

[0030] **Fig. 9** exemplifies the binary coding of analytes. Analytes in a 3-plex CY3 assay were coded using binary spaced primer concentrations. A TaqMan positive control sequence was amplified in each reaction to confirm that the PCR performed successfully.

[0031] **Fig. 10** exemplifies an amplification or polymerization reaction using a polymerase chain reaction (PCR) to detect an analyte. Fig. 10A illustrates the sequence of the analyte (Analyte 01) to be detected. Fig. 10B illustrates a forward (FWD) and a reverse (RWD) primer pair specific for Analyte 01. Fig. 10C illustrates the annealing step during the first cycle of the PCR reaction. Fig. 10D illustrates the annealing step during the second cycle of the PCR reaction. Fig. 10E illustrates the extended PCR products after the second extension cycle.

[0032] **Fig. 11** exemplifies how a detection method described herein can be used in a multiplex reaction to detect quenched signals in three channels.

[0033] **Fig. 12** exemplifies the incorporation of multiple chromophores into an analyte in order to determine its morphology.

[0034] **Fig. 13** exemplifies signal generation as a function of temperature for an analyte containing multiple chromophores. Fig. 13A illustrates the morphology of the oligonucleotide at a first temperature (T_1). Three fluorophores (F_1 , F_2 and F_3) are attached to the oligonucleotide and one quencher (Q_1) is attached at the 3' end of the oligonucleotide. Fig. 13B illustrates the fluorescence signature profile of the oligonucleotide at T_1 . At T_1 , Q_1 is closest to F_3 , and a quenching effect is observed (Fig. 13B). Fig. 13C illustrates the morphology of the oligonucleotide at a second temperature (T_2). Fig. 13D illustrates the fluorescence signature profile of the oligonucleotide at T_2 . At T_2 , the oligonucleotide is fully denatured and Q_1 is further away from F_3 . As a result, the fluorescence signal of F_3 increased (Fig. 13D).

DETAILED DESCRIPTION OF THE INVENTION

[0035] Provided herein are methods, systems, compositions, and kits for the detection of one or more analytes using chromophore-attached oligonucleotides (e.g., primers and/or probes) specific for an analyte. For example, the incorporation of chromophores into an amplification or polymerization product (e.g., through the use of forward and reverse primers that are attached to different chromophores as illustrated, e.g., in Fig. 1) provides a number of advantages.

[0036] For example, methods described herein can provide for the direct detection of one or more analytes in a single reaction (such as an amplification reaction (such as a PCR

reaction or PCR reaction process) or a polymerization reaction). In addition, the direct incorporation of chromophores into the amplification or polymerization product (e.g., the analyte) allows for more accurate quantification of the analyte and for the real-time monitoring of the progression of an amplification or polymerization reaction. For example, once the analyte has been amplified to incorporate two different chromophores, upon excitation of one chromophore, an electron may travel through the pi-bond network of a DNA backbone to interact with a second chromophore, thereby generating a detectable signal or change in signal.

[0037] Methods described herein are particularly suitable for detecting genetic variations, such as single nucleotide polymorphisms (SNPs) or other qualitative information of an analyte. In some cases, a single base-pair mismatch between a chromophore-attached primer and the analyte can be detected upon amplification or polymerization of the analyte (or polymerization or extension of the primer). For example, a change in signal may occur if there is a disruption in a contiguous double-stranded DNA sequence upon amplification or polymerization of the analyte (or polymerization or extension of the primer (e.g., the primer specific for the SNP)) when a SNP is present. A single base pair misalignment (e.g. internal misalignment (such as a SNP) or terminal overhangs) can result in significant decrease in signal compared to the signal generated upon amplification or polymerization of the analyte (or polymerization or extension of the primer) without a base pair misalignment due to a disruption in electron transport between chromophores incorporated into an analyte containing a base pair misalignment.

[0038] Additionally described herein are methods to normalize signals during an amplification or polymerization reaction, such as a PCR reaction (or PCR reaction process). In some cases, the methods described herein can provide for a cycle-by-cycle normalization of a PCR reaction or process (e.g. chopper stabilization where individual cycles are reset to a reference value). For example, in some cases, when a signal measured during the denaturing step of a cycle is within a reference signal range, the denaturation signal can be used to normalize the signal measured during the annealing step of the same cycle. Thereby, the cycle-by-cycle normalization can provide a means to determine the reliability of the PCR reaction and can be used with the detection methods described herein to provide particularly accurate detection and measurements of analytes.

[0039] Also described herein are methods which allow detection of analytes present in low concentrations. In some cases, the sensitivity of the methods described herein can detect

analytes at concentrations of about 10uM to about 1aM. In some cases, the methods provided herein can be combined with a digital amplification or polymerization process (e.g. droplet digital PCR), to further enhance the detection. In some cases, the methods provided herein can be used to detect analytes that are present at a trace concentration in a sample (e.g. a rare SNP).

[0040] Further described herein are methods to detect of multiple analytes in a single reaction or experiment, without the need to resort to additional experiments or materials (e.g. reagents). Thereby, the disclosed methods can reduce or eliminate the associated cost of additional reagents or materials and increase time and efficiency.

I. Certain Terminology

[0041] It is to be understood that the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of any subject matter claimed. In this application, the use of the singular includes the plural unless specifically stated otherwise. It must be noted that, as used in the specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise. In this application, the use of "or" means "and/or" unless stated otherwise. Furthermore, use of the term "including" as well as other forms, such as "include", "includes," and "included," is not limiting.

[0042] The term "about," as used herein, generally refers to a range that is 15% greater than or less than a stated numerical value within the context of the particular usage. For example, "about 10" would include a range from 8.5 to 11.5.

[0043] The term "primer", as used herein, generally refers to a short linear oligonucleotide that hybridizes to a target nucleic acid sequence (e.g., a DNA template to be amplified) to prime a nucleic acid synthesis reaction. The primer may be an RNA oligonucleotide, a DNA oligonucleotide, or a chimeric sequence. The primer may contain natural, synthetic, or nucleotide analogues (e.g., those that increase T_m). Both the upper and lower limits of the length of the primer are empirically determined. The lower limit on primer length is the minimum length that is required to form a stable duplex upon hybridization with the target nucleic acid under nucleic acid amplification or polymerization reaction conditions. Very short primers (usually less than 3 nucleotides long) do not form thermodynamically stable duplexes with target nucleic acid under such hybridization conditions. The upper limit is often determined by the possibility of having a duplex formation in a region other than the

pre-determined nucleic acid sequence in the target nucleic acid. Generally, suitable primer lengths are in the range of about 3 nucleotides long to about 40 nucleotides long. The "primers" used in the methods of amplification or polymerization of a target nucleic acid described herein will be of a length appropriate for a particular set of experimental conditions. The determination of primer length is well within the routine capabilities of those of skilled in the art.

[0044] The terms "polynucleotide," "oligonucleotide," or "nucleic acid," as used herein, are used herein to refer to biological molecules comprising a plurality of nucleotides. Exemplary polynucleotides include deoxyribonucleic acids, ribonucleic acids, and synthetic analogues thereof, including peptide nucleic acids.

II. Methods of Detection

[0045] Described herein are methods for detecting the presence or absence of at least one polynucleotide analyte in a sample. Detection methods provided herein may use an amplification or polymerization technique (e.g., polymerase chain reaction (PCR) or PCR process) to incorporate chromophores directly onto the product (e.g., analyte) templates. An exemplary detection method is shown in Fig. 1, in which a pair of primers, each attached to either a fluorophore or a quencher at its 5' end respectively, is used to detect an analyte by amplification or polymerization process. In some cases, a first signal is measured prior to the start of the amplification or polymerization reaction. During the initial amplification or polymerization cycle, a duplex DNA separates, allowing primers to bind to specific regions of the individual template strands (Figs. 1A-1C). A polymerase (e.g. Taq polymerase) can be used to extend the primers along the template strand (Fig. 1D). In some cases, a second signal can be measured after the initial cycle. In some cases, a change in signal is not observed after the initial cycle (Fig. 1E) since a single chromophore is incorporated into the template. In some cases, upon completion of a second cycle, a change in signal can be observed due to the incorporation of both chromophores in the synthesized template (Fig. 1F). In some cases, a second signal can be measured after the second cycle. In some cases, the first and second signals are used to detect the presence or absence of the amplified product (e.g. the polynucleotide analyte). In some cases, a change is observed between the first and second signal. In some cases, the change in signal indicates the presence of the amplified product (e.g. the polynucleotide analyte).

[0046] In some cases, the change in signal is an increase in signal (e.g., an increased quenching of fluorescence when a fluorophore and quencher are incorporated into the amplified product). In some cases, the change in signal is a decrease in signal (e.g., a decrease in fluorescence intensity). In some cases, an increase in signal indicates a presence of the product or analyte. In some cases, a decrease in signal indicates a presence of the product or analyte. In some cases, the lack of a change in signal (e.g., no significant change in fluorescence intensity) indicates the absence of the product or analyte. In some cases, a denaturation signal is measured either before, during or after the denaturation step. In some cases, an annealing signal is measured either before, during or after the annealing step. In some cases, an annealing signal is measured either before, during or after the extension step. In some cases, the denaturation signal is compared with a reference signal or reference signal range as described elsewhere herein to determine if the amplification or polymerization reaction has proceeded reliably or correctly. In some cases, when the denaturation signal falls outside of the reference signal range, it can indicate that the reaction has failed. In some cases, when the denaturation signal falls within the reference signal range, the denaturation signal can be used to normalize the annealing signal. In some cases, the denaturation signal from each cycle can be used for a cycle-by-cycle normalization of the annealing signals (e.g. chopper stabilization). In some cases, the change in annealing signals is referred to as a relative quantitation of signals. In some cases, the signal is not limited to a signal generated by a fluorophore and quencher pair. In some cases, the signal can be generated by different chromophores.

[0047] In some cases, the change in signal can be defined by a percentage change. In some cases, the change in signal can be about 0.001%, 0.01%, 0.1%, 1%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 400%, 500%, 600%, 700%, 800%, 900%, 1000% or more. In some cases, the change in signal can be greater than 0.001%, 0.01%, 0.1%, 1%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 400%, 500%, 600%, 700%, 800%, 900% or 1000%. In some cases, the change in signal can be less than 0.001%, 0.01%, 0.1%, 1%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 400%, 500%, 600%, 700%, 800%, 900% or 1000%. In some cases, the change in signal can be about 50%.

[0048] Various combinations of oligonucleotides (e.g. primers and/or probes) can be used to detect an analyte. In some cases, a primer is used with a probe or a plurality of probes. In some cases, a plurality of primers is used with a plurality of probes. In some cases, a plurality

of primers is used with a single probe. In some cases, a primer pair is used to detect an analyte. In some cases, a primer pair is used to detect multiple analytes. In some cases, multiple primer pairs are used to detect an analyte. In some cases, a primer and a probe is used to detect an analyte. In some cases, a primer and a probe is used to detect multiple analytes. In some cases, a combination of primers and probes is used to detect an analyte. In some cases, a combination of primers and probes is used to detect multiple analytes. In some cases, a probe is used to detect an analyte. In some cases, a probe is used to detect multiple analytes. In some cases, multiple probes are used to detect an analyte.

[0049] In some cases, at least one chromophore (e.g. fluorophore, quencher, intercalating dye) is used to detect an analyte. In some cases, a chromophore pair (e.g. fluorophore/quencher pairs) is used to detect an analyte. In some cases, a single chromophore pair is used to detect an analyte. In some cases, a single chromophore pair is used to detect multiple analytes. In some cases, multiple chromophore pairs are used to detect an analyte. In some cases, a chromophore pair comprises a first chromophore and a second chromophore. In some cases, each primer in a primer pair comprises either a first chromophore (e.g. a fluorophore) or a second chromophore (e.g. a quencher). In some cases, each primer in the primer pair does not comprise both first and second chromophores. In some cases, a chromophore pair is not used to detect an analyte. In some cases, a chromophore is not used to detect an analyte. In some cases, a chromophore is an intercalating dye. In some cases, an intercalating dye is not used to detect an analyte. In some cases, the first chromophore and the second chromophore are the same. In some cases, the first chromophore and the second chromophore are the same fluorophore.

[0050] In some cases, a plurality of primers is used in the detection methods. In some cases, a primer is used with an oligonucleotide (e.g. a probe) or a plurality of oligonucleotides (e.g. a plurality of probes). In some cases, a primer is used with a probe or a plurality of probes. In some cases, a plurality of primers is used with a plurality of probes. In some cases, a plurality of primers is used with a single probe. In some cases, the number of primers used equals the number of probes used in the detection methods. In some cases, the primer is not attached to the oligonucleotide.

[0051] In some cases, a probe is not used in the detection methods. In some cases, a probe with chromophores attached is not used in the detection methods. In some cases, a probe (such as a self-quenching probe) with a fluorophore and quencher pair attached is not

used in the detection methods. In some cases, a probe with a fluorophore attached at its 5' end and a quencher attached at its 3' end is not used in the detection methods.

[0052] In some cases, the method is used with a second method. In some cases, the second method is an amplification or polymerization method, an electrophoresis (e.g. gel electrophoresis, capillary electrophoresis), a mass spectroscopy method, a chromatography method or an assay (e.g. *in vitro* cell based assay). In some cases, the amplification or polymerization method is an isothermal reaction method or a polymerase chain reaction method. In some cases, the polymerase chain reaction process is a multiplex-PCR, a quantitative PCR (qPCR), an end point PCR or a digital PCR (e.g. droplet digital PCR) process. In some cases, the polymerase chain reaction is a droplet digital PCR. In some cases, the second method is a droplet digital PCR method. In some cases, the second method is an electrophoresis method (e.g. a gel electrophoresis method for DNA sequencing). In some cases, the method is used in combination with a second and a third method. In some cases, the third method is an amplification or polymerization method, an electrophoresis (e.g. gel electrophoresis, capillary electrophoresis), a mass spectroscopy method, a chromatography method or an assay (e.g. *in vitro* cell based assay). In some cases, the method is used without a second or a third method. In some cases, the method is used without a second method. In some cases, the method is used without an electrophoresis method. In some cases, the method is used without a sequencing method.

A. Detection of Analytes from Intensity-Length Relationship

[0053] Described herein is a method of detecting the presence of one or more analytes in a sample. Methods provided herein involve, e.g., the measurement of the change in signal intensity when at least two chromophores interact with each other. For example, in the case of a fluorophore and quencher interaction, the further the fluorophore is from the quencher, the brighter the fluorescence signature of a particular nucleic acid analyte will typically be. So long that the analyte lengths are small, the persistence length of the nucleic acid analyte typically determines the intensity of fluorescence. For example, for a given fluorophore-quencher pair, the intensity of the fluorescence can be correlated with the persistence length. Further, the intensity of fluorescence often indicative of an energy transfer between the fluorophore and the quencher. The efficiency of this energy transfer is described by the following equation:

$$E = \frac{1}{1 + (r/R_0)^6} \quad (1)$$

where r is the distance between the fluorophore and the quencher (Fig. 2) and R_0 is a constant related to each fluorophore/quencher pair where it can be calculated from certain parameters of the absorption and emission spectra of each chromophore. (See Biophysical Chemistry, D. Freifelder, ed., W.H. Freeman and Company, San Francisco (1976) at page 426-28). Further, R_0 is described by the following equation:

$$R_0^6 = \frac{9 Q_0 (\ln 10) \kappa^2 J}{128 \pi^3 n^4 N_A} \quad (2)$$

where Q_0 is the fluorescence quantum yield of the donor in the absence of the acceptor, κ^2 is the dipole orientation factor, n is the refractive index of the medium, N_A is Avogadro's number and J is the spectral overlap integral (Fig. 2).

[0054] Therefore, the changes in fluorescence signal typically vary with the length of the synthesized strand. In some cases, a decrease in fluorescence signal is inversely proportional with the length of the synthesized strand. Utilizing this relationship, the presence of an analyte can be detected based on the signal correlated with its length. For example, a set of fluorescence signal ladders reminiscent of molecular weight ladders based on DNA length can be established as a control. In a sample, multiple pairs of primers attached with the same fluorophore/quencher pair are amplified. Upon completion of the amplification or polymerization process, the observed signals can be correlated with the controls, thereby detecting the presence or absence of a particular analyte. In some cases, the presence or absence of a particular analyte can be monitored throughout the amplification or polymerization process, by taking measurements during each amplification or polymerization cycle and comparing with the control ladder. In some cases, the ladder comprises a plurality of signals. In some cases, the plurality of signals generates multiple curves. In some cases, the ladder is represented by a plurality of curves. In some cases, the ladder comprises multiple sets of endpoint fluorescence. In some cases, the ladder comprises multiple sets of initial and end point fluorescence. In some cases, each step of the ladder comprises a plurality of signals. In some cases, the plurality of signals generates a curve. In some cases, each step of the ladder is represented by a curve. In some cases, each step of the ladder comprises an endpoint fluorescence measurement. In some cases, each step of the ladder comprises a set of initial and endpoint fluorescence measurements. In some cases, each step of the ladder comprises an initial and endpoint fluorescence. In some cases, the curve represents a

signature profile of an analyte based on its length. In some cases, the endpoint fluorescence represents a signature profile of an analyte based on its length. In some cases, the set of initial and endpoint fluorescence represents a signature profile of an analyte based on its length. In some cases, the initial and endpoint fluorescence represents a signature profile of an analyte based on its length. In some cases, each step of the ladder generates a signature profile of an analyte based on its length. In some cases, the ladder comprises multiple steps or multiple signature profiles of analytes. In some cases, the ladder comprises a single step or a single signature profile of an analyte. In some cases, the ladder comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500 or more steps. In some cases, the ladder comprises more than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450 or 500 steps. In some cases, the ladder comprises less than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450 or 500 steps. In some cases, multiple analytes are detected by a single fluorophore/quencher pair. In some cases, a single analyte is detected by a single fluorophore/quencher pair. In some cases, the signal is not limited to a signal generated by a fluorophore and quencher pair. In some cases, the signal can be generated by different chromophores.

B. Detection of Genetic Variations

[0055] Disclosed herein is a method of determining the presence or absence of a genetic variation, e.g., based on the change in signal due, e.g., to a disruption in the electron transport mechanism described herein. Genetic variations include deletion and insertion of one or more nucleotides, translocations of different nucleotide occurrences (e.g. single point mutations such as SNPs or a base-pair substitution), or variations in the number of multiple nucleotide repetitions. For example, to detect the presence of a single deletion or alteration (e.g. a SNP) in a template (e.g. an analyte), a first primer is designed to hybridize to a region comprising the deletion. A second primer comprises a sequence complementary to the region of the

analyte about less than 500bp away from the first primer. Upon amplification or polymerization, a change in signal is observed. However, since a kink is present in the product template, an inefficient electron transport results in a decrease in the change of signal, e.g., when compared to the change in signal observed for an analyte without the genetic variation.

[0056] In some cases, the genetic variation detected is a different nucleotide occurrence in the analyte. In some cases, the different nucleotide occurrence is a single-nucleotide polymorphism (SNP). A SNP is a DNA sequence variation that occurs when a single nucleotide (e.g. A, T, C or G) in the genome is altered. In some cases, this alteration leads to either a presence of disease or is associated with (or a marker for) the presence of a disease or diseases. For example, a single nucleotide mutation from GAG to GTG in the β -globin gene that encodes haemoglobin results in development of sickle-cell anaemia.

[0057] In general, each individual has many SNPs that create a unique human DNA pattern. In some cases, a SNP is a common SNP or a rare SNP. In some cases, a SNP is a common SNP. In some cases, a common SNP has a minor allele frequency of about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20% or more. In some cases, a common SNP has a minor allele frequency of greater than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19% or 20%. In some cases, a common SNP has a minor allele frequency of less than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19% or 20%. In some cases, a SNP is a rare SNP. In some cases, a rare SNP has a minor allele frequency of about 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20% or more. In some cases, a rare SNP has a minor allele frequency of greater than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19% or 20%. In some cases, a rare SNP has a minor allele frequency of less than 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19% or 20%.

[0058] In some cases, provided herein is a method to detect the presence of a SNP. In some cases, the method described herein is used to detect the presence of a common SNP. In some cases, the method described herein is used to detect the presence of a rare SNP. In some cases, the method described herein is used to detect the presence of a combination of common and rare SNPs.

[0059] In some cases, the method described herein is used to detect the presence of SNP in a sample. In some cases, the method described herein is used to detect multiple SNPs in a sample. In some cases, the method described herein is used to detect multiple common SNPs in a sample. In some cases, the method described herein is used to detect multiple rare SNPs in a sample. In some cases, the method described herein is used to detect a combination of common and rare SNPs in a sample. In some cases, the method described herein is used to detect a single SNP in a sample. In some cases, the method described herein is used to detect a single common SNP in a sample. In some cases, the method described herein is used to detect a single rare SNP in a sample.

[0060] In some cases, a plurality of SNPs is detected in a sample using a single chromophore pair (e.g. fluorophore/quencher pair). In some cases, the plurality of SNPs can be detected based in part on the length of each analyte containing a specific SNP. In some cases, a set of fluorescence ladder comprising SNP analytes (an analyte containing at least one SNP) of known length as described herein is generated. In some cases, the set of fluorescence ladder is used as a control. In some cases, the presence or absence of a particular SNP analyte can be determined by taking measurements during the amplification or polymerization reaction and comparing with the control ladder. In some cases, the presence or absence of a particular SNP analyte can be monitored throughout the amplification or polymerization process, by taking measurements during each amplification or polymerization cycle and comparing with the control ladder. In some cases, a plurality of SNPs is detected in a sample using a relative quantification method.

[0061] In some cases, the presence of SNPs correlates directly with the development of a disease. In some cases, the presence of SNPs increases the chances of developing a disease. In some cases, the disease comprises a genetic disorder, an autoimmune disease, a neurological disease, a cardiovascular disease and cancer.

C. Monitoring an Amplification or Polymerization Reaction

[0062] Disclosed herein is a method for detecting a change in signal generated by a set of chromophores for monitoring a reaction. In some cases, the method described herein can be used to monitor the progress of a PCR reaction. For example, at cycle 1, a set of fluorescence signals are measured, one measurement at the denaturing step and one measurement at the annealing step. During cycle 2, a second set of fluorescence signals are measured at the denaturing and annealing steps. A change in fluorescence between the signals taken at the

two annealing step indicate an occurrence of a PCR reaction, while the signals taken during the denaturing steps are used both as a control and a normalization parameter.

[0063] In some cases, signals measured from the annealing steps are used to monitor the progress of a reaction. In some cases, the change in signal from two annealing steps is measured. The change in signal can be, e.g., an increase in signal or a decrease in signal. In some cases, the change in signal is defined by a percentage change. In some cases, the change in signal can be about 0.001%, 0.01%, 0.1%, 1%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 400%, 500%, 600%, 700%, 800%, 900%, 1000% or more. In some cases, the change in signal can be greater than 0.001%, 0.01%, 0.1%, 1%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 400%, 500%, 600%, 700%, 800%, 900% or 1000%. In some cases, the change in signal can be less than 0.001%, 0.01%, 0.1%, 1%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 400%, 500%, 600%, 700%, 800%, 900% or 1000%. In some cases, signals are measured before, during or after the annealing step.

[0064] In some cases, signals measured from the extension steps are used to monitor the progress of a reaction. In some cases, the change in signal from two extension steps is measured. The change in signal can be, e.g., an increase in signal or a decrease in signal. In some cases, the change in signal is defined by a percentage change. In some cases, the change in signal can be about 0.001%, 0.01%, 0.1%, 1%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 400%, 500%, 600%, 700%, 800%, 900%, 1000% or more. In some cases, the change in signal can be greater than 0.001%, 0.01%, 0.1%, 1%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 400%, 500%, 600%, 700%, 800%, 900% or 1000%. In some cases, the change in signal can be less than 0.001%, 0.01%, 0.1%, 1%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, 200%, 300%, 400%, 500%, 600%, 700%, 800%, 900% or 1000%. In some cases, the signals are measured before, during or after the extension step.

[0065] In some cases, the signals measured from the denaturing steps are used to monitor the progress of a reaction. In some cases, the denaturation signals are compared with a reference signal or reference signal range. As described elsewhere herein, when a denaturation signal is outside of the reference signal range, the denaturation signals can indicate that the reaction has failed. In some cases, when the denaturation signal is within the reference signal range, the denaturation signals can indicate that the reaction has succeeded. In some cases, the denaturation signals serve as a control for each amplification or

polymerization cycle. In some cases, the denaturation signal is used for normalization during an amplification or polymerization experiment. As described elsewhere herein, when the denaturation signal is within the reference signal range, the denaturation signal from each amplification or polymerization cycle can be used to normalize the annealing signal from that cycle. In some cases, the denaturation signals are used for cycle-by-cycle self-normalization of a reaction. In some cases, signals are measured before, during or after the denaturation step.

[0066] In some cases, multiple reactions are monitored. In some cases, multiple reactions from a single sample are monitored. In some cases, multiple reactions from multiple samples are monitored. In some cases, a single reaction is monitored. In some cases, a single reaction from a single sample is monitored. In some cases, the reaction is used to detect the presence of a genetic variation. In some cases, the reaction for detecting the presence of a genetic variation is monitored. In some cases, the reaction is used to detect the presence of a SNP. In some cases, the reaction for detecting the presence of a SNP is monitored. In some cases, multiple oligonucleotides (e.g. primers and/or probes) are used. In some cases, multiple primers are used. In some cases, multiple probes are used. In some cases, the number of primers equals the number of probes.

D. Detection of Morphological Change

[0067] Disclosed herein is a method for detecting or monitoring a morphological change in an analyte based on changes in signals. In some cases, an analyte is a protein, a polynucleotide, a lipid, a carbohydrate or an antibody. In some cases, an analyte is a polynucleotide. In some cases, the polynucleotide is a DNA or an RNA. In some cases, DNA and RNA can adopt different conformations such as a hairpin, tetraloop or pseudoknot. For example, to detect the different morphological state of a DNA containing a hairpin, a fluorophore/quencher pair can be attached to the respective stem of the hairpin. Since the fluorophore is in close proximity to the quencher, a signal may not be observed. As the temperature increases, the DNA hairpin unwinds and a fluorescence signal may be observed. In some cases, multiple signals are measured as the DNA unwinds. In some cases, only an initial and an end-point signals are measured as the DNA unwinds. In some cases, the multiple signals can generate a curve. In some cases, the multiple signals are used to generate a signature profile of a DNA containing a hairpin. In some cases, the signature profile is a curve. In some cases, an initial and an end-point signals are used to generate a signature

profile. In some cases, the signature profile obtained from the DNA denaturation study is used as a control to detect the presence of a hairpin in a target DNA. In some cases, the method described herein is used to monitor the stability of a DNA or RNA conformation after introduction of addition, deletion, substitution or base modifications (e.g. unnatural bases) within the DNA or RNA. In some cases, the stability is affected by external factors. In some cases, the external factors include pH, organic or inorganic agents (e.g. salt, intercalating dye) or additional analytes. In some cases, the additional analyte is a DNA, RNA, protein or an antibody. In some cases, the method described herein is used to monitor the stability of a DNA or RNA conformation after introduction of the external factors.

[0068] In some cases, the method described herein is used to monitor a morphological change of a protein. For example, a protein residing in a native state can be a folded protein, a partially folded protein or a disordered protein. Folding or unfolding occurs due to the presence of binding partners, organic or inorganic agents, pH, and temperature. For a folded protein, an increase in temperature induces the protein to undergo an unfolding state. By attaching proteins to a plurality of fluorophores and/or quenchers, a fluorescence signal can be measured with each iterative temperature increase and can be compared to the signals taken at its native state. In some cases, multiple signals are measured as the protein unfolds. In some cases, only an initial and an end-point signals are measured as the protein unfolds. In some cases, multiple signals can generate a curve. In some cases, multiple signals are used to generate a signature profile of the protein. In some cases, the signature profile is a curve. In some cases, an initial and an end-point signals are used to generate a signature profile. In some cases, the signature profile obtained from the protein unfolding study is used as a control to detect the morphology of proteins containing similar folds. In some cases, the method described herein is used to monitor the stability of a protein. In some cases, unfolding of the protein can be induced upon addition of an external factor. In some cases, the external factors include pH, organic or inorganic agents (e.g. salt, intercalating dye) or additional analytes. In some cases, the additional analyte is a DNA, RNA, protein or an antibody.

[0069] In some cases, the method described herein is used to monitor the morphology of an analyte-analyte interaction such as a protein-protein, protein-antibody or protein-polynucleotide (e.g. protein-DNA or protein-RNA) interactions. For example, during a protein-DNA interaction, a protein can adopt a different conformation upon binding of the DNA. In some cases, the change in signal associated with binding can be used to compare with the protein at its apo or unbound state. In some cases, multiple signals are measured as

the protein-DNA complex forms. In some cases, only an initial and an end-point signals are measured as the complex forms. In some cases, the multiple signals can generate a curve. In some cases, the multiple signals are used to generate a signature profile of the protein. In some cases, the signature profile is a curve. In some cases, an initial and an end-point signals are used to generate a signature profile. In some cases, the signature profile obtained from the protein-DNA study is used as a control to detect the formation of protein complex with additional DNAs. In some cases, the methods described herein can monitor the stability of the protein complex with addition of another external factor. In some cases, the methods described herein can be used to monitor the morphological change of an analyte with multiple binding partners.

III. Multiplex Detection

[0070] Disclosed herein are examples of determining the presence of a plurality of analytes using a plurality of chromophores to indicate the presence or absence of these analytes. For example, a multiplex detection method can combines the use of color, signal, and/or mathematical strategies to circumvent degeneracy and ensure an infinite number of unique codes that can be unambiguously decoded in any combination of occurrences. For example, in detecting a sample containing four analytes, each analyte can be assigned a fluorophore (blue, green, yellow or red) and a quencher attached to analyte-specific oligonucleotides (e.g., a forward PCR primer and a reverse PCR primer). Upon amplification or polymerization, the presence or absence of an analyte is determined based on the presence or absence of a signal in that particular color.

[0071] In some cases, multiple color codes are generated using a plurality of chromophores. In some cases, multiple color codes are generated using a plurality of fluorophores and quenchers. In some cases, multiple color codes are generated using combinations of fluorophore/quencher pairs. In some cases, multiple chromophores are assigned to multiple analytes. In some cases, a single chromophore is assigned to multiple analytes. In some cases, a single chromophore is assigned to one analyte.

[0072] In some cases, one color code or chromophore combination is assigned to one analyte. In some cases, one color code or chromophore combination is assigned to multiple analytes (e.g., to discriminate multiple analytes of varying lengths in a single detection reaction). In some cases, one color code or chromophore combination is assigned to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30,

31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 100, 500, 1000, 5000, 10,000 or 100,000 analytes. In some cases, multiple color codes or chromophore combinations are assigned to one or more analytes. In some cases, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 100, 500, 1000, 5000, 10,000 or 100,000 color codes or chromophore combinations are assigned to one or more analytes (e.g. 1, 5, 10, 100, 500, 10,000 analytes). In some cases, at most 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 100, 500, 1000, 5000, 10,000 or 100,000 color codes or chromophore combinations are assigned to one or more analytes (e.g. 1, 5, 10, 100, 500, 10,000 analytes).

[0073] In some cases, one color is assigned as a control. In some cases, the control is a positive control or a negative control.

A. Multiplex Detection for Genetic Variation

[0074] In some cases, the methods disclosed herein can be used to detect the presence of multiple genetic variations (e.g., SNPs). In some cases, an analyte contains a plurality of genetic variations. In some cases, an analyte contains one genetic variation. In some cases, one color is assigned to one genetic variation. In some cases, a sample contains a plurality of genetic variations, wherein a color code or chromophore combination is assigned to each genetic variation. In some cases, a sample contains one genetic variation.

B. Multiplex Detection for SNP

[0075] In some cases, disclosed herein are methods of detecting the presence or absence of a SNP in an analyte. In some cases, an analyte contains a plurality of SNPs. In some cases, an analyte contains 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500 or more SNPs. In some cases, an analyte contains at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, or 500 SNPs. In some cases, an analyte contains no more than 1, 2, 3, 4,

5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, or 500 SNPs. In some cases, an analyte contains 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500 or more common SNPs. In some cases, an analyte contains at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, or 500 common SNPs. In some cases, an analyte contains no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, or 500 common SNPs. In some cases, an analyte contains 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500 or more rare SNPs. In some cases, an analyte contains at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, or 500 rare SNPs. In some cases, an analyte contains no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, or 500 rare SNPs.

[0076] In some cases, a sample contains a plurality of analytes. In some cases, multiple SNPs are detected from a plurality of analytes in the sample. In some cases, multiple common SNPs are detected from a plurality of analytes in the sample. In some cases, multiple rare SNPs are detected from a plurality of analytes in the sample. In some cases, multiple SNPs are detected from an analyte in the sample. In some cases, multiple common SNPs are detected from an analyte in the sample. In some cases, multiple rare SNPs are

detected from an analyte in the sample. In some cases, one SNP is detected from an analyte in the sample. In some cases, one common SNP is detected from an analyte in the sample. In some cases, one rare SNP is detected from an analyte in the sample. In some cases, one SNP is detected in the sample. In some cases, one common SNP is detected in the sample. In some cases, one rare SNP is detected in the sample.

[0077] In some cases, a sample contains 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1000, 5000, 10,000, 50,000, 100,000 or more SNPs. In some cases, a sample contains at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1000, 5000, 10,000, 50,000, 100,000 or more SNPs. In some cases, a sample contains no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1000, 5000, 10,000, 50,000, 100,000 or more SNPs. In some cases, a sample contains 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1000, 5000, 10,000, 50,000, 100,000 or more common SNPs. In some cases, a sample contains at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1000, 5000, 10,000, 50,000, 100,000 or more common SNPs. In some cases, a sample contains no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1000, 5000, 10,000, 50,000, 100,000 or more common SNPs. In some cases, a sample contains 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23,

24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1000, 5000, 10,000, 50,000, 100,000 or more rare SNPs. In some cases, a sample contains at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1000, 5000, 10,000, 50,000, 100,000 or more rare SNPs. In some cases, a sample contains no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1000, 5000, 10,000, 50,000, 100,000 or more rare SNPs.

[0078] In some cases, the methods described herein comprise detecting 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1000, 5000, 10,000, 50,000, 100,000 or more SNPs. In some cases, the methods described herein comprise detecting at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1000, 5000, 10,000, 50,000, 100,000 or more SNPs. In some cases, the methods described herein comprise detecting no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1000, 5000, 10,000, 50,000, 100,000 or more SNPs. In some cases, the methods described herein comprise detecting 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1000, 5000, 10,000, 50,000, 100,000 or more common SNPs. In some cases, the methods described herein comprise detecting at

least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1000, 5000, 10,000, 50,000, 100,000 or more common SNPs. In some cases, the methods described herein comprise detecting no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1000, 5000, 10,000, 50,000, 100,000 or more common SNPs. In some cases, the methods described herein comprise detecting 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1000, 5000, 10,000, 50,000, 100,000 or more rare SNPs. In some cases, the methods described herein comprise detecting at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1000, 5000, 10,000, 50,000, 100,000 or more rare SNPs. In some cases, the methods described herein comprise detecting no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1000, 5000, 10,000, 50,000, 100,000 or more rare SNPs.

