fluorophore) with an intensity level of at least about 4Y. Alternatively or in addition, a third nucleic acid may comprise four or more fluorophores each with an intensity level of about Y.

[0047] In some cases, a fourth nucleic acid probe has an intensity level of at least about 8Y, about 9Y, about 10Y, about 11Y, about 12Y, about 13Y, about 14Y, about 15Y, about 20Y, or more. In some cases, a fourth nucleic acid probe has an intensity level of at most about 20Y, about 15Y, about 14Y, about 13Y, about 12Y, about 11Y, about 10Y, about 9Y, or about 8Y. In some cases, a fourth nucleic acid probe has an intensity level of about 8Y, about 9Y, about 10Y, about 11Y, about 12Y, about 13Y, about 14Y, about 15Y, or about 20Y. A fourth nucleic acid may comprise a single luminescent signal tag (e.g., fluorophore) with brightness of at least about 8Y. Alternatively or in addition, a fourth nucleic acid may comprise eight or more fluorophores each with an intensity level of about Y.

[0048] Y may be a signal intensity level produced by a nucleic acid probe comprising one or more signal tags (e.g., fluorophores). Y may be any signal intensity capable of being detected in an optical channel.

[0049] A nucleic acid probe may be a nucleic acid complementary to a region of a given nucleic acid target. A nucleic acid probe may comprise a signal tag. A signal tag may comprise a means of generating a signal such as, for example, a fluorophore. The fluorophore may be, for example, FAM, TET, HEX, JOE, Cy3, or Cy5. A nucleic acid probe may further comprise one or more

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quenchers. A quencher may inhibit signal generation from a fluorophore. A quencher may be, for example, TAMRA, BHQ-1, BHQ-2, or Dabcy.

Samples and targets

[0050] A nucleic acid target of the present disclosure may be derived from any source including, for example, viruses, bacterial cells, and eukaryotic cells. A nucleic acid target may be derived from one or more cells. A cell may be a tumor cell. A cell may be a cell suspected of comprising a viral pathogen. In some cases, a nucleic acid target is derived from a cell-free sample (e.g., serum, plasma). A nucleic acid target may be cell-free nucleic acid. Cell-free nucleic acid may be, for example, cell-free tumor DNA, cell-free fetal DNA, cell-free RNA, etc. A nucleic acid target may comprise deoxyribonucleic acid (DNA). DNA may be any kind of DNA, including genomic DNA. A nucleic acid target may be viral DNA. A nucleic acid target may comprise ribonucleic acid (RNA). RNA may be any kind of RNA, including messenger RNA, transfer RNA, ribosomal RNA, and microRNA. RNA may be viral RNA. A nucleic acid target may comprise a gene whose detection may be useful in diagnosing one or more diseases. A gene may be a viral gene or bacterial gene whose detection may be useful in identifying the presence or absence of a pathogen in a subject. In some cases, the methods of the present disclosure are useful in detecting the presence or absence or one or more infectious agents (e.g., viruses) in a subject. In some cases, the methods of the present disclosure are useful in detecting the relative amount of a fetal nucleic acid in a cell-free nucleic acid sample from a subject, thereby diagnosing the fetus for one or more genetic abnormalities. In some cases, the methods of the present disclosure are useful in detecting the presence or absence of tumor DNA in a cell-free nucleic acid sample from a subject, thereby diagnosing the subject for cancer.

EXAMPLES

Example 1

[0051] A mixture of primers, nucleic acid probes, and polymerase was added to a sample containing synthetic DNA targets for Pan InfA, InfB, and RsvB at equal concentrations of 10,000 copies per reaction. The concentration of each primer, nucleic acid probe, and polymerase is shown in Table 2.

Table 2

Reagent	Concentration
Pan InfA forward primer	3.0 μM
Pan InfA reverse primer	3.0 μM
Pan InfA probe	200 nM

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InfB forward primer	3.0 μM
InfB reverse primer	3.0 μM
InfB probe	400 nM
RsvB forward primer	3.0 μM
RsvB reverse primer	3.0 μM
RsvB probe	1.6 μM
Bio-Rad Digital PCR polymerase enzyme	2 units

[0052] 24 μL of this total reaction volume was dispersed into 80 μL of digital droplet PCR oil, generating a population of approximately 15,000 droplets. These droplets were thermally cycled using a Bio-Rad C1000 thermal cycler with the following PCR protocol: 95°C hotstart for 10min; 35 cycles at 95°C denature for 30 seconds and 57°C annealing/extension for 120 seconds. Following PCR, the droplets were then measured using the Bio-Rad QX200 droplet reader. The results of this experiment are shown in **FIG. 3**. The presence of all three targets in the ddPCR reaction mixture generated eight distinct population clusters of droplets, ranging from clusters with no targets (intensity peak of 350 AFU; "No Targets"), to clusters will all targets (intensity peak of 2800 AFU; "InfA + InfB + RsvB") as shown in **FIG. 3**. These data illustrate the ability of the disclosed methods to resolve multiple targets in a single-color channel of a digital PCR instrument.

Example 2

[0053] A probe dilution series was performed using primers, probes, and synthetic templates for a single human rhinovirus (HRV) target. The concentrations of the reactants (primers, probes, and templates) for each experiment are listed in Table 3. Seven experiments were performed (a-g), as marked, each with a different concentration of HRV probe.

Table 3

Experiment	HRV Forward Primer	HRV Reverse Primer	HRV Probe	HRV Target
a	10.0 μM	10.0 μM	0 nM	50,000 copies/rxn
b	10.0 μM	10.0 μM	100 nM	50,000 copies/rxn
c	10.0 μM	10.0 μM	200 nM	50,000 copies/rxn
d	10.0 μM	10.0 μM	500 nM	50,000 copies/rxn
e	10.0 μM	10.0 μM	1.0 μM	50,000 copies/rxn
f	10.0 μM	10.0 μM	2.0 μM	50,000 copies/rxn

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g	10.0 μM	10.0 μM	5.0 μM	50,000 copies/rxn

[0054] These reactants were added to a ddPCR polymerase enzyme mixture. 21 μL of this total reaction volume was dispersed into 80 μL of digital droplet PCR oil, generating a population of approximately 1,000 droplets. These droplets were thermally cycled using a Bio-Rad C1000 thermal cycler with the following PCR protocol: 95°C hotstart for 10min; 35 cycles at 95°C denature for 30 seconds and 57°C annealing/extension for 120 seconds. Following PCR, the droplets were then measured using the Bio-Rad QX200 droplet reader. The results of these experiments are shown in **FIG. 4**. Each reaction generated two population clusters of droplets: one at low intensity for droplets containing no amplified targets, and one at a higher intensity containing droplets with amplified target, as shown in **FIG. 4**. In experiment a, the population of empty and target-occupied droplets could not be distinguished, as no reporting probes were added, serving as a negative control. For all other experiments, distinct populations were observed for empty and target-occupied droplets. The signal intensity of the target-occupies droplets increased with increasing HRV probe concentration. These data highlight the large optical dynamic range of the disclosed multiplexing ddPCR methods.

Example 3

[0055] Targets for RsvA, RsvB, and HRV were labeled with TaqMan probes fluorescing in the FAM channel, while targets for the Pan InfA markers as well as the H1 and H3 serotypes were labeled with probes fluorescing in the VIC channel. The primer and probe mixture was designed to generate signatures specific to the presences or absence of each target. For each experiment, 8μL of the primer mixture was added to 12μL of a Bio-Rad Digital PCR polymerase enzyme master mix and 6μL of sample volume, for a total reaction volume of 24μL. The number of copies used in each reaction is shown in Table 4.

Table 4

Experiment	RsvA	HRV	RsvB	Pan InfA	InfA H1	InfA H3
a	10,000 copies/rxn	-	-	-	-	-
b	-	-	-	10,000 copies/rxn	-	10,000 copies/rxn
c	-	-	10,000 copies/rxn	10,000 copies/rxn	10,000 copies/rxn	10,000 copies/rxn
d	10,000 copies/rxn	10,000 copies/rxn	10,000 copies/rxn	10,000 copies/rxn	-	-

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e	10,000 copies/rxn	10,000 copies/rxn	10,000 copies/rxn	10,000 copies/rxn	10,000 copies/rxn	10,000 copies/rxn

[0056] This volume was dispersed in 80μL of droplet reader oil and thermally cycled under the following conditions: 95°C hotstart for 10min; 35 cycles at 95°C denature for 30 seconds and 57°C annealing/extension for 120 seconds. The droplets were then read using Bio-Rad QX200 droplet reader.

[0057] The results of these experiments are shown in **FIG. 5**. Two-dimensional signal readouts are shown on the left for each experiment (a-e, as marked), with corresponding one-dimensional signal readouts for each optical channel on the right. Each signal identifying a unique combination of nucleic acid targets in the reaction is labeled with a vertical line. These data demonstrate the detection of six targets across two color channels in a single ddPCR reaction.

[0058] The term "about" when referring to a measurable value such as an amount, a temporal duration, and the like, is meant to encompass variations of ±20% or in some instances ±10%, or in some instances ±5%, or in some instances ±1%, or in some instances ±0.1 % from the specified value, as such variations are appropriate to perform the disclosed methods. Further, “about” can mean plus or minus less than 1 or 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, or greater than 30 percent, depending upon the situation and known or knowable by one skilled in the art. About also includes the exact amount. Hence “about 200 nM” means “about 200 nM” and also “200 nM”.

[0059] 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. It is not intended that the invention be limited by the specific examples provided within the specification. While the invention has been described with reference to the aforementioned specification, the descriptions and illustrations of the embodiments herein are not meant to be construed in a limiting sense. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. Furthermore, it shall be understood that all aspects of the invention are not limited to the specific depictions, configurations or relative proportions set forth herein which depend upon a variety of conditions and variables. It should be understood that various alternatives to the embodiments of the invention described herein may be employed in practicing the invention. It is therefore contemplated that the invention shall also cover any such alternatives, modifications, variations or equivalents. 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 for performing a digital assay, comprising:
   (a) partitioning into a plurality of partitions (i) three or more nucleic acid targets and (ii) a set of nucleic acid probes comprising a first nucleic acid probe, a second nucleic acid probe, and a third nucleic acid probe;
   (b) amplifying the three or more nucleic acid targets in the partitions;
   (c) generating N signals from the set of nucleic acid probes, each of which N signals corresponds to the presence of a unique combination of nucleic acid targets of the three or more nucleic acid targets in a partition of the plurality of partitions; and
   (d) detecting the N signals in a single optical channel;
   wherein, in (a), the concentration of the first nucleic acid probe is about X, the concentration of the second nucleic acid probe is at least about 2X, and the concentration of the third nucleic acid probe is at least about 4X.
2. The method of claim 1, wherein N is 3 or more.
3. The method of any of claims 1-2 wherein N is 4 or more.
4. The method of any of claims 1-3, wherein the three or more nucleic acid targets are four or more nucleic acid targets.
5. The method of any of claims 1-4, wherein the set of nucleic acid probes further comprises a fourth nucleic acid probe.
6. The method of claim 5, wherein, in (a), the concentration of the fourth nucleic acid probe is at least about 8X.
7. The method of any of claims 1-6, wherein each of the N signals corresponds to a fluorescence intensity level.
8. The method of any of claims 1-7, wherein the N signals are generated from the set of nucleic acid probes.
9. The method of any of claims 1-8, wherein each of the set of nucleic acid probes comprises an identical fluorophore.
10. The method of any of claims 1-8, wherein each of the set of nucleic acid probes comprises a different fluorophore.
11. The method of any of claims 1-10, wherein the amplifying comprises polymerase chain reaction.
12. The method of any of claims 1-10, wherein the amplifying comprises linear amplification.

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13. The method of any of claims 1-10, wherein the amplifying comprises a nucleic acid extension reaction.

14. A method for performing a digital assay, comprising:

(a) partitioning into a plurality of partitions (i) three or more nucleic acid targets and (ii) a set of nucleic acid probes comprising a first nucleic acid probe, a second nucleic acid probe, and a third nucleic acid probe;

(b) amplifying the three or more nucleic acid targets in the partitions;

(c) generating N signals from the set of nucleic acid probes, each of which N signals corresponds to the presence of a unique combination of nucleic acid targets of the three or more nucleic acid targets in a partition of the plurality of partitions; and

(d) detecting the N signals in a single optical channel;

wherein, in (a), the first nucleic acid probe has an intensity level of about Y, the second nucleic acid probe has an intensity level of at least about 2Y, and the third nucleic acid probe has an intensity level of at least about 4Y.

15. The method of claim 14, wherein the first nucleic acid probe comprises one fluorophore with an intensity level of about Y, the second nucleic acid probe comprises at least two fluorophores each with an intensity level of about Y, and the third nucleic acid probe comprises at least four fluorophores each with an intensity level of about Y.

16. The method of claim 14, wherein the first nucleic acid probe comprises a fluorophore with an intensity level of about Y, the second nucleic acid probe comprises a fluorophore with an intensity level of at least about 2Y, and the third nucleic acid probe comprises a fluorophore with an intensity level of at least about 4Y.

17. The method of any of claims 14-16, wherein N is 3 or more.

18. The method of any of claims 14-17, wherein N is 4 or more.

19. The method of any of claims 14-18, wherein the three or more nucleic acid targets are four or more nucleic acid targets.

20. The method of any of claims 14-19, wherein the set of nucleic acid probes further comprises a fourth nucleic acid probe.

21. The method of claim 20, wherein the fourth nucleic acid probe has an intensity level of 8X.

22. The method of claim 21, wherein the fourth nucleic acid probe comprises a fluorophore with an intensity level of at least about 8X.

23. The method of any of claims 14-22, wherein each of the N signals corresponds to a fluorescence intensity level.

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24. The method of any of claims 14-23, wherein the N signals are generated from the set of nucleic acid probes.

25. The method of any of claims 14-24, wherein each of the set of nucleic acid probes comprises an identical fluorophore.

26. The method of any of claims 14-24, wherein each of the set of nucleic acid probes comprises a different fluorophore.

27. The method of any of claims 14-26, wherein the amplifying comprises polymerase chain reaction.

28. The method of any of claims 14-26, wherein the amplifying comprises linear amplification.

29. The method of any of claims 14-26, wherein the amplifying comprises a nucleic acid extension reaction.

30. A method for performing a digital assay, comprising:

(a) partitioning three or more nucleic acid targets into a plurality of partitions;

(b) amplifying the three or more nucleic acid targets in the partitions;

(c) generating N signals each of which corresponds to the presence of a unique combination of nucleic acid targets of the three or more nucleic acid targets in a partition of the plurality of partitions; and

(d) detecting the signals in a single optical channel.

31. The method of claim 30, wherein N is 3 or more.

32. The method of any of claims 30-31, wherein N is 4 or more.

33. The method of any of claims 30-32, wherein the three or more nucleic acid targets are four or more nucleic acid targets.

34. The method of any of claims 30-33, wherein each signal of the N signals corresponds to a fluorescence intensity level.

35. The method of any of claims 30-34, wherein (a) further comprises partitioning a set of nucleic acid probes comprising three or more nucleic acid probes into the plurality of partitions.

36. The method of claim 35, wherein the N signals are generated from the set of nucleic acid probes.

37. The method of claim 36, wherein the set of nucleic acid probes each comprise one or more fluorophores.

38. The method of claim 37, wherein the set of nucleic acid probes are provided in (a) at concentrations such that each of the N signals corresponds to the presence of a unique combination of nucleic acid targets of the three or more nucleic acid targets in the partition.

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39. The method of claim 37, wherein each nucleic acid probe of the set of nucleic acid probes comprises a different number of fluorophores, such that the signal corresponds to the presence of a unique combination of nucleic acid targets of the three or more nucleic acid targets in the partition.

40. The method of claim 35, wherein each nucleic acid probe of the set of nucleic acid probes in (a) is provided at a different concentration.

41. The method of claim 35, wherein the set of nucleic acid probes comprises a first nucleic acid probe, a second nucleic acid probe, and a third nucleic acid probe.

42. The method of claim 41, wherein, in (a), the concentration of the first nucleic acid probe is about X, the concentration of the second nucleic acid probe is at least about 2X, and the concentration of the third nucleic acid probe is at least about 4X.

43. The method of claim 42, wherein the set of nucleic acid probes further comprises a fourth nucleic acid probe and wherein in (a), the concentration of the fourth nucleic acid probe is at least about 8X.

44. The method of claim 41, wherein, in (a), the first nucleic acid probe comprises one fluorophore, the second nucleic acid probe comprises at least two fluorophores, and the third nucleic acid probe comprises at least four fluorophores.

45. The method of claim 44, wherein the set of nucleic acid probes further comprises a fourth nucleic acid probe and wherein in (a), the fourth nucleic acid probe comprises at least six fluorophores.

46. The method of claim 35, wherein each of the set of nucleic acid probes comprises an identical fluorophore.

47. The method of claim 35, wherein each of the set of nucleic acid probes comprises a different fluorophore.

48. The method of any of claims 30-47, wherein the amplifying comprises polymerase chain reaction.

49. The method of any of claims 30-47, wherein the amplifying comprises linear amplification.

50. The method of any of claims 30-47, wherein the amplifying comprises a nucleic acid extension reaction.

51. A method of performing a digital assay, comprising:

(a) amplifying at least three nucleic acid targets from a sample in a plurality of droplets comprising a set of nucleic acid probes comprising three or more nucleic acid probes labeled with fluorophores capable of being detected in a single

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optical channel, wherein each nucleic acid probe is complementary to a target of the at least three nucleic acid targets;

(b) detecting N signals from the plurality of droplets if one or more of the at least three nucleic acid targets is present, wherein each of the N signals corresponds to a unique combination of one or more of the at least three nucleic acid targets present in the droplet; and

(c) determining, from the N signals, the presence or absence of each of the at least three nucleic acid targets in the sample.

52. The method of claim 51, wherein N is 3 or more.

53. The method of any of claims 51-52, wherein N is 4 or more.

54. The method of any of claims 51-53, wherein the three or more nucleic acid targets are four or more nucleic acid targets.

55. The method of any of claims 51-54, wherein each of the N signals corresponds to a fluorescence intensity level.

56. The method of any of claims 51-55, wherein the N signals are generated from the set of nucleic acid probes.

57. The method of claim 56, wherein the set of nucleic acid probes are provided in (a) at concentrations such that the signal corresponds to the presence of a unique combination of nucleic acid targets of the three or more nucleic acid targets in the partition.

58. The method of any of claims 51-57, wherein each nucleic acid probe of the set of nucleic acid probes comprises a different number of fluorophores, such that the signal corresponds to the presence of a unique combination of nucleic acid targets of the three or more nucleic acid targets in the partition.

59. The method of any of claims 51-58, wherein the set of nucleic acid probes comprise a first nucleic acid probe, a second nucleic acid probe, and a third nucleic acid probe.

60. The method of claim 59, wherein, in (a), the concentration of the first nucleic acid probe is about X, the concentration of the second nucleic acid probe is at least about 2X, and the concentration of the third nucleic acid probe is at least about 4X.

61. The method of claim 60, wherein the set of nucleic acid probes further comprise a fourth nucleic acid probe and wherein in (a), the concentration of the fourth nucleic acid probe is at least about 8X.

62. The method of claim 59, wherein the first nucleic acid probe comprises a fluorophore with an intensity level of about X, the second nucleic acid probe comprises a fluorophore with an intensity level of at least about 2X, and the third nucleic acid probe comprises a fluorophore with an intensity level of at least about 4X.

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63. The method of claim 62, wherein the set of nucleic acid probes further comprises a fourth nucleic acid probe and wherein in (a), the fourth nucleic acid probe comprises a fluorophore with an intensity level of at least about 8X.

64. The method of claim 56, wherein the set of nucleic acid probes each comprise one or more fluorophores.

65. The method of claim 64, wherein the fluorophores are identical fluorophores.

66. The method of claim 64, wherein the fluorophores are different fluorophores.

67. The method of any of claims 51-66, wherein the amplifying comprises polymerase chain reaction.

68. The method of any of claims 51-66, wherein the amplifying comprises linear amplification.

69. The method of any of claims 51-66, wherein the amplifying comprises a nucleic acid extension reaction.

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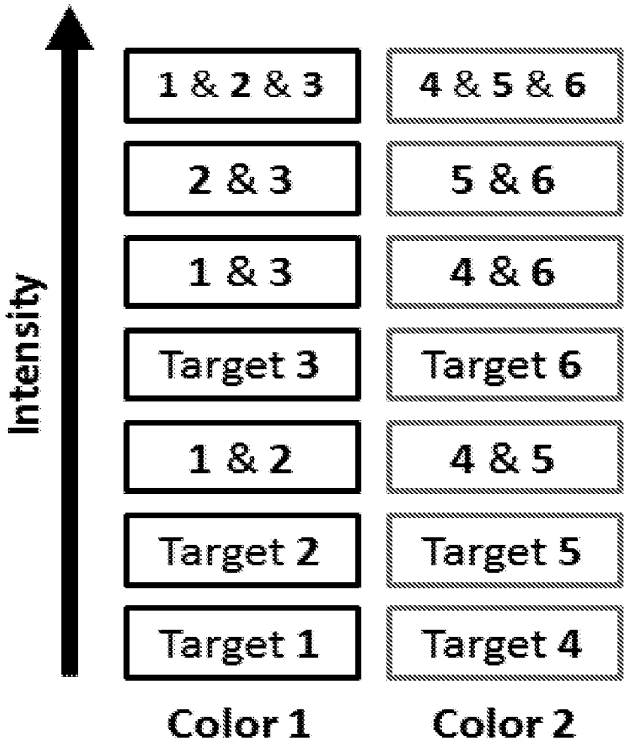

Intensity
1 & 2 & 3
2 & 3
1 & 3
Target 3
1 & 2
Target 2
Target 1
4 & 5 & 6
5 & 6
4 & 6
Target 6
4 & 5
Target 5
Target 4
Color 1
Color 2

FIG. 1

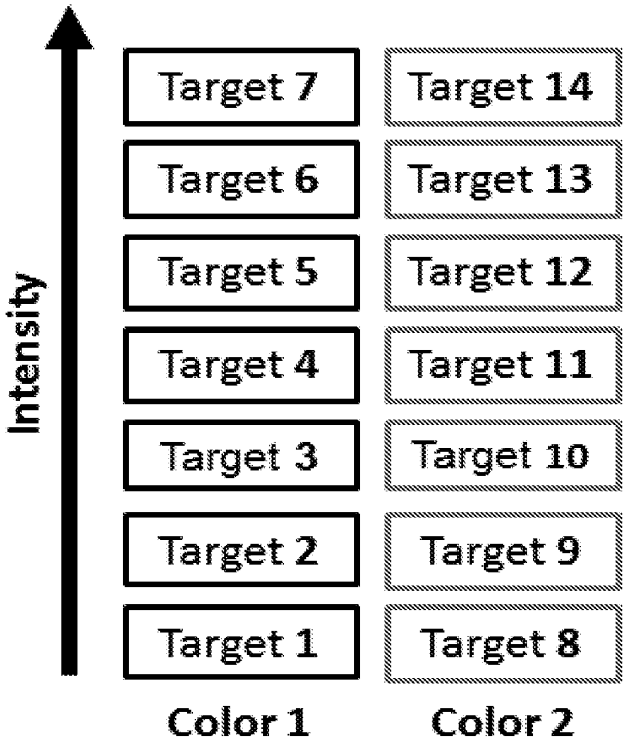

Intensity
Target 7
Target 6
Target 5
Target 4
Target 3
Target 2
Target 1
Target 14
Target 13
Target 12
Target 11
Target 10
Target 9
Target 8
Color 1
Color 2

FIG. 2

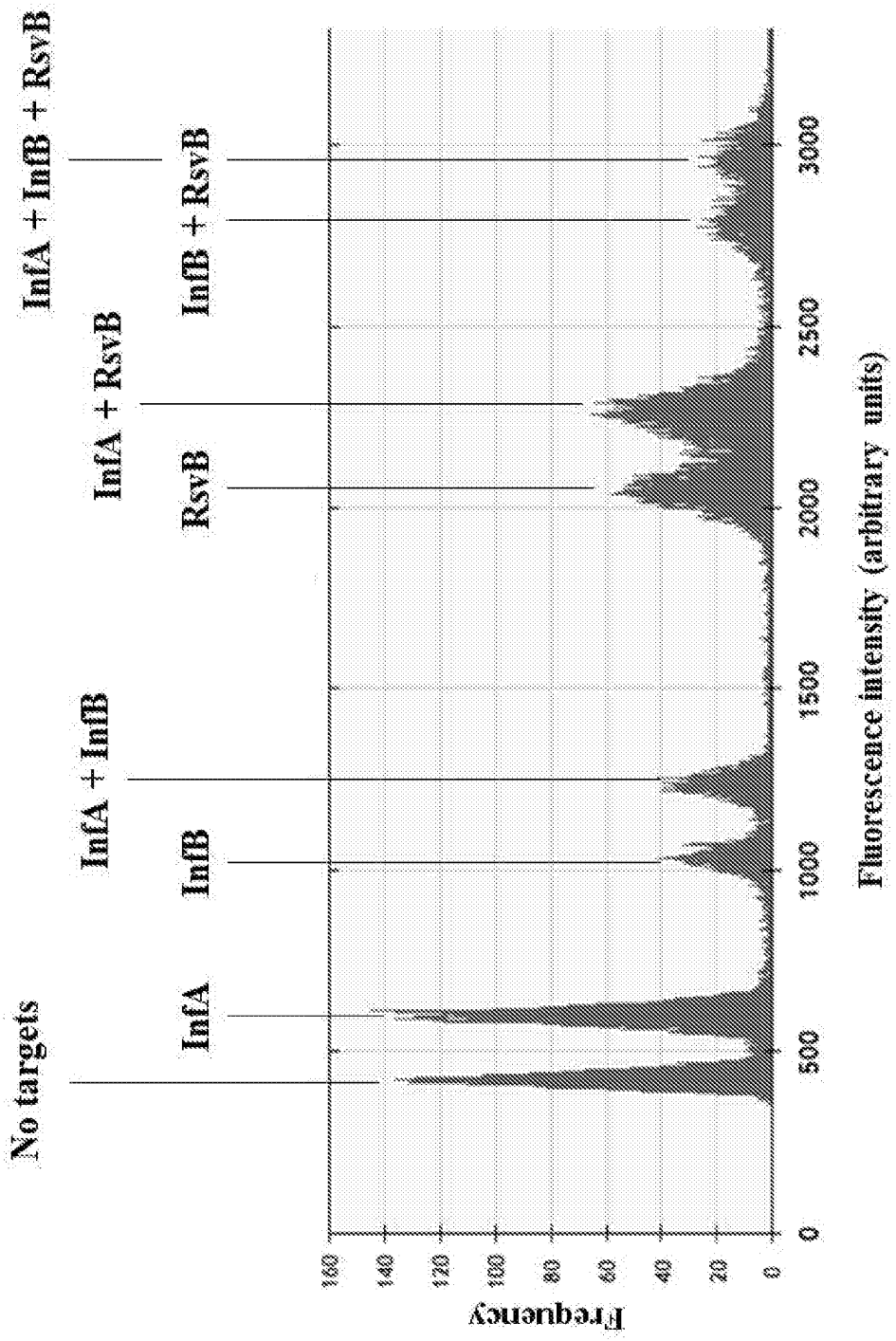

No targets
InfA
InfB
InfA + InfB
RsvB
InfA + RsvB
InfB + RsvB
InfA + InfB + RsvB
Frequency
Fluorescence intensity (arbitrary units)
0
20
40
60
80
100
120
140
160
0
500
1000
1500
2000
2500
3000

FIG. 3

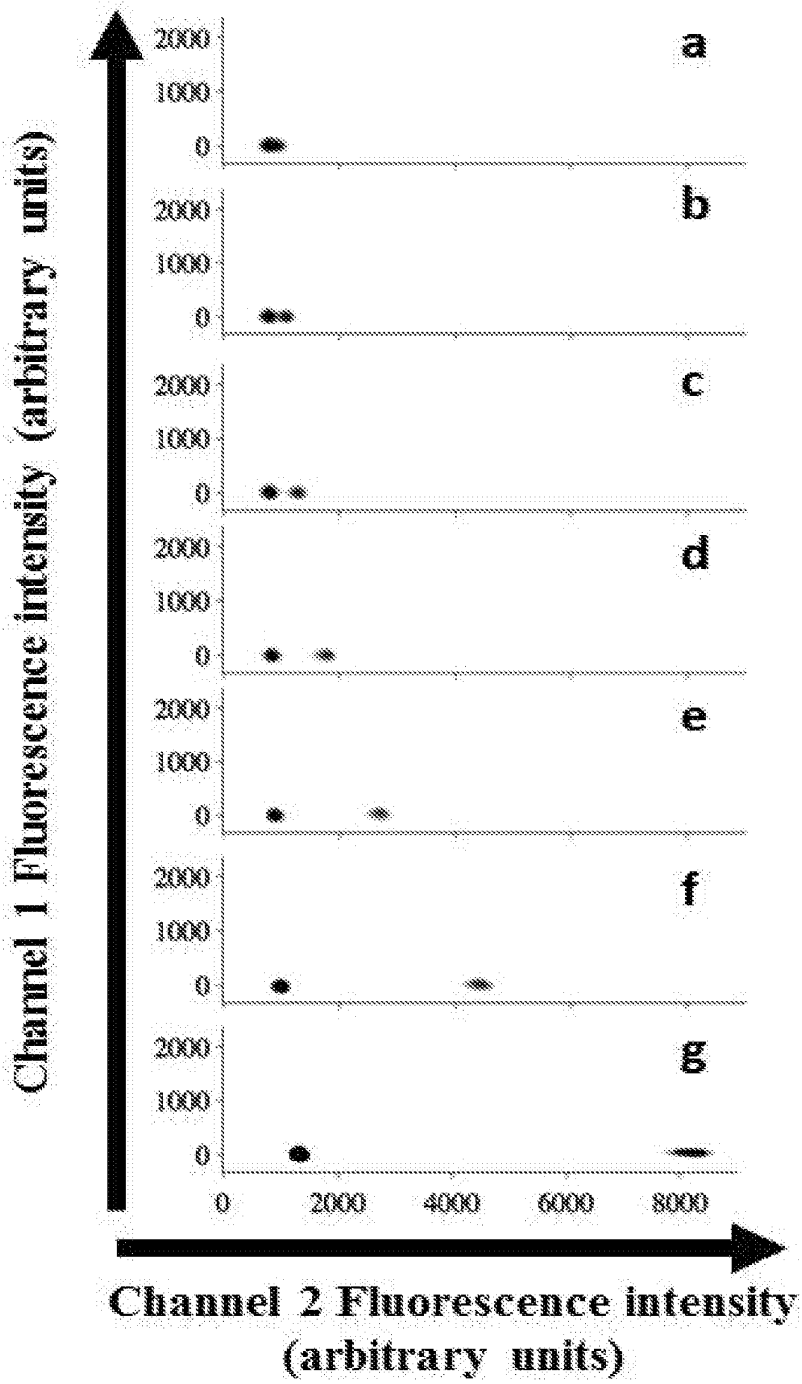

Channel 1 Fluorescence intensity (arbitrary units)
2000
1000
0
a
b
c
d
e
f
g
0
2000
4000
6000
8000
Channel 2 Fluorescence intensity
(arbitrary units)

FIG. 4

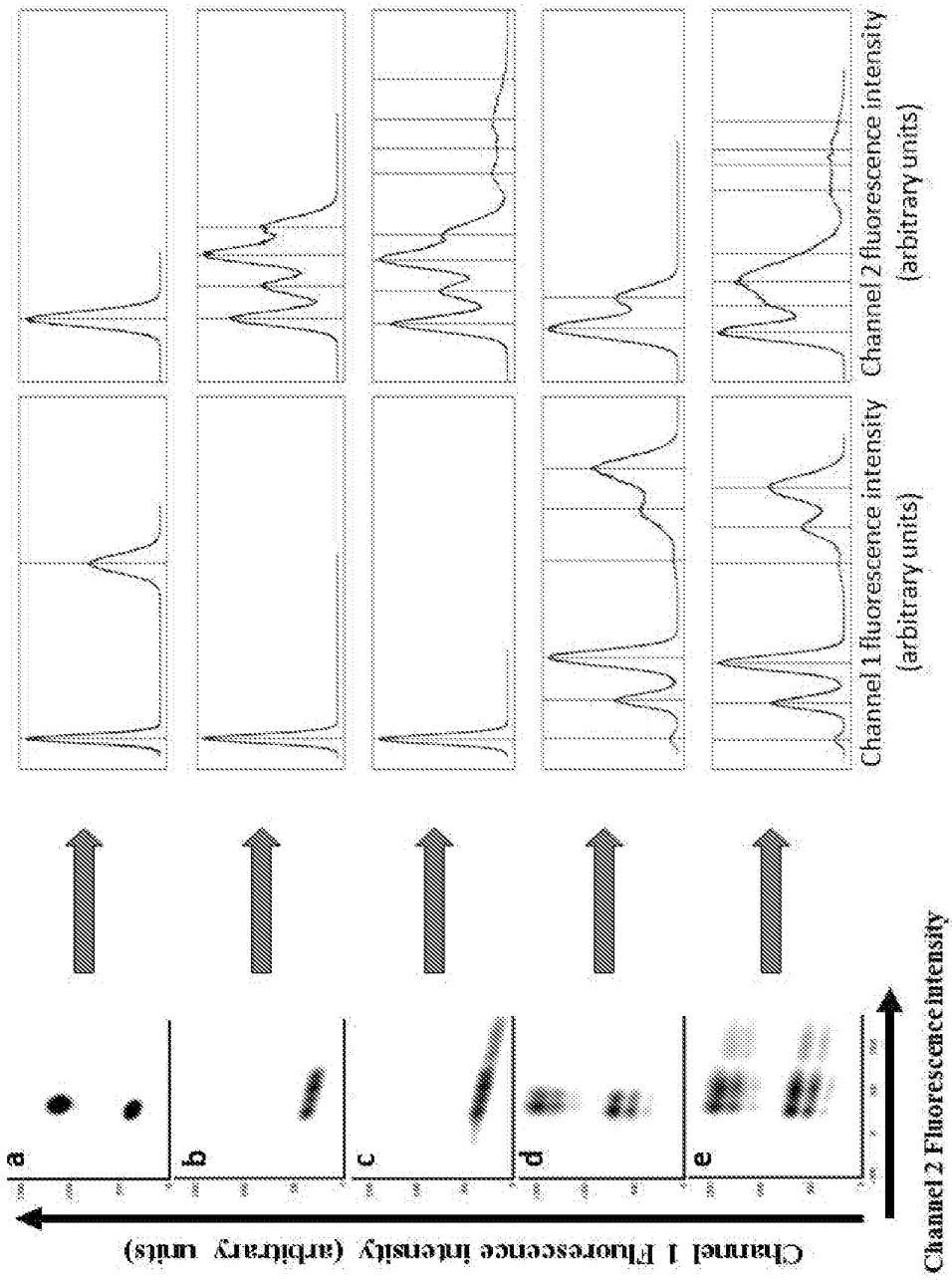

Channel 1 fluorescence intensity (arbitrary units)
Channel 2 fluorescence intensity (arbitrary units)
Channel 1 Fluorescence intensity (arbitrary units)
Channel 2 Fluorescence intensity (arbitrary units)
a
b
c
d
e

FIG. 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 18/39846

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.: 4-13, 18-29, 33-50, 54-69
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:

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.:

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 18/39846

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12Q 1/70, C12Q 1/70 (2018.01)
CPC - C12Q 1/701, C12Q 1/6818, C12Q 1/68, C12Q 1/6851, C12Q 1/686, C12Q 1/703, Y10T 436/143333, C12Q 2563/103, C12Q 2563/159

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 --- Y	US 2014/0213471 A1 (CALIFORNIA INSTITUTE OF TECHNOLOGY) 31 July 2014 (31.07.2014) Claim 3; para [0003]; para [0007]; para [0010-0011]; para [0030]; para [0035]; para [0048-0049]; para [0105] ; para [0154]; para [0176]	1-3, 30-32, 51-53 --- 14-17
Y	US 2016/0108464 A1 (BIO-RAD LABORATORIES INC.) 21 April 2016 (21.04.2016) para [0068]; para [0196]; para [0198]; para [0201]	14-17

☐ 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
21 August 2018	14 SEP 2018
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

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15 October 2018 (15.10.2018)

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15/892,245 08 February 2018 (08.02.2018) US

(63) Related by continuation (CON) or continuation-in-part (CIP) to earlier application:
US 15/892,245 (CON)
Filed on 08 February 2018 (08.02.2018)

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(74) Agent: **BRUNER, Kyle**; WILSON SONSINI GOODRICH & ROSATI, 650 Page Mill Road, Palo Alto, California 94304 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ,

(54) **Title:** METHODS AND COMPOSITIONS FOR NUCLEIC ACID DETECTION

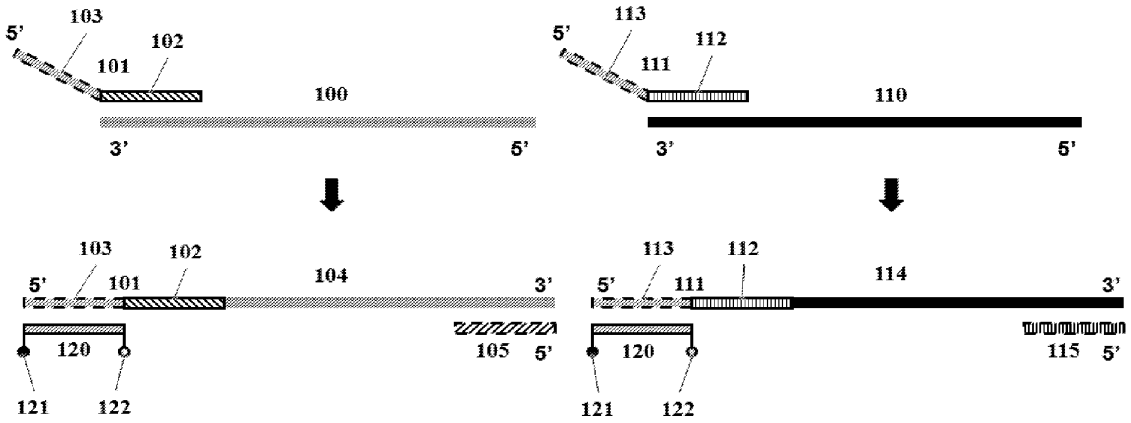


FIG. 1

(57) **Abstract:** The present disclosure provides methods and compositions for nucleic acid detection. Nucleic acids may be derived from any source including, for example, viruses, bacterial cells, and eukaryotic cells. The methods of the present disclosure may be used to detect the presence of at least one member of a plurality of nucleic acids in a sample. The methods of the present disclosure may be used to detect the presence of both a first and second member of a plurality of nucleic acids in a sample. Nucleic acids may be detected by the generation of one or more signals using, for example, polymerase chain reaction.

[Continued on next page]

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TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

Published:

— *with international search report (Art. 21(3))*

METHODS AND COMPOSITIONS FOR NUCLEIC ACID DETECTION

CROSS-REFERENCE

[0001] This application is a continuation of U.S. Patent Application No. 15/892,245, filed on February 8, 2018; and claims the benefit of U.S. Provisional Patent Application No. 62/573,046, filed on October 16, 2017; which applications are incorporated herein by reference in their entirety.

BACKGROUND

[0002] Real-Time Polymerase Chain Reaction (PCR) is a process of monitoring a PCR reaction by recording the fluorescence generated either by an intercalating dye such as SYBR Green or a reporter probe at each cycle. This is generally performed on a Real-Time PCR instrument that executes thermal cycling of the sample to complete the PCR cycles and at a specified point in each cycle measures the fluorescence of the sample in each channel through a series of excitation/emission filter sets.

SUMMARY

[0003] Quantitative real-time PCR (qPCR) is a useful technique for measuring a limited number of target analytes in a sample. Current limitations of standard qPCR instruments lie in the inability to accurately measure distinct analytes in a single reaction. Multiplex qPCR technology allows for a limited number of analytes to be detected in a reaction using individual fluorescent reporters. Additional analytes can be measured by the use of identical fluorescent reporters at different concentrations, however the requirements of deconvolution of this fluorescent signal keeps the number of analytes that can be measured in a single reaction to a minimum. Therefore, a need remains for a method of detecting the presence of any subset of a large number of target analytes from a single sample.

[0004] Disclosed herein are methods and compositions for detection of one or more nucleic acid analytes from a sample. In some aspects, one or more subsets of a large number of target analytes from a single sample may be detected using a single fluorescent signal. This is a key improvement over current multiplexing technologies.

[0005] In an aspect, the present disclosure provides a method of detecting the presence or absence of at least one member of a plurality of target nucleic acids in a sample, the method comprising: (a) providing a sample comprising, or potentially comprising, at least one member of the plurality of target nucleic acids; (b) forming a mixture comprising the sample and (i) a signal oligonucleotide probe; (ii) a first forward oligonucleotide primer comprising: a first region complementary to a first region of a first member of the plurality of target nucleic acids;

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and a second region complementary or homologous to the signal oligonucleotide probe; (iii) a first reverse oligonucleotide primer comprising a region complementary to a second region of the first member of the plurality of target nucleic acids; (iv) a second forward oligonucleotide primer comprising: a first region complementary to a first region of a second member of the plurality of target nucleic acids; and a second region complementary or homologous to the signal oligonucleotide probe; and (v) a second reverse oligonucleotide primer comprising a region complementary to a second region of the second member of the plurality of target nucleic acids; (c) thermally cycling the mixture under conditions appropriate to amplify each member of the plurality of target nucleic acids with polymerase chain reaction (PCR), such that the signal oligonucleotide probe is degraded and a signal is generated if at least one member of the plurality of target nucleic acids is present in the mixture; and (d) detecting the presence or absence of the signal. In some embodiments, one or both of the second region of the first oligonucleotide primer and the second region of the second forward oligonucleotide primer are complementary to the signal oligonucleotide probe. In some embodiments, at least one of the second region of the first oligonucleotide primer and the second region of the second forward oligonucleotide primer are homologous to the signal oligonucleotide probe. In some embodiments, the mixture further comprises a universal oligonucleotide primer. In some embodiments, the first forward oligonucleotide primer and the second forward oligonucleotide primer each further comprise a third region complementary to the universal oligonucleotide primer. In some embodiments, the mixture further comprises a nucleic acid enzyme. In some embodiments, the signal oligonucleotide probe is degraded by the nucleic acid enzyme. In some embodiments, the nucleic acid enzyme has endonuclease activity. In some embodiments, the nucleic acid enzyme has exonuclease activity. In some embodiments, the signal oligonucleotide probe is degraded by the exonuclease activity of the nucleic acid enzyme. In some embodiments, the nucleic acid enzyme is a deoxyribonucleic acid polymerase. In some embodiments, the signal oligonucleotide probe comprises a signal tag. In some embodiments, in (c), the signal is generated by the signal tag. In some embodiments, the signal tag generates the signal upon degradation of the signal oligonucleotide probe. In some embodiments, the first region of the first forward oligonucleotide primer is 5' relative to the second region of the first forward oligonucleotide primer. In some embodiments, in (c), each thermal cycle is performed at an annealing temperature appropriate for annealing of the first region of the first forward oligonucleotide primer to the first target nucleic acid and annealing of the second region of the first forward oligonucleotide primer to the signal oligonucleotide probe. In some embodiments, the first region of the second forward oligonucleotide primer is 5' relative to the second region of the second forward oligonucleotide primers. In some embodiments, the second region of the

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first forward oligonucleotide primer has between about 50% and about 100%, between about 60% and about 100%, between about 70% and about 100%, between about 80% and about 100%, between about 90% and about 100%, between about 95% and about 100%, between about 99% and about 100%, or about 100% identity with the second region of the second forward oligonucleotide primer. In some embodiments, in (c), each thermal cycle is performed at an annealing temperature appropriate for annealing of the first region of the second forward oligonucleotide primer to the second target nucleic acid and annealing of the second region of the second forward oligonucleotide primer to the signal oligonucleotide probe. In some embodiments, the method further comprises correlating the presence of the signal to the presence of at least one member of the plurality of target nucleic acids or correlating the absence of the signal with the absence of each member of the plurality of target nucleic acids.

[0006] In an aspect, the present disclosure provides a method of detecting the presence or absence of at least one member of a plurality of target nucleic acids in a sample, the method comprising: (a) providing a sample comprising, or potentially comprising, at least one member of the plurality of target nucleic acids; (b) forming a mixture of the sample and (i) a signal oligonucleotide probe; (ii) a first forward oligonucleotide primer comprising a region with complementarity to a first member of the plurality of target nucleic acids; (iii) a first reverse oligonucleotide primer comprising a region with complementarity to the first member of the plurality of target nucleic acids; (iv) a second forward oligonucleotide primer comprising a region with complementarity to a second member of the plurality of target nucleic acids; and (v) a second reverse oligonucleotide primer comprising a region with complementarity to the second member of the plurality of target nucleic acids; (c) thermally cycling the mixture under conditions appropriate to amplify each member of the plurality of target nucleic acids with polymerase chain reaction (PCR) and generating a signal if at least one member of the plurality of target nucleic acids is present in the mixture; and (d) detecting the presence or absence of the signal. In some embodiments, the mixture comprises a plurality of the first and second forward oligonucleotide primers and a plurality of the first and second oligonucleotide reverse primers. In some embodiments, the mixture further comprises a nucleic acid enzyme. In some embodiments, the signal oligonucleotide probe is degraded by the nucleic acid enzyme. In some embodiments, the signal oligonucleotide probe comprises a signal tag. In some embodiments, in (c), the signal is generated by the signal tag. In some embodiments, the signal tag generates the signal upon degradation of the signal oligonucleotide probe. In some embodiments, the region of the first forward oligonucleotide primer has complementarity to a first region of the first member of the plurality of target nucleic acids. In some embodiments, the region of the second forward oligonucleotide primer has complementarity to a second region of the first member of

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the plurality of target nucleic acids. In some embodiments, the first forward oligonucleotide primer further comprises an additional region with complementarity or homology to the signal oligonucleotide probe. In some embodiments, the additional region of the first forward oligonucleotide primer has complementarity to the signal oligonucleotide probe. In some embodiments, the additional region of the first forward oligonucleotide primer has homology to the signal oligonucleotide probe. In some embodiments, the region of the first forward oligonucleotide primer is 5' relative to the additional region of the first forward oligonucleotide primers. In some embodiments, the additional region of the first forward oligonucleotide primer has between about 50% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the additional region of the first forward oligonucleotide primer has between about 60% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the additional region of the first forward oligonucleotide primer has between about 70% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the additional region of the first forward oligonucleotide primer has between about 80% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the additional region of the first forward oligonucleotide primer has between about 90% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the additional region of the first forward oligonucleotide primer has between about 95% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the additional region of the first forward oligonucleotide primer has between about 99% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the additional region of the first forward oligonucleotide primer has about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the additional region of the first forward oligonucleotide primer has length of between about 5 nucleotides and about 500 nucleotides. In some embodiments, the additional region of the first forward oligonucleotide primer has length of between about 5 nucleotides and about 300 nucleotides. In some embodiments, the additional region of the first forward oligonucleotide primer has length of between about 5 nucleotides and about 100 nucleotides. In some embodiments, the additional region of the first forward oligonucleotide primer has length of between about 5 nucleotides and about 50 nucleotides. In some embodiments, the additional region of the first forward oligonucleotide primer has length of between about 5 nucleotides and about 30 nucleotides. In some embodiments, the additional region of the first forward oligonucleotide primer has length of between about 5 nucleotides and about 20 nucleotides. In some embodiments, the additional region of the first forward oligonucleotide primer has length of between about 5 nucleotides and about 15 nucleotides. In some embodiments, in (c), each thermal cycle is performed at an

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annealing temperature appropriate for annealing of the region of the first forward oligonucleotide primer to the first member of the plurality of target nucleic acids and annealing of the additional region of the first forward oligonucleotide primer to the signal oligonucleotide probe. In some embodiments, the second forward oligonucleotide primer further comprises an additional region with complementarity or homology to the signal oligonucleotide probe. In some embodiments, the additional region of the second forward oligonucleotide primer has complementarity to the signal oligonucleotide probe. In some embodiments, the additional region of the second forward oligonucleotide primer has homology to the signal oligonucleotide probe. In some embodiments, the region of the second forward oligonucleotide primer is 5' relative to the additional region of the second forward oligonucleotide primers. In some embodiments, the additional region of the second forward oligonucleotide primer has between about 50% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the additional region of the second forward oligonucleotide primer has between about 60% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the additional region of the second forward oligonucleotide primer has between about 70% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the additional region of the second forward oligonucleotide primer has between about 80% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the additional region of the second forward oligonucleotide primer has between about 90% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the additional region of the second forward oligonucleotide primer has between about 95% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the additional region of the second forward oligonucleotide primer has between about 99% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the additional region of the second forward oligonucleotide primer has about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the additional region of the first forward oligonucleotide primer has between about 50% and about 100% identity with the additional region of the second forward oligonucleotide primer. In some embodiments, the additional region of the second forward oligonucleotide primer has length of between about 5 nucleotides and about 500 nucleotides. In some embodiments, the additional region of the second forward oligonucleotide primer has length of between about 5 nucleotides and about 300 nucleotides. In some embodiments, the additional region of the second forward oligonucleotide primer has length of between about 5 nucleotides and about 100 nucleotides. In some embodiments, the additional region of the second forward oligonucleotide primer has length of between about 5 nucleotides and about 50 nucleotides. In some embodiments, the

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additional region of the second forward oligonucleotide primer has length of between about 5 nucleotides and about 30 nucleotides. In some embodiments, the additional region of the second forward oligonucleotide primer has length of between about 5 nucleotides and about 20 nucleotides. In some embodiments, the additional region of the second forward oligonucleotide primer has length of between about 5 nucleotides and about 15 nucleotides. In some embodiments, the additional region of the first forward oligonucleotide primer has between about 50% and about 100% identity with the additional region of the second forward oligonucleotide primer. In some embodiments, the additional region of the first forward oligonucleotide primer has between about 60% and about 100% identity with the additional region of the second forward oligonucleotide primer. In some embodiments, the additional region of the first forward oligonucleotide primer has between about 70% and about 100% identity with the additional region of the second forward oligonucleotide primer. In some embodiments, the additional region of the first forward oligonucleotide primer has between about 80% and about 100% identity with the additional region of the second forward oligonucleotide primer. In some embodiments, the additional region of the first forward oligonucleotide primer has between about 90% and about 100% identity with the additional region of the second forward oligonucleotide primer. In some embodiments, the additional region of the first forward oligonucleotide primer has between about 95% and about 100% identity with the additional region of the second forward oligonucleotide primer. In some embodiments, the additional region of the first forward oligonucleotide primer has between about 99% and about 100% identity with the additional region of the second forward oligonucleotide primer. In some embodiments, the additional region of the first forward oligonucleotide primer has about 100% identity with the additional region of the second forward oligonucleotide primer. In some embodiments, in (c), each thermal cycle is performed at an annealing temperature appropriate for annealing of the first region of the second forward oligonucleotide primer to the second member of the plurality of target nucleic acids and annealing of the additional region of the second forward oligonucleotide primer to the signal oligonucleotide probe. In some embodiments, the nucleic acid enzyme is a nucleic acid polymerase. In some embodiments, the nucleic acid enzyme has exonuclease activity. In some embodiments, the signal oligonucleotide probe is degraded by the exonuclease activity of the nucleic acid enzyme. In some embodiments, the nucleic acid enzyme has endonuclease activity. In some embodiments, the method further comprises correlating the absence of the signal with the absence of each member of the plurality of target nucleic acids. In some embodiments, the signal oligonucleotide probe does not have complementarity to any member of the plurality of target nucleic acids. In some embodiments, the method further comprises correlating the

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presence of the signal to the presence of at least one member of the plurality of target nucleic acids. In some embodiments, the nucleic acid enzyme is a deoxyribonucleic acid polymerase. In some embodiments, the deoxyribonucleic acid polymerase is a Taq polymerase. In some embodiments, the one or more target nucleic acids are deoxyribonucleic acids. In some embodiments, the one or more target nucleic acids are ribonucleic acids. In some embodiments, the signal is a fluorescent signal. In some embodiments, the signal tag comprises a fluorophore and a quencher. In some embodiments, the fluorophore is FAM, TET, HEX, JOE, Cy3, Cy5, or any combination thereof. In some embodiments, the mixture further comprises a universal oligonucleotide primer. In some embodiments, the first forward oligonucleotide primer further comprises a region with complementarity or homology to the universal oligonucleotide primer. In some embodiments, the second forward oligonucleotide primer further comprises a region with complementarity or homology to the universal oligonucleotide primer. In some embodiments, the mixture formed at (b) further comprises: an nth forward oligonucleotide primer comprising: a first region with complementarity to an nth member of the plurality of target nucleic acids; and an nth reverse oligonucleotide primer comprising a region with complementarity to a region of an nth member of the plurality of target nucleic acids; wherein n is an integer greater than 2. In some embodiments, the mixture comprises a plurality of the nth forward and nth reverse oligonucleotide primers. In some embodiments, the region of the nth forward oligonucleotide primer has complementarity to a first region of the nth member of the plurality of target nucleic acids. In some embodiments, the region of the nth reverse oligonucleotide primer has complementarity to a second region of the nth member of the plurality of target nucleic acids. In some embodiments, the nth forward oligonucleotide primer further comprises an additional region with complementarity to the signal oligonucleotide probe. In some embodiments, the region of the nth forward oligonucleotide primer is 5' relative to the additional region of the nth forward oligonucleotide primer. In some embodiments; the mixture formed at (b) further comprises: an xth signal oligonucleotide probe; a yth forward oligonucleotide primer comprising a first region with complementarity to a yth member of the plurality of target nucleic acids; and a yth reverse oligonucleotide primer comprising a region with complementarity to a second region of the yth member of the plurality of target nucleic acids; in (c), the xth signal oligonucleotide probe is degraded by the nucleic acid polymerase such that an xth signal is generated if at least one of the yth member of the plurality of target nucleic acids is present in the mixture; (e) further comprises correlating the presence of the xth signal to the presence of at least one of the yth members of the plurality target nucleic acids in the sample; and x is an integer greater than 1 and y is an integer greater than n. In some embodiments, the xth signal oligonucleotide probe comprises an xth signal tag. In some

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embodiments, the mixture comprises a plurality of the yth forward and yth reverse oligonucleotide primers. In some embodiments, the region of the yth forward oligonucleotide primer has complementarity to a first region of the yth member of the plurality of target nucleic acids. In some embodiments, the region of the yth reverse oligonucleotide primer has complementarity to a second region of the yth member of the plurality of target nucleic acids. In some embodiments, the yth forward oligonucleotide primer further comprises an additional region with complementarity to the signal oligonucleotide probe. In some embodiments, the first region of each of the plurality of yth forward oligonucleotide primers is 5' relative to the additional region of each of the plurality of yth forward oligonucleotide primers. In some embodiments, the signal tags are different. In some embodiments, the signal tags are the same. In some embodiments, the signals are different signal types. In some embodiments, the signals are different intensities of the same signal type.

