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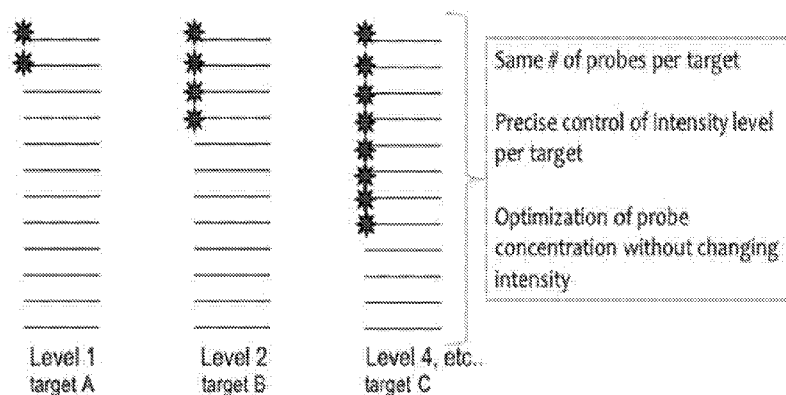
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FIG. 1



(57) Abstract: A method of identifying a first target nucleic acid comprising, providing a sample comprising the first target nucleic acid, providing a first set of paired oligonucleotides with complementarity to the first target nucleic acid, the first set of paired oligonucleotides comprising a first ratio of (a) first competitive oligonucleotides to (b) first signal oligonucleotides comprising a signal tag, wherein the competitive oligonucleotides compete with the signal oligonucleotides for binding to the first target nucleic acid, amplifying the first target nucleic acid with the polymerase chain reaction, thereby degrading the first signal oligonucleotide and permitting generation of a first signal, generating the first signal, measuring intensity of the first signal, and correlating the intensity of the first signal to the first ratio, thereby identifying the first target nucleic acid.

COMPETITIVE PROBES FOR ENGINEERING SIGNAL GENERATION**CROSS-REFERENCE**

[001] This application claims the benefit of U.S. Provisional Patent Application No. 62/317,151, filed April 1, 2016, which is hereby incorporated by reference in its entirety.

BACKGROUND

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

[003] In the age of whole genome sequencing and high-throughput analysis, there is a need for methods of detecting multiple qPCR targets at once. A limitation of current qPCR machines is the number of channels available for target detection. Modern instruments have between two and six channels, meaning that only two to six reporters can be detected in each experiment. Contemporary multiplexing technology provides for two to four different fluorescent reporters, and therefore two to four targets, to be detected in a single reaction. Unfortunately, accurately distinguishing each of the two to four targets from one another is thwarted by overlapping fluorescence signals of the different reporters. Therefore, a need still remains for a reliable and precise method for distinguishing multiple reporter signals during multiplex qPCR reactions.

SUMMARY

[004] The real-time polymerase chain reaction or quantitative PCR (qPCR) is a common molecular biology technique that uses fluorescent reporters to monitor the progress of the exponential amplification of target nucleic acids in a reaction. Traditionally the process is limited to differentiating the presence of one target per fluorescent emission filter (color channel) on a qPCR instrument.

[005] Recent advances made at the California Institute of Technology nanofabrication lab allow for detection of multiple analytes per emission filter using mathematical algorithms on real-time PCR amplification curve characteristics such as amplitude and curve shape (US Patent 8,838,394 issued September 16, 2014 and US Patent Application Publication Number US2015-0057178 published February 26, 2015, both of which are incorporated herein in their entirety). Targets in the same color channel are “coded” to exhibit these amplification curve characteristics. Coding includes setting the intensity level of each target in a color channel.

[006] Disclosed herein are methods by which the effective relative fluorescent intensity generated by a qPCR probe is adjusted independent of the total probe concentration for each target. This is a key improvement to the multi-analyte coding technique.

[007] Disclosed herein are methods and compositions for uniquely and unambiguously detecting multiple targets in a single color detection channel. One aspect of the disclosed methods is the ability to differentiate multiple targets by ratio-metric coding of signal intensity (brightness) of any given color for each target and mathematical strategies to circumvent degeneracy. When used within each of the available color channels on a qPCR instrument, the disclosed methods significantly increase the number of target measurements that can be interrogated.