[0079] In some cases, the method of detection utilizes an amplification or polymerization method. In some cases, an amplification or polymerization method (or process) comprises a polymerase chain reaction (PCR) method and an isothermal reaction method. In some cases, a PCR reaction process comprises a multiplex PCR, a real-time PCR, a quantitative PCR and a digital PCR (e.g. droplet digital PCR) process. In some cases, the method of detection utilizes a quantitative PCR method. In some cases, the quantitative PCR method is used in combination with a second method. In some cases, the second method is a digital PCR method. In some cases, the second method is a droplet digital PCR method.

[0080] In some cases, a signature profile is used to detect the presence of a SNP. In some cases, a signature profile is used to pinpoint the nucleotide mutation. In some cases, a signature profile is unique for each nucleotide mismatch, UU, UT, UG, UC, UA, AA, TT, GG, CC, AG, AC, TC, TC, and distinct from the wild-type. In some cases, a signature profile of a nucleotide mismatch is compared to that of a wild-type. In some cases, the signature profile of a SNP is compared to that of a wild-type. In some cases, a fluorescence signal of a SNP is compared to a fluorescence signal of a wild-type. In some cases, a change in fluorescence signal is detected between the signals of a SNP and a wild-type. In some cases, the change in signal can be calculated as a percentage. In some cases, the percentage of signal change is 0.01, 0.1, 0.2, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100%. In some cases, the percentage of signal change is about 0.01, 0.1, 0.2, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100%. In some cases, a change in signal is detected between the fluorescence signals of an AG mismatch and a wild-type. In some cases, the change in signal is calculated as a percentage. In some cases, the percentage of signal change is 0.01, 0.1, 0.2, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100%. In some cases, the percentage of signal change is about 0.01, 0.1, 0.2, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100%. In some cases, a change in signal is detected between the fluorescence signals of an AC mismatch and a wild-type. In some cases, the change in signal is calculated as a percentage. In some cases, the percentage of signal change is 0.01, 0.1, 0.2, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100%. In some cases, the percentage of signal change is about 0.01, 0.1, 0.2, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100%. In some cases, a change in signal is detected between the fluorescence signals of a TG mismatch and a wild-type. In some cases, the change in signal is

calculated as a percentage. In some cases, the percentage of signal change is 0.01, 0.1, 0.2, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100%. In some cases, the percentage of signal change is about 0.01, 0.1, 0.2, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100%. In some cases, a change in signal is detected between the fluorescence signals of a TC mismatch and a wild-type. In some cases, the change in signal is calculated as a percentage. In some cases, the percentage of signal change is 0.01, 0.1, 0.2, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100%. In some cases, the percentage of signal change is about 0.01, 0.1, 0.2, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100%.

[0081] In some cases, a pair of primers is utilized to detect a SNP. In some cases, the first primer comprises a sequence encoding the SNP. In some cases, the first primer hybridizes to a region of the analyte encoding the SNP. In some cases, the second primer comprises a sequence not encoding the SNP. In some cases, the second primer comprises a sequence complementary to a region of the analyte not encoding the SNP. In some cases, the first primer encodes a region on the analyte greater than 500 base pairs apart from a region of the analyte encoded by the second primer. In some cases, the first primer encodes a region on the analyte less than 500 base pairs apart from a region encoded by the second primer. In some cases, the first primer encodes a region on the analyte less than 490, 480, 470, 460, 450, 440, 430, 420, 410, 400, 390, 380, 370, 360, 350, 340, 330, 320, 310, 300, 290, 280, 270, 260, 250, 240, 230, 220, 210, 200, 195, 190, 185, 180, 175, 170, 165, 160, 155, 150, 145, 140, 135, 130, 125, 120, 115, 110, 105, 100, 99, 98, 97, 96, 95, 94, 93, 92, 91, 90, 89, 88, 87, 86, 85, 84, 83, 82, 81, 80, 79, 78, 77, 76, 75, 74, 73, 72, 71, 70, 69, 68, 67, 66, 65, 64, 63, 62, 61, 60, 59, 58, 57, 56, 55, 54, 53, 52, 51, 50, 49, 48, 47, 46, 45, 44, 43, 42, 41, 40, 39, 38, 37, 36, 35, 34, 33, 32, 31, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20 base pairs apart from a region encoded by the second primer. In some cases, the first primer encodes a region on the analyte no more than 490, 480, 470, 460, 450, 440, 430, 420, 410, 400, 390, 380, 370, 360, 350, 340, 330, 320, 310, 300, 290, 280, 270, 260, 250, 240, 230, 220, 210, 200, 195, 190, 185, 180,

175, 170, 165, 160, 155, 150, 145, 140, 135, 130, 125, 120, 115, 110, 105, 100, 99, 98, 97, 96, 95, 94, 93, 92, 91, 90, 89, 88, 87, 86, 85, 84, 83, 82, 81, 80, 79, 78, 77, 76, 75, 74, 73, 72, 71, 70, 69, 68, 67, 66, 65, 64, 63, 62, 61, 60, 59, 58, 57, 56, 55, 54, 53, 52, 51, 50, 49, 48, 47, 46, 45, 44, 43, 42, 41, 40, 39, 38, 37, 36, 35, 34, 33, 32, 31, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20 base pairs apart from a region encoded by the second primer. In some cases, the first primer encodes a region on the analyte about 490, 480, 470, 460, 450, 440, 430, 420, 410, 400, 390, 380, 370, 360, 350, 340, 330, 320, 310, 300, 290, 280, 270, 260, 250, 240, 230, 220, 210, 200, 195, 190, 185, 180, 175, 170, 165, 160, 155, 150, 145, 140, 135, 130, 125, 120, 115, 110, 105, 100, 99, 98, 97, 96, 95, 94, 93, 92, 91, 90, 89, 88, 87, 86, 85, 84, 83, 82, 81, 80, 79, 78, 77, 76, 75, 74, 73, 72, 71, 70, 69, 68, 67, 66, 65, 64, 63, 62, 61, 60, 59, 58, 57, 56, 55, 54, 53, 52, 51, 50, 49, 48, 47, 46, 45, 44, 43, 42, 41, 40, 39, 38, 37, 36, 35, 34, 33, 32, 31, 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20 base pairs apart from a region encoded by the second primer.

IV. Analytes

[0082] An analyte may be any suitable analyte that can be analyzed using the methods and compositions of the present disclosure, where the analyte is capable of interacting with a reagent (e.g., an oligonucleotide such as a primer or probe attached to a chromophore) in order to generate a signal that can be measured. An analyte may be naturally-occurring or synthetic. An analyte may be present in a sample obtained using any methods known in the art. In some cases, a sample may be processed before analyzing it for an analyte. The methods and compositions presented in this disclosure may be used in solution phase assays, without the need for particles (such as beads) or a solid support.

[0083] In some cases, an analyte may be a polynucleotide, such as DNA, RNA, peptide nucleic acids, and any hybrid thereof, where the polynucleotide contains any combination of deoxyribo- and/or ribo-nucleotides. Polynucleotides may be single stranded or double stranded, or contain portions of both double stranded or single stranded sequence. Polynucleotides may contain any combination of nucleotides or bases, including, for example, uracil, adenine, thymine, cytosine, guanine, inosine, xanthine, hypoxanthine, isocytosine, isoguanine and any nucleotide derivative thereof. As used herein, the term "nucleotide" may include nucleotides and nucleosides, as well as nucleoside and nucleotide analogs, and modified nucleotides, including both synthetic and naturally occurring species. Polynucleotides may be any suitable polynucleotide for which one or more reagents as

described herein may be produced, including but not limited to cDNA, mitochondrial DNA (mtDNA), messenger RNA (mRNA), ribosomal RNA (rRNA), transfer RNA (tRNA), nuclear RNA (nRNA), small interfering RNA (siRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), small Cajal body-specific RNA (scaRNA), microRNA (miRNA), double stranded (dsRNA), ribozyme, riboswitch or viral RNA. Polynucleotides may be contained within any suitable vector, such as a plasmid, cosmid, fragment, chromosome, or genome. In some cases, the analyte is referred to as a polynucleotide analyte. In some cases, the analyte is referred to as a nucleic acid analyte. In some cases, the analyte is referred to as a nucleic acid target.

[0084] Genomic DNA may be obtained from naturally occurring or genetically modified organisms or from artificially or synthetically created genomes. Analytes comprising genomic DNA may be obtained from any source and using any methods known in the art. For example, genomic DNA may be isolated with or without amplification or polymerization. Amplification or polymerization may include PCR amplification, multiple displacement amplification (MDA), rolling circle amplification and other amplification or polymerization methods. Genomic DNA may also be obtained by cloning or recombinant methods, such as those involving plasmids and artificial chromosomes or other conventional methods (see Sambrook and Russell, *Molecular Cloning: A Laboratory Manual*, cited *supra*.) Polynucleotides may be isolated using other methods known in the art, for example as disclosed in *Genome Analysis: A Laboratory Manual Series* (Vols. I-IV) or *Molecular Cloning: A Laboratory Manual*. If the isolated polynucleotide is an mRNA, it may be reverse transcribed into cDNA using conventional techniques, as described in Sambrook and Russell, *Molecular Cloning: A Laboratory Manual*, cited *supra*.

[0085] An analyte may be a protein, polypeptide, lipid, carbohydrate, sugar, small molecule, or any other suitable molecule that can be detected with the methods and compositions provided herein. An analyte may be an enzyme or other protein. An analyte may be a drug or metabolite (e.g. anti-cancer drug, chemotherapeutic drug, anti-viral drug, antibiotic drug, or biologic). An analyte may be any molecule, such as a co-factor, receptor, receptor ligand, hormone, cytokine, blood factor, antigen, steroid, or antibody.

[0086] An analyte may be any molecule from any pathogen, such as a virus, bacteria, parasite, fungus, archaea or prion (e.g., PrP^{Sc}). Exemplary viruses include those from the families Adenoviridae, Flaviviridae, Hepadnaviridae, Herpesviridae, Orthomyxoviridae, Papovaviridae, Paramyxoviridae, Picornaviridae, Polyomavirus, Retroviridae, Rhabdoviridae,

and Togaviridae. Specific examples of viruses include adenovirus, astrovirus, bocavirus, BK virus, coxsackievirus, cytomegalovirus, dengue virus, Ebola virus, enterovirus, Epstein-Barr virus, feline leukemia virus, hepatitis virus, hepatitis A virus, hepatitis B virus, hepatitis C virus, hepatitis D virus, hepatitis E virus, herpes simplex virus (HSV), HSV type 1, HSV type 2, human immunodeficiency virus (HIV), HIV type 1, HIV type 2, human papilloma virus (HPV), HPV type 1, HPV type 2, HPV type 3, HPV type 4, HPV type 6, HPV type 10, HPV type 11, HPV type 16, HPV type 18, HPV type 26, HPV type 27, HPV type 28, HPV type 29, HPV type 30, HPV type 31, HPV type 33, HPV type 34, HPV type 35, HPV type 39, HPV type 40, HPV type 41, HPV type 42, HPV type 43, HPV type 44, HPV type 45, HPV type 49, HPV type 51, HPV type 52, HPV type 54, HPV type 55, HPV type 56, HPV type 57, HPV type 58, HPV type 59, HPV type 68, HPV type 69, influenza type A virus, influenza type B virus, JC virus, Marburg virus, measles virus, metapneumovirus, mumps virus, Norwalk virus, parovirus, polio virus, rabies virus, respiratory syncytial virus including type A and type B, retrovirus, rhinovirus, rotavirus, Rubella virus, smallpox virus, vaccinia virus, West Nile virus, yellow fever virus, and human parainfluenza virus type 3.

[0087] Exemplary bacteria include those from the genera *Bordetella*, *Borrelia*, *Brucella*, *Campylobacter*, *Chlamydia*, *Clostridium*, *Corynebacterium*, *Enterococcus*, *Escherichia*, *Francisella*, *Haemophilus*, *Helicobacter*, *Legionella*, *Leptospira*, *Listeria*, *Mycobacterium*, *Mycoplasma*, *Neisseria*, *Pseudomonas*, *Rickettsia*, *Salmonella*, *Shigella*, *Staphylococcus*, *Streptococcus*, *Treponema*, *Vibrio*, and *Yersinia*. Specific examples of bacteria include *Bordetella parvaptensis*, *Bordetella pertussis*, *Borrelia burgdorferi*, *Brucella abortus*, *Brucella canis*, *Brucella melitensis*, *Brucella suis*, *Campylobacter jejuni*, *Chlamydia pneumoniae*, *Chlamydia psittaci*, *Chlamydia trachomatis*, *Clostridium botulinum*, *Clostridium difficile*, *Clostridium perfringens*, *Clostridium tetani*, *Corynebacterium diphtheriae*, *Enterococcus faecalis*, *Enterococcus faecium*, *Escherichia coli*, *Francisella tularensis*, *Haemophilus influenzae*, *Helicobacter pylori*, *Legionella pneumophila*, *Leptospira interrogans*, *Listeria monocytogenes*, *Mycobacterium leprae*, *Mycobacterium tuberculosis*, *Mycobacterium ulcerans*, *Mycoplasma pneumoniae*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Pseudomonas aeruginosa*, *Rickettsia rickettsii*, *Salmonella choleraesuis*, *Salmonella dublin*, *Salmonella enteritidis*, *Salmonella typhi*, *Salmonella typhimurium*, *Shigella sonnei*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, *Streptococcus agalactiae*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Treponema pallidum*, *Vibrio cholerae*, *Yersinia pestis*, and *Yersinia enterocolitica*.

[0088] Exemplary parasites include those from the genera *Acanthamoeba*, *Babesia*, *Balamuthia*, *Balantidium*, *Blasocystis*, *Cryptosporidium*, *Dientamoeba*, *Entamoeba*, *Giardia*, *Isoospora*, *Leishmania*, *Naegleria*, *Pediculus*, *Plasmodium*, *Rhinosporidium*, *Sarcocystis*, *Schistosoma*, *Toxoplasma*, *Trichomonas*, and *Trypanosoma*. Specific examples of parasites include *Babesia divergens*, *Babesia bigemina*, *Babesia equi*, *Babesia microfti*, *Babesia duncani*, *Balamuthia mandrillaris*, *Balantidium coli*, *Dientamoeba fragilis*, *Entamoeba histolytica*, *Giardia lamblia*, *Isoospora belli*, *Naegleria fowleri*, *Pediculus humanus*, *Plasmodium falciparum*, *Plasmodium knowlesi*, *Plasmodium malariae*, *Plasmodium ovale*, *Plasmodium vivax*, *Rhinosporidium seeberi*, *Sarcocystis bovi hominis*, *Sarcocystis sui hominis*, *Schistosoma mansoni*, *Toxoplasma gondii*, *Trichomonas vaginalis*, *Trypanosoma brucei*, and *Trypanosoma cruzi*.

[0089] Exemplary fungi include those from the genera *Apophysomyces*, *Aspergillus*, *Blastomyces*, *Candida*, *Cladosporium*, *Coccidioides*, *Cryptococcus*, *Exserohilum*, *Fusarium*, *Histoplasma*, *Pichia*, *Pneumocystis*, *Saccharomyces*, *Sporothrix*, *Stachybotrys*, and *Trichophyton*. Specific examples of fungi include *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus clavatus*, *Blastomyces dermatitidis*, *Candida albicans*, *Coccidioides immitis*, *Cryptococcus neoformans*, *Exserohilum rostratum*, *Fusarium verticillioides*, *Histoplasma capsulatum*, *Pneumocystis jirovecii*, *Sporothrix schenckii*, *Stachybotrys chartarum*, and *Trichophyton mentagrophytes*.

[0090] Exemplary archaea include those from the genera *Acidilobus*, *Acidococcus*, *Aeropyrum*, *Archaeoglobus*, *Caldisphaera*, *Caldococcus*, *Cenarchaeum*, *Desulfurococcus*, *Geogemma*, *Geoglobus*, *Haladaptatus*, *Halomicrobium*, *Hyperthermus*, *Ignicoccus*, *Ignisphaera*, *Methanobacterium*, *Natronococcus*, *Nitrosopumilus*, *Picrophilus*, *Pyrodictium*, *Pyrolobus*, *Staphylothermus*, *Stetteria*, *Sulfophobococcus*, *Thermoplasma*, *Thermosphaera* and *Thermoplasma*. Specific examples of archaea include *A. aceticus*, *A. camini*, *A. fulgidus*, *A. infectus*, *A. lithotrophicus*, *A. pernix*, *A. profundus*, *A. veneficus*, *A. saccharovorans*, *A. sulfurreducens*, *C. dracosis*, *C. lagunensis*, *C. noboribetis*, *C. symbiosum*, *D. amylolyticus*, *D. fermentans*, *D. mobilis*, *D. mucosus*, *G. barossii*, *G. indica*, *G. pacifica*, *H. butylicus*, *N. maritimus*, *G. ahangari*, *H. paucihalophilus*, *H. mukohataei*, *H. katesii*, *H. zhouii*, *I. aggregans*, *I. islandicus*, *I. pacificus*, *I. hospitalis*, *M. aarhusense*, *M. alcaliphilum*, *M. beijingense*, *M. bryantii*, *M. congolense*, *M. curvum*, *M. espanolae*, *M. formicicum*, *M. ivanovii*, *M. oryzae*, *M. palustre*, *M. subterraneum*, *M. thermaggregans*, *M. uliginosum*, *N. amylolyticus*, *N. jeotgali*, *N. occultus*, *P. abyssi*, *P. brockii*, *P. occultum*, *P. fumarii*, *P.*

oshimae, *P. torridus*, *S. hellenicus*, *S. marinus*, *S. hydrogenophila*, *S. zilligii*, *T. maritimus*, *T. aggregans*, *T. acidophilum*, *T. sp. P61*, *T. sp. S01*, *T. sp. S02*, *T. sp. XT101*, *T. sp. XT102*, *T. sp. XT103*, *T. sp. XT107* and *T. volcanium*.

[0091] In some cases, an analyte may be any molecule derived from a mammal. In some cases, the mammal is a human, a non-human primate, mouse, rat, rabbit, goat, dog, cat, or cow. In some embodiments, the mammal is a human. In some cases, a human is a patient.

[0092] In some cases, an analyte may be any molecule derived from a plant. In some cases, a plant is any of various photosynthetic, eukaryotic, multicellular organisms of the kingdom Plantae characteristically producing embryos, containing chloroplasts, having cellulose cell walls, and lacking the power of locomotion.

[0093] In some cases, the methods provided in this disclosure may be used to detect any one of the analytes described above, or elsewhere in the specification. In some cases the methods provided in this disclosure may be used to detect panels of the analytes described above, or elsewhere in the specification. For example, a panel may comprise an analyte selected from the group consisting of any 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100, 500, 1000, 5000, 10,000, 100,000 or more analytes described above or elsewhere in the specification.

[0094] An analyte may be obtained from any suitable location, including from organisms, whole cells, cell preparations and cell-free compositions from any organism, tissue, cell, or environment. Analytes may be obtained from environmental samples, forensic samples, biopsies, aspirates, formalin fixed embedded tissues, air, agricultural samples, soil samples, petroleum samples, water samples, or dust samples. In some instances, an analyte may be obtained from bodily fluids which may include blood, urine, feces, serum, lymph, saliva, mucosal secretions, perspiration, central nervous system fluid, vaginal fluid, or semen. Analytes may also be obtained from manufactured products, such as cosmetics, foods, personal care products, and the like. Analytes may be the products of experimental manipulation including, recombinant cloning, polynucleotide amplification or polymerization, polymerase chain reaction (PCR) amplification or polymerization, isothermal amplification or polymerization, purification methods (such as purification of genomic DNA or RNA), and synthesis reactions.

[0095] More than one type of analyte may be detected in each multiplexed assay. For example, a polynucleotide, a protein, a polypeptide, a lipid, a carbohydrate, a sugar, a small

molecule, or any other suitable molecule may be detected simultaneously in the same multiplexed assay with the use of suitable reagents. Any combination of analytes may be detected at the same time.

[0096] Detection of an analyte may be useful for any suitable application, including research, clinical, diagnostic, prognostic, forensic, and monitoring applications. Exemplary applications include detection of hereditary diseases, identification of genetic fingerprints, diagnosis of infectious diseases, cloning of genes, paternity testing, criminal identification, phylogeny, anti-bioterrorism, environmental surveillance, and DNA computing. For example, an analyte may be indicative of a disease or condition. An analyte may be used to make a treatment decision, or to assess the state of a disease. The presence of an analyte may indicate an infection with a particular pathogen, or any other disease, such as cancer, autoimmune disease, cardiorespiratory disease, liver disease, digestive disease, and so on. The methods provided herein may thus be used to make a diagnosis and to make a clinical decision based on that diagnosis. For example, a result that indicates the presence of a bacterial polynucleotide in a sample taken from a subject may lead to the treatment of the subject with an antibiotic.

[0097] In some cases, the methods and compositions of the present disclosure may be used to detect at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, 10,000, 100,000 or more analytes. In some cases the methods and compositions of the present disclosure may be used to detect about 1-10,000, 1-1000, 1-100, 1-50, 1-40, 1-30, 1-20, 1-10, or 1-5 analytes.

[0098] In some cases, this disclosure provides assays that are capable of unambiguously detecting the presence or absence of each of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, 10,000 or 100,000 analytes, in any combination of presence or absence, in a single sample volume. In some cases, this disclosure provides assays that are capable of unambiguously detecting the presence or absence of each of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28,

29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, 10,000 or 100,000 analytes, in any combination of presence or absence, in a single sample volume. In some cases, this disclosure provides assays that are capable of unambiguously detecting the presence or absence of less than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, 10,000 or 100,000 analytes, in any combination of presence or absence, in a single sample volume.

A. *Distance*

[0099] In one aspect, the methods provided herein may be used to detect polynucleotide analytes containing about 1-1000 base pairs (bp). In some cases, the methods provided herein may be used to detect polynucleotide analytes containing 1-500bp, 10-450bp, 15-400bp, 20-350bp, 25-300bp, 30-250bp, 35-200bp, or 40-190bp. In some cases, the methods may be used to detect a polynucleotide analyte containing 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190 or more base pairs. In some cases, the methods may be used to detect polynucleotide analyte containing at least 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178,

179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189 or 190 base pairs. In some cases, the methods may be used to detect polynucleotide analyte containing no more than 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189 or 190 base pairs.

B. Sensitivity

[00100] In some cases, the methods disclosed herein may be used to detect polynucleotide analyte at concentrations of about 100uM to about 1fM. In some cases, the methods provided herein may be used to detect a polynucleotide analyte at concentrations of about 10uM-20fM, 1uM-40fM, 500nM-60fM, 100nM-70fM, 50nM-80fM, 30nM-90fM, 10nM-100fM. In some cases, the methods may be used to detect a polynucleotide analyte at a concentration of 10nM, 9nM, 8nM, 7nM, 6nM, 5nM, 4nM, 3nM, 2nM, 1nM, 950pM, 900pM, 850pM, 800pM, 750pM, 700pM, 650pM, 600pM, 550pM, 500pM, 450pM, 400pM, 350pM, 300pM, 250pM, 200pM, 180pM, 160pM, 140pM, 120pM, 100pM, 95pM, 90pM, 85pM, 80pM, 75pM, 70pM, 65pM, 60pM, 55pM, 50pM, 45pM, 40pM, 35pM, 30pM, 25pM, 20pM, 18pM, 16pM, 14pM, 12pM, 10pM, 8pM, 6pM, 4pM, 2pM, 1pM, 900fM, 800fM, 700fM, 600fM, 500fM, 400fM, 300fM, 200fM, 100fM, 50fM, 10fM, 1fM, 100aM, 10aM, or 1aM. In some cases, the methods may be used to detect a polynucleotide analyte at a concentration of at least 10nM, 9nM, 8nM, 7nM, 6nM, 5nM, 4nM, 3nM, 2nM, 1nM, 950pM, 900pM, 850pM, 800pM, 750pM, 700pM, 650pM, 600pM, 550pM, 500pM, 450pM, 400pM, 350pM, 300pM, 250pM, 200pM, 180pM, 160pM, 140pM, 120pM, 100pM, 95pM, 90pM, 85pM, 80pM, 75pM, 70pM, 65pM, 60pM, 55pM, 50pM, 45pM, 40pM, 35pM, 30pM, 25pM, 20pM, 18pM, 16pM, 14pM, 12pM, 10pM, 8pM, 6pM, 4pM, 2pM, 1pM, 900fM, 800fM, 700fM, 600fM, 500fM, 400fM, 300fM, 200fM, 100fM, 50fM, 10fM, 1fM, 100aM, 10aM, or 1aM. In some cases, the methods may be used to detect a polynucleotide analyte at a concentration of no more than 10nM, 9nM, 8nM, 7nM, 6nM, 5nM, 4nM, 3nM, 2nM, 1nM, 950pM, 900pM, 850pM, 800pM, 750pM, 700pM, 650pM, 600pM, 550pM, 500pM, 450pM, 400pM, 350pM,

300pM, 250pM, 200pM, 180pM, 160pM, 140pM, 120pM, 100pM, 95pM, 90pM, 85pM, 80pM, 75pM, 70pM, 65pM, 60pM, 55pM, 50pM, 45pM, 40pM, 35pM, 30pM, 25pM, 20pM, 18pM, 16pM, 14pM, 12pM, 10pM, 8pM, 6pM, 4pM, 2pM, 1pM, 900fM, 800fM, 700fM, 600fM, 500fM, 400fM, 300fM, 200fM, 100fM, 50fM, 10fM, 1fM, 100aM, 10aM, or 1aM.

C. *Specificity*

[00101] In some methods provided herein, a primer pair may be specific for one or a plurality of analytes. In some cases, a primer pair is specific to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 analytes. In some cases, a primer pair is specific to at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 analytes. In some cases, a primer pair is specific to less than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 analytes. In some cases, a primer pair is specific to one analyte. In some cases, a primer pair is universal to all analytes.

V. **Probes and Primers**

[00102] Some of the methods provided in this disclosure utilize a reagent (e.g. an oligonucleotide such as a primer or a probe that is attached to a chromophore) that can generate a signal in the presence of an analyte. Any suitable reagent may be used with the present disclosure. Generally, a reagent will have an analyte-specific component and a component that generates a signal in the presence of the analyte. In some cases, these reagents are referred to as probes and primers. In some cases, the probes are hybridization probes. In some cases, the hybridization probes are oligonucleotide probes attached to chromophores. In some cases, the probes are antibodies that detect an analyte, with a fluorescent label that emits or is quenched upon binding of the antibody to an analyte. In some cases, the reagent is a primer. In some cases, the primer is attached to a chromophore. In some cases, the primer is attached to a fluorophore. In some cases, the primer is attached to a quencher.

[00103] The methods of the present disclosure may use one or more reagents (e.g., an oligonucleotide such as a primer or a probe that is attached to a chromophore) to detect the presence or absence of each analyte. For example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14,

15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more reagents may be used to detect the presence or absence of each analyte. In some cases, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 reagents may be used to detect the presence or absence of each analyte. In some cases, fewer than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, , 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 reagents may be used to detect the presence or absence of each analyte.

[00104] In some cases, a sample is contacted with 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more reagents to detect the presence or absence of all analytes. In some cases, a sample is contacted with at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more reagents to detect the presence or absence of all analytes. In some cases, a sample is contacted with fewer than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 reagents to detect the presence or absence of all analytes.

[00105] As described above, primers attached to a fluorophore or a quencher may be used to detect the presence of an analyte in a polynucleotide amplification or polymerization assay. The quencher can quench the fluorescence emitted by the fluorophore upon excitation by a light source when the quencher and fluorophore are in close proximity. The sequence of the primer can be designed to be complementary to or may contain nucleotide mutations to a polynucleotide sequence present in an analyte, and the primer is capable of hybridizing to the analyte. The sequence of the primer can also be designed to contain one or more nucleotide variations in a polynucleotide sequence of an analyte, and the primer is capable of hybridizing to the analyte. A fluorophore can be attached to the 5' end of one of a primer pair. A quencher can be attached to the 5' end of the second primer of the primer pair. Hybridization of the primers may be performed in a nucleic acid amplification or polymerization reaction comprising primers (e.g., a polymerase chain reaction). Upon extension of the primers by a DNA polymerase, the fluorophore and quenchers are incorporated in the amplicon or amplification (or polymerization) product (e.g., an analyte).

The incorporation of the quencher and the fluorophore in the newly generated amplicon can lead to signal generation (e.g., quenching of fluorescence intensity from the fluorophore). With each iterative amplification or polymerization reaction, the fluorescence intensity is reduced by a factor of about 2. The amount of fluorescence detected can be used to directly determine the amount of analyte present. If no analyte is present, little or no quenching will be observed.

[00106] In some cases, a sample to be analyzed is combined with 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more pairs of primers (e.g. a forward primer and a reverse primer). In some cases a sample to be analyzed is contacted with at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more pairs of primers. In some cases a sample to be analyzed is contacted with fewer than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 pairs of primers. In some cases, the number of pairs of primers is 2-10, 3-15, 4-20, 3-10, 4-10, 5-10, 6-8, or 6-10. In some cases, a sample to be analyzed is contacted with 1 pair of primers.

[00107] In some cases, a sample may contain one or more analytes. In some cases, one primer pair may be used to detect the presence or absence of each analyte. In some cases, a sample is contacted with 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 100, 500, 1000, 5000, 10,000 or more different pairs of primers with each primer pair detecting a single analyte. In some cases, a sample is contacted with at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 100, 500, 1000, 5000, 10,000 or more different pairs of primers with each primer pair detecting a single analyte. In some cases, a sample is contacted with fewer than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 100, 500, 1000, 5000, 10,000 or more different pairs of primers with each primer pair detecting a single analyte. In some cases, the number of pairs of primers is 2-10, 3-15, 4-20, 3-10, 4-10, 5-10, 6-8, or 6-10.

[00108] In some cases, primers may be specific for a particular analyte and capable of amplifying a region complementary to a probe. In some cases, the number of primers used is

equivalent to the number of probes. In other cases, the number of probes used may exceed the number of primer used. In some cases, the number of primers and probes is defined by a ratio. In some cases, the ratio of primer to probe is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, or 1000. In some cases, the ratio of probe to primer is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, or 1000.

[00109] As disclosed elsewhere herein, primers may have one or a plurality of fluorophores or quenchers per primer. For example, in some cases a primer may comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more fluorophores. A primer may comprise at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 fluorophores. A primer may comprise fewer than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 fluorophores.

[00110] A primer may comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more quenchers. A primer may comprise at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 quenchers. A primer may comprise fewer than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 quenchers.

[00111] Attachment of fluorophores and quenchers to a probe or a primer may be performed in the same reaction or in serial reactions. A series of reactions may be performed to attach probes or primers to at least one fluorophore and the reaction products may be mixed to generate a mixture of probes or primers with different fluorophores.

[00112] Although many aspects of the present disclosure are exemplified using nucleic acid-based probes and primers, one of ordinary skill in the art will readily recognize that other forms of probes and primers would work equally well with the examples described in this disclosure. For example, a binding molecule specific to an analyte could be used as a probe. Non-limiting exemplary binding molecules include an antibody recognizing an analyte, and generating a signal in the presence of an analyte.

[00113] The fluorescent labels of the present disclosure may be attached to a probe or primer at any location. In some cases, a single chromophore is attached to the primer at the 5' end. In some examples, multiple chromophores are attached to the primer with at least one chromophore attached at the 5' end. Methods of chromophore labeling are well defined in the art. See, e.g. Pesce *et al*, editors, Fluorescence Spectroscopy, Marcel Dekker, New York, (1971); White *et al*, Fluorescence Analysis: A Practical Approach, Marcel Dekker, New York, (1970); and the like. Further, there is extensive guidance in the literature for derivatizing reporter and quencher molecules for covalent attachment via common reactive groups that can be added to an oligonucleotide. See, e.g. U.S. Pat. No. 3,996, 345; and U.S. Pat. No. 4,351,760. In examples that utilize chromophore labels as described herein, any suitable labeling techniques may be used.

VI. Chromophores

[00114] Chromophores are molecules capable of selective light absorption resulting in the coloration of these molecule containing compounds. The color arises when a molecule at an excited state releases energy in the form of light with a defined spectrum. Exemplary chromophores include, but are not limited to, a fluorochrome, a non-fluorochrome chromophore, a quencher (e.g. fluorescence quencher and a dark quencher), an absorption chromophore, a fluorophore, any organic or inorganic dye, metal chelate, or any fluorescent enzyme substrate. In some cases, the chromophore is a fluorochrome. In some cases, the fluorochrome is a fluorophore. In some cases, the chromophore is a quencher. In some cases, the chromophore is a dark quencher.

[00115] Several chromophores are described in the art, e.g. Berlman, Handbook of Fluorescence Spectra of Aromatic Molecules, 2nd Edition, Academic Press, New York, (1971). In examples that utilize fluorescent labels as described herein, any suitable fluorescent label may be used.

[00116] Exemplary fluorophores suitable for use with the present disclosure includes rhodamine, rhodol, fluorescein, thiofluorescein, aminofluorescein, carboxyfluorescein, chlorofluorescein, methylfluorescein, sulfofluorescein, aminorhodol, carboxyrhodol, chlororhodol, methylrhodol, sulforhodol; aminorhodamine, carboxyrhodamine, chlororhodamine, methylrhodamine, sulforhodamine, and thiorhodamine; cyanine, indocarbocyanine, oxacarbocyanine, thiocarbocyanine, merocyanine, cyanine 2, cyanine 3, cyanine 3.5, cyanine 5, cyanine 5.5, cyanine 7, oxadiazole derivatives, pyridyloxazole, nitrobenzoxadiazole, benzoxadiazole, pyren derivatives, cascade blue, oxazine derivatives, Nile red, Nile blue, cresyl violet, oxazine 170, acridine derivatives, proflavin, acridine orange, acridine yellow, arylmethine derivatives, auramine, crystal violet, malachite green, tetrapyrrole derivatives, porphyrin, phthalocyanine and bilirubin; 1-dimethylaminonaphthyl-5-sulfonate, 1-anilino-8-naphthalene sulfonate, 2-p-touidiny-6-naphthalene sulfonate, 3-phenyl-7-isocyanatocoumarin, N-(p-(2-benzoxazolyl)phenyl)maleimide, stilbenes, pyrenes, 6-FAM (Fluorescein), 6-FAM (NHS Ester), Fluorescein dT, HEX, JOE (NHS Ester), MAX, TET, ROX, TAMRA, TARMA™ (NHS Ester), TEX 615, ATTO™ 488, ATTO™ 532, ATTO™ 550, ATTO™ 565, ATTO™ Rho101, ATTO™ 590, ATTO™ 633, ATTO™ 647N, TYE™ 563, TYE™ 665, TYE™ 705 and the like.

[00117] Exemplary quenchers suitable for use with the present disclosure includes black hole quenchers, such as BHQ-0, BHQ-1, BHQ-2, BHQ-3; ATTO quenchers, such as ATTO 540Q, ATTO580Q, and ATTO612Q; Qx1 quenchers; Iowa Black FG, Iowa Black RG, Iowa Black FQ and Iowa Black RQ; IRDye QC-1; 1.4 nm Nanogold; and the like.

[00118] The fluorophores that may be used with the disclosure are not limited to any of the fluorophores described herein. For example, fluorophores with improved properties are continually developed, and these fluorophores could readily be used with the methods provided in this disclosure. Such improved fluorophores include quantum dots, which may emit energy at different wavelengths after being excited at a single wavelength.