[0007] In an aspect, the present disclosure provides a kit for detecting the presence or absence of at least one member of a plurality of target nucleic acids in a sample, comprising: (a) a signal oligonucleotide probe; (b) a first forward oligonucleotide primer comprising: a first region with complementarity to a first member of the plurality of target nucleic acids; and a second region with complementarity or homology to the signal oligonucleotide probe; (c) a first reverse oligonucleotide primer comprising a region with complementarity to the first member of the plurality of target nucleic acids; (d) a second forward oligonucleotide primer comprising: a first region with complementarity to a second member of the plurality of target nucleic acids; and a second region with complementarity or homology to the signal oligonucleotide probe; and (e) a second reverse oligonucleotide primer comprising a region with complementarity to the second member of the plurality of target nucleic acids. In some embodiments, the kit comprises a plurality of the first and second forward oligonucleotide primers and a plurality of the first and second oligonucleotide reverse primers. In some embodiments, the signal oligonucleotide probe comprises a signal tag. In some embodiments, the second region of the first forward oligonucleotide primer has complementarity to the signal oligonucleotide probe. In some embodiments, the second region of the first forward oligonucleotide primer has homology to the signal oligonucleotide probe. In some embodiments, the second region of the second forward oligonucleotide primer has complementarity to the signal oligonucleotide probe. In some embodiments, the second region of the second forward oligonucleotide primer has homology to the signal oligonucleotide probe. In some embodiments, the kit further comprises a universal oligonucleotide primer. In some embodiments, the first forward oligonucleotide primer further comprises a region with complementarity or homology to the universal oligonucleotide primer. In some embodiments, the first forward oligonucleotide primer further comprises a region with

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complementarity or homology to the universal oligonucleotide primer. In some embodiments, the kit further comprises: a plurality of an nth forward oligonucleotide primer comprising: a first region with complementarity to a first region of an nth member of the plurality of target nucleic acids; and a second region with complementarity to the signal oligonucleotide probe; and a plurality of an nth reverse oligonucleotide primer comprising a region with complementarity to a second region of an nth member of the plurality of target nucleic acids; wherein n is an integer greater than 2. In some embodiments, the kit further comprises: an xth signal oligonucleotide probe comprising an xth signal tag; a plurality of a yth forward oligonucleotide primer comprising: a first region with complementarity to a first region of a yth member of the plurality of target nucleic acids; and a second region with complementarity to the xth signal oligonucleotide probe; and a plurality of a yth reverse oligonucleotide primer comprising a region with complementarity to a second region of the yth member of the plurality of target nucleic acids; wherein x is an integer greater than 1 and y is an integer greater than n. In some embodiments, the signal tags are different. In some embodiments, the signal tags are the same. In some embodiments, the signal oligonucleotide probes are present in different concentrations. In some embodiments, at least one of the oligonucleotide primers is lyophilized. In some embodiments, each of the oligonucleotide primers is lyophilized. In some embodiments, the signal oligonucleotide probe is lyophilized. In some embodiments, the signal tag comprises a fluorophore and a quencher. In some embodiments, the fluorophore is FAM, TET, HEX, JOE, Cy3, Cy5, or any combination thereof. In some embodiments, the second region of the first forward oligonucleotide primer has between about 50% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the second region of the first forward oligonucleotide primer has between about 60% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the second region of the first forward oligonucleotide primer has between about 70% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the second region of the first forward oligonucleotide primer has between about 80% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the second region of the first forward oligonucleotide primer has between about 90% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the second region of the first forward oligonucleotide primer has between about 95% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the second region of the first forward oligonucleotide primer has between about 99% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the second region of the first forward oligonucleotide primer has about 100% complementarity to the signal oligonucleotide probe. In

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some embodiments, the second region of the second forward oligonucleotide primer has between about 50% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the second region of the second forward oligonucleotide primer has between about 60% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the second region of the second forward oligonucleotide primer has between about 70% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the second region of the second forward oligonucleotide primer has between about 80% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the second region of the second forward oligonucleotide primer has between about 90% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the second region of the second forward oligonucleotide primer has between about 95% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the second region of the second forward oligonucleotide primer has between about 99% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the second region of the second forward oligonucleotide primer has about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the second region of the first forward oligonucleotide primer has between about 50% and about 100% identity with the second region of the second forward oligonucleotide primer. In some embodiments, the second region of the first forward oligonucleotide primer has between about 50% identity with the second region of the second forward oligonucleotide primer. In some embodiments, the second region of the first forward oligonucleotide primer has between about 60% and about 100% identity with the second region of the second forward oligonucleotide primer. In some embodiments, the second region of the first forward oligonucleotide primer has between about 70% and about 100% identity with the second region of the second forward oligonucleotide primer. In some embodiments, the second region of the first forward oligonucleotide primer has between about 80% and about 100% identity with the second region of the second forward oligonucleotide primer. In some embodiments, the second region of the first forward oligonucleotide primer has between about 90% and about 100% identity with the second region of the second forward oligonucleotide primer. In some embodiments, the second region of the first forward oligonucleotide primer has between about 95% and about 100% identity with the second region of the second forward oligonucleotide primer. In some embodiments, the second region of the first forward oligonucleotide primer has between about 99% and about 100% identity with the second region of the second forward oligonucleotide primer. In some embodiments, the second region of the first forward oligonucleotide primer has about 100% identity with the second region of the second forward oligonucleotide primer. In

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some embodiments, the second region of the first forward oligonucleotide primer has length of between about 5 nucleotides and about 500 nucleotides. In some embodiments, the second region of the first forward oligonucleotide primer has length of between about 5 nucleotides and about 300 nucleotides. In some embodiments, the second region of the first forward oligonucleotide primer has length of between about 5 nucleotides and about 100 nucleotides. In some embodiments, the second region of the first forward oligonucleotide primer has length of between about 5 nucleotides and about 50 nucleotides. In some embodiments, the second region of the first forward oligonucleotide primer has length of between about 5 nucleotides and about 30 nucleotides. In some embodiments, the second region of the first forward oligonucleotide primer has length of between about 5 nucleotides and about 20 nucleotides. In some embodiments, the second region of the first forward oligonucleotide primer has length of between about 5 nucleotides and about 15 nucleotides. In some embodiments, the second region of the second forward oligonucleotide primer has length of between about 5 nucleotides and about 500 nucleotides. In some embodiments, the second region of the second forward oligonucleotide primer has length of between about 5 nucleotides and about 300 nucleotides. In some embodiments, the second region of the second forward oligonucleotide primer has length of between about 5 nucleotides and about 100 nucleotides. In some embodiments, the second region of the second forward oligonucleotide primer has length of between about 5 nucleotides and about 50 nucleotides. In some embodiments, the second region of the second forward oligonucleotide primer has length of between about 5 nucleotides and about 30 nucleotides. In some embodiments, the second region of the second forward oligonucleotide primer has length of between about 5 nucleotides and about 20 nucleotides. In some embodiments, the second region of the second forward oligonucleotide primer has length of between about 5 nucleotides and about 15 nucleotides.

[0008] In an aspect, the present disclosure provides a method of detecting the presence or absence of a first and second member of a plurality of target nucleic acids in a sample, the method comprising: (a) providing a sample comprising, or potentially comprising, the first and second members of the plurality of target nucleic acids; (b) forming a mixture comprising the sample and (i) a template nucleic acid; (ii) a signal oligonucleotide probe with complementarity to the template nucleic acid; (iii) a first oligonucleotide primer comprising a region with complementarity to the first member of the plurality of target nucleic acids; and (iv) a second oligonucleotide primer comprising a region with complementarity to the second member of the plurality of target nucleic acids; (c) subjecting the mixture to conditions sufficient to anneal the first oligonucleotide primer to the first member of the plurality of target nucleic acids and the second oligonucleotide primer to the second member of the plurality of target nucleic acids, such

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that the first and second oligonucleotide primers are degraded; (d) thermally cycling the mixture under reaction conditions appropriate to amplify the template nucleic acid with polymerase chain reaction (PCR), such that a signal is generated if both the first and second members of the plurality of target nucleic acids are present in the mixture; and (e) detecting the presence or absence of the signal. In some embodiments, the mixture comprises a plurality of the first and second oligonucleotide primers. In some embodiments, the region of the first oligonucleotide primer has complementarity to a first region of the first member of the plurality of target nucleic acids. In some embodiments, the region of the second oligonucleotide primer has complementarity to a first region of the second member of the plurality of target nucleic acids. In some embodiments, the signal oligonucleotide probe has complementarity to a first region of the template nucleic acid. In some embodiments, the first oligonucleotide primer further comprises an additional region with complementarity to a second region of the template nucleic acid. In some embodiments, the additional region of the first oligonucleotide primer comprises deoxyribonucleic acid. In some embodiments, in (c), the conditions are insufficient for annealing of the additional region of the first oligonucleotide primer to the first member of the plurality of target nucleic acids. In some embodiments, the additional region of the first oligonucleotide primer has between about 50% and about 100% complementarity to the template nucleic acid. In some embodiments, the additional region of the first oligonucleotide primer has between about 60% and about 100% complementarity to the template nucleic acid. In some embodiments, the additional region of the first oligonucleotide primer has between about 70% and about 100% complementarity to the template nucleic acid. In some embodiments, the additional region of the first oligonucleotide primer has between about 80% and about 100% complementarity to the template nucleic acid. In some embodiments, the additional region of the first oligonucleotide primer has between about 90% and about 100% complementarity to the template nucleic acid. In some embodiments, the additional region of the first oligonucleotide primer has between about 95% and about 100% complementarity to the template nucleic acid. In some embodiments, the additional region of the first oligonucleotide primer has between about 99% and about 100% complementarity to the template nucleic acid. In some embodiments, the additional region of the first oligonucleotide primer has about 100% complementarity to the template nucleic acid. In some embodiments, the additional region of the first oligonucleotide primer has length of between about 5 nucleotides and about 500 nucleotides. In some embodiments, the additional region of the first oligonucleotide primer has length of between about 5 nucleotides and about 300 nucleotides. In some embodiments, the additional region of the first oligonucleotide primer has length of between about 5 nucleotides and about 100 nucleotides. In some embodiments, the additional region of the first oligonucleotide primer has length of between about 5 nucleotides

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and about 50 nucleotides. In some embodiments, the additional region of the first oligonucleotide primer has length of between about 5 nucleotides and about 30 nucleotides. In some embodiments, the additional region of the first oligonucleotide primer has length of between about 5 nucleotides and about 20 nucleotides. In some embodiments, the additional region of the first oligonucleotide primer has length of between about 5 nucleotides and about 15 nucleotides. In some embodiments, the additional region of the second oligonucleotide primer has length of between about 5 nucleotides and about 500 nucleotides. In some embodiments, the second oligonucleotide primer further comprises an additional region with complementarity to a third region of the template nucleic acid. In some embodiments, the additional region of the second oligonucleotide primer comprises deoxyribonucleic acid. In some embodiments, in (c), the conditions are insufficient for annealing of the additional region of the second oligonucleotide primer to the second member of the plurality of target nucleic acids. In some embodiments, the first region of the template nucleic acid is 3' of the second region of the template nucleic acid and 5' of the third region of the template nucleic acid. In some embodiments, the additional region of the second oligonucleotide primer has between about 50% and about 100% complementarity to the template nucleic acid. In some embodiments, the additional region of the second oligonucleotide primer has between about 60% and about 100% complementarity to the template nucleic acid. In some embodiments, the additional region of the second oligonucleotide primer has between about 70% and about 100% complementarity to the template nucleic acid.

[0009] In some embodiments, the additional region of the second oligonucleotide primer has between about 80% and about 100% complementarity to the template nucleic acid. In some embodiments, the additional region of the second oligonucleotide primer has between about 90% and about 100% complementarity to the template nucleic acid. In some embodiments, the additional region of the second oligonucleotide primer has between about 95% and about 100% complementarity to the template nucleic acid. In some embodiments, the additional region of the second oligonucleotide primer has between about 99% and about 100% complementarity to the template nucleic acid. In some embodiments, the additional region of the second oligonucleotide primer has about 100% complementarity to the template nucleic acid. In some embodiments, the additional region of the first oligonucleotide primer is not identical to the additional region of the second oligonucleotide primer. In some embodiments, the additional region of the second oligonucleotide primer has length of between about 5 nucleotides and about 300 nucleotides. In some embodiments, the additional region of the second oligonucleotide primer has length of between about 5 nucleotides and about 100 nucleotides. In some embodiments, the additional region of the second oligonucleotide primer has length of between about 5 nucleotides and about

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50 nucleotides. In some embodiments, the additional region of the second oligonucleotide primer has length of between about 5 nucleotides and about 30 nucleotides. In some embodiments, the additional region of the second oligonucleotide primer has length of between about 5 nucleotides and about 20 nucleotides. In some embodiments, the additional region of the second oligonucleotide primer has length of between about 5 nucleotides and about 15 nucleotides. In some embodiments, in (c), the additional region of the first oligonucleotide primer and the additional region of the second oligonucleotide primer are released. In some embodiments, the mixture further comprises one or more nucleic acid enzymes. In some embodiments, the signal oligonucleotide probe comprises a signal tag. In some embodiments, the method further comprises (f) correlating the presence of the signal to the presence of both the first and second members of the plurality of target nucleic acids in the sample. In some embodiments, in (c), the first and second oligonucleotide primers are degraded by the one or more nucleic acid enzymes. In some embodiments, in (d), the signal is generated by the signal tag. In some embodiments, the signal tag generates the signal upon degradation of the signal oligonucleotide probe. In some embodiments, the signal oligonucleotide probe is degraded by the one or more nucleic acid enzymes. In some embodiments, the mixture in (b) further comprises (vi) a third oligonucleotide primer comprising a region with complementarity to a second region of the first member of the plurality of target nucleic acids; and (vii) a fourth oligonucleotide primer comprising a region with complementarity to a second region of the second member of the plurality of target nucleic acids. In some embodiments, the mixture comprises a plurality of the third and fourth oligonucleotide primers. In some embodiments, in (c), the conditions are sufficient to denature the plurality of target nucleic acids. In some embodiments, the method further comprises in (c), thermally cycling the mixture under reaction conditions appropriate to amplify the first and second members of the plurality of target nucleic acids with polymerase chain reaction. In some embodiments, the one or more nucleic acid enzymes comprise a nucleic acid polymerase. In some embodiments, the one or more nucleic acid enzymes have exonuclease activity. In some embodiments, the signal oligonucleotide probe is degraded by the exonuclease activity of the one or more nucleic acid enzymes. In some embodiments, the one or more nucleic acid enzymes have endonuclease activity. In some embodiments, the first oligonucleotide primer and the second oligonucleotide primer are degraded by the endonuclease activity of the one or more nucleic acid enzymes. In some embodiments, the plurality of target nucleic acids comprises a plurality of deoxyribonucleic acids. In some embodiments, the plurality of target nucleic acids comprises a plurality of ribonucleic acids. In some embodiments, the plurality of ribonucleic acids comprises a plurality of messenger ribonucleic acids. In some embodiments, the template nucleic acid is a

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deoxyribonucleic acid. In some embodiments, the template nucleic acid is a ribonucleic acid. In some embodiments, the one or more nucleic acid enzymes have RNase activity. In some embodiments, the first oligonucleotide primer and the second oligonucleotide primer are degraded by RNase activity of the one or more nucleic acid enzymes. In some embodiments, the one or more nucleic acid enzymes comprise an RNase H. In some embodiments, the one or more nucleic acid enzymes comprise an RNase III. In some embodiments, the RNase III is Dicer. In some embodiments, the one or more nucleic acid enzymes comprise an endonuclease I. In some embodiments, the endonuclease I is T7 endonuclease I. In some embodiments, the one or more nucleic acid enzymes are capable of degrading a nucleic acid comprising a non-natural nucleotide. In some embodiments, the one or more nucleic acid enzymes comprise an endonuclease V. In some embodiments, the endonuclease V is E. coli endonuclease V. In some embodiments, the region of the first oligonucleotide primer comprises one or more ribonucleotide bases. In some embodiments, the region of the second oligonucleotide primer comprises one or more ribonucleotide bases. In some embodiments, the first oligonucleotide primer further comprises at least one non-natural nucleotide. In some embodiments, the non-natural nucleotide is deoxyinosine. In some embodiments, the second oligonucleotide primer further comprises at least one non-natural nucleotide. In some embodiments, the non-natural nucleotide is deoxyinosine. In some embodiments, the signal oligonucleotide probe does not have complementarity to the any of the plurality of target nucleic acids. In some embodiments, the signal tag comprises a fluorophore and a quencher. In some embodiments, the fluorophore is FAM, TET, HEX, JOE, Cy3, Cy5, or any combination thereof. In some embodiments, the method further comprises correlating the absence of the signal with the absence of both the first and second member of the plurality of target nucleic acids.

[0010] In an aspect, the present disclosure provides a method of detecting the presence or absence of a first and second member of a plurality of target nucleic acids in a sample, the method comprising: (a) providing a sample comprising, or potentially comprising, the first and second members of the plurality of target nucleic acids (b) forming a mixture comprising the sample and (i) a template nucleic acid; (ii) a signal oligonucleotide probe with complementarity to the template nucleic acid; (iii) a first forward oligonucleotide primer comprising a region with complementarity to the first member of the plurality of target nucleic acids; (iv) a first reverse oligonucleotide primer comprising a region with complementarity the first member of the plurality of target nucleic acids; (v) a second forward oligonucleotide primer comprising a first region with complementarity to the second member of the plurality of target nucleic acids; and (vi) a second reverse oligonucleotide primer comprising a region with complementarity to the second member of the plurality of target nucleic acids; (c) thermally cycling the mixture under

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reaction conditions appropriate to amplify the first and second members of the plurality of target nucleic acids with polymerase chain reaction (PCR), such that the first forward oligonucleotide primer and the second forward oligonucleotide primer are degraded; (d) thermally cycling the mixture under reaction conditions appropriate to amplify the template nucleic acid with PCR, such that a signal is generated if both the first and second members of the plurality of target nucleic acids are present in the mixture; and (e) detecting the presence or absence of the signal. In some embodiments, the method further comprises (f) correlating the presence of the signal to the presence of both the first and second members of the plurality of target nucleic acids in the sample. In some embodiments, the signal oligonucleotide probe comprises a signal tag. In some embodiments, in (d), the signal is generated by the signal tag. In some embodiments, the signal tag generates the signal upon degradation of the signal oligonucleotide probe. In some embodiments, the signal oligonucleotide probe has complementarity to a first region of the template nucleic acid. In some embodiments, the first forward oligonucleotide primer further comprises an additional region with complementarity to a second region of the template nucleic acid. In some embodiments, in (c), the additional region of the first forward oligonucleotide primer is released. In some embodiments, in (c), each thermal cycle is performed at an annealing temperature appropriate for annealing of the region of the first forward oligonucleotide primer to the first member of the plurality of target nucleic acids and inappropriate for annealing of the additional region of the first forward oligonucleotide primer to the first member of the plurality of target nucleic acids. In some embodiments, the additional region of the first forward oligonucleotide primer has between about 50% and about 100% complementarity to the template nucleic acid. In some embodiments, the additional region of the first forward oligonucleotide primer has between about 60% and about 100% complementarity to the template nucleic acid. In some embodiments, the additional region of the first forward oligonucleotide primer has between about 70% and about 100% complementarity to the template nucleic acid. In some embodiments, the additional region of the first forward oligonucleotide primer has between about 80% and about 100% complementarity to the template nucleic acid. In some embodiments, the additional region of the first forward oligonucleotide primer has between about 90% and about 100% complementarity to the template nucleic acid. In some embodiments, the additional region of the first forward oligonucleotide primer has between about 95% and about 100% complementarity to the template nucleic acid. In some embodiments, the additional region of the first forward oligonucleotide primer has between about 99% and about 100% complementarity to the template nucleic acid. In some embodiments, the additional region of the first forward oligonucleotide primer has about 100% complementarity to the template nucleic acid. In some

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embodiments, the additional region of the first forward oligonucleotide primer has length of between about 5 nucleotides and about 500 nucleotides. In some embodiments, the additional region of the first forward oligonucleotide primer has length of between about 5 nucleotides and about 300 nucleotides. In some embodiments, the additional region of the first forward oligonucleotide primer has length of between about 5 nucleotides and about 100 nucleotides. In some embodiments, the additional region of the first forward oligonucleotide primer has length of between about 5 nucleotides and about 50 nucleotides. In some embodiments, the additional region of the first forward oligonucleotide primer has length of between about 5 nucleotides and about 30 nucleotides. In some embodiments, the additional region of the first forward oligonucleotide primer has length of between about 5 nucleotides and about 20 nucleotides. In some embodiments, the additional region of the first forward oligonucleotide primer has length of between about 5 nucleotides and about 15 nucleotides. In some embodiments, the second forward oligonucleotide primer further comprises an additional region with complementarity to a third region of the template nucleic acid. In some embodiments, in (c), the additional region of the second forward oligonucleotide primer is released. In some embodiments, in (c), each thermal cycle is performed at an annealing temperature appropriate for annealing of the region of the second forward oligonucleotide primer to the second member of the plurality of target nucleic acids and inappropriate for annealing of the additional region of the second oligonucleotide primer to the second member of the plurality of target nucleic acids. In some embodiments, the first region of the template nucleic acid is 3' of the additional region of the template nucleic acid and 5' of the third region of the template nucleic acid. In some embodiments, the additional region of the second forward oligonucleotide primer has between about 50% and about 100% complementarity to the template nucleic acid. In some embodiments, the additional region of the second forward oligonucleotide primer has between about 60% and about 100% complementarity to the template nucleic acid. In some embodiments, the additional region of the second forward oligonucleotide primer has between about 70% and about 100% complementarity to the template nucleic acid. In some embodiments, the additional region of the second forward oligonucleotide primer has between about 80% and about 100% complementarity to the template nucleic acid. In some embodiments, the additional region of the second forward oligonucleotide primer has between about 90% and about 100% complementarity to the template nucleic acid. In some embodiments, the additional region of the second forward oligonucleotide primer has between about 95% and about 100% complementarity to the template nucleic acid. In some embodiments, the additional region of the second forward oligonucleotide primer has between about 99% and about 100% complementarity to the template nucleic acid. In some

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embodiments, the additional region of the second forward oligonucleotide primer has about 100% complementarity to the template nucleic acid. In some embodiments, the additional region of the first forward oligonucleotide primer is not identical to the additional region of the second forward oligonucleotide primer. In some embodiments, the additional region of the second forward oligonucleotide primer has length of between about 5 nucleotides and about 500 nucleotides. In some embodiments, the additional region of the second forward oligonucleotide primer has length of between about 5 nucleotides and about 300 nucleotides. In some embodiments, the additional region of the second forward oligonucleotide primer has length of between about 5 nucleotides and about 100 nucleotides. In some embodiments, the additional region of the second forward oligonucleotide primer has length of between about 5 nucleotides and about 50 nucleotides. In some embodiments, the additional region of the second forward oligonucleotide primer has length of between about 5 nucleotides and about 30 nucleotides. In some embodiments, the additional region of the second forward oligonucleotide primer has length of between about 5 nucleotides and about 20 nucleotides. In some embodiments, the additional region of the second forward oligonucleotide primer has length of between about 5 nucleotides and about 15 nucleotides. In some embodiments, the region of the first forward oligonucleotide primer has complementarity to a first region of the first member of the plurality of target nucleic acids. In some embodiments, the region of the first reverse oligonucleotide primer has complementarity to a second region of the first member of the plurality of target nucleic acids. In some embodiments, the region of the second forward oligonucleotide primer has complementarity to a first region of the second member of the plurality of target nucleic acids. In some embodiments, the region of the second reverse oligonucleotide primer has complementarity to a second region of the second member of the plurality of target nucleic acids. In some embodiments, mixture further comprises a nucleic acid enzyme. In some embodiments, in (c), the first and second oligonucleotide primers are degraded by the nucleic acid enzyme. In some embodiments, in (d), the signal oligonucleotide probe is degraded by the nucleic acid enzyme. In some embodiments, (c) and (d) are performed substantially simultaneously. In some embodiments, the signal oligonucleotide probe does not have complementarity to the any of the plurality of target nucleic acids. In some embodiments, the nucleic acid enzyme is a nucleic acid polymerase. In some embodiments, the nucleic acid enzyme has exonuclease activity. In some embodiments, the nucleic acid enzyme has endonuclease activity. In some embodiments, the first forward oligonucleotide primer and the second forward oligonucleotide primer are degraded by the endonuclease activity of the nucleic acid enzyme. In some embodiments, the signal is a fluorescent signal. In some embodiments, the signal tag comprises a fluorophore and a quencher. In some embodiments, the fluorophore is

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FAM, TET, HEX, JOE, Cy3, Cy5, or any combination thereof. In some embodiments, the method further comprises correlating the absence of the signal with the absence of both the first and second member of the plurality of target nucleic acids. In an aspect, the present disclosure provides a kit for detecting the presence or absence of a first and second member of a plurality of target nucleic acids in a sample, comprising: (a) a template nucleic acid; (b) a signal oligonucleotide probe with complementarity to a first region of the template nucleic acid; (c) a first oligonucleotide primer comprising: a first region with complementarity to a region of the first member of the plurality of target nucleic acids; and a second region with complementarity to a second region of the template nucleic acid; and (d) a second oligonucleotide primer comprising: a first region with complementarity to a region of the second member of the plurality of target nucleic acids; and a second region with complementarity to a third region of the template nucleic acid. In some embodiments, the kit comprises a plurality of the first and second oligonucleotide primers. In some embodiments, the signal oligonucleotide probe comprises a signal tag. In some embodiments, the signal oligonucleotide probe has complementarity to a first region of the template nucleic acid. In some embodiments, the kit further comprises (e) a third oligonucleotide primer comprising a region with complementarity to an additional region of the first member of the plurality of target nucleic acids; and (f) a fourth oligonucleotide primer comprising a region with complementarity to an additional region of the second member of the plurality of target nucleic acids. In some embodiments, the mixture comprises a plurality of the third and fourth oligonucleotide primers. In some embodiments, the kit further comprises a nucleic acid enzyme. In some embodiments, the nucleic acid enzyme is a nucleic acid polymerase. In some embodiments, the nucleic acid enzyme is an RNase. In some embodiments, the RNase is an RNase III. In some embodiments, the RNase III is Dicer. In some embodiments, the nucleic acid enzyme is an endonuclease. In some embodiments, the endonuclease is an endonuclease I. In some embodiments, the endonuclease I is T7 endonuclease I. In some embodiments, the signal oligonucleotide probe does not have complementarity to the any of the plurality of target nucleic acids. In some embodiments, the first region of the template nucleic acid is 3' of the second region of the template nucleic acid and 5' of the third region of the template nucleic acid. In some embodiments, the signal tag comprises a fluorophore and a quencher. In some embodiments, the fluorophore is FAM, TET, HEX, JOE, Cy3, Cy5, or any combination thereof. In some embodiments, the second region of the first oligonucleotide primer has between about 50% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of the first oligonucleotide primer has between about 60% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of the first oligonucleotide primer has between about

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70% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of the first oligonucleotide primer has between about 80% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of the first oligonucleotide primer has between about 90% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of the first oligonucleotide primer has between about 95% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of the first oligonucleotide primer has between about 99% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of the first oligonucleotide primer has about 100% complementarity to the template nucleic acid. In some embodiments, the second region of the second oligonucleotide primer has between about 50% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of the second oligonucleotide primer has between about 60% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of the second oligonucleotide primer has between about 70% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of the second oligonucleotide primer has between about 80% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of the second oligonucleotide primer has between about 90% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of the second oligonucleotide primer has between about 95% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of the second oligonucleotide primer has between about 99% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of the second oligonucleotide primer has about 100% complementarity to the template nucleic acid. In some embodiments, the second region of the first oligonucleotide primer is not identical to the second region of the second oligonucleotide primer. In some embodiments, the second region of the first oligonucleotide primer has length of between about 5 nucleotides and about 500 nucleotides. In some embodiments, the second region of the first oligonucleotide primer has length of between about 5 nucleotides and about 300 nucleotides. In some embodiments, the second region of the first oligonucleotide primer has length of between about 5 nucleotides and about 100 nucleotides. In some embodiments, the second region of the first oligonucleotide primer has length of between about 5 nucleotides and about 50 nucleotides. In some embodiments, the second region of the first oligonucleotide primer has length of between about 5 nucleotides and about 30 nucleotides. In some embodiments, the second region of the first oligonucleotide primer has length of between about 5 nucleotides and about 20 nucleotides. In some embodiments, the second region of the first oligonucleotide primer has length of between

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about 5 nucleotides and about 15 nucleotides. In some embodiments, the second region of the second oligonucleotide primer has length of between about 5 nucleotides and about 500 nucleotides. In some embodiments, the second region of the second oligonucleotide primer has length of between about 5 nucleotides and about 300 nucleotides. In some embodiments, the second region of the second oligonucleotide primer has length of between about 5 nucleotides and about 100 nucleotides. In some embodiments, the second region of the second oligonucleotide primer has length of between about 5 nucleotides and about 50 nucleotides. In some embodiments, the second region of the second oligonucleotide primer has length of between about 5 nucleotides and about 30 nucleotides. In some embodiments, the second region of the second oligonucleotide primer has length of between about 5 nucleotides and about 20 nucleotides. In some embodiments, the second region of the second oligonucleotide primer has length of between about 5 nucleotides and about 15 nucleotides. In some embodiments, at least one of the oligonucleotide primers is lyophilized. In some embodiments, each of the oligonucleotide primers is lyophilized. In some embodiments, the signal oligonucleotide probe is lyophilized. In some embodiments, the template nucleic acid is lyophilized.

[0011] In an aspect, the present disclosure provides a method of detecting the presence or absence of at least one member of a plurality of target nucleic acids in a sample, the method comprising: (a) providing a sample comprising, or potentially comprising, at least one member of the plurality of target nucleic acids; (b) forming a mixture of the sample and (i) a signal oligonucleotide probe comprising a signal tag; (ii) a plurality of a first forward oligonucleotide primer comprising: a first region with complementarity to a first region of a first member of the plurality of target nucleic acids; and a second region with complementarity to the signal oligonucleotide probe; (iii) a plurality of a first reverse oligonucleotide primer comprising a region with complementarity to a second region of the first member of the plurality of target nucleic acids; (iv) a plurality of a second forward oligonucleotide primer comprising: a first region with complementarity to a first region of a second member of the plurality of target nucleic acids; and a second region with complementarity to the signal oligonucleotide probe; (v) a plurality of a second reverse oligonucleotide primer comprising a region with complementarity to a second region of the second member of the plurality of target nucleic acids; and (vi) a nucleic acid enzyme; (c) thermally cycling the mixture under reaction conditions appropriate to amplify each member of the plurality of target nucleic acids with polymerase chain reaction (PCR), such that the signal oligonucleotide probe is degraded by the nucleic acid enzyme such that a signal is generated if at least one member of the plurality of target nucleic acids is present in the mixture; (d) detecting the presence or absence of the signal; and (e) correlating the presence of the signal to the presence of at least one of the members of the plurality of target

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nucleic acids in the sample. In some embodiments, the mixture formed at (b) further comprises: a plurality of an nth forward oligonucleotide primer comprising: a first region with complementarity to a first region of an nth member of the plurality of target nucleic acids; and a second region with complementarity to the signal oligonucleotide probe; and a plurality of an nth reverse oligonucleotide primer comprising a region with complementarity to a second region of an nth member of the plurality of target nucleic acids; wherein n is an integer greater than 2. In some embodiments; the mixture formed at (b) further comprises: an xth signal oligonucleotide probe comprising an xth signal tag; a plurality of a yth forward oligonucleotide primer comprising: a first region with complementarity to a first region of a yth member of the plurality of target nucleic acids; and a second region with complementarity to the xth signal oligonucleotide probe; and a plurality of a yth reverse oligonucleotide primer comprising a region with complementarity to a second region of the yth member of the plurality of target nucleic acids; in (c), the xth signal oligonucleotide probe is degraded by the nucleic acid polymerase such that an xth signal is generated if at least one of the yth member of the plurality of target nucleic acids is present in the mixture; (e) further comprises correlating the presence of the xth signal to the presence of at least one of the yth members of the plurality target nucleic acids in the sample; and x is an integer greater than 1 and y is an integer greater than n. In some embodiments, the nucleic acid enzyme is a nucleic acid polymerase. In some embodiments, the nucleic acid enzyme has exonuclease activity. In some embodiments, the signal oligonucleotide probe is degraded by the exonuclease activity of the nucleic acid enzyme. In some embodiments, the nucleic acid enzyme has endonuclease activity. In some embodiments, the method further comprises correlating the absence of the signal with the absence of each member of the plurality of target nucleic acids. In some embodiments, the signal oligonucleotide probe does not have complementarity to any member of the plurality of target nucleic acids. In some embodiments, the first region of each of the plurality of first forward oligonucleotide primers is 5' relative to the second region of each of the plurality of first forward oligonucleotide primers. In some embodiments, the first region of each of the plurality of second forward oligonucleotide primers is 5' relative to the second region of each of the plurality of second forward oligonucleotide primers. In some embodiments, the first region of each of the plurality of nth forward oligonucleotide primers is 5' relative to the second region of each of the plurality of nth forward oligonucleotide primers. In some embodiments, the first region of each of the plurality of yth forward oligonucleotide primers is 5' relative to the second region of each of the plurality of yth forward oligonucleotide primers. In some embodiments, in (c), each thermal cycle is performed at an annealing temperature appropriate for annealing of the first region of each of the plurality of first forward oligonucleotide primers to the first member of the plurality of target nucleic

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acids and annealing of the second region of each of the plurality of first forward oligonucleotide primers to the signal oligonucleotide probe. In some embodiments, in (c), each thermal cycle is performed at an annealing temperature appropriate for annealing of the first region of each of the plurality of second forward oligonucleotide primers to the second member of the plurality of target nucleic acids and annealing of the second region of each of the plurality of second forward oligonucleotide primers to the signal oligonucleotide probe. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has between about 50% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has between about 60% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has between about 70% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has between about 80% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has between about 90% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has between about 95% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has between about 99% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the second region of each of the plurality of second forward oligonucleotide primers has between about 50% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the second region of each of the plurality of second forward oligonucleotide primers has between about 60% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the second region of each of the plurality of second forward oligonucleotide primers has between about 70% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the second region of each of the plurality of second forward oligonucleotide primers has between about 80% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the second region of each of the plurality of second forward oligonucleotide primers has between about 90% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the second region of each of the plurality of second forward oligonucleotide primers has between about 95% and

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about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the second region of each of the plurality of second forward oligonucleotide primers has between about 99% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the second region of each of the plurality of second forward oligonucleotide primers has about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has between about 50% and about 100% identity with the second region of each of the plurality of second forward oligonucleotide primers. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has between about 50% and about 100% identity with the second region of each of the plurality of second forward oligonucleotide primers. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has between about 60% and about 100% identity with the second region of each of the plurality of second forward oligonucleotide primers. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has between about 70% and about 100% identity with the second region of each of the plurality of second forward oligonucleotide primers. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has between about 80% and about 100% identity with the second region of each of the plurality of second forward oligonucleotide primers. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has between about 90% and about 100% identity with the second region of each of the plurality of second forward oligonucleotide primers. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has between about 95% and about 100% identity with the second region of each of the plurality of second forward oligonucleotide primers. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has between about 99% and about 100% identity with the second region of each of the plurality of second forward oligonucleotide primers. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has about 100% identity with the second region of each of the plurality of second forward oligonucleotide primers. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has length of between about 5 nucleotides and about 500 nucleotides. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has length of between about 5 nucleotides and about 300 nucleotides. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has length of between about 5 nucleotides and about 100 nucleotides. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has length of

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between about 5 nucleotides and about 50 nucleotides. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has length of between about 5 nucleotides and about 30 nucleotides. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has length of between about 5 nucleotides and about 20 nucleotides. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has length of between about 5 nucleotides and about 15 nucleotides. In some embodiments, the second region of each of the plurality of second forward oligonucleotide primers has length of between about 5 nucleotides and about 500 nucleotides. In some embodiments, the second region of each of the plurality of second forward oligonucleotide primers has length of between about 5 nucleotides and about 300 nucleotides. In some embodiments, the second region of each of the plurality of second forward oligonucleotide primers has length of between about 5 nucleotides and about 100 nucleotides. In some embodiments, the second region of each of the plurality of second forward oligonucleotide primers has length of between about 5 nucleotides and about 50 nucleotides. In some embodiments, the second region of each of the plurality of second forward oligonucleotide primers has length of between about 5 nucleotides and about 30 nucleotides. In some embodiments, the second region of each of the plurality of second forward oligonucleotide primers has length of between about 5 nucleotides and about 20 nucleotides. In some embodiments, the second region of each of the plurality of second forward oligonucleotide primers has length of between about 5 nucleotides and about 15 nucleotides. In some embodiments, the nucleic acid enzyme is a deoxyribonucleic acid polymerase. In some embodiments, the deoxyribonucleic acid polymerase is a Taq polymerase. In some embodiments, the one or more target nucleic acids are deoxyribonucleic acids. In some embodiments, the one or more target nucleic acids are ribonucleic acids. In some embodiments, the signal is a fluorescent signal. In some embodiments, the signal tag comprises a fluorophore and a quencher. In some embodiments, the fluorophore is FAM, TET, HEX, JOE, Cy3, Cy5, or any combination thereof. In some embodiments, the signal tags are different. In some embodiments, the signal tags are the same. In some embodiments, the signals are different signal types. In some embodiments, the signals are different intensities of the same signal type.

[0012] In an aspect, the present disclosure provides a kit for detecting the presence or absence of at least one member of a plurality of target nucleic acids in a sample, comprising: (a) a signal oligonucleotide probe comprising a signal tag; (b) a plurality of a first forward oligonucleotide primer comprising: a first region with complementarity to a first region of a first member of the plurality of target nucleic acids; and a second region with complementarity to the signal oligonucleotide probe; (c) a plurality of a first reverse oligonucleotide primer comprising a

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region with complementarity to a second region of the first member of the plurality of target nucleic acids; (d) a plurality of a second forward oligonucleotide primer comprising: a first region with complementarity to a first region of a second member of the plurality of target nucleic acids; and a second region with complementarity to the signal oligonucleotide probe; (e) a plurality of a second reverse oligonucleotide primer comprising a region with complementarity to a second region of the second member of the plurality of target nucleic acids. In some embodiments, the kit further comprises: a plurality of an nth forward oligonucleotide primer comprising: a first region with complementarity to a first region of an nth member of the plurality of target nucleic acids; and a second region with complementarity to the signal oligonucleotide probe; and a plurality of an nth reverse oligonucleotide primer comprising a region with complementarity to a second region of an nth member of the plurality of target nucleic acids; wherein n is an integer greater than 2. In some embodiments, the kit further comprises: an xth signal oligonucleotide probe comprising an xth signal tag; a plurality of a yth forward oligonucleotide primer comprising: a first region with complementarity to a first region of a yth member of the plurality of target nucleic acids; and a second region with complementarity to the xth signal oligonucleotide probe; and a plurality of a yth reverse oligonucleotide primer comprising a region with complementarity to a second region of the yth member of the plurality of target nucleic acids; wherein x is an integer greater than 1 and y is an integer greater than n. In some embodiments, the signal tags are different. In some embodiments, the signal tags are the same. In some embodiments, the signal oligonucleotide probes are present in different concentrations. In some embodiments, at least one of the oligonucleotide primers is lyophilized. In some embodiments, each of the oligonucleotide primers is lyophilized. In some embodiments, the signal oligonucleotide probe is lyophilized. In some embodiments, the signal tag comprises a fluorophore and a quencher. In some embodiments, the fluorophore is FAM, TET, HEX, JOE, Cy3, Cy5, or any combination thereof. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has between about 50% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has between about 60% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has between about 70% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has between about 80% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has between about 90% and

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about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has between about 95% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has between about 99% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the second region of each of the plurality of second forward oligonucleotide primers has between about 50% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the second region of each of the plurality of second forward oligonucleotide primers has between about 60% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the second region of each of the plurality of second forward oligonucleotide primers has between about 70% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the second region of each of the plurality of second forward oligonucleotide primers has between about 80% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the second region of each of the plurality of second forward oligonucleotide primers has between about 90% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the second region of each of the plurality of second forward oligonucleotide primers has between about 95% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the second region of each of the plurality of second forward oligonucleotide primers has between about 99% and about 100% complementarity to the signal oligonucleotide probe. In some embodiments, the second region of each of the plurality of second forward oligonucleotide primers has about 100% complementarity to the signal oligonucleotide probe.. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has between about 50% and about 100% identity with the second region of each of the plurality of second forward oligonucleotide primers. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has between about 50% identity with the second region of each of the plurality of second forward oligonucleotide primers. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has between about 60% and about 100% identity with the second region of each of the plurality of second forward oligonucleotide primers. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has between about 70% and about 100% identity with the second region of each of the plurality of second forward oligonucleotide primers. In some embodiments, the

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second region of each of the plurality of first forward oligonucleotide primers has between about 80% and about 100% identity with the second region of each of the plurality of second forward oligonucleotide primers. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has between about 90% and about 100% identity with the second region of each of the plurality of second forward oligonucleotide primers. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has between about 95% and about 100% identity with the second region of each of the plurality of second forward oligonucleotide primers. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has between about 99% and about 100% identity with the second region of each of the plurality of second forward oligonucleotide primers. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has about 100% identity with the second region of each of the plurality of second forward oligonucleotide primers. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has length of between about 5 nucleotides and about 500 nucleotides. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has length of between about 5 nucleotides and about 300 nucleotides. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has length of between about 5 nucleotides and about 100 nucleotides. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has length of between about 5 nucleotides and about 50 nucleotides. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has length of between about 5 nucleotides and about 30 nucleotides. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has length of between about 5 nucleotides and about 20 nucleotides. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has length of between about 5 nucleotides and about 15 nucleotides. In some embodiments, the second region of each of the plurality of second forward oligonucleotide primers has length of between about 5 nucleotides and about 500 nucleotides. In some embodiments, the second region of each of the plurality of second forward oligonucleotide primers has length of between about 5 nucleotides and about 300 nucleotides. In some embodiments, the second region of each of the plurality of second forward oligonucleotide primers has length of between about 5 nucleotides and about 100 nucleotides. In some embodiments, the second region of each of the plurality of second forward oligonucleotide primers has length of between about 5 nucleotides and about 50 nucleotides. In some embodiments, the second region of each of the plurality of second forward oligonucleotide primers has length of between about 5 nucleotides and about 30 nucleotides. In some

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embodiments, the second region of each of the plurality of second forward oligonucleotide primers has length of between about 5 nucleotides and about 20 nucleotides. In some embodiments, the second region of each of the plurality of second forward oligonucleotide primers has length of between about 5 nucleotides and about 15 nucleotides.

[0013] In an aspect, the present disclosure provides a method of detecting the presence or absence of a first and second member of a plurality of target nucleic acids in a sample, the method comprising: (a) providing a sample comprising, or potentially comprising, the first and second members of the plurality of target nucleic acids (b) forming a mixture comprising the sample and (i) a template nucleic acid; (ii) a signal oligonucleotide probe with complementarity to a first region of the template nucleic acid, the signal oligonucleotide probe comprising a signal tag; (iii) a plurality of a first oligonucleotide primer comprising: a first region with complementarity to a region of the first member of the plurality of target nucleic acids; and a second region with complementarity to a second region of the template nucleic acid; (iv) a plurality of a second forward oligonucleotide primer comprising: a first region with complementarity to a region of the second member of the plurality of target nucleic acids; and a second region with complementarity to a third region of the template nucleic acid; and (v) one or more nucleic acid enzymes; (c) subjecting the mixture to conditions sufficient to anneal the first oligonucleotide primer to the first member of the plurality of target nucleic acids and the second oligonucleotide primer to the second member of the plurality of target nucleic acids, such that the first and second oligonucleotide primers are degraded by the nucleic acid enzyme, releasing the second region of the first oligonucleotide primer and the second region of the second oligonucleotide primer; (d) thermally cycling the mixture under reaction conditions appropriate to amplify the template nucleic acid with polymerase chain reaction (PCR), such that the signal oligonucleotide probe is degraded such that a signal is generated if both the first and second members of the plurality of target nucleic acids are present in the mixture; (e) detecting the presence or absence of the signal; and (f) correlating the presence of the signal to the presence of both the first and second members of the plurality of target nucleic acids in the sample. In some embodiments, the mixture in (b) further comprises (vi) a plurality of a third oligonucleotide primer comprising a region with complementarity to a second region of the first member of the plurality of target nucleic acids; and (vii) a plurality of a fourth oligonucleotide primer comprising a region with complementarity to a second region of the second member of the plurality of target nucleic acids. In some embodiments, in (c), the conditions are sufficient to denature the plurality of target nucleic acids. In some embodiments, the method further comprises in (c), thermally cycling the mixture under reaction conditions appropriate to amplify the first and second members of the plurality of target nucleic acids with polymerase chain

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reaction. In some embodiments, the nucleic acid enzyme is a nucleic acid polymerase. In some embodiments, the nucleic acid enzyme has exonuclease activity. In some embodiments, the signal oligonucleotide probe is degraded by the exonuclease activity of the nucleic acid enzyme. In some embodiments, the nucleic acid enzyme has endonuclease activity. In some embodiments, the first oligonucleotide primer and the second oligonucleotide primer are degraded by the endonuclease activity of the nucleic acid enzyme. In some embodiments, the plurality of target nucleic acids comprises a plurality of deoxyribonucleic acids. In some embodiments, the plurality of target nucleic acids comprises a plurality of ribonucleic acids. In some embodiments, the plurality of ribonucleic acids comprises a plurality of messenger ribonucleic acids. In some embodiments, the template nucleic acid is a deoxyribonucleic acid. In some embodiments, the template nucleic acid is a ribonucleic acid. In some embodiments, the nucleic acid enzyme has RNase activity. In some embodiments, the first oligonucleotide primer and the second oligonucleotide primer are degraded by RNase activity of the nucleic acid enzyme. In some embodiments, the nucleic acid enzyme is an RNase H. In some embodiments, the nucleic acid enzyme is an RNase III. In some embodiments, the RNase III is Dicer. In some embodiments, the nucleic acid enzyme is an endonuclease I. In some embodiments, the endonuclease I is T7 endonuclease I. In some embodiments, the nucleic acid enzyme is capable of degrading a nucleic acid comprising a non-natural nucleotide. In some embodiments, the enzyme is an endonuclease V. In some embodiments, the endonuclease V is E. coli endonuclease V. In some embodiments, the first region of each of the plurality of first oligonucleotide primers comprises one or more ribonucleotide bases. In some embodiments, the second region of each of the plurality of the first oligonucleotide primers comprises deoxyribonucleic acid. In some embodiments, the first region of each of the plurality of second oligonucleotide primers comprises one or more ribonucleotide bases. In some embodiments, the second region of each of the plurality of the second oligonucleotide primers comprises deoxyribonucleic acid. In some embodiments, the plurality of first oligonucleotide primers further comprises at least one non-natural nucleotide. In some embodiments, the non-natural nucleotide is deoxyinosine. In some embodiments, the plurality of second oligonucleotide primers further comprises at least one non-natural nucleotide. In some embodiments, the non-natural nucleotide is deoxyinosine. In some embodiments, in (c), the conditions are insufficient for annealing of the second region of the first oligonucleotide primer to the first member of the plurality of target nucleic acids. In some embodiments, in (c), the conditions are insufficient for annealing of the second region of the second oligonucleotide primer to the second member of the plurality of target nucleic acids. In some embodiments, the signal oligonucleotide probe does not have complementarity to the any of the plurality of target nucleic acids. In some

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embodiments, the first region of the template nucleic acid is 3' of the second region of the template nucleic acid and 5' of the third region of the template nucleic acid. In some embodiments, the signal tag comprises a fluorophore and a quencher. In some embodiments, the fluorophore is FAM, TET, HEX, JOE, Cy3, Cy5, or any combination thereof. In some embodiments, the second region of each of the plurality of first oligonucleotide primers has between about 50% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of each of the plurality of first oligonucleotide primers has between about 60% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of each of the plurality of first oligonucleotide primers has between about 70% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of each of the plurality of first oligonucleotide primers has between about 80% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of each of the plurality of first oligonucleotide primers has between about 90% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of each of the plurality of first oligonucleotide primers has between about 95% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of each of the plurality of first oligonucleotide primers has between about 99% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of each of the plurality of first oligonucleotide primers has about 100% complementarity to the template nucleic acid. In some embodiments, the second region of each of the plurality of second oligonucleotide primers has between about 50% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of each of the plurality of second oligonucleotide primers has between about 60% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of each of the plurality of second oligonucleotide primers has between about 70% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of each of the plurality of second oligonucleotide primers has between about 80% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of each of the plurality of second oligonucleotide primers has between about 90% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of each of the plurality of second oligonucleotide primers has between about 95% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of each of the plurality of second oligonucleotide primers has between about 99% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of each of the plurality of second oligonucleotide primers has about 100%

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complementarity to the template nucleic acid. In some embodiments, the second region of each of the plurality of first oligonucleotide primers is not identical to the second region of each of the plurality of second oligonucleotide primers. In some embodiments, the second region of each of the plurality of first oligonucleotide primers has length of between about 5 nucleotides and about 500 nucleotides. In some embodiments, the second region of each of the plurality of first oligonucleotide primers has length of between about 5 nucleotides and about 300 nucleotides. In some embodiments, the second region of each of the plurality of first oligonucleotide primers has length of between about 5 nucleotides and about 100 nucleotides. In some embodiments, the second region of each of the plurality of first oligonucleotide primers has length of between about 5 nucleotides and about 50 nucleotides. In some embodiments, the second region of each of the plurality of first oligonucleotide primers has length of between about 5 nucleotides and about 30 nucleotides. In some embodiments, the second region of each of the plurality of first oligonucleotide primers has length of between about 5 nucleotides and about 20 nucleotides. In some embodiments, the second region of each of the plurality of first oligonucleotide primers has length of between about 5 nucleotides and about 15 nucleotides. In some embodiments, the second region of each of the plurality of second oligonucleotide primers has length of between about 5 nucleotides and about 500 nucleotides. In some embodiments, the second region of each of the plurality of second oligonucleotide primers has length of between about 5 nucleotides and about 300 nucleotides. In some embodiments, the second region of each of the plurality of second oligonucleotide primers has length of between about 5 nucleotides and about 100 nucleotides. In some embodiments, the second region of each of the plurality of second oligonucleotide primers has length of between about 5 nucleotides and about 50 nucleotides. In some embodiments, the second region of each of the plurality of second oligonucleotide primers has length of between about 5 nucleotides and about 30 nucleotides. In some embodiments, the second region of each of the plurality of second oligonucleotide primers has length of between about 5 nucleotides and about 20 nucleotides. In some embodiments, the second region of each of the plurality of second oligonucleotide primers has length of between about 5 nucleotides and about 15 nucleotides. In some embodiments, the method further comprises correlating the absence of the signal with the absence of both the first and second member of the plurality of target nucleic acids.

[0014] In an aspect, the present disclosure provides a method of detecting the presence or absence of a first and second member of a plurality of target nucleic acids in a sample, the method comprising: (a) providing a sample comprising, or potentially comprising, the first and second members of the plurality of target nucleic acids (b) forming a mixture comprising the sample and (i) a template nucleic acid; (ii) a signal oligonucleotide probe with complementarity

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to a first region of the template nucleic acid, the signal oligonucleotide probe comprising a signal tag; (iii) a plurality of a first forward oligonucleotide primer comprising: a first region with complementarity to a first region of the first member of the plurality of target nucleic acids; and a second region with complementarity to a second region of the template nucleic acid; (iv) a plurality of a first reverse oligonucleotide primer comprising a region with complementarity to a second region of the first member of the plurality of target nucleic acids; (v) a plurality of a second forward oligonucleotide primer comprising: a first region with complementarity to a first region of the second member of the plurality of target nucleic acids; and a second region with complementarity to a third region of the template nucleic acid; (vi) a plurality of a second reverse oligonucleotide primer comprising a region with complementarity to a second region of the second member of the plurality of target nucleic acids; and (vii) a nucleic acid enzyme; (c) thermally cycling the mixture under reaction conditions appropriate to amplify the first and second members of the plurality of target nucleic acids with polymerase chain reaction (PCR), such that the first forward oligonucleotide primer and the second forward oligonucleotide primer are degraded by the nucleic acid enzyme, releasing the second region with complementarity to the second region of the template nucleic acid and the second region with complementarity to the third region of the template nucleic acid; (d) thermally cycling the mixture under reaction conditions appropriate to amplify the template nucleic acid with PCR, such that the signal oligonucleotide probe is degraded such that a signal is generated if both the first and second members of the plurality of target nucleic acids are present in the mixture; (e) detecting the presence or absence of the signal; and (f) correlating the presence of the signal to the presence of both the first and second members of the plurality of target nucleic acids in the sample. In some embodiments, in (c), each thermal cycle is performed at an annealing temperature appropriate for annealing of the first region of the first oligonucleotide primer to the first member of the plurality of target nucleic acids and inappropriate for annealing of the second region of the first oligonucleotide primer to the first member of the plurality of target nucleic acids. In some embodiments, in (c), each thermal cycle is performed at an annealing temperature appropriate for annealing of the first region of the second oligonucleotide primer to the second member of the plurality of target nucleic acids and inappropriate for annealing of the second region of the second oligonucleotide primer to the second member of the plurality of target nucleic acids. In some embodiments, (c) and (d) are performed substantially simultaneously. In some embodiments, the signal oligonucleotide probe does not have complementarity to the any of the plurality of target nucleic acids. In some embodiments, the first region of the template nucleic acid is 3' of the second region of the template nucleic acid and 5' of the third region of the template nucleic acid. In some embodiments, the nucleic acid enzyme is a nucleic acid

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polymerase. In some embodiments, the nucleic acid enzyme has exonuclease activity. In some embodiments, the nucleic acid enzyme has endonuclease activity. In some embodiments, the first forward oligonucleotide primer and the second forward oligonucleotide primer are degraded by the endonuclease activity of the nucleic acid enzyme. In some embodiments, the signal is a fluorescent signal. In some embodiments, the signal tag comprises a fluorophore and a quencher. In some embodiments, the fluorophore is FAM, TET, HEX, JOE, Cy3, Cy5, or any combination thereof. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has between about 50% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has between about 60% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has between about 70% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has between about 80% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has between about 90% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has between about 95% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has between about 99% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has about 100% complementarity to the template nucleic acid. In some embodiments, the second region of each of the plurality of second forward oligonucleotide primers has between about 50% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of each of the plurality of second forward oligonucleotide primers has between about 60% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of each of the plurality of second forward oligonucleotide primers has between about 70% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of each of the plurality of second forward oligonucleotide primers has between about 80% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of each of the plurality of second forward oligonucleotide primers has between about 90% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of each of the plurality of second forward oligonucleotide primers has between about 95% and about 100% complementarity to the template nucleic acid. In some

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embodiments, the second region of each of the plurality of second forward oligonucleotide primers has between about 99% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of each of the plurality of second forward oligonucleotide primers has about 100% complementarity to the template nucleic acid. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers is not identical to the second region of each of the plurality of second forward oligonucleotide primers. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has length of between about 5 nucleotides and about 500 nucleotides. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has length of between about 5 nucleotides and about 300 nucleotides. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers length of between about 5 nucleotides and about 100 nucleotides. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has length of between about 5 nucleotides and about 50 nucleotides. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has length of between about 5 nucleotides and about 30 nucleotides. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has length of between about 5 nucleotides and about 20 nucleotides. In some embodiments, the second region of each of the plurality of first forward oligonucleotide primers has length of between about 5 nucleotides and about 15 nucleotides. In some embodiments, the second region of each of the plurality of second forward oligonucleotide primers has length of between about 5 nucleotides and about 500 nucleotides. In some embodiments, the second region of each of the plurality of second forward oligonucleotide primers has length of between about 5 nucleotides and about 300 nucleotides. In some embodiments, the second region of each of the plurality of second forward oligonucleotide primers has length of between about 5 nucleotides and about 100 nucleotides. In some embodiments, the second region of each of the plurality of second forward oligonucleotide primers has length of between about 5 nucleotides and about 50 nucleotides. In some embodiments, the second region of each of the plurality of second forward oligonucleotide primers has length of between about 5 nucleotides and about 30 nucleotides. In some embodiments, the second region of each of the plurality of second forward oligonucleotide primers has length of between about 5 nucleotides and about 20 nucleotides. In some embodiments, the second region of each of the plurality of second forward oligonucleotide primers has length of between about 5 nucleotides and about 15 nucleotides. In some embodiments, the method further comprises correlating the absence of the signal with the absence of both the first and second members of the plurality of target nucleic acids.

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[0015] In an aspect, the present disclosure provides a kit for detecting the presence or absence of a first and second member of a plurality of target nucleic acids in a sample, comprising: (a) a template nucleic acid; (b) a signal oligonucleotide probe with complementarity to a first region of the template nucleic acid, the signal oligonucleotide probe comprising a signal tag; (c) a plurality of a first oligonucleotide primer comprising: a first region with complementarity to a region of the first member of the plurality of target nucleic acids; and a second region with complementarity to a second region of the template nucleic acid; and (d) a plurality of a second oligonucleotide primer comprising: a first region with complementarity to a region of the second member of the plurality of target nucleic acids; and a second region with complementarity to a third region of the template nucleic acid. In some embodiments, the kit further comprises (e) a plurality of a third oligonucleotide primer comprising a region with complementarity to a second region of the first member of the plurality of target nucleic acids; and (f) a plurality of a fourth oligonucleotide primer comprising a region with complementarity to a second region of the second member of the plurality of target nucleic acids. In some embodiments, the kit further comprises a nucleic acid enzyme. In some embodiments, the nucleic acid enzyme is a nucleic acid polymerase. In some embodiments, the nucleic acid enzyme is an RNase. In some embodiments, the RNase is an RNase III. In some embodiments, the RNase III is Dicer. In some embodiments, the nucleic acid enzyme is an endonuclease. In some embodiments, the endonuclease is an endonuclease I. In some embodiments, the endonuclease I is T7 endonuclease I. In some embodiments, the signal oligonucleotide probe does not have complementarity to the any of the plurality of target nucleic acids. In some embodiments, the first region of the template nucleic acid is 3' of the second region of the template nucleic acid and 5' of the third region of the template nucleic acid. In some embodiments, the signal tag comprises a fluorophore and a quencher. In some embodiments, the fluorophore is FAM, TET, HEX, JOE, Cy3, Cy5, or any combination thereof. In some embodiments, the second region of each of the plurality of first oligonucleotide primers has between about 50% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of each of the plurality of first oligonucleotide primers has between about 60% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of each of the plurality of first oligonucleotide primers has between about 70% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of each of the plurality of first oligonucleotide primers has between about 80% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of each of the plurality of first oligonucleotide primers has between about 90% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of each

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of the plurality of first oligonucleotide primers has between about 95% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of each of the plurality of first oligonucleotide primers has between about 99% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of each of the plurality of first oligonucleotide primers has about 100% complementarity to the template nucleic acid. In some embodiments, the second region of each of the plurality of second oligonucleotide primers has between about 50% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of each of the plurality of second oligonucleotide primers has between about 60% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of each of the plurality of second oligonucleotide primers has between about 70% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of each of the plurality of second oligonucleotide primers has between about 80% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of each of the plurality of second oligonucleotide primers has between about 90% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of each of the plurality of second oligonucleotide primers has between about 95% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of each of the plurality of second oligonucleotide primers has between about 99% and about 100% complementarity to the template nucleic acid. In some embodiments, the second region of each of the plurality of second oligonucleotide primers has about 100% complementarity to the template nucleic acid. In some embodiments, the second region of each of the plurality of first oligonucleotide primers is not identical to the second region of each of the plurality of second oligonucleotide primers. In some embodiments, the second region of each of the plurality of first oligonucleotide primers has length of between about 5 nucleotides and about 500 nucleotides. In some embodiments, the second region of each of the plurality of first oligonucleotide primers has length of between about 5 nucleotides and about 300 nucleotides. In some embodiments, the second region of each of the plurality of first oligonucleotide primers has length of between about 5 nucleotides and about 100 nucleotides. In some embodiments, the second region of each of the plurality of first oligonucleotide primers has length of between about 5 nucleotides and about 50 nucleotides. In some embodiments, the second region of each of the plurality of first oligonucleotide primers has length of between about 5 nucleotides and about 30 nucleotides. In some embodiments, the second region of each of the plurality of first oligonucleotide primers has length of between about 5 nucleotides and about 20 nucleotides. In some embodiments, the second region of each of the plurality of first oligonucleotide primers has length of between about 5 nucleotides and

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about 15 nucleotides. In some embodiments, the second region of each of the plurality of second oligonucleotide primers has length of between about 5 nucleotides and about 500 nucleotides. In some embodiments, the second region of each of the plurality of second oligonucleotide primers has length of between about 5 nucleotides and about 300 nucleotides. In some embodiments, the second region of each of the plurality of second oligonucleotide primers has length of between about 5 nucleotides and about 100 nucleotides. In some embodiments, the second region of each of the plurality of second oligonucleotide primers has length of between about 5 nucleotides and about 50 nucleotides. In some embodiments, the second region of each of the plurality of second oligonucleotide primers has length of between about 5 nucleotides and about 30 nucleotides. In some embodiments, the second region of each of the plurality of second oligonucleotide primers has length of between about 5 nucleotides and about 20 nucleotides. In some embodiments, the second region of each of the plurality of second oligonucleotide primers has length of between about 5 nucleotides and about 15 nucleotides. In some embodiments, at least one of the oligonucleotide primers is lyophilized. In some embodiments, each of the oligonucleotide primers is lyophilized. In some embodiments, the signal oligonucleotide probe is lyophilized. In some embodiments, the template nucleic acid is lyophilized.