[008] Provided herein are methods of identifying a first target nucleic acid comprising, providing a sample comprising the first target nucleic acid, providing a first set of paired oligonucleotides with complementarity to the first target nucleic acid, said first set of paired oligonucleotides comprising a first ratio of (a) first competitive oligonucleotides to (b) first signal oligonucleotides comprising a first signal tag, wherein the first competitive oligonucleotides compete with the first signal oligonucleotides for binding to the first target nucleic acid, amplifying the first target nucleic acid with the polymerase chain reaction, thereby degrading the first signal oligonucleotide and permitting generation of a first signal, generating the first signal, measuring intensity of the first signal, and correlating the intensity of the first signal to the first ratio, thereby identifying the first target nucleic acid. In some aspects, the sample comprises a second target nucleic acid. In some aspects, the method further comprises identifying the second target nucleic acid by providing a second set of paired oligonucleotides with complementarity to the second target nucleic acid, said second set of paired oligonucleotides comprising a second ratio of (a) second competitive oligonucleotides to (b) second signal oligonucleotides comprising a second signal tag, wherein the second competitive oligonucleotides compete with the second signal oligonucleotides for binding to the second target nucleic acid, amplifying the second target sequence with the polymerase chain reaction, thereby degrading the second signal oligonucleotide and permitting generation of a second signal, generating the second signal, measuring intensity of the second signal, and correlating the intensity of the second signal to the second ratio, thereby identifying the second target nucleic acid. In some aspects, the first signal oligonucleotides and the second signal oligonucleotides comprise the same signal tag. In some aspects, the first target nucleic acid and the second target nucleic acid are identified from differing signal intensities generated during the same polymerase chain reaction. In some aspects, the first signal intensity is different from the second signal intensity. In some aspects, the paired oligonucleotides comprise qPCR probes. In

some aspects, the signal oligonucleotides comprise a signal tag and a signal quenching tag. In some aspects, the signal tag comprises a fluorophore. In some aspects, the signal quenching tag blocks the generation of the signal corresponding with the signal tag until the signal quenching tag is separated from the signal tag by oligonucleotide degradation during the polymerase chain reaction, thereby permitting the signal to be generated. In some aspects, generating the signal comprises exciting the signal tag. In some aspects, exciting comprises directing at the signal tag at least one of illumination, electromagnetic radiation, and energy emitted from an energy source. In some aspects, the competitive oligonucleotides do not comprise a signal tag. In some aspects, the competitive oligonucleotides comprise at least one modified end. In some aspects, the modified end prevents enzymatic extension during amplification. In some aspects, the modified end comprises a 3' end lacking a hydroxyl group. In some aspects, the modified end comprises a 5' end lacking a phosphate group. In some aspects, the modified end comprises a 5' capped end. In some aspects, the modified end comprises a 3' capped end. In some aspects, the ratio of signal oligonucleotides to competitive oligonucleotides or the ratio of competitive oligonucleotides to signal oligonucleotides is about 1:1. In some aspects, the ratio of signal oligonucleotides to competitive oligonucleotides or the ratio of competitive oligonucleotides to signal oligonucleotides is about 1:2. In some aspects, the ratio of signal oligonucleotides to competitive oligonucleotides or the ratio of competitive oligonucleotides to signal oligonucleotides is about 1:3. In some aspects, the ratio of signal oligonucleotides to competitive oligonucleotides or the ratio of competitive oligonucleotides to signal oligonucleotides is about 1:4. In some aspects, the ratio of signal oligonucleotides to competitive oligonucleotides or the ratio of competitive oligonucleotides to signal oligonucleotides is about 1:5. In some aspects, the ratio of signal oligonucleotides to competitive oligonucleotides or the ratio of competitive oligonucleotides to signal oligonucleotides is about 1:6. In some aspects, the ratio of signal oligonucleotides to competitive oligonucleotides or the ratio of competitive oligonucleotides to signal oligonucleotides is about 1:7. In some aspects, the ratio of signal oligonucleotides to competitive oligonucleotides or the ratio of competitive oligonucleotides to signal oligonucleotides is about 1:8. In some aspects, the ratio of signal oligonucleotides to competitive oligonucleotides or the ratio of competitive oligonucleotides to signal oligonucleotides is about 1:9. In some aspects, the ratio of signal oligonucleotides to competitive oligonucleotides or the ratio of competitive oligonucleotides to signal oligonucleotides is about 1:10. In some aspects, the ratio of signal oligonucleotides to competitive oligonucleotides or the ratio of competitive oligonucleotides to signal oligonucleotides is about 1:16. In some aspects, the ratio of signal oligonucleotides to competitive oligonucleotides or the ratio of competitive oligonucleotides to signal oligonucleotides is about 1:20. In some aspects, the paired oligonucleotides comprise only