A. Chromophore Combinations

[00119] In some cases, a plurality of chromophores is attached to an oligonucleotide (e.g. a probe or a primer). In some cases, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more chromophores are attached to an oligonucleotide. In some cases, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24,

25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more chromophores are attached to an oligonucleotide. In some cases, less than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50 chromophores are attached to an oligonucleotide. In some cases, one chromophore is attached to an oligonucleotide.

[00120] In some cases, the oligonucleotide comprises a probe. In some cases, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more chromophores are attached to a probe. In some cases, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more chromophores are attached to a probe. In some cases, less than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50 chromophores are attached to a probe. In some cases, one chromophore is attached to a probe.

[00121] In some cases, the oligonucleotide is a primer. In some cases, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more chromophores are attached to a primer. In some cases, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more chromophores are attached to a primer. In some cases, less than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50 chromophores are attached to a primer. In some cases, one chromophore is attached to a primer. In some cases, one chromophore is attached at the 5' end of a primer. In some cases, a plurality of chromophores is attached to a primer with at least one chromophore attached at the 5' end of the primer.

[00122] In some cases, a plurality of fluorophores and quenchers is attached to a probe. In some cases, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more fluorophores are attached to a probe. In some cases, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34,

35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more fluorophores are attached to a probe. In some cases, less than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 fluorophores are attached to a probe. In some cases, one fluorophore is attached to a probe. In some cases, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more quenchers are attached to a probe. In some cases, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more quenchers are attached to a probe. In some cases, less than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49 or 50 quenchers are attached to on a probe. In some cases, one quencher is attached to a probe.

[00123] In some cases, a combination of fluorophores and quenchers are attached to a probe. In some cases, the number of fluorophores and quenchers on a probe is defined by a ratio. In some cases, the ratio of fluorophore to quencher is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, or 1000. In some cases, the ratio of fluorophore to quencher is about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, or 1000. In some cases, the ratio of quencher to fluorophore is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, or 1000. In some cases, the ratio of quencher to fluorophore is about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, or 1000.

[00124] In some cases, a plurality of fluorophores and quenchers is attached to a primer. In some cases, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more fluorophores are attached to a primer. In some cases, about 1, 2, 3, 4, 5, 6, 7, 8, 9,

10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more fluorophores are attached to a primer. In some cases, one fluorophore is attached to a primer. In some cases, less than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 fluorophores are attached to a primer. In some cases, one fluorophore is attached at the 5' end of a primer. In some cases, a plurality of fluorophores is attached to a primer with at least one fluorophore attached to the 5' end of the primer. In some cases, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more quenchers are attached to a primer. In some cases, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more quenchers are attached to a primer. In some cases, less than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 quenchers are attached to a primer. In some cases, one quencher is attached to a primer. In some cases, one quencher is attached at the 5' end of a primer. In some cases, a plurality of quenchers is attached to a primer with at least one quencher attached at the 5' end of the primer.

[00125] In some cases, a combination of fluorophores and quenchers are attached to a primer. In some cases, the number of fluorophores and quenchers on a primer is defined by a ratio. In some cases, the ratio of fluorophore to quencher is about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, or 1000. In some cases, the ratio of quencher to fluorophore is about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500, 600, 700, 800, 900, or 1000.

[00126] In some cases, multiple fluorophores are paired with one quencher. In some cases 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more fluorophores are paired with one quencher. In some cases, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more fluorophores are paired with

one quencher. In some cases, less than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 fluorophores are paired with one quencher. In some cases, multiple fluorophore and quencher pairs are used. In some cases, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more fluorophore and quencher pairs are used. In some cases, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more fluorophore and quencher pairs are used. In some cases, less than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more fluorophore and quencher pairs are used. In some cases, 1 fluorophore and quencher pair is used.

[00127] In some cases, a pair of primers comprises a first primer and a second primer. In some cases, multiple fluorophores are attached to a first primer and one quencher is attached to a second primer. In some cases, at least one fluorophore is attached to the 5' end of the first primer. In some cases, the quencher is attached to the 5' end of the second primer. In some cases, multiple fluorophores are attached to a second primer and one quencher is attached to a first primer. In some cases, at least one fluorophore is attached to the 5' end of the second primer. In some cases, the quencher is attached to the 5' end of the first primer. In some cases, a fluorophore and a quencher are not attached to the same primer.

[00128] The skilled artisan will realize that the advantages of the present disclosed probes or primers may be retained while modifying various aspects of its structure. For example, but not by way of limitation, the number of donor/quencher pairs may be modified. The addition of more donor/quencher pairs to the probe or primer is expected to increase the amount of total fluorescence observable prior to initiation of amplification or polymerization reaction. There is no upper limit to the number of donor/quencher pairs that may be added to the probe or primer. In one example, the number of donor/quencher pairs is at least two. In other examples, the detector contains at least three or more donor/quencher pairs. In some examples, the detector may contain at least 10, 20, 30, or 50 pairs, or it may contain hundreds of donor/quencher pairs, as needed to produce, for example, an optimal signal-to-noise ratio and assay sensitivity.

[00129] In some cases, the methods provided in this disclosure may include the use of fluorophore/quencher pair as a control. The control fluorophore/quencher pair may be attached to one or more probe or primer pairs binding a positive control analyte, and each analyte to be detected, in a sample. If the same sequence occurs in the positive control analyte and each analyte to be detected, a single control primer pair may be used. If the same sequence does not occur in the positive control analyte and each analyte to be detected, different primer pairs may be used, but each primer pair may still be attached to the control fluorophore/quencher pair.

[00130] For example, building on the methods described above, one fluorophore/quencher pair may be used to encode the presence of a control analyte that is always present in the sample. The control analyte may be added to the sample, or may be inherently present in the sample. Additional fluorophore/quencher pairs may be used to encode the presence of additional analytes.

B. Signals

[00131] Disclosed herein is a method of utilizing the signal to identify the presence or absence of an analyte. In some cases, the signal is an increase in signal. In some cases, the signal is a decrease in signal. In some cases, a signature profile is generated based on the changes in signal at a specific distance. In some cases, once the length of an analyte is known, additional information can be extrapolated. In some cases, additional information includes the molecular weight of an analyte.

[00132] In some cases, a signal can be compared to a threshold value. A threshold can be defined as the average standard deviation of a baseline (or background) signal multiplied by an adjustable factor. In some cases, during a PCR experiment, reaction and environmental conditions related to each tube can influence fluorescence, and the fluorescence signal may fluctuate over time creating a background signal or baseline signal. This baseline signal can be determined, e.g, using the initial cycles of the PCR experiment used to detect an analyte. Alternatively, the background or baseline signal can be determined using a separate experiment. A standard deviation calculated from the mean of the baseline signal can then be used to establish a threshold. In some cases, the adjustable factor can be a factor of about 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50, 100 or more. In some cases, the adjustable factor can be calculated prior to the start of an experiment. In some cases, the adjustable factor can vary with the type of chromophore used. In some cases, the

adjustable factor can vary from experiment to experiment. In some cases, the adjustable factor can vary from instrument to instrument. In some cases, the adjustable factor is calculated for each instrument. In some cases, a threshold is established above the baseline signal. In some cases, a threshold is established below the baseline signal. In some cases, an observed signal (e.g. the signal that is measured to detect an analyte) crosses the threshold level. In some cases, an observed signal does not cross the threshold level. In some cases, the observed signal that crosses the threshold indicates a presence of the product analyte. In some cases, the observed signal that does not cross the threshold indicates an absence of the product analyte.

[00133] In some cases, a reference signal or reference signal range is established which can be used, for example, as a control for any of the methods described herein. In some cases, the reference signal or reference signal range can be used to determine whether an amplification or polymerization reaction described herein is performed successfully. For example, prior to initiating a PCR experiment involving the use of a chromophore, a reference signal range for the chromophore can be generated. The reference signal range can be generated by determining the values of two components, X and Y, such that the reference signal range is $X \pm Y$. In some cases, the X component is the mean fluorescence signal of the chromophore with respect to temperature and time, and the Y component is the standard deviation of X. When used as a control during a PCR reaction, a signal from each denaturation step can be compared with the reference signal or reference signal range. A signal outside of the reference signal range can indicate that the reaction has failed while a signal within the reference signal range can indicate that the reaction has succeeded. In some cases, the reference signal or reference signal range can be used as a cycle-by-cycle control. In some cases, the reference signal or reference signal range can be used as an internal control. In some cases, the method of calculating the reference signal or reference signal range is the same as calculating the baseline signal, e.g. establishing the mean of a fluorescence signal and calculating its standard deviation.

[00134] In some cases, the denaturation signal is used for normalization during an amplification or polymerization experiment. For example, when the denaturation signal is within the reference signal range, the denaturation signal can be used for normalization. During the course of each amplification or polymerization cycle, the denaturation signal can be used to normalize the signal measured during the annealing step, thereby generating an internal normalization for each cycle, e.g. similar to the chopper stabilization where the signal

can be reset to a particular value or unit, for example, a value of 1. In some cases, the denaturation signal is used for internal or self normalization. In some cases, the methods described herein use the denaturation signal of each cycle for a cycle-by-cycle self-normalization.

[00135] In some cases, the annealing signal is used to determine the presence or absence of an analyte. In some cases, when the annealing signals are compared between consecutive steps using the cycle-by-cycle self-normalization method described previously, a relative change in signals can be determined. In some cases, the relative signal change can be referred to as a relative quantitation of signals. In some cases, the relative quantitation of signals is used to determine the presence or absence of an analyte. In some cases, the annealing signal is compared to a standard to determine the presence of an analyte. In some cases, the standard is referred to as a standard ladder or a control signature profile, described elsewhere herein in this disclosure. In some cases, the comparison of the annealing signal to a standard ladder is referred to as a relative quantification of the analyte. In some cases, the methods described herein use a relative quantification method to determine the presence or absence of an analyte.

[00136] In some cases, the methods presented in this disclosure may be used with any quantifiable signal. As described herein and elsewhere herein, a coding scheme may be utilized to indicate a multiplicity of signals based on color. In some cases, the coding scheme is equally applicable to any other method providing a quantifiable signal, including an electrochemical signal and a chemiluminescent signal.

[00137] In some cases, if a fluorescent signal is employed, the number of analytes that can be encoded may be further expanded by utilizing additional fluorophores. For example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more fluorophores may be used. In some cases, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 fluorophores may be used. In some cases, fewer than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 fluorophores may be used.

[00138] Signals can be measured and compared at various points during a detection method described herein. For example, during an amplification or polymerization reaction (e.g., a PCR reaction or process), pairwise signals can be measured. In some cases, a signal can be measured: before annealing, during or after annealing of the primers to the template (e.g., analyte); before, during or after denaturing the double stranded template; or before the annealing step or after the denaturing step. A signal signature as described herein can be generated using these measurements.

C. *Signature Profiles*

[00139] A signature profile typically comprises a plurality of signals. In some cases, a signal includes an electrochemical signal, a chemiluminescence signal and a fluorescence signal. In some cases, a signature profile contains a plurality of fluorescence signals. In some cases, a profile curve is generated from the plurality of fluorescence signals. In some cases, a signature profile contains an initial fluorescence signal and an end-point fluorescence signal. In some cases, a signature profile contains signals measured during the annealing step of an amplification or polymerization reaction. In some cases, a fluorescence signal is influenced by external factors. In some cases, the external factors include temperature, pH, organic and inorganic agents (e.g. salts, urea, DMSO) and addition or removal of chromophores.

[00140] In some cases, signature profiles are generated from different types of detection experiments. In some cases, a signature profile generated from a polynucleotide morphology study is referred to as a morphology curve. In some cases, a signature profile generated from a denaturation study is referred to as a melt curve. In some cases, a signature profile generated from a persistence length study is referred to as a length curve. In some cases, a signature profile generated from a single-nucleotide polymorphism (SNP) study is referred to as a SNP curve.

[00141] In some cases, the change in signal can be calculated as a percentage of change. In some cases, the percentage of signal change is 0.01, 0.1, 0.2, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 150, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 10,000%. In some cases, the percentage of signal change is about 0.01, 0.1, 0.2, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38,

39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 150, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 10,000%.

VII. Analytical Techniques and Instrumentation

[00142] The methods described in this disclosure are compatible with a variety of amplification or polymerization methods, including polymerase chain reaction (PCR), ligase chain reaction (LCR), replicase-mediated amplification, strand-displacement amplification (SDA), “rolling circle” types of amplification, and various transcription associated amplification or polymerization methods. See, e.g., PCR amplification: U.S. Pat. Nos. 4,683,195, 4,683,202, and 4,800,159; LCR amplification: U.S. Pat. No. 5,516,663 and EP 0320308 B1; replicase-mediated amplification: U.S. Pat. No. 4,786,600; SDA amplification: U.S. Pat. Nos. 5,422,252 and 5,547,861; rolling circle types of amplification: U.S. Pat. Nos. 5,714,320 and 5,834,252; and transcription associated amplification: U.S. Pat. Nos. 5,399,491, 5,554,516, 5,130,238, 5,437,990, 4,868,105 and 5,124,246, PCT Pub. WO 1988/010315 A1 and US Pub. 2006-0046265 A1, which are hereby incorporated by reference.

[00143] In some cases, the polymerase chain reaction (PCR) is a multiplex-PCR, a variable number of tandem repeats (VNTR) PCR, an asymmetric PCR, long PCR, a nested PCR, a hot-start PCR, a Touchdown PCR, an assembly PCR, a colony PCR, a quantitative PCR (qPCR), an end point PCR, a reverse transcriptase PCR, a digital PCR or a droplet digital PCR. In some cases, the PCR process is a quantitative PCR process.

[00144] In some cases, the PCR amplification or polymerization step of the present disclosure can be performed by standard techniques well known in the art (See, e.g., Sambrook, E.F. Fritsch, and T. Maniatis, *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press (1989); U.S. Patent No. 4,683,202; and PCR Protocols: A Guide to Methods and Applications, Innis et al., eds., Academic Press, Inc., San Diego (1990) which are hereby incorporated by reference). PCR cycling conditions typically consist of an initial denaturation step, which can be performed by heating the PCR reaction mixture to a temperature ranging from about 80°C to about 105°C for times ranging from about 1 to about 10 min. Heat denaturation is typically followed by a number of cycles, ranging from about 1 to about 80 cycles, each cycle usually comprising an initial denaturation step, followed by a primer annealing step and concluding with a primer extension step. Enzymatic extension of the primers by a nucleic acid polymerase, e.g. Taq polymerase,

produces copies of the template (e.g., an analyte) that can be used as templates in subsequent cycles. In some cases, the denaturation temperature is about 85°C to about 100°C. In some cases, the denaturation temperature is 85°C, 86°C, 87°C, 88°C, 89°C, 90°C, 91°C, 92°C, 93°C, 94°C, 95°C, 96°C, 97°C, 98°C, 99°C or 100°C. In some cases, the denaturation temperature is at least 85°C, 86°C, 87°C, 88°C, 89°C, 90°C, 91°C, 92°C, 93°C, 94°C, 95°C, 96°C, 97°C, 98°C, 99°C or 100°C. In some cases, the denaturation temperature is no more than 85°C, 86°C, 87°C, 88°C, 89°C, 90°C, 91°C, 92°C, 93°C, 94°C, 95°C, 96°C, 97°C, 98°C, 99°C or 100°C. In some cases, the annealing temperature is about 25°C to about 80°C. In some cases, the annealing temperature is 25°C, 26°C, 27°C, 28°C, 29°C, 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C, 37°C, 38°C, 39°C, 40°C, 41°C, 42°C, 43°C, 44°C, 45°C, 46°C, 47°C, 48°C, 49°C, 50°C, 51°C, 52°C, 53°C, 54°C, 55°C, 56°C, 57°C, 58°C, 59°C, 60°C, 61°C, 62°C, 63°C, 64°C, 65°C, 66°C, 67°C, 68°C, 69°C, 70°C, 71°C, 72°C, 73°C, 74°C, 75°C, 76°C, 77°C, 78°C, 79°C or 80°C. In some cases, the annealing temperature is at least 25°C, 26°C, 27°C, 28°C, 29°C, 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C, 37°C, 38°C, 39°C, 40°C, 41°C, 42°C, 43°C, 44°C, 45°C, 46°C, 47°C, 48°C, 49°C, 50°C, 51°C, 52°C, 53°C, 54°C, 55°C, 56°C, 57°C, 58°C, 59°C, 60°C, 61°C, 62°C, 63°C, 64°C, 65°C, 66°C, 67°C, 68°C, 69°C, 70°C, 71°C, 72°C, 73°C, 74°C, 75°C, 76°C, 77°C, 78°C, 79°C or 80°C. In some cases, the annealing temperature is no more than 25°C, 26°C, 27°C, 28°C, 29°C, 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C, 37°C, 38°C, 39°C, 40°C, 41°C, 42°C, 43°C, 44°C, 45°C, 46°C, 47°C, 48°C, 49°C, 50°C, 51°C, 52°C, 53°C, 54°C, 55°C, 56°C, 57°C, 58°C, 59°C, 60°C, 61°C, 62°C, 63°C, 64°C, 65°C, 66°C, 67°C, 68°C, 69°C, 70°C, 71°C, 72°C, 73°C, 74°C, 75°C, 76°C, 77°C, 78°C, 79°C or 80°C. In some cases, the extension temperature is 25°C, 26°C, 27°C, 28°C, 29°C, 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C, 37°C, 38°C, 39°C, 40°C, 41°C, 42°C, 43°C, 44°C, 45°C, 46°C, 47°C, 48°C, 49°C, 50°C, 51°C, 52°C, 53°C, 54°C, 55°C, 56°C, 57°C, 58°C, 59°C, 60°C, 61°C, 62°C, 63°C, 64°C, 65°C, 66°C, 67°C, 68°C, 69°C, 70°C, 71°C, 72°C, 73°C, 74°C, 75°C, 76°C, 77°C, 78°C, 79°C or 80°C. In some cases, the extension temperature is at least 25°C, 26°C, 27°C, 28°C, 29°C, 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C, 37°C, 38°C, 39°C, 40°C, 41°C, 42°C, 43°C, 44°C, 45°C, 46°C, 47°C, 48°C, 49°C, 50°C, 51°C, 52°C, 53°C, 54°C, 55°C, 56°C, 57°C, 58°C, 59°C, 60°C, 61°C, 62°C, 63°C, 64°C, 65°C, 66°C, 67°C, 68°C, 69°C, 70°C, 71°C, 72°C, 73°C, 74°C, 75°C, 76°C, 77°C, 78°C, 79°C or 80°C. In some cases, the extension temperature is no more than 25°C, 26°C, 27°C, 28°C, 29°C, 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C, 37°C, 38°C, 39°C, 40°C, 41°C, 42°C, 43°C, 44°C, 45°C, 46°C,

47°C, 48°C, 49°C, 50°C, 51°C, 52°C, 53°C, 54°C, 55°C, 56°C, 57°C, 58°C, 59°C, 60°C, 61°C, 62°C, 63°C, 64°C, 65°C, 66°C, 67°C, 68°C, 69°C, 70°C, 71°C, 72°C, 73°C, 74°C, 75°C, 76°C, 77°C, 78°C, 79°C or 80°C. In some cases, the number of cycles ranges from about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75 to about 80 cycles.

[00145] The methods provided herein are suitable for use with a variety of detection methods. For example, the methods may be applied using an analytical technique that measures a fluorescent signal. For example, many real-time PCR, quantitative PCR and droplet digital PCR instruments comprising an excitation light source that enable the detection of fluorescent signals can be used. Therefore, the methods of the present disclosure can be readily applied using instruments widely used in the art.

VIII. Diseases

[00146] The methods described herein can be used, for example, to detect one or more analytes associated with a disease or one or more genetic variations (e.g., a SNP) associated with a disease. A disease is an abnormal condition of an organism. In some cases, the organism is a mammal, such as a human, non-human primate, mouse, rat, rabbit, goat, dog, cat, or cow. In some cases, the mammal is a human. In some cases, the human is a patient or subject. In some cases, the disease is a genetic disorder, an autoimmune disease, a neurological disease, a cardiovascular disease or a cancer.

[00147] A genetic disorder is a disease caused by one or more abnormalities in the genome. Exemplary genetic disorders include 22q11.2 deletion syndrome, Acrocephaly, Acute cerebral Gaucher's disease, Adrenal gland disorders, Adrenogenital syndrome, Alzheimer's disease, Amelogenesis imperfect, androgen insensitivity syndrome, anemia, Angelman syndrome, Apert syndrome, ataxia telangiectasia, Canavan disease, Charcot–Marie–Tooth disease, Color blindness, Cri du chat, Cystic fibrosis, Down syndrome, Duchenne muscular dystrophy, Haemochromatosis, Haemophilia, Klinefelter syndrome, Neurofibromatosis, Phenylketonuria, Polycystic kidney disease, Prader–Willi syndrome, Sickle-cell disease, Tay–Sachs disease and Turner syndrome.

[00148] An autoimmune disease is a disease caused when the immune system mistakenly attacks and destroys healthy body tissue. Exemplary autoimmune diseases include alopecia areata, autoimmune hemolytic anemia, autoimmune hepatitis, dermatomyositis, diabetes (type 1), several forms of juvenile idiopathic arthritis, glomerulonephritis, Graves' disease,

Guillain-Barré syndrome, idiopathic thrombocytopenic purpura, myasthenia gravis, several forms of myocarditis, multiple sclerosis, pemphigus/pemphigoid, pernicious anemia, polyarteritis nodosa, polymyositis, primary biliary cirrhosis, psoriasis, rheumatoid arthritis, scleroderma/systemic sclerosis, Sjögren's syndrome, systemic lupus erythematosus, several forms of thyroiditis, several forms of uveitis, vitiligo, and granulomatosis with polyangiitis (Wegener's).

[00149] Exemplary neurological diseases include attention deficit hyperactivity disorder (ADHD), ALS, Alzheimer's disease, bipolar disorder, Bell's palsy, birth defects of the brain and spinal cord, cerebral palsy, chronic fatigue syndrome, dyslexia, epilepsy, Guillain-Barré syndrome, multiple sclerosis, muscular dystrophy, neuropathy, neuromuscular and related diseases, Parkinson's disease, schizophrenia, scoliosis and spinal deformity.

[00150] Exemplary cardiovascular disease include acute myocardial infarction, angina, arrhythmia, atherosclerosis, cardiomegaly, cardiomyopathy, carotid artery disease, congenital heart disease, congestive heart failure, coronary artery disease, endocarditis, fluid around the heart, hypertension, infective endocarditis, mitral valve prolapsed, peripheral artery disease, stroke, and valvular heart disease.

[00151] Cancer is characterized by an abnormal growth of cells. Exemplary cancer include bladder, brain, breast, bladder, bone, cervical, colon, esophageal, kidney, liver, lung, ovarian, pancreatic, proximal or distal bile duct, prostate, skin, stomach, thyroid, and uterine cancer.

[00152] In some cases, the presence of an analyte or a genetic variation in an analyte (e.g., a SNP) can serve as a disease marker. In some cases, the method disclosed herein can be used to detect a disease marker. In some cases, the method disclosed herein can be applicable in determining the presence or absence or the type of diseases affecting a patient. For example, **Fig. 3** illustrates an overview of a method of providing a treatment in conjunction with a detection method described herein. **601** illustrates a clinician preparing to take a sample from a patient. In some cases, the sample can be a blood sample. In some cases, the sample can be a tissue sample. **602** illustrates a sample that is diluted into three concentrations (e.g. a separate concentration in each tube). **603** indicates the amplification or polymerization step (e.g., PCR). **604** illustrates products from the amplification or polymerization step. **605** depicts a clinician returning the results of an analysis to a patient.

IX. Compositions and Kits

[00153] This disclosure also provides compositions and kits for use with the methods described herein. The compositions may comprise any component, reaction mixture and/or intermediate described herein, as well as any combination thereof. For example, the disclosure provides detection reagents for use with the methods provided herein. Any suitable detection reagents may be provided, including a primer pair attached to two different chromophores (e.g., a fluorophore and a quencher), as described elsewhere in the specification.

[00154] In some cases, compositions comprise a first and a second primer or probe for the detection of at least one analyte wherein the primers are attached to either a fluorophore or a quencher at the 5' end. In some cases, compositions comprise primers attached at the 5' end with either a fluorophore or a quencher for the detection of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 100, 500, 1000, 5000, or 10000 analytes. In some cases, compositions comprise primers attached to multiple different fluorophores or quenchers wherein at least one fluorophore or quencher is at the 5' end for the detection of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 100, 500, 1000, 5000, or 10000 analytes. In some cases the compositions comprise multiple pairs of first and second primers, wherein each pair of first and second primers comprise either a fluorophore or a quencher at the 5' end. In some cases each pair of first and second primers comprise a different fluorophore and quencher from the remaining set of primers. In some cases the compositions comprise at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 100, 500, 1000, 5000, or 10000 pairs of first and second primers.

[00155] The present disclosure also provides kits for carrying out the methods of the invention. Accordingly, a variety of kits are provided in suitable packaging. The kits may be used for any one or more of the uses described herein, and, accordingly, may contain instructions for detecting the presence or absence of each analyte or a plurality of analytes. A kit may be a diagnostic kit, for example, a diagnostic kit suitable for the detection of one or more analytes, including the analytes recited herein. A kit may contain any of the compositions provided in this disclosure, including those recited above.

X. Services

[00156] The methods provided herein may also be performed as a service. For example, a service provider may obtain the identity of a plurality of analytes that a customer wishes to analyze. The service provider may then encode each analyte to be detected by any of the methods described herein and provide appropriate reagents to the customer for the assay. The customer may perform the assay and provide the results to the service provider for decoding. The service provider may then provide the decoded results to the customer. The customer may also encode analytes, generate probes, and/or decode results by interacting with software installed locally (at the customer's location) or remotely (e.g., on a server reachable through a network). Exemplary customers include clinical laboratories, physicians, manufacturers of food and consumer products, industrial manufacturers (e.g., petroleum companies) and the like. A customer or party may be any suitable customer or party with a need or desire to use the methods, systems, compositions, and kits of the invention.

A. Server

[00157] The methods provided herein may be processed on a server or a computer server (Fig. 4). The server **1101** includes a central processing unit (CPU, also "processor") **1105** which can be a single core processor, a multi core processor, or plurality of processors for parallel processing. A processor used as part of a control assembly may be a microprocessor. The server **1101** also includes memory **1110** (e.g. random access memory, read-only memory, flash memory); electronic storage unit **1115** (e.g. hard disk); communications interface **1120** (e.g. network adaptor) for communicating with one or more other systems; and peripheral devices **1125** which may include cache, other memory, data storage, and/or electronic display adaptors. The memory **1110**, storage unit **1115**, interface **1120**, and peripheral devices **1125** are in communication with the processor **1105** through a communications bus (solid lines), such as a motherboard. The storage unit **1115** can be a data storage unit for storing data. The server **1101** is operatively coupled to a computer network ("network") **1130** with the aid of the communications interface **1120**. A processor with the aid of additional hardware may also be operatively coupled to a network. The network **1130** can be the Internet, an intranet and/or an extranet, an intranet and/or extranet that is in communication with the Internet, a telecommunication or data network. The network **1130** in some cases, with the aid of the server **1101**, can implement a peer-to-peer network, which may enable devices coupled to the server **1101** to behave as a client or a

server. In general, the server may be capable of transmitting and receiving computer-readable instructions (e.g., device/system operation protocols or parameters) or data (e.g., sensor measurements, raw data obtained from detecting nucleic acids, analysis of raw data obtained from detecting nucleic acids, interpretation of raw data obtained from detecting nucleic acids, etc.) via electronic signals transported through the network **1130**. Moreover, a network may be used, for example, to transmit or receive data across an international border.

[00158] The server **1101** may be in communication with one or more output devices **1135** such as a display or printer, and/or with one or more input devices **1140** such as, for example, a keyboard, mouse, or joystick. The display may be a touch screen display, in which case it may function as both a display device and an input device. Different and/or additional input devices may be present such as an enunciator, a speaker, or a microphone. The server may use any one of a variety of operating systems, such as for example, any one of several versions of Windows, or of MacOS, or of Unix, or of Linux.

[00159] The storage unit **1115** can store files or data associated with the operation of a device or method described herein.

[00160] The server can communicate with one or more remote computer systems through the network **1130**. The one or more remote computer systems may be, for example, personal computers, laptops, tablets, telephones, Smart phones, or personal digital assistants.

[00161] In some situations a control assembly includes a single server **1101**. In other situations, the system includes multiple servers in communication with one another through an intranet, extranet and/or the Internet.

[00162] The server **1101** can be adapted to store device operation parameters, protocols, methods described herein, and other information of potential relevance. Such information can be stored on the storage unit **1115** or the server **1101** and such data can be transmitted through a network.

EXAMPLES

Example 1: DNA Sequences, Primers and Probes

[00163] Synthetic nucleic acid analytes from the Human Immunodeficiency Virus 1 (HIV-1) poly protease gene were chosen for exemplary detection studies using an amplification or polymerization technique. Templates from this gene, of lengths varying from 40bp-80bp, were synthesized.

[00164] Seven nucleic acid analytes from organisms of clinical relevance were chosen for exemplary detection studies using a multiplex PCR technique. These analytes include Influenza type A (InfA), influenza type B (InfB), respiratory syncytial virus type A (RsvA), respiratory syncytial virus type B (RsvB), human rhinovirus (Hrv), and human metapneumovirus (Hmpv). An additional analyte for human parainfluenza virus type 3 (PIV-3) was included to run as an orthogonal control. Multiplex analytes were coded using a binary scheme described elsewhere herein in this disclosure.

[00165] All oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, Iowa). Diagnostic sequences were input into IDT's OligoAnalyzer 3.1 tool. Probes and primer pairs for each analyte were chosen, from the OligoAnalyzer's set of generated sequences, to minimize homology with un-intended analytes, probes and primers. Forward primers for all analytes were synthesized with a fluorophore at the 5' end, while the reverse primes for all analytes were synthesized with a quencher at the 5' end. Sequence information is tabulated in Tables 1 to 8. Nucleic acid products were synthesized and lyophilized by IDT. All nucleic acid analytes were reconstituted in TE buffer, pH7 (Life Technologies, Carlsbad, CA). Dilutions were done using UltraPure RNase-free Water (Life Technologies, Carlsbad, CA).

Example 2: Primer Design

[00166] Specific primers and probes were designed using conserved regions of the analyte genes. Primers for Influenza A and B were designed in the nucleoprotein genes; Primers for RSV A and RSV B were designed in the conserved Fusion protein gene; Primers for HRV were designed in the 5'-UTR region; Primers for HMPV were designed in the Fusion protein gene; Primers for human PIV-3 were designed in conserved regions of the haemagglutinin genes.

[00167] Established GenBank Accession nos. were used for Influenza A, Influenza B, HRV, HMPV, and PIV-3. For RSV A and RSV B, primers used in previous studies (Jansen *et al.* "Development and evaluation of a four-tube real time multiplex PCR assay covering fourteen respiratory viruses, and comparison to its corresponding single target counterparts." *Journal of Clinical Virology* 51.3 (2011): 179-185; and Chun *et al.* "Dual priming oligonucleotide system for the multiplex detection of respiratory viruses and SNP genotyping of CYP2C19 gene." *Nucleic Acids Research* 35.6 (2007): e40) were inputted into BLAST and Accession nos. corresponding to high homology with complete coding sequence

information were chosen. Accession nos. for virus sequences used in primer design can be found in Tables 2-8. Sequences were uploaded into Primer Quest software (Integrated DNA Technologies, Coralville, Iowa) with parameters set to take into account primer size, amplicon size, G-C%, similarity of reaction kinetics. A BLAST search was performed to check the specificity of the sequences of the primers and probes. Chosen primer pairs were entered into OligoAnalyzer 3.1 and analyzed for self-dimerization and hetero-dimerization. Primer sequences and their properties can be found in Tables 2-8.