BRIEF DESCRIPTION OF THE DRAWINGS

[0016] FIG. 1 shows an example method for generating a signal for detecting the presence of one or more of multiple members of a plurality of target nucleic acids.

[0017] FIG. 2 shows an example method for generating a signal for detecting the presence of one member of a plurality of target nucleic acids.

[0018] FIG. 3 shows an example method for generating a signal for detecting the presence of a first and second member of a plurality of target nucleic acids.

[0019] FIG. 4 shows another example method for generating a signal for detecting the presence of a first and second member of a plurality of target nucleic acids.

[0020] FIG. 5 illustrates an example analysis of the present disclosure.

[0021] FIG. 6 shows an example method for generating a signal for detecting the presence of one or more of multiple target nucleic acids using a universal primer.

[0022] FIG. 7 shows results from an example quantitative polymerase chain reaction experiment measuring the presence or absence of at least one of two targets.

[0023] FIG. 8 shows peak fluorescence intensity results from the example experiment referred to in FIG. 7.

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DETAILED DESCRIPTION

[0024] Polymerase Chain Reaction (PCR) is a method of exponential amplification of specific target nucleic acid in a reaction mix with a nucleic acid polymerase and primers. Primers are short single stranded oligonucleotides which are complementary to the 3' sequences of the positive and negative strand of the target sequence. The reaction mix is cycled in repeated heating and cooling steps. The heating cycle denatures or splits a double stranded nucleic acid target into single stranded templates. In the cooling cycle, the primers bind to complementary sequence on the template. After the template is primed the nucleic acid polymerase creates a copy of the original template. Repeated cycling exponentially amplifies the target 2 fold with each cycle leading to approximately a billion-fold increase of the target sequence in 30 cycles (Saiki et al 1988).

[0025] Real-Time PCR is a process of monitoring a PCR reaction by recording the fluorescence generated either by an intercalating dye such as SYBR Green or a target-specific reporter probe at each cycle. This is generally performed on a Real-Time PCR instrument that executes thermal cycling of the sample to complete the PCR cycles and at a specified point in each cycle measures the fluorescence of the sample in each channel through a series of excitation/emission filter sets.

[0026] Frequently, the target specific reporter probe is a short oligonucleotide complementary to one strand of the amplified target. The probe lacks a 3' hydroxyl and therefore is not extendable by the DNA polymerase. TaqMan (ThermoFisher Scientific) chemistry is a common reporter probe method used for multiplex Real-Time PCR (Holland et al. 1991). The TaqMan oligonucleotide probe is covalently modified with a fluorophore and a quenching tag (i.e., quencher). In this configuration the fluorescence generated by the fluorophore is quenched and is not detected by the real time PCR instrument. When the target of interest is present, the probe oligonucleotide base pairs with the amplified target. While bound, it is digested by the 5'->3' exonuclease activity of the Taq polymerase thereby physically separating the fluorophore from the quencher and liberating signal for detection by the real time PCR instrument.

[0027] Current methods that use a unique probe for each target of interest limit the number of targets that can be measured in a single reaction. While current multiplex technology allows for measurement of multiple targets using multiple unique probes, this technology is costly and is limited by the number of available fluorescent channels. There remains a need for methods which allow for measurement of any subset of a number of targets in a single sample. Disclosed herein are methods and compositions for detecting the presence or absence of one or more members of a plurality of target nucleic acids in a sample.

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[0028] In one example, multiple sets of PCR primers are provided, each with specificity for a different target nucleic acid. One primer from each set (e.g., a forward primer) comprises a first region complementary to a unique target nucleic acid and a second region complementary to a single, universal probe (see FIG. 1). A sample potentially comprising one or more targets is subjected to thermal cycling under appropriate conditions for target amplification. If at least one target nucleic acid complementary to any of the provided primers is present, the second region of the primers is incorporated into the amplification product and the universal probe binds to this region, thereby generating a signal. The presence of a signal serves to identify the presence of at least one target in a sample.

[0029] In another example, two sets of PCR primers are provided, each with specificity for one of two target nucleic acids. One primer from each set (e.g., a forward primer) comprises a first region complementary to a target nucleic acid and a second region complementary to a single, template nucleic acid (see FIG. 4). A sample potentially comprising the target nucleic acids is subjected to thermal cycling under appropriate conditions for target amplification. If both targets are present in the sample, the second region of the two primers is released via enzymatic digestion. Once released, the second regions bind to and enable amplification of the template nucleic acid (i.e., act as forward and reverse primers). A reporter probe complementary to the template nucleic acid is provided. Amplification of the template nucleic acid in the presence of the reporter probe generates a signal. The presence of a signal may serve to identify the presence of both targets in a sample.

Definitions

[0030] The term "complementarity," or "complementary," as used herein, can refer to a property of a nucleic acid or the region of a nucleic acid wherein the nucleic acid is capable of binding, annealing, or otherwise attaching to another nucleic acid under a given set of conditions. A region of a nucleic acid which has complementarity to a region of another nucleic acid can comprise nucleotides which are complementary to those on the other nucleic acid. Complementarity may be of varying degrees. For example, a region of a nucleic acid can have between about 50% and about 100%, between about 60% and about 100%, between about 70% and about 100%, between about 80% and about 100%, between about 90% and about 100%, between about 95% and about 100%, between about 99% and about 100%, or about 100% complementarity to a region of another nucleic acid. Degrees of complementarity can describe the portion of a nucleic acid which is capable of attaching to another nucleic acid under appropriate conditions. Degrees of complementarity can describe the amount of a nucleic acid or region of a nucleic acid comprising nucleotides which are the complement of those on another nucleic acid. A nucleic acid with complementarity can be a double-stranded nucleic acid. A

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nucleic acid with complementarity can be a single-stranded nucleic acid. A single-stranded nucleic acid which is complementary to another single-stranded nucleic acid can bind to a region of another single-stranded nucleic acid under appropriate conditions. Appropriate conditions for binding of one complementary nucleic acid to another can include appropriate buffer conditions and appropriate temperature conditions. Appropriate buffer conditions can include appropriate salt concentrations, such as concentrations of sodium salts, magnesium salts, or potassium salts. Appropriate temperature conditions can include an annealing temperature which is appropriate for binding of one nucleic acid to another. While a nucleic acid with complementarity to another nucleic acid may bind to the other nucleic acid under appropriate conditions, the same nucleic acid may not bind under inappropriate conditions, such as inappropriate salt concentrations or temperature conditions.

[0031] The term "homologous" or "homology", as used herein, can refer to a property of a nucleic acid or the region of a nucleic acid wherein the nucleic acid comprises nucleotides which are identical to those comprised in another nucleic acid. A nucleic acid which is homologous to another nucleic acid can comprise one or more nucleotides which are identical to those on the other nucleic acid. Homology may be of varying degrees, depending on the number of nucleotides which are identical. A region of a nucleic acid which is homologous to a region of another nucleic acid may have a certain degree of homology. For example, a region of a nucleic acid can have between about 50% and about 100%, between about 60% and about 100%, between about 70% and about 100%, between about 80% and about 100%, between about 90% and about 100%, between about 95% and about 100%, between about 99% and about 100%, or about 100% homology to a region of another nucleic acid.

[0032] The term "primer," as used herein, can refer to an oligonucleotide or nucleic acid which can bind to another nucleic acid and facilitate transcription and/or nucleic acid synthesis. A primer can be double-stranded. A primer can be single-stranded. A primer can be a forward primer or a reverse primer. A forward primer and a reverse primer can be those which bind to opposite strands of a double-stranded nucleic acid. For example, a forward primer can bind to a region of a first strand (e.g., Watson strand) derived from a nucleic acid, and a reverse primer can bind to a region of a second strand (e.g., Crick strand) derived from the nucleic acid. A forward primer may bind to a region closer to the start site of a gene relative to a reverse primer or may bind closer to the end site of a gene relative to a reverse primer. A forward primer may bind to the coding strand of a nucleic acid, or may bind to the non-coding strand of a nucleic acid. A reverse primer may bind to the coding strand of a nucleic acid, or may bind to the non-coding strand of a nucleic acid.

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Nucleic Acid Detection

[0033] In a first broad aspect, the present disclosure provides methods for detecting the presence or absence of at least one target nucleic acid in a sample. The sample may comprise one or more target nucleic acids, or no target nucleic acids. Methods may comprise forming a mixture of the sample, a signal oligonucleotide probe, and multiple sets of oligonucleotide primers. FIG. 1 shows an example mixture comprising target nucleic acids 100 and 110, a signal oligonucleotide probe 120, and oligonucleotide primers 101, 111, 105, and 115. FIG. 6 shows another example mixture comprising target nucleic acids 600 and 610, a signal oligonucleotide probe 660, and oligonucleotide primers 601, 611, 605, and 615. Each set of oligonucleotide primers may comprise a forward oligonucleotide primer and a reverse oligonucleotide primer. Each oligonucleotide primer may comprise a region with complementarity to a target nucleic acid. FIG. 1 shows two example sets of oligonucleotide primers. A first set of oligonucleotide primers includes forward primer 101, comprising a region 102 with complementarity to a first target nucleic acid 100, and reverse primer 105. A second set of oligonucleotide primers includes forward primer 111, comprising a region 112 with complementarity to a second target nucleic acid 110, and reverse primer 115. FIG. 6 shows two additional example sets of oligonucleotide primers. A first set of oligonucleotide primers includes forward primer 601, comprising a region 603 with complementarity to a first target nucleic acid 600, and reverse primer 605. A second set of oligonucleotide primers includes forward primer 611, comprising a region 613 with complementarity to a second target nucleic acid 610, and reverse primer 615. Once formed, a mixture may be thermally cycled under conditions appropriate to amplify each target nucleic acid from the sample. Amplification may be performed by, for example, polymerase chain reaction (PCR). Subsequent to or simultaneous with thermal cycling, a signal may be generated if at least one target nucleic acid is present in the mixture. The signal may be detected, thereby detecting the presence or absence of at least one target nucleic acid in the sample.

[0034] Each forward oligonucleotide primer may comprise an additional region with complementarity to a signal oligonucleotide probe. FIG. 1 shows regions 103 and 113, each with complementarity to signal oligonucleotide probe 120. Under appropriate conditions, the first oligonucleotide primer may be incorporated into an amplification product of a target nucleic acid, which may be produced by a nucleic acid enzyme. FIG. 1 shows forward primer 101 incorporated into an amplification product 104 and forward primer 111 incorporated into an amplification product 114. Once incorporated, a signal oligonucleotide probe in a mixture may bind to the additional region of the forward oligonucleotide primer, thereby allowing for signal generation. FIG. 1 shows one copy of signal oligonucleotide probe 120 bound to region 103 of forward primer 103 and a second copy of signal oligonucleotide probe 120 bound to region 113

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of forward primer 111. A signal oligonucleotide probe may comprise a signal tag. A signal tag may comprise a fluorophore and a quencher, which may act in the suppression and generation of a fluorescent signal. FIG. 1 shows signal probe 120 comprising a fluorophore 122 and a quencher 121. FIG. 6 shows a signal oligonucleotide probe comprising a fluorophore 662 and a quencher 661. A nucleic acid enzyme (e.g., nucleic acid polymerase) may be used to degrade the signal oligonucleotide probe and release the quencher, thereby generating a signal.

[0035] Alternatively or in addition, each forward oligonucleotide primer may comprise an additional region homologous to (i.e., with homology to) a signal oligonucleotide probe. FIG. 6 shows regions 602 and 612, each homologous to signal oligonucleotide probe 660. In some cases, a mixture may further comprise a universal primer. Each forward oligonucleotide primer may comprise a third region homologous to a universal primer. FIG. 6 shows regions 604 and 614, each complementary to universal primer 650. Under appropriate conditions, the first forward oligonucleotide primer may be incorporated into an amplification product of a target nucleic acid, which may be produced by a nucleic acid enzyme. FIG. 6 shows forward primer 601 incorporated into an amplification product 620 and forward primer 611 incorporated into an amplification product 630. Once incorporated, a reverse primer may be used to generate a reverse complement product. FIG. 6 shows reverse primers 605 and 615 used to generate reverse complement products 640 and 650 from products 620 and 630. Product 640 comprises reverse complement sequences 622, 623, and 624 generated from regions 602, 603, and 604. Product 650 comprises reverse complement sequences 632, 633, and 634 generated from regions 612, 613, and 614. Universal primer 650 may bind to sequences 624 and 634. The signal oligonucleotide probe 660 may bind to sequences 622 and 632. FIG. 6 shows one copy of a signal oligonucleotide probe 660 bound to sequence 622 of product 640 and a second copy of signal oligonucleotide probe 660 bound to sequence 632 of product 650. The universal primer may be used to amplify a reverse complement product, thereby allowing for signal generation, for example, by degrading the signal oligonucleotide probe to release the quencher.

[0036] FIG. 2 shows another example mixture of the present disclosure comprising target nucleic acid 200, a first forward oligonucleotide primer 201 comprising a first region 202 with complementarity to the target nucleic acid 200, and a second region 203 with complementarity to a signal oligonucleotide probe 220. The mixture also comprises a second forward oligonucleotide primer 211 comprising a first region 212 with complementarity to a second member (not present in the mixture) and a second region 213 with complementarity to signal oligonucleotide probe 220. First forward oligonucleotide primer 201 is incorporated into amplification product 204. Signal oligonucleotide probe 220 comprising a quencher 221 and a fluorophore 222 binds to the second region 203 of the first forward oligonucleotide primer. A

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reverse oligonucleotide primer 205 is used to extend the first nucleic acid product 204 using a nucleic acid polymerase (not shown in FIG. 2). The signal oligonucleotide probe 220 is degraded by the exonuclease activity of the nucleic acid polymerase, thereby releasing the quencher 221 and generating a signal.

[0037] In another broad aspect, the present disclosure provides methods for detecting the presence or absence of two target nucleic acids in a sample. The sample may comprise one, both, or neither target nucleic acid. Methods may comprise forming a mixture of the sample, a template nucleic acid, a signal oligonucleotide probe, and two oligonucleotide primers. FIG. 3 shows an example mixture comprising target nucleic acids 300 and 310, a signal oligonucleotide probe 320, oligonucleotide primers 301 and 311, and a template nucleic acid 330. The signal oligonucleotide probe may be complementary to the template nucleic acid. Each oligonucleotide primer may comprise a region with complementarity to one of the two target nucleic acids. This region may comprise one or more non-natural nucleotides. FIG. 3 shows two example primers. Primer 301 comprises a region 302 with complementarity to target nucleic acid 300. Primer 311 comprises a region 312 with complementarity to target nucleic acid 310.Once formed, a mixture may be subjected to conditions sufficient to anneal the oligonucleotide primers to their complementary target nucleic acids, as shown in FIG. 3. These conditions may be such that the primers are degraded. A mixture may be thermally cycled under conditions appropriate to amplify the template nucleic acid. Amplification may be performed by, for example, polymerase chain reaction (PCR). Subsequent to or simultaneous with thermal cycling, a signal may be generated if both target nucleic acids are present in the mixture. The signal may be detected, thereby detecting the presence or absence of two target nucleic acids in the sample.

[0038] The region of each oligonucleotide primer with complementarity to a target nucleic acid may comprise one or more non-natural nucleotides. A non-natural nucleotide may be, for example, deoxyinosine. FIG. 3 shows a primer 301 comprising a non-natural nucleotide 304 and a primer 311 comprising a non-natural nucleotide 314. Each oligonucleotide primer may comprise an additional region with complementarity to a template nucleic acid. FIG. 3 shows regions 303 and 313, each with complementarity to a template nucleic acid 330. Under appropriate conditions, the region of the oligonucleotide primers with complementarity to a target nucleic acid may anneal to the target, as shown in FIG. 3. Conditions may be such that the region with complementarity to the template nucleic acid does not anneal to the target. FIG. 3 shows regions 302 and 312 annealed to target nucleic acids 300 and 310. Once annealed, the primers may be degraded by, for example, a nucleic acid enzyme. A nucleic acid enzyme may be capable of degrading a non-natural nucleotide. The nucleic acid enzyme may be an endonuclease V such as, for example, an E. coli endonuclease V. Primer degradation may

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release the additional region with complementarity to the template nucleic acid. The regions from the two primers may anneal to the template nucleic acid and, under appropriate conditions, serve to amplify the template nucleic acid (e.g., may act as forward and reverse primers). A signal oligonucleotide probe may bind to the template nucleic acid. FIG. 3 shows signal probe 320 bound to template nucleic acid 330. A signal oligonucleotide probe may comprise a fluorophore and a quencher, which may act in the suppression or generation of a fluorescent signal. A nucleic acid enzyme (e.g., nucleic acid polymerase) may be used to degrade the signal oligonucleotide probe and release the quencher, thereby generating a signal.

[0039] In another broad aspect, the present disclosure provides methods for detecting the presence or absence of two target nucleic acids in a sample. The sample may comprise one, both, or neither target nucleic acid. Methods may comprise forming a mixture of the sample, a template nucleic acid, a signal oligonucleotide probe, and two sets of oligonucleotide primers. FIG. 4 shows an example mixture comprising target nucleic acids 400 and 410, a signal oligonucleotide probe 420, oligonucleotide primers 401, 404, 411, and 414, and a template nucleic acid 430. Each set of oligonucleotide primers may comprise a forward oligonucleotide primer and a reverse oligonucleotide primer. The signal oligonucleotide probe may have complementarity to the template nucleic acid. Each oligonucleotide primer may comprise a region with complementarity to one of the two target nucleic acids. FIG. 4 shows two example primers. Primer 401 comprises a region 402 with complementarity to target nucleic acid 400. Primer 411 comprises a region 412 with complementarity to target nucleic acid 410. Once formed, a mixture may be thermally cycled under conditions sufficient to amplify the target nucleic acids. These conditions may be such that the primers are degraded. A mixture may be thermally cycled under conditions appropriate to amplify the template nucleic acid. Amplification may be performed by, for example, polymerase chain reaction (PCR). Subsequent to or simultaneous with thermal cycling, a signal may be generated if both target nucleic acids are present in the mixture. The signal may be detected, thereby detecting the presence or absence of two target nucleic acids in the sample.

[0040] Each oligonucleotide primer may comprise an additional region with complementarity to a template nucleic acid. FIG. 4 shows regions 403 and 413, each with complementarity to template nucleic acid 430. Under appropriate conditions, the region of the oligonucleotide primers with complementarity to a target nucleic acid may anneal to the target, as shown in FIG. 4. Conditions may be such that the region with complementarity to the template nucleic acid does not anneal to the target. FIG. 4 shows regions 402 and 412 annealed to target nucleic acids 400 and 410. Once annealed, the target nucleic acids may be amplified. During amplification, the oligonucleotide primers may be degraded, releasing the regions complementary to the

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template nucleic acid. Primers may be degraded by the endonuclease activity of a nucleic acid polymerase. The regions from the two primers may anneal to the template nucleic acid and, under appropriate conditions, serve to amplify the template nucleic acid (e.g., may act as forward and reverse primers). A signal oligonucleotide probe may bind to the template nucleic acid. FIG. 4 shows signal probe 420 bound to template nucleic acid 430. A signal oligonucleotide probe may comprise a fluorophore and a quencher, which may act in the suppression or generation of a fluorescent signal. A nucleic acid enzyme (e.g., nucleic acid polymerase) may be used to degrade the signal oligonucleotide probe and release the quencher, thereby generating a signal.

Oligonucleotide Probes

[0041] Mixtures and compositions of the present disclosure may comprise a signal oligonucleotide probe. The concentration of a signal oligonucleotide probe may be such that it is in excess relative to other components in the mixture. A mixture may comprise more than one signal oligonucleotide probe. Multiple signal oligonucleotide probes may be the same, or may be different. In some examples, a mixture comprises a first signal oligonucleotide probe and one or more additional signal oligonucleotide probes. An additional signal oligonucleotide probe may be an xth signal oligonucleotide probe. x may be any number which describes a signal oligonucleotide probe as one of multiple signal oligonucleotide probes. x may be an integer greater than one. x may be at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, or higher. x may be at most 20, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, or 2. In one example, x may be 2, such that an xth signal oligonucleotide probe is a 2nd signal oligonucleotide probe. A signal oligonucleotide probe may be a nucleic acid (e.g., DNA, RNA, etc.). A signal oligonucleotide probe may be at least 5, 10, 15, 20, 30, or 50 nucleotides in length, or more. A signal oligonucleotide probe may be at most 50, 30, 20, 15, 10 or, 5, nucleotides in length. A signal oligonucleotide probe may have less than 50%, 40%, 30%, 20%, 10%, 5%, or 1% complementarity to any member of the plurality of target nucleic acids. A signal oligonucleotide probe may have no complementarity to any member of a plurality of target nucleic acids (i.e. no complementarity to any target nucleic acid). A signal oligonucleotide probe may comprise a signal tag. A signal tag may comprise a means of generating a signal such as, for example, a fluorophore. A fluorophore may be, for example, FAM, TET, HEX, JOE, Cy3, or Cy5. A signal tag may further comprise one or more quenchers. A quencher may inhibit signal generation from a fluorophore. A quencher may be, for example, TAMRA, BHQ-1, BHQ-2, or Dabcyl.

Oligonucleotide Primers

[0042] An oligonucleotide primer of the present disclosure may be a deoxyribonucleic acid. An oligonucleotide primer may be a ribonucleic acid. An oligonucleotide primer may comprise one

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or more non-natural nucleotides. A non-natural nucleotide may be, for example, deoxyinosine. An oligonucleotide primer may be a forward primer. An oligonucleotide primer may be a reverse primer. An oligonucleotide primer may be between about 5 and about 100 nucleotides in length. An oligonucleotide primer may be at least 5, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, or 100 base pairs in length, or more. An oligonucleotide primer may be at most 100, 90, 80, 70, 60, 50, 45, 40, 35, 30, 25, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, or 5 nucleotides in length.

[0043] An oligonucleotide primer may comprise one or more regions. In some cases, an oligonucleotide primer has two regions. In some cases, an oligonucleotide primer has three regions. A region may be between about 5 and about 50 nucleotides in length. A region may be at least 5, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, or 50 nucleotides in length, or more. A region may be at most 50, 45, 40, 35, 30, 25, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, or 5 nucleotides in length. A region may have complementarity to a region of a target nucleic acid. A region may have complementarity to a signal oligonucleotide probe. A region may be homologous to a signal oligonucleotide probe. A region may have complementarity to a universal primer. A region may be homologous to a universal primer. A region with complementarity or homology to a universal primer region may have little or no complementarity or homology to either a region of a target nucleic acid or a signal oligonucleotide probe. A region may have complementarity to a region of a template nucleic acid. A first region of a first forward oligonucleotide primer may be 5' relative to a second region of the first forward oligonucleotide primer. A first region of a second forward oligonucleotide primer may be 5' relative to a second region of the second forward oligonucleotide primer. A third region of a first forward oligonucleotide primer may be 5' relative to a first and second region of the first forward oligonucleotide primer. A third region of a second forward oligonucleotide primer may be 5' relative to a first and second region of the second forward oligonucleotide primer.

[0044] Mixtures of the present disclosure may comprise one or more oligonucleotide primers. An oligonucleotide primer may be an nth oligonucleotide primer. n may be any number which describes an oligonucleotide primer as one of multiple oligonucleotide primers. n may be an integer greater than two. n may be at least 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, or higher. n may be at most 20, 15, 10, 9, 8, 7, 6, 5, 4, or 3. In one example, n may be 3, such that an nth oligonucleotide primer is a 3rd oligonucleotide primer. An oligonucleotide primer may be a yth oligonucleotide primer. y may be any number which describes an oligonucleotide primer as one of multiple oligonucleotide primers. y may be an integer greater than n. y may be at least 4, 5, 6,

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7, 8, 9, 10, 11, 15, 20, or higher. y may be at most 20, 15, 10, 9, 8, 7, 6, 5, or 4. In one example, y may be 4, such that a yth oligonucleotide primer is a 4th oligonucleotide primer.

Nucleic Acid Enzymes

[0045] Mixtures and compositions of the present disclosure may comprise one or more nucleic acid enzymes. A nucleic acid enzyme may have exonuclease activity. A nucleic acid enzyme may have endonuclease activity. A nucleic acid enzyme may have RNase activity. A nucleic acid enzyme may be capable of degrading a nucleic acid comprising one or more ribonucleotide bases. A nucleic acid enzyme may be, for example, RNase H or RNase III. An RNase III may be, for example, Dicer. A nucleic acid may be an endonuclease I such as, for example, a T7 endonuclease I. A nucleic acid enzyme may be capable of degrading a nucleic acid comprising a non-natural nucleotide. A nucleic acid enzyme may be an endonuclease V such as, for example, an *E. coli* endonuclease V. A nucleic acid enzyme may be a polymerase (e.g., a DNA polymerase). A polymerase may be a Taq polymerase. A nucleic acid enzyme may be capable, under appropriate conditions, of degrading a signal oligonucleotide probe. A nucleic acid enzyme may, for example, be capable of releasing a quencher from a signal oligonucleotide probe by exonuclease activity.

Thermal Cycling

[0046] Methods of the present disclosure may comprise thermal cycling. Thermal cycling may comprise one or more thermal cycles. Thermally cycling may be performed under reaction conditions appropriate to amplify one or more nucleic acids (e.g., one or more members of a plurality of target nucleic acids, one or more target nucleic acids, etc.). Amplification may comprise, for example, polymerase chain reaction (PCR). Appropriate reaction conditions may include appropriate temperature conditions, appropriate buffer conditions, and the presence of appropriate reagents. Appropriate temperature conditions may be such that each thermal cycle is performed at a desired annealing temperature. A desired annealing temperature may be sufficient for annealing of a region of an oligonucleotide primer to a target nucleic acid. A desired annealing temperature may be sufficient for annealing of a region of an oligonucleotide primer to a signal oligonucleotide probe. A desired annealing temperature may be insuffucieent for a region (e.g., a region with complementarity to a template nucleic acid) to anneal to a target nucleic acid. Appropriate buffer conditions may be such that the appropriate salts are present in a buffer used during thermal cycling. Appropriate salts may include magnesium salts, potassium salts, ammonium salts. Appropriate buffer conditions may be such that the appropriate salts are present in appropriate concentrations. Appropriate reagents for amplification of each member of a plurality of target nucleic acids with PCR may include deoxytriphosphates (dNTPs). dNTPs

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may comprise natural or non-natural dNTPs including, for example, dATP, dCTP, dGTP, dTTP, dUTP, and variants thereof.

[0047] In some aspects, a mixture may be thermally cycled under reaction conditions appropriate to amplify a template nucleic acid. Amplification may comprise, for example, PCR. Amplification of a template nucleic acid may require binding or annealing of both a region released from a first oligonucleotide and a region released from a second oligonucleotide primer to the template nucleic acid. A region released from an oligonucleotide primer may enable amplification of a template nucleic acid by acting as a forward and reverse primer for PCR amplification.

Signal Generation and Detection

[0048] Methods of the present disclosure may be such that one or more signal oligonucleotide probes are degraded by a nucleic acid enzyme. A signal oligonucleotide probe may be degraded by the exonuclease activity of a nucleic acid enzyme. A signal oligonucleotide probe may generate a signal upon degradation. In some cases, a signal oligonucleotide probe may generate a signal only if at least one member of a plurality of target nucleic acids is present in a mixture. For example, a signal oligonucleotide probe may be degraded only when bound (i.e., annealed) to a region of an oligonucleotide primer (e.g., a second region of a forward oligonucleotide primer). In some cases, a signal oligonucleotide probe may generate a signal only if both a first and second member of a plurality of target nucleic acids is present in a mixture. For example, a signal oligonucleotide probe may be degraded only when bound (i.e. annealed) to a region of a template nucleic acid. In cases where a signal oligonucleotide probe comprises a signal tag, the signal oligonucleotide probe may be degraded when bound to a region of an oligonucleotide primer, thereby generating a signal. A signal may be a fluorescent signal. A signal tag may comprise a quencher and a fluorophore, such that the quencher is released from a signal tag upon degradation of a signal oligonucleotide probe, thereby generating a fluorescent signal. Thermal cycling of the present disclosure may generate one signal, or more than one signal. Thermal cycling may generate at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 signals, or more. Thermal cycling may generate at most 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 signal. Multiple signals may be of the same type or of different types. Signals of different types may be fluorescent signals with different fluorescent wavelengths. Signals of different types may be generated by signal tags comprising different fluorophores. Signals of the same type may be of different intensities (e.g., different intensities of the same fluorescent wavelength). Signals of the same type may be generated by signal tags comprising the same fluorophore. Signal tags comprising the same fluorophore may generate different signals by nature of being at different concentrations, thereby generating different intensities of the same signal type.

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[0049] The presence or absence of one or more signals may be detected. One signal may be detected, or multiple signals may be detected. Multiple signals may be detected simultaneously. Alternatively, multiple signals may be detected sequentially. A signal may be detected by any means, including, for example, detection of a fluorescent signal. A signal may be detected throughout the process of thermal cycling, for example, at the end of each thermal cycle. In some cases, the signal intensity increases with each thermal cycle. The signal intensity may increase in a sigmoidal fashion.

[0050] The presence of a signal may be correlated to the presence of at least one member of a plurality of target nucleic acids. Alternatively, the presence of a signal may be correlated to the presence of both a first and second member of a plurality of target nucleic acids. Correlating the presence of a signal to the presence of one or more target nucleic acids may comprise establishing a signal intensity threshold. A signal intensity threshold may be different for different signals. Making a correlation may comprise determining whether the intensity of a signal increases beyond a signal intensity threshold. The presence of a signal may be correlated with the presence of at least one of all members of a plurality of target nucleic acids. The presence of a first signal may be correlated with the presence of at least one of a first subset of members of a plurality of target nucleic acids, and the presence of a second signal may be correlated with the presence of at least one of a second subset of members of a plurality of target nucleic acids. Multiple signals may be detected, and the presence of each may be correlated with the presence of at least one of a subset of members of a plurality of target nucleic acids. The presence of least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more signals may be correlated with the presence of at least one of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more subsets of members of a plurality of target nucleic acid molecules.

[0051] The absence of a signal may be correlated with the absence of each of at least a subset of members of a plurality of target nucleic acids. Alternatively, the absence of a signal may be correlated with the absence of either a first or a second member of a plurality of target nucleic acids. The absence of a signal may be correlated with the absence of each member of the plurality of target nucleic acids. The absence of a first signal may be correlated with the absence of each of a first subset of members of a plurality of target nucleic acids, and the absence of a second signal may be correlated with the absence of each of a second subset of members of a plurality of target nucleic acids. Multiple signals may be detected, and the absence of each may be correlated with the absence of each of a subset of members of a plurality of target nucleic acids. The absence of least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more signals may be correlated with the absence of each of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more subsets of members of a plurality of target nucleic acid molecules.

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[0052] Detection and analysis of one or more signals may be useful in, for example, determining the presence or absence of a large number of targets in a sample. Analysis of these methods may be useful in clinical applications, where detection of one or more targets in a subject may inform clinical decisions. FIG. 5 shows a schematic of an example analysis that may be performed. Target nucleic acids are provided as described herein in operation 500. Nucleic acids are detected, generating fluorescent readouts in operation 501. In operation 502, analysis of the fluorescent readouts results in the determination of the presence or absence of a given viral pathogen based on the presence or absence of one or more targets. Based on the presence or absence of one or more viral pathogens, clinical actions are taken in operation 503, such as treatment of a subject with a clinically appropriate drug.

Samples and Targets

[0053] Target nucleic acids of the present disclosure may be derived from any source including, for example, viruses, bacterial cells, and eukaryotic cells. Target nucleic acids may be derived from one or more cells. A cell may be a tumor cell. A cell may be a cell suspected of comprising a viral pathogen. In some cases, target nucleic acids are derived from a cell-free sample (e.g., plasma). Target nucleic acids may be cell-free nucleic acid. Cell-free nucleic acid may be, for example, cell-free tumor DNA, cell-free fetal DNA, cell-free RNA, etc. Target nucleic acids may comprise deoxyribonucleic acid (DNA). Target nucleic acids may comprise ribonucleic acid (RNA). RNA may be any kind of RNA, including messenger RNA, transfer RNA, ribosomal RNA, and microRNA. RNA may be viral RNA. A target nucleic acid sample may comprise one or more members (e.g., may comprise one or more target nucleic acids). A member may be any region of a target nucleic acid. A member may be of any length. A member may be, for example, up to 1, 2, 3, 4, 5, 10, 20, 50, 100, 500, 1000, 5000, 10000, 50000, 100000 nucleotides in length, or more. In some instances, a member may be a gene. A member may be any region of a target nucleic acid which is of interest. For example, a member may be a gene whose detection may be useful in diagnosing one or more diseases. A gene may be a viral gene or bacterial gene whose detection may be useful in identifying the presence or absence of a pathogen in a subject.

Template Nucleic Acids

The present disclosure provides, in some aspects, one or more template nucleic acids. A template nucleic acid may be added to a sample as described herein. A template nucleic acid may be useful in, for example, ensuring the generation of a signal only if both a first and second target nucleic acid are present in a sample, thereby verifying the presence of both targets in a single sample using a single signal. A template nucleic acid may comprise DNA, RNA, or a combination thereof. A template nucleic acid may comprise a first region, a second region, and a

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third region. The first region may be 3' relative to the second region. The first region may be 5' relative to the third region. A template nucleic acid may be derived from any source (i.e. a natural source). A template nucleic acid may be synthetically generated, for example, by chemical synthesis.

Kits

[0054] Also provided herein are kits for detecting the presence or absence of at least one member of a plurality of target nucleic acids in a sample. Kits may be used for detecting the presence of a first and second member of a plurality of target nucleic acids in a sample. Kits may include one or more signal oligonucleotide probes. Signal oligonucleotide probes may be lyophilized. Signal oligonucleotide probes may be present at different concentrations. Signal oligonucleotide probes may comprise a signal tag, which may comprise, for example, a fluorophore and one or more quenchers. Kits may include one or more oligonucleotide primers as described herein, including, for example, a first oligonucleotide primer (e.g., a first forward oligonucleotide primer), a second oligonucleotide primer (e.g., a second forward oligonucleotide primer), a third oligonucleotide primer (e.g., a first reverse oligonucleotide primer), and/or a fourth oligonucleotide primer (e.g., a second reverse oligonucleotide primer). The oligonucleotide primers may comprise various regions as disclosed herein. One or more of the oligonucleotide primers may be lyophilized. In some cases, all of the oligonucleotide primers may be lyophilized. Kits may comprise one or more nucleic acid enzymes. A nucleic acid enzyme may be a nucleic acid polymerase. A nucleic acid polymerase may be a deoxyribonucleic acid polymerase. A nucleic acid enzyme may be an RNase. An RNase may be an RNase III. An RNase III may be Dicer. The nucleic acid enzyme may be an endonuclease. An endonuclease may be an endonuclease I. An endonuclease I may be a T7 endonuclease I. Kits may comprise instructions for using any of the foregoing in the methods described herein.

EXAMPLES

Example 1

[0055] A biological sample is obtained from a subject suspected of having human immunodeficiency virus (HIV) and/or hepatitis C virus (HCV). The sample is mixed with a TaqMan signal oligonucleotide probe, two sets of forward and reverse primers, and a Taq DNA polymerase.

[0056] The first forward primer comprises two regions: a first region complementary to HIV and a second region complementary to the signal oligonucleotide probe. The first reverse primer is complementary to HIV. The second forward primer comprises two regions, a first region

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complementary to a region of HCV and a second region complementary to the signal oligonucleotide probe. The second reverse primer is complementary to HCV.

[0057] The mixture is thermally cycled under appropriate buffer conditions and temperature conditions, and comprising the appropriate reagents, such that the primers anneal to their respective targets, which are amplified by polymerase chain reaction. During this process, the regions complementary to the signal oligonucleotide probe are incorporated into the amplification product. The signal oligonucleotide probe binds to the amplification products by annealing to the complementary regions from the forward primers. The Taq polymerase degrades the signal oligonucleotide probes via its exonuclease activity, thus releasing the quencher. A fluorescent signal is generated by the fluorophore upon release of the quencher. The fluorescent signal is detected, indicating the sample to contain HIV, HCV, or both.

Example 2

[0058] A biological sample is obtained from a subject suspected of having human immunodeficiency virus (HIV), hepatitis C virus (HCV), hepatitis B virus (HBV), and/or hepatitis A virus. The sample is mixed with two different TaqMan signal oligonucleotide probes, four sets of forward and reverse primers, and a Taq DNA polymerase.

[0059] The first forward primer comprises two regions: a first region complementary to HIV and a second region complementary to the first signal oligonucleotide probe. The first reverse primer is complementary to HIV. The second forward primer comprises two regions, a first region complementary to a region of HCV and a second region complementary to the first signal oligonucleotide probe. The second reverse primer is complementary to HCV. The third forward primer comprises two regions, a first region complementary to a region of HBV and a second region complementary to the second signal oligonucleotide probe. The third reverse primer is complementary to HBV. The fourth forward primer comprises two regions, a first region complementary to a region of HCV and a fourth region complementary to the second signal oligonucleotide probe. The second reverse primer is complementary to HCV.

[0060] The mixture is thermally cycled under appropriate buffer conditions and temperature conditions, and comprising the appropriate reagents, such that the primers anneal to their respective targets, which are amplified by polymerase chain reaction. During this process, the regions complementary to the signal oligonucleotide probes are incorporated into the amplification product. The signal oligonucleotide probes binds to the amplification products by annealing to the respective complementary regions from the forward primers. The Taq polymerase degrades the signal oligonucleotide probes via its exonuclease activity, thus releasing the quencher. A fluorescent signal of a given intensity is generated by the fluorophores

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upon release of the quencher. The fluorescent signal generated from both probes is detected, indicating the sample to contain HIV, HCV, or both and HBV, HAV, or both.

Example 3.

[0061] A biological sample is obtained from a subject suspected of having human immunodeficiency virus (HIV) and hepatitis C virus (HCV). The sample is mixed with a TaqMan signal oligonucleotide probe, two sets of forward and reverse primers, a chemically synthesized template nucleic acid, and a Taq DNA polymerase.

[0062] The signal oligonucleotide probe is complementary to the template nucleic acid. The first forward primer comprises two regions: a first region complementary to HIV and a second region complementary to the template nucleic acid. The first reverse primer is complementary to HIV. The second forward primer comprises two regions, a first region complementary to a region of HCV and a second region complementary to the template nucleic acid. The second reverse primer is complementary to HCV.

[0063] The mixture is thermally cycled under appropriate buffer conditions and temperature conditions, and comprising the appropriate reagents, such that the primers anneal to their respective targets, which are amplified by polymerase chain reaction.. The Taq polymerase releases the second region of the first forward oligonucleotide primer and the second region of the second oligonucleotide primer via its endonuclease activity. The second region of the first forward oligonucleotide primer and the second region of the second oligonucleotide primer are used as forward and reverse primers to amplify the template nucleic acid by thermally cycling the mixture under reaction conditions sufficient for template nucleic acid amplification. The signal oligonucleotide probe binds to the template nucleic acid. The Taq polymerase degrades the signal oligonucleotide probe via its exonuclease activity, thus releasing the quencher. A fluorescent signal is generated by the fluorophore upon release of the quencher. The fluorescent signal is detected, indicating the presence of both HIV and HCV in the sample.

Example 4

[0064] Synthetic DNA comprising a sequence from Influenza A (FluA) or Influenza B (FluB) was generated. Three mixtures were generated comprising either 10^5 copies of FluA alone, 10^5 copies of FluB alone, or 10^5 copies of both FluA and FluB. To each of these mixtures, primer pairs were added with regions complementary to either FluA or FluB. Each of the first forward primers from the primer pairs comprised, in addition to a region complementary to a target, a region complementary to a signal oligonucleotide probe (universal probe). A signal oligonucleotide probe (universal probe) was also added to each mixture. Table 1 shows the concentration added for each primer and the universal probe. Finally, reagents necessary to perform polymerase chain reaction were added.

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Table 1: Reaction components

Reagent	Concentration
FluA forward primer with reverse complement of universal probe	10nM
FluA reverse primer	100nM
FluB forward primer with reverse complement of universal probe	10nM
FluB reverse primer	100nM
Universal probe comprising a FAM fluorophore	50nM

[0065] Each mixture was subjected to thermal cycling under conditions appropriate to amplify each target nucleic acid with polymerase chain reaction using an Applied Biosystems 7500 Fast Real-Time PCR system. Each mixture was subjected to 60 seconds at 95°C, followed by a series of forty-five thermal cycles each consisting of 30 seconds at 95°C followed by 120 seconds at 57°C. Fluorescence was measured at the end of each thermal cycle, and is plotted in FIG. 7. FIG. 7 shows that amplification and measurement of signals from each of the three mixtures results in a final fluorescence intensity in the same region ("FluA/FluB Acceptance Band"). FIG. 8 shows a bar graph of peak fluorescence intensity from FIG. 7. Error bars indicate 95% confidence bounds. Following fluorescence measurement, the presence of a signal in the mixtures was correlated to the presence of either or both of FluA or FluB in the mixture.

[0066] 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. It is not intended that the invention be limited by the specific examples provided within the specification. While the invention has been described with reference to the aforementioned specification, the descriptions and illustrations of the embodiments herein are not meant to be construed in a limiting sense. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. Furthermore, it shall be understood that all aspects of the invention are not limited to the specific depictions, configurations or relative proportions set forth herein which depend upon a variety of conditions and variables. It should be understood that various alternatives to the embodiments of the invention described herein may be employed in practicing the invention. It is therefore contemplated that the invention shall also cover any such alternatives, modifications, variations or equivalents. 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 at least one member of a plurality of target nucleic acids in a sample, the method comprising:

(a) providing a sample comprising, or potentially comprising, at least one member of the plurality of target nucleic acids;

(b) forming a mixture comprising the sample and

(i) a signal oligonucleotide probe;

(ii) a first forward oligonucleotide primer comprising: a first region complementary to a first region of a first member of the plurality of target nucleic acids; and a second region complementary or homologous to the signal oligonucleotide probe;

(iii) a first reverse oligonucleotide primer comprising a region complementary to a second region of the first member of the plurality of target nucleic acids;

(iv) a second forward oligonucleotide primer comprising: a first region complementary to a first region of a second member of the plurality of target nucleic acids; and a second region complementary or homologous to the signal oligonucleotide probe; and

(v) a second reverse oligonucleotide primer comprising a region complementary to a second region of the second member of the plurality of target nucleic acids;

(c) thermally cycling the mixture under conditions appropriate to amplify each member of the plurality of target nucleic acids with polymerase chain reaction (PCR), such that the signal oligonucleotide probe is degraded and a signal is generated if at least one member of the plurality of target nucleic acids is present in the mixture; and

(d) detecting the presence or absence of the signal.

2. The method of claim 1, wherein the mixture further comprises a nucleic acid enzyme.

3. The method of claim 1 or 2, wherein the signal oligonucleotide probe comprises a signal tag.

4. The method of claim 3, wherein, in (c), the signal is generated by the signal tag.

5. The method of claim 4, wherein the signal tag generates the signal upon degradation of the signal oligonucleotide probe.

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6. The method of any of claims 1-5, wherein, in (c), each thermal cycle is performed at an annealing temperature appropriate for annealing of the first region of the first forward oligonucleotide primer to the first target nucleic acid and annealing of the second region of the first forward oligonucleotide primer to the signal oligonucleotide probe.

7. The method of any of claims 1-6, further comprising correlating the presence of the signal to the presence of at least one member of the plurality of target nucleic acids or correlating the absence of the signal with the absence of each member of the plurality of target nucleic acids.

8. A method of detecting the presence or absence of at least one member of a plurality of target nucleic acids in a sample, the method comprising:

(a) providing a sample comprising, or potentially comprising, at least one member of the plurality of target nucleic acids;

(b) forming a mixture of the sample and

(i) a signal oligonucleotide probe;

(ii) a first forward oligonucleotide primer comprising a region with complementarity to a first member of the plurality of target nucleic acids;

(iii) a first reverse oligonucleotide primer comprising a region with complementarity to the first member of the plurality of target nucleic acids;

(iv) a second forward oligonucleotide primer comprising a region with complementarity to a second member of the plurality of target nucleic acids; and

(v) a second reverse oligonucleotide primer comprising a region with complementarity to the second member of the plurality of target nucleic acids;

(c) thermally cycling the mixture under conditions appropriate to amplify each member of the plurality of target nucleic acids with polymerase chain reaction (PCR) and generating a signal if at least one member of the plurality of target nucleic acids is present in the mixture; and

(d) detecting the presence or absence of the signal.

9. The method of claim 8, wherein the mixture further comprises a nucleic acid enzyme.

10. The method of claim 8 or 9, wherein the first forward oligonucleotide primer further comprises an additional region with complementarity or homology to the signal oligonucleotide probe.

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11. The method of any of claims 8-10, wherein the second forward oligonucleotide primer further comprises an additional region with complementarity or homology to the signal oligonucleotide probe.

12. The method of claim 11, wherein, in (c), each thermal cycle is performed at an annealing temperature appropriate for annealing of the first region of the second forward oligonucleotide primer to the second member of the plurality of target nucleic acids and annealing of the additional region of the second forward oligonucleotide primer to the signal oligonucleotide probe.

13. The method of any of claims 8-12, wherein the mixture formed at (b) further comprises:

(a) an nth forward oligonucleotide primer comprising: a first region with complementarity to an nth member of the plurality of target nucleic acids; and

(b) an nth reverse oligonucleotide primer comprising a region with complementarity to a region of an nth member of the plurality of target nucleic acids;

wherein n is an integer greater than 2.

14. The method of claim 13, wherein the nth forward oligonucleotide primer further comprises an additional region with complementarity to the signal oligonucleotide probe.

15. The method of claim 13 or 14;

wherein the mixture formed at (b) further comprises:

(a) an xth signal oligonucleotide probe;

(b) a yth forward oligonucleotide primer comprising a first region with complementarity to a yth member of the plurality of target nucleic acids; and

(c) a yth reverse oligonucleotide primer comprising a region with complementarity to a second region of the yth member of the plurality of target nucleic acids;

wherein, in (c), the xth signal oligonucleotide probe is degraded by the nucleic acid polymerase such that an xth signal is generated if at least one of the yth member of the plurality of target nucleic acids is present in the mixture;

wherein (e) further comprises correlating the presence of the xth signal to the presence of at least one of the yth members of the plurality target nucleic acids in the sample; and

wherein x is an integer greater than 1 and y is an integer greater than n.

16. The method of claim 15, wherein the yth forward oligonucleotide primer further comprises an additional region with complementarity to the signal oligonucleotide probe.

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17. The method of claim 15 or 16, wherein the yth forward oligonucleotide primer further comprises an additional region with complementarity to the signal oligonucleotide probe.

18. A kit for detecting the presence or absence of at least one member of a plurality of target nucleic acids in a sample, comprising:

(a) a signal oligonucleotide probe;

(b) a first forward oligonucleotide primer comprising: a first region with complementarity to a first member of the plurality of target nucleic acids; and a second region with complementarity or homology to the signal oligonucleotide probe;

(c) a first reverse oligonucleotide primer comprising a region with complementarity to the first member of the plurality of target nucleic acids;

(d) a second forward oligonucleotide primer comprising: a first region with complementarity to a second member of the plurality of target nucleic acids; and a second region with complementarity or homology to the signal oligonucleotide probe; and

(e) a second reverse oligonucleotide primer comprising a region with complementarity to the second member of the plurality of target nucleic acids.

19. A method of detecting the presence or absence of a first and second member of a plurality of target nucleic acids in a sample, the method comprising:

(a) providing a sample comprising, or potentially comprising, the first and second members of the plurality of target nucleic acids;

(b) forming a mixture comprising the sample and

(i) a template nucleic acid;

(ii) a signal oligonucleotide probe with complementarity to the template nucleic acid;

(iii) a first oligonucleotide primer comprising a region with complementarity to the first member of the plurality of target nucleic acids; and

(iv) a second oligonucleotide primer comprising a region with complementarity to the second member of the plurality of target nucleic acids;

(c) subjecting the mixture to conditions sufficient to anneal the first oligonucleotide primer to the first member of the plurality of target nucleic acids and the second oligonucleotide primer to the second member of the plurality of target nucleic acids, such that the first and second oligonucleotide primers are degraded;

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(d) thermally cycling the mixture under reaction conditions appropriate to amplify the template nucleic acid with polymerase chain reaction (PCR), such that a signal is generated if both the first and second members of the plurality of target nucleic acids are present in the mixture; and

(e) detecting the presence or absence of the signal.

20. The method of claim 19, wherein the first oligonucleotide primer further comprises an additional region with complementarity to a second region of the template nucleic acid.

21. The method of claim 19 or 20, wherein the second oligonucleotide primer further comprises an additional region with complementarity to a third region of the template nucleic acid.

22. The method of claim 21, wherein, in (c), the additional region of the first oligonucleotide primer and the additional region of the second oligonucleotide primer are released.

23. The method of any of claims 19-22, wherein the mixture further comprises one or more nucleic acid enzymes.

24. The method of any of claims 19-23, wherein the signal oligonucleotide probe comprises a signal tag.

25. The method of any of claims 19-24, further comprising (f) correlating the presence of the signal to the presence of both the first and second members of the plurality of target nucleic acids in the sample.

26. The method of any of claims 19-25, wherein the mixture in (b) further comprises (vi) a third oligonucleotide primer comprising a region with complementarity to a second region of the first member of the plurality of target nucleic acids; and (vii) a fourth oligonucleotide primer comprising a region with complementarity to a second region of the second member of the plurality of target nucleic acids.

27. The method of any of claims 19-26, further comprising, in (c), thermally cycling the mixture under reaction conditions appropriate to amplify the first and second members of the plurality of target nucleic acids with polymerase chain reaction.

28. A method of detecting the presence or absence of a first and second member of a plurality of target nucleic acids in a sample, the method comprising:

(a) providing a sample comprising, or potentially comprising, the first and second members of the plurality of target nucleic acids

(b) forming a mixture comprising the sample and

(i) a template nucleic acid;

(ii) a signal oligonucleotide probe with complementarity to the template nucleic acid;

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(iii) a first forward oligonucleotide primer comprising a region with complementarity to the first member of the plurality of target nucleic acids;

(iv) a first reverse oligonucleotide primer comprising a region with complementarity the first member of the plurality of target nucleic acids;

(v) a second forward oligonucleotide primer comprising a first region with complementarity to the second member of the plurality of target nucleic acids; and

(vi) a second reverse oligonucleotide primer comprising a region with complementarity to the second member of the plurality of target nucleic acids;

(c) thermally cycling the mixture under reaction conditions appropriate to amplify the first and second members of the plurality of target nucleic acids with polymerase chain reaction (PCR), such that the first forward oligonucleotide primer and the second forward oligonucleotide primer are degraded;

(d) thermally cycling the mixture under reaction conditions appropriate to amplify the template nucleic acid with PCR, such that a signal is generated if both the first and second members of the plurality of target nucleic acids are present in the mixture; and

(e) detecting the presence or absence of the signal.

29. The method of claim 28, further comprising (f) correlating the presence of the signal to the presence of both the first and second members of the plurality of target nucleic acids in the sample.

30. The method of claim 28 or 29, wherein the signal oligonucleotide probe comprises a signal tag.

31. The method of any of claims 28-30, wherein the first forward oligonucleotide primer further comprises an additional region with complementarity to a second region of the template nucleic acid.

32. The method of claim 31, wherein, in (c), the additional region of the first forward oligonucleotide primer is released.

33. The method of any of claims 28-32, wherein the second forward oligonucleotide primer further comprises an additional region with complementarity to a third region of the template nucleic acid.

34. The method of claim 33, wherein, in (c), the additional region of the second forward oligonucleotide primer is released.

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35. The method of any of claims 28-34, wherein mixture further comprises a nucleic acid enzyme.

36. The method of claim 35, wherein, in (c), the first and second oligonucleotide primers are degraded by the nucleic acid enzyme.

37. A kit for detecting the presence or absence of a first and second member of a plurality of target nucleic acids in a sample, comprising:

(a) a template nucleic acid;

(b) a signal oligonucleotide probe with complementarity to a first region of the template nucleic acid;

(c) a first oligonucleotide primer comprising: a first region with complementarity to a region of the first member of the plurality of target nucleic acids; and a second region with complementarity to a second region of the template nucleic acid; and

(d) a second oligonucleotide primer comprising: a first region with complementarity to a region of the second member of the plurality of target nucleic acids; and a second region with complementarity to a third region of the template nucleic acid.

38. A method of detecting the presence or absence of at least one member of a plurality of target nucleic acids in a sample, the method comprising:

(a) providing a sample comprising, or potentially comprising, at least one member of the plurality of target nucleic acids;

(b) forming a mixture of the sample and

(i) a signal oligonucleotide probe comprising a signal tag;

(ii) a plurality of a first forward oligonucleotide primer comprising: a first region with complementarity to a first region of a first member of the plurality of target nucleic acids; and a second region with complementarity to the signal oligonucleotide probe;

(iii) a plurality of a first reverse oligonucleotide primer comprising a region with complementarity to a second region of the first member of the plurality of target nucleic acids;

(iv) a plurality of a second forward oligonucleotide primer comprising: a first region with complementarity to a first region of a second member of the plurality of target nucleic acids; and a second region with complementarity to the signal oligonucleotide probe;

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(v) a plurality of a second reverse oligonucleotide primer comprising a region with complementarity to a second region of the second member of the plurality of target nucleic acids; and

(vi) a nucleic acid enzyme;

(c) thermally cycling the mixture under reaction conditions appropriate to amplify each member of the plurality of target nucleic acids with polymerase chain reaction (PCR), such that the signal oligonucleotide probe is degraded by the nucleic acid enzyme such that a signal is generated if at least one member of the plurality of target nucleic acids is present in the mixture;

(d) detecting the presence or absence of the signal; and

(e) correlating the presence of the signal to the presence of at least one of the members of the plurality of target nucleic acids in the sample.

39. The method of claim 38, wherein the mixture formed at (b) further comprises:

(c) a plurality of an nth forward oligonucleotide primer comprising: a first region with complementarity to a first region of an nth member of the plurality of target nucleic acids; and a second region with complementarity to the signal oligonucleotide probe; and

(d) a plurality of an nth reverse oligonucleotide primer comprising a region with complementarity to a second region of an nth member of the plurality of target nucleic acids;

wherein n is an integer greater than 2.

40. The method of claim 39;

wherein the mixture formed at (b) further comprises:

(d) an xth signal oligonucleotide probe comprising an xth signal tag;

(e) a plurality of a yth forward oligonucleotide primer comprising: a first region with complementarity to a first region of a yth member of the plurality of target nucleic acids; and a second region with complementarity to the xth signal oligonucleotide probe; and

(f) a plurality of a yth reverse oligonucleotide primer comprising a region with complementarity to a second region of the yth member of the plurality of target nucleic acids;

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wherein, in (c), the xth signal oligonucleotide probe is degraded by the nucleic acid polymerase such that an xth signal is generated if at least one of the yth member of the plurality of target nucleic acids is present in the mixture;

wherein (e) further comprises correlating the presence of the xth signal to the presence of at least one of the yth members of the plurality target nucleic acids in the sample; and

wherein x is an integer greater than 1 and y is an integer greater than n.