signal oligonucleotides or only competitive oligonucleotides. In some aspects, the total concentration of the first set of paired oligonucleotides is the same as the total concentration of the second set of paired oligonucleotides. In some aspects, the methods further comprise identifying up to thirty target nucleic acid sequences using up to thirty sets of paired oligonucleotides, wherein each set of paired oligonucleotides possesses complementarity to one of the up to thirty target nucleic acid sequences, wherein each of the up to thirty sets of paired oligonucleotides comprises a unique ratio of (a) competitive oligonucleotides to (b) signal oligonucleotides comprising a signal tag, wherein the competitive oligonucleotides within each set compete with the signal oligonucleotides within the same set for binding to a corresponding target nucleic acid sequence, amplifying the up to thirty target nucleic acid sequences with the polymerase chain reaction, thereby degrading the signal oligonucleotides and permitting generation of up to thirty signals, generating up to thirty signals, measuring the intensities of the up to thirty signals, and correlating the intensities of the up to thirty signals to the up to thirty ratios, thereby identifying the up to thirty target nucleic acid sequences. In some aspects, the signal tag of the signal oligonucleotides within each of the up to thirty sets of paired oligonucleotides are the same. In some aspects, the up to thirty target nucleic acid sequences are identified from signal intensities generated during the same polymerase chain reaction. In some aspects, each of the up to thirty ratios differs from every other of the up to thirty ratios. In some aspects, each of the up to thirty signal intensities differs from every other of the up to thirty signal intensities. In some aspects, the polymerase chain reaction is carried out by use of a nucleic acid polymerase having 5' to 3' exonuclease activity, an exo-polymerase, or a molecular beacon. In some aspects, the up to five signals are measured in a single fluorescence channel. In some aspects, the total concentration of each of the up to five sets of paired oligonucleotides is the same. In some aspects, a total number of molecules in the reaction remains constant

[009] Provided herein are compositions comprising a first set of paired oligonucleotides with complementarity to a first target nucleic acid, said first set of paired oligonucleotides comprising a first ratio of (a) competitive oligonucleotides to (b) signal oligonucleotides comprising a signal tag, wherein the competitive oligonucleotides are configured to compete with the signal oligonucleotides for binding to the first target nucleic acid during amplification of the first target nucleic acid with the polymerase chain reaction, wherein the signal oligonucleotides are adapted to generate a first signal having a first intensity when degraded by a nucleic acid polymerase during the polymerase chain reaction and excited by an energy source, and wherein the first intensity correlates with the first ratio.

[010] Provided herein are methods of identifying a first target molecule comprising, providing a sample comprising the first target molecule, providing a first set of paired capture molecules