Table 1. HIV-1 Poly Protease Sequence Information

Sequence Information	HIV-1 40mer, 60mer, and 80mer
Source	HIV-1 Reference Sequence, Los Alamos National Laboratory
40Mer Template	5'- GGA AGC TCT ATT AGA TAC AGA CAC CTG TCA ACA TAA TTG G -3'
60Mer Template	5'- GGA AGC TCT ATT AGA TAC AGA TGA TAC AGT ATT AGA AGA AAC ACC TGT CAA CAT AAT TGG -3'
80Mer Template	5'- GGA AGC TCT ATT AGA TAC AGA TGA TAC AGT ATT AGA AGA AAT GAG TTT GCC AGG AAG ATG ACA CCT GTC AAC ATA ATT GG -3'
FWD Primer	5'- /5Cy3/GG AAG CTC TAT TAG ATA CAG -3'
RWD Primer	5'- /5IABkFQ/CC AAT TAT GTT GAC AGG TGT -3'

Table 2. Influenza type A sequence information

Sequence Information	Influenza A
Source	GenBank Accession #M23976.1 Influenza A
85Mer Template	5'- GTA GGG ATA GAC CCT TTC AAA CTG CTT CAA AAC AGC CAA GTA TAC AGC CTA ATC AGA CCG AAT GAG AAT CCA GCA CAC AAG AGT C -3'
FWD Primer	5'- GTA GGG ATA GAC CCT TTC AAA CTG -3'
RWD Primer	5'- GAC TCT TGT GTG CTG GAT TCT C -3'

TaqMan Probe	5'- /56-FAM/AG CCA AGT ATA CAG CCT AAT CAG ACC GA/3BHQ_1/ -3'
FWD qPrimer	5'- /5Cy3/GT AGG GAT AGA CCC TTT CAA ACT G -3'
RWD qPrimer	5'- /5IABkFQ/GA CTC TTG TGT GCT GGA TTC TC -3'
FWD Mistake qPrimer	5'- /5Cy3/GT AGG GAG AGA CCC TTT CAA ACT G -3'

Table 3. Influenza type B sequence information

Sequence Information	Influenza B
Source	GenBank Accession # AB036876 Influenza B
81Mer Template	5'- GTG CTT CCC ATA AGC ATT TAC GCC AAA ATA CCT CAA CTA GGG TTC AAC GTT GAA GAG TAC TCT ATG GTT GGG TAT GAA GCC -3'
FWD Primer	5'- GTG CTT CCC ATA AGC ATT TAC G -3'
RWD Primer	5'- GGC TTC ATA CCC AAC CAT AGA G -3'
TaqMan Probe	5'- /56-FAM/CC TCA ACT AGG GTT CAA CGT TGA AGA GT/3BHQ_1/ -3'
FWD qPrimer	5'- /5Cy3/GT GCT TCC CAT AAG CAT TTA CG -3'
RWD qPrimer	5'- /5IABkFQ/GG CTT CAT ACC CAA CCA TAG AG -3'

Table 4. Respiratory syncytial virus type A sequence information

Sequence Information	Respiratory syncytial virus type A
Source	GenBank Accession # JX627336.1 :5726-7450 Human respiratory syncytial virus strain RSVA/GN435/11, complete genome
85Mer Template	5'- GTT GGA AAC TAC ACA CAT CTC CTC TAT GTA CAA CCA ACA CAA AGG AAG GAT CCA ACA TCT GCT TAA CAA GAA CCG ACA GAG GAT G -3'

FWD Primer	5'- GTT GGA AAC TAC ACA CAT CTC CTC -3'
RWD Primer	5'- CAT CCT CTG TCG GTT CTT GTT AAG -3'
TaqMan Probe	5'- /56-FAM/CC AAC ACA AAG GAA GGA TCC AAC ATC TG/3BHQ_1/ -3'
FWD qPrimer	5'- /5Cy3/GT TGG AAA CTA CAC ACA TCT CCT C -3'
RWD qPrimer	5'- /5IABkFQ/CA TCC TCT GTC GGT TCT TGT TAA G -3'

Table 5. Respiratory syncytial virus type B sequence information

Sequence Information	Respiratory syncytial virus type B
Source	GenBank Accession # JX682822.1
81Mer Template	5'- CCT CAC CTC AAG TCA GAA CAT AAC TGA GGA GTT TTA CCA ATC GAC ATG TAG TGC AGT TAG CAG AGG TTA CTT GAG TGC TTT -3'
FWD Primer	5'- CCT CAC CTC AAG TCA GAA CAT AAC -3'
RWD Primer	5'- AAA GCA CTC AAG TAA CCT CTG C -3'
TaqMan Probe	5'- /56-FAM/AC CAA TCG ACA TGT AGT GCA GT/3BHQ_1/ -3'
FWD qPrimer	5'- /5Cy3/CC TCA CCT CAA GTC AGA ACA TAA C -3'
RWD qPrimer	5'- /5IABkFQ/AA AGC ACT CAA GTA ACC TCT GC -3'

Table 6. Human rhinovirus sequence information

Sequence Information	Human rhinovirus
Source	GenBank Accession # AF108174.1
81Mer Template	5'- ACA ATG GAC AAG GTG TGA AGA GCC CCG TGT GCT CGC TTT GAG TCC TCC GGC CCC TGA ATG TGG CTA ACC TTA ACC CTG CAG CTA G- 3'
FWD Primer	5'- ACA ATG GAC AAG GTG TGA AGA G -3'

RWD Primer	5'- CTA GCT GCA GGG TTA AGG TTA G -3'
TaqMan Probe	5'- /56-FAM/TG TGC TCG CTT TGA GTC CTC CG/3BHQ_1/-3'
FWD qPrimer	5'- /5Cy3/AC AAT GGA CAA GGT GTG AAG AG -3'
RWD qPrimer	5'- /5IABkFQ/CT AGC TGC AGG GTT AAG GTT AG -3'

Table 7. Human metapneumovirus sequence information

Sequence Information	Human metapneumovirus
Source	GenBank Accession # AF371337.2 :3052-4671 Human metapneumovirus isolate 00-1, complete genome)
88Mer Template	5'- GAG AGC ATT GAG AAC AGT CAG GCC TTG GTG GAT CAA TCA AAC AGA ATC CTA AGC AGT GCA GAG AAA GGA AAC ACT GGC TTC ATC ATT G- 3'
FWD Primer	5'- GAG AGC ATT GAG AAC AGT CAG G -3'
RWD Primer	5'- CAA TGA TGA AGC CAG TGT TTC C -3'
TaqMan Probe	5'- /56-FAM/AC AGA ATC CTA AGC AGT GCA GAG A/3BHQ_1/ -3'
FWD qPrimer	5'- /5Cy3/GA GAG CAT TGA GAA CAG TCA GG -3'
RWD qPrimer	5'- /5IABkFQ/CA ATG ATG AAG CCA GTG TTT CC -3'

Table 8. Parainfluenza virus type 3 sequence information

Sequence Information	Parainfluenza virus type 3
Source	GenBank Accession # gi 403376 emb Z26523.1 Human parainfluenza virus type 3 HN gene for hemagglutinin-neuraminidase)
86Mer Template	5'- TCG AGA GTG AAC CCA GTC ATA ACT TAC TCA ACA GCA ACC GAA AGA GTA AAC GAG CTG GCC ATC CGA AAC AGA ACA CTC TCA GCT GG -3'

FWD Primer	5' - TCG AGA GTG AAC CCA GTC ATA A -3'
RWD Primer	5' - CCA GCT GAG AGT GTT CTG TTT C -3'
TaqMan Probe 1	5' - /56-FAM/CC GAA AGA GTA AAC GAG CTG GCC A/3BHQ_1/ -3'
TaqMan Probe 2	5' - /5Cy3/CC GAA AGA GTA AAC GAG CTG GCC A/3BHQ_2/ -3'
TaqMan Probe 3	5' - /56-ROXN/CC GAA AGA GTA AAC GAG CTG GCC A/3BHQ_2/ -3'
TaqMan Probe 4	5' - /5Cy5/CC GAA AGA GTA AAC GAG CTG GCC A/3IAbRQSp/ -3'

Example 3: Polymerase Chain Reactions

[00168] PCR reactions were performed on a Roche 480 LightCycler instrument (Roche Applied Science, Penzberg, Germany). The PCR cycling reaction was run for 45 cycles, with a 60 sec hot-start at 95°C. The cycling conditions were: denaturation for 45sec at 95°C, annealing for 120sec at 45°C, and extension for 120sec at 58°C. Each experiment was run in sextuplicate, with a reaction volume of 15µL. Fluorescence measurements in 523nm-568nm (Cy3) and 615nm-670nm (Cy5) were taken at the end of annealing for every cycle. The change in fluorescence intensity between the first and last measurements (the quenched signal) was measured for each experiment.

[00169] Positive control experiments were performed to determine base-line quenching levels for each analyte and set of primers. Only analytes with their associated probes were cycled in experiments 1-10, and tabulated in Tables 9 - 18.

[00170] In each experiment, the uncertainty in the cycling data was determined by the spread of values in the last five cycles of the particular amplification reaction. This uncertainty did not scale with the value of the total signal, which implied that the source of uncertainty was instrumental rather than experimental. A 1x fluorophore (200nM) baseline was determined by statistical analysis of a set of data on 200nM concentration. The expected multiplicative signal levels were determined by multiplying this baseline by the multiplicity. The change in fluorescence intensity was used to assemble the expected signal levels in the chromatograms depicted in Figs. 5-9.

Example 4: Detection of HIV TPP analytes

[00171] Fig. 5 illustrates the successful detection of HIV TPP analytes using the analytes, primers and methods described in Examples 1-3. Initial experiments showed the quantitative PCR (qPCR) detection of 80bp HIV TPP analytes at varying concentrations from 10nM to 10pM (A-D). All successful detection curves exhibited the inverse-sigmoidal characteristic. Fig. 6 indicates the detection assay can be used to detect low concentrations of HIV TPP. The experiments demonstrated the detection assay was able to detect between 1pM and 100fM (Fig. 6A and Fig. 6B) of HIV TPP. Fig. 7 illustrates the length dependence of quenching. Experiments showed that the extent of quenching is strongly correlated to the separation length between a quencher and a fluorophore (Fig. 7A-7C). Analytes were varied in length from 40bp to 120bp for a single set of primers.

Example 5: Detection of single nucleotide polymorphisms

[00172] Fig. 8 illustrates the detection of a single nucleotide polymorphism. Signal level changed significantly (approximately a 35% reduction in signal) when a mis-priming event occurred (Fig. 8A and Fig. 8B). The observed effect suggests the mechanism of signal change involved the electron transport between chromophores of the labeled analyte.

Example 6: Multiplex detection using binary coding of analytes

[00173] Fig. 9 illustrates the binary coding of the analytes. Analytes in a 3-plex CY3 assay were coded using binary spaced primer concentrations. A TaqMan positive control sequence was amplified in every reaction to confirm that the PCR completed successfully.

Example 7: Tagged Primer PCR

[00174] This example describes a method of directly detecting a DNA analyte in a sample by incorporating chromophores onto primers and performing a PCR reaction. This method directly detects a PCR product, and does not require a secondary detection technique, such as a gel electrophoresis. For instance, an exemplary analyte is illustrated in Fig. 10A. A forward (FWD) and reverse or rewind (RWD) primers are designed such that a quencher and fluorophore are ligated to the 5' of each primer, respectively (Fig. 10B). The primers are selected in appropriate concentrations to limit unbound quenching. Before the first cycle of the PCR reaction is run, the total fluorescence intensity is 10. During the first cycle of the

PCR, this intensity level is typically unchanged, as there is no DNA analyte present that incorporates both a fluorophore and a quencher (Fig. 10C). A decrease in signal is observed upon completion of a second PCR cycle (Figs. 10D and 10E). As the cycles progress, on average, the total fluorescent signal decreases by a factor of 2 per cycle. Therefore, the fluorescent intensity at the end of cycle two is approximately $10/2$ because the fluorophore on the anti-sense strand is quenched by the quencher on the sense strand. This attenuation of the fluorescent signal continues until the supply of primers for that analyte is exhausted. In this way, a “dark-field” measurement for the DNA analyte is obtained by looking at the intensity of fluorescence after each cycle.

Example 8: Multiplex detection using various chromophore combinations

[00175] This example describes a technique for distinguishing multiple DNA analytes in a single PCR reaction using various chromophore combinations for each pair of analyte-specific primers. Color multiplexing different strands as illustrated in Fig. 11 is used to distinguish between multiple analytes. Dark-field curves are generated after performing PCR. From these dark-field curves, the initial concentrations are interpolated.

Example 9: Multiplex detection using a single chromophore combination

[00176] This example describes a technique for distinguishing multiple DNA analytes of varying lengths in a single PCR reaction using the same chromophore combination for each pair of analyte-specific primers. Analytes of differing lengths (e.g., 40bp, 60bp, and 80bp) are detected using a single fluorophore/quencher combination. Longer analytes have a greater separation between the quencher and the fluorophore, and thus exhibit different end point fluorescence levels. In superposition, different combinations of analytes code for different fluorescence levels. Further discrimination of nucleic acid analytes is performed using a melt curve analysis. Lower molecular weight analytes denature at lower temperatures. By measuring fluorescence as a function of increasing temperature, multiple analytes in a single color channel are quantified.

Example 10: Detection of morphology

[00177] This example describes the characterization of the morphology of an analyte (e.g., a DNA analyte) using multiple chromophores. The DNA analyte illustrated in Fig. 12 has a particular morphology at a given temperature. This morphology results in a fluorescence

signature. Changing the temperature results in a different morphology of the DNA analyte, which in turn results in a different fluorescence signature (Fig. 13). The signatures for the morphology of analytes (such as the DNA molecule shown in Fig. 12) are obtained by ramping of the temperature and determining fluorescent signatures. By superimposing these signatures, a three dimensional folding model for the DNA molecule is obtained.

[00178] While preferred disclosures of the present disclosure have been shown and described herein, it will be obvious to those skilled in the art that such disclosures are provided by way of example only. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the present disclosure. It should be understood that various alternatives to the disclosures described herein may be employed in practicing the invention. It is intended that the following claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.

Table 9. Experiment 01 Cocktail

Reagents	Concentration	Volume Added
UltraPure H ₂ O	-	70μL
Taq 5x Master Mix	-	25μL
Templates	Concentration	Volume Added
Poly Protease 40mer	12nM	5μL
Primers	Concentration	Volume Added
Poly Protease FWD qPrimer	1μM	5μL
Poly Protease RWD qPrimer	1μM	5μL

Table 10. Experiment 02 Cocktail

Reagents	Concentration	Volume Added
UltraPure H ₂ O	-	70μL
Taq 5x Master Mix	-	25μL
Templates	Concentration	Volume Added
Poly Protease 60mer	10nM	5μL
Primers	Concentration	Volume Added

Poly Protease FWD qPrimer	1 μ M	5 μ L
Poly Protease RWD qPrimer	1 μ M	5 μ L

Table 11. Experiment 03 Cocktail

Reagents	Concentration	Volume Added
UltraPure H ₂ O	-	70 μ L
Taq 5x Master Mix	-	25 μ L
Templates	Concentration	Volume Added
Poly Protease 80mer	10nM	5 μ L
Primers	Concentration	Volume Added
Poly Protease FWD qPrimer	1 μ M	5 μ L
Poly Protease RWD qPrimer	1 μ M	5 μ L

Table 12. Experiment 04 Cocktail

Reagents	Concentration	Volume Added
UltraPure H ₂ O	-	70 μ L
Taq 5x Master Mix	-	25 μ L
Templates	Concentration	Volume Added
Poly Protease 45mer	10nM	5 μ L
Primers	Concentration	Volume Added
Poly Protease FWD qPrimer	1 μ M	5 μ L
Poly Protease RWD qPrimer	1 μ M	5 μ L

Table 13. Experiment 05 Cocktail

Reagents	Concentration	Volume Added
UltraPure H ₂ O	-	70 μ L
Taq 5x Master Mix	-	25 μ L
Templates	Concentration	Volume Added
Poly Protease 50mer	10nM	5 μ L

Primers	Concentration	Volume Added
Poly Protease FWD qPrimer	1 μ M	5 μ L
Poly Protease RWD qPrimer	1 μ M	5 μ L

Table 14. Experiment 06 Cocktail

Reagents	Concentration	Volume Added
UltraPure H ₂ O	-	70 μ L
Taq 5x Master Mix	-	25 μ L
Templates	Concentration	Volume Added
Poly Protease 55mer	10nM	5 μ L
Primers	Concentration	Volume Added
Poly Protease FWD qPrimer	1 μ M	5 μ L
Poly Protease RWD qPrimer	1 μ M	5 μ L

Table 15. Experiment 07 Cocktail

Reagents	Concentration	Volume Added
UltraPure H ₂ O	-	70 μ L
Taq 5x Master Mix	-	25 μ L
Templates	Concentration	Volume Added
Poly Protease 100mer	10nM	5 μ L
Primers	Concentration	Volume Added
Poly Protease FWD qPrimer	1 μ M	5 μ L
Poly Protease RWD qPrimer	1 μ M	5 μ L

Table 16. Experiment 08 Cocktail

Reagents	Concentration	Volume Added
UltraPure H ₂ O	-	70 μ L
Taq 5x Master Mix	-	25 μ L
Templates	Concentration	Volume Added

Poly Protease 120mer	10nM	5μL
Primers	Concentration	Volume Added
Poly Protease FWD qPrimer	1μM	5μL
Poly Protease RWD qPrimer	1μM	5μL

Table 17. Experiment 09 Cocktail

Reagents	Concentration	Volume Added
UltraPure H ₂ O	-	105μL
Taq 5x Master Mix	-	75μL
Templates	Concentration	Volume Added
Influenza A 85mer	10nM	5μL
Primers	Concentration	Volume Added
Influenza A FWD qPrimer	300nM	5μL
Influenza A RWD qPrimer	300nM	5μL

Table 18. Experiment 10 Cocktail

Reagents	Concentration	Volume Added
UltraPure H ₂ O	-	105μL
Taq 5x Master Mix	-	75μL
Templates	Concentration	Volume Added
Influenza B 81mer	10nM	5μL
Primers	Concentration	Volume Added
Influenza B FWD qPrimer	300nM	5μL
Influenza B RWD qPrimer	300nM	5μL

CLAIMS**WHAT IS CLAIMED IS:**

1. A method of detecting at least one polynucleotide analyte in a sample, comprising:
 - a) combining the sample with a first primer and a first oligonucleotide, wherein a first chromophore is attached to the first primer, a second chromophore is attached to the first oligonucleotide, the first primer and the first oligonucleotide are specific for a first polynucleotide analyte and the first chromophore is different from the second chromophore;
 - b) measuring a first signal generated by the first and second chromophores;
 - c) performing at least one polymerization reaction with the first primer using the first polynucleotide analyte as a template; and
 - d) measuring a second signal generated by the first and second chromophores; wherein the first and second signals are used to detect the first polynucleotide analyte.
2. The method of claim 1, wherein the first oligonucleotide is a second primer.
3. The method of claim 1, wherein the first chromophore is attached to the 5' end of the first primer.
4. The method of claim 1, wherein the first chromophore is an inorganic or organic dye, a fluorophore or a quencher.
5. The method of claim 4, wherein the first chromophore is a fluorophore.
6. The method of claim 5, wherein the fluorophore is 6-FAM (Fluorescein), 6-FAM (NHS Ester), Fluorescein dT, HEX, JOE (NHS Ester), MAX, TET, ROX, TAMRA, TARMMA (NHS Ester), TEX 615, ATTO 488, ATTO 532, ATTO 550, ATTO 565, ATTO Rho101, ATTO 590, ATTO 633, ATTO 647N, TYE 563, TYE 665 or TYE 705.
7. The method of claim 2, wherein the second chromophore is attached to the 5' end of the second primer.
8. The method of claims 1 or 7, wherein the second chromophore is an inorganic or organic dye, a fluorophore or a quencher.
9. The method of claim 8, wherein the second chromophore is a quencher.
10. The method of claim 9, wherein the quencher is Iowa Black FG, Iowa Black RG, BHQ1, BHQ2 or BHQ3.
11. The method of any of claims 1-10, wherein the first chromophore is a fluorophore and the second chromophore is a quencher.

12. The method of any of claims 1-11, further comprising comparing the first and second signals, wherein a change in the first and second signals indicates the presence of the first polynucleotide analyte.
13. The method of any of claims 1-12, wherein the change in the first and second signals is a decrease in fluorescent intensity.
14. The method of claim 13, wherein the decrease in fluorescent intensity is at least about a 30% decrease in signal.
15. The method of any of claims 1-14, wherein the second signal is measured after a second polymerization reaction.
16. The method of any of claims 1-15, wherein the polymerization reaction is a polymerase chain reaction process or an isothermal process.
17. The method of claim 16, wherein the polymerase chain reaction process is an end-point polymerase chain reaction process, a real-time polymerase chain reaction process, a digital polymerase chain reaction process, a droplet digital polymerase chain reaction process, or a quantitative polymerase chain reaction process.
18. The method of any of claims 1-17, wherein the first primer is a forward primer and the second primer is a reverse primer.
19. The method of any of claims 1-18, wherein the first primer is a reverse primer and the second primer is a forward primer.
20. The method of any of claims 1-19, wherein the first and second chromophores interact through an electron-transfer process.
21. The method of any of claims 1-20, wherein the first polynucleotide analyte is from about 10 to about 500 nucleotides in length.
22. The method of any of claims 1-21, wherein the concentration of the first polynucleotide analyte is from about 10 μ M to about 10aM.
23. The method of any of claims 1-22, wherein the first polynucleotide analyte is a DNA polynucleotide analyte.
24. The method of any of claims 1-22, wherein the first polynucleotide analyte is an RNA polynucleotide analyte.
25. The method of any of claims 1-24, wherein the first polynucleotide analyte comprises a genetic variation.
26. The method of claim 25, wherein the genetic variation comprises a substitution, an addition, a deletion or a translocation.

27. The method of claim 25, wherein the genetic variation comprises a single-nucleotide polymorphism (SNP).
28. The method of claim 1, wherein the at least one polynucleotide analyte is from a source selected from a human, a non-human mammal, a plant, a bacteria, a fungus, an archaea, a parasite, or a virus.
29. The method of claim 28, wherein the virus is a human immunodeficiency virus, an influenza type A virus, an influenza type B virus, a respiratory syncytial virus type A (RsvA), a respiratory syncytial virus type B virus (RsvB), a human rhinovirus (Hrv), a human metapneumovirus (Hmpv) or a human parainfluenza virus type 3 (PIV-3).
30. The method of any of claims 1-29, wherein the sample is a forensic sample, a clinical sample, a food sample, an environmental sample, a pharmaceutical sample, or a sample from a consumer product.
31. The method of any of claims 1-30, further comprising detecting at least one additional polynucleotide analyte within the sample with an additional fluorophore attached to a primer, wherein the first chromophore is a fluorophore with the same color as the additional fluorophore.
32. The method of any of claims 1-31, wherein the first primer is not attached to two or more chromophores.
33. The method of any of claims 1-32, wherein the first oligonucleotide is not attached to two or more chromophores.
34. The method of claim 1, wherein:
step b) further comprises measuring the first signal at a first denaturing step and a third signal at a first annealing step;
step d) further comprises measuring the second signal at a second denaturing step and a fourth signal at a second annealing step; and
further comprising comparing the first signal with the second signal to obtain a first ratio and comparing the third signal with the fourth signal to obtain a second ratio;
a presence of the first polynucleotide analyte is indicated when the first ratio is about 1 and the second ratio is greater than or less than 1.
35. The method of any of claims 1-33, wherein:
step a) further comprises combining the sample with a third primer and a second oligonucleotide, wherein a third chromophore is attached to the third primer and a

- fourth chromophore is attached to the second oligonucleotide, and the third primer and the second oligonucleotide are specific for a second polynucleotide analyte;
- step b) further comprises measuring a fifth signal generated by the third and fourth chromophores;
- step d) further comprises measuring a sixth signal generated by the third and fourth chromophores;
- wherein the fifth and sixth signals are used to detect the second polynucleotide analyte.
36. The method of claim 35, further comprising comparing the fifth and sixth signals, wherein a change in the fifth and sixth signals indicates the presence of the second polynucleotide analyte.
37. The method of claim 35 or 36, wherein the second oligonucleotide is a fourth primer.
38. A method of detecting at least one genetic variation in an analyte comprising:
- combining a first analyte with a first primer and a second primer, wherein a first chromophore is attached to the first primer, a second chromophore is attached to the second primer, at least one of the first and the second primers are specific for a first genetic variation in the first analyte and the first chromophore is different from the second chromophore;
 - measuring a first signal generated by the first and second chromophores;
 - performing at least one polymerization reaction with the first primer and the second primer using the first analyte as a template; and
 - measuring a second signal generated by the first and second chromophores; wherein the first and second signals are used to detect the first genetic variation in the first analyte.
39. The method of claim 38, further comprising comparing the first and second signals, wherein a change in the first and second signals indicates the presence of the genetic variation in the first analyte.
40. The method of claim 38 or 39, wherein the analyte is a polynucleotide analyte.
41. The method of any of claims 38-40, wherein the genetic variation comprises a substitution, an addition, a deletion or a translocation.
42. The method of any of claims 38-40, wherein the genetic variation comprises a single-nucleotide polymorphism (SNP).
43. The method of claim 42, wherein the first primer comprises a sequence encoding the SNP or the first primer binds to a region of the analyte encoding the SNP.

44. The method of claim 42 or 43, wherein the second primer comprises a sequence not encoding the SNP or the second primer comprises a sequence complementary to a region of the analyte not encoding the SNP.
45. The method of any of claims 38-44, wherein the first primer encodes a region of the analyte less than 500 base pairs apart from a region of the analyte encoded by the second primer.
46. The method of any of claims 38-45, wherein the change in signal is distinct for at least two of the mismatched base pairs selected from the group consisting of UU, UT, UG, UC, UA, AA, TT, GG, CC, AG, AC, TG and TC.
47. The method of any of claims 38-46, wherein the change in signal from a mismatched base pair is distinct from a change in signal from a complementary base pair.
48. The method of any of claims 38-47, wherein:
 - step a) further comprises combining the first analyte with a third primer and a fourth primer, wherein a third chromophore is attached to the third primer, a fourth chromophore is attached to the fourth primer, the third and the fourth primers are specific for a second genetic variation in the first analyte and the third chromophore is different from the fourth chromophore;
 - step b) further comprises measuring a third signal;
 - step d) further comprises measuring a fourth signal; and
 - further comprising comparing the third and fourth signals;wherein a change in the third and fourth signals indicates the presence of the second single genetic variation in the first analyte.
49. The method of any of claims 38-48, wherein:
 - step a) further comprises combining a second analyte with a third primer and a fourth primer, wherein a third chromophore is attached to the third primer, a fourth chromophore is attached to the fourth primer comprises, the third and the fourth primers are specific for a second genetic variation in the second analyte and the third chromophore is different from the fourth chromophore;
 - step b) further comprises measuring a third signal;
 - step d) further comprises measuring a fourth signal; and
 - further comprising comparing the third and fourth signals;wherein a change in the third and fourth signals indicates the presence of the second single genetic variation in the second analyte.

50. The method of any of claims 38-49, wherein step a) further comprises combining a second analyte with a third primer and a fourth primer, wherein the first chromophore is attached to the third primer, the second chromophore is attached to the fourth primer, and the third and the fourth primers are specific for a second genetic variation in the second analyte.
51. The method of any of claims 38-50, wherein the polymerization reaction is a PCR process or an isothermal reaction.
52. The method of claim 51, wherein the PCR process is an end-point PCR process, a digital PCR process, a real-time PCR process, a droplet digital PCR process, or a quantitative PCR process.
53. The method of any of claims 38-52, wherein the polymerization reaction is a quantitative PCR process.
54. The method of any of claims 38-53, wherein the detecting comprises a quantitative PCR method.
55. The method of any of claims 38-54, wherein the detecting comprises a quantitative PCR method and a second method.
56. The method of claim 55, wherein the second method is a digital PCR process.
57. The method of any of claims 38-56, wherein at least one SNP is detected in a gene.
58. The method of any of claims 38-57, wherein at least one SNP is associated with a disease.
59. The method of claim 58, wherein the disease is a genetic disorder, an autoimmune disease, a neurological disease, a cardiovascular disease, or a cancer.
60. A method of detecting a plurality of analytes, comprising:
 - a) combining the plurality of analytes with a plurality of primer pairs, wherein each primer pair is specific to a single analyte and each primer of the primer pair is attached to at least one chromophore;
 - b) measuring a first set of signals generated by the chromophores;
 - c) performing at least one polymerization reaction with the plurality of primer pairs using the plurality of analytes as templates; and
 - d) measuring a second set of signals generated by the chromophores;wherein the first and second set of signals are used to detect each analyte of the plurality of analytes.

61. The method of claim 60, further comprising:
 - e) repeating step c and d at least once; and
 - f) generating a set of signature profiles;wherein the presence of each analyte of the plurality of analytes is detected by comparing the set of signature profiles to a control set of signature profiles.
62. The method of claim 61, wherein the signature profile is an end-point signature profile or a signature curve.
63. The method of any of claims 60-62, wherein the plurality of analytes has different lengths.
64. The method of any of claims 60-63, wherein each of the plurality of primer pairs comprises a forward primer and a reverse primer.
65. The method of claim 64 wherein one, two or more chromophores are attached to the forward primer.
66. The method of claim 65, wherein one chromophore is attached to the 5' end of the forward primer.
67. The method of claim 64, wherein at least one chromophore is attached to the reverse primer.
68. The method of claim 67, wherein one chromophore is attached to the 5' end of the reverse primer.
69. The method of any of claims 60-68, wherein the chromophore is an inorganic or organic dye, a fluorophore or a quencher.
70. The method of claim 69, wherein the chromophore is a fluorophore.
71. The method of claim 69, wherein the chromophore is a quencher.
72. A method of generating a signature curve profile for a polynucleotide analyte, comprising:
 - a) contacting the polynucleotide analyte with a first primer and a second primer, wherein a first chromophore is attached to the first primer and a second chromophore is attached to the second primer, the first primer and the second primer are specific for the polynucleotide analyte and the first chromophore is different from the second chromophore;
 - b) measuring a first signal at a first temperature;
 - c) performing at least one polymerization reaction with the first primer and the second primer using the polynucleotide analyte as a template;

- d) measuring a second signal at the first temperature; and
 - e) repeating step c and d at least once;
wherein the signals create the signature curve profile of the polynucleotide analyte.
- 73. The method of claim 72, further comprising: f) changing the temperature; g) measuring a third signal at a second temperature; and h) repeating steps f and g at least once;
wherein the signals create the signature curve profile of the polynucleotide analyte.
 - 74. The method of claim 72 or 73, wherein the signature curve is a length curve, a morphology curve, a melt curve, or a SNP curve.
 - 75. The method of any of claims 72-74, wherein the polymerization reaction is a polymerase chain reaction process.
 - 76. The method of claim 75, wherein the polymerase chain reaction process is an end-point polymerase chain reaction process, a real-time polymerase chain reaction process, a digital polymerase chain reaction process or a quantitative polymerase chain reaction process.
 - 77. The method of any of claims 72-76, wherein the first chromophore is an inorganic or organic dye, a fluorophore or a quencher.
 - 78. The method of claim 77, wherein the first chromophore is a fluorophore.
 - 79. The method of claim 78, wherein the fluorophore is 6-FAM (Fluorescein), 6-FAM (NHS Ester), Fluorescein dT, HEX, JOE (NHS Ester), MAX, TET, ROX, TAMRA, TARMMA (NHS Ester), TEX 615, ATTO 488, ATTO 532, ATTO 550, ATTO 565, ATTO Rho101, ATTO 590, ATTO 633, ATTO 647N, TYE 563, TYE 665 or TYE 705.
 - 80. The method of any of claims 72-79, wherein the second chromophore is an inorganic or organic dye, a fluorophore or a quencher.
 - 81. The method of claim 80, wherein the second chromophore is a quencher.
 - 82. The method of claim 81, wherein the quencher is Iowa Black FG, Iowa Black RG, BHQ1, BHQ2 or BHQ3.
 - 83. The method of any of claims 72-82, wherein the polynucleotide analyte is a DNA polynucleotide analyte.
 - 84. The method of any of claims 72-82, wherein the polynucleotide analyte is an RNA polynucleotide analyte.

85. The method of claim 72, wherein the polynucleotide analyte is from a source selected from the group consisting of a human, a non-human mammal, a plant, a bacteria, an archaea, a fungus, a parasite, and a virus.
86. The method of claim 85, wherein the virus is a human immunodeficiency virus, an influenza type A virus, an influenza type B virus, a respiratory syncytial virus type A (RsvA), a respiratory syncytial virus type B virus (RsvB), a human rhinovirus (Hrv), a human metapneumovirus (Hmpv) or a human parainfluenza virus type 3 (PIV-3).
87. A method of monitoring an amplification reaction comprising:
- a) contacting a first polynucleotide analyte with: i) a first primer and a second primer, wherein the first primer and the second primer are specific for the first polynucleotide analyte; and ii) at least two chromophores capable of specific incorporation into a product amplified from the first polynucleotide analyte;
 - b) subjecting the combination in step (a) to a temperature capable of denaturing double-stranded DNA;
 - c) measuring a first signal in step (b) generated by the at least two chromophores;
 - d) subjecting the combination in step b) to a temperature capable of annealing polynucleotides;
 - e) measuring a second signal in step (d) generated by the at least two chromophores; and
 - f) repeating steps b) – e) to obtain a third signal and a fourth signal; and
 - g) comparing the first and the third signals to obtain a first ratio and the second and the fourth signals to obtain a second ratio;
wherein an amplification reaction occurs when the first ratio is about 1 and the second ratio is greater than or less than 1.
88. The method of claim 87, wherein one, two or more chromophores are attached to the first primer.
89. The method of claim 88, wherein one chromophore is attached to the 5' end of the first primer.
90. The method of claim 87, wherein one, two or more chromophores are attached to the second primer.
91. The method of claim 90, wherein one chromophore is attached to the 5' end of the second primer.
92. The method of any of claims 87-91, wherein the chromophore is an inorganic or organic dye, a fluorophore or a quencher.

93. A method of detecting a morphology of an analyte, comprising:
- a) providing a sample comprising the analyte, wherein a first chromophore, a second chromophore and a third chromophore are attached to the analyte, wherein the first, second and third chromophores are different;
 - b) measuring a signal from the first chromophore, the second chromophore and the third chromophore at a first and second temperature, wherein the first and second temperatures are different; and
 - c) using the measured signals to detect the morphology of the analyte.
94. The method of claim 93, wherein the analyte is a protein, a polypeptide, a lipid or a polynucleotide.
95. The method of claim 94, wherein the analyte is a polynucleotide.
96. The method of claim 95, wherein the polynucleotide is a DNA polynucleotide.
97. The method of claim 95, wherein the polynucleotide is an RNA polynucleotide.
98. The method of any of claims 93-97, wherein the first chromophore is an inorganic or organic dye, a fluorophore or a quencher.
99. The method of claim 98, wherein the first chromophore is a first fluorophore.
100. The method of claim 99, wherein the first fluorophore is 6-FAM (Fluorescein), 6-FAM (NHS Ester), Fluorescein dT, HEX, JOE (NHS Ester), MAX, TET, ROX, TAMRA, TARMA (NHS Ester), TEX 615, ATTO 488, ATTO 532, ATTO 550, ATTO 565, ATTO Rho101, ATTO 590, ATTO 633, ATTO 647N, TYE 563, TYE 665 or TYE 705.
101. The method of any of claims 93-100, wherein the second chromophore is an inorganic or organic dye, a fluorophore or a quencher.
102. The method of claim 101, wherein the second chromophore is a second fluorophore.
103. The method of claim 102, wherein the second fluorophore is 6-FAM (Fluorescein), 6-FAM (NHS Ester), Fluorescein dT, HEX, JOE (NHS Ester), MAX, TET, ROX, TAMRA, TARMA (NHS Ester), TEX 615, ATTO 488, ATTO 532, ATTO 550, ATTO 565, ATTO Rho101, ATTO 590, ATTO 633, ATTO 647N, TYE 563, TYE 665 or TYE 705.
104. The method of any of claims 93-103, wherein the third chromophore is an inorganic or organic dye, a fluorophore or a quencher.
105. The method of claim 104, wherein the third chromophore is a quencher.