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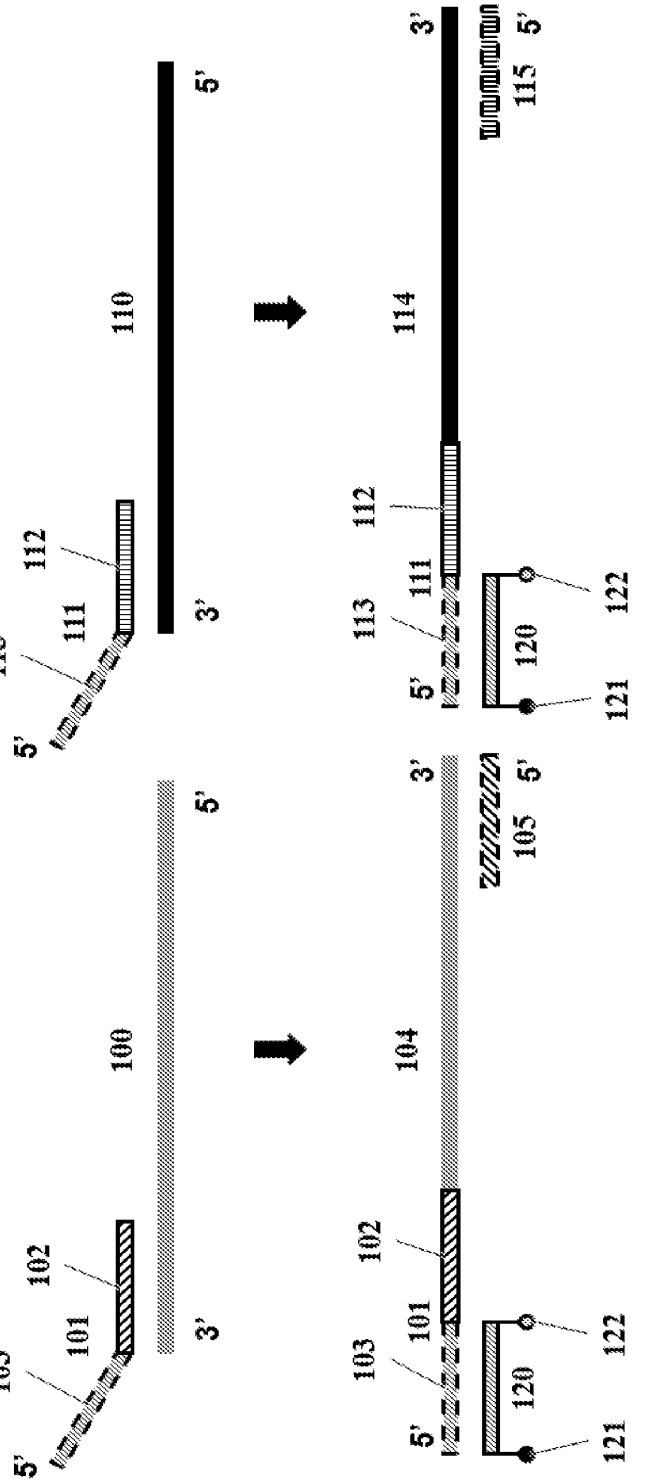


FIG. 1

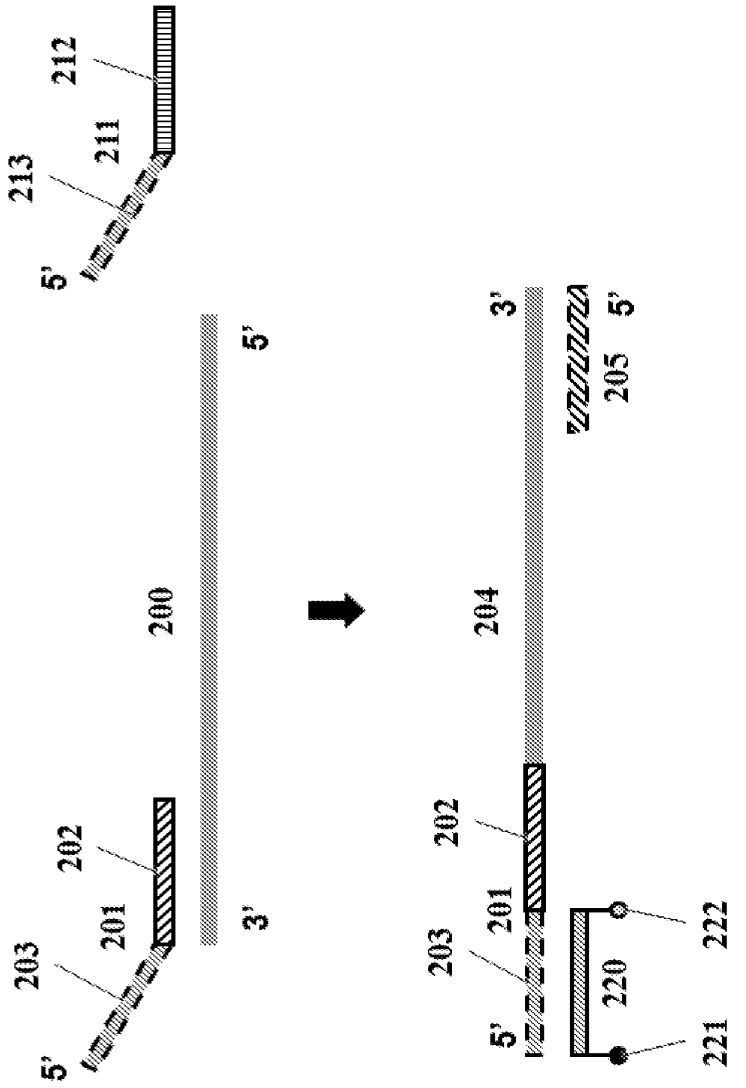

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FIG. 2

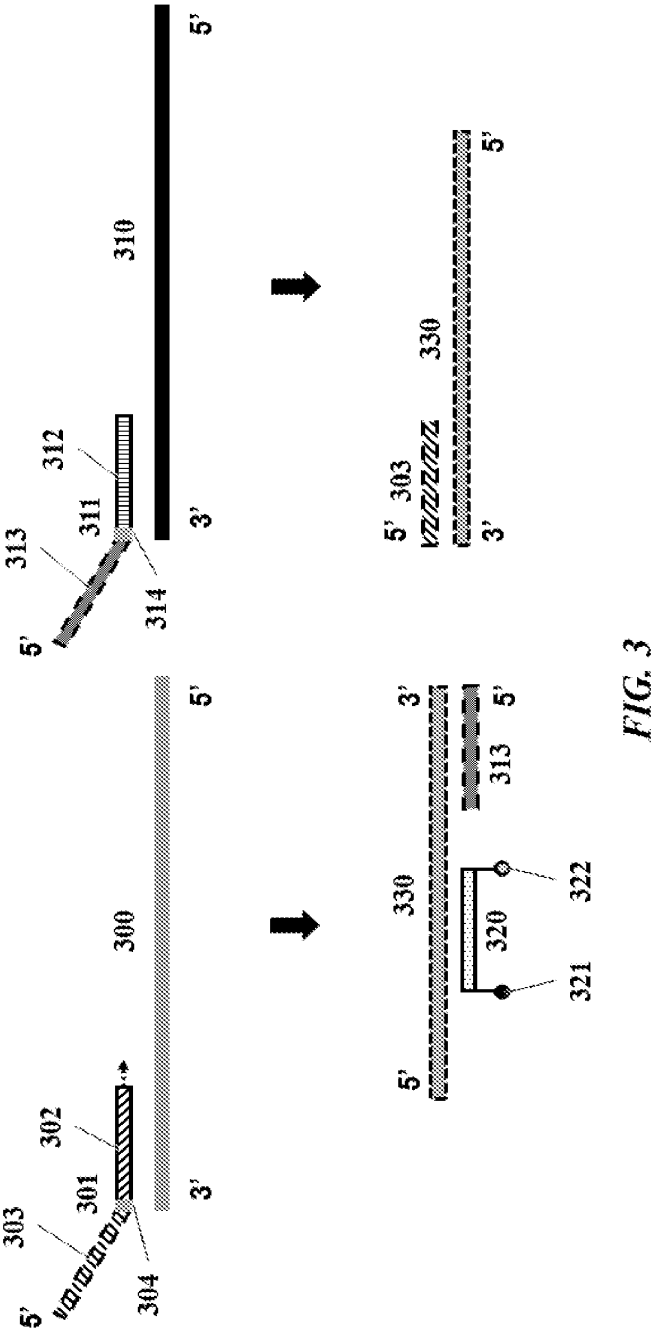

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FIG. 3

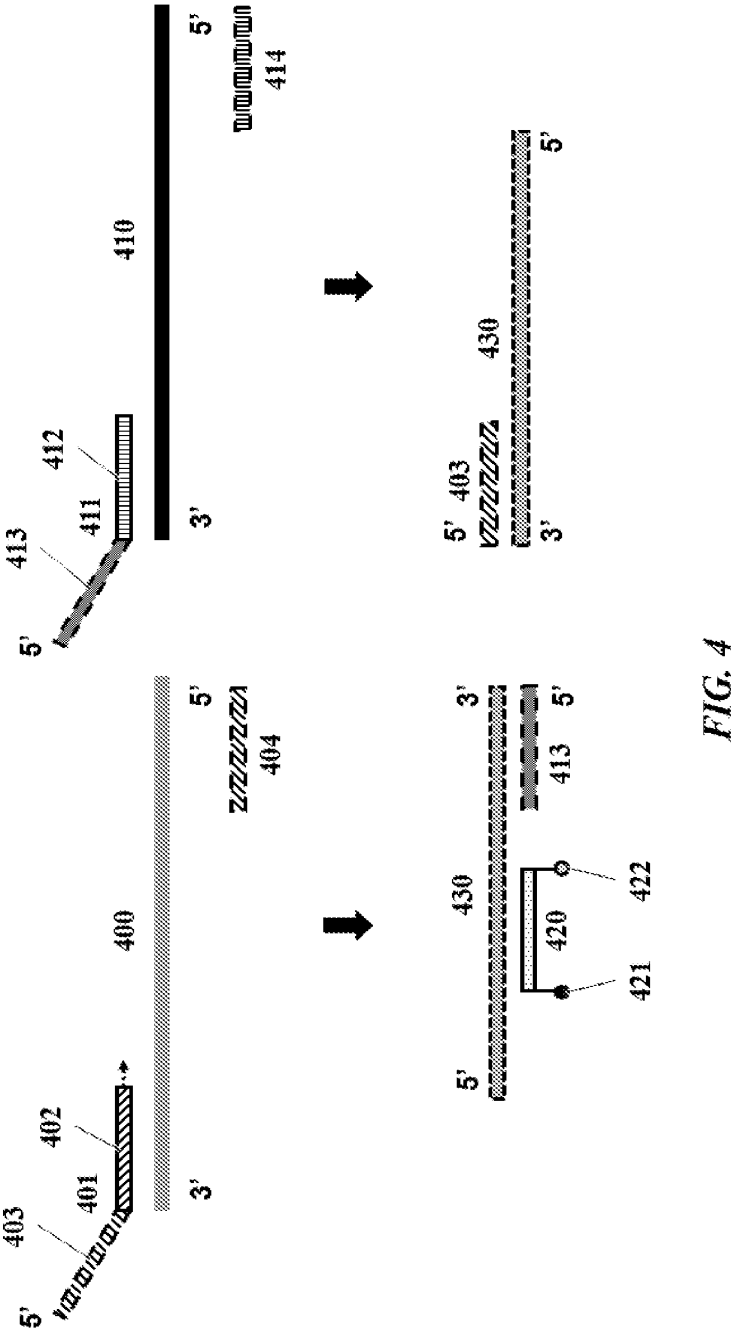

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FIG. 4

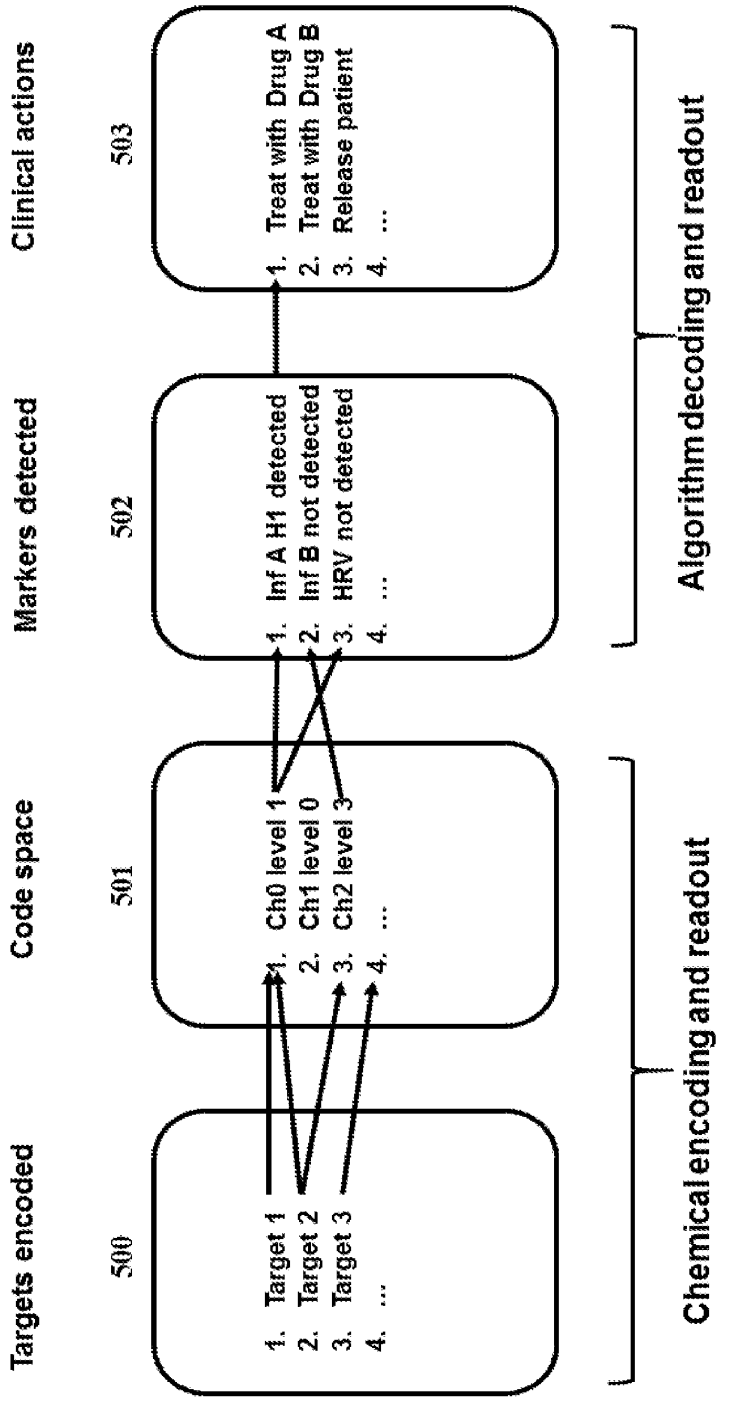

Targets encoded
Code space
Markers detected
Clinical actions
500
501
502
503
1. Target 1
2. Target 2
3. Target 3
4. ...
1. Ch0 level 1
2. Ch1 level 0
3. Ch2 level 3
4. ...
1. Inf A H1 detected
2. Inf B not detected
3. HRV not detected
4. ...
1. Treat with Drug A
2. Treat with Drug B
3. Release patient
4. ...
Chemical encoding and readout
Algorithm decoding and readout

FIG. 5

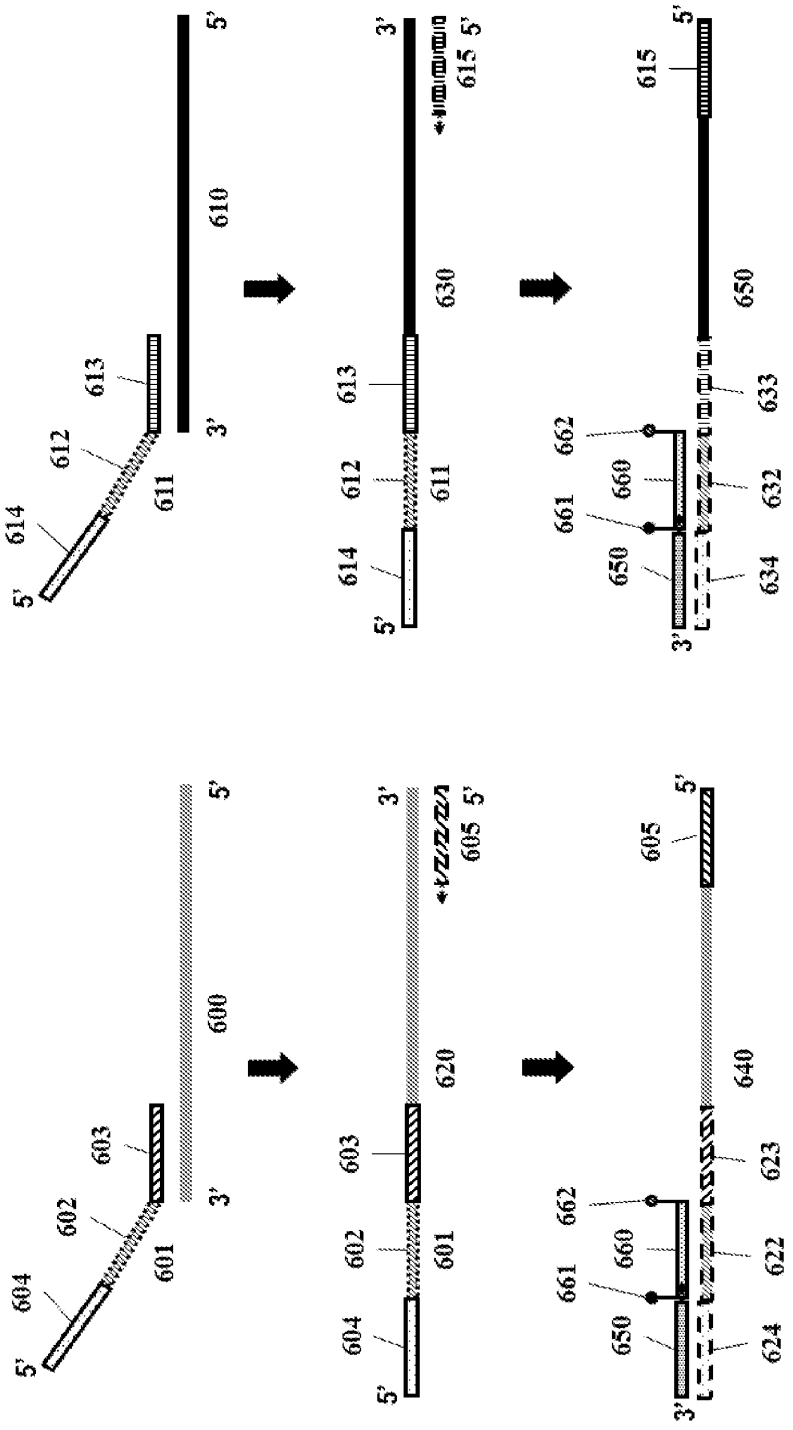

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FIG. 6

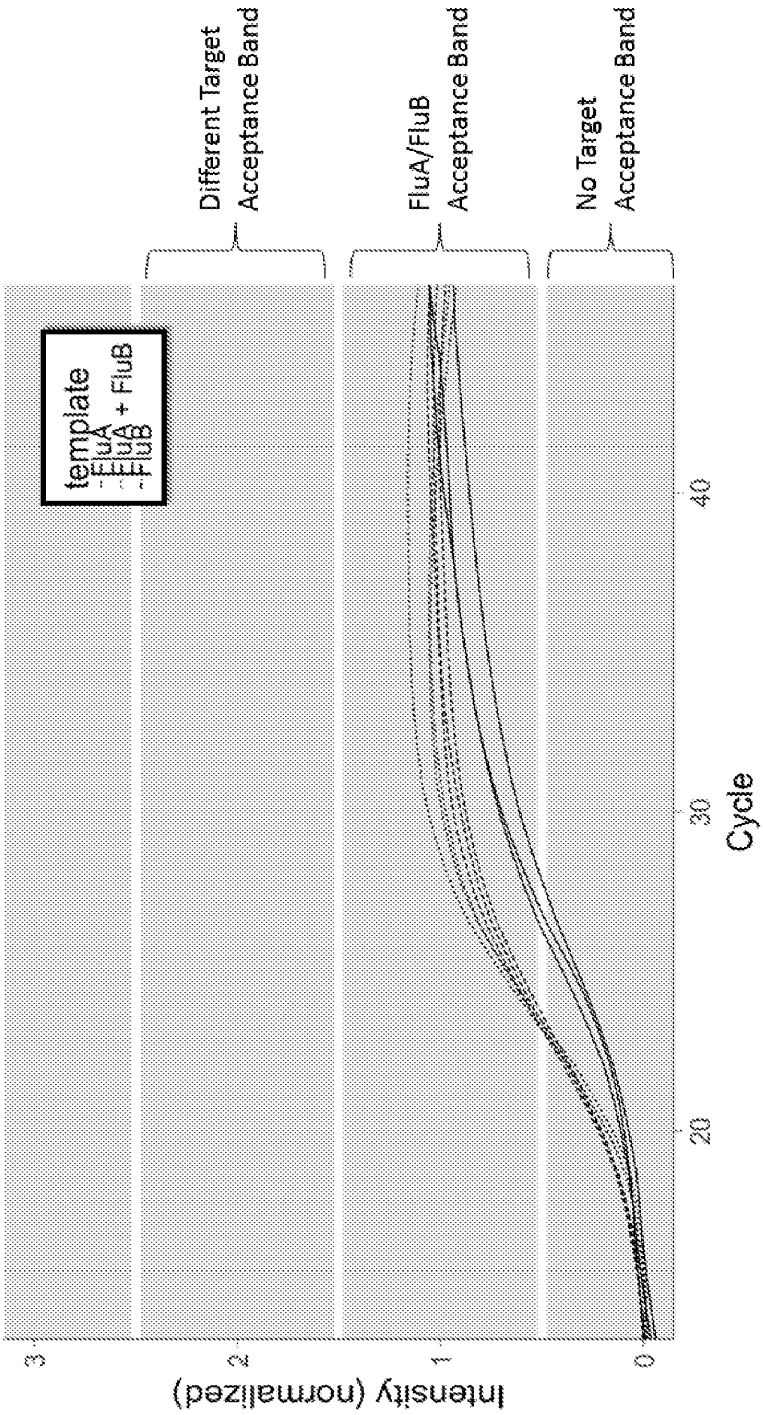

Different Target
Acceptance Band
FluA/FluB
Acceptance Band
No Target
Acceptance Band
template
FluA
FluA + FluB
FluB
Intensity (normalized)
0
1
2
3
20
30
40
Cycle

FIG. 7

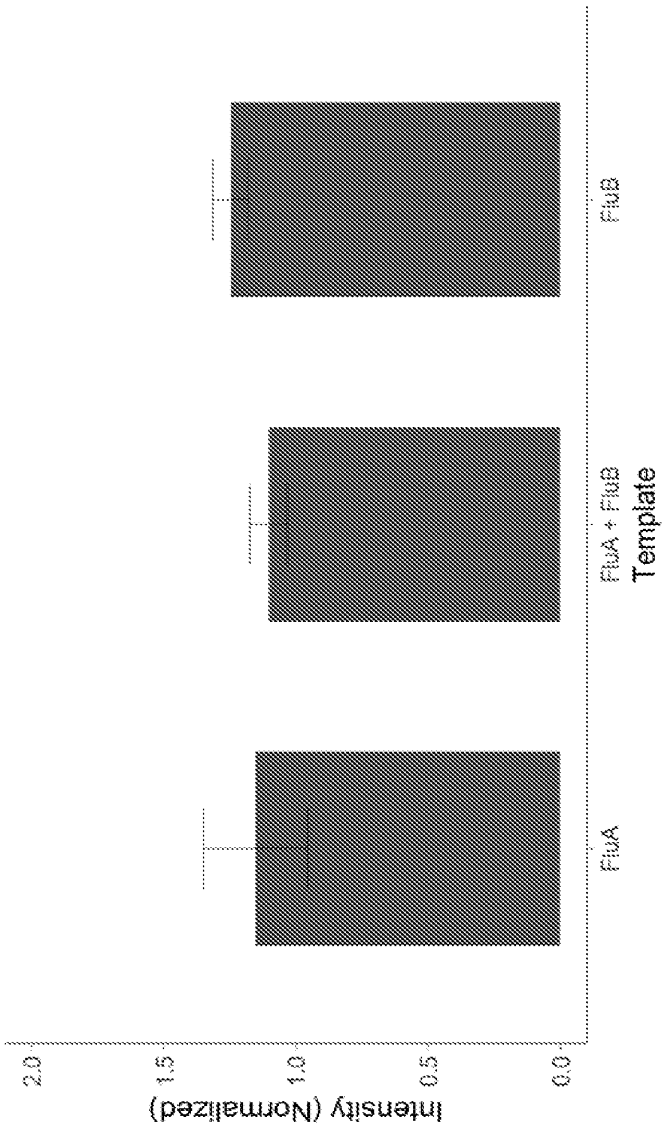

Intensity (Normalized)
2.0
1.5
1.0
0.5
0.0
FluA
FluA + FluB
FluB
Template

FIG. 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US18/55927

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☒ forming part of the international application as filed:

      ☐ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

An invitation to Furnish Nucleotide and/or Amino Acid Sequence Listing and to Pay, Where Applicable, Late Furnishing Fee ("ISA/225") was mailed on 26 October 2018 (26.10.2018). No approved electronic sequence listing was received in response to the ISA/225. Therefore, the international search was carried out only to the extent possible.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US18/55927

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.: 6-7, 11-17, 23-27, 31-36
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:

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.:

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/US18/55927

A. CLASSIFICATION OF SUBJECT MATTER
IPC - C12Q 1/686, 1/68 ; G01N 21/64, 33/58, 33/533 (2019.01)
CPC - C12Q 1/686, 1/6851, 1/6811, 1/6816, 1/6827, 1/6888; G01N 21/64, 21/6428

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 – Y	WO 2017/ 173035 A1 (CHROMACODE, INC) 5 October 2017; paragraphs [007], [008], [022], claims 1, 3.	8, 19, 28-29, 30/28-29 1, 3/1, 4/3/1, 5/4/3/1, 18, 2, 3/2, 4/3/2, 5/4/3/2, 9, 10/9, 20
Y	(ROBIN, JD et al.) Comparison of DNA Quantification Methods for Next Generation Sequencing. Scientific Reports. 6 April 2016. Vol. 6; figure 1; page 3, 2nd paragraph; DOI: 10.1038/srep24067	1, 2, 3/1-2, 4/3/1-2, 5/4/3/1-2, 18
Y	(MOKANY, E et al.) MNAzyme qPCR with Superior Multiplexing Capacity. Clinical Chemistry. February 2013. Epub 11 December 2012. Vol. 59, No. 2, pages 419-426; abstract; DOI: 10.1373/clinchem.2012.192930	2, 3/2, 4/3/2, 5/4/3/2, 9, 10/9
Y	WO 2008/118998 A2 (PRIMERA BIOSYSTEMS, INC) 2 October 2008; claim 1	10/8, 20
A	(ANDROVIC, P et al.) Two-tailed RT-qPCR: a novel method for highly accurate miRNA quantification. Nucleic Acids Research. 6 September 2017, Vol. 45, No. 15; abstract; DOI: 10.1093/nar/gkx588	21/19-20, 22/21/19-20
A	(WANG, JG et al.) An improved protocol with a highly degenerate primer targeting copper-containing membrane-bound monooxygenase genes for community analysis of methane- and ammonia-oxidizing bacteria. FEMS Microbiology Ecology. March 2017. Epub 8 December 2016. Vol. 93, No. 3; page 3, 1st column, 2nd paragraph; DOI: 10.1093/femsec/fiw244	21/19-20, 22/21/19-20

☐ 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
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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 December 2018 (20.12.2018)	08 FEB 2019
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

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Declarations under Rule 4.17:
— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*
— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

(54) Title: MOLECULAR TARGETS FOR FETAL NUCLEIC ACID ANALYSIS

(57) Abstract: The present disclosure provides methods and compositions for evaluation of nucleic acid size distribution and genetic abnormalities. The disclosed methods may be useful in determining size distribution of nucleic acids in a sample, for example, fetal fraction in a plasma sample. The disclosed methods may be useful in identifying or detecting genetic abnormalities from a subject, for example, fetal aneuploidy (e.g., trisomy 21).

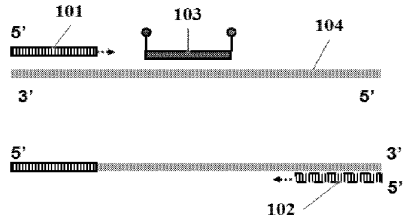


FIG. 1A

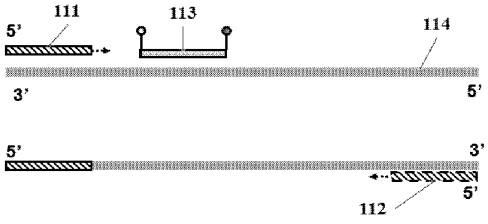


FIG. 1B

[Continued on next page]

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MOLECULAR TARGETS FOR FETAL NUCLEIC ACID ANALYSIS

CROSS-REFERENCE

[0001] This application claims the benefit of U.S. Provisional Patent Application No. 62/636,632, filed on February 28, 2018, which application is incorporated herein by reference in its entirety.

BACKGROUND

[0002] Digital PCR (dPCR) is a useful method for detection and quantification of nucleic acid targets. The use of labeled oligonucleotide probes enables specific detection of a target present in a partition (e.g., droplet, microwell). dPCR may be used in a variety of nucleic acid detection methods.

SUMMARY

[0003] Disclosed herein, in some aspects, is a method for analyzing a size distribution of nucleic acids, the method comprising: (A) providing a sample comprising: (i) a first plurality of nucleic acids and a second plurality of nucleic acids, wherein the first plurality of nucleic acids each comprise a first nucleic acid sequence of a given length and the second plurality of nucleic acids each comprise a second nucleic acid sequence longer than the given length; (ii) a first set of paired oligonucleotide primers configured to amplify the first nucleic acid sequence; and (iii) a second set of paired oligonucleotide primers, configured to amplify the second nucleic acid sequence; (B) performing an amplification reaction on (a) the first nucleic acid sequence to generate a first signal and (b) the second nucleic acid sequence to generate a second signal; and (C) determining a ratio of a first value derived from the first signal to a second value derived from the second signal, thereby analyzing the size distribution. In some embodiments, the sample further comprises (iv) a first oligonucleotide probe configured to hybridize to a region of the first nucleic acid sequence and (v) a second oligonucleotide probe configured to hybridize to a region of the second nucleic acid sequence. In some embodiments, the first signal is generated from the first oligonucleotide probe and the second signal is generated from the second oligonucleotide probe. In some embodiments, the sample further comprises an intercalating dye. In some embodiments, the first signal or the second signal is generated from the intercalating dye. In some embodiments, the first signal and the second signal are generated from the intercalating dye. In some embodiments, the intercalating dye is SYBR® Green or EvaGreen®. In some embodiments, the first signal or the second signal is generated by mass spectrometry.

[0004] Disclosed herein, in some aspects, is a method for analyzing a size distribution of nucleic acids, the method comprising: (A) providing a sample comprising: (i) a first plurality of nucleic

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acids and a second plurality of nucleic acids, wherein the first plurality of nucleic acids each comprise a first nucleic acid sequence of a given length and the second plurality of nucleic acids each comprise a second nucleic acid sequence longer than the given length; (ii) a first set of paired amplification oligomers configured to amplify the first nucleic acid sequence; (iii) a second set of paired amplification oligomers, configured to amplify the second nucleic acid sequence; (iv) a first detection probe configured to anneal to a region of the first nucleic acid sequence; and (v) a second detection probe configured to anneal to a region of the second nucleic acid sequence; (B) performing an amplification reaction on (a) the first nucleic acid sequence to generate a first signal from the first detection probe and (b) the second nucleic acid sequence to generate a second signal from the second detection probe; and (C) determining a ratio of a first value derived from the first signal to a second value derived from the second signal, thereby analyzing the size distribution. In some embodiments, the first set of paired amplification oligomers comprises: a first forward amplification oligomer; and a first reverse amplification oligomer. In some embodiments, the first set of paired amplification oligomers comprises: a plurality of first forward amplification oligomers; and a plurality of first reverse amplification oligomers. In some embodiments, each of the plurality of first forward amplification oligomers has a different nucleic acid sequence. In some embodiments, a first forward amplification oligomer of the plurality of first forward amplification oligomers is configured to hybridize to a region of the first sequence. In some embodiments, each of the plurality of first reverse amplification oligomers has a different nucleic acid sequence. In some embodiments, a first reverse amplification oligomer of the plurality of first reverse amplification oligomers is configured to hybridize to a region of the first sequence. In some embodiments, the second set of paired amplification oligomers comprises: a second forward amplification oligomer; and a second reverse amplification oligomer. In some embodiments, the second set of paired amplification oligomers comprises: a plurality of second forward amplification oligomers and a plurality of second reverse amplification oligomers. In some embodiments, each of the plurality of second forward amplification oligomers has a different nucleic acid sequence. In some embodiments, a second forward amplification oligomer of the plurality of second forward amplification oligomers is configured to hybridize to a region of the second sequence. In some embodiments, each of the plurality of second reverse amplification oligomers has a different nucleic acid sequence. In some embodiments, a second reverse amplification oligomer of the plurality of second reverse amplification oligomers is configured to hybridize to a region of the second sequence. In some embodiments, the first value and the second value provide a quantitative ratio measurement corresponding to an abundance of the first plurality of nucleic acids and the second plurality of nucleic acids in the sample. In some embodiments, the first

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detection probe or the second detection probe comprises a non-target-hybridizing sequence. In some embodiments, the first detection probe or the second detection probe is a hairpin detection probe. In some embodiments, the hairpin detection probe is a molecular beacon or a molecular torch. In some embodiments, the sample comprises: genomic DNA, mRNA, cDNA, or a combination thereof. In some embodiments, the sample is derived from plasma from a pregnant woman. In some embodiments, the sample comprises maternal nucleic acid and fetal nucleic acid. In some embodiments, the first plurality of nucleic acids comprises the fetal nucleic acid and the second plurality of nucleic acids comprises the maternal nucleic acid. In some embodiments, the determining of the ratio provides a fetal fraction. In some embodiments, the sample is from an individual having or suspected of having cancer.

[0005] In some embodiments, the first signal and the second signal are generated in a single fluorescence channel. In some embodiments, (B) is performed in at least one partition of a plurality of partitions. In some embodiments, the plurality of partitions is a plurality of droplets. In some embodiments, the plurality of partitions is a plurality of wells. In some embodiments, the second nucleic acid sequence comprises at least a portion of the first nucleic acid sequence. In some embodiments, the second nucleic acid sequence comprises the first nucleic acid sequence. In some embodiments, the amplification reaction comprises polymerase chain reaction (PCR). In some embodiments, the PCR is quantitative PCR (qPCR) or digital PCR (dPCR). In some embodiments, the first detection probe comprises a first detectable label and the second detection probe comprises a second detectable label. In some embodiments, the first detection probe and the second detection probe each further comprise a quencher. In some embodiments, during the amplification reaction, the first detectable label is released from the first detection probe and the second detectable label is released from the second detection probe, thereby generating the first signal and the second signal. In some embodiments, the first detectable label and the second detectable label are each selected from the group consisting of a chemiluminescent label, a fluorescent label, and any combination thereof. In some embodiments, the first signal or the second signal is a chemiluminescent signal, a fluorescent signal, or any combination thereof. In some embodiments, the first detection probe and the second detection probe are TaqMan® detection probes. In some embodiments, the method further comprises comparing the ratio to a reference value. In some embodiments, the comparing identifies the presence or absence of a genetic abnormality in the sample. In some embodiments, the reference value corresponds to a ratio of a third value generated from a third nucleic acid sequence and a fourth value generated from a fourth nucleic acid sequence. In some embodiments, the third nucleic acid sequence and the fourth nucleic acid sequence each correspond to a region of nucleic acid not associated with the genetic abnormality. In some

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embodiments, the reference value is derived from a plurality of third values generated from a plurality of third nucleic acid sequences and a plurality of fourth values generated from a plurality of fourth nucleic acid sequences. In some embodiments, the plurality of third nucleic acid sequences and the plurality of fourth nucleic acid sequences each correspond to a region of nucleic acid not associated with the genetic abnormality. In some embodiments, the genetic abnormality is a fetal aneuploidy. In some embodiments, the second nucleic acid sequence comprises at least a portion of the first nucleic acid sequence. In some embodiments, the method further comprises comparing the ratio to a reference value. In some embodiments, the first value is a quantity of the first plurality of nucleic acids. In some embodiments, the second value is a quantity of the second plurality of nucleic acids. In some embodiments, the ratio is determined without quantifying the first plurality of nucleic acids and the second plurality of nucleic acids. In some embodiments, the amplification reaction comprises qPCR, wherein the first value is derived from amplification kinetics of the first plurality of nucleic acids. In some embodiments, the amplification reaction comprises qPCR, wherein the second value is derived from amplification kinetics of the second plurality of nucleic acids. In some embodiments, the amplification reaction comprises dPCR, wherein the first value is derived from a number of partitions containing the first nucleic acid sequence. In some embodiments, the amplification reaction comprises dPCR, wherein the second value is derived from a number of partitions containing the second nucleic acid sequence.

[0006] In some embodiments, in (A), the sample comprises: (vi) one or more additional pluralities of nucleic acids comprising one or more additional nucleic acid sequences; (vii) one or more additional sets of paired amplification oligomers configured to amplify the one or more additional nucleic acid sequences; and (viii) one or more additional detection probes configured to anneal to a region of the one or more additional nucleic acid sequences; in (B), the amplification reaction is performed on the one or more additional nucleic acid sequences to generate one or more additional signals from the one or more additional sets of detection probes; and in (C), determining an additional ratio of the first value or the second value to one or more additional values derived from the one or more additional signals, thereby analyzing the size distribution. In some embodiments, the one or more additional sets of paired amplification oligomers comprise n amplification oligomers; and the one or more additional sets of detection probes comprise n additional detection probes. In some embodiments, n is an integer between 1 and 30. In some embodiments, the first value is a quantity of the first plurality of nucleic acids. In some embodiments, the second value is a quantity of the second plurality of nucleic acids. In some embodiments, the ratio is determined without quantifying the first plurality of nucleic acids and the second plurality of nucleic acids.

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[0007] Disclosed herein, in some aspects, is a method for identifying the presence or absence of a fetal aneuploidy, comprising: (A) providing a sample comprising: (i) a plurality of fetal nucleic acids, each comprising a first nucleic acid sequence of a given length; (ii) a plurality of maternal nucleic acids, each comprising a second nucleic acid sequence longer than the given length; (iii) a first set of oligonucleotide primers configured to amplify the first nucleic acid sequence; (iv) a second set of oligonucleotide primers configured to amplify the second nucleic acid sequence; (v) a first oligonucleotide probe configured to hybridize to the first nucleic acid sequence; and (vi) a second oligonucleotide probe configured to hybridize to the second nucleic acid sequence; (B) amplifying (a) the first nucleic acid sequence to generate a first signal from the first oligonucleotide probe and (b) the second nucleic acid sequence to generate a second signal from the second oligonucleotide probe; (C) determining a ratio of a value derived from the the first signal to a second value derived from the second signal; and (D) comparing the ratio to a reference value, thereby identifying the presence or absence of the fetal aneuploidy. In some embodiments, the first nucleic acid sequence corresponds to a region of nucleic acid potentially associated with the fetal aneuploidy. In some embodiments, the region comprises a region of chromosome 22, chromosome 21, chromosome 18, chromosome 13, chromosome 9, chromosome 8, or an X chromosome. In some embodiments, the region comprises a region of chromosome 21. In some embodiments, the region comprises a region of chromosome 18. In some embodiments, the region comprises a region of chromosome 13. In some embodiments, the region comprises a region of an X chromosome. In some embodiments, the reference value corresponds to a ratio of a third value generated from a third nucleic acid sequence and a fourth value generated from a fourth nucleic acid sequence. In some embodiments, the third nucleic acid sequence and the fourth nucleic acid sequence each correspond to a region of nucleic acid not associated with the fetal aneuploidy. In some embodiments, the reference value is derived from a plurality of third values generated from a plurality of third nucleic acid sequences and a plurality of fourth values generated from a plurality of fourth nucleic acid sequences. In some embodiments, the plurality of third nucleic acid sequences and the plurality of fourth nucleic acid sequences each correspond to a region of nucleic acid not associated with the fetal aneuploidy. In some embodiments, the region is a region of a housekeeping gene. In some embodiments, the housekeeping gene is β-globin. In some embodiments, the ratio is larger than the reference value, thereby indicating the presence of the fetal aneuploidy. In some embodiments, the ratio is smaller than the reference value, thereby identifying the presence of the fetal aneuploidy. In some embodiments, the plurality of fetal nucleic acids and the plurality of maternal nucleic acids are obtained from plasma from a pregnant woman. In some embodiments, the plurality of fetal nucleic acids comprises fetal deoxyribonucleic acid (DNA)

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and the plurality of maternal nucleic acids comprises maternal DNA. In some embodiments, the amplifying in (b) comprises polymerase chain reaction (PCR). In some embodiments, the PCR is quantitative PCR (qPCR) or digital PCR (dPCR). In some embodiments, the first oligonucleotide probe comprises a first detectable label and the second oligonucleotide probe comprises a second detectable label. In some embodiments, the first oligonucleotide probe and the second oligonucleotide probe each further comprise a quencher. In some embodiments, during the amplifying, the first detectable label is released from the first oligonucleotide probe and the second detectable label is released from the second oligonucleotide probe, thereby generating the first signal and the second signal. In some embodiments, the first detectable label and the second detectable label are each selected from the group consisting of a chemiluminescent label, a fluorescent label, and any combination thereof. In some embodiments, the first signal or the second signal is a chemiluminescent signal, a fluorescent signal, or any combination thereof. In some embodiments, the first oligonucleotide probe and the second oligonucleotide probe are TaqMan® detection probes. In some embodiments, the first set of oligonucleotide primers comprises a first forward primer and a first reverse primer. In some embodiments, the second set of oligonucleotide primers comprises a second forward primer and a second reverse primer. In some embodiments, the fetal aneuploidy is trisomy 21, trisomy 18, trisomy 13, trisomy 9, or trisomy 8. In some embodiments, the fetal aneuploidy is trisomy 21. In some embodiments, the fetal aneuploidy is trisomy 18. In some embodiments, the fetal aneuploidy is trisomy 13. In some embodiments, the fetal aneuploidy is a sex chromosome aneuploidy. In some embodiments, the sex chromosome aneuploidy is Turner syndrome, Klinefelter syndrome, trisomy X, XXY, or XYY.

[0008] In some embodiments, the second nucleic acid sequence does not comprise any of the first nucleic acid sequence. In some embodiments, the second nucleic acid sequence comprises at least a portion of the first nucleic acid sequence. In some embodiments, the second nucleic acid sequence comprises the first nucleic acid sequence. In some embodiments, the reference value corresponds to a ratio of a third value generated from a third nucleic acid sequence and a fourth value generated from a fourth nucleic acid sequence. In some embodiments, the second nucleic acid comprises at least a portion of the first nucleic acid sequence. In some embodiments, the first value is a quantity of the first plurality of nucleic acids. In some embodiments, the second value is a quantity of the second plurality of nucleic acids. In some embodiments, the ratio is determined without quantifying the first plurality of nucleic acids and the second plurality of nucleic acids. In some embodiments, the amplification reaction comprises qPCR, wherein the first value is derived from amplification kinetics of the first plurality of nucleic acids. In some embodiments, the amplification reaction comprises qPCR,

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wherein the second value is derived from amplification kinetics of the second plurality of nucleic acids. In some embodiments, the amplification reaction comprises dPCR, wherein the first value is derived from a number of partitions containing the first nucleic acid sequence. In some embodiments, the amplification reaction comprises dPCR, wherein the second value is derived from a number of partitions containing the second nucleic acid sequence.

[0009] In some embodiments, in (A), the sample comprises: (vi) one or more additional pluralities of fetal nucleic acids comprising one or more additional first nucleic acid sequences of a given length; (vii) one or more additional pluralities of maternal nucleic acids comprising one or more additional second nucleic acid sequences longer than the given length; (viii) one or more additional first sets of oligonucleotide primers configured to amplify the one or more additional first nucleic acid sequences; (xi) one or more additional second sets of oligonucleotide primers configured to amplify the one or more additional second nucleic acid sequences; (x) one or more additional first oligonucleotide probes configured to anneal to a region of the one or more additional first nucleic acid sequences; and(xi) one or more additional second oligonucleotide probes configured to anneal to a region of the one or more additional second nucleic acid sequences; in (B), the amplification reaction is performed on: the one or more additional first nucleic acid sequences to generate one or more additional first signals from the one or more additional first oligonucleotide probes; and the one or more additional second nucleic acid sequences to generate one or more additional second signals from the one or more additional second oligonucleotide probes; in (C), an additional ratio of one or more additional first values derived from the one or more additional first signals to one or more additional second values derived from the one or more additional second signals is determined; and in (D), the additional ratio is compared to the reference value. In some embodiments, the one or more additional first sets of oligonucleotide primers comprise n oligonucleotide primers; and the one or more additional first oligonucleotide probes comprise n additional detection probes. In some embodiments, the one or more additional second sets of oligonucleotide primers comprise n oligonucleotide primers; and the one or more additional second oligonucleotide probes comprise n additional detection probes. In some embodiments, n is an integer between 1 and 30. In some embodiments, the first value is a quantity of the first plurality of nucleic acids. In some embodiments, the second value is a quantity of the second plurality of nucleic acids. In some embodiments, the ratio is determined without quantifying the first plurality of nucleic acids and the second plurality of nucleic acids. In some embodiments, the plasma is subjected to conditions sufficient to enrich for fetal nucleic acids. In some embodiments, the plasma is not subjected to conditions sufficient to enrich for fetal nucleic acids. In some embodiments, the plasma is subjected to conditions sufficient to enrich for fetal nucleic acids. In some

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embodiments, the plasma is not subjected to conditions sufficient to enrich for fetal nucleic acids. In some embodiments, the first plurality of nucleic acids and the second plurality of nucleic acids are derived from the same source. In some embodiments, the first plurality of nucleic acids and the second plurality of nucleic acids are derived from different sources.

BRIEF DESCRIPTION OF THE DRAWINGS

[0010] 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:

[0011] **FIGs. 1A** and **1B** illustrate an example method for amplifying nucleic acid sequences of different lengths for identifying a differential in nucleic acid size distributions.

[0012] **FIGs. 2A** and **2B** illustrate another example method for amplifying nucleic acid sequences of different lengths for identifying a differential in nucleic acid size distributions.

[0013] **FIG. 3A** shows a simulated distribution of fetal fraction in cell free deoxyribonucleic acid (DNA). **FIG. 3B** shows a Receiver Operating Characteristic (ROC) curve of a simulated digital polymerase chain reaction (dPCR) assay. **FIG. 3C** shows the true positive (TP) rate for a simulated dPCR assay relative to the fetal fraction in a sample.

[0014] **FIG. 4** shows ROC curves for a simulated digital PCR assay with target fetal DNA enriched over maternal DNA by 70% (**FIG. 4A**) or 20% (**FIG. 4B**).

DETAILED DESCRIPTION

[0015] Polymerase Chain Reaction (PCR) is a method of exponential amplification of specific target nucleic acid in a reaction mix with a nucleic acid polymerase and primers. Primers are short single stranded oligonucleotides which are complementary to the 3' sequences of the positive and negative strand of the target sequence. The reaction mix is cycled in repeated heating and cooling steps. The heating cycle denatures or splits a double stranded nucleic acid target into single stranded templates. In the cooling cycle, the primers bind to complementary sequence on the template. After the template is primed the nucleic acid polymerase creates a copy of the original template. Repeated cycling exponentially amplifies the target 2 fold with each cycle leading to approximately a billion-fold increase of the target sequence in 30 cycles.

[0016] Digital PCR is a process of partitioning a sample containing one or more targets into a plurality of partitions (e.g., wells, droplets, etc.), performing a PCR reaction in each partition, and recording the fluorescence generated by, for example, a target-specific reporter probe. This

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is generally performed on a digital PCR instrument that measures the fluorescence from each partition in an optical channel through one or more excitation/emission filter sets.

[0017] Frequently, the target-specific nucleic acid probe is a short oligonucleotide complementary to one strand of the amplified target. The probe lacks a 3' hydroxyl and therefore is not extendable by the DNA polymerase. TaqMan® (ThermoFisher Scientific) chemistry is a common reporter probe method used for multiplex Real-Time PCR. The TaqMan® oligonucleotide probe is covalently modified with a fluorophore and a quenching tag (i.e., quencher). In this configuration the fluorescence generated by the fluorophore is quenched and is not detected by the real time PCR instrument. When the target of interest is present, the probe oligonucleotide base pairs with the amplified target. While bound, it is digested by the 5' to 3' exonuclease activity of the Taq polymerase thereby physically separating the fluorophore from the quencher and liberating signal for detection by the real time PCR instrument.

[0018] One tool for diagnosing fetal aneuploidy (e.g., trisomy 21) is digital PCR-based noninvasive prenatal screening (NIPS) testing using cell-free fetal DNA sequences isolated from a maternal blood sample. Standard NIPS can often report false negatives and may vary in sensitivity and/or specificity depending on the target. Furthermore, as only 4-10% of DNA in maternal plasma is of fetal origin, existing methods to detect fetal aneuploidy may be limited by the amount of fetal DNA present in a sample. Recognized herein is a need for noninvasive means of accurately detecting, measuring, and evaluating trace amounts of fetal DNA in maternal plasma.

Definitions

[0019] The term "primer," or "amplification oligomer," used herein interchangeably, can refer to an oligonucleotide or nucleic acid configured to bind to another nucleic acid and facilitate one or more reactions, for example, transcription, nucleic acid synthesis, and nucleic acid amplification. A primer can be double-stranded. A primer can be single-stranded. A primer can be a forward primer or a reverse primer. A forward primer and a reverse primer can be those which bind to opposite strands of a double-stranded nucleic acid. For example, a forward primer can bind to a region of a first strand (e.g., Watson strand) derived from a nucleic acid, and a reverse primer can bind to a region of a second strand (e.g., Crick strand) derived from the nucleic acid. A forward primer may bind to a region closer to the start site of a gene relative to a reverse primer or may bind closer to the end site of a gene relative to a reverse primer. A forward primer may bind to the coding strand of a nucleic acid, or may bind to the non-coding strand of a nucleic acid. A reverse primer may bind to the coding strand of a nucleic acid, or may bind to the non-coding strand of a nucleic acid.

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Overview

[0020] Targeted amplification and subsequent differentiation of nucleic acids based on length is a useful tool in molecular diagnostics, especially where a target nucleic acid sequence is present in trace amounts in a sample. The existence of short fragments of nucleic acid may be indicative of various conditions, including disorders resulting from viral, transplant, and/or cancerous ailments. Accordingly, amplifying known target nucleic acid sequences may establish the existence of circulating, short-fragment nucleic acids. Nucleic acids derived from the same source may be used, for example, to confirm the presence of viral and/or cancerous mutations. Nucleic acids from multiple individuals may be used, for example, for transplant and/or pregnancy diagnostics. Described herein are methods for differentiating nucleic acid fragments of varying length, for detection and analysis of nucleic acids of low abundance in a sample (e.g., fetal nucleic acid in a plasma sample).

[0021] In one aspect, the present disclosure provides a method for detecting a fetal aneuploidy. Plasma obtained from a pregnant woman will comprise cell-free fragments of both fetal and maternal nucleic acid (e.g., DNA). Maternal nucleic acids in a cell-free sample from a pregnant woman have a higher average fragment length compared with fetal nucleic acids from the same sample. This differential may be utilized to detect fetal aneuploidy with high accuracy. In general, the disclosed methods comprise the use of multiple sets of oligonucleotide primers, each configured to amplify nucleic acid fragments of different length. For example, one set of oligonucleotide primers may be configured to amplify nucleic acid fragments of smaller length (e.g., fetal nucleic acids), and another set of oligonucleotide primers may be configured to amplify fragments of longer length (e.g., maternal nucleic acids). Each set of oligonucleotide primers may be paired with an oligonucleotide probe to generate a signal associated with each set of primers. Oligonucleotide primers and probes designed in this manner can be used to identify a differential in fragment size distribution (e.g., fetal nucleic acid vs. maternal nucleic acid), by identifying the difference between the signals associated with each set of primers. For example, in samples with little to no fetal nucleic acid fraction, the signals generated from the two sets of oligonucleotide primers would be about identical. By contrast, in samples with high fetal nucleic acid fraction, the signal associated with the fetal (i.e., shorter) nucleic acid fragments would be significantly greater than the signal associated with the maternal (i.e., longer) nucleic acid fragments. In addition, this signal differential, or ratio, can be used to identify fetal aneuploidy by comparing the ratio of a test subject with a reference value, such as the ratio from a healthy subject. A significant difference (e.g., increase or decrease) in a measured ratio compared with such a reference value may positively identify a subject as having a fetal aneuploidy.

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[0022] **FIGs. 1A** and **1B** illustrate an example method for targeting different sizes of nucleic acid sequences for nucleic acid analysis. **FIG. 1A** shows forward primer **101**, reverse primer **102**, oligonucleotide probe **103**, and nucleic acid **104**. Forward primer **101** and reverse primer **102** are designed to amplify nucleic acid **104**, as shown. Oligonucleotide probe **103** is designed to hybridize to a region of nucleic acid **104**, as shown, and is configured to generate a signal following amplification of nucleic acid **104** with forward primer **101** and reverse primer **102**. Nucleic acid **104** may be a nucleic acid fragment. Nucleic acid **104** may be a cell-free nucleic acid. Nucleic acid **104** may be a fetal nucleic acid. Nucleic acid **104** is of a given length. For example, nucleic acid **104** may be a cell-free, fetal nucleic acid fragment of a given length. **FIG. 1B** shows forward primer **111**, reverse primer **112**, oligonucleotide probe **113**, and nucleic acid **114**. Forward primer **111** and reverse primer **112** are designed to amplify nucleic acid **114**, as shown. Oligonucleotide probe **113** is designed to hybridize to a region of nucleic acid **114**, as shown, and is configured to generate a signal following amplification of nucleic acid **114** with forward primer **111** and reverse primer **112**. Nucleic acid **114** may be a nucleic acid fragment. Nucleic acid **114** may be a cell-free nucleic acid. Nucleic acid **114** may be a maternal nucleic acid. Nucleic acid **114** is of a length longer than nucleic acid **104**. Nucleic acid **114** may comprise a portion of the sequence of nucleic acid **104**. Nucleic acid **114** may comprise all of the sequence of nucleic acid **104**. Nucleic acid **114** may be of a different sequence than nucleic acid **104**. Nucleic acids **104** and **114** may be identified by detection of the signals generated by oligonucleotide probes **103** and **113**, respectively, following nucleic acid amplification. A value derived from the signal generated by nucleic acid **104** (e.g., cycle threshold value, partition count, etc.) may be compared to a value derived from the signal generated by nucleic acid **114** (e.g., cycle threshold value, partition count, etc.), thereby generating a ratio. Analysis of this signal ratio may be used, for example, to differentiate nucleic acid **104** from nucleic acid **114** and/or to estimate the size distribution of nucleic acid fragments of varying lengths (e.g., fetal fraction). This ratio can be compared to a reference value, thereby identifying a genetic abnormality (e.g., aneuploidy). For example, the ratio obtained from a subject suspected of having a genetic abnormality can be compared to a ratio obtained from a healthy subject, such that a significant difference in the ratios identifies the subject as having a genetic abnormality.

[0023] In some cases, a plurality of different nucleic acids may be analyzed using methods of the present disclosure. For example, a first set of paired oligonucleotide primers may comprise a plurality of forward primers and a plurality of reverse primers, each configured to amplify a nucleic acid sequence of a given length. A first set of paired oligonucleotide primers may be configured to amplify, for example, fetal nucleic acid. A second set of paired oligonucleotide primers may comprise a plurality of forward primers and a plurality of reverse primers, each

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configured to amplify a nucleic acid sequence of a longer length than the first set of paired oligonucleotide primers. A first set of paired oligonucleotide primers may be configured to amplify, for example, maternal nucleic acid. Signals generated from amplification of fetal nucleic acid and maternal nucleic acid using the first set of paired oligonucleotide primers and the second set of paired oligonucleotide primers may be detected and analyzed, thereby evaluating fetal fraction or identifying a fetal aneuploidy.

[0024] FIGs. 2A and **2B** illustrate an example method for targeting different sizes of nucleic acid sequences for nucleic acid analysis. **FIG. 2A** shows nucleic acids **204**, **208**, and **212**; forward primers **201**, **205**, and **209**; and reverse primers **203**, **207**, and **211**. The forward and reverse primers are each configured to amplify a given nucleic acid, as shown. **FIG. 2A** also shows oligonucleotide probes **202**, **206**, and **210**, each configured to hybridize to a given nucleic acid and to generate a signal following amplification. Nucleic acids **204**, **208**, and **212** are each of a given length. Nucleic acids **204**, **208**, and **212** may be fetal nucleic acid fragments. **FIG. 2B** shows nucleic acids **224**, **228**, and **232**; forward primers **221**, **225**, and **229**; and reverse primers **223**, **227**, and **211**. The forward and reverse primers are each configured to amplify a given nucleic acid, as shown. **FIG. 2B** also shows oligonucleotide probes **222**, **226**, and **230**, each configured to hybridize to a given nucleic acid and to generate a signal following amplification. Nucleic acids **224**, **228**, and **232** are each of a longer length than nucleic acids **204**, **208**, and **212**. Nucleic acids **224**, **228**, and **232** may be maternal nucleic acid fragments. Nucleic acids **202**, **206**, **210**, **222**, **226**, and **230** may be identified by detection of signals generated from each of oligonucleotide probes **222**, **226**, and **230**. The signals (e.g., signal intensities) generated by nucleic acids **224, 228,** and **232** may be compared to the signals (e.g., signal intensities) generated by nucleic acids **204**, **208**, and **212**, thereby generating a ratio. Analysis of this signal ratio may be used, for example, estimate the size distribution of nucleic acid fragments of varying lengths (e.g., fetal fraction). This ratio can be compared to a reference value, thereby identifying a genetic abnormality (e.g., aneuploidy). For example, the ratio obtained from a subject suspected of having a genetic abnormality can be compared to a ratio obtained from a healthy subject, such that a significant difference in the ratios identifies the subject as having a genetic abnormality.

Analyzing nucleic acid size distribution

[0025] In some aspects, disclosed herein are methods for analyzing nucleic acid size distribution. First, a sample may be provided comprising: (i) a first plurality of nucleic acids and a second plurality of nucleic acids, wherein the first plurality of nucleic acids each comprise a first nucleic acid sequence of a given length and the second plurality of nucleic acids each comprise a second nucleic acid sequence longer than the given length; (ii) a first set of paired

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amplification oligomers configured to amplify the first nucleic acid sequence; (iii) a second set of paired amplification oligomers, configured to amplify the second nucleic acid sequence; (iv) a first detection probe configured to anneal to a region of the first nucleic acid sequence; and (v) a second detection probe configured to anneal to a region of the second nucleic acid sequence. In some cases, a first detection probe and a second detection probe are not provided. Next, amplification may be performed on the first nucleic acid sequence and the second nucleic acid sequence. The amplification may generate a first signal from the first detection probe and a second signal from the second detection probe. Alternatively, in cases where a first detection probe and a second detection probe are not provided, the amplification may generate a first signal and/or a second signal from an intercalating dye (e.g., SYBR® Green, EvaGreen®) and/or from mass spectrometry. Next, a ratio of a value derived from the first signal to value derived from the second signal may be determined, thereby analyzing nucleic acid size distribution.