that bind to the first target molecule, said first set of paired capture molecules comprising a first ratio of (a) first competitive capture molecules to (b) first signal capture molecules comprising a signal tag, wherein the competitive capture molecules compete with the signal capture molecules for binding to the first target molecule, generating the first signal, measuring intensity of the first signal, and correlating the intensity of the first signal to the first ratio, thereby identifying the first target molecule. In some aspects, the sample comprises a second target molecule. In some aspects, the methods further comprise identifying the second target molecule by providing a second set of paired capture molecules that binds to the second target molecule, said second set of paired capture molecules comprising a second ratio of (a) second competitive capture molecules to (b) second signal capture molecules comprising the signal tag, wherein the second competitive capture molecules compete with the second signal capture molecules for binding to the second target molecule, generating the second signal, measuring intensity of the second signal, and correlating the intensity of the second signal to the second ratio, thereby identifying the second target molecule. In some aspects, the first signal capture molecules and the second signal capture molecules comprise the same signal tag. In some aspects, the first target capture molecules and the second target capture molecules are identified from differing signal intensities generated. In some aspects, the first signal intensity is different from the second signal intensity. In some aspects, the signal tag comprises a fluorophore. In some aspects, generating the signal comprises exciting the signal tag. In some aspects, exciting comprises directing at the signal tag at least one of illumination, electromagnetic radiation, and energy emitted from an energy source. In some aspects, the competitive capture molecules do not comprise a signaling tag. In some aspects, the ratio of signal capture molecules to competitive capture molecules or the ratio of competitive capture molecules to signal capture molecules is about 1:1. In some aspects, the ratio of signal capture molecules to competitive capture molecules or the ratio of competitive capture molecules to signal capture molecules is about 1:2. In some aspects, the ratio of signal capture molecules to competitive capture molecules or the ratio of competitive capture molecules to signal capture molecules is about 1:3. In some aspects, the ratio of signal capture molecules to competitive capture molecules or the ratio of competitive capture molecules to signal capture molecules is about 1:4. In some aspects, the ratio of signal capture molecules to competitive capture molecules or the ratio of competitive capture molecules to signal capture molecules is about 1:5. In some aspects, the ratio of signal capture molecules to competitive capture molecules or the ratio of competitive capture molecules to signal capture molecules is about 1:6. In some aspects, the ratio of signal capture molecules to competitive capture molecules or the ratio of competitive capture molecules to signal capture molecules is about 1:7. In some aspects, the ratio of signal capture molecules to competitive capture molecules or the

ratio of competitive capture molecules to signal capture molecules is about 1:8. In some aspects, the ratio of signal capture molecules to competitive capture molecules or the ratio of competitive capture molecules to signal capture molecules is about 1:9. In some aspects, the ratio of signal capture molecules to competitive capture molecules or the ratio of competitive capture molecules to signal capture molecules is about 1:10. In some aspects, the ratio of signal capture molecules to competitive capture molecules or the ratio of competitive capture molecules to signal capture molecules is about 1:16. In some aspects, the ratio of signal capture molecules to competitive capture molecules or the ratio of competitive capture molecules to signal capture molecules is about 1:20. In some aspects, the first paired capture molecules comprise only signal capture molecules or only competitive capture molecules. In some aspects, the methods further comprise identifying up to one hundred target molecules using up to one hundred sets of paired capture molecules, wherein each set of paired capture molecules that binds to one of the up to one hundred target molecules, wherein each of the up to one hundred sets of paired capture molecules comprises a unique ratio of (a) competitive capture molecules to (b) signal capture molecules comprising a signal tag, wherein the competitive capture molecules within each set compete with the signal capture molecules within the same set for binding to a corresponding target molecule, generating up to one hundred signals, measuring the intensities of the up to one hundred signals, and correlating the intensities of the up to one hundred signals to the up to one hundred ratios, thereby identifying the up to one hundred target molecules. In some aspects, the signal tag of the signal capture molecules within each of the up to one hundred sets of capture molecules are the same. In some aspects, the up to one hundred target molecules are identified from signal intensities generated at the same time. In some aspects, each of the up to one hundred ratios differs from every other of the up to one hundred ratios. In some aspects, each of the up to one hundred signal intensities differs from every other of the up to one hundred signal intensities. In some aspects, the up to one hundred signals are measured in a single fluorescence channel. In some aspects, the target molecule is fixed to a surface. In some aspects, the capture molecule is fixed to a surface. In some aspects, the target molecule comprises a protein or a functional fragment thereof. In some aspects, the target molecule comprises an antigen. In some aspects, the target molecule comprises an aptamer. In some aspects, the target molecule comprises a nucleic acid. In some aspects, the capture molecule comprises a protein a protein or a functional fragment thereof. In some aspects, the capture molecule comprises an antibody a protein or a functional fragment thereof. In some aspects, the capture molecule comprises an aptamer. In some aspects, a total number of molecules in the reaction remains constant.

[011] Provided herein are compositions comprising a first set of paired capture molecules with complementarity to a first target molecule, said first set of paired capture molecules comprising

a first ratio of (a) competitive capture molecules to (b) signal capture molecules comprising a signal tag, wherein the competitive capture molecules are configured to compete with the signal capture molecules for binding to the target molecule, wherein the signal capture molecules are adapted to generate a first signal having a first intensity when excited by an energy source, and wherein the first intensity correlates with the first ratio. In some aspects, the target molecule comprises a protein or a functional fragment thereof. In some aspects, the target molecule comprises a antigen. In some aspects, the target molecule comprises a aptamer. In some aspects, the target molecule comprises a nucleic acid. In some aspects, the capture molecule comprises a protein or a functional fragment thereof. In some aspects, the capture molecule comprises an antibody or a functional fragment thereof. In some aspects, the capture molecule comprises an aptamer.