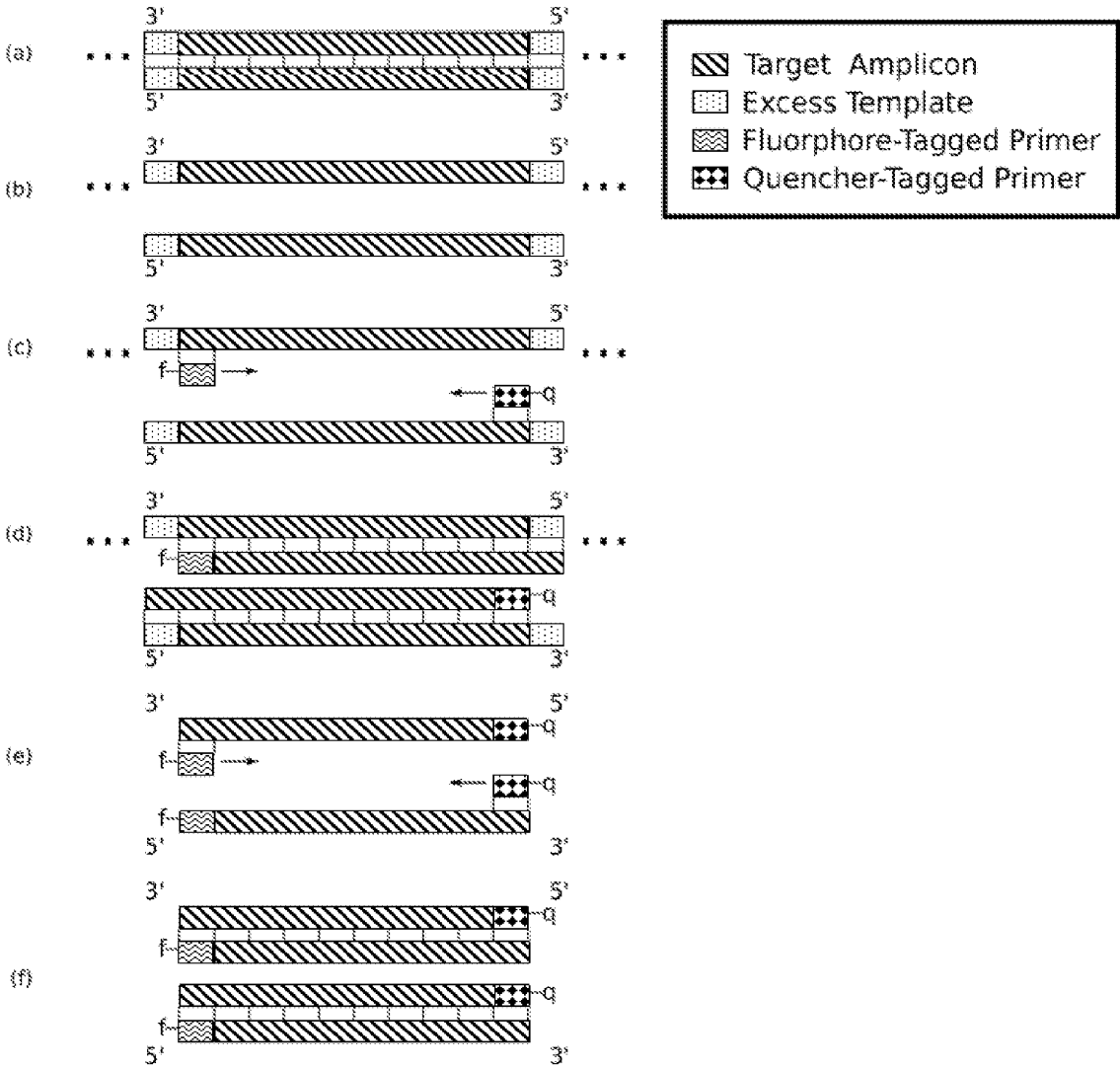
106. The method of claim 105, wherein the quencher is Iowa Black FG, Iowa Black RG, BHQ1, BHQ2 or BHQ3.
107. The method of any of claims 93-106, wherein the analyte comprises more than one fluorophore.
108. The method of any of claims 93-106, wherein the analyte comprises more than one quencher.
109. The method of any of claims 93-108, wherein the polynucleotide comprises a quencher at its 3' end.
110. A method of detecting a polynucleotide analyte comprising:
- a. combining the polynucleotide analyte with at least two chromophores, wherein the at least two chromophores are each attached to a separate polynucleotide that is complementary to a region within the polynucleotide analyte;
 - b. performing at least one polymerization reaction to incorporate the at least two chromophores into products of the polymerization reaction; and
 - c. detecting a fluorescent intensity from the at least two chromophores at a first timepoint and a second timepoint, wherein the second timepoint is later than the first timepoint and wherein a decrease in fluorescent intensity at the second timepoint relative to the first timepoint is indicative of the presence of the polynucleotide analyte.
111. The method of claim 110, wherein the polymerization reaction is a polymerase chain reaction.
112. The method of claim 110, wherein the first timepoint is after step a) and the second timepoint is after step b).
113. The method of claim 110, wherein the products of the at least one polymerization reaction each comprise a first polynucleotide strand and a second polynucleotide strand, wherein the first polynucleotide strand and the second polynucleotide strand are complementary.
114. The method of claim 110, wherein the at least two chromophores are different.
115. The method of claim 110, wherein the at least two chromophores comprise a fluorophore.
116. The method of claim 110, wherein the at least two chromophores comprise a fluorophore and a quencher.

117. The method of claim 116, wherein the fluorophore is incorporated into the first polynucleotide strand and the quencher is incorporated into the second polynucleotide strand.
118. The method of claim 116, wherein the fluorophore is incorporated at the 5' end of first polynucleotide strand and the quencher is incorporated at the 5' end of the second polynucleotide strand.
119. A kit comprising: (a) a first primer attached to a first chromophore; and (b) a second primer attached to a second chromophore, wherein the first and second chromophores are different chromophores.
120. The kit of claim 119, wherein the first chromophore is a first fluorophore.
121. The kit of claim 119 or 120, wherein the second chromophore is a second fluorophore.
122. The kit of claim 119 or 120, wherein the second chromophore is a quencher.
123. The kit of claim 120 or 121, wherein the first or second fluorophore is 6-FAM (Fluorescein), 6-FAM (NHS Ester), Fluorescein dT, HEX, JOE (NHS Ester), MAX, TET, ROX, TAMRA, TARMA (NHS Ester), TEX 615, ATTO 488, ATTO 532, ATTO 550, ATTO 565, ATTO Rho101, ATTO 590, ATTO 633, ATTO 647N, TYE 563, TYE 665 or TYE 705.
124. The kit of claim 122, wherein the quencher is Iowa Black FG, Iowa Black RG, BHQ1, BHQ2 or BHQ3.
125. The kit of any one of claims 119-124, wherein the kit comprises at least three primers, wherein each primer is attached to a different chromophore.
126. The kit of any one of claims 119-125, wherein the first primer comprises a sequence complementary to a target analyte.
127. The kit of any one of claims 119-125, wherein the second primer comprises a sequence complementary to a target analyte.
128. A kit comprising a first oligonucleotide probe attached to exactly one first chromophore and a second oligonucleotide probe attached to exactly one second chromophore, wherein the first and second chromophores are different.
129. The kit of claim 128, wherein the first chromophore is a fluorophore.
130. The kit of claims 128 or 129, wherein the second chromophore is a fluorophore.
131. The kit of claim 128, wherein the second chromophore is a quencher.
132. The kit of claims 129, or 130, wherein the first or second chromophore is a fluorophore selected from the group consisting of: the first or second fluorophore is 6-FAM

(Fluorescein), 6-FAM (NHS Ester), Fluorescein dT, HEX, JOE (NHS Ester), MAX, TET, ROX, TAMRA, TARMMA (NHS Ester), TEX 615, ATTO 488, ATTO 532, ATTO 550, ATTO 565, ATTO Rho101, ATTO 590, ATTO 633, ATTO 647N, TYE 563, TYE 665 or TYE 705.

133. The kit of claim 131, wherein the quencher is Iowa Black FG, Iowa Black RG, BHQ1, BHQ2 or BHQ3.

Fig. 1



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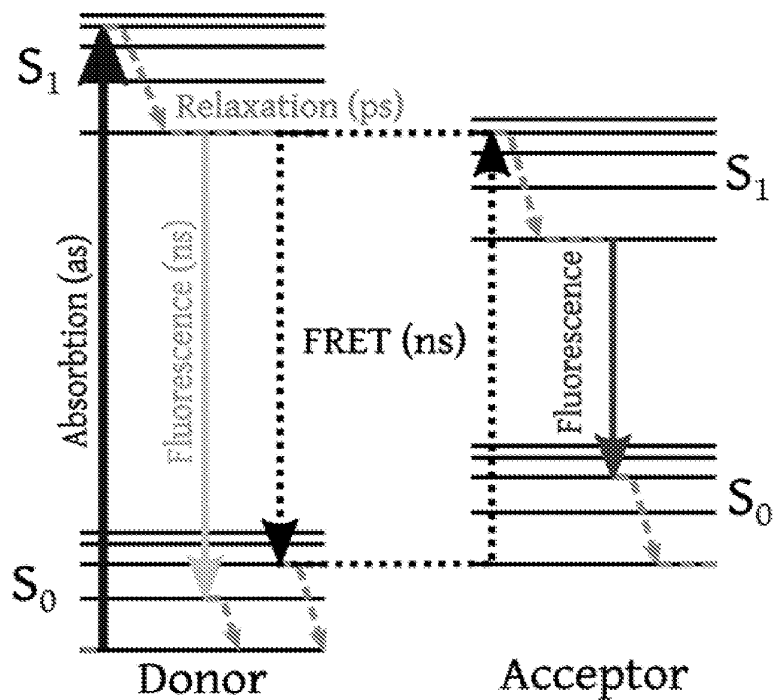
Fig. 2

A

$$R_0^6 = \frac{9 Q_0 (\ln 10) \kappa^2 J}{128 \pi^5 n^4 N_A}$$

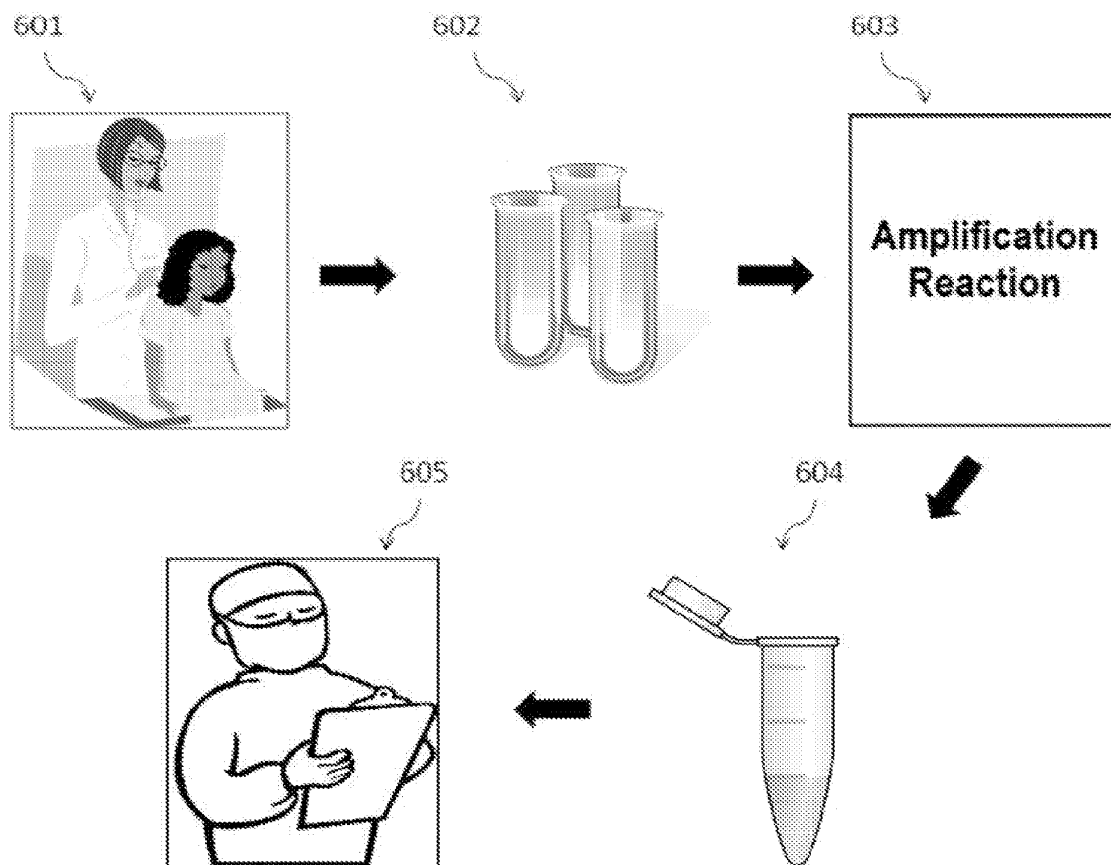
$$E = \frac{1}{1 + (r/R_0)^6}$$

B



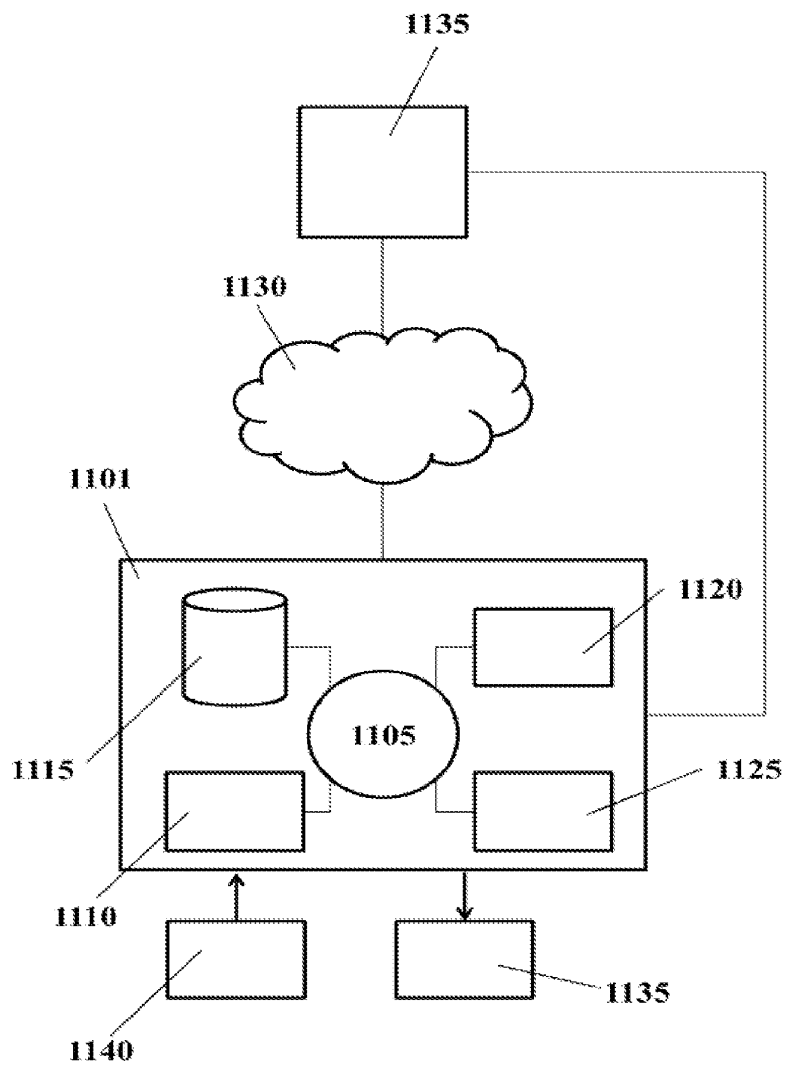
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Fig. 3

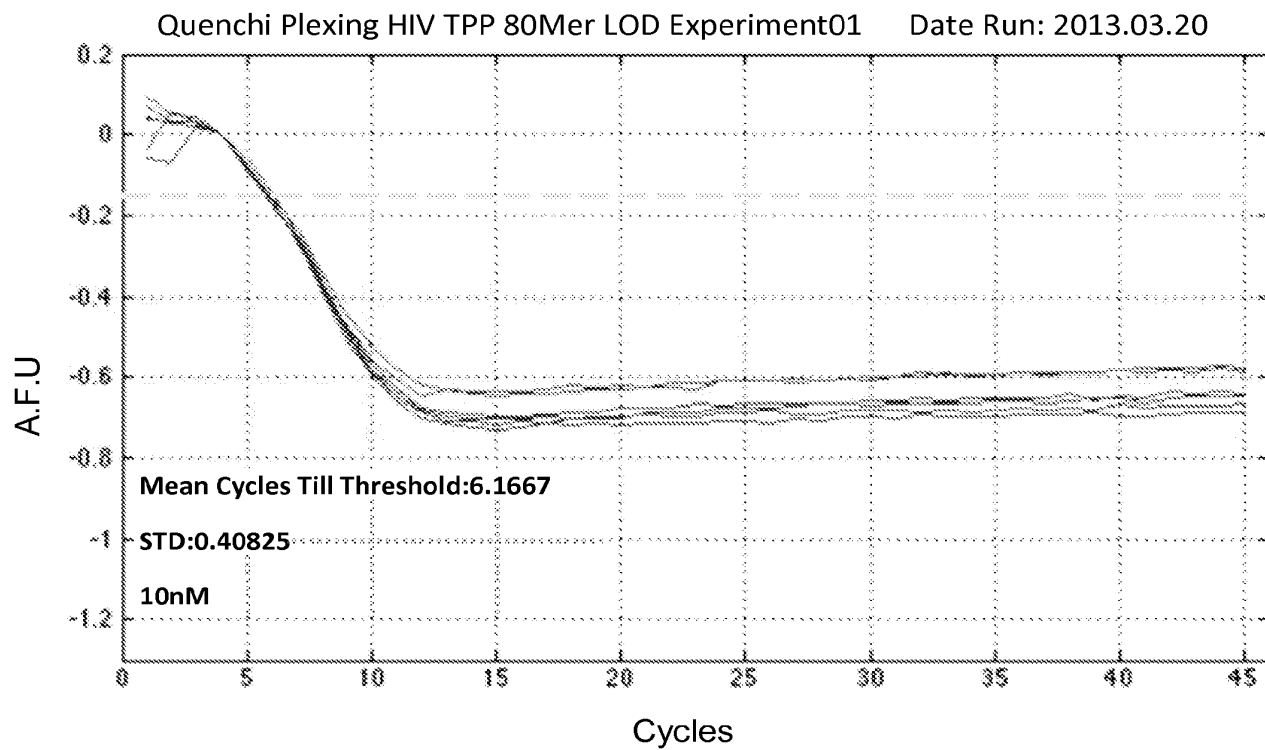


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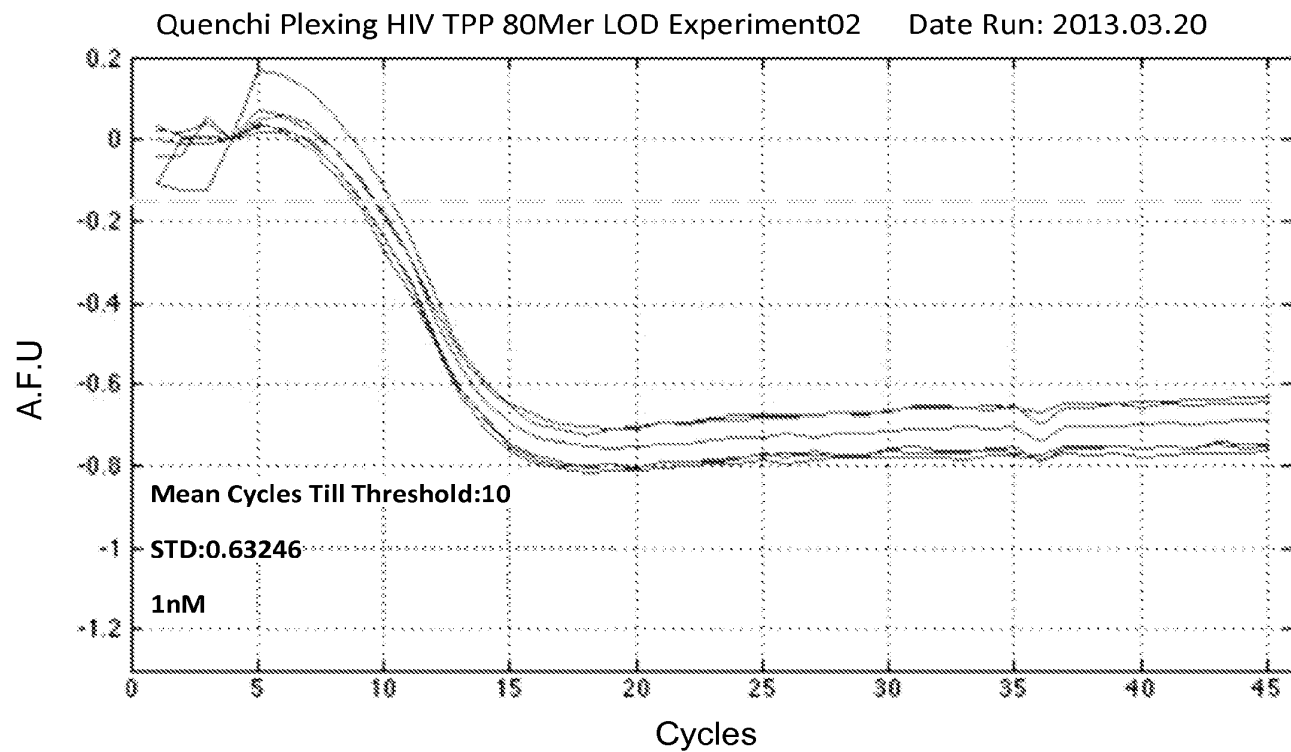
Fig. 4



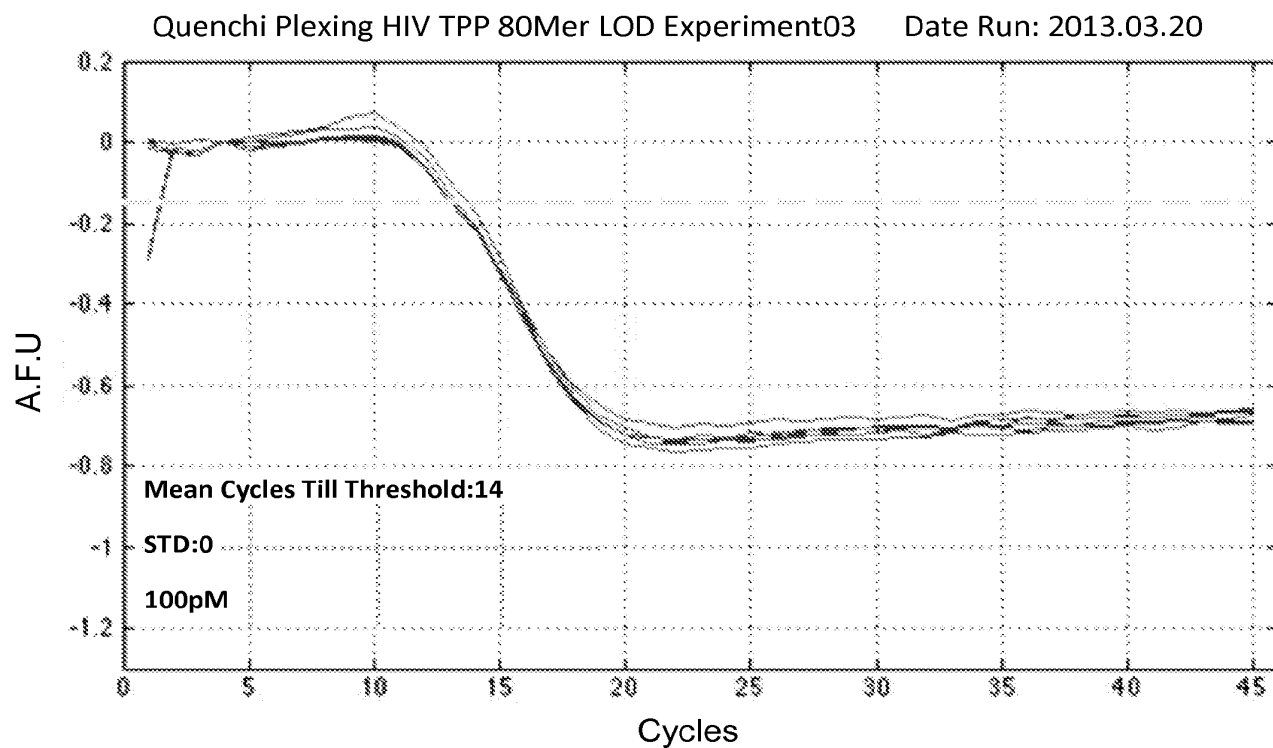
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Fig. 5A

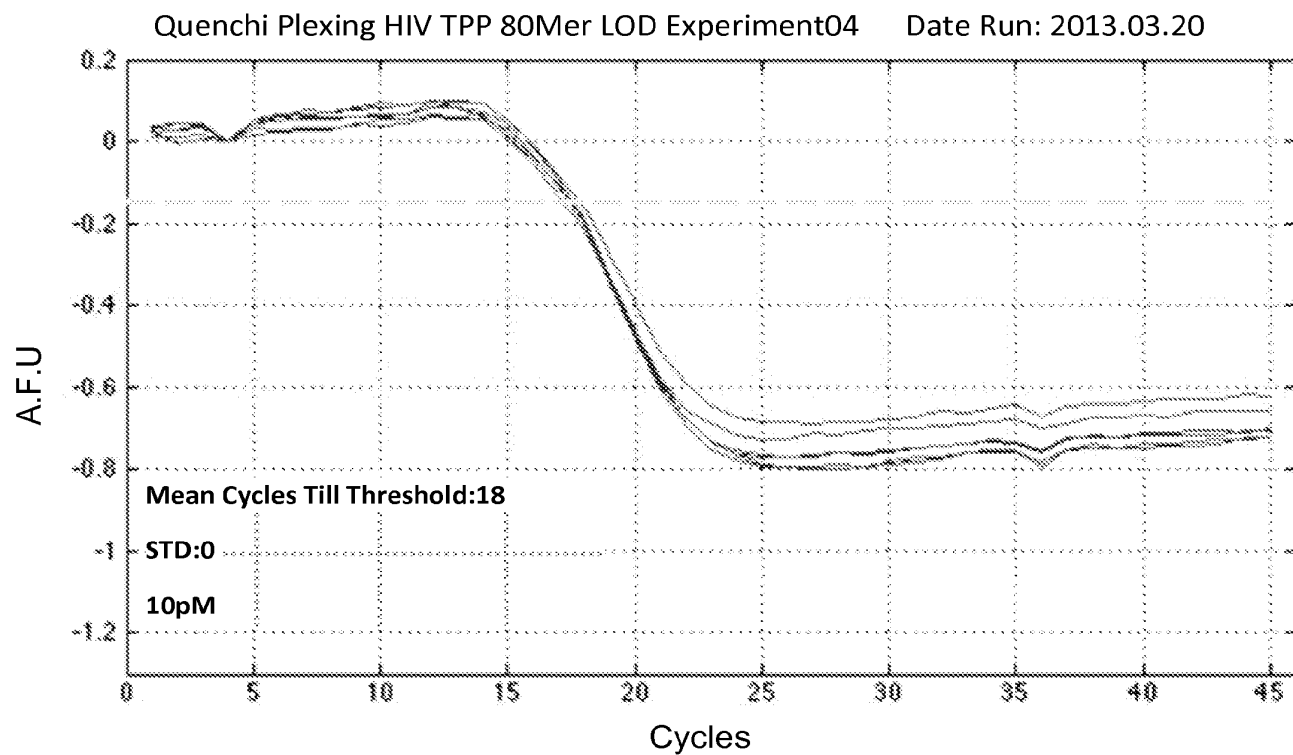
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Fig. 5B

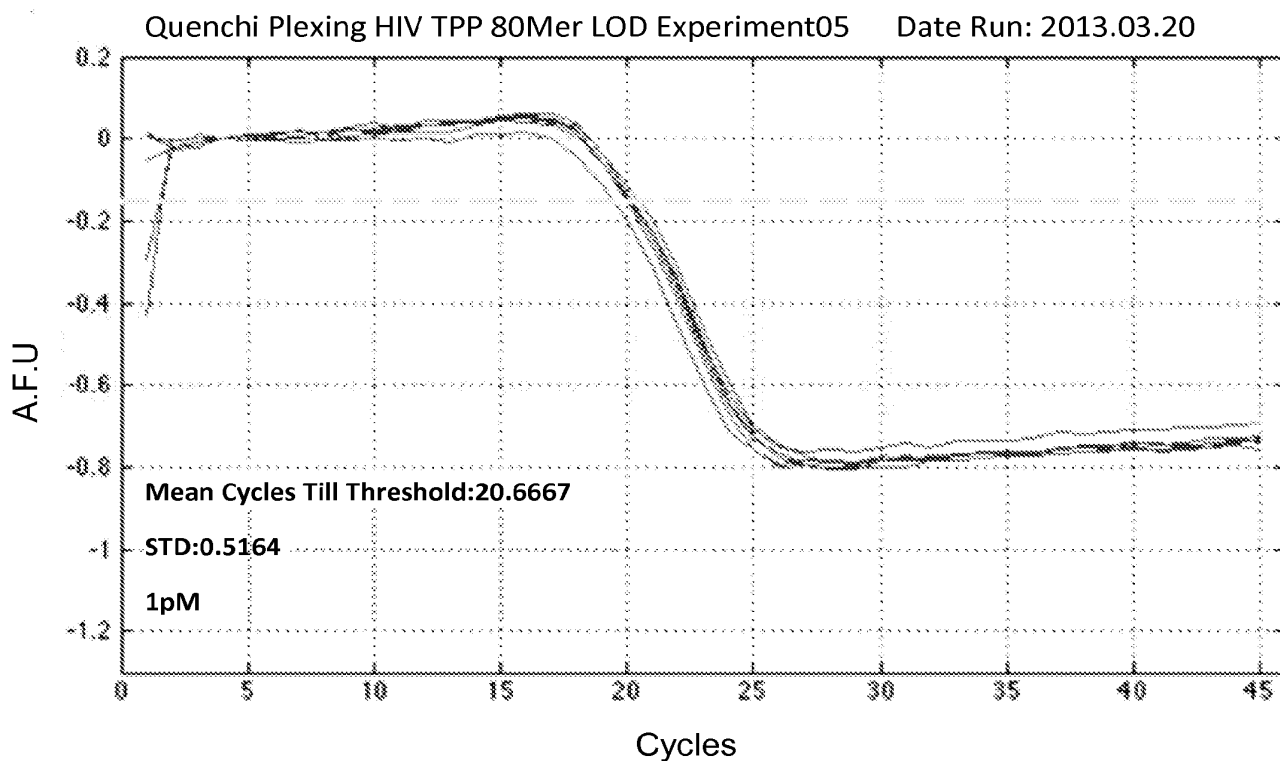
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Fig. 5C

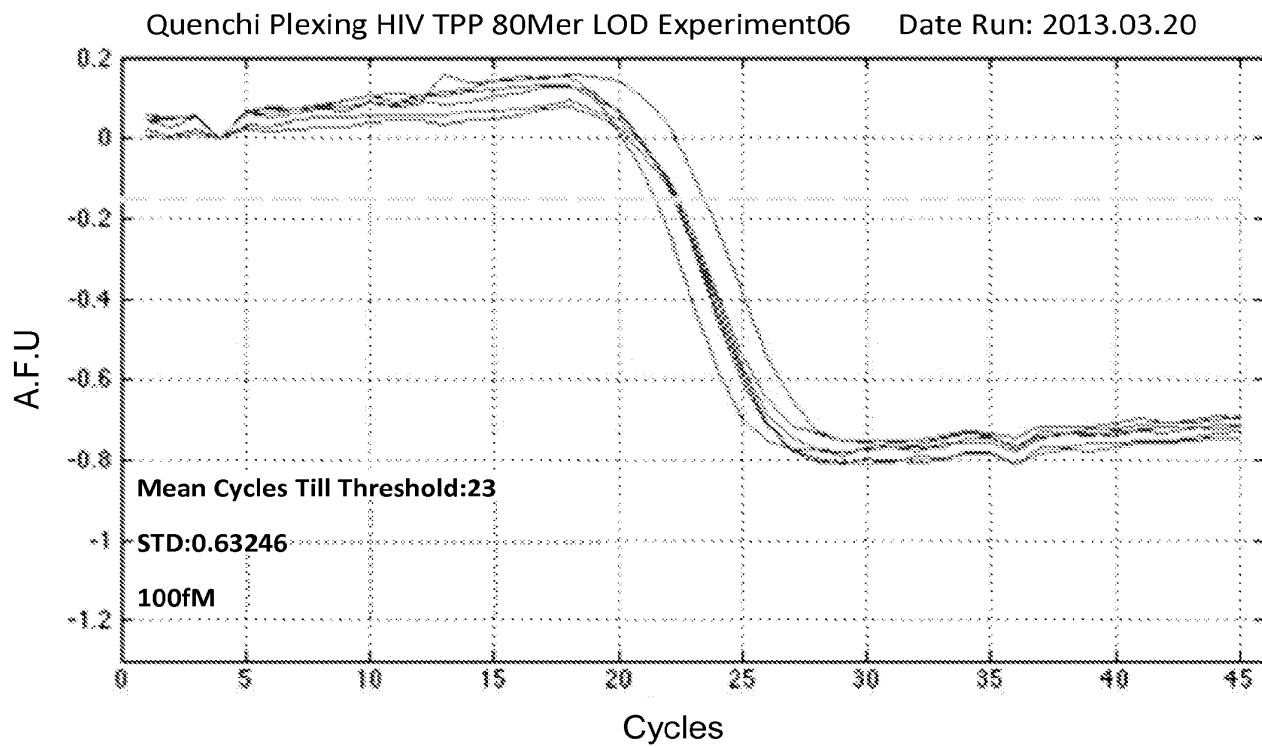
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Fig. 5D

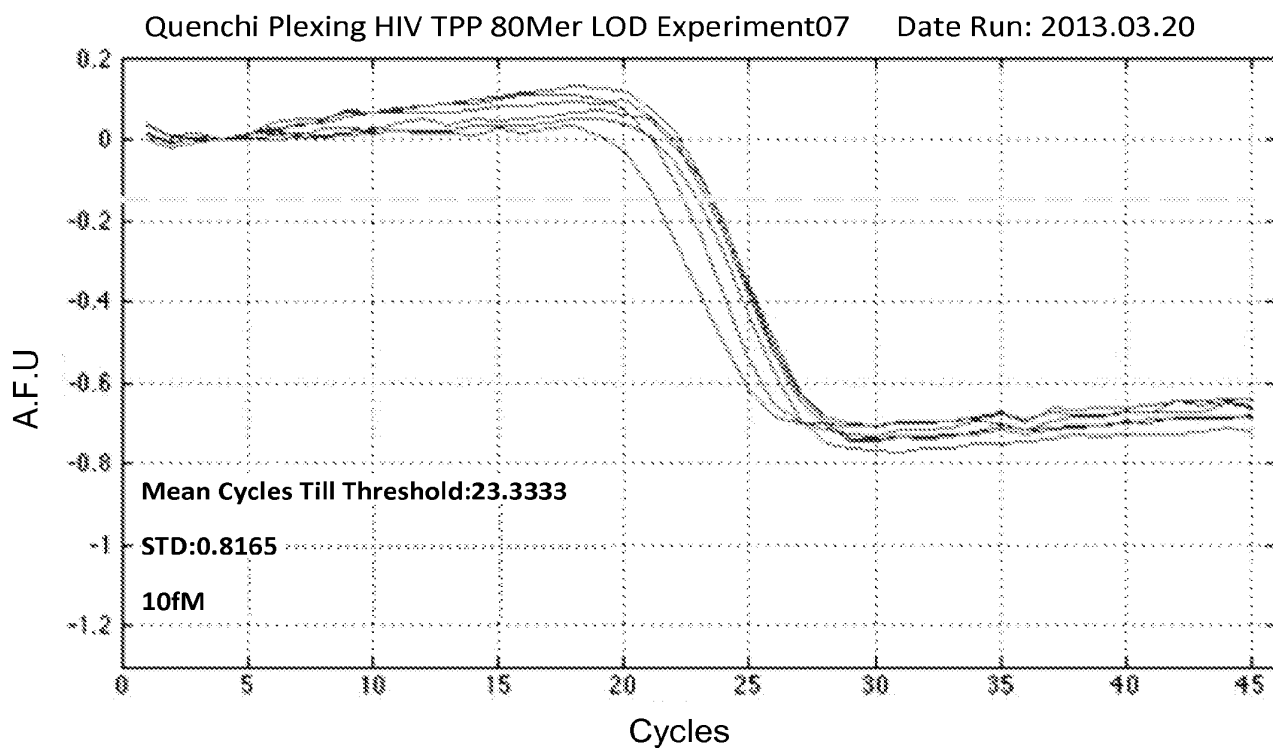
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Fig. 6A