[0026] The first set of paired oligonucleotide primers may comprise a first forward primer and a first reverse primer (i.e., a first pair of oligonucleotide primers). A first pair of oligonucleotide primers (e.g., a first forward primer and a first reverse primer) may be configured to amplify a nucleic acid sequence of a given length (e.g., may hybridize to regions of a nucleic acid sequence a given distance apart). A first pair of oligonucleotide primers may be configured to amplify a nucleic acid sequence of a length of at least 50, at least 75, at least 100, at least 125, at least 150, at least 175, at least 200, at least 225, at least 250, at least 275, or at least 300 base pairs (bp), or more. A first pair of oligonucleotide primers may be configured to amplify a nucleic acid sequence of a length of at most 300, at most 275, at most 250, at most 225, at most 200, at most 175, at most 150, at most 125, at most 100, at most 75, or at most 50 bp, or less. A first pair of oligonucleotide primers may be configured to amplify a nucleic acid sequence of a length of about 50, about 75, about 100, about 125, about 150, about 175, about 200, about 225, about 250, about 275, or about 300 bp. In some cases, a first pair of oligonucleotide primers is configured to amplify a nucleic acid sequence of a length of about 70 bp. In some cases, a first pair of oligonucleotide primers is configured to amplify a nucleic acid sequence of a length of about 100 bp. In some cases, a first pair of oligonucleotide primers is configured to amplify a nucleic acid sequence of a length of about 150 bp. The first set of paired oligonucleotide primers may comprise a plurality of first forward primers and a plurality of first reverse primers (i.e., a first plurality of paired oligonucleotide primers). The first set of paired oligonucleotide primers may comprise at least 2, at least 3, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or at least 10 pairs of oligonucleotide primers, or more. The first set of paired oligonucleotide primers may comprise about 2, about 3, about 3, about 4, about 5, about 6, about 7, about 8, about 9, or about 10 pairs of oligonucleotide primers. The first set of paired

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oligonucleotide primers may comprise n pairs of oligonucleotide primers. n may be an integer. n may be an integer from 2-30. n 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, or 30. n may be an integer greater than 30. Each pair of oligonucleotide primers in a first set of paired oligonucleotide primers may be configured to amplify a nucleic acid sequence of a given length. In some cases, each pair of oligonucleotide primers in a first set of paired oligonucleotide primers is configured to amplify a nucleic acid sequence of about the same length (e.g., about 70 bp, about 100 bp, about 150 bp, or more). In some cases, some or all of the pairs of oligonucleotide primers in a first set of paired oligonucleotide primers are configured to amplify a nucleic acid sequence of a different length. For example, a pair of oligonucleotide primers in a first set of oligonucleotide primers may be configured to amplify a nucleic acid sequence of a length of about 70 bp, and another pair of oligonucleotide primers in a first set of oligonucleotide primers may be configured to amplify a nucleic acid sequence of a length of about 100 bp.

[0027] The second set of paired oligonucleotide primers may comprise a second forward primer and a second reverse primer (i.e., a second pair of oligonucleotide primers). A second pair of oligonucleotide primers (e.g., a second forward primer and a second reverse primer) may be configured to amplify a nucleic acid sequence of a given length (e.g., may hybridize to regions of a nucleic acid sequence a given distance apart). A second pair of oligonucleotide primers may be configured to amplify a nucleic acid sequence of a length longer than a nucleic acid sequence amplified by a first pair of oligonucleotide primers. A second pair of oligonucleotide primers may be configured to amplify a nucleic acid sequence of a length of at least 300, at least 325, at least 350, at least 375, at least 400, at least 425, at least 450, at least 500, at least 550, at least 600, at least 650, at least 700, or at least 750 base pairs (bp), or more. A second pair of oligonucleotide primers may be configured to amplify a nucleic acid sequence of a length of at most 750, at most 700, at most 650, at most 600, at most 550, at most 500, at most 750, at most 425, at most 400, at most 375, at most 350, at most 325, at most 350, at most 325, or at most 300 bp, or less. A second pair of oligonucleotide primers may be configured to amplify a nucleic acid sequence of a length of about 300, about 325, about 350, about 375, about 400, about 425, about 450, about 500, about 550, about 600, about 650, about 700, or about 750 bp. In some cases, a second pair of oligonucleotide primers is configured to amplify a nucleic acid sequence of a length of about 300 bp. In some cases, a second pair of oligonucleotide primers is configured to amplify a nucleic acid sequence of a length of about 500 bp. In some cases, a second pair of oligonucleotide primers is configured to amplify a nucleic acid sequence of a length of about 750 bp. The second set of paired oligonucleotide primers may comprise a plurality of second forward primers and a plurality of second reverse primers (i.e., a second

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plurality of paired oligonucleotide primers). The second set of paired oligonucleotide primers may comprise at least 2, at least 3, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or at least 10 pairs of oligonucleotide primers, or more. The second set of paired oligonucleotide primers may comprise about 2, about 3, about 3, about 4, about 5, about 6, about 7, about 8, about 9, or about 10 pairs of oligonucleotide primers. The second set of paired oligonucleotide primers may comprise n pairs of oligonucleotide primers. n may be an integer. n may be an integer from 2-30. n 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, or 30. n may be an integer greater than 30. Each pair of oligonucleotide primers in a second set of paired oligonucleotide primers may be configured to amplify a nucleic acid sequence of a given length. Each pair of oligonucleotide primers in a second set of paired oligonucleotide primers may be configured to amplify a nucleic acid sequence of a length longer than a nucleic acid sequence amplified by a first set of paired oligonucleotide primers. In some cases, each pair of oligonucleotide primers in a second set of paired oligonucleotide primers is configured to amplify a nucleic acid sequence of about the same length (e.g., about 300 bp, about 500 bp, about 750 bp, or more). In some cases, some or all of the pairs of oligonucleotide primers in a second set of paired oligonucleotide primers are each configured to amplify a nucleic acid sequence of a different length. For example, a pair of oligonucleotide primers in a second set of oligonucleotide primers may be configured to amplify a nucleic acid sequence of a length of about 300 bp, and another pair of oligonucleotide primers in a second set of oligonucleotide primers may be configured to amplify a nucleic acid sequence of a length of about 500 bp.

[0028] The first detection probe or the second detection probe may comprise a non-target hybridizing sequence. A non-target hybridizing sequence may be a region which is not complementary to any target nucleic acid. The first detection probe or the second detection probe may be a molecular beacon. The first detection probe or the second detection probe may be a molecular torch. The first detection probe or the second detection probe may be a molecular beacon. The first detection probe or the second detection probe may comprise a detectable label. A detectable label may be a chemiluminescent label. A detectable label may be a fluorescent label. The first detection probe and the second detection probe may each comprise a different detectable label. For example, the first detection probe may comprise a fluorophore of a first color, and the second detection probe may comprise a fluorophore of a second color. The first detection probe and the second detection probe may each comprise an identical detectable label. The first detection probe and the second detection probe may each comprise a quencher. The first detection probe and the second detection probe may be TaqMan® detection probes.

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[0029] The amplification may be linear amplification. The amplification may comprise polymerase chain reaction (PCR). The amplification may be digital PCR. The amplification may be quantitative PCR. The amplification may be performed in a partition of a plurality of partitions. The amplification may be performed in a droplet in an emulsion. The amplification may be performed in a microwell.

[0030] Determining the ratio of the first value to the second value may provide a fetal fraction. The ratio may be compared to a reference value. Comparing the ratio to the reference value may determine an estimated fetal fraction in a sample. Comparing the ratio to the reference value may identify the presence or absence of a genetic abnormality (e.g., an aneuploidy) in a sample. A reference value may correspond to a ratio of a third value generated from a third nucleic acid sequence and a fourth value generated from a fourth nucleic acid sequence. The third nucleic acid sequence and the fourth nucleic acid sequence may each correspond to a region of nucleic acid not associated with a genetic abnormality.

[0031] The first plurality of nucleic acids may be a plurality of fetal nucleic acids. The second plurality of nucleic acids may be a plurality of maternal nucleic acids. The first nucleic acid sequence may correspond to a region of a fetal nucleic acid associated with a fetal aneuploidy. The region associated with a fetal aneuploidy may be, for example, a region of chromosome 21, a region of chromosome 18, a region of chromosome 13, or a region of an X chromosome.

[0032] A method for analyzing nucleic acid size distribution may comprise providing one or more additional pluralities of nucleic acids comprising one or more additional nucleic acid sequences, one or more additional sets of amplification oligomers, and one or more additional detection probes. One or more additional sets of amplification oligomers 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, or 30 additional sets of amplification oligomers. One or more additional sets of amplification oligomers may be n additional sets of amplification oligomers. One or more additional pluralities of nucleic acids 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, or 30 additional pluralities of nucleic acids. One or more additional pluralities of nucleic acids may be n additional pluralities of nucleic. One or more additional detection probes 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, or 30 additional detection probes. One or more additional detection probes may be n additional detection probes. n may be an integer. n may be an integer between 1 and 30. n 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, or 30. n may be an integer greater than 30.

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Detecting fetal aneuploidy

[0033] In some aspects, disclosed herein are methods for identifying the presence or absence of a fetal aneuploidy. First, a sample may be provided comprising: (i) a plurality of fetal nucleic acids comprising a sequence of a given length; (ii) a plurality of maternal nucleic acids comprising a sequence longer than the given length; (iii) a first set of oligonucleotide primers configured to amplify the first nucleic acid sequence; (iv) a second set of oligonucleotide primers, configured to amplify the second nucleic acid sequence; (v) a first oligonucleotide probe configured to anneal to a region of the first nucleic acid sequence; and (vi) a second oligonucleotide probe configured to anneal to a region of the second nucleic acid sequence. In some cases, a first oligonucleotide probe and a second oligonucleotide probe are not provided. Next, amplification may be performed on the first nucleic acid sequence and the second nucleic acid sequence. The amplification may generate a first signal from the first oligonucleotide probe and a second signal from the second oligonucleotide probe. Alternatively, in cases where a first oligonucleotide probe and a second oligonucleotide probe are not provided, the amplification may generate a first signal and/or a second signal from an intercalating dye (e.g., SYBR® Green, EvaGreen®) and/or from mass spectrometry. Next, a ratio of a value derived from the first signal to a value generated from the second signal may be determined. Next, the ratio may be compared to a reference value, thereby identifying the presence of absence of the fetal aneuploidy.

[0034] The first set of paired oligonucleotide primers may comprise a first forward primer and a first reverse primer (i.e., a first pair of oligonucleotide primers). A first pair of oligonucleotide primers (e.g., a first forward primer and a first reverse primer) may be configured to amplify a nucleic acid sequence of a given length (e.g., may hybridize to regions of a nucleic acid sequence a given distance apart). A first pair of oligonucleotide primers may be configured to amplify a nucleic acid sequence of a length of at least 50, at least 75, at least 100, at least 125, at least 150, at least 175, at least 200, at least 225, at least 250, at least 275, or at least 300 base pairs (bp), or more. A first pair of oligonucleotide primers may be configured to amplify a nucleic acid sequence of a length of at most 300, at most 275, at most 250, at most 225, at most 200, at most 175, at most 150, at most 125, at most 100, at most 75, or at most 50 bp, or less. A first pair of oligonucleotide primers may be configured to amplify a nucleic acid sequence of a length of about 50, about 75, about 100, about 125, about 150, about 175, about 200, about 225, about 250, about 275, or about 300 bp. In some cases, a first pair of oligonucleotide primers is configured to amplify a nucleic acid sequence of a length of about 70 bp. In some cases, a first pair of oligonucleotide primers is configured to amplify a nucleic acid sequence of a length of about 100 bp. In some cases, a first pair of oligonucleotide primers is configured to amplify a

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nucleic acid sequence of a length of about 150 bp. The first set of paired oligonucleotide primers may comprise a plurality of first forward primers and a plurality of first reverse primers (i.e., a first plurality of paired oligonucleotide primers). The first set of paired oligonucleotide primers may comprise at least 2, at least 3, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or at least 10 pairs of oligonucleotide primers, or more. The first set of paired oligonucleotide primers may comprise about 2, about 3, about 3, about 4, about 5, about 6, about 7, about 8, about 9, or about 10 pairs of oligonucleotide primers. Each pair of oligonucleotide primers in a first set of paired oligonucleotide primers may be configured to amplify a nucleic acid sequence of a given length. In some cases, each pair of oligonucleotide primers in a first set of paired oligonucleotide primers is configured to amplify a nucleic acid sequence of about the same length (e.g., about 70 bp, about 100 bp, about 150 bp, or more). In some cases, some or all of the pairs of oligonucleotide primers in a first set of paired oligonucleotide primers are each configured to amplify a nucleic acid sequence of a different length. For example, a pair of oligonucleotide primers in a first set of oligonucleotide primers may be configured to amplify a nucleic acid sequence of a length of about 70 bp, and another pair of oligonucleotide primers in a first set of oligonucleotide primers may be configured to amplify a nucleic acid sequence of a length of about 100 bp.

[0035] The second set of paired oligonucleotide primers may comprise a second forward primer and a second reverse primer (i.e., a second pair of oligonucleotide primers). A second pair of oligonucleotide primers (e.g., a second forward primer and a second reverse primer) may be configured to amplify a nucleic acid sequence of a given length (e.g., may hybridize to regions of a nucleic acid sequence a given distance apart). A second pair of oligonucleotide primers may be configured to amplify a nucleic acid sequence of a length longer than a nucleic acid sequence amplified by a first pair of oligonucleotide primers. A second pair of oligonucleotide primers may be configured to amplify a nucleic acid sequence of a length of at least 300, at least 325, at least 350, at least 375, at least 400, at least 425, at least 450, at least 500, at least 550, at least 600, at least 650, at least 700, or at least 750 base pairs (bp), or more. A second pair of oligonucleotide primers may be configured to amplify a nucleic acid sequence of a length of at most 750, at most 700, at most 650, at most 600, at most 550, at most 500, at most 750, at most 425, at most 400, at most 375, at most 350, at most 325, at most 350, at most 325, or at most 300 bp, or less. A second pair of oligonucleotide primers may be configured to amplify a nucleic acid sequence of a length of about 300, about 325, about 350, about 375, about 400, about 425, about 450, about 500, about 550, about 600, about 650, about 700, or about 750 bp. In some cases, a second pair of oligonucleotide primers is configured to amplify a nucleic acid sequence of a length of about 300 bp. In some cases, a second pair of oligonucleotide primers is

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configured to amplify a nucleic acid sequence of a length of about 500 bp. In some cases, a second pair of oligonucleotide primers is configured to amplify a nucleic acid sequence of a length of about 750 bp. The second set of paired oligonucleotide primers may comprise a plurality of second forward primers and a plurality of second reverse primers (i.e., a second plurality of paired oligonucleotide primers). The second set of paired oligonucleotide primers may comprise at least 2, at least 3, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or at least 10 pairs of oligonucleotide primers, or more. The second set of paired oligonucleotide primers may comprise about 2, about 3, about 3, about 4, about 5, about 6, about 7, about 8, about 9, or about 10 pairs of oligonucleotide primers. Each pair of oligonucleotide primers in a second set of paired oligonucleotide primers may be configured to amplify a nucleic acid sequence of a given length. Each pair of oligonucleotide primers in a second set of paired oligonucleotide primers may be configured to amplify a nucleic acid sequence of a length longer than a nucleic acid sequence amplified by a first set of paired oligonucleotide primers. In some cases, each pair of oligonucleotide primers in a second set of paired oligonucleotide primers is configured to amplify a nucleic acid sequence of about the same length (e.g., about 300 bp, about 500 bp, about 750 bp, or more). In some cases, some or all of the pairs of oligonucleotide primers in a second set of paired oligonucleotide primers are configured to amplify a nucleic acid sequence of a different length. For example, a pair of oligonucleotide primers in a second set of oligonucleotide primers may be configured to amplify a nucleic acid sequence of a length of about 300 bp, and another pair of oligonucleotide primers in a second set of oligonucleotide primers may be configured to amplify a nucleic acid sequence of a length of about 500 bp.

[0036] The plurality of fetal nucleic acids and the plurality of maternal nucleic acids may be obtained from plasma from a pregnant woman. The fetal nucleic acids may be fetal deoxyribonucleic acids (DNA). The maternal nucleic acids may be maternal DNA. The first nucleic acid sequence may correspond to a region of a fetal nucleic acid associated with a fetal aneuploidy. The region associated with a fetal aneuploidy may be, for example, a region of chromosome 21, a region of chromosome 18, a region of chromosome 13, or a region of an X chromosome.

[0037] The ratio may be larger than the reference value, thereby indicating the presence of a fetal aneuploidy. The ratio may be smaller than the reference value, thereby indicating the presence of a fetal aneuploidy. The ratio may be about the same as the reference value, thereby indicating the absence of a fetal aneuploidy. The reference value may correspond to a ratio of a third value generated from a third nucleic acid sequence and a fourth value generated from a fourth nucleic acid sequence. A third nucleic acid sequence and a fourth nucleic acid sequence may each correspond to a region of nucleic acid not associated with a fetal aneuploidy. A region

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of nucleic acid not associated with fetal aneuploidy may be a region of a housekeeping gene. A housekeeping gene may be, for example, β-globin.

[0038] The first oligonucleotide probe or the second oligonucleotide probe may comprise a non-target hybridizing sequence. A non-target hybridizing sequence may be a region which is not complementary to any target nucleic acid. The first oligonucleotide probe or the second oligonucleotide probe may be a molecular beacon. The first oligonucleotide probe or the second oligonucleotide probe may be a molecular torch. The first oligonucleotide probe or the second oligonucleotide probe may be a molecular beacon. The first oligonucleotide probe or the second oligonucleotide probe may comprise a detectable label. A detectable label may be a chemiluminescent label. A detectable label may be a fluorescent label. The first oligonucleotide probe and the second oligonucleotide probe may each comprise a different detectable label. For example, the first oligonucleotide probe may comprise a fluorophore of a first color, and the second oligonucleotide probe may comprise a fluorophore of a second color. The first oligonucleotide probe and the second oligonucleotide probe may each comprise an identical detectable label. The first oligonucleotide probe and the second oligonucleotide probe may each comprise a quencher. The first detection probe and the second oligonucleotide probe may be TaqMan® oligonucleotide probes.

[0039] The amplification may be linear amplification. The amplification may comprise polymerase chain reaction (PCR). The amplification may be digital PCR. The amplification may be quantitative PCR. The amplification may be performed in a partition of a plurality of partitions. The amplification may be performed in a droplet in an emulsion. The amplification may be performed in a microwell.

[0040] A fetal aneuploidy may be an abnormal number of chromosomes. The fetal aneuploidy may be a trisomy. A trisomy may be trisomy 21. A trisomy may be trisomy 18. A trisomy may be trisomy 13. The fetal aneuploidy may be a monosomy. A monosomy may be Turner syndrome. A fetal aneuploidy may be a sex chromosome aneuploidy. A sex chromosome aneuploidy may be, for example, XO, XXX, XXY, or XYY.

[0041] A method for analyzing nucleic acid size distribution may comprise providing one or more additional pluralities of nucleic acids comprising one or more additional nucleic acid sequences, one or more additional sets of amplification oligomers, and one or more additional detection probes. One or more additional sets of amplification oligomers 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, or 30 additional sets of amplification oligomers. One or more additional sets of amplification oligomers may be n additional sets of amplification oligomers. One or more additional pluralities of nucleic acids may be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19,

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20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 additional pluralities of nucleic acids. One or more additional pluralities of nucleic acids may be n additional pluralities of nucleic. One or more additional detection probes 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, or 30 additional detection probes. One or more additional detection probes may be n additional detection probes. n may be an integer. n may be an integer between 1 and 30. n 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, or 30. n may be an integer greater than 30.

Genetic Abnormalities

[0042] The disclosed methods may be used in diagnosing, detecting, or otherwise identifying one or more genetic abnormalities from a nucleic acid sample. The disclosed methods may be used in a test that diagnoses, detects, or otherwise identifies one or more genetic abnormalities from a nucleic acid sample, where data obtained from methods described herein (e.g., nucleic acid size distribution, estimated fetal fraction, etc.) is used to aid the detection or filter out samples unlikely to result in an accurate result. A genetic abnormality may be, for example, a chromosomal abnormality (e.g., chromosomal translocation, aneuploidy, etc), a genetic mutation (e.g., insertion, deletion, etc.), or a nucleic acid variant (e.g., single nucleotide polymorphism). In some cases, a genetic abnormality identified by the disclosed methods is an aneuploidy. An aneuploidy may be a fetal aneuploidy. In some cases, the disclosed methods comprise amplification of nucleic acid sequences from a chromosome associated with a fetal aneuploidy. Chromosomes associated with fetal aneuploidy include, for example, chromosome 21 (e.g., associated with trisomy 21), chromosome 18 (e.g., associated with trisomy 18), chromosome 13 (e.g., associated with trisomy 13), and an X chromosome (e.g., associated with sex chromosome aneuploidies, for example, Turner syndrome, Klinefelter syndrome, trisomy X, XXY, XYY, etc.).

[0043] An aneuploidy may describe the presence of an abnormal number of chromosomes in a sample or subject (e.g., a fetus). An aneuploidy may be a trisomy. In some cases, a trisomy identified via the disclosed methods is trisomy 21 (i.e., Down syndrome). In some cases, a trisomy identified via the disclosed methods is trisomy 13. In some cases, a trisomy identified via the disclosed methods is trisomy 13. In some cases, a trisomy identified via the disclosed methods is trisomy X. In some cases, a trisomy identified via the disclosed methods is XYY. In some cases, a trisomy identified via the disclosed methods is Klinefelter syndrome. An aneuploidy may be a monosomy. In some cases, a monosomy identified via the disclosed methods is monosomy X (i.e., Turner syndrome).

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Oligonucleotide primers

[0044] An oligonucleotide primer (or "amplification oligomer") of the present disclosure may be a deoxyribonucleic acid. An oligonucleotide primer may be a ribonucleic acid. An oligonucleotide primer may comprise one or more non-natural nucleotides. A non-natural nucleotide may be, for example, deoxyinosine. An oligonucleotide primer may be a forward primer. An oligonucleotide primer may be a reverse primer. An oligonucleotide primer may be between about 5 and about 50 nucleotides in length. An oligonucleotide primer may be at least 5, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, or 50 base pairs in length, or more. An oligonucleotide primer may be at most 50, 45, 40, 35, 30, 25, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, or 5 nucleotides in length. An oligonucleotide primer may be about 5, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, or 50 base pairs in length.

[0045] A set of oligonucleotide primers may comprise paired oligonucleotide primers. Paired oligonucleotide primers may comprise a forward oligonucleotide primer and a reverse oligonucleotide primer. A set of oligonucleotide primers may be configured to amplify a nucleic acid sequence of a given length. For example, a forward oligonucleotide primer may be configured to hybridize to a first region (e.g., a 3' end) of a nucleic acid sequence, and a reverse oligonucleotide primer may be configured to hybridize to a second region (e.g., a 5' end) of the nucleic acid sequence, thereby being configured to amplify the nucleic acid sequence of given length under conditions sufficient for nucleic acid amplification. Different sets of oligonucleotide primers may be configured to amplify nucleic acid sequences of different lengths. In one example, a first set of oligonucleotide primers may be configured to amplify a first nucleic acid sequence of a given length, and a second set of oligonucleotide primers may be configured to amplify a second nucleic acid sequence of shorter length than the first nucleic acid sequence. In another example, a first set of oligonucleotide primers may be configured to amplify a first nucleic acid sequence of a given length, and a second set of oligonucleotide primers may be configured to amplify a second nucleic acid sequence of longer length than the first nucleic acid sequence.

A set of paired oligonucleotide primers may comprise a forward primer and a reverse primer (i.e., a pair of oligonucleotide primers). A pair of oligonucleotide primers (e.g., a forward primer and a reverse primer) may be configured to amplify a nucleic acid sequence of a given length (e.g., may hybridize to regions of a nucleic acid sequence a given distance apart). A pair of oligonucleotide primers may be configured to amplify a nucleic acid sequence of a length of at least 50, at least 75, at least 100, at least 125, at least 150, at least 175, at least 200, at least 225, at least 250, at least 275, or at least 300 base pairs (bp), or more. A pair of oligonucleotide primers may be configured to amplify a nucleic acid sequence of a length of at most 300, at most

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275, at most 250, at most 225, at most 200, at most 175, at most 150, at most 125, at most 100, at most 75, or at most 50 bp, or less. A pair of oligonucleotide primers may be configured to amplify a nucleic acid sequence of a length of about 50, about 75, about 100, about 125, about 150, about 175, about 200, about 225, about 250, about 275, or about 300 bp. In some cases, a pair of oligonucleotide primers is configured to amplify a nucleic acid sequence of a length of about 70 bp. In some cases, a pair of oligonucleotide primers is configured to amplify a nucleic acid sequence of a length of about 100 bp. In some cases, a pair of oligonucleotide primers is configured to amplify a nucleic acid sequence of a length of about 150 bp. A pair of oligonucleotide primers may be configured to amplify a nucleic acid sequence of a length of at least 300, at least 325, at least 350, at least 375, at least 400, at least 425, at least 450, at least 500, at least 550, at least 600, at least 650, at least 700, or at least 750 base pairs (bp), or more. A pair of oligonucleotide primers may be configured to amplify a nucleic acid sequence of a length of at most 750, at most 700, at most 650, at most 600, at most 550, at most 500, at most 750, at most 425, at most 400, at most 375, at most 350, at most 325, at most 350, at most 325, or at most 300 bp, or less. A pair of oligonucleotide primers may be configured to amplify a nucleic acid sequence of a length of about 300, about 325, about 350, about 375, about 400, about 425, about 450, about 500, about 550, about 600, about 650, about 700, or about 750 bp. In some cases, a pair of oligonucleotide primers is configured to amplify a nucleic acid sequence of a length of about 300 bp. In some cases, a pair of oligonucleotide primers is configured to amplify a nucleic acid sequence of a length of about 500 bp. In some cases, a pair of oligonucleotide primers is configured to amplify a nucleic acid sequence of a length of about 750 bp. A set of paired oligonucleotide primers may comprise a plurality of forward primers and a plurality of reverse primers (i.e., a plurality of paired oligonucleotide primers). A set of paired oligonucleotide primers may comprise at least 2, at least 3, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, or at least 10 pairs of oligonucleotide primers, or more. A set of paired oligonucleotide primers may comprise about 2, about 3, about 3, about 4, about 5, about 6, about 7, about 8, about 9, or about 10 pairs of oligonucleotide primers. Each pair of oligonucleotide primers in a set of paired oligonucleotide primers may be configured to amplify a nucleic acid sequence of a given length. In some cases, each pair of oligonucleotide primers in a set of paired oligonucleotide primers is configured to amplify a nucleic acid sequence of about the same length (e.g., about 70 bp, about 100 bp, about 150 bp, about 300 bp, about 500 bp, about 750 bp or more). In some cases, some or all of the pairs of oligonucleotide primers in a set of paired oligonucleotide primers are each configured to amplify a nucleic acid sequence of a different length. For example, a pair of oligonucleotide primers in a set of oligonucleotide primers may be configured to amplify a nucleic acid sequence of a length of about 70 bp, and

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another pair of oligonucleotide primers in a set of oligonucleotide primers may be configured to amplify a nucleic acid sequence of a length of about 100 bp. Nucleic acid enzymes

[0046] Mixtures and compositions of the present disclosure may comprise one or more nucleic acid enzymes. A nucleic acid enzyme may have exonuclease activity. A nucleic acid enzyme may have endonuclease activity. A nucleic acid enzyme may have RNase activity. A nucleic acid enzyme may be capable of degrading a nucleic acid comprising one or more ribonucleotide bases. A nucleic acid enzyme may be, for example, RNase H or RNase III. An RNase III may be, for example, Dicer. A nucleic acid may be an endonuclease I such as, for example, a T7 endonuclease I. A nucleic acid enzyme may be capable of degrading a nucleic acid comprising a non-natural nucleotide. A nucleic acid enzyme may be an endonuclease V such as, for example, an *E. coli* endonuclease V. A nucleic acid enzyme may be a polymerase (e.g., a DNA polymerase). A polymerase may be *Taq* polymerase or a variant thereof. A nucleic acid enzyme may be capable, under appropriate conditions, of degrading an oligonucleotide probe. A nucleic acid enzyme may be capable, under appropriate conditions, of releasing a quencher from an oligonucleotide probe.

Thermal cycling and amplification

[0047] Methods of the present disclosure may comprise thermal cycling. Thermal cycling may comprise one or more thermal cycles. Thermally cycling may be performed under reaction conditions sufficient to amplify one or more nucleic acids. Sufficient reaction conditions may include sufficient temperature conditions, sufficient buffer conditions, and the presence of sufficient reagents. Sufficient temperature conditions may be such that each thermal cycle is performed at a desired annealing temperature. A desired annealing temperature may be sufficient for annealing of a region of an oligonucleotide primer to a target nucleic acid. A desired annealing temperature may be sufficient for annealing of a region of an oligonucleotide probe to a target nucleic acid. Sufficient buffer conditions may be such that the required salts are present in a buffer used during thermal cycling. Required salts may include magnesium salts, potassium salts, and/or ammonium salts. Sufficient buffer conditions may be such that the appropriate salts are present at required concentrations. Sufficient reagents for amplification of nucleic acids with PCR may include deoxytriphosphates (dNTPs). dNTPs may comprise natural or non-natural dNTPs including, for example, dATP, dCTP, dGTP, dTTP, dUTP, and variants thereof.

[0048] Amplification of nucleic acid targets may be performed using a suitable amplification method. Amplification may comprise linear amplification. Amplification may comprise polymerase chain reaction (PCR). Amplification may comprise a nucleic acid extension reaction. Amplification may be performed before or after partitioning nucleic acids. In some

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cases, amplification of nucleic acids is performed in a plurality of partitions. For example, nucleic acid targets may be partitioned into a plurality of droplets, and amplification performed within each droplet.

Signal generation and detection

[0049] In some cases, a signal may be generated simultaneous with hybridization of an oligonucleotide probe to a region of a nucleic acid. For example, an oligonucleotide probe (e.g., a molecular beacon probe or molecular torch) may generate a signal (e.g., a fluorescent signal) following hybridization to a nucleic acid. In some cases, a signal may be generated subsequent to hybridization of an oligonucleotide probe to a region of a nucleic acid, following degradation of the oligonucleotide probe by a nucleic acid enzyme. For example, an oligonucleotide probe (e.g., a TaqMan® probe) may generate a signal following hybridization of the oligonucleotide probe to a nucleic acid and subsequent degradation by a polymerase (e.g., during amplification, such as PCR amplification). An oligonucleotide probe may be degraded by the exonuclease activity of a nucleic acid enzyme. A signal may be a chemiluminescent signal. A signal may be a fluorescent signal. A detectable label may comprise a quencher and a fluorophore, such that the quencher is released from a detectable label upon degradation of an oligonucleotide probe, thereby generating a fluorescent signal. Thermal cycling of the present disclosure may generate a signal. Thermal cycling may generate at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 signals, or more. Thermal cycling may generate at most 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 signal. Multiple signals may be of the same type or of different types. Signals of different types may be fluorescent signals with different fluorescent wavelengths. Signals of different types may be generated by detectable labels comprising different fluorophores. Signals of the same type may be of different intensities (e.g., different intensities of the same fluorescent wavelength). Signals of the same type may be generated by detectable labels comprising the same fluorophore. Detectable labels comprising the same fluorophore may generate different signals by nature of being at different concentrations, thereby generating different intensities of the same signal type. A signal may be generated by an intercalating dye (e.g., SYBR® Green, EvaGreen®). A signal may be generated by mass spectrometry (e.g., may be a mass spectrum corresponding to a nucleic acid).

[0050] The presence or absence of one or more signals may be detected. One signal may be detected, or multiple signals may be detected. Multiple signals may be detected simultaneously. Alternatively, multiple signals may be detected sequentially. A signal may be detected throughout the process of thermal cycling, for example, at the end of each thermal cycle. In some cases, a signal intensity increases with each thermal cycle. A signal intensity may increase in a sigmoidal fashion. A signal may be generated and used to derive (e.g., calculate) a cycle threshold value. A signal may be generated and used to derive (e.g., calculate) a partition count.

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Partitioning

[0051] Methods of the present disclosure may comprise partitioning nucleic acids, oligonucleotide probes, and, in some cases, additional reagents into a plurality of partitions. A partition may be a droplet (e.g., a droplet in an emulsion). A partition may be a well. A partition may be a microwell. Partitioning may be performed using a microfluidic device. In some cases, partitioning is performed using a droplet generator. Partitioning may comprise dividing a sample or mixture into water-in-oil droplets. A droplet may comprise one or more nucleic acids. A droplet may comprise a single nucleic acid. A droplet may comprise two or more nucleic acids. A droplet may comprise no nucleic acids.

Oligonucleotide probes

[0052] Samples, mixtures, kits, and and compositions of the present disclosure may comprise an oligonucleotide probe, also referenced herein as a detection probe. An oligonucleotide probe may be a nucleic acid (e.g., DNA, RNA, etc.). An oligonucleotide probe may comprise a region complementary to a region of a target nucleic acid. The concentration of an oligonucleotide probe may be such that it is in excess relative to other components in a sample. A sample may comprise more than one oligonucleotide probe. Multiple oligonucleotide probes may be the same, or may be different. An oligonucleotide probe may be at least 5, at least 10, at least 15, at least 20, or at least 30 nucleotides in length, or more. An oligonucleotide probe may be at most 30, at most 20, at most 15, at most 10 or at most 5 nucleotides in length.

[0053] An oligonucleotide probe may comprise a non-target-hybridizing sequence. A non-target-hybridizing sequence may be a sequence which is not complementary to any region of a target nucleic acid sequence. An oligonucleotide probe comprising a non-target-hybridizing sequence may be a hairpin detection probe. An oligonucleotide probe comprising a non-target-hybridizing sequence may be a molecular beacon probe. Examples of molecular beacon probes are provided in, for example, U.S. Patent 7,671,184, incorporated herein by reference in its entirety. An oligonucleotide probe comprising a non-target-hybridizing sequence may be a molecular torch. Examples of molecular torches are provided in, for example, U.S. Patent 6,534,274, incorporated herein by reference in its entirety.

[0054] An oligonucleotide probe may comprise a detectable tag. A detectable label may be a chemiluminescent label. A detectable label may comprise a fluorescent label. A detectable label may comprise a fluorophore. A fluorophore may be, for example, FAM, TET, HEX, JOE, Cy3, or Cy5. A fluorophore may be FAM. A fluorophore may be HEX. An oligonucleotide probe may further comprise one or more quenchers. A quencher may inhibit signal generation from a fluorophore. A quencher may be, for example, TAMRA, BHQ-1, BHQ-2, or Dabcy. A quencher may be BHQ-1. A quencher may be BHQ-2.

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Target Nucleic Acids

[0055] A nucleic acid of the present disclosure may be derived from any source including, for example, viruses, bacterial cells, and eukaryotic cells. A nucleic acid may be derived from one or more cells. A cell may be a tumor cell. A cell may be a cell suspected of comprising a viral pathogen. In some cases, a nucleic acid is derived from a cell-free sample (e.g., serum, plasma). Nucleic acid may be derived from plasma of a subject. For example, nucleic acid (e.g., maternal nucleic acid and fetal nucleic acid) may be derived from a plasma sample of a pregnant woman. A nucleic acid may be cell-free nucleic acid. Cell-free nucleic acid may be, for example, cell-free tumor DNA, cell-free fetal DNA, cell-free RNA, etc. A nucleic acid may comprise deoxyribonucleic acid (DNA). DNA may be any kind of DNA, including genomic DNA. A nucleic acid may be viral DNA. A nucleic acid may comprise ribonucleic acid (RNA). RNA may be any kind of RNA, including messenger RNA, transfer RNA, ribosomal RNA, and microRNA. RNA may be viral RNA. A nucleic acid may comprise a gene whose detection may be useful in diagnosing one or more diseases. A gene may be a viral gene or bacterial gene whose detection may be useful in identifying the presence or absence of a pathogen in a subject.

[0056] A nucleic acid may be a fetal nucleic acid. A nucleic acid may be a maternal nucleic acid. A nucleic acid may comprise one or more nucleic acid sequences which may be detected or amplified by the methods of the present disclosure. A nucleic acid sequence may correspond to a region of a nucleic acid potentially associated with an aneuploidy. For example, a nucleic acid sequence of a fetal nucleic acid may be associated with a fetal aneuploidy. A nucleic acid sequence may be a region of a chromosome associated with a fetal aneuploidy. Chromosomes associated with fetal aneuploidy include, for example, chromosome 21 (e.g., associated with trisomy 21), chromosome 18 (e.g., associated with trisomy 18), chromosome 13 (e.g., associated with trisomy 13), and an X chromosome (e.g., associated with sex chromosome aneuploidies, for example, Turner syndrome, Klinefelter syndrome, and trisomy X). In some cases, the methods of the present disclosure are useful in identifying and estimating a fetal fraction in a sample. In some cases, the methods of the present disclosure are useful in identifying the presence or absence of a fetal aneuploidy in a sample. In some cases, the methods of the present disclosure are useful in detecting the presence or absence or one or more infectious agents (e.g., viruses) in a subject. In some cases, the methods of the present disclosure are useful in detecting the relative amount of a fetal nucleic acid in a cell-free nucleic acid sample from a subject, thereby diagnosing the fetus for one or more genetic abnormalities (e.g., fetal aneuploidy). In some cases, the methods of the present disclosure are useful in detecting the presence or absence of tumor DNA in a cell-free nucleic acid sample from a subject, thereby diagnosing the subject for cancer.

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[0057] A sample may be processed concurrently with, prior to, or subsequent to the methods of the present disclosure. A sample may be processed to purify or enrich for nucleic acids (e.g., to purify nucleic acids from a plasma sample). A sample comprising nucleic acids may be processed to purity or enrich for nucleic acid of interest. A sample comprising nucleic acids may be processed to enrich for fetal nucleic acid. A sample comprising nucleic acids may be processed to enrich for nucleic acid fragments smaller than a given size. A sample may be enriched for nucleic acid of interest (e.g., fetal nucleic acid) by various methods including, for example, by size exclusion filtration, sequence-specific enrichment (e.g., via use of capture sequences), epigenetic-specific enrichment (e.g., via use of methylation-specific capture moieties, such as antibodies). Enrichment may comprise isolation of nucleic acid of interest and/or depletion of nucleic acid that is not of interest. In some cases, a sample is not processed to purify or enrich for nucleic acid of interest prior to performing methods of the present disclosure (e.g., amplification of nucleic acids from a sample). For example, a sample may not be processed to enrich for fetal nucleic acid prior to mixing a sample with oligonucleotide primers and oligonucleotide probes, as described elsewhere herein. The disclosed methods may be capable of, for example, identifying fetal fraction and/or identifying a fetal aneuploidy regardless of whether a sample has been purified or enriched for fetal nucleic acid.

Kits

[0058] Also provided herein are kits useful in, for example, analyzing nucleic acid size distribution (e.g., fetal fraction) and/or identifying the presence or absence of a fetal aneuploidy in a sample. Kits may comprise one or more oligonucleotide probes. Oligonucleotide probes may be lyophilized. Different oligonucleotide probes may be present at different concentrations in a kit. Oligonucleotide probes may comprise a detectable label, which may comprise, for example, a fluorophore and one or more quenchers.

[0059] Kits may comprise one or more sets of oligonucleotide primers (or "amplification oligomers") as described herein. A set of oligonucleotide primers may comprise paired oligonucleotide primers. Paired oligonucleotide primers may comprise a forward oligonucleotide primer and a reverse oligonucleotide primer. A set of oligonucleotide primers may be configured to amplify a nucleic acid sequence of a given length. For example, a forward oligonucleotide primer may be configured to hybridize to a first region (e.g., a 3' end) of a nucleic acid sequence, and a reverse oligonucleotide primer may be configured to hybridize to a second region (e.g., a 5' end) of the nucleic acid sequence, thereby being configured to amplify the nucleic acid sequence of given length under conditions sufficient for nucleic acid amplification. Different sets of oligonucleotide primers may be configured to amplify nucleic acid sequences of different lengths. In one example, a first set of oligonucleotide primers may be

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configured to amplify a first nucleic acid sequence of a given length, and a second set of oligonucleotide primers may be configured to amplify a second nucleic acid sequence of shorter length than the first nucleic acid sequence. In another example, a first set of oligonucleotide primers may be configured to amplify a first nucleic acid sequence of a given length, and a second set of oligonucleotide primers may be configured to amplify a second nucleic acid sequence of longer length than the first nucleic acid sequence. Oligonucleotide primers configured to amplify sequences of different lengths may be provided together in a kit, which may be used in performing the disclosed methods (e.g., size distribution analysis, fetal aneuploidy detection, etc.). Oligonucleotide primers may be lyophilized. In some cases, all of the oligonucleotide primers may be lyophilized.

[0060] Kits may comprise one or more nucleic acid enzymes. A nucleic acid enzyme may be a nucleic acid polymerase. A nucleic acid polymerase may be a deoxyribonucleic acid polymerase (DNase). A DNase may be a Taq polymerase or variant thereof. A nucleic acid enzyme may be a ribonucleic acid polymerase (RNase). An RNase may be an RNase III. An RNase III may be Dicer. The nucleic acid enzyme may be an endonuclease. An endonuclease may be an endonuclease I. An endonuclease I may be a T7 endonuclease I. Kits may comprise instructions for using any of the foregoing in the methods described herein.

EXAMPLES

Example 1 – Simulated digital PCR assay for fetal nucleic acid analysis

[0061] Current attempts at using digital PCR to detect fetal aneuploidy (e.g., trisomy 21) or other fetal conditions can be limited by the amount of fetal DNA present in a sample. **FIG. 3A** shows a simulated distribution of fetal fraction in cell free deoxyribonucleic acid (DNA). To evaluate the sensitivity and specificity of digital PCR assays, a simulated digital PCR for detection of fetal aneuploidy was generated. This simulated assay contains 8 wells, an average of 4,125 maternal counts of DNA, two targets per chromosome and a mean fetal fraction of 0.16 with a standard deviation of 0.06. **FIG. 3B** shows a Receiver Operating Characteristic (ROC) curve for the simulated assay, showing true positive (TP) rate versus false positive (FP) rate. The region within the dotted line represents the area with >90% true positive rate and <5% false positive rate. The area within the solid line represents area with >99% true positive rate and <1% false positive rate. This data demonstrates limited sensitivity, driven primarily by samples with low fetal fraction. **FIG. 3C** shows the true positive (TP) rate for the simulated digital PCR assay of **FIG. 3B**, relative to the fetal fraction in a sample. These data demonstrate that the success rate of a simulated digital PCR assay for detection of fetal aneuploidy is highly dependent on the fetal fraction of the sample.

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Example 2 – Simulated digital PCR assay for fetal nucleic acid analysis using a cutoff filter

[0062] To evaluate the sensitivity and specificity of a digital PCR assay using a cutoff filter for low fetal fraction samples, a simulated digital PCR for detection of fetal aneuploidy was generated. The simulation consisted of a Monte Carlo approach consisting of trials of distributing the input maternal counts of DNA and randomly chosen fetal fraction according to the following distribution. DNA counts were then randomly partitioned into 20,000 virtual partitions in two wells to simulate the performance of digital PCR instrumentation. This simulated assay contains 2 wells, an average of 4,125 maternal counts of DNA, ten targets per chromosome, and a mean fetal fraction of 0.16 with a standard deviation of 0.06. **FIG. 4A** shows an ROC curve for the simulated assay with a target enriched in maternal DNA by 70% relative to a housekeeping gene. Here, a cutoff filter was used to filter out low accuracy samples with low fetal fraction. The cutoff filter was varied such that it excluded a given percentage of the simulated trials, as shown in **FIG. 4A** and **FIG. 4B**. By increasing the amount of "No Calls" using such a filter, significant increases in sensitivity and specificity of the simulated assay were observed. **FIG. 4B** shows an ROC curve for the simulated assay with a target enriched in maternal DNA by 20% relative to a housekeeping gene, using a cutoff filter as in **FIG. 4A**. Even with only a 20% difference in fetal DNA, increasing the amount of "No Calls" using a filter resulted in significant gains in specificity and sensitivity of the simulated assay.

Example 3 – Determining fetal fraction using two pairs of oligonucleotide primers

[0063] A plasma sample from a pregnant woman is obtained. The plasma sample comprises fetal cell-free nucleic acid and maternal cell-free nucleic acid. The fetal and maternal nucleic acid are mixed with a pair of oligonucleotide primers and a second pair of oligonucleotide primers, each specific for a sequence on chromosome 21. The first pair of oligonucleotide primers is designed to amplify a nucleic acid sequence from chromosome 21 of a length of about 100 base pairs (bp). The second pair of oligonucleotide primers is designed to amplify a nucleic acid sequence from chromosome 21 of a length of about 300 bp. Also provided are first and second TaqMan® oligonucleotide probes, corresponding to the first and second pairs of oligonucleotide primers, designed for detecting each nucleic acid sequence.

[0064] The fetal and maternal nucleic acid are subjected to droplet digital polymerase chain reaction using the provided oligonucleotide primers and probes. A signal corresponding to each nucleic acid sequence from chromosome 21 is detected. A quantity of nucleic acid is derived from each signal. The ratio of the quantity derived from the signal generated from the first pair of oligonucleotide primers to the quantity derived from the signal generated from the second pair of oligonucleotide primers is calculated. This ratio is compared to a reference ratio obtained from signals similarly generated by amplification of nucleic acid sequences from β-globin. The

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ratio of signal generated from chromosome 21 is higher than the reference ratio, thereby identifying the sample as containing a fetal fraction. The comparison is used to calculate an estimated fetal fraction in the sample.

Example 4 – Determining fetal fraction using two sets of oligonucleotide primers each comprising two pairs of oligonucleotide primers

[0065] A plasma sample from a pregnant woman is obtained. The plasma sample comprises fetal cell-free nucleic acid and maternal cell-free nucleic acid. The fetal and maternal nucleic acid are mixed with a first set of paired oligonucleotide primers and a second set of paired oligonucleotide primers. Each set comprises two pairs of oligonucleotide primers (i.e., two forward primers, each paired with a reverse primer). Each pair of oligonucleotide primers in the first and second set is specific for a different sequence on chromosome 21. The first set of paired oligonucleotide primers comprises two pairs of oligonucleotide primers, each designed to amplify a different nucleic acid sequence from chromosome 21 of a length of about 70 base pairs (bp). The second set of paired oligonucleotide primers comprises two pairs of oligonucleotide primers, each designed to amplify a different nucleic acid sequence from chromosome 21 of a length of about 500 bp. Also provided are a first set of TaqMan® oligonucleotide probes and second set of TaqMan® oligonucleotide probes designed for detecting each nucleic acid sequence. Each set of TaqMan® oligonucleotide probes comprises two oligonucleotide probes corresponding to the two primer pairs of each of the first and second sets of oligonucleotide primers.

[0066] The fetal and maternal nucleic acid are subjected to droplet digital polymerase chain reaction using the provided oligonucleotide primers and probes. A signal corresponding to each nucleic acid sequence from chromosome 21 is detected. A quantity of nucleic acid is derived from each signal. The ratio of the quantity derived from the signals generated from the first set of oligonucleotide primers to the quantity derived from the signals generated from the second set of oligonucleotide primers is calculated. This ratio is compared to a reference ratio obtained from signals similarly generated by amplification of nucleic acid sequences from β-globin. The ratio of signal generated from chromosome 21 is higher than the reference ratio, thereby identifying the sample as containing a fetal fraction. The comparison is used to calculate an estimated fetal fraction in the sample.

[0067] The term "about" when referring to a measurable value such as an amount, a temporal duration, and the like, is meant to encompass variations of ±20% or in some instances ±10%, or in some instances ±5%, or in some instances ±1%, or in some instances ±0.1% from the specified value, as such variations are appropriate to perform the disclosed methods. Further, "about" can mean plus or minus less than 1 or 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16,

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17, 18, 19, 20, 25, 30, or greater than 30 percent, depending upon the situation and known or knowable by one skilled in the art. About also includes the exact amount. Hence "about 200 nM" means "about 200 nM" and also "200 nM".

[0068] 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. It is not intended that the invention be limited by the specific examples provided within the specification. While the invention has been described with reference to the aforementioned specification, the descriptions and illustrations of the embodiments herein are not meant to be construed in a limiting sense. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. Furthermore, it shall be understood that all aspects of the invention are not limited to the specific depictions, configurations or relative proportions set forth herein which depend upon a variety of conditions and variables. It should be understood that various alternatives to the embodiments of the invention described herein may be employed in practicing the invention. It is therefore contemplated that the invention shall also cover any such alternatives, modifications, variations or equivalents. 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 for analyzing a size distribution of nucleic acids, the method comprising:

(A) providing a sample comprising:

(i) a first plurality of nucleic acids and a second plurality of nucleic acids, wherein said first plurality of nucleic acids each comprise a first nucleic acid sequence of a given length and said second plurality of nucleic acids each comprise a second nucleic acid sequence longer than said given length;

(ii) a first set of paired amplification oligomers configured to amplify said first nucleic acid sequence;

(iii) a second set of paired amplification oligomers, configured to amplify said second nucleic acid sequence;

(iv) a first detection probe configured to anneal to a region of said first nucleic acid sequence; and

(v) a second detection probe configured to anneal to a region of said second nucleic acid sequence;

(B) performing an amplification reaction on (a) said first nucleic acid sequence to generate a first signal from said first detection probe and (b) said second nucleic acid sequence to generate a second signal from said second detection probe; and

(C) determining a ratio of a first value derived from said first signal to a second value derived from said second signal, thereby analyzing said size distribution.

2. The method of claim 1, wherein said first set of paired amplification oligomers comprises: a first forward amplification oligomer; and a first reverse amplification oligomer.

3. The method of claim 2, wherein said first set of paired amplification oligomers comprises: a plurality of first forward amplification oligomers; and a plurality of first reverse amplification oligomers.

4. The method of claim 3, wherein each of said plurality of first forward amplification oligomers has a different nucleic acid sequence.

5. The method of claim 4, wherein a first forward amplification oligomer of said plurality of first forward amplification oligomers is configured to hybridize to a region of said first sequence.

6. The method of claim 3, wherein each of said plurality of first reverse amplification oligomers has a different nucleic acid sequence.

7. The method of claim 6, wherein a first reverse amplification oligomer of said plurality of first reverse amplification oligomers is configured to hybridize to a region of said first sequence.

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8. The method of claim 1, wherein said second set of paired amplification oligomers comprises: a second forward amplification oligomer; and a second reverse amplification oligomer.

9. The method of claim 8, wherein said second set of paired amplification oligomers comprises: a plurality of second forward amplification oligomers and a plurality of second reverse amplification oligomers.

10. The method of claim 9, wherein each of said plurality of second forward amplification oligomers has a different nucleic acid sequence.

11. The method of claim 10, wherein a second forward amplification oligomer of said plurality of second forward amplification oligomers is configured to hybridize to a region of said second sequence.

12. The method of claim 9, wherein each of said plurality of second reverse amplification oligomers has a different nucleic acid sequence.

13. The method of claim 12, wherein a second reverse amplification oligomer of said plurality of second reverse amplification oligomers is configured to hybridize to a region of said second sequence.

14. The method of claim 1, wherein said first value and said second value provide a quantitative ratio measurement corresponding to an abundance of said first plurality of nucleic acids and said second plurality of nucleic acids in said sample.

15. The method of any of claims 1-14, wherein said first detection probe or said second detection probe comprises a non-target-hybridizing sequence.

16. The method of claim 15, wherein said first detection probe or said second detection probe is a hairpin detection probe.

17. The method of claim 16, wherein said hairpin detection probe is a molecular beacon or a molecular torch.

18. The method of any of claims 1-17, wherein said sample comprises: genomic DNA, mRNA, cDNA, or a combination thereof.

19. The method of any of claims 1-18, wherein said sample is derived from plasma from a pregnant woman.

20. The method of claim 19, wherein said sample comprises maternal nucleic acid and fetal nucleic acid.

21. The method of claim 20, wherein said first plurality of nucleic acids comprises said fetal nucleic acid and wherein said second plurality of nucleic acids comprises said maternal nucleic acid.

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22. The method of claim 20 or 21, wherein said determining of said ratio provides a fetal fraction.

23. The method of any of claims 1-22, wherein said sample is from an individual having or suspected of having cancer.

24. The method of any of claims 1-23, wherein said first signal and said second signal are generated in a single fluorescence channel.

25. The method of any of claims 1-24, wherein (B) is performed in at least one partition of a plurality of partitions.

26. The method of claim 25, wherein said plurality of partitions is a plurality of droplets.

27. The method of claim 25, wherein said plurality of partitions is a plurality of wells.

28. The method of claim 1, wherein said second nucleic acid sequence comprises at least a portion of said first nucleic acid sequence.

29. The method of claim 28, wherein said second nucleic acid sequence comprises said first nucleic acid sequence.

30. The method of any of claims 1-29, wherein said amplification reaction comprises polymerase chain reaction (PCR).

31. The method of claim 30, wherein said PCR is quantitative PCR (qPCR) or digital PCR (dPCR).

32. The method of any of claims 1-31, wherein said first detection probe comprises a first detectable label and said second detection probe comprises a second detectable label.

33. The method of claim 32, wherein said first detection probe and said second detection probe each further comprise a quencher.

34. The method of claim 32 or 33, wherein, during said amplification reaction, said first detectable label is released from said first detection probe and said second detectable label is released from said second detection probe, thereby generating said first signal and said second signal.

35. The method of any of claims 32-34, wherein said first detectable label and said second detectable label are each selected from the group consisting of a chemiluminescent label, a fluorescent label, and any combination thereof.

36. The method of claim 35, wherein said first signal or said second signal is a chemiluminescent signal, a fluorescent signal, or any combination thereof.

37. The method of claim 32-36, wherein said first detection probe and said second detection probe are TaqMan® detection probes.

38. The method of claim 1, further comprising comparing said ratio to a reference value.

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39. The method of claim 38, wherein said comparing identifies the presence or absence of a genetic abnormality in said sample.

40. The method of claim 39, wherein said reference value corresponds to a ratio of a third value generated from a third nucleic acid sequence and a fourth value generated from a fourth nucleic acid sequence.

41. The method of claim 40, wherein said third nucleic acid sequence and said fourth nucleic acid sequence each correspond to a region of nucleic acid not associated with said genetic abnormality.

42. The method of claim 41, wherein said reference value is derived from a plurality of third values generated from a plurality of third nucleic acid sequences and a plurality of fourth values generated from a plurality of fourth nucleic acid sequences.

43. The method of claim 42, wherein said plurality of third nucleic acid sequences and said plurality of fourth nucleic acid sequences each correspond to a region of nucleic acid not associated with said genetic abnormality.

44. The method of any of claims 39-43, wherein said genetic abnormality is a fetal aneuploidy.

45. The method of any of claims 1-27, wherein said second nucleic acid sequence comprises at least a portion of said first nucleic acid sequence.

46. The method of any of claims 1-38, further comprising comparing said ratio to a reference value.

47. The method of any of claims 1-46, wherein said first value is a quantity of said first plurality of nucleic acids.

48. The method of any of claims 1-47, wherein said second value is a quantity of said second plurality of nucleic acids.

49. The method of any of claims 1-46, wherein said ratio is determined without quantifying said first plurality of nucleic acids and said second plurality of nucleic acids.

50. The method of any of claims 1-49, wherein said amplification reaction comprises qPCR, wherein said first value is derived from amplification kinetics of said first plurality of nucleic acids.

51. The method of any of claims 1-49, wherein said amplification reaction comprises qPCR, wherein said second value is derived from amplification kinetics of said second plurality of nucleic acids.

52. The method of any of claims 1-49, wherein said amplification reaction comprises dPCR, wherein said first value is derived from a number of partitions containing said first nucleic acid sequence.

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53. The method of any of claims 1-49, wherein said amplification reaction comprises dPCR, wherein said second value is derived from a number of partitions containing said second nucleic acid sequence.

54. The method of any of claims 1-53, wherein:

in (A), said sample comprises:

(vi) one or more additional pluralities of nucleic acids comprising one or more additional nucleic acid sequences;

(vii) one or more additional sets of paired amplification oligomers configured to amplify said one or more additional nucleic acid sequences; and

(viii) one or more additional detection probes configured to anneal to a region of said one or more additional nucleic acid sequences;

in (B), said amplification reaction is performed on said one or more additional nucleic acid sequences to generate one or more additional signals from said one or more additional sets of detection probes; and

in (C), determining an additional ratio of said first value or said second value to one or more additional values derived from said one or more additional signals, thereby analyzing said size distribution.

55. The method of claim 54, wherein: said one or more additional sets of paired amplification oligomers comprise n amplification oligomers; and said one or more additional sets of detection probes comprise n additional detection probes.

56. The method of claim 55, wherein n is an integer between 1 and 30.

57. The method of claim 1, wherein said first value is a quantity of said first plurality of nucleic acids.

58. The method of claim 1, wherein said second value is a quantity of said second plurality of nucleic acids.

59. The method of claim 1, wherein said ratio is determined without quantifying said first plurality of nucleic acids and said second plurality of nucleic acids.

60. A method for identifying the presence or absence of a fetal aneuploidy, comprising:

(A) providing a sample comprising:

(i) a plurality of fetal nucleic acids, each comprising a first nucleic acid sequence of a given length;

(ii) a plurality of maternal nucleic acids, each comprising a second nucleic acid sequence longer than said given length;

(iii) a first set of oligonucleotide primers configured to amplify said first nucleic acid sequence;

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(iv) a second set of oligonucleotide primers configured to amplify said second nucleic acid sequence;

(v) a first oligonucleotide probe configured to hybridize to said first nucleic acid sequence; and

(vi) a second oligonucleotide probe configured to hybridize to said second nucleic acid sequence;

(B) amplifying (a) said first nucleic acid sequence to generate a first signal from said first oligonucleotide probe and (b) said second nucleic acid sequence to generate a second signal from said second oligonucleotide probe;

(C) determining a ratio of a value derived from said said first signal to a second value derived from said second signal; and

(D) comparing said ratio to a reference value, thereby identifying said presence or absence of said fetal aneuploidy.