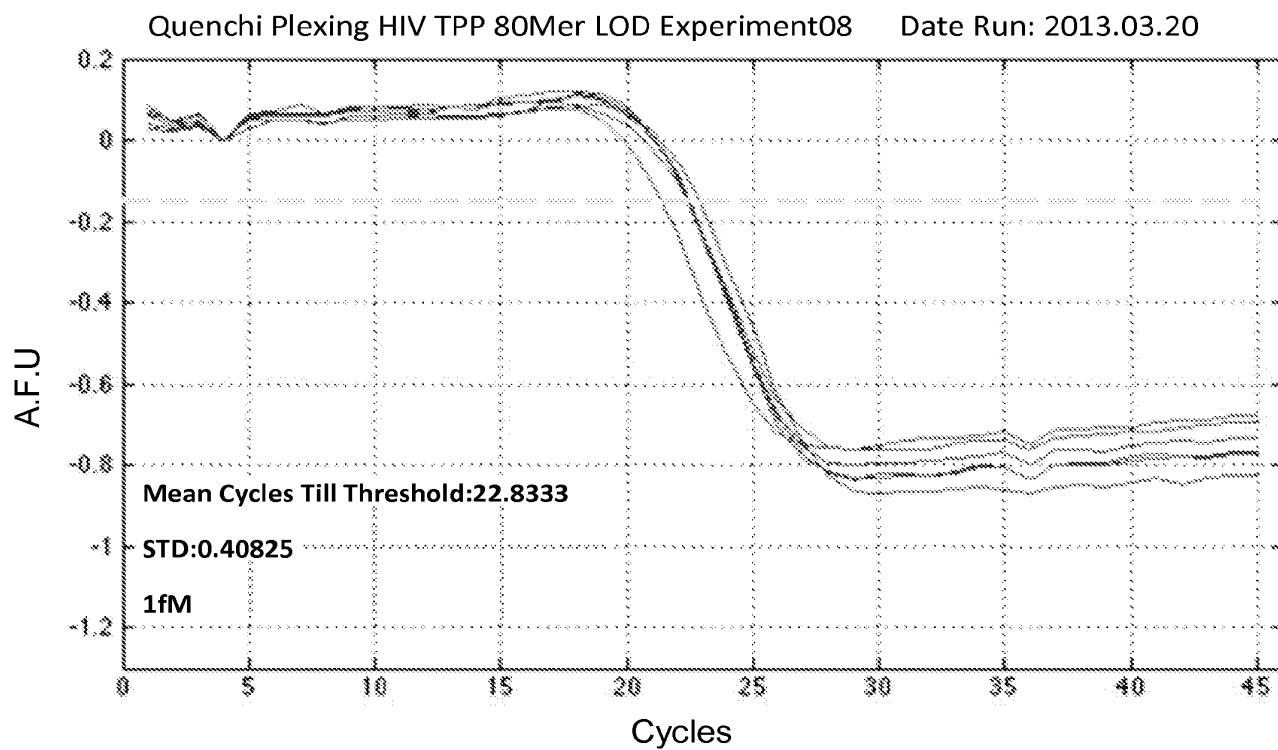
10/22

Fig. 6B

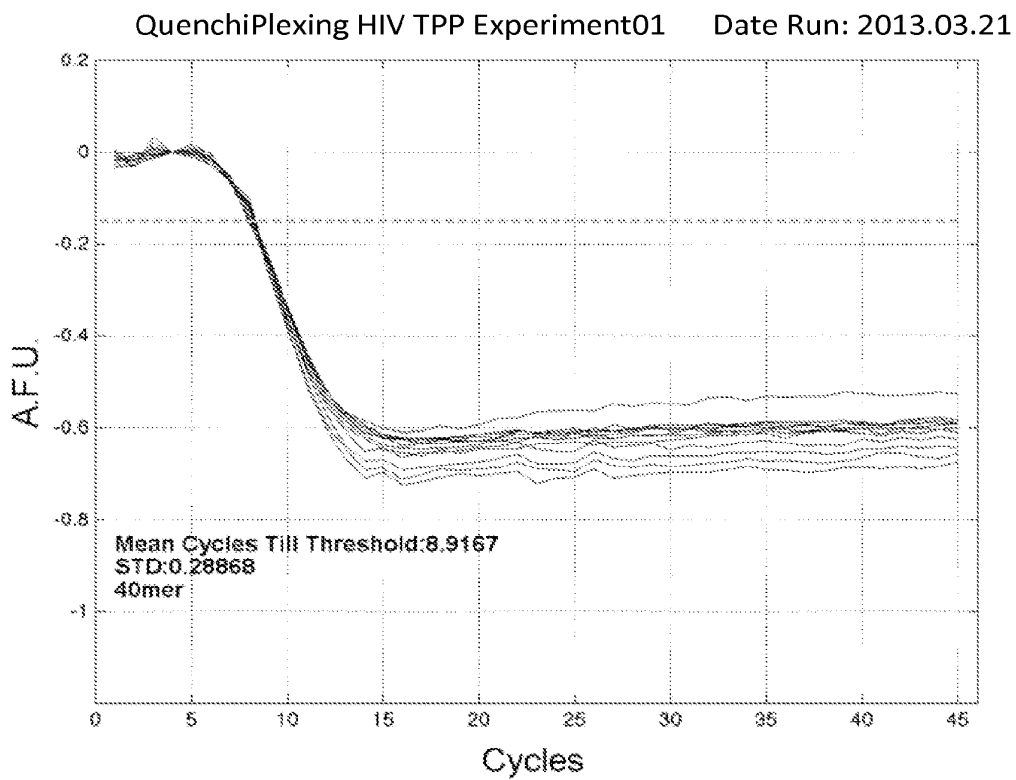
11/22

Fig. 6C

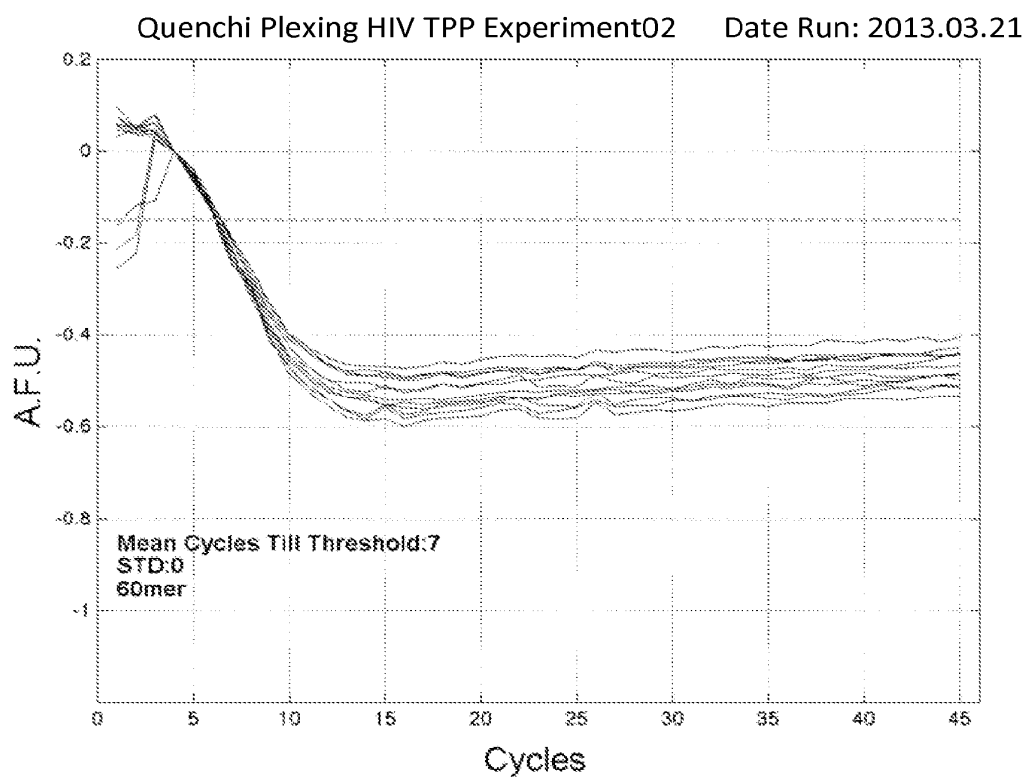
12/22

Fig. 6D

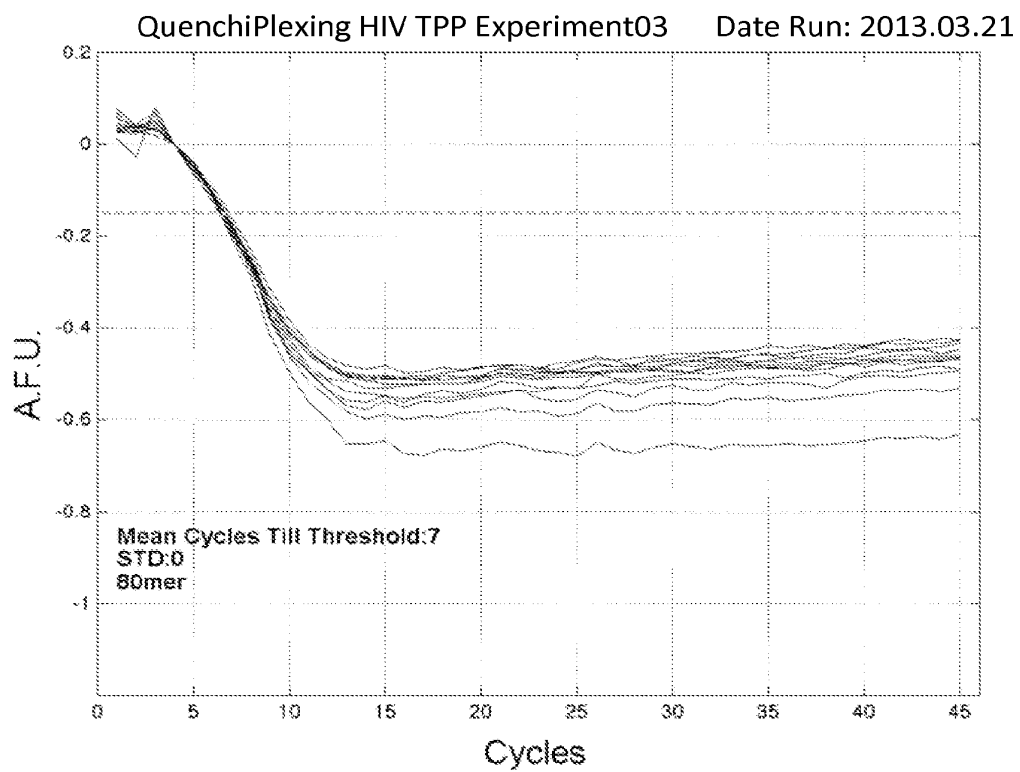
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Fig. 7A

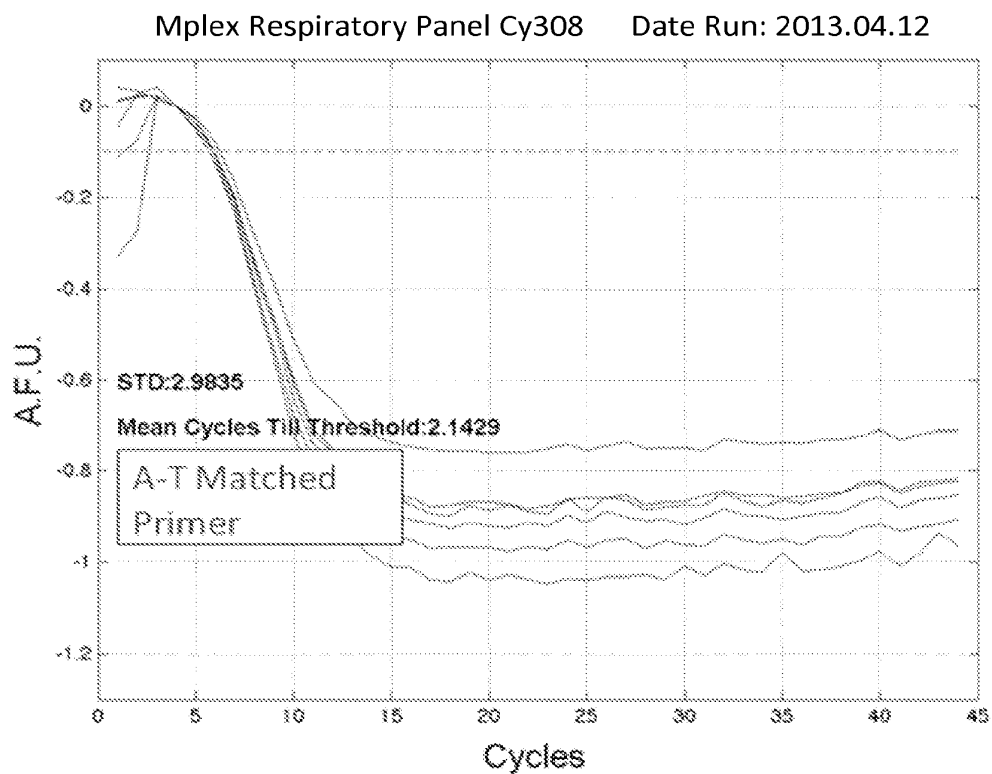
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Fig. 7B

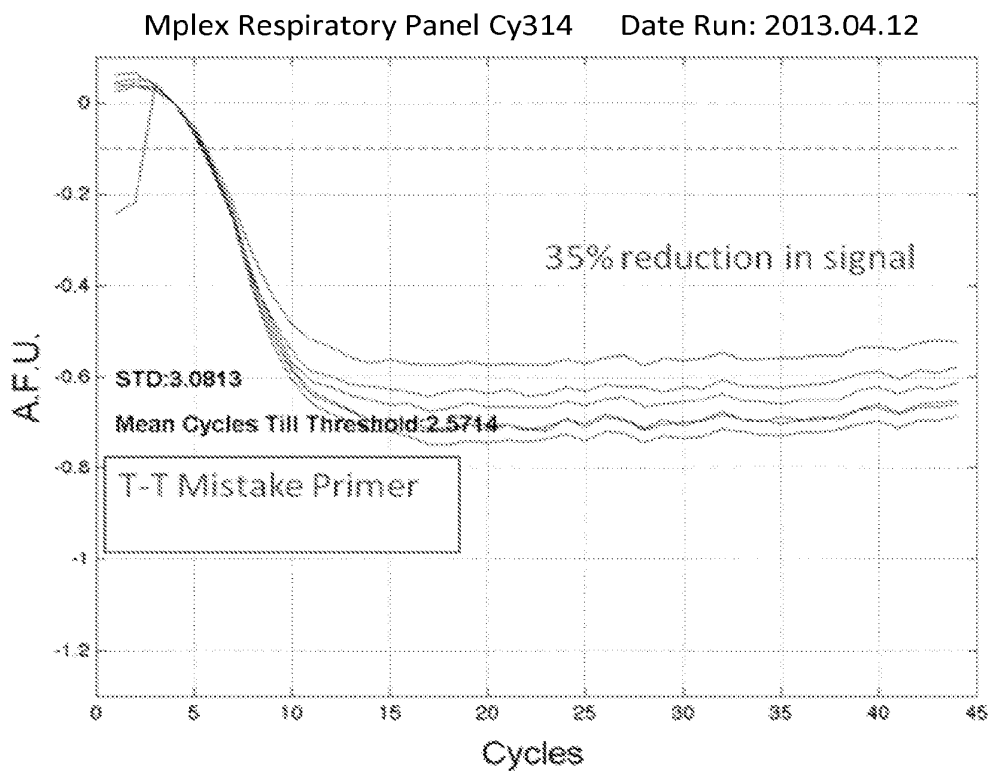
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Fig. 7C

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Fig. 8A

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Fig. 8B

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Fig. 9

Target	Cy3	Cy5
HIV TPP 80mer	1x [1um]	–
InfA 80mer	2x [2um]	–
InfB 80mer	4x [4um]	–
PIV-3 80mer	–	1x [3um]

Fig. 10A

Fig. 10B

Fig. 10C

Fig. 10D

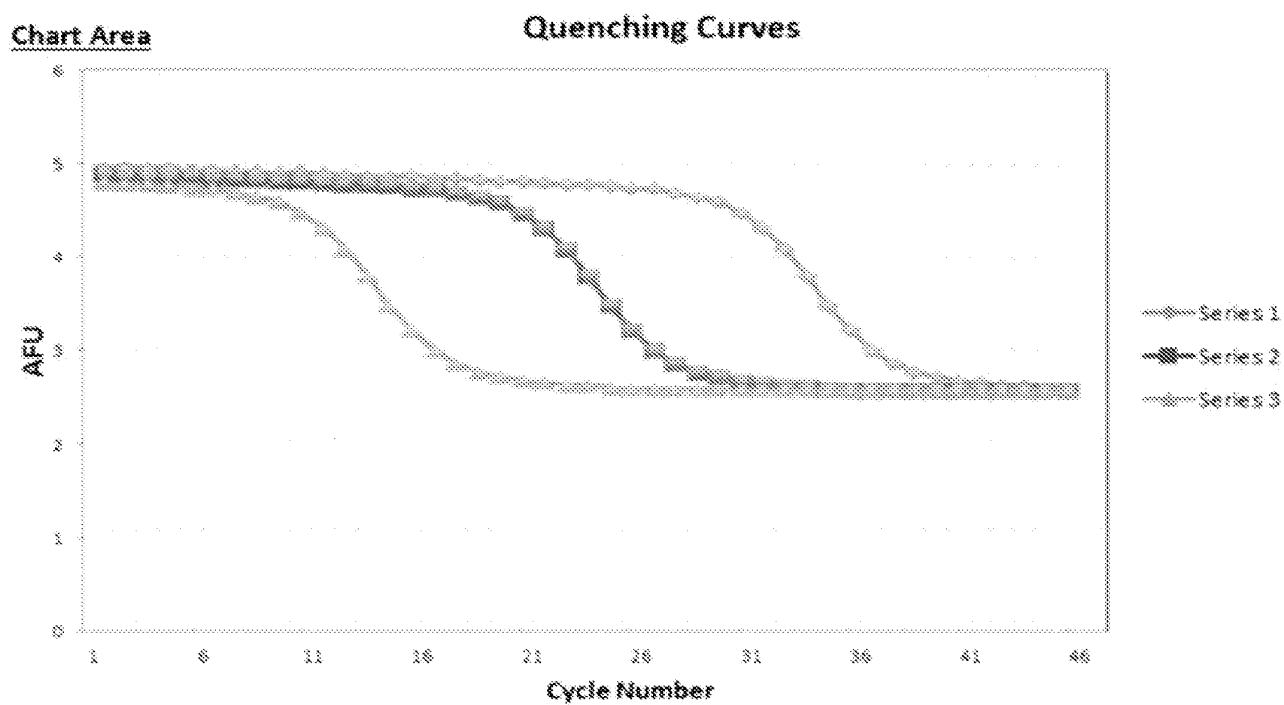
Fig. 10E

/Q/5' ATGGGGGGGGGGGGGGGGGGATGGGGGGGGAAGGGGGGATGGGGGGGGATGGGGATGGATG3'
 3' TACCCCCCCCCCCCCCCCCCTACCCCCCCCCTTCCCCCTACCCCCCCCCTACCCCTACCTAC5' /F/

/Q/5' ATGGGGGGGGGGGGGGGGGGATGGGGGGGGAAGGGGGGATGGGGGGGGATGGGGATGGATG3'
 3' TACCCCCCCCCCCCCCCCCCTACCCCCCCCCTTCCCCCTACCCCCCCCCTACCCCTACCTAC5' /F/

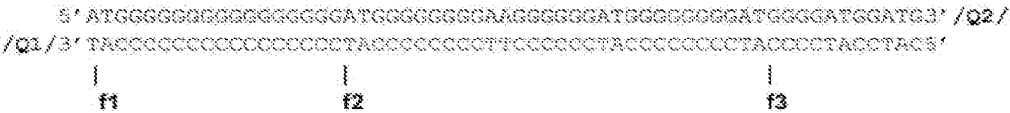
20/22

Fig. 11



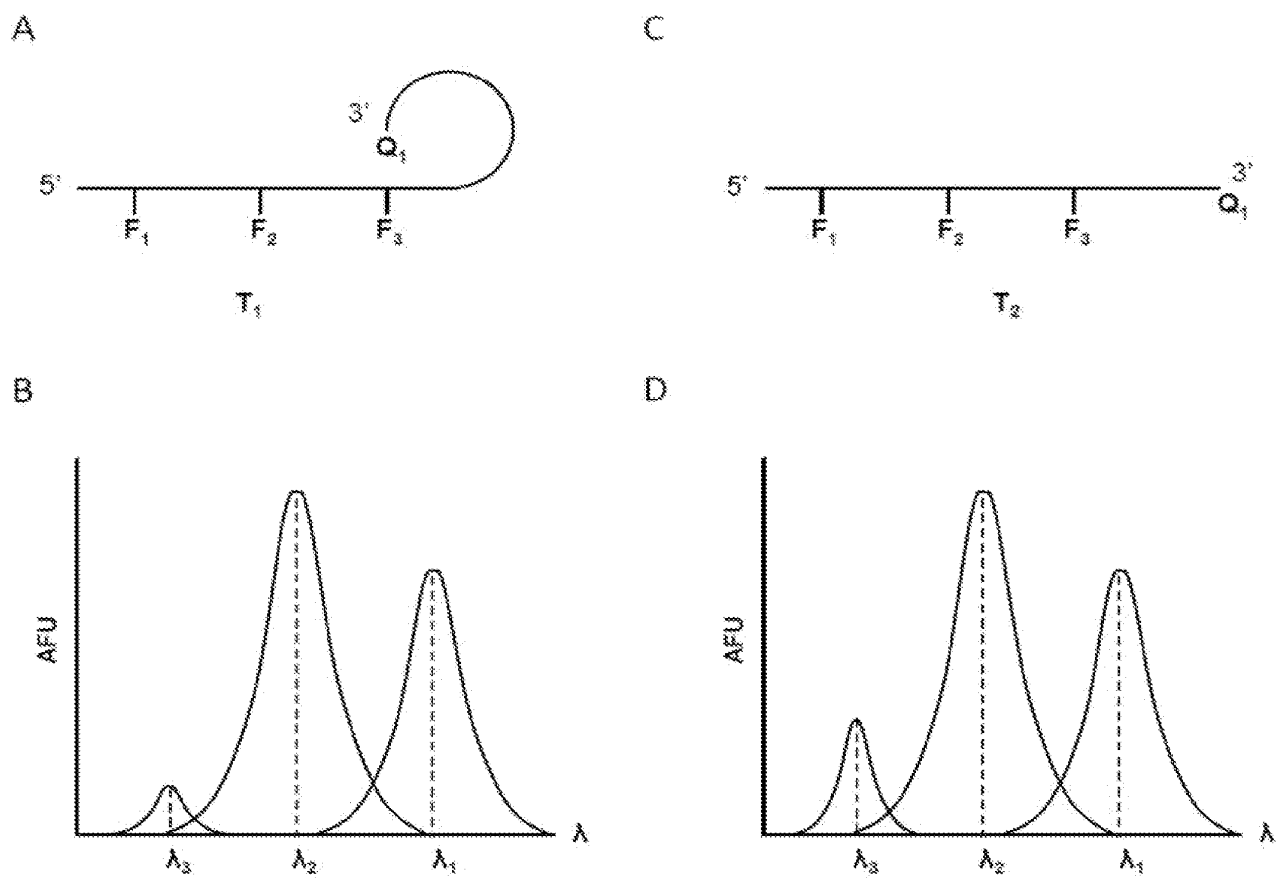
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Fig. 12



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Fig. 13



Box No. 1 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 filed or furnished:
- a. (means)
- ☐ on paper
- ☒ in electronic form
- b. (time)
- ☐ in the international application as filed
- ☐ together with the international application in electronic form
- ☒ subsequently to this Authority for the purposes of search
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 in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

PCT/US2014/012836 13.05.2014

International application No.

PCT/US 14/12836

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.: 11-27, 30-33, 35-37, 41-59, 64-71, 75-86, 101-109, 123-127, 132
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.

Form PCT/ISA/210 (continuation of first sheet (2)) (July 2009)

INTERNATIONAL SEARCH REPORT

PCT/US2014/012836 13.05.2014

International application No.

PCT/US 14/12836

A. CLASSIFICATION OF SUBJECT MATTER IPC(8) - C12Q 1/68 (2014.01) USPC - 435/6.11 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) USPC: 435/6.11 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched USPC: 435/6.11, 6.12, 91.2 (text search) Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) Electronic data bases: PatBase; Google Scholar; Google Patents Search terms: fluorophore, two fluorescent primers, three fluorescent probes, fluorescent primer extension, 6-FAM (fluorescein), TAMRA (tetramethyl rhodamine) [fluorophore and quencher], BHQ1 [quencher] genetic variant or polymorphism, background fluorescence before PCR, PCR cycling		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	6,022,686 A (GARMAN et al.) 08 February 2000 (08.02.2000). Especially col 7 In 36-52, col 8 23-41 and 47-50, col 9 In 24-31 and 46-47, col 13 In 3-5, 29-31 and 43-45.	1-9, 28, 34, 38-40, 60-63, 72-74, 87-100, 110-122, 128-131 10, 29, 133
X	CHEHAB et al. Detection of specific DNA sequences by fluorescence amplification: a color complementation assay. Proc Nat Acad Sci December 1989 Vol 86 No 23 Pages 9178-9182. Especially pg 9179 col 2 para 2, pg 9180 fig 2.	38-49
Y	YEUNG et al. Evaluation of dual-labeled fluorescent DNA probe purity versus performance in real-time PCR. Biotechniques February 2004 Vol 36 No 2 Pages 266-270. Especially pg 237 col 3 para 2.	10, 133
Y	MERZOUKI et al. Accurate and differential quantitation of HIV-1 tat, rev and nef mRNAs by competitive PCR. J Virol Methods December 1994 Vol 50 No 1-3 Pages 115-128.	29
A	PERDOK et al. PROTOZOA INHIBITORS TO REDUCE METHANE EXCRETION FROM DAIRY COWS [online] 29 May 2007 [retrieved 10 April 2014]. Available on the internet: <http://scholar.google.co.th/scholar?q=PROTOZOA+INHIBITORS+TO+REDUCE+METHANE+EXCRETION+FROM+DAIRY+COWS&btnG=&hl=en&as_sdt=0%2C5>. Especially pg 9 para 7, pg 10 fig 4.4	1, 38, 60, 72, 87, 93, 110
☒ Further documents are listed in the continuation of Box C. ☐		
* 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 10 April 2014 (10.04.2014)		Date of mailing of the international search report 13 MAY 2014
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. 571-273-3201		Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

IPR2024-01178

INTERNATIONAL SEARCH REPORT

PCT/US2014/012836 13.05.2014

International application No.

PCT/US 14/12836

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2012/0264643 A1 (CHUN et al.) 18 October 2012 (18.10.2012). Especially para [0027-0031], sheet 1 fig 1A, sheet 2 fig 1B.	1, 38, 60, 72, 87, 93,110
A	LI et al.Antiprimer quenching-based real-time PCR and its application to the analysis of clinical cancer samples. Clin Chem April 2006 Vol 52 No 4 Pages 624-633. Especially abstract, pg 625 fig 1, pg 629 fig 2.	1, 38, 60, 72, 87, 93,110

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(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,

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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))

Published:

- with international search report (Art. 21(3))

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

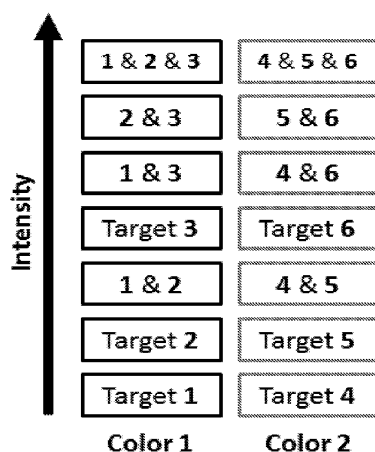


FIG. 1

(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.

WO 2019/006023 A1

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

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.

[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.

[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

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,

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.

[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 of 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.

[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 $m \times n$, 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.

[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.

[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.

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

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

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-

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

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 μ M
Pan InfA reverse primer	3.0 μ M
Pan InfA probe	200 nM

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

[0052] 24 μ L of this total reaction volume was dispersed into 80 μ L 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 with 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 μ M	10.0 μ M	0 nM	50,000 copies/rxn
b	10.0 μ M	10.0 μ M	100 nM	50,000 copies/rxn
c	10.0 μ M	10.0 μ M	200 nM	50,000 copies/rxn
d	10.0 μ M	10.0 μ M	500 nM	50,000 copies/rxn
e	10.0 μ M	10.0 μ M	1.0 μ M	50,000 copies/rxn
f	10.0 μ M	10.0 μ M	2.0 μ M	50,000 copies/rxn

g	10.0 μ M	10.0 μ M	5.0 μ M	50,000 copies/rxn
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[0054] These reactants were added to a ddPCR polymerase enzyme mixture. 21 μ L of this total reaction volume was dispersed into 80 μ L 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, 8 μ L of the primer mixture was added to 12 μ L of a Bio-Rad Digital PCR polymerase enzyme master mix and 6 μ L of sample volume, for a total reaction volume of 24 μ L. 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	-	-

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
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[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 $\pm 20\%$ or in some instances $\pm 10\%$, or in some instances $\pm 5\%$, or in some instances $\pm 1\%$, or in some instances $\pm 0.1\%$ 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.

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.

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.

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.

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

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.

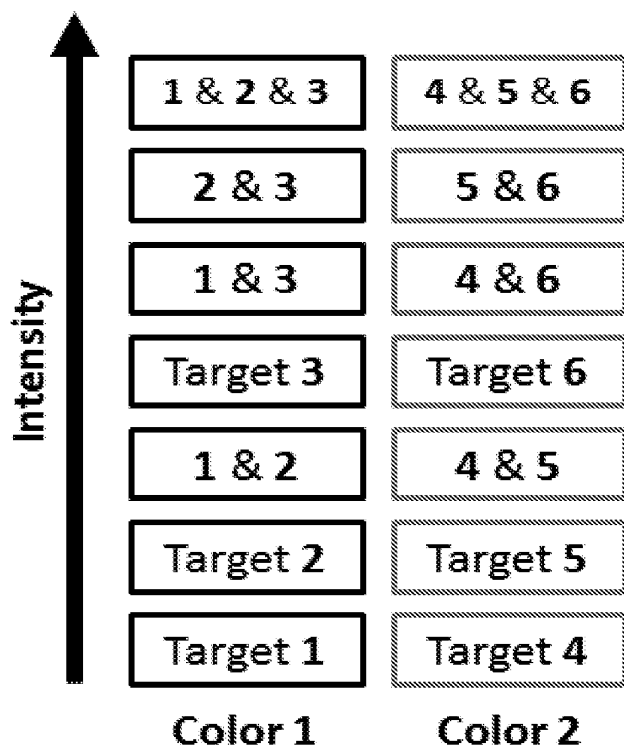
59. The method of any of claims 51-58, wherein the set of nucleic acid probes comprise a first nucleic acid probe, a second nucleic acid probe, and a third nucleic acid probe.

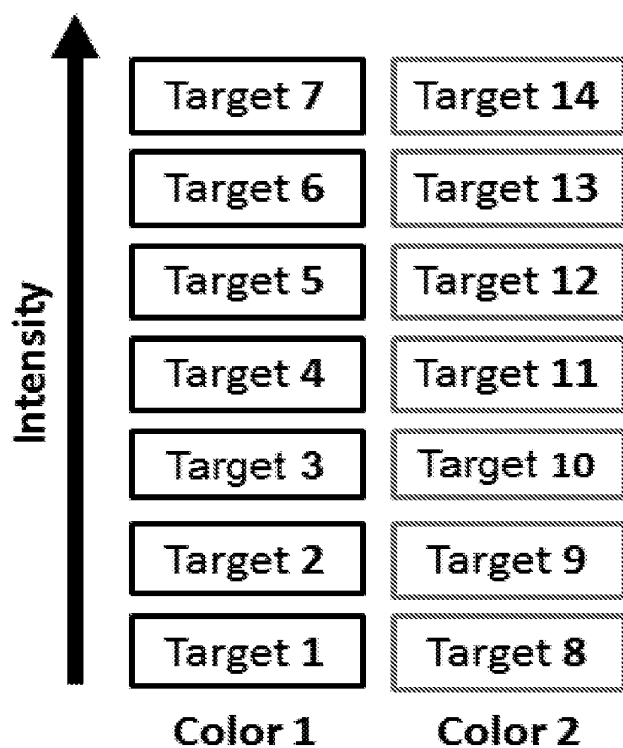
60. The method of claim 59, wherein, in (a), the concentration of the first nucleic acid probe is about X, the concentration of the second nucleic acid probe is at least about 2X, and the concentration of the third nucleic acid probe is at least about 4X.

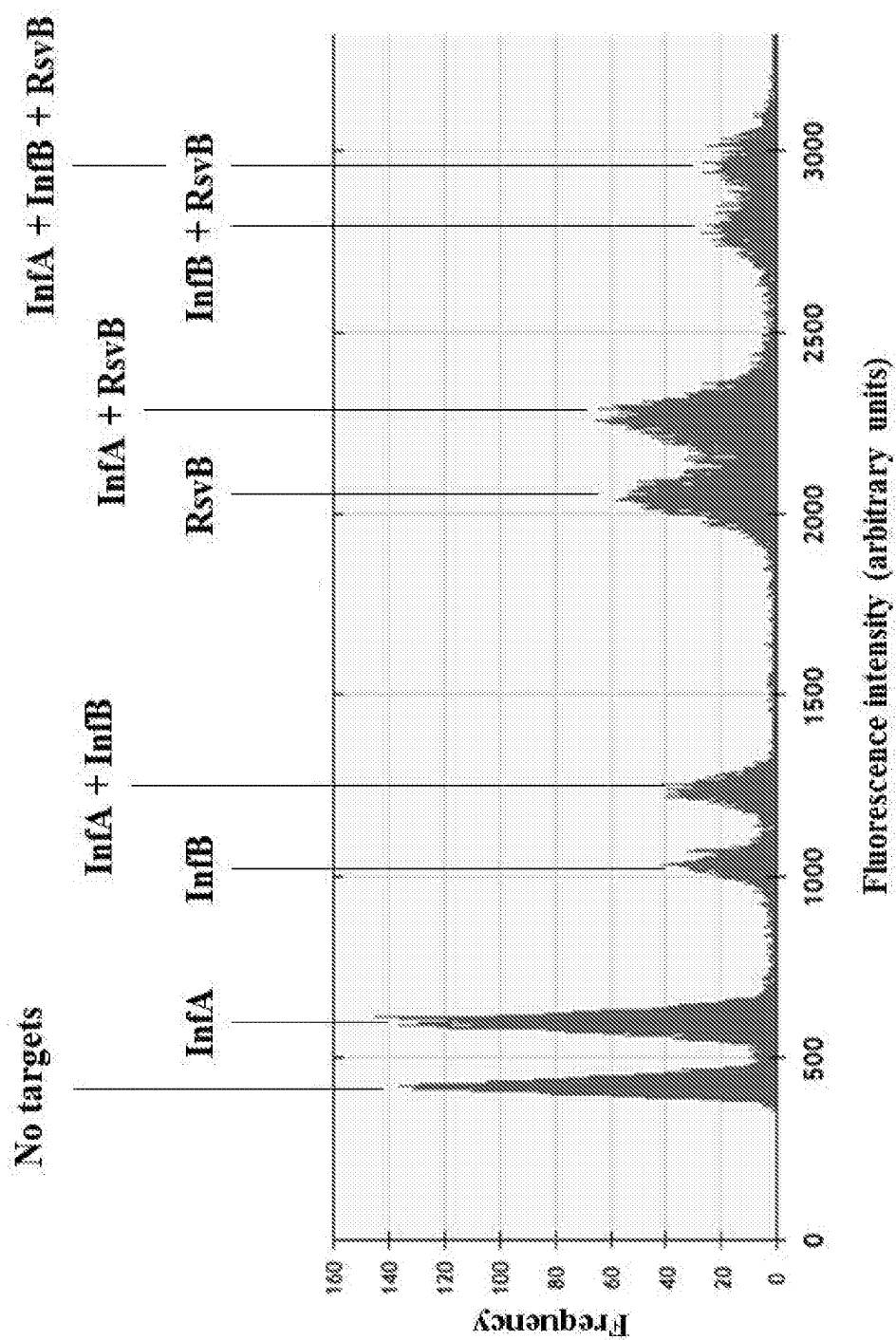
61. The method of claim 60, wherein the set of nucleic acid probes further comprise a fourth nucleic acid probe and wherein in (a), the concentration of the fourth nucleic acid probe is at least about 8X.

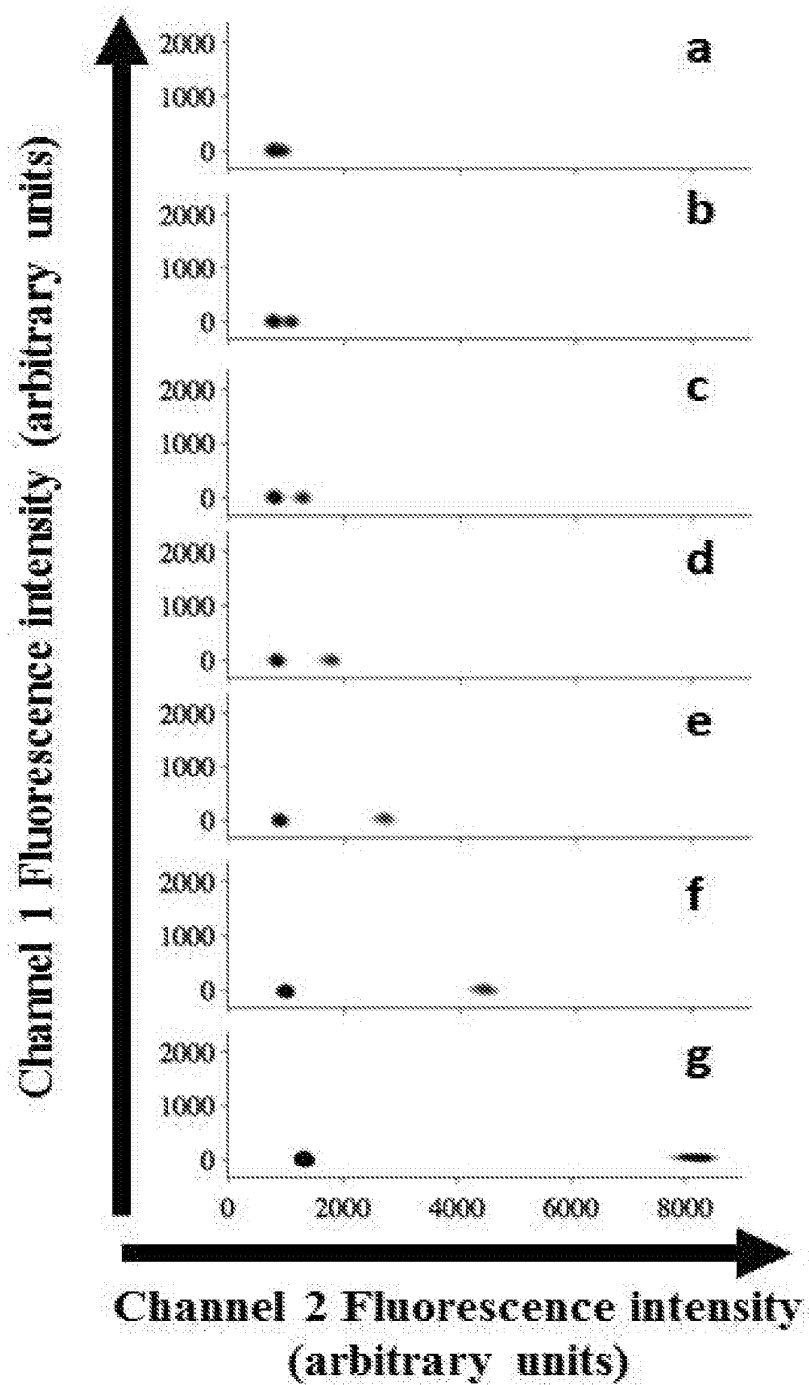
62. The method of claim 59, wherein the first nucleic acid probe comprises a fluorophore with an intensity level of about X, the second nucleic acid probe comprises a fluorophore with an intensity level of at least about 2X, and the third nucleic acid probe comprises a fluorophore with an intensity level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

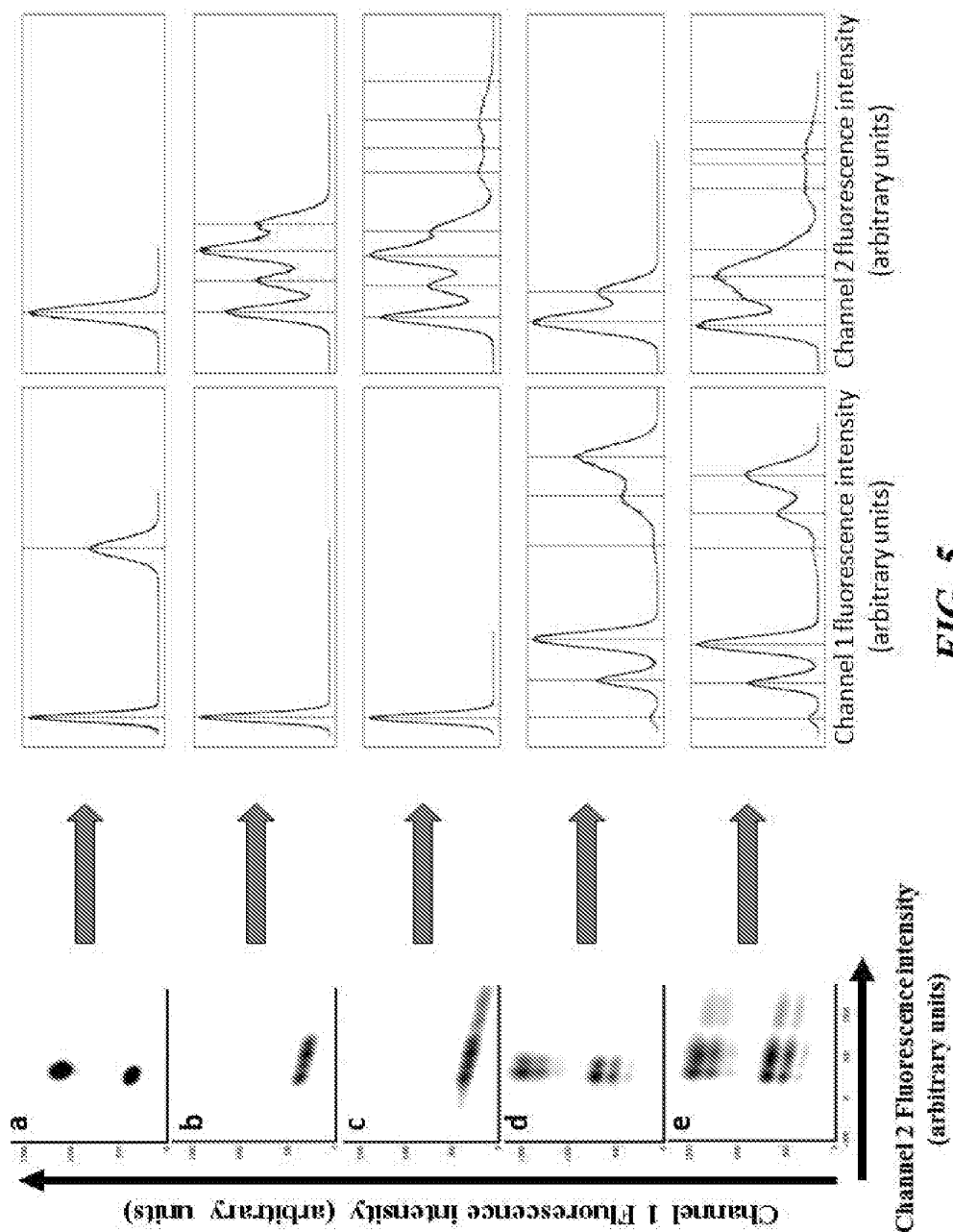
63. The method of claim 62, wherein the set of nucleic acid probes further comprises a fourth nucleic acid probe and wherein in (a), the fourth nucleic acid probe comprises a fluorophore with an intensity level of at least about 8X.
64. The method of claim 56, wherein the set of nucleic acid probes each 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.

***FIG. 1***

**FIG. 2**

**FIG. 3**

**FIG. 4**



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.

Form PCT/ISA/210 (continuation of first sheet (2)) (January 2015)

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

21 August 2018

Date of mailing of the international search report

14 SEP 2018

Name and mailing address of the ISA/US

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Authorized officer:

Lee W. Young

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PCT OSP: 571-272-7774

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

Electronic Acknowledgement Receipt	
EFS ID:	38444226
Application Number:	15914356
International Application Number:	
Confirmation Number:	2457
Title of Invention:	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS
First Named Inventor/Applicant Name:	Emil P. Kartalov
Customer Number:	21971
Filer:	Vernon A. Norviel/Lesley Borrayo
Filer Authorized By:	Vernon A. Norviel
Attorney Docket Number:	38075-716.302
Receipt Date:	30-JAN-2020
Filing Date:	07-MAR-2018
Time Stamp:	12:53:06
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no
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File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		IDS38075-716-302-01-30-2020.pdf	361667	yes	11
			9d363e62e2bdf2bf443f02df5a8b7df6ca2f7b2c		

	Multipart Description/PDF files in .zip description				
	Document Description		Start	End	
	Transmittal Letter		1	4	
	Information Disclosure Statement (IDS) Form (SB08)		5	11	
Warnings:					
Information:					
2	Non Patent Literature	BLACKET- MOLECULARECOLOGYRES-456- 463-2012.PDF	1054530 bb0213835a1b67f12f45ba39a78c391f58cd a2e6	no	8
Warnings:					
Information:					
3	Non Patent Literature	CN2013800498449_1STOA_28 DEC2015.PDF	509802 330decb38a041698a9ee857c036ee2cf063 e110d	no	11
Warnings:					
Information:					
4	Non Patent Literature	CN2013800498449_2NDOA_19 SEPT2016.PDF	564829 66636c1d852fb68b55dae95612fc8feecb 971b	no	9
Warnings:					
Information:					
5	Foreign Reference	EP0519338A1_EFS.PDF	1464771 b0ebfd0fe5d010f9be7acf38a6dff971fa8fb 69	no	20
Warnings:					
Information:					
6	Non Patent Literature	EP138247440_EESR_26FEB201 6.PDF	221287 afc8901e81877928a1ade35d7439fe07504d 8bdf	no	7
Warnings:					
Information:					
7	Non Patent Literature	EP138247440_OA_6NOV2018. PDF	94789 7de3486505e4b1e84e20d5bc17e025b7c2 2d0c72	no	4
Warnings:					

Information:					
8	Non Patent Literature	ESR-07-23-2019-EP18181686.PDF	836110	no	20
			fac5218113f0afd9dddf4c92dc03d937937e d2c0		
Warnings:					
Information:					
9	Non Patent Literature	ESR-12-02-2019-EP17814096.PDF	277651	no	8
			1f6a8fcd571ef8d0af023519f3086bfa126e7 bff		
Warnings:					
Information:					
10	Non Patent Literature	FU_MULTIPLEXDETECTION_E30340.PDF	725423	no	8
			9f329198681d100bd6e9b4fb14fd843c5fb2 546f		
Warnings:					
Information:					
11	Non Patent Literature	HAUSTEIN_FLUORESCENCECORRELATION_151-169.PDF	654368	no	20
			dfbf67a879e5b9d186b3937296f0faa9e658 f13d		
Warnings:					
Information:					
12	Non Patent Literature	ISRWO-PCTUS1839846-14SEP2018.PDF	1465658	no	17
			49020e052296d3be6ea2a5f996e40f94c57 b9db4		
Warnings:					
Information:					
13	Non Patent Literature	OA-US15892245-19JULY2018.PDF	126610	no	4
			f7c4cb8d6e2c9878680a9692d7a3532e087 4bf18		
Warnings:					
Information:					
14	Non Patent Literature	PCTUS2013053512_ISRWO_16OCT2013.PDF	437308	no	12
			64fd7e26d3451ba909b2e8430f1dff9d524 9fe9		
Warnings:					
Information:					

15	Non Patent Literature	RAJAGOPAL_SUPERCOLORCOD ING_7629-7636.PDF	840476	no	8
			6d8c65e436228e847c9fa99fd5a73db5d039205f		
Warnings:					
Information:					
16	Non Patent Literature	SAIKI_PRIMERDIRECTEDENZYM ATIC_487-491.PDF	969266	no	5
			70f60adb372f1695c9f9b50c85c9dc37fb94208c		
Warnings:					
Information:					
17	Non Patent Literature	THERMOFISHER_2015.PDF	25192	no	1
			b862e52d898563342487e8ea9c1ae7e91e18402a		
Warnings:					
Information:					
18	Non Patent Literature	US13958479_OA_1MARCH201 6.PDF	543010	no	14
			40acdffd8d19246b7a64a192c3f9a5f553104fcac		
Warnings:					
Information:					
19	Non Patent Literature	US16128343_1STACTIONINTER VIEW_18APRIL2019.PDF	870144	no	25
			a767b76712efeb1ceb8ec25d9af36a9ad8e5702f		
Warnings:					
Information:					
20	Non Patent Literature	US16128343-OA-15OCT2019. PDF	932054	no	25
			8964ef572f381a3a8eff187a57606b21a552d4fc		
Warnings:					
Information:					
21	Non Patent Literature	US16537415.PDF	4745310	no	84
			9ebbdaf591195233d9c2df93553c39c5826d7d96		
Warnings:					
Information:					

22	Foreign Reference	WO9746714A1_EFS.PDF	7002526	no	217
			6ae4974d9165e2df00f37804ce062c7cd1111232		
Warnings:					
Information:					
23	Foreign Reference	WO2009036514A2_EFS.PDF	6818423	no	100
			cb0369e2f63dc285383254f615cb7af0b714e99c		
Warnings:					
Information:					
24	Foreign Reference	WO2010007355A1_EFS.PDF	1802572	no	42
			5927fa00fb58df4856c158acb3c961145ad4893b		
Warnings:					
Information:					
25	Foreign Reference	WO2010013017A1_EFS.PDF	5484431	no	113
			af5491a3dfe54ef9a08113c9b90062dbc59336d		
Warnings:					
Information:					
26	Foreign Reference	WO2010017543A1_EFS.PDF	1363705	no	33
			78e727b1878d6c7b8a531d1f613c8781a91bee46		
Warnings:					
Information:					
27	Foreign Reference	WO2014022827A1_EFS.PDF	2195774	no	52
			5e1026db171e6de28c7b5e705b1ae96bd2cd5dfc		
Warnings:					
Information:					
28	Foreign Reference	WO2014116884A1_EFS.PDF	6173924	no	114
			d6922d081981448ea91c13e1510c7fcad17eff89		
Warnings:					
Information:					

29	Foreign Reference	WO2019006023A1_EFS.PDF	1832060	no	32
			7a77e60c38305e10c5cf89bd83e971314edcc0b		
Warnings:					
Information:					
30	Non Patent Literature	Z-HAN-QUANTUM-DOT-631_EFS.PDF	2723306	no	5
			fc1cdef791c1fc29d4813211ebe851834f35e3b4		
Warnings:					
Information:					
31	Non Patent Literature	ZHAO_COMPREHENSIVEALGORITHM_1047-1064.PDF	1360581	no	18
			841944932a4966bf39c15e415899b7bb348c6e40		
Warnings:					
Information:					
Total Files Size (in bytes):			54477557		
This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503. <u>New Applications Under 35 U.S.C. 111</u> If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application. <u>National Stage of an International Application under 35 U.S.C. 371</u> If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course. <u>New International Application Filed with the USPTO as a Receiving Office</u> If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.					

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventor:	KARTALOV; Emil P. et al.	Group Art Unit:	1631
Serial Number:	15/914,356	Examiner:	NEGIN, RUSSELL SCOTT
Filing or 371 (c) Date:	2018-03-07	CONFIRMATION NO:	2457
Title:	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS		

FILED ELECTRONICALLY ON: January 30, 2020

Commissioner for Patents
P.O. Box 1450
Alexandria VA 22313-1450

INFORMATION DISCLOSURE STATEMENT
UNDER 37 CFR § 1.97

Commissioner for Patents:

An Information Disclosure Statement along with attached PTO/SB/08 is hereby submitted. A copy of each listed publication is submitted, if required, pursuant to 37 CFR §§1.97-1.98, as indicated below.

The Examiner is requested to review the information provided and to make the information of record in the above-identified application. The Examiner is further requested to initial and return the attached PTO/SB/08 in accordance with MPEP § 609.

The right to establish the patentability of the claimed invention over any of the information provided herewith, and/or to prove that this information may not be prior art, and/or to prove that this information may not be enabling for the teachings purportedly offered, is hereby reserved.

This statement is not intended to represent that a search has been made or that the information cited in the statement is, or is considered to be, prior art or material to patentability as defined in § 1.56.

A. ☒ 37 CFR § 1.97 (b). This Information Disclosure Statement should be considered by the Office because:

☐ (1) It is being filed within 3 months of the filing date of a national application and is other than a continued prosecution application under § 1.53 (d);

-- OR --

☐ (2) It is being filed within 3 months of entry of the national stage as set forth in § 1.491 in an international application;

-- OR --

☐ (3) It is being filed before the mailing of a first Office action on the merits;

-- OR --

☒ (4) It is being filed before the mailing of a first Office action after the filing of a request for continued examination under § 1.114.

B. ☐ 37 CFR § 1.97(c). Although this Information Disclosure Statement is being filed after the period specified in 37 CFR § 1.97(b), above, it is filed before the mailing date of the earlier of (1) a final office action under § 1.113, (2) a notice of allowance under § 1.311, or (3) an action that otherwise closes prosecution in the application, this Information Disclosure Statement should be considered because it is accompanied by one of:

☐ a statement as specified in §1.97 (e) provided concurrently herewith;

-- OR --

☐ a fee of \$240.00 as set forth in § 1.17 (p) authorized below, enclosed, or included with the payment of other papers filed together with this statement.

C. ☐ 37 CFR § 1.97 (d). Although this Information Disclosure Statement is being filed after the mailing date of the earlier of (1) a final office action under § 1.113, (2) a notice of allowance under § 1.311, or (3) an action that otherwise closes prosecution in the application, it is being filed before payment of the issue fee and should be considered because it is accompanied by:

i. a statement as specified in § 1.97 (e);

-- AND --

ii. a fee of \$240.00 as set forth in §1.17(p) is authorized below, enclosed, or included with the payment of other papers filed together with this Statement.

D. ☐ 37 CFR §1.97 (e). Statement.

☐ A statement is provided herewith to satisfy the requirement under 37 CFR §§ 1.97 (c);

-- AND/OR --

☐ A statement is provided herewith to satisfy the requirement under 37 CFR §§ 1.97 (d);

-- AND/OR --

☐ A copy of a dated communication from a foreign patent office clearly showing that the information disclosure statement is being submitted within 3 months of the filing date on the communication is provided in lieu of a statement under 37 C.F.R. § 1.97(e) (1) as provided for under MPEP 609.04(b) V.

- E. ☐ *Statement Under 37 C.F.R. §1.704(d).* Each item of information contained in the information disclosure statement was first cited in any communication from a patent office in a counterpart foreign or international application or from the Office or is a communication that was issued by a patent office in a counterpart foreign or international application or by the Office that was received by an individual designated in § 1.56(c) not more than thirty (30) days prior to the filing of this information disclosure statement. This statement is made pursuant to the requirements of 37 C.F.R. §1.704(d) to avoid reduction of the period of adjustment of the patent term for Applicant(s) delay.
- F. ☒ *37 CFR §1.98 (a) (2).* The content of the Information Disclosure Statement is as follows:
- ☐ Copies of each of the references listed on the attached Form PTO/SB/08 are enclosed herewith.
- OR --
- ☒ Copies of U.S. Patent Documents (issued patents and patent publications) listed on the attached Form PTO/SB/08 are not enclosed.
- AND/OR --
- ☒ Copies of Foreign Patent Documents and/or Non Patent Literature Documents listed on the attached Form PTO/SB/08 are enclosed in accordance with 37 CFR §1.98 (a)(2).
- AND/OR --
- ☒ Copies of pending unpublished U.S. patent applications are enclosed in accordance with 37 CFR §1.98 (a) (2) (iii).
- G. ☐ *37 CFR §1.98(a)(3).* The Information Disclosure Statement includes non-English patents and/or references.
- ☐ Pursuant to 37 CFR §1.98(a)(3)(i), a concise explanation of the relevance of each patent, publication or other information provided that is not in English is provided herewith.
- ☐ Pursuant to MPEP 609(B), an English language copy of a foreign search report is submitted herewith to satisfy the requirement for a concise explanation where non-English language information is cited in the search report.
- OR --
- ☐ A concise explanation of the relevance of each patent, publication or other information provided that is not in English is as follows: _____
- ☐ Pursuant to 37 CFR §1.98(a) (3) (ii), a copy of a translation, or a portion thereof, of the non-English language reference(s) is provided herewith.
- H. ☐ *37 CFR §1.98(d).* Copies of patents, publications and pending U.S. patent applications, or other information specified in 37 C.F.R. § 1.98(a) are not provided herewith because:
- ☐ Pursuant to 37 CFR §1.98(d)(1) the information was previously submitted in an Information Disclosure Statement, or cited by examiner for another application under which this application claims priority for an earlier effective filing date under 35 U.S.C. 120.
- Application in which the information was submitted: _____
- Information Disclosure Statement(s) filed on: _____
- AND
- ☐ The information disclosure statement submitted in the earlier application complied with paragraphs (a) through (c) of 37 CFR §1.98.

- I. ☒ *Fee Authorization.* The Commissioner is hereby authorized to charge the above-referenced fees of \$0.00 and charge any additional fees or credit any overpayment associated with this communication to Deposit Account No. 23-2415 (Docket No.38075-716.302).

Respectfully submitted,

WILSON SONSINI GOODRICH & ROSATI

Dated: January 23, 2020

By: /Nicholas Tice/
Nicholas Tice, Ph.D.
Reg. No. 77,907

650 Page Mill Road
Palo Alto, CA 94304-1050
(650) 493-9300
Customer No. 21971



UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

NOTICE OF ALLOWANCE AND FEE(S) DUE

21971 7590 05/04/2020
WILSON, SONSINI, GOODRICH & ROSATI
650 PAGE MILL ROAD
PALO ALTO, CA 94304-1050

EXAMINER

NEGIN, RUSSELL SCOTT

ART UNIT

PAPER NUMBER

1631

DATE MAILED: 05/04/2020

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/914,356	03/07/2018	Emil P. Kartalov	38075-716.302	2457

TITLE OF INVENTION: SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	\$1000	\$0.00	\$0.00	\$1000	08/04/2020

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.

THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.

HOW TO REPLY TO THIS NOTICE:

I. Review the ENTITY STATUS shown above. If the ENTITY STATUS is shown as SMALL or MICRO, verify whether entitlement to that entity status still applies.

If the ENTITY STATUS is the same as shown above, pay the TOTAL FEE(S) DUE shown above.

If the ENTITY STATUS is changed from that shown above, on PART B - FEE(S) TRANSMITTAL, complete section number 5 titled "Change in Entity Status (from status indicated above)".

For purposes of this notice, small entity fees are 1/2 the amount of undiscounted fees, and micro entity fees are 1/2 the amount of small entity fees.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Maintenance fees are due in utility patents issuing on applications filed on or after Dec. 12, 1980. It is patentee's responsibility to ensure timely payment of maintenance fees when due. More information is available at www.uspto.gov/PatentMaintenanceFees.

PART B - FEE(S) TRANSMITTAL

Complete and send this form, together with applicable fee(s), by mail or fax, or via EFS-Web.

By mail, send to: Mail Stop ISSUE FEE
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

By fax, send to: (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

21971 7590 05/04/2020
WILSON, SONSINI, GOODRICH & ROSATI
650 PAGE MILL ROAD
PALO ALTO, CA 94304-1050

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

Certificate of Mailing or Transmission

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being transmitted to the USPTO via EFS-Web or by facsimile to (571) 273-2885, on the date below.

(Typed or printed name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/914,356	03/07/2018	Emil P. Kartalov	38075-716.302	2457

TITLE OF INVENTION: SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	\$1000	\$0.00	\$0.00	\$1000	08/04/2020

EXAMINER	ART UNIT	CLASS-SUBCLASS
NEGIN, RUSSELL SCOTT	1631	702-019000

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-09 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list

(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,

(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 _____

2 _____

3 _____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document must have been previously recorded, or filed for recordation, as set forth in 37 CFR 3.11 and 37 CFR 3.81(a). Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE

(B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent): ☐ Individual ☐ Corporation or other private group entity ☐ Government

4a. Fees submitted: ☐ Issue Fee ☐ Publication Fee (if required) ☐ Advance Order - # of Copies _____

4b. Method of Payment: (Please first reapply any previously paid fee shown above)

☐ Electronic Payment via EFS-Web ☐ Enclosed check ☐ Non-electronic payment by credit card (Attach form PTO-2038)

☐ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment to Deposit Account No. _____

5. Change in Entity Status (from status indicated above)

☐ Applicant certifying micro entity status. See 37 CFR 1.29

☐ Applicant asserting small entity status. See 37 CFR 1.27

☐ Applicant changing to regular undiscounted fee status.

NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.

NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.

NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature _____

Date _____

Typed or printed name _____

Registration No. _____



UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/914,356	03/07/2018	Emil P. Kartalov	38075-716.302	2457
21971 7590 05/04/2020 WILSON, SONSINI, GOODRICH & ROSATI 650 PAGE MILL ROAD PALO ALTO, CA 94304-1050			EXAMINER NEGIN, RUSSELL SCOTT	
			ART UNIT 1631	PAPER NUMBER
DATE MAILED: 05/04/2020				

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b) (Applications filed on or after May 29, 2000)

The Office has discontinued providing a Patent Term Adjustment (PTA) calculation with the Notice of Allowance.

Section 1(h)(2) of the AIA Technical Corrections Act amended 35 U.S.C. 154(b)(3)(B)(i) to eliminate the requirement that the Office provide a patent term adjustment determination with the notice of allowance. See Revisions to Patent Term Adjustment, 78 Fed. Reg. 19416, 19417 (Apr. 1, 2013). Therefore, the Office is no longer providing an initial patent term adjustment determination with the notice of allowance. The Office will continue to provide a patent term adjustment determination with the Issue Notification Letter that is mailed to applicant approximately three weeks prior to the issue date of the patent, and will include the patent term adjustment on the patent. Any request for reconsideration of the patent term adjustment determination (or reinstatement of patent term adjustment) should follow the process outlined in 37 CFR 1.705.

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

OMB Clearance and PRA Burden Statement for PTOL-85 Part B

The Paperwork Reduction Act (PRA) of 1995 requires Federal agencies to obtain Office of Management and Budget approval before requesting most types of information from the public. When OMB approves an agency request to collect information from the public, OMB (i) provides a valid OMB Control Number and expiration date for the agency to display on the instrument that will be used to collect the information and (ii) requires the agency to inform the public about the OMB Control Number's legal significance in accordance with 5 CFR 1320.5(b).

The information collected by PTOL-85 Part B is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 30 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. **DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450.** Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

Privacy Act Statement

The Privacy Act of 1974 (P.L. 93-579) requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

Notice of Allowability	Application No. 15/914,356	Applicant(s) Kartalov et al.	
	Examiner RUSSELL S NEGIN	Art Unit 1631	AIA (FITF) Status No

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to the communication of 1/21/2020.
☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.

2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.

3. ☒ The allowed claim(s) is/are 1-20 . As a result of the allowed claim(s), you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.

4. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

Certified copies:

a) ☐ All b) ☐ Some *c) ☐ None of the:

1. ☐ Certified copies of the priority documents have been received.

2. ☐ Certified copies of the priority documents have been received in Application No. _____.

3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

* Certified copies not received: _____.

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.

5. ☐ CORRECTED DRAWINGS (as "replacement sheets") must be submitted.
☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____.

Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).

6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Attachment(s)

1. ☐ Notice of References Cited (PTO-892)	5. ☒ Examiner's Amendment/Comment
2. ☒ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date 1/30/2020.	6. ☒ Examiner's Statement of Reasons for Allowance
3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material _____.	7. ☐ Other _____.
4. ☐ Interview Summary (PTO-413), Paper No./Mail Date. _____.	

/RUSSELL S NEGIN/ Primary Examiner, Art Unit 1631	
--	--

CONTINUED EXAMINATION UNDER 37 CFR 1.114

A request for continued examination under 37 CFR 1.114, including the fee set forth in 37 CFR 1.17(e), was filed in this application after final rejection. Since this application is eligible for continued examination under 37 CFR 1.114, and the fee set forth in 37 CFR 1.17(e) has been timely paid, the finality of the previous Office action has been withdrawn pursuant to 37 CFR 1.114. Applicant's submission filed on 21 January 2020 has been entered.

INFORMATION DISCLOSURE STATEMENT

The IDS of 1/30/2020 has been considered. The non-patent literature document of Han et al. has been crossed out since the document has been considered on a previous IDS.

REASONS FOR ALLOWANCE

The following is an examiner's statement of reasons for allowance:

The claims recite the new and non-obvious limitation of determining a unique presence and absence pattern of at least five polynucleotides in a sample using a cumulative intensity value of fluorophores and without using immobilization or mass spectrometry.

Any comments considered necessary by applicant must be submitted no later than the payment of the issue fee and, to avoid processing delays, should preferably accompany the issue fee. Such submissions should be clearly labeled "Comments on Statement of Reasons for Allowance."

CONCLUSION

Any inquiry concerning this communication or earlier communications from the Examiner should be directed to Russell Negin, whose telephone number is (571) 272-1083. This Examiner can normally be reached from Monday through Thursday from 9:00 am to 5 pm and variable hours on Fridays.

If attempts to reach the Examiner by telephone are unsuccessful, the Examiner's Supervisor, Karlheinz Skowronek, Supervisory Patent Examiner, can be reached at (571) 272-9047.

Information regarding the status of the application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only.

/RUSSELL S NEGIN/
Primary Examiner, Art Unit 1631
30 April 2020

<i>Search Notes</i> 	Application/Control No. 15/914,356	Applicant(s)/Patent Under Reexamination Kartalov et al.
	Examiner RUSSELL S NEGIN	Art Unit 1631

CPC - Searched*		
Symbol	Date	Examiner

CPC Combination Sets - Searched*		
Symbol	Date	Examiner

US Classification - Searched*			
Class	Subclass	Date	Examiner

* See search history printout included with this form or the SEARCH NOTES box below to determine the scope of the search.

Search Notes		
Search Notes	Date	Examiner
see attached search history printouts	04/30/2020	RSN
EAST prior art search	04/30/2020	RSN
EAST inventor search on all inventors	04/30/2020	RSN
PALM inventor search on all inventors	04/30/2020	RSN

Interference Search			
US Class/CPC Symbol	US Subclass/CPC Group	Date	Examiner
	see attached interference search history printouts	04/30/2020	RSN

/RUSSELL S NEGIN/ Primary Examiner, Art Unit 1631	
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Issue Classification 	Application/Control No. 15/914,356	Applicant(s)/Patent Under Reexamination Kartalov et al.	
	Examiner RUSSELL S NEGIN	Art Unit 1631	

CPC						
Symbol					Type	Version
G16B	/	25	/	00	F	2019-02-01
G16B	/	5	/	00	I	2019-02-01
C12Q	/	1	/	6851	I	2013-01-01
G16B	/	25	/	20	I	2019-02-01
G16B	/	40	/	10	I	2019-02-01
C12Q	/	1	/	68	I	2013-01-01
G01N	/	21	/	6486	I	2013-01-01
C12Q	/	1	/	6825	I	2013-01-01
G06F	/	17	/	10	A	2013-01-01
G06F	/	17	/	11	A	2013-01-01
G16B	/	40	/	00	A	2019-02-01
Y02A	/	50	/	53	A	2018-01-01

CPC Combination Sets						
Symbol					Type	Set
C12Q	/	1	/	6851	I	1
C12Q	/	2537	/	101	I	1
C12Q	/	2537	/	143	I	1
C12Q	/	2537	/	165	I	1
C12Q	/	2563	/	107	I	1

NONE		Total Claims Allowed:	
(Assistant Examiner)	(Date)	20	
/RUSSELL S NEGIN/ Primary Examiner, Art Unit 1631	30 April 2020	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	1

U.S. Patent and Trademark Office

Part of Paper No.: 20200430

Issue Classification 	Application/Control No. 15/914,356	Applicant(s)/Patent Under Reexamination Kartalov et al.
	Examiner RUSSELL S NEGIN	Art Unit 1631

INTERNATIONAL CLASSIFICATION			
CLAIMED			
G01N		33	48
G01N		33	50
NON-CLAIMED			

US ORIGINAL CLASSIFICATION	
CLASS	SUBCLASS
702	19

CROSS REFERENCES(S)					
CLASS	SUBCLASS (ONE SUBCLASS PER BLOCK)				

NONE		Total Claims Allowed:	
(Assistant Examiner)	(Date)	20	
/RUSSELL S NEGIN/ Primary Examiner, Art Unit 1631	30 April 2020	O.G. Print Claim(s)	O.G. Print Figure
(Primary Examiner)	(Date)	1	1

U.S. Patent and Trademark Office

Part of Paper No.: 20200430

Issue Classification 	Application/Control No. 15/914,356	Applicant(s)/Patent Under Reexamination Kartalov et al.
	Examiner RUSSELL S NEGIN	Art Unit 1631

☒ Claims renumbered in the same order as presented by applicant ☐ CPA ☒ T.D. ☐ R.1.47															
CLAIMS															
Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original	Final	Original

NONE (Assistant Examiner) _____ (Date) _____		Total Claims Allowed: 20	
/RUSSELL S NEGIN/ Primary Examiner, Art Unit 1631 (Primary Examiner) _____ (Date) 30 April 2020		O.G. Print Claim(s) 1	O.G. Print Figure 1

/RSN/ 4/30/2020

EAST Search History

EAST Search History (Prior Art)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	99	kartalov.in. near emil.in.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/30 07:51
L2	164	rajagopal.in. near aditya.in.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/30 07:51
L3	815	scherer.in. near axel.in.	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/30 07:51
L4	905	l1 l2 l3	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/30 07:52
L5	224437	polynucleotides	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/30 07:52
L6	57	l4 and l5	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/30 07:52
L7	64307	fluorophores	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/30 07:52
L8	537	cumulative adj intensity	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/30 07:52
L9	19	l7 and l8	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/30 07:52
L10	796876	presence near absence	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/30 07:53
L11	77	l8 and l10	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/30 07:53
L12	348034	hybridization	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/30 07:53
L13	32470	l7 and l12	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/30 07:53

L14	463217	droplets	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/30 07:53
L15	3886	l13 and l14	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/30 07:53
L16	7119219	signals	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/30 07:54
L17	3054	l15 and l16	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/30 07:54
L18	601983	probes	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/30 07:54
L19	2787	l17 and l18	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/30 07:54
L20	1185613	cancer	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/30 07:54
L21	2142	l19 and l20	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/30 07:54
L22	831967	infection	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/30 07:54
L23	1297	l21 and l22	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/30 07:54
L24	559572	viral	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/30 07:54
L25	1161	l23 and l24	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/30 07:55
L26	276780	fetal	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/30 07:55
L27	622	l25 and l26	US-PGPUB; USPAT; USOCR; FPRS; EPO; JPO; DERWENT	OR	OFF	2020/04/30 07:55

4/30/2020 7:55:29 AM

C:\Users\rnegin\Documents\EAST\Workspaces\15914356.wsp

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /RSN/

Approved for use through 11/30/2020. OMB 0651-0031

U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

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Substitute for form 1449/PTO INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Use as many sheets as necessary)			Complete if Known		
			Application Number	15914356	
			Filing Date	03-07-2018	
			First Named Inventor	KARTALOV; Emil P.	
			Art Unit	1631	
			Examiner Name	NEGIN, RUSSELL SCOTT	
Sheet	1	of	7	Attorney Docket Number	38075-716.302

U. S. PATENT DOCUMENTS					
Examiner Initials*	Cite No. ¹	Document Number	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
		Number-Kind Code ² (if known)			
	001	US-6103463	08-15-2000	CHETVERIN; Alexander B. et al.	
	002	US-7507575	03-24-2009	BEDINGHAM; William [Us] et al.	
	003	US-9791372	10-17-2017	MALIK; Imran R. et al.	
	004	US-20040053230	03-18-2004	SCHAFFER; Michael Edward [Us] et al.	
	005	US-20050053950	03-10-2005	ZUDAIRE; Ubani Enrique. et al.	
	006	US-20050106607	05-19-2005	YIN; Li-Te. et al.	
	007	US-20080096767	04-24-2008	KOHN; Heinz G.	
	008	US-20100120043	05-13-2010	SOOD; Anup. et al.	
	009	US-20100324834	12-23-2010	TREPTOW; Rainer [De] et al.	
	010	US-20110223602	09-15-2011	WHITMAN; Douglas F. [Us] et al.	
	011	US-20120040352	02-16-2012	WANGH; Lawrence J. [Us] et al.	
	012	US-20120101740	04-26-2012	ORPANA; Arto [Se] et al.	
	013	US-20140004520	01-02-2014	MOHAPATRA; Shyam S. et al.	
	014	US-20150211054	07-30-2015	KOSTEM; Emrah. et al.	
	015	US-20160108464	04-21-2016	SAXONOV; Serge. et al.	
	016	US-20160201122	07-14-2016	BUSHKIN; Yuri. et al.	
	017	US-20180052110	02-22-2018	MALIK; Imran R. et al.	
	018	US-20190002963	01-03-2019	RAJAGOPAL; Aditya.	