61. The method of claim 60, wherein said first nucleic acid sequence corresponds to a region of nucleic acid potentially associated with said fetal aneuploidy.

62. The method of claim 61, wherein said region comprises a region of chromosome 22, chromosome 21, chromosome 18, chromosome 13, chromosome 9, chromosome 8, or an X chromosome.

63. The method of claim 61, wherein said region comprises a region of chromosome 21.

64. The method of claim 62, wherein said region comprises a region of chromosome 18.

65. The method of claim 62, wherein said region comprises a region of chromosome 13.

66. The method of claim 62, wherein said region comprises a region of an X chromosome.

67. The method of claim 60, wherein said reference value corresponds to a ratio of a third value generated from a third nucleic acid sequence and a fourth value generated from a fourth nucleic acid sequence.

68. The method of claim 67, wherein said third nucleic acid sequence and said fourth nucleic acid sequence each correspond to a region of nucleic acid not associated with said fetal aneuploidy.

69. The method of claim 60, wherein said reference value is derived from a plurality of third values generated from a plurality of third nucleic acid sequences and a plurality of fourth values generated from a plurality of fourth nucleic acid sequences.

70. The method of claim 69, wherein said plurality of third nucleic acid sequences and said plurality of fourth nucleic acid sequences each correspond to a region of nucleic acid not associated with said fetal aneuploidy.

71. The method of claim 67-70, wherein said region is a region of a housekeeping gene.

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72. The method of claim 71, wherein said housekeeping gene is β-globin.

73. The method of any of claims 60-72, wherein said ratio is larger than said reference value, thereby indicating the presence of said fetal aneuploidy.

74. The method of any of claims 60-72, wherein said ratio is smaller than said reference value, thereby identifying the presence of said fetal aneuploidy.

75. The method of any of claims 60-74, wherein said plurality of fetal nucleic acids and said plurality of maternal nucleic acids are obtained from plasma from a pregnant woman.

76. The method of any of claims 60-75, wherein said plurality of fetal nucleic acids comprises fetal deoxyribonucleic acid (DNA) and said plurality of maternal nucleic acids comprises maternal DNA.

77. The method of any of claims 60-76, wherein said amplifying in (b) comprises polymerase chain reaction (PCR).

78. The method of claim 77, wherein said PCR is quantitative PCR (qPCR) or digital PCR (dPCR).

79. The method of any of claims 60-78, wherein said first oligonucleotide probe comprises a first detectable label and said second oligonucleotide probe comprises a second detectable label.

80. The method of claim 79, wherein said first oligonucleotide probe and said second oligonucleotide probe each further comprise a quencher.

81. The method of claim 79 or 80, wherein, during said amplifying, said first detectable label is released from said first oligonucleotide probe and said second detectable label is released from said second oligonucleotide probe, thereby generating said first signal and said second signal.

82. The method of any of claims 79-81, wherein said first detectable label and said second detectable label are each selected from the group consisting of a chemiluminescent label, a fluorescent label, and any combination thereof.

83. The method of claim 82, wherein said first signal or said second signal is a chemiluminescent signal, a fluorescent signal, or any combination thereof.

84. The method of any of claims 60-83, wherein said first oligonucleotide probe and said second oligonucleotide probe are TaqMan® detection probes.

85. The method of any of claims 60-84, wherein said first set of oligonucleotide primers comprises a first forward primer and a first reverse primer.

86. The method of any of claims 60-85, wherein said second set of oligonucleotide primers comprises a second forward primer and a second reverse primer.

87. The method of any of claims 60-86, wherein said fetal aneuploidy is trisomy 21, trisomy 18, trisomy 13, trisomy 9, or trisomy 8.

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88. The method of claim 87, wherein said fetal aneuploidy is trisomy 21.

89. The method of claim 87, wherein said fetal aneuploidy is trisomy 18.

90. The method of claim 87, wherein said fetal aneuploidy is trisomy 13.

91. The method of claim 87, wherein said fetal aneuploidy is a sex chromosome aneuploidy.

92. The method of claim 91, wherein said sex chromosome aneuploidy is Turner syndrome, Klinefelter syndrome, trisomy X, XXY, or XYY.

93. The method of any of claims 60-92, wherein said second nucleic acid sequence does not comprise any of said first nucleic acid sequence.

94. The method of claim 60, wherein said second nucleic acid sequence comprises at least a portion of said first nucleic acid sequence.

95. The method of claim 94, wherein said second nucleic acid sequence comprises said first nucleic acid sequence.

96. The method of any of claims 60-66, wherein said reference value corresponds to a ratio of a third value generated from a third nucleic acid sequence and a fourth value generated from a fourth nucleic acid sequence.

97. The method of any of claims 60-92, wherein said second nucleic acid comprises at least a portion of said first nucleic acid sequence.

98. The method of any of claims 60-97, wherein said first value is a quantity of said first plurality of nucleic acids.

99. The method of any of claims 60-98, wherein said second value is a quantity of said second plurality of nucleic acids.

100. The method of any of claims 60-97, wherein said ratio is determined without quantifying said first plurality of nucleic acids and said second plurality of nucleic acids.

101. The method of any of claims 60-100, wherein said amplification reaction comprises qPCR, wherein said first value is derived from amplification kinetics of said first plurality of nucleic acids.

102. The method of any of claims 60-100, wherein said amplification reaction comprises qPCR, wherein said second value is derived from amplification kinetics of said second plurality of nucleic acids.

103. The method of any of claims 60-100, wherein said amplification reaction comprises dPCR, wherein said first value is derived from a number of partitions containing said first nucleic acid sequence.

104. The method of any of claims 60-100, wherein said amplification reaction comprises dPCR, wherein said second value is derived from a number of partitions containing said second nucleic acid sequence.

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105. The method of any of claims 60-104, wherein:

in (A), said sample comprises:

(vi) one or more additional pluralities of fetal nucleic acids comprising one or more additional first nucleic acid sequences of a given length;

(vii) one or more additional pluralities of maternal nucleic acids comprising one or more additional second nucleic acid sequences longer than said given length;

(viii) one or more additional first sets of oligonucleotide primers configured to amplify said one or more additional first nucleic acid sequences;

(xi) one or more additional second sets of oligonucleotide primers configured to amplify said one or more additional second nucleic acid sequences;

(x) one or more additional first oligonucleotide probes configured to anneal to a region of said one or more additional first nucleic acid sequences; and

(xi) one or more additional second oligonucleotide probes configured to anneal to a region of said one or more additional second nucleic acid sequences;

in (B), said amplification reaction is performed on: said one or more additional first nucleic acid sequences to generate one or more additional first signals from said one or more additional first oligonucleotide probes; and said one or more additional second nucleic acid sequences to generate one or more additional second signals from said one or more additional second oligonucleotide probes;

in (C), an additional ratio of one or more additional first values derived from said one or more additional first signals to one or more additional second values derived from said one or more additional second signals is determined; and

in (D), said additional ratio is compared to said reference value.

106. The method of claim 105, wherein: said one or more additional first sets of oligonucleotide primers comprise n oligonucleotide primers; and said one or more additional first oligonucleotide probes comprise n additional detection probes.

107. The method of claim 105, wherein: said one or more additional second sets of oligonucleotide primers comprise n oligonucleotide primers; and said one or more additional second oligonucleotide probes comprise n additional detection probes.

108. The method of claim 106 or 107, wherein n is an integer between 1 and 30.

109. The method of claim 60, wherein said first value is a quantity of said first plurality of nucleic acids.

110. The method of claim 60, wherein said second value is a quantity of said second plurality of nucleic acids.

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111. The method of claim 60, wherein said ratio is determined without quantifying said first plurality of nucleic acids and said second plurality of nucleic acids.

112. The method of claim 19, wherein said plasma is subjected to conditions sufficient to enrich for fetal nucleic acids.

113. The method of claim 19, wherein said plasma is not subjected to conditions sufficient to enrich for fetal nucleic acids.

114. The method of claim 59, wherein said plasma is subjected to conditions sufficient to enrich for fetal nucleic acids.

115. The method of claim 59, wherein said plasma is not subjected to conditions sufficient to enrich for fetal nucleic acids.

116. The method of any of claims 1-60, wherein said first plurality of nucleic acids and said second plurality of nucleic acids are derived from the same source.

117. The method of any of claims 1-60, wherein said first plurality of nucleic acids and said second plurality of nucleic acids are derived from different sources.

118. A method for analyzing a size distribution of nucleic acids, the method comprising:

(A) providing a sample comprising:

(i) a first plurality of nucleic acids and a second plurality of nucleic acids, wherein said first plurality of nucleic acids each comprise a first nucleic acid sequence of a given length and said second plurality of nucleic acids each comprise a second nucleic acid sequence longer than said given length;

(ii) a first set of paired oligonucleotide primers configured to amplify said first nucleic acid sequence; and

(iii) a second set of paired oligonucleotide primers, configured to amplify said second nucleic acid sequence;

(B) performing an amplification reaction on (a) said first nucleic acid sequence to generate a first signal and (b) said second nucleic acid sequence to generate a second signal; and

(C) determining a ratio of a first value derived from said first signal to a second value derived from said second signal, thereby analyzing said size distribution.

119. The method of claim 118, wherein said sample further comprises (iv) a first oligonucleotide probe configured to hybridize to a region of said first nucleic acid sequence and (v) a second oligonucleotide probe configured to hybridize to a region of said second nucleic acid sequence.

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120. The method of claim 119, wherein said first signal is generated from said first oligonucleotide probe and said second signal is generated from said second oligonucleotide probe.

121. The method of claim 118, wherein said sample further comprises an intercalating dye.

122. The method of claim 121, wherein said first signal or said second signal is generated from said intercalating dye.

123. The method of claim 121, wherein said first signal and said second signal are generated from said intercalating dye.

124. The method of claim 118, wherein said intercalating dye is SYBR® Green or EvaGreen®.

125. The method of claim 118, wherein said first signal or said second signal are generated by mass spectrometry.

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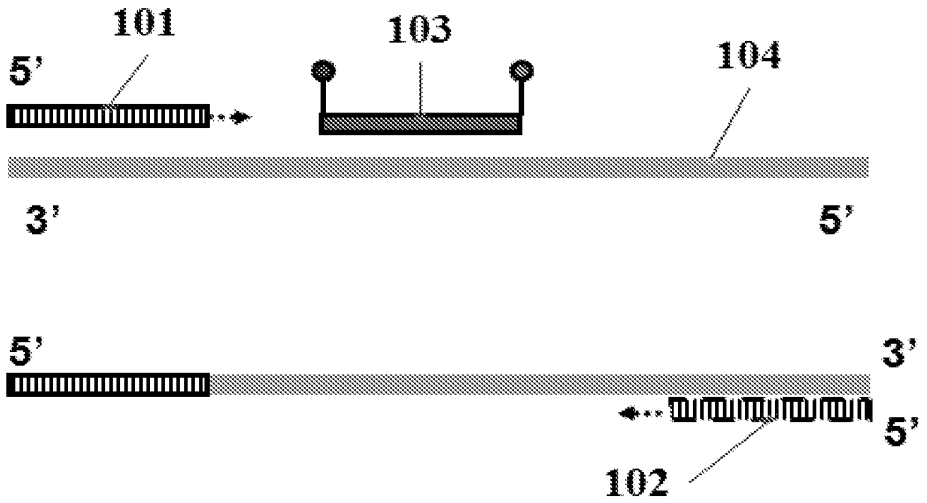


FIG. 1A

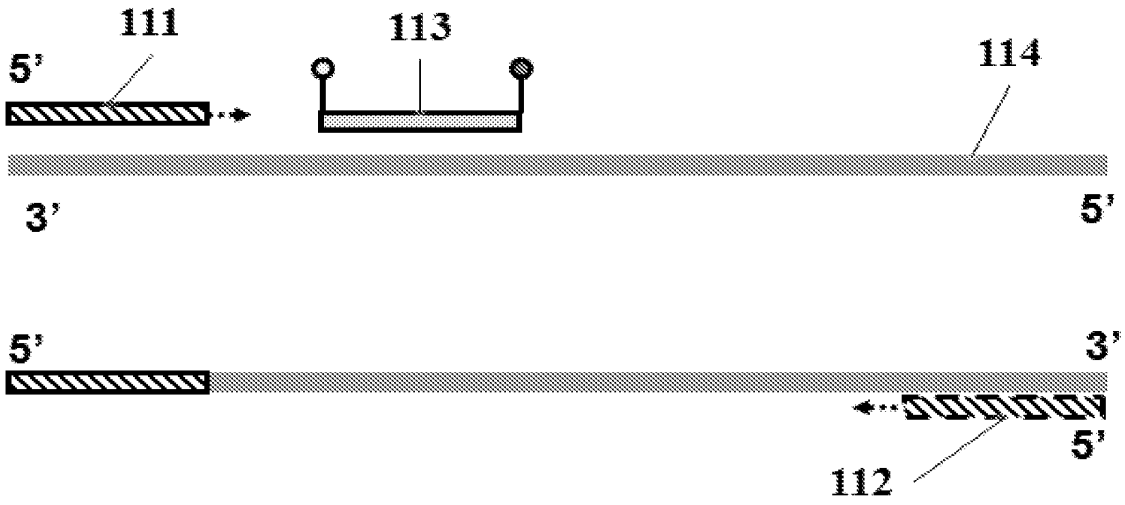


FIG. 1B

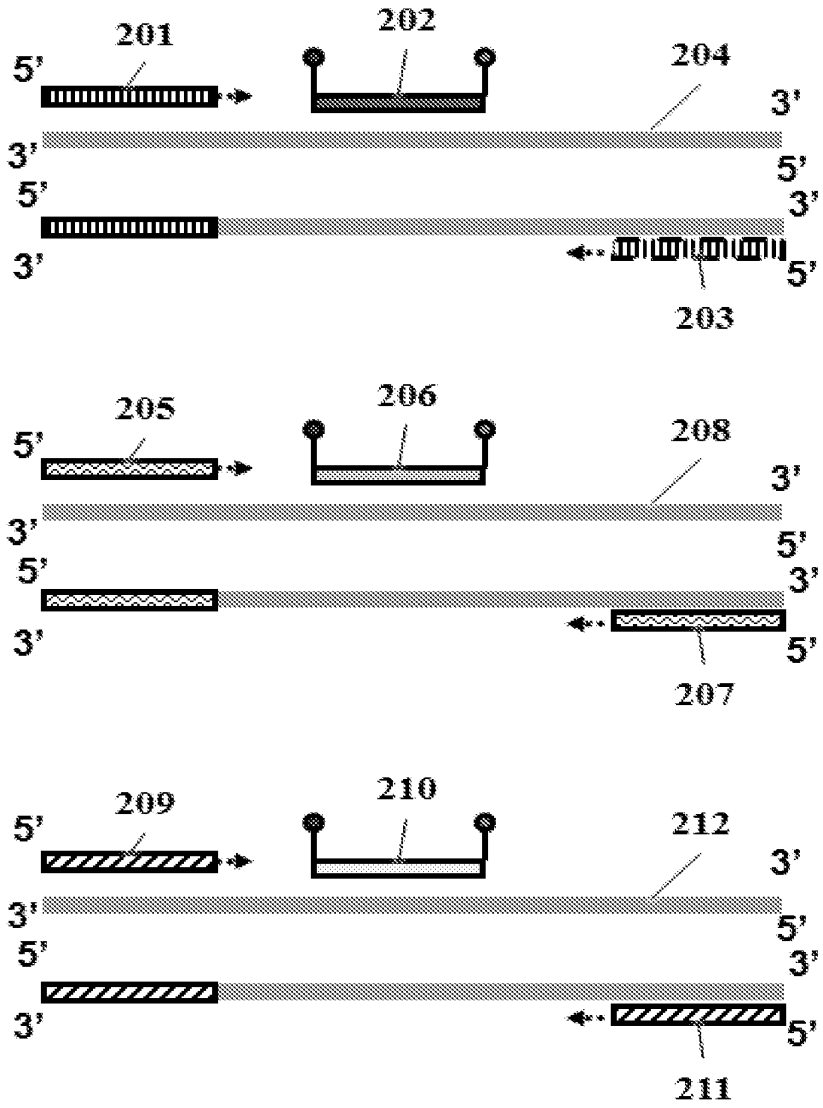

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211

FIG. 2A

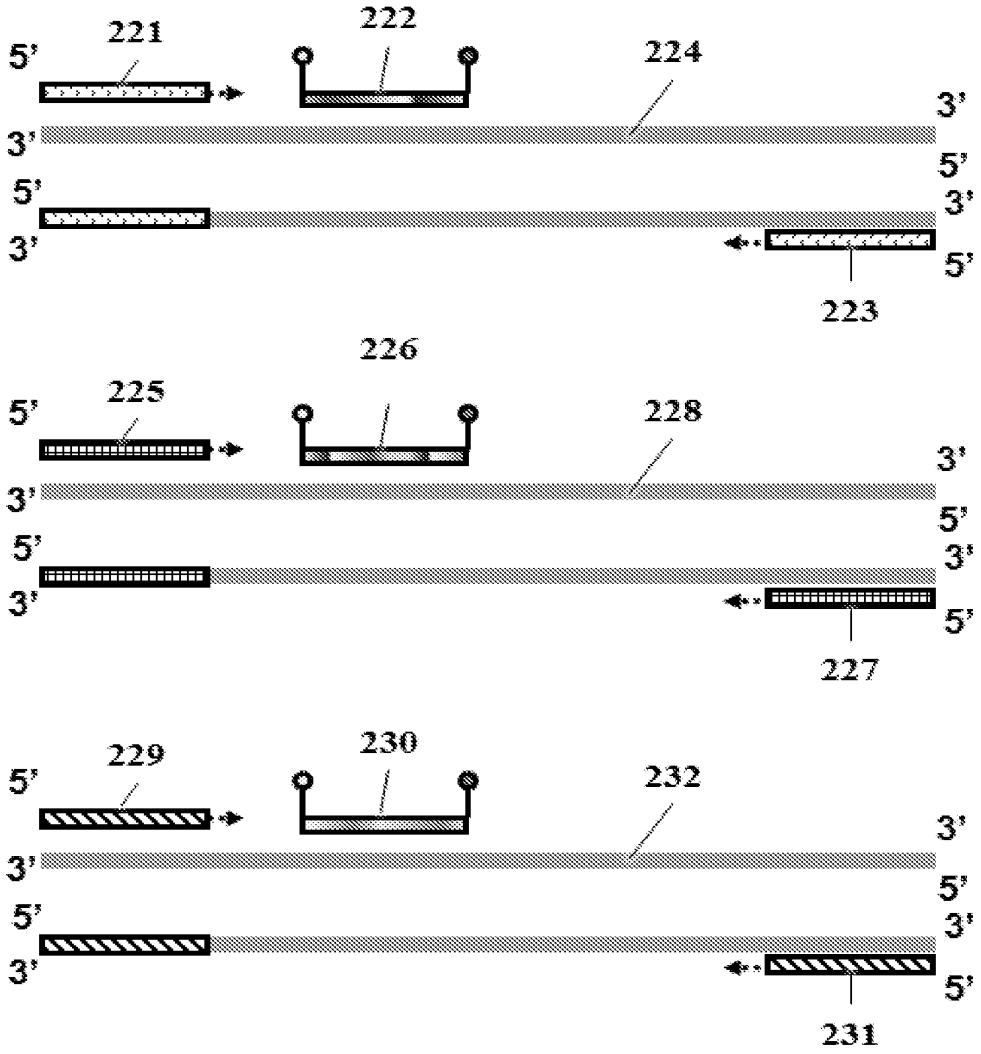

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FIG. 2B

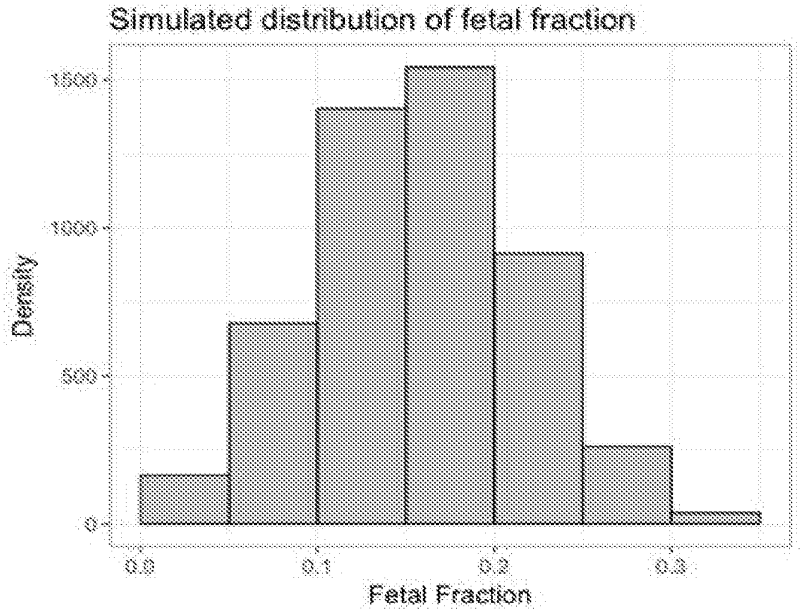

Simulated distribution of fetal fraction
1500
1000
500
0
Density
0.0
0.1
0.2
0.3
Fetal Fraction

FIG. 3A

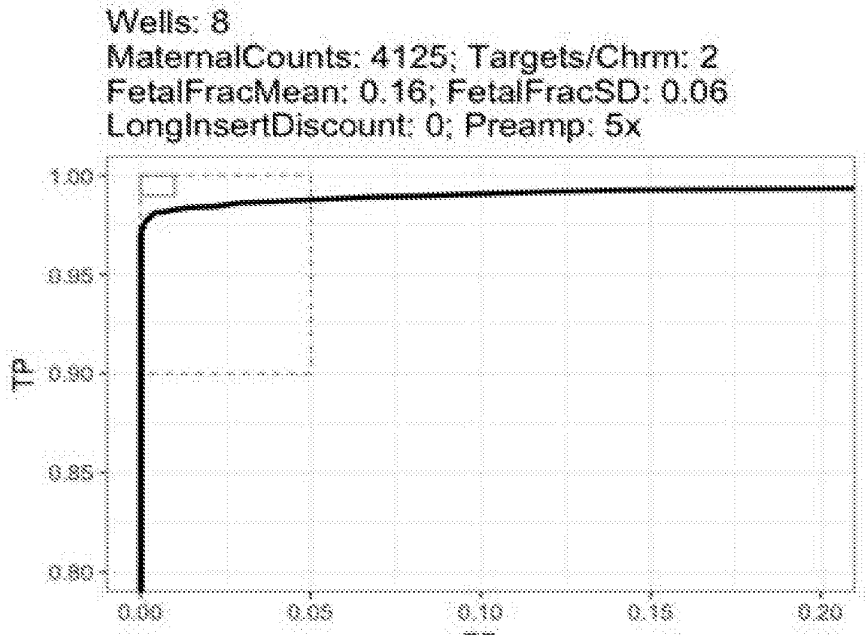

Wells: 8
MaternalCounts: 4125; Targets/Chrm: 2
FetalFracMean: 0.16; FetalFracSD: 0.06
LongInsertDiscount: 0; Preamp: 5x
1.00
0.95
0.90
0.85
0.80
TP
0.00
0.05
0.10
0.15
0.20

FIG. 3B

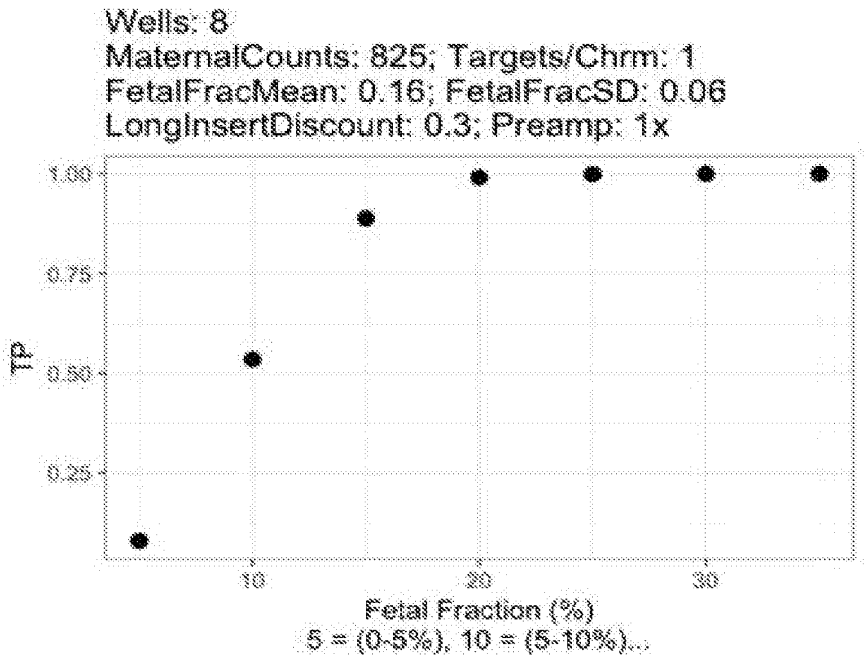

Wells: 8
MaternalCounts: 825; Targets/Chrm: 1
FetalFracMean: 0.16; FetalFracSD: 0.06
LongInsertDiscount: 0.3; Preamp: 1x
1.00
0.75
0.50
0.25
TP
10
20
30
Fetal Fraction (%)
5 = (0-5%], 10 = (5-10%]...

FIG. 3C

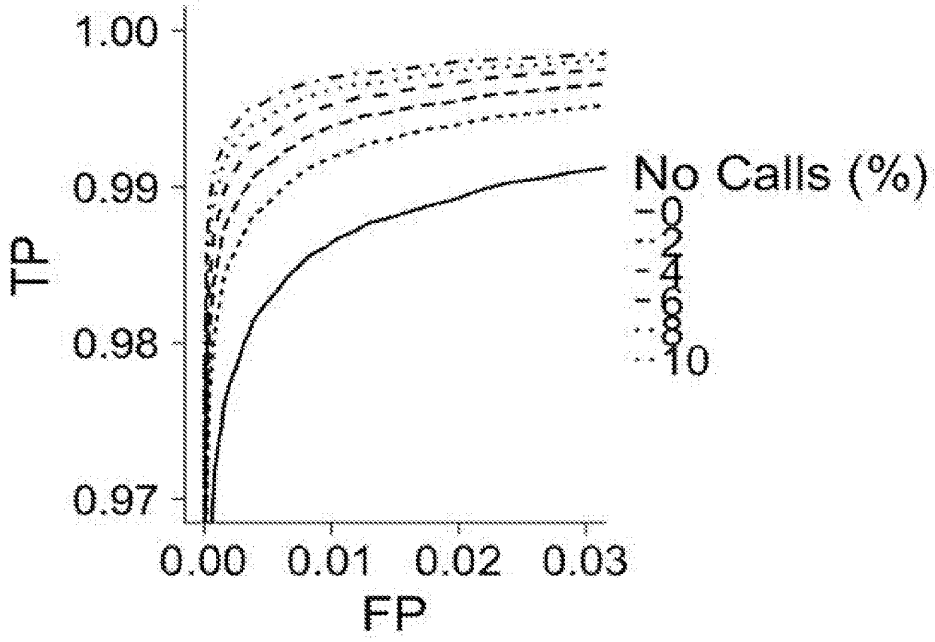

1.00
0.99
0.98
0.97
TP
0.00
0.01
0.02
0.03
FP
No Calls (%)
0
2
4
6
8
10

FIG. 4A

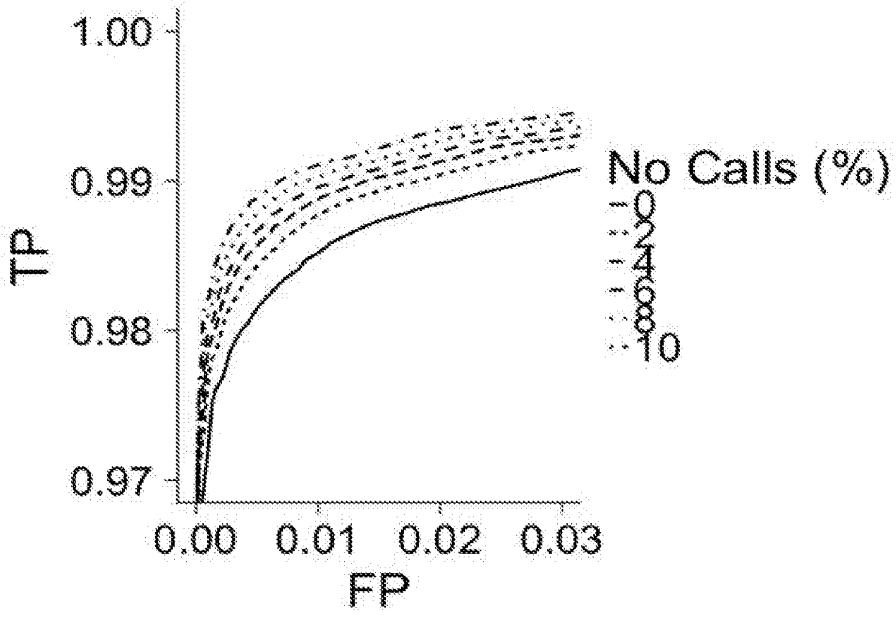

1.00
0.99
0.98
0.97
TP
0.00
0.01
0.02
0.03
FP
No Calls (%)
0
2
4
6
8
10

FIG. 4B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US19/19906

A. CLASSIFICATION OF SUBJECT MATTER
IPC - C12Q 1/6846, 1/6851, 1/6872, 1/6876; G01N 33/48 (2019.01)
CPC - C12Q 1/6846, 1/6851, 1/6872, 1/6876; G01N 33/48

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.
Y	US 2013/0288244 A1 (SEQUENOM, INC.) 31 October, 2013; paragraphs [0098]-[0100], [0232], [0236], [0277], [0279], [0290], [0294], [0302], [1103], [1166]	1-14, 15/1-14, 16/15/1-14, 17/16/15/1-14, 28-29, 38-43, 44/39-43, 57-70, 71/67-70, 72/71/67-70, 94-95, 96/60-66, 109-111, 114-155, 118-125
Y	US 2013/0078626 A1 (WASSERSTROM, A et al.) 28 March, 2013; paragraph [0050]; claims 1, 31	1-14, 15/1-14, 16/15/1-14, 17/16/15/1-14, 28-29, 38-43, 44/39-43, 57-70, 71/67-70, 72/71/67-70, 94-95, 96/60-66, 109-111, 114-115, 118-125
Y	US 2016/0273048 A1 (ONCODIAG) 22 September, 2016; paragraph [0108]	72/71/67-70

☐ 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
17 April 2019 (17.04.2019)	06 MAY 2019
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas 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/US19/19906

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.: 18-27, 30-37, 45-56, 73-93, 97-108, 112-113, 116-117
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:

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.:

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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(54) Title: MONITORING HYBRIDIZATION DURING PCR

(57) Abstract

Methods of monitoring hybridization during polymerase chain reaction are disclosed. These methods are achieved with rapid thermal cycling and use of double stranded DNA dyes or specific hybridization probes. A fluorescence resonance energy transfer pair comprises fluorescein and Cy5 or Cy5.5. Methods for quantitating amplified DNA and determining its purity are carried out by analysis of melting and reannealing curves.

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MONITORING HYBRIDIZATION DURING PCR

CROSS REFERENCE TO RELATED APPLICATIONS

U.S. application Serial No. 08/818,267, filed March 17, 1997, entitled Method for Detecting the Factor V Leiden Mutation, which is a continuation-in-part of U.S. Patent Application Serial No. 08/658,993, filed June 4, 1996, entitled System And Method For Monitoring PCR Processes, which is a continuation-in-part of U.S. Patent Application Serial No. 08/537,612, filed October 2, 1995, entitled Method For Rapid Thermal Cycling of Biological Samples, which is a continuation-in-part of U.S. Patent Application Serial No. 08/179,969, filed January 10, 1994, (now U.S. Patent No. 5,455,175), entitled Rapid Thermal Cycling Device, which is a continuation-in-part of U.S. Patent Application Serial No. 07/815,966 filed January 2, 1992, (now abandoned) entitled Rapid Thermal Cycling Device which is a continuation-in-part of U.S. Patent Application Serial No. 07/534,029 filed June 4, 1990, (now abandoned) entitled Automated Polymerase Chain Reaction Device, each of the above-identified applications are now each individually incorporated herein by reference in their entireties. The copending PCT application filed in the Receiving Office/United States (RO/US) on June 4, 1997, entitled System and Method for Carrying Out and Monitoring Biological Processes as serial no. PCT/US97/_____ and naming University of Utah Research Foundation as applicant, with Carl T. Wittwer, Kirk M. Ririe, Randy P. Rasmussen, and David R. Hillyard as inventors and applicants for the United States, is also hereby incorporated by reference in its entirety.

BACKGROUND OF THE INVENTION

This invention relates generally to observing fluorescence signals resulting from hybridization in conjunction with the polymerase chain reaction. More specifically, the present invention relates to observing hybridization with fluorescence during and/or immediately after PCR and using this information for product identification, sequence alteration detection, and quantification.

The polymerase chain reaction (PCR) is fundamental to molecular biology and is the first practical molecular technique for the clinical laboratory. Despite its usefulness and popularity, current understanding of PCR is not highly advanced. Adequate conditions for successful amplifications must be found by trial and error and optimization is empirical. Even those skilled in the art are

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required to utilize a powerful technique without a comprehensive or predictive theory of the process.

PCR is achieved by temperature cycling of the sample, causing DNA to denature (separate), specific primers to attach (anneal), and replication to occur (extend). One cycle of PCR is usually performed in 2 to 8 min, requiring 1 to 4 hours for a 30-cycle amplification. The sample temperature response in most PCR instrumentation is very slow compared to the times required for denaturation, annealing, and extension. The physical (denaturation and annealing) and enzymatic (extension) reactions in PCR occur very quickly. Amplification times for PCR can be reduced from hours to less than 15 min. Incorporated herein by reference in its entirety is U.S. Serial No. 08/537,612, filed October 2, 1995, which discloses such a rapid cycling system. Rapid cycling techniques are made possible by the rapid temperature response and temperature homogeneity possible for samples in high surface area-to-volume sample containers such as capillary tubes. For further information, see also: C.T. Wittwer, G.B. Reed, and K.M. Ririe, Rapid cycle DNA amplification, in K.B. Mullis, F. Ferre, and R.A. Gibbs, The polymerase chain reaction, Birkhauser, Boston, 174-181, (1994). Improved temperature homogeneity allows the time and temperature requirements of PCR to be better defined and understood. Improved temperature homogeneity also increases the precision of any analytical technique used to monitor PCR during amplification.

Fluorimetry is a sensitive and versatile technique with many applications in molecular biology. Ethidium bromide has been used for many years to visualize the size distribution of nucleic acids separated by gel electrophoresis. The gel is usually transilluminated with ultraviolet light and the red fluorescence of double stranded nucleic acid observed. Specifically, ethidium bromide is commonly used to analyze the products of PCR after amplification is completed. Furthermore, EPA 0 640 828 A1 to Higuchi & Watson, hereby incorporated by reference, discloses using ethidium bromide during amplification to monitor the amount of double stranded DNA by measuring the fluorescence each cycle. The fluorescence intensity was noted to rise and fall inversely with temperature, was greatest at the annealing/extension temperature (50°C), and least at the denaturation temperature (94°C). Maximal fluorescence was acquired each cycle as a measure of DNA amount. The Higuchi & Watson application does not teach using fluorescence to monitor hybridization events, nor does it suggest acquiring fluorescence over different temperatures to follow the extent of

hybridization. Moreover, Higuch & Watson fails to teach or suggest using the temperature dependence of PCR product hybridization for identification or quantification of PCR products.

The Higuchi & Watson application, however, does mention using other fluorophores, including dual-labeled probe systems that generate flourescence when hydrolyzed by the 5'-exonuclease activity of certain DNA polymerases, as disclosed in US Patent No. 5,210,015 to Gelfand et al. The fluorescence observed from these probes primarily depends on hydrolysis of the probe between its two fluorophores. The amount of PCR product is estimated by acquiring fluorescence once each cycle. Although hybridization of these probes appears necessary for hydrolysis to occur, the fluorescence signal primarily results from hydrolysis of the probes, not hybridization, wherein an oligonucleotide probe with fluorescent dyes at opposite ends thereof provides a quenched probe system useful for detecting PCR product and nucleic acid hybridization, K.J. Livak et al., 4 PCR Meth. Appl. 357-362 (1995). There is no suggestion of following the temperature dependence of probe hybridization with fluorescence to identify sequence alterations in PCR products.

The specific hybridization of nucleic acid to a complementary strand for identification has been exploited in many different formats. For example, after restriction enzyme digestion, genomic DNA can be size fractionated and hybridized to probes by Southern blotting. As another example, single base mutations can be detected by "dot blots" with allele-specific oligonucleotides. Usually, hybridization is performed for minutes to hours at a single temperature to achieve the necessary discrimination. Alternately, the extent of hybridization can be dynamically monitored while the temperature is changing by using fluorescence techniques. For example, fluorescence melting curves have been used to monitor hybridization. L.E. Morrison & L.M. Stols, Sensitive fluorescence-based thermodynamic and kinetic measurements of DNA hybridization in solution, 32 Biochemistry 3095-3104, 1993). The temperature scan rates are usually 10°C/hour or less, partly because of the high thermal mass of the fluorimeter cuvette.

Current methods for monitoring hybridization require a lot of time. If hybridization could be followed in seconds rather than hours, hybridization could be monitored during PCR amplification, even during rapid cycle PCR. The many uses of monitoring hybridization during PCR, as will be fully disclosed herein,

include, product identification and quantification, sequence alteration detection, and automatic control of temperature cycling parameters by fluorescence feedback

The prior art, as explained above, carries out temperature cycling slowly and empirically. When analysis of PCR products by hybridization is needed, additional time consuming steps are required. Thus, it would be a great advance in the art to provide methods for monitoring hybridization during PCR and analyzing the reaction while it is taking place, that is, during or immediately after temperature cycling without manipulation of the sample. By monitoring hybridization during PCR, the underlying principles that allow PCR to work can be followed and used to analyze and optimize the reaction during amplification.

BRIEF SUMMARY OF THE INVENTION

It is an object of the present invention to provide a double-strand-specific DNA dye for monitoring product hybridization during PCR.

It is another object of the invention to provide a system for identifying PCR-amplified products by their fluorescence melting curves.

It is also an object of the invention to provide a method for improving the sensitivity of PCR quantification with double-strand-specific DNA dyes.

It is still another objection of the invention for determining the amount of specific product amplified by PCR by melting curves to correct for nonspecific amplification detected with the double-strand-specific DNA dye.

It is a further object of the invention to provide a method of relative quantification of different PCR products with double-strand-specific dyes.

It is yet another object of the invention to provide a method of product quantification by the reannealing kinetics of the product in the presence of a double-strand-specific DNA dye.

It is a still further object of the invention to provide a novel resonance energy transfer pair to monitor primer and/or probe hybridization.

It is still another object of the invention to provide a method of product quantification by the reannealing kinetics of a probe to the product using a resonance energy transfer pair.

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It is also an object of the present invention to provide a method to determine initial template copy number by following the fluorescence of a hybridization probe or probes each cycle during PCR amplification.

It is another object of the invention to provide a system for homogeneous detection of PCR products by resonance energy transfer between two labeled probes that hybridize internal to the PCR primers.

It is still another object of the invention to provide a system for homogeneous detection of PCR products by resonance energy transfer between one labeled primer and one labeled probe that hybridizes internal to the PCR primers.

It is yet another object of the invention to provide a system for detection of sequence alterations internal to PCR primers by resonance energy transfer and probe melting curves.

It is a further object of the invention to provide a system for relative quantification of different PCR products by probe melting curves.

It is yet another object of the invention to provide methods to determine the initial template copy number by curve fitting the fluorescence vs cycle number plot.

It is still another object of the invention to provide a system and method for performing PCR rapidly and also continuously monitoring the reaction and adjusting the reaction parameters while the reaction is ongoing.

It is another object of the invention to replace the nucleic acid probes by synthetic nucleic acid analogs or derivatives, e.g. by peptide nucleic acids (PNA), provided that they can also be labeled with fluorescent compounds.

These and other objects and advantages of the invention will become more fully apparent from the description and claims which follow, or may be learned by the practice of the invention.

The present invention particularly decreases the total time required for PCR amplification and analysis over prior art techniques while at the same time allowing the option of significantly increasing the quality of the reaction by optimizing amplification conditions.

The present invention provides methods and applications for continuous fluorescence monitoring of DNA amplification. Required instrumentation combines optical components with structures to

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provide rapid temperature cycling to continuously monitor DNA amplification by a variety of different fluorescence techniques. In one illustrative embodiment, fluorescence is acquired continuously from a single sample or alternately from multiple samples on a rotating carousel with all of the samples being simultaneously subjected to rapid thermal cycling. Further information on associated instrumentation can be found in the U.S. patent applications referenced above.

In accordance with one aspect of the present invention, fluorescence during DNA amplification was monitored by: 1) the double strand-specific dye SYBR Green I, and 2) resonance energy transfer of fluorescein to Cy5™ or Cy5.5™ with hybridization probes. Fluorescence data acquired once per cycle allow quantification of initial template copy number.

Furthermore, in contrast to measuring fluorescence once per cycle, embodiments of the present invention are disclosed which monitor temperature, time and fluorescence continuously throughout each cycle thus producing a 3-dimensional spiral. This 3-dimensional spiral can be reduced to temperature vs. time, fluorescence vs. time, and fluorescence vs. temperature plots. Fluorescence vs. temperature plots of the fluorescence from hybridization probes can be used to detect sequence alterations in the product. These sequence alterations may be natural, as in mutations or polymorphisms, or artificial, as in an engineered alternative template for quantitative PCR.

In accordance with another aspect of the present invention, fluorescence monitoring is used to acquire product melting curves during PCR by fluorescence monitoring with double-strand-specific DNA specific dyes. Plotting fluorescence as a function of temperature as the thermal cycler heats through the dissociation temperature of the product gives a PCR product melting curve. The shape and position of this DNA melting curve is a function of GC/AT ratio, length, and sequence, and can be used to differentiate amplification products separated by less than 2°C in melting temperature. Desired products can be distinguished from undesired products, including primer dimers. Analysis of melting curves can be used to extend the dynamic range of quantitative PCR and to differentiate different products in multiplex amplification. Using double strand dyes, product denaturation, reannealing and extension can be followed within each cycle. Continuous monitoring of

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fluorescence allows acquisition of melting curves and product annealing curves during temperature cycling.

The present invention provides reagents and methods for rapid cycle PCR with combined amplification and analysis by fluorescence monitoring in under thirty minutes, more preferably in under fifteen minutes, and most preferably in under ten minutes.

A method for analyzing a target DNA sequence of a biological sample comprises

amplifying the target sequence by polymerase chain reaction in the presence of two nucleic acid probes that hybridize to adjacent regions of the target sequence, one of the probes being labeled with an acceptor fluorophore and the other probe labeled with a donor fluorophore of a fluorescence energy transfer pair such that upon hybridization of the two probes with the target sequence, the donor and acceptor fluorophores are within 25 nucleotides of one another, the polymerase chain reaction comprising the steps of adding a thermostable polymerase and primers for the targeted nucleic acid sequence to the biological sample and thermally cycling the biological sample between at least a denaturation temperature and an elongation temperature;

exciting the biological sample with light at a wavelength absorbed by the donor fluorophore and detecting the emission from the fluorescence energy transfer pair.

A method for analyzing a target DNA sequence of a biological sample comprises

amplifying the target sequence by polymerase chain reaction in the presence of two nucleic acid probes that hybridize to adjacent regions of the target sequence, one of the probes being labeled with an acceptor fluorophore and the other probe labeled with a donor fluorophore of a fluorescence energy transfer pair such that upon hybridization of the two probes with the target sequence, the donor and acceptor fluorophores are within 25 nucleotides of one another, the polymerase chain reaction comprising the steps of adding a thermostable polymerase and primers for the targeted nucleic acid sequence to the biological sample and thermally cycling the biological sample between at least a denaturation temperature and an elongation temperature;

exciting the sample with light at a wavelength absorbed by the donor fluorophore; and

monitoring the temperature dependent fluorescence from the fluorescence energy transfer pair.

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A method of real time monitoring of a polymerase chain reaction amplification of a target nucleic acid sequence in a biological sample comprises

(a) adding to the biological sample an effective amount of two nucleic acid primers and a nucleic acid probe, wherein one of the primers and the probe are each labeled with one member of a fluorescence energy transfer pair comprising an acceptor fluorophore and a donor fluorophore, and wherein the labeled probe hybridizes to an amplified copy of the target nucleic acid sequence within 15 nucleotides of the labeled primer;

(b) amplifying the target nucleic acid sequence by polymerase chain reaction;

(c) illuminating the biological sample with light of a selected wavelength that is absorbed by said donor fluorophore; and

(d) detecting the fluorescence emission of the sample.

An improved method of amplifying a target nucleic acid sequence of a biological sample comprises

(a) adding to the biological sample an effective amount of a nucleic-acid-binding fluorescent entity;

(b) amplifying the target nucleic acid sequence using polymerase chain reaction, comprising thermally cycling the biological sample using initial predetermined temperature and time parameters, and then

(i) illuminating the biological sample with a selected wavelength of light that is absorbed by the fluorescent entity during the polymerase chain reaction;

(ii) monitoring fluorescence from the sample to determine the optimal temperature and time parameters for the polymerase chain reaction; and

(iii) adjusting the initial temperature and time parameters in accordance with the fluorescence.

In one illustrative embodiment, the fluorescent entity comprises a double strand specific nucleic acid binding dye, and in another illustrative embodiment the fluorescent entity comprises a fluorescently labeled oligonucleotide probe that hybridizes to the targeted nucleic acid sequence.

A method for detecting a target nucleic acid sequence of a biological sample comprises

(a) adding to the biological sample an effective amount of a pair of oligonucleotide probes that hybridize to the

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target nucleic acid sequence, one of the probes being labeled with an acceptor fluorophore and the other probe labeled with a donor fluorophore of a fluorescence energy transfer pair, wherein an emission spectrum of the donor fluorophore and an absorption spectrum of the acceptor fluorophore overlap less than 25%, the acceptor fluorophore has a peak extinction coefficient greater than 100,000 $M^{-1}cm^{-1}$ and upon hybridization of the two probes, the donor and acceptor fluorophores are within 25 nucleotides of one another;

(b) illuminating the biological sample with a selected wavelength of light that is absorbed by said donor fluorophore; and

(c) detecting the emission of the biological sample.

An illustrative resonance energy transfer pair comprises fluorescein as the donor and Cy5 or Cy5.5 as the acceptor.

A method of real time monitoring of a polymerase chain reaction amplification of a target nucleic acid sequence in a biological sample comprises

amplifying the target sequence by polymerase chain reaction in the presence of two nucleic acid probes that hybridize to adjacent regions of the target sequence, one of the probes being labeled with an acceptor fluorophore and the other probe labeled with a donor fluorophore of a fluorescence energy transfer pair such that upon hybridization of the two probes with the target sequence, the donor and acceptor fluorophores are within 25 nucleotides of one another, the polymerase chain reaction comprising the steps of adding a thermostable polymerase and primers for the targeted nucleic acid sequence to the biological sample and thermally cycling the biological sample between at least a denaturation temperature and an elongation temperature;

exciting the biological sample with light at a wavelength absorbed by the donor fluorophore and detecting the emission from the biological sample; and

monitoring the temperature dependent fluorescence from the fluorescence energy transfer pair.

A method of real time monitoring of a polymerase chain reaction amplification of a target nucleic acid sequence in a biological sample comprises

amplifying the target sequence by polymerase chain reaction in the presence of SYBR™ Green I, the polymerase chain reaction comprising the steps of adding a thermostable polymerase and primers for the targeted nucleic acid sequence to the biological

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sample and thermally cycling the biological sample between at least a denaturation temperature and an elongation temperature;

exciting the biological sample with light at a wavelength absorbed by the SYBR™ Green I and detecting the emission from the biological sample; and

monitoring the temperature dependent fluorescence from the SYBR™ Green I. Preferably, the monitoring step comprises determining a melting profile of the amplified target sequence.

A method for analyzing a target DNA sequence of a biological sample comprises

(a) adding to the biological sample an effective amount of two nucleic acid primers and a nucleic acid probe, wherein one of the primers and the probe are each labeled with one member of a fluorescence energy transfer pair comprising an acceptor fluorophore and a donor fluorophore, and wherein the labeled probe hybridizes to an amplified copy of the target nucleic acid sequence within 15 nucleotides of the labeled primer;

(b) amplifying the target nucleic acid sequence by polymerase chain reaction;

(c) illuminating the biological sample with light of a selected wavelength that is absorbed by said donor fluorophore and detecting the fluorescence emission of the sample. In another illustrative embodiment, the method further comprises the step of monitoring the temperature dependent fluorescence of the sample, preferably by determining a melting profile of the amplified target sequence.

A method of detecting a difference at a selected locus in a first nucleic acid as compared to a second nucleic acid comprises

(a) providing a pair of oligonucleotide primers configured for amplifying, by polymerase chain reaction, a selected segment of the first nucleic acid and a corresponding segment of the second nucleic acid, wherein the selected segment and corresponding segment each comprises the selected locus, to result in amplified products containing a copy of the selected locus;

(b) providing a pair of oligonucleotide probes, one of the probes being labeled with an acceptor fluorophore and the other probe being labeled with a donor fluorophore of a fluorogenic resonance energy transfer pair such that upon hybridization of the two probes with the amplified products the donor and acceptor are in resonance energy transfer relationship, wherein one of the probes is configured for hybridizing to the amplified products such that said

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one of the probes spans the selected locus and exhibits a melting profile when the difference is present in the first nucleic acid that is distinguishable from a melting profile of the second nucleic acid;

(c) amplifying the selected segment of first nucleic acid and the corresponding segment of the second nucleic acid by polymerase chain reaction in the presence of effective amounts of probes to result in an amplified selected segment and an amplified corresponding segment, at least a portion thereof having both the probes hybridized thereto with the fluorogenic resonance energy transfer pair in resonance energy transfer relationship;

(d) illuminating the amplified selected segment and the amplified corresponding segment with the probes hybridized thereto with a selected wavelength of light to elicit fluorescence by the fluorogenic resonance energy transfer pair;

(e) measuring fluorescence emission as a function of temperature to determine in a first melting profile of said one of the probes melting from the amplified selected segment of first nucleic acid and a second melting profile of said one of the probes melting from the amplified corresponding segment of second nucleic acid; and

(f) comparing the first melting profile to the second melting profile, wherein a difference therein indicates the presence of the difference in the sample nucleic acid.

A method of detecting a difference at a selected locus in a first nucleic acid as compared to a second nucleic acid comprises

(a) providing a pair of oligonucleotide primers configured for amplifying, by polymerase chain reaction, a selected segment of the first nucleic acid and a corresponding segment of the second nucleic acid, wherein the selected segment and corresponding segment each comprises the selected locus, to result in amplified products containing a copy of the selected locus;

(b) providing an oligonucleotide probe, wherein one of the primers and the probe are each labeled with one member of a fluorescence energy transfer pair comprising an donor fluorophore and an acceptor fluorophore, and wherein the labeled probe and labeled primer hybridize to the amplified products such that the donor and acceptor are in resonance energy transfer relationship, and wherein the probe is configured for hybridizing to the amplified products such that said probe spans the selected locus and exhibits a melting profile when the difference is present in the first

nucleic acid that is distinguishable from a melting profile of the second nucleic acid;

(c) amplifying the selected segment of first nucleic acid and the corresponding segment of the second nucleic acid by polymerase chain reaction in the presence of effective amounts of primers and probe to result in an amplified selected segment and an amplified corresponding segment, at least a portion thereof having the labled primer and probe hybridized thereto with the fluorogenic resonance energy transfer pair in resonance energy transfer relationship;

(d) illuminating the amplified selected segment and the amplified corresponding segment with the labeled primer and probe hybridized thereto with a selected wavelength of light to elicit fluorescence by the fluorogenic resonance energy transfer pair;

(e) measuring fluorescence emission as a function of temperature to determine in a first melting profile of said probe melting from the amplified selected segment of first nucleic acid and a second melting profile of said probe melting from the amplified corresponding segment of second nucleic acid; and

(f) comparing the first melting profile to the second melting profile, wherein a difference therein indicates the presence of the difference in the sample nucleic acid.

A method of detecting heterozygosity at a selected locus in the genome of an individual, wherein the genome comprises a mutant allele and a corresponding reference allele, each comprising the selected locus, comprises

(a) obtaining sample genomic DNA from the individual;

(b) providing a pair of oligonucleotide primers configured for amplifying, by polymerase chain reaction, a first selected segment of the mutant allele and a second selected segment of the corresponding reference allele wherein both the first and second selected segments comprise the selected locus;

(c) providing a pair of oligonucleotide probes, one of the probes being labeled with an acceptor fluorophore and the other probe being labeled with a donor fluorophore of a fluorogenic resonance energy transfer pair such that upon hybridization of the two probes with the amplified first and second selected segments one of the probes spans the selected locus and exhibits a first melting profile with the amplified first selected segment that is distinguishable from a second melting profile with the amplified second selected segment;

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(d) amplifying the first and second selected segments of sample genomic DNA by polymerase chain reaction in the presence of effective amounts of probes to result in amplified first and second selected segments, at least a portion thereof having both the probes hybridized thereto with the fluorogenic resonance energy transfer pair in resonance energy transfer relationship;

(e) illuminating the amplified first and second selected segments having the probes hybridized thereto with a selected wavelength of light to elicit fluorescence by the donor and acceptor;

(f) measuring a fluorescence emission as a function of temperature to determine a first melting profile of said one of the probes melting from the amplified first selected segment and a second melting profile of said one of the probes melting from the amplified second selected segment; and

(g) comparing the first melting profile to the second melting profile, wherein distinguishable melting profiles indicate heterozygosity in the sample genomic DNA.

A method of detecting heterozygosity at a selected locus in the genome of an individual, wherein the genome comprises a mutant allele and a corresponding reference allele, each comprising the selected locus, comprises

(a) obtaining sample genomic DNA from the individual;

(b) providing a pair of oligonucleotide primers configured for amplifying, by polymerase chain reaction, a first selected segment of the mutant allele and a second selected segment of the corresponding reference allele wherein both the first and second selected segments comprise the selected locus;

(c) providing an oligonucleotide probe, wherein one of the primers and the probe are each labeled with one member of a fluorescence energy transfer pair comprising an donor fluorophore and an acceptor fluorophore, and wherein the labeled probe and labeled primer hybridize to the amplified first and second selected segments such that one of the probes spans the selected locus and exhibits a first melting profile with the amplified first selected segment that is distinguishable from a second melting profile with the amplified second selected segment;

(d) amplifying the first and second selected segments of sample genomic DNA by polymerase chain reaction in the presence of effective amounts of primers and probe to result in amplified first and second selected segments, at least a portion thereof having both

the labeled primer and probe hybridized thereto with the fluorogenic resonance energy transfer pair in resonance energy transfer relationship;

(e) illuminating the amplified first and second selected segments having the labeled primer and probe hybridized thereto with a selected wavelength of light to elicit fluorescence by the donor and acceptor;

(f) measuring a fluorescence emission as a function of temperature to determine a first melting profile of said probe melting from the amplified first selected segment and a second melting profile of said probe melting from the amplified second selected segment; and

(g) comparing the first melting profile to the second melting profile, wherein distinguishable melting profiles indicate heterozygosity in the sample genomic DNA.

A method of determining completion of a polymerase chain reaction in a polymerase chain reaction mixture comprising (1) a nucleic acid wherein the nucleic acid or a polymerase-chain-reaction-amplified product thereof consists of two distinct complementary strands, (2) two oligonucleotide primers configured for amplifying by polymerase chain reaction a selected segment of the nucleic acid to result in an amplified product, and (3) a DNA polymerase for catalyzing the polymerase chain reaction, comprises

(a) adding to the mixture (1) an effective amount of an oligonucleotide probe labeled with a resonance energy transfer donor or a resonance energy transfer acceptor of a fluorogenic resonance energy transfer pair, wherein the probe is configured for hybridizing to the amplified product under selected conditions of temperature and monovalent ionic strength, and (2) an effective amount of a reference oligonucleotide labeled with the donor or the acceptor, with the proviso that as between the probe and reference oligonucleotide one is labeled with the donor and the other is labeled with the acceptor, wherein the reference oligonucleotide is configured for hybridizing to the amplified product under the selected conditions of temperature and monovalent ionic strength such that the donor and the acceptor are in resonance energy transfer relationship when both the probe and the reference oligonucleotide hybridize to the amplified product;

(b) amplifying the selected segment of nucleic acid by polymerase chain reaction to result in the amplified product, at least a portion thereof having both the probe and the reference

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oligonucleotide hybridized thereto with the fluorogenic resonance energy transfer pair in resonance energy transfer relationship; and

(c) illuminating the amplified product having the probe and reference oligonucleotide hybridized thereto with a selected wavelength of light for eliciting fluorescence by the fluorogenic resonance energy pair and monitoring fluorescence emission and determining a cycle when the fluorescence emission reaches a plateau phase, indicating the completion of the reaction.

A method of determining completion of a polymerase chain reaction in a polymerase chain reaction mixture comprising (1) a nucleic acid wherein the nucleic acid or a polymerase-chain-reaction-amplified product thereof consists of two distinct complementary strands, (2) two oligonucleotide primers configured for amplifying by polymerase chain reaction a selected segment of the nucleic acid to result in an amplified product, and (3) a DNA polymerase for catalyzing the polymerase chain reaction, comprises

(a) adding to the mixture an effective amount of a nucleic-acid-binding fluorescent dye;

(b) amplifying the selected segment of nucleic acid by polymerase chain reaction in the mixture to which the nucleic-acid-binding fluorescent dye has been added to result in the amplified product with nucleic-acid-binding fluorescent dye bound thereto; and

(c) illuminating amplified product with nucleic-acid-binding fluorescent dye bound thereto with a selected wavelength of light for eliciting fluorescence therefrom and monitoring fluorescence emission and determining a cycle when the fluorescence emission reaches a plateau phase, indicating the completion of the reaction. Preferably, the nucleic-acid-binding fluorescent dye is a member selected from the group consisting of SYBR™ GREEN I, ethidium bromide, pico green, acridine orange, thiazole orange, YO-PRO-1, and chromomycin A3, and more preferably is SYBR™ GREEN I.

A method of controlling temperature cycling parameters of a polymerase chain reaction comprising repeated cycles of annealing, extension, and denaturation phases of a polymerase chain reaction mixture comprising a double-strand-specific fluorescent dye, wherein the parameters comprise duration of the annealing phase, duration of the denaturation phase, and number of cycles, comprises

(a) illuminating the reaction with a selected wavelength of light for eliciting fluorescence from the fluorescent dye and continuously monitoring fluorescence during the repeated annealing, extension, and denaturation phases;

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(b) determining at least

(i) duration for fluorescence to stop increasing during the extension phase, or

(ii) Duration for fluorescence to decrease to a baseline level during the denaturation phase, or

(iii)number of cycles for fluorescence to reach a preselected level during the extension phase; and

(c) adjusting the length of the extension phase according to the length of time for fluorescence to stop increasing during the extension phase, the length of the denaturation phase according to the length of time for fluorescence to decrease to the baseline level during the denaturation phase, or the number of cycles according to the number of cycles for fluorescence to reach the preselected level during the extension phase.

A method of determining a concentration of an amplified product in a selected polymerase chain reaction mixture comprises

(a) determining a second order rate constant for the amplified product at a selected temperature and reaction conditions by monitoring rate of hybridization of a known concentration of the amplified product;

(b) determining rate of annealing for an unknown concentration of the amplified product; and

(c) calculating the concentration of the amplified product from the rate of annealing and the second order rate constant.

Preferably, the rate of annealing is determined after multiple cycles of amplification. One illustrative method of determining the second oder rate constant comprises the steps of

raising the temperature of a first polymerase chain reaction mixture comprising a known concentration of the amplified product and an effective amount of a double-strand specific fluorescent dye, above the denaturation temperature of the amplified product to result in a denatured amplified product;

rapidly reducing the temperature of the first polymerase chain reaction mixture comprising the known amount of denatured amplified product to a selected temperature below the denaturation temperature of the amplified product while continuously monitoring the fluorescence of the first polymerase chain reaction mixture as a function of time;

plotting fluorescence as a function of time for determining maximum fluorescence, minimum fluorescence, the time at

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minimum fluorescence, and a second order rate constant for the known concentration of amplified product from the equation

$$F = F_{max} - \frac{F_{max} - F_{min}}{k(t-t_0)\,[DNA] + 1}$$

wherein F is fluorescence, F_{max} is maximum fluorescence, F_{min} is minimum fluorescence, k is the second order rate constant, t_0 is the time at F_{min}, and [DNA] is the known concentration of the amplified product.

A method of determining a concentration of a selected nucleic acid template by competitive quantitative polymerase chain reaction comprises the steps of:

(a) in a reaction mixture comprising:

(i) effective amounts of each of a pair of oligonucleotide primers configured for amplifying, in a polymerase chain reaction, a selected segment of the selected template and a corresponding selected segment of a competitive template to result in amplified products thereof,

(ii) an effective amount of an oligonucleotide probe labeled with a resonance energy transfer donor or a resonance energy transfer acceptor of a fluorogenic resonance energy transfer pair, wherein the probe is configured for hybridizing to the amplified products such that the probe melts from the amplified product of the selected template at a melting temperature that is distinguishable from the melting temperature at which the probe melts from the amplified product of the competitive template,

(iii) an effective amount of a reference oligonucleotide labeled with the donor or the acceptor, with the proviso that as between the probe and transfer oligonucleotide one is labeled with the donor and the other is labeled with the acceptor, wherein the reference oligonucleotide is configured for hybridizing to the amplified products such that the donor and the acceptor are in resonance energy transfer relationship when both the probe and the reference oligonucleotide hybridize to the amplified products;

amplifying, by polymerase chain reaction, an unknown amount of the selected template and a known amount of the competitive template to result in the amplified products thereof;

(b) illuminating the reaction mixture with a selected wavelength of light for eliciting fluorescence by the fluorogenic

resonance energy transfer pair and determining a fluorescence emission as a function of temperature as the temperature of the reaction mixture is changed to result in a first melting curve of the probe melting from the amplified product of the selected template and a second melting curve of the probe melting from the competitive template;

(c) converting the first and second melting curves to first and second melting melting peaks and determining relative amounts of the selected template and the competitive template from such melting peaks; and

(d) calculating the concentration of the selected template based on the known amount of the competitive template and the relative amounts of selected template and competitive template.

A fluorogenic resonance energy transfer pair consists of fluorescein and Cy5 or Cy5.5.

A method of determining a concentration of a selected nucleic acid template in a polymerase chain reaction comprises the steps of:

(a) in a reaction mixture comprising:

(i) effective amounts of each of a first pair of oligonucleotide primers configured for amplifying, in a polymerase chain reaction, a selected first segment of the selected template to result in an amplified first product thereof,

(ii) effective amounts of each of a second pair of oligonucleotide primers configured for amplifying, in a polymerase chain reaction, a selected second segment of a reference template to result in an amplified second product thereof,

(iii) an effective amount of a nucleic-acid-binding fluorescent dye;

amplifying, by polymerase chain reaction, an unknown amount of the selected template to result in the amplified first product and a known amount of the reference template to result in the amplified second product thereof;

(b) illuminating the reaction mixture with a selected wavelength of light for eliciting fluorescence by the nucleic-acid-binding fluorescent dye and continuously monitoring the fluorescence emitted as a function of temperature to result in a melting curve of the amplified products wherein the first product and second product melt at different temperatures;

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(c) converting the melting curves to melting melting peaks and determining relative amounts of the selected template and the reference template from such melting peaks; and

(d) calculating the concentration of the selected template based on the known amount of the reference template and the relative amounts of selected template and reference template.

A method of monitoring amplification of a selected template in a polymerase chain reaction that also comprises a positive control template comprises the steps of:

(a) in a reaction mixture comprising:

(i) effective amounts of each of a first pair of oligonucleotide primers configured for amplifying, in a polymerase chain reaction, a selected first segment of the selected template to result in an amplified first product thereof,

(ii) effective amounts of each of a second pair of oligonucleotide primers configured for amplifying, in a polymerase chain reaction, a selected second segment of the positive control template to result in an amplified second product thereof,

(iii) an effective amount of a nucleic-acid-binding fluorescent dye;

subjecting the selected template and the positive control template to conditions for amplifying the selected template and the positive control template by polymerase chain reaction; and

(b) illuminating the reaction mixture with a selected wavelength of light for eliciting fluorescence by the nucleic-acid-binding fluorescent dye and continuously monitoring the fluorescence emitted as a function of temperature during an amplification cycle of the polymerase chain reaction to result in a first melting peak of the amplified first product, if the selected template is amplified, and a second melting peak of the amplified second product, if the positive control template is amplified;

wherein obtaining of the second melting curve indicates that the polymerase chain reaction was operative, obtaining the first melting curve indicates that the selected first segment was amplifiable, and absence of the first melting curve indicates that the selected first segment was not amplifiable.

A method of detecting the factor V Leiden mutation in an individual, wherein the factor V Leiden mutation consists of a

single base change at the factor V Leiden mutation locus as compared to wild type, comprises the steps of:

(a) obtaining sample genomic DNA from the individual;

(b) providing wild type genomic DNA as a control;

(c) providing a pair of oligonucleotide primers configured for amplifying by polymerase chain reaction a selected segment of the sample genomic DNA and of the wild type genomic DNA wherein the selected segment comprises the factor V Leiden mutation locus to result in amplified products containing a copy of the factor V Leiden mutation locus;

(d) providing an oligonucleotide probe labeled with a resonance energy transfer donor or a resonance energy transfer acceptor of a fluorogenic resonance energy transfer pair, wherein the probe is configured for hybridizing to the amplified products such that the probe spans the mutation locus and exhibits a melting profile when the factor V Leiden mutation is present in the sample genomic DNA that is differentiable from a melting profile of the wild type genomic DNA;

(e) providing a transfer oligonucleotide labeled with the resonance energy transfer donor or the resonance energy transfer acceptor, with the proviso that as between the probe and transfer oligonucleotide one is labeled with the resonance energy transfer donor and the other is labeled with the resonance energy transfer acceptor, wherein the transfer oligonucleotide is configured for hybridizing to the amplified products such that the resonance energy transfer donor and the resonance energy transfer acceptor are in resonance energy transfer relationship when both the probe and the transfer oligonucleotide hybridize to the amplified products;

(f) amplifying the selected segment of sample genomic DNA and wild type genomic DNA by polymerase chain reaction in the presence of effective amounts of oligonucleotide probe and transfer oligonucleotide to result in amplified selected segments, at least a portion thereof having both the probe and the transfer oligonucleotide hybridized thereto with the fluorogenic resonance energy transfer pair in resonance energy transfer relationship;

(g) determining fluorescence as a function of temperature during an amplification cycle of the polymerase chain reaction to result in a melting profile of the probe melting from the amplified segment of sample genomic DNA and a melting profile of the probe melting from the amplified segment of wild type genomic DNA; and

(h) comparing the melting profile for the sample genomic DNA to the melting profile for the wild type genomic DNA, wherein a difference therein indicates the presence of the factor V Leiden mutation in the sample genomic DNA.

A method of analyzing nucleic acid hybridization comprises the steps of

(a) providing a mixture comprising a nucleic acid sample to be analyzed and a nucleic acid binding fluorescent entity; and

(b) monitoring fluorescence while changing temperature at a rate of $\geq$ 0.1°C/second.

A method of quantitating an initial copy number of a sample containing an unknown amount of nucleic acid comprises the steps of

(a) amplifying by polymerase chain reaction at least one standard of known concentration in a mixture comprising the standard and a nucleic acid binding fluorescent entity;

(b) measuring fluorescence as a function of cycle number to result in a set of data points;

(c) fitting the data points to a given predetermined equation describing fluorescence as a function of initial nucleic acid concentration and cycle number;

(d) amplifying the sample containing the unknown amount of nucleic acid in a mixture comprising the sample and the nucleic acid binding fluorescent entity and monitoring fluorescence thereof; and

(e) determining initial nucleic acid concentration from the equation determined in step (c).

A fluorescence resonance energy transfer pair is disclosed wherein the pair comprises a donor fluorophore having an emission spectrum and an acceptor fluorophore having an absorption spectrum and an extinction coefficient greater than 100,000 $M^{-1}cm^{-1}$, wherein the donor fluorophore's emission spectrum and the acceptor fluorophore's absorption spectrum overlap less than 25%. One illustrative fluorescence resonance energy transfer pair described is where the donor fluorophore is fluorescein and the acceptor fluorophore is Cy5 or Cy5.5.

A method for analyzing a target DNA sequence of a biological sample comprises

amplifying the target sequence by polymerase chain reaction in the presence of a nucleic acid binding fluorescent entity, said polymerase chain reaction comprising the steps of adding a thermostable polymerase and primers for the targeted nucleic acid sequence to the biological sample and thermally cycling

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the biological sample between at least a denaturation temperature and an elongation temperature;

exciting the sample with light at a wavelength absorbed by the nucleic acid binding fluorescent entity; and

monitoring the temperature dependent fluorescence from the nucleic acid binding fluorescent entity as temperature of the sample is changed. Preferably, the nucleic acid binding fluorescent entity comprises a double stranded nucleic acid binding fluorescent dye, such as SYBR™ Green I. The temperature dependent fluorescence can be used to identify the amplified products, preferably by melting curve analysis. Relative amounts fo two or more amplified products can be determined by analysis of melting curves. For example, areas under the melting curves are found by non-linear least squares regression of the sum of multiple gaussians.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Figures 1A&B are graphs representing an equilibrium PCR paradigm (A) and a kinetic PCR paradigm (B).

Figure 2 illustrates useful temperature v. time segments for fluorescence hybridization monitoring.

Figure 3 is a temperature v. time chart exemplary of rapid temperature cycling for PCR.

Figure 4 shows the results of four different temperature/time profiles (A-D) and their resultant amplification products after thirty cycles (inset).

Figures 5A, B & C illustrate the mechanism of fluorescence generation for three different methods of fluorescence monitoring of PCR: (A) double-stranded DNA dye, (B) hydrolysis probe, and (C) hybridization probes.

Figure 6 shows the chemical structure of the monovalent N-hydroxysuccinimide ester of Cy5™.

Figure 7 shows the chemical structure of the monovalent N-hydroxysuccinimide ester of Cy5.5™.

Figure 8 shows the emission spectrum of fluorescein (solid line) and the excitation spectrum of Cy5 (broken line).

Figure 9 shows resonance energy transfer occurring between fluorescein- and Cy5-labeled adjacent hybridization probes at each cycle during PCR.

Figure 10 shows the effect of varying the ratio of the Cy5 probe to the fluorescein probe on the resonance energy transfer signal generated during PCR.

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Figure 11 shows the effect of varying the probe concentration at a given probe ratio on the resonance energy transfer signal generated during PCR.

Figure 12 shows the effect of spacing between the labeled oligonucleotides on the resonance energy transfer signal generated during PCR.

Figure 13 shows the time course of adjacent probe hybridization by fluorescence energy transfer immediately after 30 cycles of amplification with Taq DNA polymerase (exo$^+$; solid line) and the Stoffel fragment of Taq DNA polymerase (exo$^-$; dotted line) of temperature cycling and the type of polymerase on fluorescence development during PCR with adjacent hybridization probes; temperature is shown with a bold line.

Figure 14 is a fluorescence ratio v. cycle number plot for amplification with Taq DNA polymerase (exo$^+$; solid line), Stoffel fragment of Taq DNA polymerase (exo$^-$; broken line), and KlenTaq DNA polymerase (exo$^-$; dotted line): top panel - cycling is between 94°C and 60°C with a 20 second hold at 60°C; middle panel - cycling is between 94°C and 60°C with a 120 second hold at 60°C; bottom panel - cycling is between 94°C and 60°C with a slow increase from 60°C to 75°C.

Figure 15 is a fluorescence v. cycle number plot for a number of different initial template copy reactions monitored with Sybr™ Green I: 0, (△); 1, (■); 10, (—); 10^2, (-); 10^3, (+); 10^4, (●); 10^5, (◇); 10^6, (×); 10^7, (▲); 10^8, (□); 10^9, (◆).

Figure 16 is a fluorescence ratio v. cycle number plot for a number of different initial template copy reactions monitored with a dual-labeled hydrolysis probe: 0, (-); 1, (▲); 10, (○); 10^2, (*)); 10^3, (●); 10^4, (□); 10^5, (+); 10^6, (■); 10^7, (◇); 10^8, (×); 10^9, (◆).

Figure 17 is a fluorescence ratio v. cycle number plot for a number of different initial template copy reactions monitored with adjacent hybridization probes: 0, (-); 1, (▲); 10, (○); 10^2, (*)); 10^3, (●); 10^4, (□); 10^5, (+); 10^6, (■); 10^7, (◇); 10^8, (×); 10^9, (◆).

Figure 18 is a fluorescence ratio v. cycle number plot distinguishing two hybridization probe designs monitored by resonance energy transfer: (◇) two hybridization probes labeled respectively with fluorescein and Cy5; and (◆)a primer labeled with Cy5 and a probe labeled with fluorescein.

Figures 19A-C provide a comparison of three fluorescence monitoring techniques for PCR, including the double-strand specific DNA dye SYBR Green I (A), a dual-labeled fluorescein/rhodamine

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hydrolysis probe (B), and a fluorescein-labeled hybridization probe with a Cy5-labeled primer (C); Figure 19D shows the coefficient of variation for the three monitoring techniques represented in Figures 19A-C.

Figure 20 shows a typical log fluorescence vs cycle number plot of a standard amplification monitored with SYBR Green I.

Figure 21 shows an expontial curve fit to cycles 20-27 of the data from Figure 20.

Figure 22 shows an exponential fit to an unknown to determine initial copy number from amplification data.

Figure 23 shows a typical fluorescence v. cycle number plot of five standards monitored each cycle with adjacent hybridization probes, wherein initial copy numbers are represented as follows: 10^3, (●); 10^4, (◇); 10^5, (▲); 10^6, (□); 10^7, (◆).

Figure 24 shows a curve fit to the standard data of Figure 23.

Figure 25 shows a typical fluorescence vs cycle number plot of five standards monitored each cycle with a hydrolysis probe, wherein initial copy numbers are represented as follows: 1.5, (●); 15, (◇); 150, (▲); 1500, (□); 15,000, (◆).

Figure 26 shows a curve fit to the standard data of Figure 25.

Figure 27 shows a typical log fluorescence vs cycle number plot of three standard amplifications monitored with SYBR Green I, wherein: (■); (●); (▲).

Figure 28 shows different curve fit to the standard data of Figure 27.

Figures 29A&B show plots of (A) time v. fluorescence and (B) time v. temperature demonstrating the inverse relationship between temperature and fluorescence.

Figure 30 is a chart showing 2D plots of temperature v. time, fluorescence v. time, and fluorescence v. temperature, also shown as a 3D plot, for the amplification of a 180 base pair fragment of the hepatitis B genome in the presence of SYBR Green I.

Figure 31 is a fluorescence v. temperature projection for the amplification of a 536 base pair fragment of the human beta-globin gene in the presence of SYBR Green I.

Figures 32A&B provide a plot showing (A) a linear change in fluorescence ratio with temperature for hydrolysis probes, and (B) a radical change with temperature for hybridization probes.

Figure 33 shows a fluorescence ratio v. temperature plot of amplification with an exo$^-$ polymerase in the presence of adjacent hybridization probes.

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Figure 34 shows a fluorescence ratio v. temperature plot of amplification with an exo$^+$ polymerase in the presence of adjacent hybridization probes.

Figure 35 shows a 3-dimensional plot of temperature, time and fluorescence during amplification with an exo$^-$ polymerase in the presence of adjacent hybridization probes.

Figure 36 shows a 3-dimensional plot of temperature, time, and fluorescence during amplification with an exo$^+$ polymerase in the presence of adjacent hybridization probes.

Figure 37 shows melting curves for PCR-amplified products of hepatitis B virus (●; 50% GC, 180 bp); beta-globin (▲; 53.2% GC, 536 bp); and prostate specific antigen (X; 60.3% GC, 292 bp).

Figure 38 shows melting curves for PCR-amplified product of hepatitis B virus at heating rates of 0.1°C to 5.0°C.

Figure 39 shows melting curves for PCR-amplified product of hepatitis B virus at various SYBR™ Green I concentrations.

Figures 40A&B show (A) melting curves and (B) electrophoretically fractionated bands of products of a beta-globin fragment amplified with (a) no added template, (b) 10^6 copies of added template under low stringency conditions, and (c) 10^6 copies of added template under higher stringency conditions.

Figures 41A&B show (A) melting curves and (B) melting peaks of hepatitis B virus fragment (HBV), β-globin, and a mixture thereof.

Figures 42A-D show (A) a relative fluorescence v. cycle number plot for PCR amplified products from various amounts of β-globin template, (B) melting peaks and (C) electrophoretic bands of the various products, and (D) corrected fluorescence of the data of (A).

Figures 43A&B show (A) melting curves and (B) melting peaks from PCR amplified products of a mixture of the cystic fibrosis gene and the c-erbB-2 oncogene.

Figure 44 show melting peaks at various cycle numbers for the cystic fibrosis gene (CFTR) and c-erbB-2 (neu).

Figure 45 shows a graph of integrated melting peaks of CFTR and neu PCT products.

Figures 46A&B show (A) melting curves and (B) melting peaks for PCR products of a person heterozygous for the factor V Leiden mutation (solid line), homozygous for the factor V Leiden mutation (dotted line), homozygous wild type (broken line), and no DNA control (alternating dot and dash).

Figure 47 shows a fluorescence ratio v. temperature plot of continuous monitoring during cycle 40 of PCR products of a sample

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homozygous for the factor V Leiden mutation (solid line), heterozygous for the factor V Leiden mutation (dotted line), and homozygous wild type (alternating dot and dash).

Figure 48 shows melting peaks of a homozygous mutant of the methylenetatrahydrofolate gene (solid line), homozygous wild type (broken line), heterozygous mutant (dotted line), and no DNA control (alternating dot and dash).

Figure 49 shows the shape of reannealing curves of amplified β-globin PCR products from various initial template amounts.

Figure 50 shows the determination of a second order rate constant for determining initial template concentration.

Figure 51 shows a block diagram for controlling thermal cycling from fluorescence data.

Figures 52A&B show (A) a temperature v. time plot acquired after 20 cycles and (B) a fluorescence v. time plot acquired after 25 cycles wherein thermal cycling was controlled from fluorescence data.

DETAILED DESCRIPTION

Before the present methods for monitoring hybridization during PCR are disclosed and described, it is to be understood that this invention is not limited to the particular configurations, process steps, and materials disclosed herein as such configurations, process steps, and materials may vary somewhat. It is also to be understood that the terminology employed herein is used for the purpose of describing particular embodiments only and is not intended to be limiting since the scope of the present invention will be limited only by the appended claims and equivalents thereof.

It must be noted that, as used in this specification and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise.

In describing and claiming the present invention, the following terminology will be used in accordance with the definitions set out below.

As used herein, "nucleic acid," "DNA," and similar terms also include nucleic acid analogs, i.e. analogs having other than a phosphodiester backbone. For example, the so-called "peptide nucleic acids," which are known in the art and have peptide bonds

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instead of phosphodiester bonds in the backbone, are considered within the scope of the present invention.

As used herein, "continuous monitoring" and similar terms refer to monitoring multiple times during a cycle of PCR, preferably during temperature transitions, and more preferably obtaining at least one data point in each temperature transition.

As used herein, "cycle-by-cycle" monitoring means monitoring the PCR reaction once each cycle, preferably during the annealing phase of PCR.

As used herein, "fluorescence resonance energy transfer relationship" and similar terms refer to adjacent hybridization of an oligonucleotide labeled with a donor fluorophore and another oligonucleotide labeled with an acceptor fluorophore to a target nucleic acid such that the donor fluorophore can transfer resonance energy to the acceptor fluorophore such that the acceptor fluorophore produces a measurable fluorescence emission. If the donor fluorophore and acceptor fluorophore are spaced apart by too great a distance, then the donor fluorophore cannot transfer resonance energy to the acceptor fluorophore such that the acceptor fluorophore emits measurable fluorescence, and hence the donor fluorophore and acceptor fluorophore are not in resonance energy transfer relationship. Preferably, when the two labeled oligonucleotides are both probes and neither functions as a PCR primer ("probe-probe"), then the donor and acceptor fluorophores are within about 0-25 nucleotides, more preferably within about 0-5 nucleotides, and most preferably within about 0-2 nucleotides. A particularly preferred spacing is 1 nucleotide. When one of the labeled oligonucleotides also functions as a PCR primer ("probe-primer"), then the donor and acceptor fluorophores are preferably within about 0-15 nucleotides and more preferably within about 4-6 nucleotides.

As used herein, "effective amount" means an amount sufficient to produce a selected effect. For example, an effective amount of PCR primers is an amount sufficient to amplify a segment of nucleic acid by PCR provided that a DNA polymerase, buffer, template, and other conditions, including temperature conditions, known in the art to be necessary for practicing PCR are also provided.

PCR requires repetitive template denaturation and primer annealing. These hybridization transitions are temperature-dependent. The temperature cycles of PCR that drive amplification alternately denature accumulating product at a high temperature and

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anneal primers to the product at a lower temperature. The transition temperatures of product denaturation and primer annealing depend primarily on GC content and length. If a probe is designed to hybridize internally to the PCR product, the melting temperature of the probe also depends on GC content, length, and degree of complementarity to the target. Fluorescence probes compatible with PCR can monitor hybridization during amplification.

In accordance with the present invention, which is preferably used in connection with rapid cycling (fully described in the above-mentioned U.S. Serial No. 08/658,993, filed June 4, 1996, entitled System And Method For Monitoring PCR Processes, and U.S. Serial No. 08/537,612, filed October 2, 1995, entitled Method For Rapid Thermal Cycling of Biological Samples), a kinetic paradigm for PCR is appropriate. Instead of thinking about PCR as three reactions (denaturation, annealing, extension) occurring at three different temperatures for three time periods (Figure 1A), a kinetic paradigm for PCR is more useful (Figure 1B). With a kinetic paradigm, the temperature vs. time curve consists of continuous transitions between overlapping reactions. Denaturation and annealing are so rapid that no holding time at a particular temperature is necessary. Extension occurs over a range of temperatures at varying rates. A complete analysis would require knowledge of all relevant rate constants over all temperatures. If rate constants of all reactions were known, a "physicochemical description of PCR" could be developed. Determining these rates would require precise sample temperature control and is greatly simplified by reaction monitoring during temperature cycling.

Figure 2 illustrates useful temperature v. time segments for fluorescence hybridization monitoring. Product melting curves are obtained during a slow temperature increase to denaturation. By quickly lowering the temperature after denaturation to a constant temperature, product, probe, or primer annealing can optionally be followed. Probe melting curves are obtained by slowly heating through temperatures around the probe Tm. The embodiment represented in Figure 2 provides all analysis during temperature cycling with immediate real time display. Fluorescent probes are included as part of the amplification solution for continuous monitoring of primer, probe, or product hybridization during temperature cycling.

The fluorescence hybridization techniques contained herein are based on rapid cycling, with its advantages in speed and specificity.

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A sample temperature profile during rapid cycle PCR is shown in Figure 3. Denaturation and annealing appear as temperature "spikes" on these figures, as opposed to the broad plateaus of conventional temperature cycling for PCR, e.g. Figure 1A. Rapid temperature cycling is contrasted to conventional temperature cycling in Figure 4, wherein it is shown that 30 cycles of amplification can be completed in 15 minutes and the resulting PCR products contain many fewer side products. Thus, with rapid cycling the required times for amplification are reduced approximately 10-fold, and specificity is improved.

Example 1

Figure 4 shows the results of four different temperature/time profiles (A-D) and their resultant amplification products after thirty cycles (A-D). The profiles A and B in Figure 4 were obtained using a prior art heating block device using a prior art microfuge tube. As can be seen in Figure 4, the transitions between temperatures are slow and many nonspecific bands are present in profiles A and B. Profile B shows improvement in eliminating some of the nonspecific bands (in contrast to profile A) by limiting the time each sample remains at each temperature, thus indicating that shorter times produce more desirable results.

Profiles C and D were obtained using a rapid temperature cycler. As can be seen in Figure 4, amplification is specific and, even though yield is maximal with 60-second elongation times (C), it is still entirely adequate with 10-second elongation times (D).

The optimal times and temperatures for the amplification of a 536 bp fragment of beta-globin from human genomic DNA were also determined. Amplification yields and product specificity were optimal when denaturation (93°C) and annealing (55°C) were less than 1 second. No advantage was found to longer denaturation or annealing times. The yield increased with longer elongation times at 77°C, but there was little change with elongation times longer than 10-20 seconds. These unexpected results indicate that the previously available devices used for DNA amplification are not maximizing the conditions needed to optimize the physical and enzymatic requirements of the reaction.

Further information can be obtained from: C.T. Wittwer et al., Rapid Cycle Allele-Specific Amplification with Cystic Fibrosis delta F(508) Locus, 39 Clinical Chemistry 804 (1993) and C.T. Wittwer et

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al., Rapid DNA Amplification, THE POLYMERASE CHAIN REACTION 174 (1994), which are both now incorporated herein by this reference. The instrumentation used for fluorescence acquisition and rapid temperature cycling is fully disclosed in Serial No. 08/537,612, *supra*.

As indicated earlier, the polymerase chain reaction can be performed rapidly. In addition to facilitating rapid heat transfer, the use of optically clear capillary tubes allows for continuous fluorescence monitoring of DNA amplification in accordance with the present invention.

Fluorescent probes can be used to detect and monitor DNA amplification. Useful probes include double-stranded-DNA-specific dyes and sequence-specific probes. Three different fluorescence techniques for following DNA amplification are compared in Figure 5. In Figure 5A, fluorescence depends on the hybridization of PCR product as detected with a double-strand-specific DNA dye. In figure 5B, fluorescence depends on the hydrolysis of a 5'-exonuclease quenching probe, which is well known in the art as discussed above. Figure 5C diagrams a hybridization scheme based on resonance energy transfer between fluorophores on two adjacent probes. The method of Figure 5A is not sequence specific, although product specificity can be determined by melting curves, one aspect of the current invention. Both Figures 5B and 5C are sequence specific. However, the hybridization method also allows analysis with melting curves, another aspect of the current invention.

In monitoring fluorescence from reactions involving hydrolysis probes as in Figure 5B and from reactions involving hybridization probes as in Figure 5C, it is advantageous to measure fluorescence emitted by both the donor fluorophore and the acceptor fluorophore. In practice, the majority of the fluorescence emitted by hydrolysis probes is from the donor fluorophore, and the majority of the fluorescence emitted by hybridization probes is from the acceptor fluorophore.

Double-strand-specific DNA dye selection. Those skilled in the art will be familiar with the use of ethidium bromide in fluorescence techniques. When a double strand-specific fluorescent dye is present during amplification, fluorescence generally increases as more double stranded product is made, see R. Higuchi et al., Simultaneous amplification and detection of specific DNA sequences, 10 Bio/Technology 413-417 (1992). A fluorescence PCR assay for

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hepatitis C RNA using the intercalater, YO-PRO-1 is also known in the art. See T. Ishiguro et al., Homogeneous quantitative assay of hepatitis C virus RNA by polymerase chain reaction in the presence of a fluorescent intercalater, 229 Anal. Biochem. 207-213 (1995). It is preferred that SYBR™ Green I, which is well known in the art and available from Molecular Probes of Eugene, Oregon, be used as a double-strand-specific dye. The molecular structure of this dye is a trade secret, but it is recommended by the manufacturer as a more sensitive double-strand-specific dye for DNA detection on gels. SYBR™ Green I is heat labile, however, and thus is not useful for fluorescence monitoring of PCR according to conventional methods where the temperature of the reaction mixture is maintained at melting temperatures for extended periods of time. Because of this heat lability, it was unexpected to discover that SYBR™ Green I can be used to monitor PCR reactions when melting temperatures are not maintained for extended periods, i.e. when PCR is carried out by rapid cycling according to the kinetic paradigm described above.

Example 2

Different double-strand-specific DNA dyes were compared by monitoring the amplification of a 110 base pair fragment from the PCO3/PCO4 primer pair of the human beta-globin gene from 10,000 template copies. Primers were synthesized by standard phosphoramidite chemistry as known in the art, namely, using Pharmacia Biotech Gene Assembler Plus (Piscataway, New Jersey). The human beta-globin primers PC03/PC04 (110 base pairs) are described in C.T. Wittwer et al., Automated polymerase chain reaction in capillary tubes with hot air, 17 Nucl. Acids. Res. 4353-4357 (1989), which is now incorporated herein by reference. DNA amplification was performed in 50 mM Tris, pH 8.5 (25°C), 3 mM $MgCl_2$, 500 μg/ml bovine serum albumin, 0.5 μM of each primer, 0.2 mM of each deoxynucleoside triphosphate and 0.2 U of Taq polymerase per 5 μl sample unless otherwise stated in the following examples. Purified amplification product was used as DNA template and was obtained by phenol/chloroform extraction and ethanol precipitation, see D.M. Wallace, Large- and small-scale phenol extractions and precipitation of nucleic acids, 152 Methods in Enzymology 33-48 (1987), followed by removal of primers by repeated washing through a Centricon 30 microconcentrator (Amicon, Danvers, Massachusetts). Template concentrations were determined by absorbance at 260 nm. A(260):A(280) ratios of templates were greater than 1.7.

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SYBR™ Green I (Molecular Probes, Eugene, Oregon) was used at a 1:10,000 dilution, ethidium bromide was at 5 μg/ml, and acridine orange was at 3 μg/ml. These concentrations were determined to be optimal concentrations for maximizing the fluorescence signal observed during amplification for each dye. Excitation was through a 450-490 nm interference filter from a xenon arc source, except for ethidium bromide, where a 520-550 nm excitation was used. For SYBR™ Green I, the emmision at 520-550 was monitored. Ethidium bromide fluorescence was observed through a 580-620 nm bandpass. The acridine orange signal was taken as the ratio of green (520-550 nm) to red (>610 nm) fluorescence. The fluorescence of the sample before amplification was compared to the fluorescence after 35 cycles (94°C max, 60°C for 20 sec) at 60°C. The fluorescence increase was 5.3-fold for SYBR™ Green I, 1.7-fold for ethidium bromide, and 1.2-fold for acridine orange. In separate experiments, the fluorescence from SYBR™ Green I was stable for greater than 30 min at 70°C. It is also conveniently excited with visable light and is claimed to be less of a mutagen than ethidium bromide. Background fluorescence in all cases arose primarily from the primers.

SYBR™ Green I is a preferred double-strand-specific dye for fluorescence monitoring of PCR, primarily because of superior sensitivity, arising from greater discrimination between double stranded and single stranded nucleic acid. SYBR™ Green I can be used in any amplification and is inexpensive. In addition, product specificity can be obtained by analysis of melting curves, as will be described momentarily.

Resonance energy transfer dye selection for hybridization probes. Fluorescence resonance energy transfer can occur between 2 fluorophores if they are in physical proximity and the emission spectrum of one fluorophore overlaps the excitation spectrum of the other. Introductory theory on fluorescence resonance energy transfer can be found in many recent review articles. The rate of resonance energy transfer is:

$(8.785E-5)(t^{-1})(k^2)(n^{-4})(q_D)(R^{-6})(J_{DA})$, where:

t = excited state lifetime of the donor in the absence of the acceptor;

k^2 = is an orientation factor between the donor and acceptor;

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n = refractive index of visible light in the intervening medium;

q_D = quantum efficiency of the donor in the absence of the acceptor;

R = distance between the donor and acceptor (in angstroms);

J_{DA} = the integral of $(F_D)(e_A)(W^4)$ with respect to W at all overlapping wavelengths with:

F_D = peak normalized fluorescence spectrum of the donor,

e_A = molar absorption coefficient of the acceptor ($M^{-1}cm^{-1}$), and

W = wavelength (nm).

For any given donor and acceptor, a distance where 50% resonance energy transfer occurs can be calculated and is abbreviated R_0. Because the rate of resonance energy transfer depends on the 6th power of the distance between donor and acceptor, resonance energy transfer changes rapidly as R varies from R_0. At $2R_0$, very little resonance energy transfer occurs, and at $0.5R_0$, the efficiency of transfer is nearly complete, unless other forms of de-excitation predominate (i.e., collisional quenching). R_0 values for many different donor and acceptor pairs have been compiled and vary between 22 and 72 angstroms.

In double helical DNA, 10 bases are separated by about 34 angstroms. By labeling the bases of DNA with donor and acceptor fluorophores, resonance energy transfer has be used as a spectroscopic ruler to observe the helical geometry of DNA and analyze the structure of a four-way DNA junction. Resonance energy transfer can also be used as a monitor of hybridization. If a labeled oligonucleotide is hybridized to a labeled template strand, R can be brought from much greater than R_0 to well below R_0, increasing resonance energy transfer dramatically. Alternately, 2 labeled probes can be hybridized to the same template strand for a similar change in fluorescence energy transfer.

The practical use of resonance energy transfer to monitor hybridization depends on the sensitivity required and how much time is available. Using a competitive hybridization technique with 1 nM labeled probes, PCR-amplified DNA was detected after 15 min at 40°C. Faster signal generation is desirable. If only seconds were required for hybridization, PCR products could conveniently be quantified each cycle of amplification. Even further, the extent of probe hybridization could be monitored within a temperature cycle.

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Hybridization is a second order process (see B. Young & M. Anderson, Quantitative analysis of solution hybridization, In: Nucleic Acid Hybridization: A Practical Approach 47-71, (B. Hames, S. Higgins eds., 1985). When the concentration of the probe is much greater than the concentration of the target, the hybridization rate is inversely proportional to concentration of probe. For example, by doubling the probe concentration, the hybridization time is cut in half. High probe concentrations would be necessary for cycle-by-cycle monitoring during PCR, because hybridization must occur before the hybridization site is covered by polymerase extension.

The high probe concentrations required for hybridization monitoring during PCR require a resonance energy transfer pair with unique characteristics. Consider excitation of a donor (D) and an acceptor (A) pair with light. The number of fluorophores of D and A directly excited will be proportional to the extinction coefficient (e) of each fluorophore at the excitation wavelength, or:

Number of D molecules directly excited = $(K)(e_D)$
Number of A molecules directly excited = $(K)(e_A)$

where K is a proportionality constant. De-excitation of the donor will occur by fluorescence, resonance energy transfer, and other processes summarized as thermal quenching. If p_F = probability of resonance energy transfer, and p_{TD} = probability of donor thermal quenching, then the probability of donor fluorescence is:

$$1-p_F-p_{TD}$$

and the number of fluorescing donor molecules is:

$$(K)(e_D)(1-p_F-p_{TD})$$

If the probability of detecting a donor emission in the donor emission window (for example, a bandpass filter window) is p_{DD}, then the number of observed donor emissions is:

$$(p_{DD})(K)(e_D)(1-p_F-p_{TD})$$

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Now, the number of excited acceptor fluorophores is the sum of those directly excited and those excited through resonance energy transfer:

$$(K)(e_A) + (K)(e_D)(p_F)$$

If p_{TA} = the probability of thermal quenching of the acceptor, then the probability of acceptor fluorescence is:

$$1-p_{TA}$$

and the number of fluorescing acceptor molecules is:

$$[(K)(e_A) + (K)(e_D)(p_F)]\ [1-(p_{TA})]$$

If the probability of detecting an acceptor emission in the acceptor emission window is p_{AA}, then the number of observed acceptor emissions is:

$$(p_{AA})[(K)(e_A) + (K)(e_D)(p_F)]\ [1-(p_{TA})]$$

Finally, if the probability of observing a donor emission in the acceptor emission window is p_{DA}, then the number of observed emissions (both D and A) in the acceptor emission window is:

$$(p_{AA})[(K)(e_A) + (K)(e_D)(p_F)]\ [1-(p_{TA})] + (p_{DA})(K)(e_D)(1-p_F-p_{TD})$$

Since fluorescence measurements are relative and K is present in all terms, if we remove K and rearrange, the observed intensity at the donor window is proportional to (donor excitation) - (energy transfer):

1) $(e_D)(p_{DD})(1-p_{TD}) - (e_D)(p_{DD})(p_F)$

and the observed intensity at the acceptor window is proportional to (acceptor excitation) + (energy transfer) + (donor emission in the acceptor window):

2) $(e_A)(p_{AA})(1-p_{TA}) + (e_D)(p_{DD})(p_F)(1-p_{TA}) + (e_D)(p_{DA})(1-p_{TD}-p_F)$

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As resonance energy transfer increases, the donor signal decreases and the acceptor signal increases. The percent signal change depends on the background light intensity in each window. With high concentrations of probes, this background light intensity is high. During PCR, when varying target (product) concentrations need to be monitored, it is not possible to match the donor concentration to the target concentration. The excess donor molecules contribute to the background light intensity in both the donor and acceptor windows and partially mask the energy transfer phenomena. Background light in the acceptor window comes from not only donor emission in the acceptor window, but also from direct excitation of the acceptor. This background can be minimized with a low e_A and a low p_{DA}.

The fluorescein/rhodamine fluorescence energy transfer pair, commonly used for nucleic acid detection, has high background fluorescence. Both direct acceptor excitation (e_A, ca. 10% e_{MAX}) and emission of the donor at wavelengths used to detect acceptor emission (p_{DA}, ca. 20% peak emission) are high. This pair can be used to monitor hybridization if the probe concentration is near to the target concentration and enough time is allowed for complete hybridization. It is not a useful pair of fluorophores for continuous monitoring of PCR because high probe concentrations are required and the template concentration in PCR is continually changing.

Monitoring product concentration during PCR by hybridization has not been possible in the past because an acceptable resonance energy transfer pair had not been found. There have been few attempts to use resonance energy transfer for direct "noncompetitive" detection of hybridization. For example, U.S. Patent No. 5,565,322 states "the observed energy transfer efficiency in terms of re-emission by the acceptor was relatively low." At probe concentrations that are high enough for significant hybridization to occur in seconds, the background fluorescence is too high.

Fluorescein is perhaps the most widely used fluorophore. Its extinction coefficient and quantum efficiency are high and it is extensively used in microscopy, immunoassays, and flow cytometry. It is the donor in a commonly used resonance energy transfer pair with rhodamine. Cy5 is a popular red-emitting fluorophore with a very high extinction coefficient. The structure of the N-hydroxysuccinimide ester of Cy5 is shown in Figure 6, and the structure of the related dye, Cy5.5, is shown in Figure 7. These

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dyes are indodicarbocyanine dyes that are used commonly in flow cytometry and automated fluorescence sequencers and are available from Amersham (Pittsburg, PA). Both fluorescein and Cy5 are commercially available as amidites for direct, automated incorporation into oligonucleotides. However, Cy5 has never been reported as a resonance energy transfer pair with fluorescein. Intuitively, fluorescein emission and Cy5 absorption do not overlap enough for resonance energy transfer to be considered. The emission spectrum of fluorescein and absorption spectrum of Cy5 attached to oligonucleotides are shown in Figure 8. When the areas under the curves are normalized, the overlap from the technical spectra is 19%. Cy5.5 excitation is shifted to the red by about 25 nm, further decreasing the overlap with fluorescein emission to about 15%. Working in the red/infrared region of the spectrum is advantageous when choosing optical components for instrumentation. Laser diodes can be used for illumination, photodiode detectors have excellent sensitivity, and most materials have minimal autofluorescence in the pertinent spectral region.

Despite low spectral overlap, it has been discovered that fluorescein and either Cy5 or Cy5.5 make an excellent resonance energy transfer pair for hybridization monitoring during PCR.

Example 3

A 110 bp beta-globin fragment was amplified from 50 ng human genomic DNA according to the procedure of Example 2 with the internal probes CAAACAGACA CCATGGTGCA CCTGACTCCT GAGGA-fluorescein (SEQ ID NO:3) and Cy5-GAAGTCTGCC GTTACTGCCC TGTGGGGCAA G-p (SEQ ID NO:18) at 0.2 μM each and 0.8 U KlenTaq1 polymerase (a 5'-exonuclease deficient variant of Taq polymerase - U.S. Patent No. 5,436, 149) in a 10 μl reaction. The probes hybridized internal to the primers on the same strand and were immediately adjacent without any intervening bases.

Probes and primers were synthesized by standard phosphoramidite chemistry as known in the art, using a Pharmacia Biotech Gene Assembler Plus (Piscataway, New Jersey). The 3'- fluorescein-labeled probe was synthesized on a fluorescein-labeled CPG cassette (Glen Research, Sterling, VA) with the final trityl-ON to assist with C18 reverse phase HPLC purification. The late eluting peak was collected and the trityl group was removed on a PolyPack (Glen Research). The fluorescein-labeled oligo was eluted with 50% acetonitrile and again purified by C18 reverse phase HPLC. The 5'-Cy5-labeled probe was synthesized with a chemical phosphorylation agent on the 3'-end (Glen

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Research) and adding a Cy5 amidite (Pharmacia) to the 5'-end during trityl-OFF synthesis. Failure sequences were removed by C18 reverse phase HPLC. Probe purity was checked with polyacrylamide electrophoresis and the absorbance of the dye and the oligo.

HPLC was perfomed on a 4 x 250 mm Hypersil ODS column (Hewlett Packard) with a 0.1 M triethanolamine:acetate mobile phase and an acetonitrile gradient at 1 ml/min. The eluate was monitored for both absorbance (A_{260}) and fluorescence (490 nm excitation, 520 nm emission for fluorescein and 650 nm excitation, 670 nm emission for Cy5). Tritylated- and fluorescein-labeled oligonucleotides were eluted with a 10-20% acetonitrile gradient, and Cy5-labeled oligonucleotides eluted over a 10-40% acetonitrile gradient.

Temperature cycling was 94°C for 0 sec with a programmed approach rate of 20°C/sec, 60°C for 20 sec with an approach rate of 20°C/sec, and 75°C for 0 sec with an approach rate of 1°C/sec in a capillary fluorescence rapid temperature cycler. During temperature cycling, fluorescein and Cy5 fluorescence were acquired each cycle at the end of the annealing/extension segment. Resonance energy transfer was observed as both a decrease in fluorescein fluorescence, and an increase in Cy5 fluorescence beginning around cycle 26 of amplification (Figure 9). In general, observing the fluorescence ratio of Cy5 to fluorescein fluorescence is perferred.

The unexpectedly good results with the fluorescein/Cy5 pair can at least partly be rationalized. The overlap integral, J_{DA} depends not only on spectral overlap, but also on the extinction coefficient of the acceptor (Cy5 has an extinction coefficient of 250,000 $M^{-1}cm^{-1}$ at 650 nm), and on the 4th power of the wavelength. Both of these factors will favor a high J_{DA} for Cy5, even given low spectral overlap. Recently, phycoerythrin and Cy7 were shown to be an effective tandem probe for immunofluorescence, despite low spectral overlap. In a later example, the utility of fluorescein and Cy5.5 as labels on hybridization probes is demonstrated. Fluorescence resonance energy transfer can be used to monitor nucleic acid hybridization even when the interacting dyes have low spectral overlap. The use of fluorescein with Cy5, Cy5.5 and other red or infrared emitting dyes as resonance energy transfer pairs for monitoring hybridization has not been previously recognized. Fluorescein has a long emission "tail" that goes out to 600 nm, 700 nm and beyond that can be used to excite these far red and infrared dyes. The rate of energy transfer is dependent on the overlap

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integral, but is also effected by the 6th power of the distance between the fluorophores. If the probes are designed so that the resonance energy transfer dyes are in close proximity, the transfer rate is high. At least with fluorescein/Cy5, fluorescein/Cy5.5 and like pairs, resonance energy transfer appears to predominate over collisional quenching and other forms of energy loss when the fluorophores are close together, as in the above example where the fluorophores are attached to adjacent probes with no intervening bases.

The potential usefulness of a resonance energy transfer pair for hybridization probes can be judged by observing the change in the ratio of light intensity in the donor and acceptor windows at minimal and maximal resonance energy transfer. One way to obtain minimal and maximal transfer is to attach both fluorophores to the same oligonucleotide and measure fluorescence ratio before and after digestion with phospodiesterase.

Example 4

The dual-labeled fluorescein/Cy5 probe Cy5-CTGCCG-F-TACT GCCCTGTGGG GCAAGGp (SEQ ID NO:19) was synthesized by standard phosphoramidite chemistry, where p is a terminal 3'-phosphate (chemical phosphorylation reagent, Glen Research), F is a fluorescein residue introduced as an amidite with a 2-aminobutyl-1,3-propanediol backbone to maintain the natural 3-carbon internucleotide phosphodiester distance (ClonTech, Palo Alto, CA), and Cy5 is added as the amidite (Pharmacia). The ratio of Cy5 to fluorescein fluorescence in 0.1 M Tris, pH 8.0 was obtained before and after exhastive hydrolysis with phosphodiesterase (Sigma, St. Louis, MO). The change in the fluorescence ratio was 220-fold after hydrolysis. A dual-labeled fluorescein/rhodamine probe F-ATGCCCT*CCC CCATGCCATC CTGCGTp (SEQ ID NO:20) was purchased from Perkin Elmer (Foster City, CA), where F is fluorescein and * is a rhodamine attached to a modified T residue by an amino-linker arm. The change in the fluorescence ratio (rhodamine to fluorescein) was 22-fold after hydrolysis with phosphodiesterase.

The potential signal from the fluorescein/Cy5 pair was 10-fold that of the fluorescein/rhodamine pair.

Example 5

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The effect of the ratio, concentration, and spacing of fluorescein and Cy5-labeled adjacent hybridization probes during PCR was studied. Amplification of the beta globin locus and probe pair of Example 3 was used and the maximum change in the fluorescence ratio of Cy5 to fluorescein was observed. The maximal signal occurred when the ratio of Cy5 to fluorescein-labeled probes was 2:1 (Figure 10). At this 2:1 ratio, the best signal occurred at a fluorescein probe concentration of 0.2 μM and a Cy5-labeled probe concentration of 0.4 μM (Figure 11). The optimal number of intervening bases between adjacent hybridization probes during PCR was also determined. Several probes of the same length but slightly shifted in their hybridization position were synthesized according to Example 3 so that when they hybridized to the beta globin target, 0, 1, 2, 3, 4, or 6 bases remained between the probes. The highest signal during PCR occurred with one intervening base (Figure 12). Although some resonance energy transfer was detected at a spacing of 15 and even 25 bases, much better transfer occurred at 0-5 bases.

Heller et al. (U.S, Patent No. 4,996,143), found that energy transfer efficiency decreased as the number of nucleotides between fluorophores decreased from 4 to 0 units. In contrast, the best energy transfer with the fluorescein/Cy5 pair was seen at 0 to 2 intervening nucleotides.

Hybridization probe method. If 2 probes are synthesized that hybridize adjacently on a target and each is labeled with one fluorophore of a resonance energy transfer pair, the resonance energy transfer increases when hybridization occurs (Figure 5C). The fluorescein/rhodamine pair is most commonly used for nucleic acid detection.

One aspect of this invention is to provide a sequence-specific homogeneous hybridization method for detection of PCR products. It is not obvious how to achieve this. Using hybridization probes during amplification is counterintuitive. It does not seem that both probe hybridization and polymerase extension can occur. To get sequence specific fluorescence, the probes must be hybridized, but the probes cannot be hybridized if the polymerase is to complete primer extension and exponentially amplify DNA.

One solution to this problem is to use a dual-labeled single probe and utilize the 5'-exonuclease activity of common heat stable DNA polymerases to cleave the probe during extension, thereby

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separating the 2 fluorophores. In this case, the fluorescence signal arises from separation of the resonance energy transfer pair upon probe hydrolysis (Figure 5B), rather than approximation of the fluorophores by adjacent hybridization (Figure 5C). However, dual-labeled probes are difficult to make, requiring manual addition of at least one fluorophore to the oligo and usually require extensive purification. The probes are expensive, and two dual-labeled probes are necessary for competitive quantification of a target or for mutation detection. A further concern is that the observed fluorescence depends on the cumulative amount of probe hydrolyzed, not directly on the amount of product present at any given cycle. This results in a continued increase in fluorescence even after the PCR plateau has been reached. Finally and most importantly, probe hydrolysis does not always occur during polymerase extension, an effect that is not well understood. For example, the dual-labeled fluorescein/Cy5 probe of Example 4 showed very poor hydrolysis during PCR when it was flanked by primers. Indeed, several dual-labeled fluorescein/Cy5 probes, including those with terminal labels, were made and all showed poor hydrolysis and signal generation during amplification.

Homogeneous detection of PCR products with adjacent hybridization probes would solve many of the problems of the 5'-exonuclease system. Synthesis of adjacent hybridization probes is relatively simple because amidites for both fluorescein and Cy5 are available for direct incorporation during automated synthesis and dual labeling of one probe is not required. Because their fluorescence results from hybridization, not hydrolysis, the temperature dependence of probe fluorescence could be used for mutation detection and quantification. However, use of adjacent hybridization probes for homogeneous detection of PCR products has not been reported previously. Surprisingly, both hybridization for signal generation and amplification by polymerase extension through the area blocked by the probes can occur.

Example 6

A 110 bp beta-globin fragment was amplified from genomic DNA with adjacent fluorescein- and Cy5-labeled probes as described in Example 3. Either 0.4 U (Taq) or 0.8 U (Stoffel fragment, Perkin Elmer, or KlenTaq1) of enzyme was used in 10 μl reactions. Unless indicated otherwise, temperature cycling was 94°C for 0 sec with a programmed approach rate of 20°C/sec, 60°C for 20 sec with an

approach rate of 20°C/sec, and 75°C for 0 sec with an approach rate of 1°C/sec. Figure 13 shows the development of fluorescence by 2 adjacent hybridization probes immediately after the template was amplified for 30 cycles. After a brief denaturation at 94°C, the temperature was lowered to 60°C and fluorescence increased for about 20 sec. The magnitude of the signal is greater with an exonuclease deficient polymerase (Stoffel fragment) than with native Taq polymerase that includes a 5'-exonuclease activity. After about 20 sec., the fluorescence drops as the polymerase displaces and/or hydrolyzes the probes. The relative decrease in fluorescence is slightly faster when the polymerase has 5'-exonuclease activity (Taq DNA polymerase) then when it lacks this activity (Stoffel fragment).

In Figure 14 (top panel), the temperature is cycled between 94°C and 60°C with a 20 sec hold at 60°C. Fluorescence is acquired at the end of the 20 sec when fluorescence is maximal. Good amplification occurs with Taq (exo$^+$), but not with Stoffel fragment (exo$^-$) as verified by both fluorescence development and agarose gels (gels not shown). However, if the time at 60°C is increased from 20 sec to 120 sec (Figure 14, middle panel), the exo$^-$ polymerase amplifies well. The slower rate of probe displacement with an exo$^-$ polymerase apparently requires more time at 60°C for efficient amplification than the exo$^+$ polymerase. The time required by exo$^-$ polymerases can be reduced by slowly increasing the temperature from 60°C to 75°C (Figure 14, bottom panel). The polymerase stalls when it reaches the probe. However, at the probe melting temperatures, the probes melt off the template and the polymerase continues unencumbered to complete polymerization of the strand. Polymerization is completed as long as the temperature is not raised too quickly after probe melting. Figure 14 (bottom panel) shows one exo$^+$ polymerase (Taq) and two exo$^-$ polymerases (Stoffel fragment and KlenTaq1).

When exonuclease activity is present, some of the probe is hydrolyzed each cycle as evidenced by an the decrease in fluorescence with extensive amplification. This is observed in Figures 13 and 14 (middle and bottom panels), but is does not occur with exo$^-$ polymerases. Because the fluorescence is stable on extensive amplification, exo$^-$ polymerases such as KlenTaq1 are preferred.

The success of using adjacent hybridization probes to monitor PCR depends on several factors. Resonance energy transfer is maximized when there is either 0 to 2 intervening bases between

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adjacent hybridization probes. To increase the fraction of strands that hybridize to the probes before the primer extends through the area of probe hybridization, the probe melting temperatures should be greater than the primer melting temperatures (preferably >5°C).

Cycle-by-cycle fluorescence. Conventional endpoint analysis of DNA amplification by gel electrophoresis identifies product size and estimates purity. However, because amplification is at first stochastic, then exponential, and finally stagnant, the utility of endpoint analysis is limited for quantification. One aspect of the present invention includes cycle-by-cycle monitoring for quantification of initial template copy number with hybridization probes. As will be appreciated by those skilled in the art, once-per-cycle monitoring of multiple samples undergoing DNA amplification is a powerful quantitative tool. Cycle-by-cycle monitoring is achieved by acquiring fluorescence during the extension or combined annealing/extension phase of each cycle and relating the fluorescence to product concentration.

Example 7

Cycle-by-cycle monitoring of PCR was performed by three different fluorescence techniques. Fluorescence was monitored by (i) the double-strand-specific dye SYBR™ Green I, (ii) a decrease in fluorescein quenching by rhodamine after exonuclease cleavage of a dual-labeled hydrolysis probe and (iii) resonance energy transfer of fluorescein to Cy5 by adjacent hybridization probes. Amplification reagents and conditions were as described in Example 2. The human beta-globin primers RS42/KM29 (536 base pairs) and PC03/PC04 (110 base pairs) are described in C.T. Wittwer et al., Automated polymerase chain reaction in capillary tubes with hot air, 17 Nucl. Acids. Res. 4353-4357 (1989), which is now incorporated herein by reference. Temperature cycling for beta-globin was 95°C maximum, 61°C minimum, 15 sec at 72°C and an average rate between temperatures of 5.2°C/sec. The beta-actin primers and fluorescein/rhodamine dual probe were obtained from Perkin Elmer (no. N808-0230). Temperature cycling for beta-actin was 94°C maximum, 60°C for 15 sec with an average rate between temperatures of 6.2°C/sec. The single labeled probes 5'-CAAACAGACA CCATGGTGCA CCTGACTCCT GAGGA-fluorescein-3' (SEQ ID NO:3) and 5'-Cy5-AAGTCTGCCG TTACTGCCCT GTGGGGCAAGp (SEQ ID NO:4) were synthesized as in Example 3. These adjacent probes hybridize internal to the PC03/PC04 beta-globin primer pair on the same DNA

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strand and are separated by one base pair. Temperature cycling was 94°C maximum, 59°C for 20 sec with an average rate between temperatures of 7.0°C/sec. Hybridization probes (beta-actin and beta-globin) were used at 0.2 μM each.

When multiple samples are monitored once each cycle with SYBR™ Green I, a 10^7-10^8 range of initial template concentration can be discerned as represented in Figure 15. This amplification is of a 536 base pair fragment of the beta-globin gene, with SYBR™ Green I as the double-strand specific dye. When the data were normalized as the percent maximal fluorescence of each sample, one hundred initial copies were clearly separated from ten copies. However, the difference between one and ten copies was marginal, and no difference was observed between zero and one average copies per sample.