Examiner Signature		Date Considered	
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*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. ¹ Applicant's unique citation designation number (optional). ² See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. ³ Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). ⁴ For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. ⁵ Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. ⁶ Applicant is to place a check mark here if English language Translation is attached. This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 2 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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				Application Number	15914356
				Filing Date	03-07-2018
				First Named Inventor	KARTALOV; Emil P.
				Art Unit	1631
				Examiner Name	NEGIN, RUSSELL SCOTT
Sheet	2	of	7	Attorney Docket Number	38075-716.302

U. S. PATENT DOCUMENTS					
Examiner Initials*	Cite No. ¹	Document Number	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
		Number-Kind Code ² (if known)			
	019	US-20190112636	04-18-2019	RAJAGOPAL; Aditya. et al.	
	020	US-20200010876-A1	01-09-2020	MACDONALD; Christopher.	
	021	US-20190032112-A1	01-31-2019	RAJAGOPAL; Aditya. et al.	

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /RSN/

FOREIGN PATENT DOCUMENTS						
Examiner Initials*	Cite No. ¹	Foreign Patent Document	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear	T ⁶
		Country Code ³ Number ⁴ Kind Code ⁵ (if known)				
	001	EP-0519338-A1	12-23-1992	HOFFMANN LA ROCHE [CH]		☐
	002	WO-2009036514-A2	03-26-2009	MOLECULAR PLANT BREEDING NOMINEES LTD [AU], et al.		☐
	003	WO-2010007355-A1	01-21-2010	LUMORA LTD [GB], et al.		☐
	004	WO-2010013017-A1	02-04-2010	OXITEC LTD [GB], et al.		☐
	005	WO-2010017543-A1	02-11-2010	SMITHS DETECTION INC [US], et al.		☐
	006	WO-2014022827-A1	02-06-2014	CALIFORNIA INST OF TECHN [US]		☐

Examiner Signature		Date Considered	
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*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. ¹ Applicant's unique citation designation number (optional). ² See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. ³ Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). ⁴ For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. ⁵ Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. ⁶ Applicant is to place a check mark here if English language Translation is attached. This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 2 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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				Application Number	15914356
				Filing Date	03-07-2018
				First Named Inventor	KARTALOV; Emil P.
				Art Unit	1631
				Examiner Name	NEGIN, RUSSELL SCOTT
Sheet	3	of	7	Attorney Docket Number	38075-716.302

FOREIGN PATENT DOCUMENTS						
Examiner Initials*	Cite No. ¹	Foreign Patent Document	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear	T ⁶
		Country Code ³ Number ⁴ Kind Code ⁵ (if known)				
	007	WO-2014116884-A1	07-31-2014	CALIFORNIA INST OF TECHN [US]		☐
	008	WO-2019006023-A1	01-03-2019	CHROMACODE INC [US]		☐
	009	WO-9746714-A1	12-11-1997	UNIV UTAH RES FOUND [US], et al.		☐

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /RSN/

Examiner Signature		Date Considered	
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*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. ¹ Applicant's unique citation designation number (optional). ² See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. ³ Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). ⁴ For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. ⁵ Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. ⁶ Applicant is to place a check mark here if English language Translation is attached. This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 2 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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Substitute for form 1449/PTO		Complete if Known			
INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Use as many sheets as necessary)		Application Number	15914356		
		Filing Date	03-07-2018		
		First Named Inventor	KARTALOV; Emil P.		
		Art Unit	1631		
		Examiner Name	NEGIN, RUSSELL SCOTT		
Sheet	4	of	7	Attorney Docket Number	38075-716.302

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /RSN/

NON-PATENT LITERATURE DOCUMENTS			
Examiner Initials*	Cite No. ¹	Include name of the author(in CAPITAL LETTERS), title of the article(when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T ²
	001	BLACKET et al. Universal primers for fluorescent labeling of PCR fragments - an efficient and cost-effective approach to genotyping by fluorescence. Molecular Ecology Resources 12(3):456-463 (2012) Epub 2012 Jan 24.	☐
	002	Co-pending US patent application No. US16/537,415, filed on 08-09-2019.	☐
	003	EP17814096.8 Extended European Search Report dated December 2, 2019.	☐
	004	EP1818686.9 Extended Search Report dated July 23, 2019.	☐
	005	European Office Action for Patent Application No. EP13824744.0 dated November 6, 2018.	☐
	006	Extended European Search Report for Patent Application No. EP13824744.0 dated February 26, 2016.	☐
	007	First Office Action for Chinese Patent Application No. 201380049844.9 dated December 28, 2015 (w/translation).	☒
	008	FU et al. Multiplex detection and SNP genotyping in a single fluorescence channel. PLoS One 7(1):Article No. e30340 (January 2012).	☐
	009	~~HAN, et al., Quantum-dot-tagged microbeads for multiplexed optical coding of biomolecules. Nature Biotechnology 19.99 (July 2001): 631-635.~~	☐
	010	HAUSTEIN et al. Fluorescence correlation spectroscopy: novel variations of an established technique. Ann Rev Biophys Biomol Struct 36:151-69 (2007).	☐
	011	PCT/US2013/053512 International Search Report and Written Opinion dated October 16, 2013.	☐

Examiner Signature		Date Considered	
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*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. 1 Applicant's unique citation designation number (optional). 2 Applicant is to place a check mark here if English language Translation is attached. This collection of information is required by 37 CFR 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 2 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

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Substitute for form 1449/PTO INFORMATION DISCLOSURE STATEMENT BY APPLICANT (Use as many sheets as necessary)		Complete if Known			
		Application Number	15914356		
		Filing Date	03-07-2018		
		First Named Inventor	KARTALOV; Emil P.		
		Art Unit	1631		
		Examiner Name	NEGIN, RUSSELL SCOTT		
Sheet	5	of	7	Attorney Docket Number	38075-716.302

NON-PATENT LITERATURE DOCUMENTS			
Examiner Initials*	Cite No. ¹	Include name of the author(in CAPITAL LETTERS), title of the article(when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T ²
	012	PCT/US2018/39846 International Search Report and Written Opinion Mailed September 14, 2018.	☐
	013	RAJAGOPAL et al. Supercolor Coding Methods for Large-Scale Multiplexing of Biochemical Assays. Analytical Chemistry 85:7629-7636 (2013).	☐
	014	SAIKI, et al. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science, 239(4839):487-491 (January 29, 1988).	☐
	015	Second Office Action for Chinese Patent Application No. 201380049844.9 dated September 19, 2016 (w/translation).	☒
	016	ThermoFisher Scientific Fluorescence SpectraViewer. Retrieved from http://www.thermofisher.com/us/en/home/life-science/cell-analysis/labeling-chemistry/fluorescence-spectraviewer.html on September 25, 2015.	☐
	017	U.S. Application No. 13/958,479 Office Action dated March 1, 2016.	☐
	018	U.S. Application No. 15/892,245 First Action Interview - Office Action mailed July 19, 2018.	☐
	019	U.S. Patent Application No. 16/128,343 Office Action dated October 15, 2019.	☐
	020	U.S. Serial No. 16/128,343 First Action Interview dated April 18, 2019.	☐
	021	ZHAO et al. Comprehensive Algorithm for Quantitative Real-Time Polymerase Chain Reaction. Journal of Computational Biology 12(8):1047-1064 (2005).	☐

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /RSN/

Examiner Signature	/RUSSELL S NEGIN/	Date Considered	04/30/2020
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*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. 1 Applicant's unique citation designation number (optional). 2 Applicant is to place a check mark here if English language Translation is attached. This collection of information is required by 37 CFR 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 2 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

If you need assistance in completing the form, call 1-800-PTO-9199 (1-800-786-9199) and select option 2.



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BIB DATA SHEET

CONFIRMATION NO. 2457

SERIAL NUMBER	FILING or 371(c) DATE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.		
15/914,356	03/07/2018 RULE	702	1631	38075-716.302		
APPLICANTS California Institute of Technology, Pasadena, CA; INVENTORS Emil P. Kartalov, Pasadena, CA; Aditya Rajagopal, Orange, CA; Axel Scherer, Pasadena, CA; ** CONTINUING DATA ***** This application is a CON of 14/451,876 08/05/2014 PAT 10068051 which is a CON of 13/756,760 02/01/2013 PAT 8838394 which claims benefit of 61/703,093 09/19/2012 and claims benefit of 61/594,480 02/03/2012 ** FOREIGN APPLICATIONS ***** ** IF REQUIRED, FOREIGN FILING LICENSE GRANTED ** 10/18/2018						
Foreign Priority claimed ☐ Yes ☒ No	35 USC 119(a-d) conditions met ☐ Yes ☒ No	☐ Met after Allowance /RSN/ Initials	STATE OR COUNTRY CA	SHEETS DRAWINGS 8	TOTAL CLAIMS 20	INDEPENDENT CLAIMS 1
Verified and Acknowledged	/RUSSELL SCOTT NEGIN/ Examiner's Signature					
ADDRESS WILSON, SONSINI, GOODRICH & ROSATI 650 PAGE MILL ROAD PALO ALTO, CA 94304-1050 UNITED STATES						
TITLE SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS						
FILING FEE RECEIVED 1720	FEES: Authority has been given in Paper No. _____ to charge/credit DEPOSIT ACCOUNT No. _____ for following:			☐ All Fees		
				☐ 1.16 Fees (Filing)		
				☐ 1.17 Fees (Processing Ext. of time)		
				☐ 1.18 Fees (Issue)		
				☐ Other _____		
				☐ Credit		

EAST Search History

EAST Search History (Interference)

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	529	hybrization	US-PGPUB; USPAT	OR	OFF	2020/04/30 08:32
L2	442551	probes	US-PGPUB; USPAT	OR	OFF	2020/04/30 08:33
L3	59542	fluorophores	US-PGPUB; USPAT	OR	OFF	2020/04/30 08:33
L4	79	l1 and l2 and l3	US-PGPUB; USPAT	OR	OFF	2020/04/30 08:33
L5	608781	presence near absence	US-PGPUB; USPAT	OR	OFF	2020/04/30 08:33
L6	28225	l3 and l5	US-PGPUB; USPAT	OR	OFF	2020/04/30 08:33
L7	210104	polynucleotide	US-PGPUB; USPAT	OR	OFF	2020/04/30 08:33
L8	16555	l6 and l7	US-PGPUB; USPAT	OR	OFF	2020/04/30 08:33
L9	309615	cumulative	US-PGPUB; USPAT	OR	OFF	2020/04/30 08:33
L10	2180	l8 and l9	US-PGPUB; USPAT	OR	OFF	2020/04/30 08:33
L11	23	intesnity	US-PGPUB; USPAT	OR	OFF	2020/04/30 08:34
L12	1489578	intensity	US-PGPUB; USPAT	OR	OFF	2020/04/30 08:34
L13	1627	l10 and l12	US-PGPUB; USPAT	OR	OFF	2020/04/30 08:34

L14	6929860	combination	US-PGPUB; USPAT	OR	OFF	2020/04/30 08:34
L15	1616	l13 and l14	US-PGPUB; USPAT	OR	OFF	2020/04/30 08:34

4/30/2020 8:34:44 AM

C:\Users\rnegin\Documents\EAST\Workspaces\15914356.wsp

15914356

Google Scholar Search

/RSN/ 4/30/2020

polynucleotide analytes hybridization

polynucleotide analytes hybridization fluorophores

polynucleotide analytes hybridization fluorophores cumulative

polynucleotide hybridization fluorophores cumulative

polynucleotide hybridization fluorophores cumulative probes

polynucleotide hybridization fluorophores signals probes

polynucleotide hybridization fluorophores signals cumulative

polynucleotide hybridization fluorophores presence absence cumulative

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application	)	
Inventors: KARTALOV, Emil P., et al.	)	Confirmation No.: 2457
Application No.: 15/914,356	)	Art Unit: 1631
Filed: March 07, 2018	)	Examiner: NEGIN, Russell Scott
Title: SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS	)	Customer No. 021971

ELECTRONICALLY FILED ON: July 23, 2020

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

AMENDMENT AFTER ALLOWANCE UNDER 37 C.F.R § 1.312

Dear Commissioner:

This Amendment under 37 C.F.R. § 1.312 is submitted after the Notice of Allowance mailed May 4, 2020. Entry of the following amendment under 37 C.F.R. § 1.312 is respectfully requested.

Amendments to the Claims begin on page **2** of this paper.

Remark begins on page **5** of this paper.

AMENDMENTS TO THE CLAIMS

1. (Currently Amended) A method of unambiguously detecting any unique combination of presence or absence of at least five polynucleotide analytes in a plurality of droplets, the method comprising:
 - (a) providing a sample comprising, or potentially comprising, at least one of said at least five polynucleotide analytes;
 - (b) forming a mixture of said sample and at least five hybridization probes, wherein each of said at least five hybridization probes further comprises at least one fluorophore and at most four fluorophores;
 - (c) partitioning said mixture into said plurality of droplets;
 - (d) exciting said at least one fluorophore to generate one or more signals if one or more of said at least five polynucleotide analytes is present in said plurality of droplets, wherein said one or more signals comprise at least one signal generated by excitement of said ~~one or more~~ at least one fluorophore[[s]];
 - (e) measuring said one or more signals to generate a cumulative intensity measurement, wherein said cumulative intensity measurement corresponds to the presence of a unique combination of presence or absence of said at least five polynucleotide analytes in said sample; and
 - (f) determining whether each of said least five polynucleotide analytes is present, in any unique combination of presence or absence, based on said cumulative intensity measurement, wherein the method does not require any step of immobilization of said at least five polynucleotide analytes or mass spectrometry.
2. (Previously Presented) The method of claim 1, wherein each of said at least five hybridization probes is complementary to one of said at least five polynucleotide analytes.
3. (Previously Presented) The method of claim 1, wherein said at least five hybridization probes comprise one or more fluorophores selected from one, two, three, or four fluorophores.
4. (Previously Presented) The method of claim 1, wherein, in (b), a droplet of said plurality of droplets comprises said at least five hybridization probes.

5. (Previously Presented) The method of claim 1, wherein, in (b), a droplet of said plurality of droplets comprises one or more of said at least five polynucleotide analytes.
6. (Previously Presented) The method of claim 1, wherein, in (b), a droplet of said plurality of droplets comprises at most one of said at least five polynucleotide analytes.
7. (Previously Presented) The method of claim 1, wherein said one or more signals is generated during a polymerase chain reaction.
8. (Previously Presented) The method of claim 1, wherein said at least five polynucleotide analytes are at least seven polynucleotide analytes.
9. (Previously Presented) The method of claim 1, wherein said at least five hybridization probes are at least seven hybridization probes.
10. (Currently Amended) The method of claim 1, wherein said one or more of said at least five polynucleotide analytes comprise cell-free nucleic acid, tumor nucleic acid, fetal nucleic acid, viral nucleic acid, or any combination thereof.
11. (Previously Presented) The method of claim 1, wherein said sample is a biological sample derived from a subject.
12. (Previously Presented) The method of claim 11, wherein detecting the presence or absence of said at least five polynucleotide analytes indicates the presence of cancer or an infection with a pathogen in said subject.
13. (Previously Presented) The method of claim 1, further comprising transmitting information concerning the presence or absence of said at least five polynucleotide analytes through a computer network.
14. (Currently Amended) The method of claim 1, further comprising providing information concerning the presence or absence of said at least five polynucleotide analytes to a health care professional, wherein said[[the]] health care professional makes a clinical decision based on said information.
15. (Previously Presented) The method of claim 14, wherein said clinical decision is a treatment decision.
16. (Currently Amended) The method of claim 15, wherein said treatment decision comprises treatment with an antibiotic or an anticancer therapeutic.

17. (Previously Presented) The method of claim 1, wherein at least part of said method is performed using a computer.
18. (Previously Presented) The method of claim 17, wherein said computer is located on a remote server.
19. (Previously Presented) The method of claim 17, wherein said computer is located on a droplet reader.
20. (Currently Amended) The method of claim 1, wherein each of said at least five hybridization probes are provided at a given concentration such that said cumulative intensity measurement corresponds to said presence of said unique combination.

REMARKS

This Amendment under 37 C.F.R. § 1.312 is submitted after the Notice of Allowance mailed May 4, 2020, and before payment of the Issue Fee.

Claims 1-20 have been allowed. Claims 1, 10, 14, 16, and 20 are herein amended for antecedent basis and typographical purposes. No new matter has been added by this amendment. Accordingly, Applicant respectfully requests entry of the amendment.

CONCLUSION

Applicant respectfully requests entry of the abovementioned amendments. Should the examiner have any requests, the Examiner is encouraged to contact the undersigned at (650) 849-3327. If any fees be required for any reason, the Commissioner is authorized to charge said fees to Deposit Account No. 23-2415 and reference Docket No. 38075-716.302.

Respectfully submitted,

WILSON SONSINI GOODRICH & ROSATI
Professional Corporation

Date: July 23, 2020

By: /Nicholas Tiee/
Nicholas Tiee, Ph.D.
Registration No. 77,907

Wilson Sonsini Goodrich & Rosati
650 Page Mill Road
Palo Alto, California 94304
Telephone No.: (650) 493-9300
Facsimile No.: (650) 493-6811

Electronic Acknowledgement Receipt	
EFS ID:	40091825
Application Number:	15914356
International Application Number:	
Confirmation Number:	2457
Title of Invention:	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS
First Named Inventor/Applicant Name:	Emil P. Kartalov
Customer Number:	21971
Filer:	Nicholas Shaofeng Tiee/Cecilia Hui
Filer Authorized By:	Nicholas Shaofeng Tiee
Attorney Docket Number:	38075-716.302
Receipt Date:	23-JUL-2020
Filing Date:	07-MAR-2018
Time Stamp:	19:53:48
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no
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File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		38075_716302_312Amendments.pdf	113846	yes	6
			4437bffb776bf20835a319631b1cc6ad728c855		

Multipart Description/PDF files in .zip description			
Document Description		Start	End
Amendment after Notice of Allowance (Rule 312)		1	1
Claims		2	4
Applicant Arguments/Remarks Made in an Amendment		5	6

Warnings:

Information:

Total Files Size (in bytes):	113846
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New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

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PART B - FEE(S) TRANSMITTAL

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Commissioner for Patents
P.O. Box 1450
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By fax, send to: (571)-273-2885

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

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21971 7590 05/04/2020
WILSON, SONSINI, GOODRICH & ROSATI
650 PAGE MILL ROAD
PALO ALTO, CA 94304-1050

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I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being transmitted to the USPTO via EFS-Web or by facsimile to (571) 273-2885, on the date below.

(Typed or printed name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/914,356	03/07/2018	Emil P. Kartalov	38075-716.302	2457

TITLE OF INVENTION: SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	\$1000	\$0.00	\$0.00	\$1000	08/04/2020

EXAMINER	ART UNIT	CLASS-SUBCLASS
NEGIN, RUSSELL SCOTT	1631	702-019000

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-09 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list

(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,

(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

1 Wilson Sonsini Goodrich & Rosati

2 _____

3 _____

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document must have been previously recorded, or filed for recordation, as set forth in 37 CFR 3.11 and 37 CFR 3.81(a). Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE

California Institute of Technology

(B) RESIDENCE: (CITY and STATE OR COUNTRY)

Pasadena, CA

Please check the appropriate assignee category or categories (will not be printed on the patent): ☐ Individual ☒ Corporation or other private group entity ☐ Government

4a. Fees submitted: ☒ Issue Fee ☐ Publication Fee (if required) ☐ Advance Order - # of Copies _____

4b. Method of Payment: (Please first reapply any previously paid fee shown above)

☐ Electronic Payment via EFS-Web ☐ Enclosed check ☐ Non-electronic payment by credit card (Attach form PTO-2038)

☒ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment to Deposit Account No. 23-2415

5. Change in Entity Status (from status indicated above)

☐ Applicant certifying micro entity status. See 37 CFR 1.29

☐ Applicant asserting small entity status. See 37 CFR 1.27

☐ Applicant changing to regular undiscounted fee status.

NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.

NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.

NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature /Nicholas Tiee/Date July 23, 2020Typed or printed name Nicholas TieeRegistration No. 77,907

Electronic Patent Application Fee Transmittal				
Application Number:		15914356		
Filing Date:		07-Mar-2018		
Title of Invention:		SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS		
First Named Inventor/Applicant Name:		Emil P. Kartalov		
Filer:		Nicholas Shaofeng Tiee/Cecilia Hui		
Attorney Docket Number:		38075-716.302		
Filed as Large Entity				
Filing Fees for Utility under 35 USC 111(a)				
Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
UTILITY APPL ISSUE FEE	1501	1	1000	1000

Description	Fee Code	Quantity	Amount	Sub-Total in USD(\$)
Extension-of-Time:				
Miscellaneous:				
Total in USD (\$)				1000

Electronic Acknowledgement Receipt	
EFS ID:	40098531
Application Number:	15914356
International Application Number:	
Confirmation Number:	2457
Title of Invention:	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS
First Named Inventor/Applicant Name:	Emil P. Kartalov
Customer Number:	21971
Filer:	Nicholas Shaofeng Tiee/Cecilia Hui
Filer Authorized By:	Nicholas Shaofeng Tiee
Attorney Docket Number:	38075-716.302
Receipt Date:	24-JUL-2020
Filing Date:	07-MAR-2018
Time Stamp:	14:31:33
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	DA
Payment was successfully received in RAM	\$1000
RAM confirmation Number	E20207NE32140130
Deposit Account	
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows:	

File Listing:					
Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Issue Fee Payment (PTO-85B)	38075_716302_IFTransmittal.pdf	163811	no	1
			5b9a4dd2b00d30b0e49c9987933fc234f0bbd183		
Warnings:					
Information:					
2	Fee Worksheet (SB06)	fee-info.pdf	30549	no	2
			bec347a975f9a12cba6d3f50abd37fd4cc0d87b2		
Warnings:					
Information:					
Total Files Size (in bytes):			194360		
This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503. <u>New Applications Under 35 U.S.C. 111</u> If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application. <u>National Stage of an International Application under 35 U.S.C. 371</u> If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course. <u>New International Application Filed with the USPTO as a Receiving Office</u> If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.					



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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/914,356	03/07/2018	Emil P. Kartalov	38075-716.302	2457
21971 7590 07/29/2020 WILSON, SONSINI, GOODRICH & ROSATI 650 PAGE MILL ROAD PALO ALTO, CA 94304-1050			EXAMINER NEGIN, RUSSELL SCOTT	
			ART UNIT 1631	PAPER NUMBER
			NOTIFICATION DATE 07/29/2020	DELIVERY MODE ELECTRONIC

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

patentdocket@wsgr.com

Response to Rule 312 Communication	Application No. 15/914,356	Applicant(s) Kartalov et al.	
	Examiner RUSSELL S NEGIN	Art Unit 1631	AIA (FITF) Status No
-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address -- 1. ☒ The amendment filed on <u>23 July 2020</u> under 37 CFR 1.312 has been considered, and has been: a) ☒ entered. b) ☐ entered as directed to matters of form not affecting the scope of the invention. c) ☐ disapproved because the amendment was filed after the payment of the issue fee. Any amendment filed after the date the issue fee is paid must be accompanied by a petition under 37 CFR 1.313(c)(1) and the required fee to withdraw the application from issue. d) ☐ disapproved. See explanation below. e) ☐ entered in part. See explanation below. _____			
/RUSSELL S NEGIN/ Primary Examiner, Art Unit 1631			

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application	)	
Inventors: KARTALOV, Emil P., et al.	)	Confirmation No.: 2457
Application No.: 15/914,356	)	Art Unit: 1631
Filed: March 07, 2018	)	Examiner: NEGIN, Russell Scott
Title: SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS	)	Customer No. 021971

ELECTRONICALLY FILED ON: July 23, 2020

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

AMENDMENT AFTER ALLOWANCE UNDER 37 C.F.R. § 1.312

Dear Commissioner:

This Amendment under 37 C.F.R. § 1.312 is submitted after the Notice of Allowance mailed May 4, 2020. Entry of the following amendment under 37 C.F.R. § 1.312 is respectfully requested.

Amendments to the Claims begin on page **2** of this paper.

Remark begins on page **5** of this paper.

OK TO ENTER
/RSN/ 7/25/2020



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APPLICATION NO.	ISSUE DATE	PATENT NO.	ATTORNEY DOCKET NO.	CONFIRMATION NO.
15/914,356	09/08/2020	10770170	38075-716.302	2457

21971 7590 08/19/2020
WILSON, SONSINI, GOODRICH & ROSATI
650 PAGE MILL ROAD
PALO ALTO, CA 94304-1050

ISSUE NOTIFICATION

The projected patent number and issue date are specified above.

Determination of Patent Term Adjustment under 35 U.S.C. 154 (b) (application filed on or after May 29, 2000)

The Patent Term Adjustment is 0 day(s). Any patent to issue from the above-identified application will include an indication of the adjustment on the front page.

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (<http://pair.uspto.gov>).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Application Assistance Unit (AAU) of the Office of Data Management (ODM) at (571)-272-4200.

APPLICANT(s) (Please see PAIR WEB site <http://pair.uspto.gov> for additional applicants):

Emil P. Kartalov, Pasadena, CA;
California Institute of Technology, Pasadena, CA;
Aditya Rajagopal, Orange, CA;
Axel Scherer, Pasadena, CA;

The United States represents the largest, most dynamic marketplace in the world and is an unparalleled location for business investment, innovation, and commercialization of new technologies. The USA offers tremendous resources and advantages for those who invest and manufacture goods here. Through SelectUSA, our nation works to encourage and facilitate business investment. To learn more about why the USA is the best country in the world to develop technology, manufacture products, and grow your business, visit SelectUSA.gov.

TRANSMITTAL FOR POWER OF ATTORNEY TO ONE OR MORE REGISTERED PRACTITIONERS

NOTE: This form is to be submitted with the Power of Attorney by Applicant form (PTO/AIA/82B) to identify the application to which the Power of Attorney is directed, in accordance with 37 CFR 1.5, unless the application number and filing date are identified in the Power of Attorney by Applicant form. If neither form PTO/AIA/82A nor form PTO/AIA/82B identifies the application to which the Power of Attorney is directed, the Power of Attorney will not be recognized in the application.

Application Number	15/914,356
Filing Date	March 7, 2018
First Named Inventor	
Title	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS
Art Unit	1631
Examiner Name	NEGIN, RUSSELL SCOTT
Attorney Docket Number	30KJ-330783-US3

SIGNATURE of Applicant or Patent Practitioner

Signature	/Jesse A. Salen/	Date (Optional)	
Name	Jesse A. Salen	Registration Number	72209
Title (if Applicant is a juristic entity)			

Applicant Name (if Applicant is a juristic entity)

California Institute of Technology

NOTE: This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4(d) for signature requirements and certifications. If more than one applicant, use multiple forms.

☐

*Total of _____ forms are submitted.

This collection of information is required by 37 CFR 1.131, 1.32, and 1.33. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 3 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

POWER OF ATTORNEY BY APPLICANT

I hereby revoke all previous powers of attorney given in the application identified in either the attached transmittal letter or the boxes below.

Application Number	Filing Date
15/914,356	2018-03-07

(Note: The boxes above may be left blank if information is provided on form PTO/AIA/82A.)

☒ I hereby appoint the Patent Practitioner(s) associated with the following Customer Number as my/our attorney(s) or agent(s), and to transact all business in the United States Patent and Trademark Office connected therewith for the application referenced in the attached transmittal letter (form PTO/AIA/82A) or identified above:

30764

OR

☐ I hereby appoint Practitioner(s) named in the attached list (form PTO/AIA/82C) as my/our attorney(s) or agent(s), and to transact all business in the United States Patent and Trademark Office connected therewith for the patent application referenced in the attached transmittal letter (form PTO/AIA/82A) or identified above. (Note: Complete form PTO/AIA/82C.)

Please recognize or change the correspondence address for the application identified in the attached transmittal letter or the boxes above to:

☒ The address associated with the above-mentioned Customer Number

OR

☐ The address associated with Customer Number:

OR

☐ Firm or Individual Name

Address

City

State

Zip

Country

Telephone

Email

I am the Applicant (if the Applicant is a juristic entity, list the Applicant name in the box):

California Institute of Technology

☐ Inventor or Joint Inventor (title not required below)

☐ Legal Representative of a Deceased or Legally Incapacitated Inventor (title not required below)

☒ Assignee or Person to Whom the Inventor is Under an Obligation to Assign (provide signer's title if applicant is a juristic entity)

☐ Person Who Otherwise Shows Sufficient Proprietary Interest (e.g., a petition under 37 CFR 1.46(b)(2) was granted in the application or is concurrently being filed with this document) (provide signer's title if applicant is a juristic entity)

SIGNATURE of Applicant for Patent

The undersigned (whose title is supplied below) is authorized to act on behalf of the applicant (e.g., where the applicant is a juristic entity).

Signature /Fred Farina/

Date (Optional) 03/29/21

Name Fred Farina

Title Chief Innovation Officer, OTTCP

NOTE: Signature - This form must be signed by the applicant in accordance with 37 CFR 1.33. See 37 CFR 1.4 for signature requirements and certifications. If more than one applicant, use multiple forms.

☒ Total of 1 forms are submitted.

This collection of information is required by 37 CFR 1.131, 1.32, and 1.33. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 3 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Electronic Acknowledgement Receipt	
EFS ID:	42339649
Application Number:	15914356
International Application Number:	
Confirmation Number:	2457
Title of Invention:	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS
First Named Inventor/Applicant Name:	Emil P. Kartalov
Customer Number:	21971
Filer:	Jesse A. Salen/Jamie Barber
Filer Authorized By:	Jesse A. Salen
Attorney Docket Number:	38075-716.302
Receipt Date:	31-MAR-2021
Filing Date:	07-MAR-2018
Time Stamp:	21:22:42
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no
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File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Power of Attorney	POA02_30KJ330783US3.pdf	259287	no	2
			ab5915dc0f3fa5fe7072fee6fdd0d7dbf0316b33		

Warnings:

Information:	
Total Files Size (in bytes):	259287
This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503. <u>New Applications Under 35 U.S.C. 111</u> If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application. <u>National Stage of an International Application under 35 U.S.C. 371</u> If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course. <u>New International Application Filed with the USPTO as a Receiving Office</u> If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.	



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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
15/914,356	03/07/2018	Emil P. Kartalov	30KJ-330783-US3

CONFIRMATION NO. 2457

30764

SHEPPARD, MULLIN, RICHTER & HAMPTON LLP
650 Town Center Drive, 10th Floor
Costa Mesa, CA 92626

POA ACCEPTANCE LETTER



OC000000124641858

Date Mailed: 04/07/2021

NOTICE OF ACCEPTANCE OF POWER OF ATTORNEY

This is in response to the Power of Attorney filed 03/31/2021.

The Power of Attorney in this application is accepted. Correspondence in this application will be mailed to the above address as provided by 37 CFR 1.33.

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at (571) 272-4000 or (571) 272-4200 or 1-888-786-0101.

/mabebe/



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Alexandria, Virginia 22313-1450
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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO./TITLE
15/914,356	03/07/2018	Emil P. Kartalov	30KJ-330783-US3

CONFIRMATION NO. 2457

POWER OF ATTORNEY NOTICE

21971
WILSON SONSINI GOODRICH & ROSATI
650 PAGE MILL ROAD
PALO ALTO, CA 94304-1050



OC000000124641848

Date Mailed: 04/07/2021

NOTICE REGARDING CHANGE OF POWER OF ATTORNEY

This is in response to the Power of Attorney filed 03/31/2021.

- The Power of Attorney to you in this application has been revoked by the applicant. Future correspondence will be mailed to the new address of record(37 CFR 1.33).

Questions about the contents of this notice and the requirements it sets forth should be directed to the Office of Data Management, Application Assistance Unit, at (571) 272-4000 or (571) 272-4200 or 1-888-786-0101.

/mabebe/



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APPLICATION NUMBER	FILING OR 371(C) DATE	FIRST NAMED APPLICANT	ATTY.DOCKET NO./TITLE	REQUEST ID
15/914,356	03/07/2018	Emil P. Kartalov	88DG-330783-US3	192363

Acknowledgement of Change to Small Entity Status

The entity status change request below filed through Private PAIR on 07/13/2023 has been accepted.

CERTIFICATIONS:

Change of Entity Status:

☒ Applicant asserting small entity status. See 37 CFR 1.27.

NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.

This portion must be completed by the signatory or signatories making the entity status change in accordance with 37 CFR 1.4(d)(4).

Signature:	/Jesse A. Salen/
Name:	Jesse A. Salen
Registration Number:	42651