In contrast, sequence-specific probes have a similar dynamic range but, appear to discriminate even a single initial template copy from negative controls. Signal generation with 5'-exonuclease probes (beta-actin fragment, Figure 16) is dependent not only on DNA synthesis, but requires hybridization and hydrolysis between the fluorophores of the dual-labeled probe. This hydrolysis reduces quenching and the fluorescence ratio of fluorescein to rhodamine emission increases. Whereas the fluorescence from double strand dyes levels off with excess cycling (Figure 15), the signal from exonuclease probes continues to increase with each cycle (Figure 16). Even though no net product is being synthesized, probe hybridization and hydrolysis continue to occur. As the template copy number decreases below 10^3, signal intensity decreases, but low copy numbers can still be quantified because the negative control signal is stable.

In Figure 17, amplification is monitored using adjacent hybridization probes and is expressed as a ratio of Cy5 to fluorescein fluorescence. The change in fluorescence ratio is largely due to an increase in Cy5 fluorescence from resonance energy transfer (Figure 9). In contrast to dual-labeled hydrolysis probes, the fluorescence signal of hybridization probes decreases at high cycle numbers if the polymerase contains an exonuclease activity (see also Figure 14).

The present invention's feasibility using two different methods for resonance energy transfer detection of hybridization during PCR will now be demonstrated. The first method uses two adjacent hybridization probes, one labeled 3' with fluorescein and the other

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labeled 5' with Cy5. As product accumulates during PCR, the probes hybridize next to each other during the annealing segment of each cycle. The second method uses a primer labeled with Cy5 and a single hybridization probe. The labeled primer is incorporated into the PCR product during amplification and only a single hybridization is necessary.

Example 8

Cycle-by-cycle monitoring of PCR was performed by resonance energy transfer between a Cy5-labeled primer and a fluorescein-labeled hybridization probe. This was compared to monitoring with adjacent Cy5/fluorescein hybridization probes. The Cy5-labeled primer was CAACTTCATC CACGT*TCACC (SEQ ID NO:21) where T* is a modified T base with Cy5 attached and the corresponding probe was GTCTGCCGTT ACTGCCCTGT GGGGCAA-fluorescein (SEQ ID NO:22). The adjacent hybridization probes were CCTCAAACAG ACACCATGGT GCACCTGACT CC-fluorescein (SEQ ID NO:23) and Cy5-GAAGTCTGCC GTTACTGCCC TGTGGGGCAAp (SEQ ID NO:24). The hybridizaton probes were synthesized according to Example 3 and used at 0.2 μM. The Cy5-labeled primer was synthesized in two steps. Automated synthesis was used to incorporate an amino-modifier C6dT (Glen Research) at the desired T position. Then, the monovalent N-hydroxysuccinimide ester of Cy5 (Figure 6) was manually conjugated to the amino linker according to the manufacturer's instructions (Amersham). HPLC purification was as described in Example 3.

The Cy5-labeled primer (0.5 μM) was used instead of PCO4 to amplify the 110 base pair beta-globin fragent from human genomic DNA as in Example 3, except that 0.4 U of Taq polymerase was used per 10 μl. The adjacent hybridization probes also monitored amplification of the same beta-globin fragment. Temperature cycling was done at 94°C for 0 sec and 60°C for 20 sec. The fluorescence was monitored once each cycle at the end of the annealing/extension segment. In both methods, fluorescence energy transfer to Cy5 increases with hybridization and is plotted as a ratio of Cy5 to fluorescein fluorescence (Figure 18).

In additional experiments, the number of bases separating the Cy5-label and the fluorescein label were varied. The best fluorescence resonance energy transfer was observed with about 4-6 bases between the fluorophores, although a signal was detectable up to at least 15 bases.

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In contrast to hydrolysis probes, the fluorescence signal from hybridization probes is not cumulative and develops anew during each annealing phase. The fluorescence is a direct measure of product concentration because the hybridization is a pseudo-first order reaction. Because the concentration of probe is much greater than the product, the fraction of product hybridized to probe is independent of product concentration. These characteristics indicate that using a single hybridization probe along with a labeled primer will provide a superior monitor of product accumulation for quantification. The inherent variance of different fluorescence techniques during cycle-by-cycle monitoring is also important for quantification.

Example 9

DNA amplification was perfomed according to Example 2 for each of three different fluorescence monitoring methods. SYBR™ Green I was used at a 1:10,000 dilution in the amplification of a 205 base pair human beta-globin fragment from primers KM29 and PC04. The hydrolysis probe and conditions are those specified in Example 7. The hybridization probe, TCTGCCGTTA CTGCCCTGTG GGGCAAG-fluorescein (SEQ ID NO:5) was used with KM29 and the Cy5-labeled primer CAACTTCATCCACGTT*CACC (SEQ ID NO:6) where T* was a Cy5-labeled T base synthesized as in example 8. All amplifications were performed in ten replicates with 15,000 template copies (50 ng of human genomic DNA/10 μl). The temperature cycles were 31 sec long (94°C maximum, 60°C for 20 sec, average rate between temperatures 6.2°C/sec). Fluorescence was acquired for each sample between seconds 15 and 20 of the annealing/extension phase.

Figure 19 allows comparison of three fluorescence monitoring techniques for PCR. The fluorescence probes are the dsDNA dye SYBR™ Green I (Figure 19A), a dual-labeled fluorescein/rhodamine hydrolysis probe (Figure 19B), and a fluorescein-labeled hybridization probe with a Cy5-labeled primer (Figure 19C). All probes had nearly the same sensitivity with detectable fluorescence occurring around cycle 20. With extended amplification, the signal continued to increase with the hydrolysis probe, was level with SYBR™ Green I, and slightly decreased with the hybridization probe. The precision of the three fluorescence monitoring techniques are compared in Figure

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19D. The mean +/- standard deviations are plotted for each point. The data are plotted as the coefficient of variation (standard deviation/mean) of the fluorescence ratio above baseline (taken as the average of cycles 11-15).

Although the change in fluorescence ratio from the hydrolysis probe is greater than that from the hybridization probe (Figures 19B and 19C), the coefficient of variation of fluorescence from the hydrolysis probes is greater (Figure 19D). That is, the fluorescence resulting from the hybridization probe method is more precise than using a hydrolysis probe, even though the absolute signal levels are lower. This is an unexpected advantage of hybridization probes over the more usual dual-labeled hydrolysis probes.

Quantification of initial template copy number. Quantitative PCR has become an important technique in both biomedical research and in the clinical laboratory. The process of quantification often includes running a standard curve of samples containing known copy numbers of the target sequence. The copy number of an unknown sample is determined by extrapolation between the known values. When a complete amplification curve is monitored cycle-by-cycle using fluorescence, radioactivity or any other method that gives a signal proportional to the amount of DNA present, many data points are available for analysis and it is not obvious which value to choose to represent a standard or unknown.. Prior art is to choose a "threshold value" of the signal and then use the cycle number when the standard or unknown crosses that threshold as the representative value (see Higuchi & Watson, EPA 0 640 828 A1). This approach uses a very small amount of the available data in an amplification curve. In addition, the assignment of the threshold value is highly subjective and is subject to conscious or unconscious bias. More of the available data could be used objectively by applying non-linear curve fitting techniques to the data in an amplification curve. Preferably, equations could be found that describe the shape of the amplification curves by modeling factors of the underlying process.

A number of different equations could be used to fit the data produced during amplification. DNA amplifications typically have a log linear segment and the data in this segment can be fit to an equation that describes an exponential increase like that expected

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in a DNA amplification. The log-linear portion of a DNA amplification can be described by the equation:

$$y = A*[DNA]*(1+E)^n$$

wherein A is a scaling factor that converts units of signal to units of DNA; [DNA] is the starting concentration of DNA in the reaction; E is the efficiency of the reaction; and n is the cycle number.

A quantification process would involve: (1) fitting the known standards to this equation allowing the parameters A and E to float, and (2) fitting the unknown samples to the equation using the values of A and E from the standards and allowing [DNA] to float. This technique uses much more of the data and uses the portion of the data, the log-linear portion, that is likely to be most informative. Figures 20, 21 and 22 show an example of this approach. Ten-fold dilutions of a purified PCR product were amplified as a standard curve and an "unknown" human genomic DNA standard was used. Figure 20 shows that the log-linear portion is easily identified either by the user or by software. Figure 21 shows a fit of the equation $y=A*[DNA]*(1+E)^n$ to the 10^4 copy standard. Figure 22 uses average values from several standards for A and E and fits [DNA]. The fit value of 16,700 is very close to the theoretical value for a single copy gene in genomic DNA (15,000 copies).

Using all the data in an amplification curve would include the background level and the plateau value. While at high copy number the plateau is uninformative, at low copy number it is often proportional to starting copy number. The background level could be useful in determining the first point that shows a significant increase in signal. At this time all the factors involved in the shape of the DNA amplification curve are not known, so one approach is to describe the shape of the curve. Figure 23 shows amplification curves using fluorescent hybridization probes to detect a five order of magnitude range of DNA template concentrations. Each curve is fit to the equation:

$$y=((as*x+ab)-(ds*x+db))/(1+(x/c)^b)+(ds*x+db)$$

wherein "as" is the background of the slope line, "ab" is the y intercept of the background line, "ds" is the slope of the plateau line, "db" is the y intercept of the slope line, "c" is cycle number where the reaction is half way from background to plateau (A_{50}), and "b" is the slope of the log-linear portion of the amplification.

This equation gives good fits to this amplification data, and Figure 24 shows that the value of the A_{50} correlates well with the log of the starting copy number across seven orders of magnitude. Figure

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25 shows the same equation fit to data from amplifications that used a hydrolysis probe to detect DNA template over a 5 order of magnitude range. This equation gives good fits to this amplification data, and Figure 26 shows that the value of the A_{50} correlates well with the log of the starting copy number. This demonstrates the flexibility of the full curve fit approach as the equation has given good fits to both the sharp plateaus of the hybridization probe amplification curves and the steadily increaseing "plateaus" of the hydrolysis probe curves.

Total curve fits are not limited to this equation. Figure 27 shows an amplification of three concentrations of DNA template fit to the equation:

y=(((as*x+ab)-(dmax*x/dd+x)) / (1+(x/c)^b))+(dmax*x/dd+x), which is similar to the first 6 parameter equations except that the plateau is defined by a hyperbolic curve rather than by a line. Figure 28 shows that the A_{50} for this equation correlates well to the starting copy number.

While the A_{50} has been used in these examples and level between the background and the plateau could be chosen if a particular technique is more robust lower or higher in the amplification profile. For example a series of amplification standard curves are evaluated for the best correlation between the starting copy number and the A_{50}, the A_{40}, the A_{30}, the A_{20} and the A_{10}. The level of amplification that best correlates with the known starting copy number is determined. This will be different for different detections systems. Figure 19 shows that coefficient of variation for various detection systems. The level of amplification that is the best predictor is likely to be the level with the lowest coefficient of variation.

As the DNA amplification reaction itself is better understood, other equations that have parameters that reflect physical processes could be used. The plateau of the DNA amplification curve has different causes in different reactions. It is often due to the inability of the primers to compete with product reannealing in the latter cycles. This effect could be captured with a parameter that is dependent on the square of the concentration of product in the reaction (as reannealing rate is proportional to the square of the product concentration). Another cause of the plateau can be the depletion of the primers. Primer limited reactions have a characteristic shape, they have a very sharp plateau that can be recognized. Primer limited reaction fits will include parameters

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that define this sharp top. Enzyme limited reactions have a very rounded plateau that can be fit accordingly. Weighting factors can be devised that reflect the known coefficients of variation for the given system to more heavily weight the more reliable data points. By fitting more of the points in an amplification profile, more accurate and robust estimates of starting copy number can be obtained. One or more of the parameters of these fits can be used to estimate the starting copy number of unknown samples.

Continuous fluorescence monitoring of PCR. The present invention's feature of continuous monitoring, that is, monitoring many times within each PCR cycle, will now be discussed. While fluorescence monitoring during PCR can be done once each cycle at a constant temperature, the present invention provides the important advantage of providing continuous monitoring throughout the PCR cycle. Temperature is important because fluorescence changes as the temperature changes. Figures 29A&B demonstrate the inverse relationship between temperature and fluorescence for SYB™ Green I. This is a confounding effect during temperature cycling that is usually eliminated by considering fluorescence once per cycle at a constant extension temperature. However, in accordance with the present invention, monitoring fluorescence during temperature changes is very informative. Prior to the present invention, continuous fluorescence monitoring within each cycle, as opposed to once each cycle, has not been carried out. In accordance with the present invention, time, temperature and fluorescence is acquired every sec, every 200 msec, every 100 msec or even at a greater frequency. Such data can reveal fine details of product denaturation, reannealing and extension, and probe annealing and melting during rapid cycling not available in previously available methods.

Example 10

A 180-base-pair fragment of the hepatitis B surface antigen gene was amplified from 10^6 copies of purified PCR product using primers 5'-CGTGGTGGAC TTCTCTCAAT-3' (SEQ ID NO:1), and 5'-AGAAGATGAG GCATAGCAGC-3' (SEQ ID NO:2)(Genbank sequence HVHEPB). The amplification conditions of Example 2 were followed except that the reaction contained a 1:20,000 dilution of SYBR™ Green I and 2 mM $MgCl_2$. Each temperature cycle was 27 sec long (92°C maximum, 59°C minimum, 5 sec at 70°C, average rate between temperatures 3.0°C/sec). Time, temperature, and 2 channels of fluorescence were acquired every 200 msec and continuously displayed as fluorescence v. cycle number

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and fluorescence v. temperature plots. Figure 30 shows a 3D trace of temperature, time and fluorescence for cycles 10 through 34. This 3D curve is also projected in Figure 30 as 2D plots of temperature v. time, fluorescence v. time, and fluorescence v. temperature. The temperature v. time projection of Figure 30 repeats each cycle and provides essentially the same information as set forth in Figure 3. Because fluorescence varies inversely with temperature, the fluorescence v. time projection shown in Figure 30 at early cycles is a scaled mirror image of the temperature v. time plot (see Figure 29). As product accumulates, the fluorescence increases at all temperatures with double stranded product. However at denaturation temperatures, fluorescence returns to baseline since only single stranded DNA is present. The fluorescence v. temperature projection of double stranded dyes shown in Figure 30 eliminates the time axis and shows the temperature dependence of strand status during DNA amplification.

Example 11

A 536 base pair fragment of the human beta-globin gene was amplified from 25 ng of genomic DNA and a 1:10,000 dilution of SYBR™ Green I in a volume of 5 μl. Each temperature cycle was 28 sec long (95°C maximum, 61°C minimum, 15 sec at 72°C with an average rate between temperatures of 5.2°C/sec). Other conditions are the same as those described in Figure 30. Cycles 15-40 are displayed. Thetemperature dependence of product strand status during PCR is revealed by fluorescence v. temperature plots using as shown in Figure 31. Early cycles represented appear identical, with a nonlinear increase in fluorescence at lower temperatures. As amplification proceeds, temperature cycles appear as rising loops between annealing and denaturation temperatures. As the sample is heated, fluorescence is high until denaturation occurs. As the sample cools, fluorescence increases, reflecting product reannealing. When the temperature is constant during extension, increasing fluorescence correlates with additional DNA synthesis.

As will be appreciated by an understanding of this disclosure, continuous monitoring within a cycle can provide insight into DNA amplification mechanics not previously available in the art. Using the present invention, many aspects of DNA amplification that have heretofore been little understood are discernable. For example,

rapid cycle amplification claims to denature the product in less than one second, while the prior art uses ten seconds to one minute of denaturation. Observing product melting by real time fluorescence monitoring with double strand dyes in accordance with the present invention (Figures 30 and 31) shows that use of the shorter denaturation times is effective. As another example, many causes of the known "plateau effect" have been proposed, but few data are available to distinguish between alternatives. As shown in Figure 31, product reannealing is very rapid. In fact, during later cycles of amplification, a majority of product is reannealed each cycle during cooling before the primer annealing temperature is reached. This occurs with cooling rates of 5-10°C/sec in rapid cycle instrumentation. The product reannealing with slower, prior art temperature cyclers will be more extensive and this undesirable effect will be greater. Product reannealing appears to be a major, and perhaps the sole, cause of the "plateau effect."

Now consider continuous monitoring of sequence specific probes. As will be appreciated by an understanding of this disclosure, continuous monitoring within a cycle can identify the nature of probe fluorescence.

Example 12

Continuous monitoring of amplification every 200 msec was performed with a dual-labeled hydrolysis probe (beta-actin) and adjacent hybridization probes (beta-globin) as in Example 7. In Figure 32A, cycles 20-45 of a reaction monitored with the hydrolysis probe is shown. Hydrolysis probes show a linear change in fluorescence ratio with temperature and a parallel increase in fluorescence as more probe is hydrolyzed. In contrast, the fluorescence ratio from hybridization probes varies radically with temperature (Figure 32B, cycles 20-40). During the annealing/extension phase, the probes hybridize to single stranded product and the fluorescence ratio (Cy5/fluorescein) increases. During heating to product denaturation temperatures, the probes dissociate around 70°C, returning the fluorescence ratio to background levels.

Example 13

A 110 base pair beta-globin fragment was amplified from 50 ng of genomic DNA in a volume of 10 μl. The amplification conditions

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and adjacent hybridization probes of Example 3 were followed with either 0.4 U of Taq polymerase or 0.8 U of KlenTaq1. Fluorescence was monitored each 100 msec. Fluorescence v. temperature plots using KlenTaq1 (Figure 33) and Taq (Figure 34) demonstrate melting of the probes at about 70°C. The maximal signal with KlenTaq1 is greater than that with Taq, because of the exonuclease activity of the latter. At later cycles with Taq, the fluorescence each cycle begins to decrease as the concentration of intact probe decreases. Three dimensional plot of temperature, time, and fluorescence are shown in Figure 35 (KlenTaq1) and Figure 36 (Taq).

The present invention's combination of (1) continuous fluorescence monitoring within each temperature cycle and (2) analysis of the temperature and time dependence of hybridization provides advantages not otherwise obtainable. Figure 2 shows that information that was previously unobtainable can be extracted by continuous monitoring throughout the cycle. Continuous fluorescence monitoring during the product melting phase of the cycle provides useful information on the purity, identity, and quantity of DNA present during that cycle.

As a PCR reaction is heated from the extension temperature to the denaturation temperature, any DNA in the sample is melted to single strands. This denaturation can be observed as a drop in the fluorescence of SYBR™ Green I. For small PCR products, the melting transition occurs over a narrow temperature range and the midpoint of that melting range is referred to as the Tm. Similar to sizing by gel electrophoresis, melting peak analysis measures a fundamental characteristic of DNA and can be used to identify amplified products. Unlike gel electrophoresis, melting curve analysis can distinguish products of the same length but different GC/AT ratio. In addition, two products with the same length and GC content, but differing in their GC distribution (for example, equally distributed vs. a GC clamp on one end) would have very different melting curves.

Thetemperature at which PCR products melt varies over a large range. Using empirical formulas known in the art, the effect of GC content on the melting temperature (Tm) of DNA predicts that a 0% GC duplex would melt 41° C lower than a 100% GC duplex. Given the same GC content, a 40 base pair primer dimer would melt 12°C below a 1000 bp product. Hence, the range of Tm for potential PCR products spans at least 50°C. This wide range allows most PCR products to be differentiated by melting curves. Thus, the combination of

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fluorescence monitoring of PCR with melting curve analysis provides simultaneous amplification, detection, and differentiation of PCR products.

Example 14

DNA melting curves for three different PCR products were acquired on a microvolume fluorimeter integrated with a 24-sample thermal cycler with optics for SYBR™ Green I fluorescence (LightCycler LC24, Idaho Technology, Idaho Falls, Idaho). The primers for the 180 base pair hepatitis B surface antigen gene amplification were 5'-CGTGGTGGAC TTCTCTCAAT-3' (SEQ ID NO:1) and 5'-AGAAGATGAG GCATAGCAGC-3'(SEQ ID NO:2). The primers for the 292 base pair prostate specific antigen (PSA) gene amplification were 5'-CTGTCCGTGA CGTGGATT-3' (SEQ ID NO:7) and 5'-AAGTCCTCCG AGTATAGC-3'(SEQ ID NO:8). The 536 base pair human beta-globin gene amplification was done as in Example 7. PCR was performed as described in Example 2. Amplification products were purified by phenol/chloroform extraction and ethanol precipitation, followed by repeated washing through a Centricon 30 microconcentrator (available from Amicon of Danvers, Massachusetts). Template concentrations were determined by absorbency at 260 nm and had A(260)/A(280) ratios greater than 1.7.

Fifty ng of purified DNA in 50 mM Tris, pH 8.5, 2 mM $MgCl_2$, and 250 μg/ml bovine serum albumin and a 5 μl volume were pipetted into the open plastic reservoir of composite glass/plastic reaction tubes, centrifuged at low speed to place the sample at the tip of the glass capillary, and sealed inside with a plastic plug. Fluorescence data for melting curves was acquired by integrating the signal over 0.25 - 2.0 seconds during a linear temperature transition to 95°C at 0.1-10.0°C/second. The fluorescence was continuously acquired and displayed at fluorescence v. temperature plots in the LabView programming environment (National Instrument, Austin, TX). Figure 37 shows the melting curves of the three purified PCR products.

The Tm's of three products in Figure 37 span only 6 degrees and two of the curves are separated by only 2 degrees. This small separation is ample to allow easy differentiation of the products. The importance of GC percentage over length on Tm is illustrated by the 292 bp PSA product melting at a higher temperature than the longer 536 bp beta-globin fragment. Melting curves are often obtained at rates of 0.5°C/minute to ensure equilibrium. Moreover, as the heating rate decreases, the melting curve shifts to lower

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temperatures and becomes sharper (Figure 38, hepatitis B fragment). Note however, that the melting curves of Figure 37 were obtained during a heating rate of 0.2°C/sec (12°C/minute) and can differentiate products differing in Tm by 2°C or less.

The apparent Tm of PCR products is also dependent on double-strand-specific DNA dye concentration (Figure 39, hepatitis B fragment). Higher concentrations of dye increase the stability of the DNA duplex and the observed Tm.

For monitoring of melting curves with SYBR™ Green I, the preferred conditions are 1:7,000-1:30,000 fold dilution of SYBR Green I with heating rates of 0.1-0.5°C/second. These conditions allow easy differentiation of products that differ in Tm by 2° C.

More precise temperature control and software for melting peak analysis will reduce the detectable difference in Tm to a fraction of a degree. This will allow the differentiation of most PCR products. Not all products can be differentiated by Tm however, just as it is possible to misread electrophoresis results because of comigration of two or more products, it is possible that some of the product melting in the expected range may not be the intended product. However, if no DNA melts in the range of the expected product, it can conclusively be said that none of the expected product is present.

Another form of product differentiation available with melting curve analysis is the distinctive patterns of domain melting seen in longer PCR products. While short products (<300 bp) usually melt in one transition, longer products can have internal melting domains that give melting curves of a complex, distinctive shape. These complex melting curves can be used as a fingerprint for product identification.

Melting curve analysis can be used to differentiate intended product from nonspecific products such as primer dimers. Primer dimers melt over a wide range of low temperatures; very different from the sharp melting curves of specific PCR amplification products. Larger heterogeneous products which resulted from running many cycles at low annealing stringency have lower and broader melting curves when compared with pure PCR product.

Example 15

Amplification of the 536 beta-globin gene fragment was performed as in Example 7 with a 1:30,000 dilution of SYBR™ Green I

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except that the conditions were varied. In reaction A (Figure 40), no template was added and the reaction was cycled at 94°C for 0 sec, 60°C for 0 sec, and 72°C for 10 sec for 30 cycles to produce small nonspecific amplification products. In B, amplification of 10^6 initial copies of purified template at low stringency (94°C for 0 sec, 50°C for 0 sec, and 72°C for 10 sec) for 55 cycles showed a broad size range of amplification products on gel electrophoresis and melts across a wide temperature range. In C, 10^6 initial copies of purified template were cycled at 94°C for 0 sec, 60°C for 0 sec, and 72°C for 10 sec for 30 times and shows a single bright band and melts in a sharp transition. The temperature transition rate was 0.2°C/sec. A Hind III digest of λ phage DNA (M) is used as a marker.

Figure 40 shows how melting curves accurately reflect the specificity of a PCR reaction. The sharp, high temperature melting curve C corresponds to a single band on a gel. The low temperature, broad melting, curve A comes from analysis of a no template control that shows only primer dimers. Over-amplification of the product in C gives the intermediate melting curve B, still clearly differentiable from the specific product.

The melting curves seen, for example, in Figure 37, can be better quantified by first taking the derivative of fluorescence (F) with respect to temperature (T). This derivative is plotted as -dF/dT v. T and converts the melting curves to melting peaks.

Example 16

The purified hepatitis B and beta-globin gene fragments of Example 14 were melted individually and together with a temperature transition rate of 0.2°C/sec and other conditions as specified in Example 14 (Figure 41). The somewhat subjective determination of Tm from the melting curves (top) is easily called by eye from the melting peaks (bottom). The area under the melting peaks can also be quantified by integration of the area under the curves. The fluorescence baseline was first subtracted from the -dF/dT v. T plot assuming that the magnitude of the baseline varies as the area under the curve. Then the peaks were fit by nonlinear least squares regression to gaussians with the mean, standard deviation, and height of the peak as the fit parameters. The area under each gaussian was taken as the peak area. All calculations were performed in the LabView programming environment (National Instruments, Austin, TX).

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Figure 41 shows an example of this conversion of melting curves to melting peaks. The code for these calculations is included as appendix A.

The ability to distinguish specific product from primer dimer and other reaction artifacts improves the use of double-strand-specific DNA dyes in the quantification of low initial copy numbers. Relatively large initial template copy numbers have been quantified using ethidium bromide (Higuchi & Watson, *supra*). However, at low initial copy numbers, the background amplification of primer dimers and other amplification artifacts interferes with the specific amplification signal. With the present invention's ability to differentiate specific products from non-specific artifacts, double-strand-specific DNA dyes can be used to quantify low initial template copy numbers. This is advantageous because of the simplicity of using these dyes. The double-strand-specific DNA dyes can be used in any amplification and custom fluorescently-labeled oligonucleotides are not necessary. Quantification of very low copy numbers with double-strand-specific DNA dyes requires very good amplification specificity or, as provided by the present invention, a means to differentiate the desired product from nonspecific amplification.

Example 17

The present invention's approach to product purity determination was used to improve quantitative PCR based on once-per-cycle monitoring of double-strand-specific DNA dye fluorescence. Fluorescence was acquired once each cycle after polymerase extension of the product for a series of reactions varying in the initial concentration of purified beta-globin template (see Figure 42A). The beta globin template and amplification conditions were as given in Example 7. The log-linear increase above background fluorescence began at a cycle number dependent on initial template concentration. The plots of the five reactions ranging from 10^6 to 10^2 copies per reaction were separated by about four cycles. The sample with an average 10^2 copies per reaction showed a decrease in reaction efficiency, and reactions with initial copy number below 100 gave fluorescence profiles that were less useful. The fluorescence profiles for the reactions containing 10 and 1 (average) copies rise in reverse order, and the negative control showed amplification after about 30 cycles. This is due to amplification of primer dimers and

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other nonspecific amplification products that cannot be distinguished from the intended product by once-per-cycle fluorescence monitoring of double-strand-specific DNA specific dyes.

Melting peaks were acquired for each sample (Figure 42B) and these were found to correlate well with electrophoresis results (Figure 42C). The reaction containing zero and one average initial template copies produced no discernible electrophoresis band at the expected 536 base pair location. The reactions containing 10 and 100 initial copies of template showed weak electrophoresis bands. This agreed well with the melting peak analysis, which showed no DNA melting in the range of the intended product (90 - 92°C) for the reactions containing zero and one initial copies and small peaks in this temperature range for 10 and 100 copies. Strong electrophoresis bands for the reactions containing 10^3 - 10^6 initial copies correlate well with large melting peaks in the expected 90 - 92°C range.

The ratio of intended product to total product, determined by melting peak integration, ranged from 0.28 for 10^5 copies to 0.0002 for zero initial template copies. Each fluorescence value in Figure 41A was multiplied by the appropriate ratio to give the corrected plot (designated "corrected fluorescence" in Figure 42D). This procedure extended the effective dynamic range of quantitation to between 10 and 1 initial template copies.

Melting peaks can distinguish specific products from non-specific products (Figure 40) and they can distinguish two purified PCR products mixed together (Figure 41) so they should also be useful for distinguishing two specific products amplified together in a single reaction tube. Melting curves obtained by continuous monitoring of PCR reactions according to the present invention are useful in multiplex PCR.

Example 18

In this example, two gene fragments were simultaneously amplified from genomic DNA and monitored with SYBR™ Green I fluorescence. During each amplification cycle, different amplification products denature at melting temperatures dependent on the length of the product, GC ratio, and other factors well known in the art. The temperature at which each product melts can be monitored with the double-strand-specific dye, SYBR™ Green I. At 81 base pair fragment from the cystic fibrosis gene was amplified using the primers described herein as SEQ ID NO:14 and SEQ ID NO:15 along

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with a 98 base pair fragment of the c-erbB-2 (HER2/neu) oncogene using the primers described herein as SEQ ID NO:16 and SEQ ID NO:17.

Amplification reactions were comprised of 50 mM Tris-HCl, pH 8.3, 3 mM $MgCl_2$, 500 μg/ml of bovine serum albumin, 200 μM of each dNTP, and 0.5 μM of the cystic fibrosis primers, 0.3 μM of the HER2/neu primers, a 1:30,000 dilution of SYBR™ Green I, 1 U AmpliTaq Gold DNA polymerase (Perkin Elmer, Foster City, CA), and 50 ng of human genomic DNA in 10 μl.

After activation of the polymerase at 95°C for 30 minutes, the samples were cycled at 94°C for 0 seconds (slope = 20), 55°C for 0 seconds (slope = 20), and 70°C for 10 seconds (slope = 20) for 35 cycles. The samples were cooled to 70°C, and the fluorescence was continuously acquired during a 0.2°C/sec ramp to 94°C. Melting curves (Figure 43) clearly showed two distinct products melting at 78°C (CFTR) and 88°C (neu). The two products differ in Tm by approximately 10°C and are easily distinguishable.

Multiplex amplification is useful in cases where an internal control is needed during amplification. For example, many translocations are detectable by PCR by placing primers on each side of the breakpoint. If no amplification occurs, the translocation is not present as long as the DNA is intact and no inhibitor is present. These possibilities can be ruled out by amplifying a positive control locus in the same reaction mixture. Such control amplifications are best done as internal controls with simultaneous amplification and detection.

Example 19

In this example, the procedure of Example 18 was followed except that after activation of the polymerase at 95°C for 30 minutes, the samples were cycled at 94°C for 0 seconds (slope = 20), 55°C for 0 seconds (slope = 20), and 70°C for 10 seconds (slope = 20) for 20 cycles, followed by 94°C for 0 seconds (slope = 1), 55°C for 0 seconds (slope = 20), and 70°C for 20 seconds (slope = 20) for 15 cycles. For cycles 26-31, fluorescence was continuously acquired during each 1°C/sec transition from 70°C to 94°C. The melting curves were converted to melting peaks and displayed (Figure 44).

Note that the amplification efficiency of the CFTR fragment appears greater than the neu fragment. The amplification efficiency can be rigorously determined by integrating the melting peak data as in Example 16.

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This kind of quantitative data referenced to a control has many applications. For instance, certain oncogenes, such as HER2/neu, are amplified *in vivo* in many tumors. That is, the genes are replicated in genomic DNA, sometimes many fold. Often, the clinical behavior of the tumor depends on the degree of oncogene replication. Amplification of the oncogene and a control template allows quantitative assessment of the relative copy number. As a further example, quantification of viral load in patients infected with HIV or hepatitis C is important in prognosis and therapy. Using a control template and monitoring the efficiency of amplification of both control and natural templates during amplification, accurate quantification of initial template copy number is achieved.

The present invention's feature of using melting curves for relative quantification will now be explained. In accordance with the present invention, an additional use for melting curves is quantitative PCR. Figure 42 showed there was a correlation between the area under the melting peak and the amount of specific product. Relative quantification of two PCR products would be possible if the two products were amplified with similar efficiency (or if the differing efficiencies were known and compensated for). Relative quantification of two products by integrating melting peak areas (see Example 16) is an aspect of the current invention.

Example 20

The cystic fibrosis and HER-2-neu gene fragments of Example 18 were amplified, purified as in Example 2. and adjusted to 175 μg/ml. The samples were mixed in various ratios (total 8 μl) and added to buffer (1 μl) and SYBR™ Green I (1 μl). Final concentrations were 50 mM Tris, pH 8.3, 3 mM $MgCl_2$, 250 μg/ml bovine serum albumin, and a 1:30,000 dilution of SYBR™ Green I. Melting curves were acquired at 0.2°C/sec, background fluorescence subtracted and the peaks integrated as described in Example 16. The results are displayed in Figure 45. Excellent correlation was found between the relative areas under melting peaks and the relative amounts of the two products.

Relative quantification of two PCR products is important in many quantitative PCR applications. Multiplex amplification of two or more products followed by integration of the areas under the

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melting peaks will be extremely useful in these areas. mRNA is often quantified relative to the amount of mRNA of a housekeeping gene.

Another important use of relative quantification is in competitive quantitative PCR. Typically a competitor is synthesized that has the same priming sites, but differs in length from the original target sequence. Known amounts of the competitor are spiked into an unknown sample and relative quantitation is performed. Competitors can be made that differ from the target sequence in Tm rather than length. The relative amounts of the products can be quantified by comparing the areas under their melting peaks. As the amount of one of the products is known, the quantity of the original target can be obtained. Using the melting peak method is significantly easier than the currently used methods which involve running multiple tubes for each unknown sample and often pulling tubes at various cycle numbers during the reaction to find the log-linear portion of the reaction. The relative amounts of the two products must then be determined. Usually this is done by labeling one of the dNTPs with a radioisotope and then quantifying the amount of label incorporated into each band after agarose gel electrophoresis. In comparison, the current invention allows the reaction to be monitored continuously so the log-linear portion of the amplification can be easily identified. Relative quantification can be done quickly by integration of melting peaks. An all day process is reduced to less than an hour.

From the foregoing discussion, it will be appreciated that fluorescence monitoring during DNA amplification is an extraordinarily powerful analytical technique. When sequence-specific detection and quantification are desired, resonance energy transfer probes can be used instead of double-strand-specific DNA dyes. The Tm of hybridization probes shifts about 4-8°C if a single base mismatch is present. If a hybridization probe is placed at a mutation site, single base mutations are detectable as a shift in the probe melting temperature.

Example 21

The factor V Leiden mutation is a single base change (G to A) that substitutes a glutamine residue for an arginine residue at amino acid residue 506 (R506Q). For further information, see R.M. Bertina et al., Mutation in Blood Coagulation Factor V Associated with Resistance to Activated Protein C, 369 Nature 64-67 (1994) and J. Voorberg et al., Association of Idiopathic Venous Thromboembolism

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with a Single Point-Mutation at Arg^{506} of Factor V, 343 Lancet 1535-36 (1994), both of which are hereby incorporated by reference. As used herein, "factor V Leiden mutation locus" means the nucleotide position in the factor V gene at which a guanine base in the wild type is replaced by an adenine base in the factor V Leiden mutant. SEQ ID NO:9 shows a portion of the wild type factor V gene, and SEQ ID NO:10 shows the corresponding portion of the factor V Leiden gene, with the relevant nucleotide at position 31 in each case. The complete nucleotide sequence of the factor V gene is described at R.J. Jenny et al., Complete cDNA and Derived Amino Acid Sequence of Human Factor V, 84 Proc. Nat'l Acad. Sci. USA 4846-50 (1987), hereby incorporated by reference, and sequences can also be obtained at Genbank locus HUMF10. The amino acid change in the mutant factor V protein makes this clotting factor resistant to degradation and increases the tendency to clotting and thrombosis. As the most common cause of inherited thrombophilia, this mutation is the target of a common laboratory test done in clinical molecular genetics laboratories.

The standard method of analysis for the factor V Leiden mutation is to amplify the gene segment by PCR, digest the resulting amplified products with a restriction endonuclease that cuts the wild type sequence but not the mutant, and distinguish digested wild type and undigested mutant products by gel electrophoresis. R.M. Bertina et al., *supra*. This is a method well known in the art for analysis for defined mutations. Such a test usually requires about 4 hours, including PCR amplification (2 hours), enzyme digestion (1 hour), and electrophoresis (1 hour). Post-amplification steps include opening the sample tube, adding the enzyme, and transferring the digested sample to the electrophoresis apparatus. Post-amplification processing increases the risk of end product contamination, and manual handling requires care to prevent mislabeling of samples. A method that simultaneously amplifies and analyzes for point mutations would eliminate these concerns.

A method for complete amplification and analysis of the factor V Leiden mutation within 30 min in the same instrument comprises asymmetrically amplifying a portion of a human genomic DNA sample containing the mutation locus, followed by obtaining and analyzing a melting curve for the amplified DNA. Genomic DNA is prepared according to methods well known in the art, e.g. J. Sambrook et al., Molecular Cloning: A Laboratory Manual (2d ed., 1989), hereby incorporated by reference. Preferably, the melting curve is obtained

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by the resonance energy transfer methodology described above with a fluorogenic hybridization probe. Such an assay easily discriminates between homozygous wild type, homozygous mutant, and heterozygous genotypes. In a preferred embodiment, the oligonucleotide probe is 3'-labeled with fluorescein and designed to hybridize on the amplified DNA near to a Cy5-labeled primer for resonance energy transfer. This method can be applied to any defined mutation.

The probe oligonucleotide is preferably about 15-40 nucleotide residues in length. The probe could conceivably contain as few as about 10 nucleotide residues, however, possible disadvantages of such short oligonucleotides include low specificity, low melting temperature, and increased background. Oligonucleotides larger than 40 residues could also be used, but would be unnecessarily expensive. Thus, the limits on the size of the probe oligonucleotide are only those imposed by functionality. The probe oligonucleotide should span the mutation, but the mutation preferably does not correspond to either the 5'- or 3'-terminal nucleotide residue of the probe. Since the present invention is based on melting curves, and lack of base pairing at the termini is known to have less of an effect on melting properties than at internal sites, the probe should be designed such that the mutation occurs at an internal position.

The oligonucleotide primers for amplification of the selected mutation locus are preferably about 15 to 30 residues in length. Primers shorter than the preferred range could be used but may not be as specific as would be desired. Similarly, primers longer than the preferred range could be used, but would be unnecessarily expensive. Thus, the limits on the sizes of the PCR primers are only those imposed by functionality.

The distance between the resonance energy transfer pair is also important for the proper functioning of the invention. The optimum distance between the resonance energy transfer pair is about 5 nucleotides. A distance of about 2 to 8 nucleotides is preferred, although a distance of up to about 10-15 nucleotides is functional. Having the resonance energy transfer pair on adjacent nucleotides is not necessarily beneficial because the distance between the resonance energy transfer pair is effected by the position on the DNA helix.

In this example, PCR amplification was carried out in 10 μl reaction mixtures comprising 50 mM Tris, pH 8.3, 3 mM $MgCl_2$, 500 μg/ml bovine serum albumin, 200 μM each dNTP, 0.5 μM Cy5-labeled primer (SEQ ID NO:11), 0.2 μM unlabeled opposing primer (SEQ ID NO: 12), 0.1 μM fluorescein-labeled probe (SEQ ID NO:13), 0.4 U Taq

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polymerase, and fifty ng human genomic DNA. Four different samples of DNA were tested: human genomic DNA from an individual homozygous for the factor V Leiden mutation; human genomic DNA from a heterozygous individual; human genomic DNA from an individual homozygous for the wild type factor V allele; and a negative control without DNA. The orientation of the Cy5-labeled primer, the fluorescein-labeled probe, and the mutation site (identified by asterisk) are shown below:

```
                    Cy5
                     |
5'-TAATCTGTAAGAGCAGATCC-3'     (SEQ ID NO:11)
                          *
   TAATCTGTAAGAGCAGATCCCTGGACAGGCGAGGAATACAGGTATT      (SEQ ID NO:9)
   ATTAGACATTCTCGTCTAGGGACCTGTCCGCTCCTTATGTCCATAA

                        3'-CTGTCCGCTCCTTATGTCCATAA-5'(SEQ ID NO:13)
                           |
                        Fluorescein
```

The sequence of the unlabeled opposing primer was TGTTATCACACTGGTGCTAA (SEQ ID NO:12) and the amplified product was 186 base pairs in length. The Cy5-labeled primer was obtained as in Example 8. Cycling conditions were 94°C for 0 sec (slope=20), 50°C for 10 sec (slope=20), and 72°C for 0 sec (slope=1) for 50 cycles, followed by cooling to 45°C and continuous fluorescence monitoring at a slope of 0.2°C/sec to 94°C for the melting curve. The highest quality melting curves were obtained at the end of amplification with a slow temperature transition rate (0.2°C/sec - Figure 46), although monitoring during each cycle at 1°C/sec between 50°C and 94°C also provided clear genotype identification (Figure 47). The melting curves are easiest to visualize by plotting the negative derivative of fluorescence with respect to temperature vs temperature (-dF/dT vs T). Such a plot allows facile visual identification of all possible genotypes from the raw fluorescence data.

The closer the Cy5 label is to the primer's 3'-end, the greater the resonance energy transfer signal. However, the 3'-end must have a free 3'-hydroxyl for polymerase extension, and placing the Cy5 too close to the 3'-end (either on the 3' or penultimate base) may inhibit polymerase attachment and extension. The 3'-fluorescein probe should hybridize as close to the primer as possible (minor overlap of 1-3 bases can be tolerated) and the mutation site should be near the middle of the probe. A 5-base separation between the hybridized fluorophores and a mutation at base 8 of a 23-mer probe

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gave a melting curve shift of 8°C between mutant and wild type sequences (Figure 46).

Mutation detection by probe melting can also be performed with 2 labeled probes instead of one labeled probe and one labeled primer. In this embodiment, one probe is labeled 5' with Cy5 and the other probe is labeled 3' with fluorescein. Since both these fluorescent probes can be synthesized directly from the amidites, a manual synthesis step is not required as it is in the primer/probe system. The fluorescein-labeled probe should be designed such that the mutation locus is near the center of the fluorescein-labeled probe. The length of the Cy5-labeled probe should be designed such that it melts at a higher temperature (>5°C) than the fluorescein-labeled probe which spans the mutation locus. Because background from fluorescein is more troublesome than that from Cy5, the concentration of the Cy5-labeled probe should preferably be 2-5 fold that of the fluorescein-labeled probe. The two probes should hybridize to the same strand of genomic DNA, and the resonance energy transfer pair should be separated by about 0 to 5 nucleotide residues. Alternately, the probe that spans the mutation site can be labeled with Cy5 and the other probe labeled with fluorescein.

It will be appreciated that the particular probes and primers disclosed herein for detection of the factor V Leiden mutation are merely illustrative, and that a person of ordinary skill in the art will be able to design other probes and primers for detection of mutations without undue experimentation by following the principles and guidelines set forth herein. It should also be recognized that although the invention is described with respect to detection of a single base mutation in genomic DNA, the same principles can be applied to detection of a mutation in cDNA. Preparation of the cDNA requires extra process steps and time, as is well known in the art, thus it is preferred to use genomic DNA because of the advantages of speed and lower cost. Further, the same technique can be used to detect insertions and deletions by designing the hybridization probe so that it melting temperature changes when the mutation or polymorphism is present. The invention can be used to detect any known mutation where a probe can be designed to differ in melting temperature when hybridized to mutant vs wild type.

Although fluorescein and Cy5 were used as resonance energy transfer labels in the example above, other acceptors, such as Cy5.5, can also be used with fluorescein.

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Example 22

The factor V locus of Example 21 was amplified as before except that the primer was labeled with Cy5.5 instead of Cy5. Cy5.5 emission was observed through a 683 nm long pass dichroic and a 683-703 nm bandpass interference filter. The Cy5.5 to fluorescein ratio increased above background at about cycle 30 and the ratio approximately doubled by 50 cycles of asymmetric amplification. When amplified with wild type DNA, the probe Tm was 65-66°C as judged by melting peaks.

Another example for detecting single base mutations will now be given.

Example 23

There is a common point mutation in the methylenetetrahydrofolate reductase (MTHFR) gene ($C_{677}T$) that converts an alanine to a valine residue and results in a thermolabile enzyme. This mutation can reduce MTHFR activity and lead to elevated homocysteine plasma levels which has been implicated as an independent risk factor for early vascular disease and thrombosis as is well known in the art. One of the primers was labeled with Cy5 (TGAAGGAGAAGGTGTCT*GCGGGA) (SEQ ID NO:25) where T* represents a modified T residue linked to Cy5 (see Example 9 for synthesis and purification). The probe sequence was fluorescein-CCTCGGCTAAATAGTAGTGCGTCGA (SEQ ID NO:26) and the other primer was AGGACGGTGCGGTGAGAGTG (SEQ ID NO:27). A 198 base pair fragment of the MTHFR gene was amplified from 50 ng of human genomic DNA in 50 mM Tris, pH 8.3, 2 mM $MgCl_2$, 500 μg/ml bovine serum albumin, 0.2 mM of each dNTP, 0.5 μM of the Cy5-labeled primer, 0.1 μM of the opposing primer, 0.1 μM of the fluorescein-labeled probe, and 0.4 U Taq DNA polymerase per 10 μl. Each cycle was 30 sec long and consisted of denaturation at 94°C followed by a 20 sec combined annealing/extension step at 60°C. The temperature transition rate between steps was 20°C/sec. After 60 cycles, a melting curve was acquired as follows: heating from 50-65°C at 0.5°C/sec, 65-75°C at 0.1°C/sec, and 75-94°C at 0.5°C/sec. After baseline subtraction and conversion to melting peaks, all possible genotypes were easily distinguished (Figure 48).

The discriminatory power of hybridization probes is also used to great advantage in multiplex or competitive PCR. For example, an artificial template is designed with a single internal base change

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and a hybridization probe designed to cover the base change as in Examples 21 and 23. Relative amplification of the competitor and natural template are determined by acquiring and integrating melting peaks as in Example 16. Alternately, if multiple detection probes are used that sequentially melt off different targets at different temperatures, relative quantification is achieved by the same analysis. In general, any quantitative technique described previously for double-strand-specific DNA dyes can be made sequence specific with hybridization probes.

Absolute Product Concentration by Product Reannealing Kinetics. Product concentration determinations are also advantageously carried out using the present invention. Continuous monitoring of double stranded DNA formation allows DNA quantification at any cycle of amplification by reannealing kinetics. The sample temperature is quickly dropped from the denaturation temperature and held constant at a lower temperature that is still high enough to prevent primer annealing (Figure 2). The rate of product reannealing follows second order kinetics (see B. Young & M. Anderson, Quantitative analysis of solution hybridization, In: Nucleic Acid Hybridization: A Practical Approach 47-71 (B. Hames & S. Higgins, eds., (1985), which is now incorporated herein by reference). For any given PCR product and temperature, a second order rate constant can be measured. Once the rate constant is known, any unknown DNA concentration can be determined from experimental reannealing data. Cooling is never instantaneous, and some reannealing occurs before a constant temperature is reached. Rapid cooling will maximize the amount of data available for rate constant and DNA concentration determination. The technique requires pure PCR product, but such can be assured by melting curves also obtained during temperature cycling using the present invention. This method of quantification by the present invention is advantageously independent of any signal intensity variations between samples.

Example 24

A 536 base pair fragment of the beta-globin gene was amplified from human genomic DNA (Example 7) and purified (see Example 2). Different amounts of the purified DNA were mixed with a 1:30,000 dilution of SYBR™ Green I in 5 μl of 50 mM Tris, pH 8.3 and 3mM $MgCl_2$. The samples were denatured at 94°C and then rapidly cooled to 85°C. The fluorescence at 520-550 nm was monitored at 85°C over

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time. When different concentrations of DNA were tested, the shape of the reannealing curve was characteristic of the DNA concentration (See Figure 49). For any given PCR product and temperature, a second order rate constant can be determined. Figure 50 shows the determination of a second order reannealing rate constant for 100 ng of the 536 base pair fragment in 5 μl at 85°C. The curve was fit by non-linear least squares regression with F_{max}, F_{min}, t_0 and k as the floating parameters using the second order rate equation shown in Figure 50. Analysis programs for this kind of curve fitting are well known in the art (for example, the user defined curve fit of Delta Graph, DeltaPoint, Inc, Monteray, CA). Once the rate constant is known, an unknown DNA concentration can be determined from experimental reannealing data.

With the rate constant (k) defined, DNA concentrations are determined on unknown samples. The fluorescence vs time curves of unknown samples are fit by non-linear least squares regression, preferably during temperature cycling in real time (for example, using the nonlinear Levenberg-Marquardt method described in the LabView programming environment, National Instruments, Austin, TX). For this fit, F_{max}, F_{min}, t_0 , and [DNA] are the floating parameters and k is constant.

Since some fluorescent dyes affect reannealing in a concentration dependent manner, the assumption of second order kinetics for product reannealing is checked by determining the rate constant at different standard DNA concentrations. The relationship is defined and alternate formula for fitting incorporated as necessary.

Also within the scope of the present invention is to use probe annealing rates to determine product concentrations. The rate of fluorescence resonance energy transfer is followed over time after a quick drop to a probe annealing temperature that is greater than the primer annealing temperature (Figure 2). For the case of amplification with a labeled primer and one labeled probe, the rate of annealing (and fluorescence generation) is second order. When using two labeled probes, the rate of fluorescence development is third order. These two arrangements are shown in Figure 18. When the concentration of the probe(s) is much greater than the product concentration, pseudo-first order and pseudo-second order equations are adequate to describe the possibilities. The appropriate rate equations for these different conditions are well known in the art

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(see Young, B. and Anderson, M., supra). For the purposes of this invention, it is adequate that the prior art suggests appropriate rate equations that are tested experimentally and corrected if necessary.

When probe annealing rates are used to determine product concentrations, possible interfering effects include product reannealing (with probe displacement by branch migration) and primer annealing and extension through the probe. The later is minimized when the probe Tm's are higher than the primer Tm's and a probe annealing temperature is chosen to minimize primer annealing. Figure 13 shows that even if extension occurs, the fluorescence increases with time for about 20 sec. During this period, the fluorescence increase depends on product concentration.

Probe annealing rates are used to determine product concentration similar to the method described above for determining product concentration by product reannealing. The steps are summarized as follows: (1) choosing the appropriate rate equation for the system, (2) running known DNA standards to determine the rate constant, (3) checking the validity of the rate equation by comparing different rate constants derived from different concentrations, and (4) using the rates constants to determine the DNA concentration of unknowns from their probe annealing data.

Fluorescence Feedback for Control of Temperature Cycling. In contrast to endpoint and cycle-by-cycle analysis, the present invention can also monitor fluorescence throughout each temperature cycle. Continuous fluorescence monitoring can be used to control temperature cycling parameters. The present invention uses fluorescence feedback for real time control and optimization of amplification. Continuous fluorescence monitoring of PCR samples containing a double-strand-specific DNA dye or fluorescently labeled oligonucleotide probes can be used to monitor hybridization and melting during individual amplification cycles. This information can be used by the temperature control algorithms within the temperature cycling apparatus to improve and customize thermal cycling conditions. Conventional PCR is performed by programming all cycling parameters before amplification. With continuous monitoring, determination of temperature cycling requirements can occur during amplification, based on continuous observation of annealing, extension, and denaturation. The potential benefits of using hybridization information to control temperature cycling include:

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1. Ensuring complete denaturation of the PCR product each cycle while:

a. Minimizing exposure to excessively high denaturation temperatures thus avoiding heat-induced damage to the amplification products and polymerase. Limiting the time product is exposed to denaturation temperatures is especially useful for amplification of long products.

b. Increasing reaction specificity by minimizing the denaturation temperature. This selects against products with a Tm higher than the intended amplification product.

2. Maximizing the amplification efficiency by ensuring adequate time for primer annealing each cycle while:

a. Minimizing the amount of time required for amplification by allowing no longer than is needed to reach a certain efficiency of primer annealing.

b. Enhancing reaction specificity by minimizing the time at the annealing temperature.

3. Maximizing the amplification efficiency by ensuring adequate time for product extension each cycle while:

a. Minimizing the amount of time required for amplification by allowing no longer than needed to complete product extension.

b. Enhancing reaction specificity by selecting against products longer than the intended amplification product. These would require longer than the allotted time to complete product extension.

4. Initiating thermal cycling changes dependent on the level of fluorescence obtained or the current efficiency of amplification. For example, over-amplification and nonspecific reaction products can be minimized by terminating thermal cycling when the efficiency drops to a certain level. As another example, temperature cycling can be modified to initiate slower temperature ramps for melting curve acquisition when the fluorescence becomes detectable. This saves time because the slower ramps need not be used on earlier cycles. Other desirable changes may become evident on continued practice of the invention.

Control is based on an estimate of reaction parameters from the fluorescence data. The original fluorescence data is either acquired as a change in fluorescence over time (temperature specific rates of

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denaturation, annealing, and extension), a change in fluorescence over temperature (product or probe Tm), or a change in extent of amplification (amplification yield and efficiency). These rates, Tm's and their first and second derivatives are used to determine optimal reaction parameters that include denaturation temperature and time, primer annealing temperature and time, probe annealing temperature and time, enzyme extension temperature and time, and number of cycles.

Double-strand-specific DNA dyes are used for the control of denaturation, control of extension, and to initiate thermal cycling changes at a certain amplification level or efficiency. Resonance energy transfer dyes are used for the control of annealing as will be described after the following example.

Example 25

A commercial fluorescence monitoring thermal cycler (LC24 LightCycler, Idaho Technology Inc., Idaho Falls, Idaho) was modified so that the software is no longer programmed with temperature/time setpoints, but is programmed to acquire fluorescence values, then to use these values for thermal cycler control.

As depicted in the Functional Block Diagram (Figure 51), the Run-Time Program communicates through serial and DAQ-board interfaces with the LightCycler. This allows high level access to either temperature or fluorescence data and either can be used by the Board-level Software for temperature control. However, in the current embodiment of the instrument, only the temperature data is converted into digital form at the Controller Hardware level. The fluorescence data is sent in analog form through the Digital acquisition board interface, is analyzed by the Run-time Program, and is sent back to the Board-level software via the serial interface.

Product melting control:

A melting peak was acquired for the intended PCR product and a baseline fluorescence was acquired for the sample containing the reaction cocktail at the temperature at which the product was completely melted.

Each cycle of the reaction then used this fluorescence value as a target. The approach to product denaturation was made in two stages to overcome the time-lag due to the requirement of sending the fluorescence value to a remote computer for analysis, then returning the instruction that the value had been reached. With each product

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melting step, the temperature was increased until the fluorescence reached an intermediate value, then the heating power was reduced so that a temperature ramp rate of roughly 3°/sec gave the computer time to analyze the fluorescence and signal the thermal cycler that product denaturation had occurred.

The resulting temperature/time plot (Fig 52) shows a characteristic increase in the melting temperature after cycle 20 as the concentration of amplification product increases. Product Tm is a function of product concentration.

Product annealing/extension:

During an extended hold at a combined annealing/extension temperature, the fluorescence was monitored and this information was used to ensure that adequate, but not excessive time had been allowed for product extension. The fluorescence was monitored at 10 second intervals, if the fluorescence increased more than a settable ratio (typically 1.00 - 1.05), then the annealing/extension step was continued. Otherwise, the next product melting step was initiated. The interval of 10 seconds was chosen to give a minimum of 20 seconds at the combined annealing/extension temperature.

The resulting fluorescence/time plot (Fig 52) shows a characteristic increase in the dwell time at the combined annealing/extension temperature as the concentration of amplification product grows. As the primer concentration and polymerase become limiting, more time is needed to complete product extension with each cycle.

Amplification plateau:

At the end of each annealing/extension step, the fluorescence value was acquired and stored. When this value had increased to 1.2 times the lowest end-cycle fluorescence value and had subsequently stopped increasing below a user settable ratio (typically 1.00 - 1.02), the thermal cycling was terminated. Alternately, a melting-curve acquisition step was initiated by entering a slow 0.1 - 0.2°C/second temperature ramp through the product Tm and monitoring the fluorescence of the sample continuously.

The resulting fluorescence/time plot (Fig 52) shows that after twenty-five cycles of amplification the ratio of cycle-by-cycle fluorescence growth fell below 1.00 and the reaction was terminated.

In one embodiment of the present invention, detection of the amplification plateau is used to acquire a high-resolution melting curves for each sample in a multiple sample run at the optimal temperature cycle for each sample. As a sample reaches its amplification plateau, a melting-curve is acquired for that sample, then regular temperature cycling is resumed until another reaction reaches its amplification plateau.

Real time monitoring and control of annealing distinct from extension is also provided by the present invention. If one of the primers is 3'-labeled with Cy5, no extension can occur. However, if labeled primer (1-10%) is mixed with unlabeled primer (90-99%), amplification efficiency will be slightly decreased, but annealing is observable as fluorescence energy transfer from a double-strand-specific dye to Cy5. The primer with the lowest Tm (as determined by nearest neighbor thermodynamics as known in the art) is labeled with Cy5 and SYBR™ Green I is included as a double-strand-specific dye. Alternately, primer annealing can be monitored indirectly with equivalent complementary oligonucleotides. An oligonucleotide of the same length and Tm as the lowest melting primer is designed with no complementarity to the amplified sequence. This oligonucleotide is 5'-labeled with Cy5 and its complement is 3'-labeled with fluorescein or some other resonance energy transfer pair. Hybridization of these oligonucleotides is followed by resonance energy transfer. The concentration of one probe is made the same as the concentration of the lowest Tm primer and the concentration of the other probe is made much less than this in order to obtain pseudo-first-order kinetics that approximates the pseudo-first-order kinetics of primer annealing to product. The efficiency of annealing is monitored and used to control annealing temperature and times by one of these methods.

It is also within the scope of the present invention to entirely replace temperature and time setpoints with fluorescence feedback control. For example, three samples are placed in a fluorescence temperature cycler with feedback capacity. The samples are:

1. A non-reacting sample containing amplified product and SYBR™ Green I.

2. A non-reacting sample containing complementary fluorescently labeled primers with a Tm equal to the lowest Tm primer and concentrations as noted above.

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