[012] Provided herein are methods of altering amplification level of a target nucleic acid comprising, providing a sample comprising the target nucleic acid, providing a set of paired oligonucleotides with complementarity to the target nucleic acid, said set of paired oligonucleotides comprising a ratio of (a) competitive oligonucleotides to (b) functional oligonucleotides, wherein the competitive oligonucleotides compete with the functional oligonucleotides for binding to the target nucleic acid and are not extendible by a nucleic acid polymerase, wherein the functional oligonucleotides are extendible by a nucleic acid polymerase as primers for amplifying the target nucleic acid by the polymerase chain reaction (PCR), and amplifying the target nucleic acid with PCR. In some aspects, the competitive oligonucleotides comprise at least one modified end. In some aspects, the modified end prevents enzymatic extension during amplification. In some aspects, the modified end comprises a 3' end lacking a hydroxyl group. In some aspects, the modified end comprises a 5' end lacking a phosphate group. In some aspects, the modified end comprises a 5' capped end. In some aspects, the modified end comprises a 3' capped end. In some aspects, a total number of molecules in the reaction remains constant.

[013] Provided herein are compositions comprising a set of paired oligonucleotides with complementarity to a target nucleic acid, said set of paired oligonucleotides comprising a ratio of (a) competitive oligonucleotides to (b) functional oligonucleotides, wherein the competitive oligonucleotides are configured to compete with the functional oligonucleotides for binding to the nucleic acid target during amplification of the first target nucleic acid with the polymerase chain reaction and are not extendible by a nucleic acid polymerase.

BRIEF DESCRIPTION OF THE DRAWINGS

[014] **Figure 1** illustrates three example probe sets with different ratios of competitive to fluorescent probes

[015] **Figure 2** illustrates an example PIV3 benchmark assay titration performed at four distinct coding levels.

[016] **Figures 3A-3B** illustrate an example RT-qPCR experiment with five distinct ratio levels of labeled and unlabeled competitive probes.

[017] **Figures 4A-4B** illustrate an example RT-qPCR experiment with three distinct ration levels of labeled and unlabeled competitive probes.

DETAILED DESCRIPTION

[018] Without being bound by theory, Polymerase Chain Reaction is a method of exponential amplification of specific target DNA in a reaction mix with a DNA polymerase and primers. Primers are short single stranded DNA 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 split the double stranded DNA target into single stranded templates. In the cooling cycle, the primers bind to complementary sequence on the template. After the template is primed the DNA 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).

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

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

activity of the Taq polymerase thereby physically separating the fluorophore from the quencher and liberating signal for detection by the real time PCR instrument. Other methods, such as molecular beacon probes, are also used in real-time PCR and amenable to this competitive probe technique.

[021] For certain qPCR, multiplexed qPCR, and coded qPCR methods, there is a need to adjust signal level in a predictable fashion. This is typically done by increasing or decreasing the number of fluorometric reporters added to a reaction. While this does adjust signal level to the first order, it also risks altering the chemical behavior of the reaction mixture as the total number of participating reactants has also changed. Often, this adversely affects the performance of other components within the reaction mixture. In particular, this is a critical failure mode for multiplexed qPCR. Disclosed herein are methods and compositions for detecting multiple target sequences with the same fluorophore within a single coded or multiplexed qPCR reaction.

[022] Disclosed herein are methods and compositions for adjusting fluorescent intensity without adjusting the reactant concentrations in a mixture. In some embodiments, combinations of both light-emitting and non-light-emitting chemical reporters are used to independently adjust the fluorescent intensity of a qPCR reaction without altering the chemical interactivity of the qPCR reaction mixture. In some embodiments, this is achieved by creating a mixture of non-fluorescent oligo probes and the TaqMan reporter probes for every target interrogated in a qPCR reaction.

[023] The herein disclosed competitive probes (also referred to as dimming probes, or non-fluorescing probes) are oligonucleotides similar to target probes with the exception of the covalently attached fluorophore and quencher. In some embodiments, the competitive probes do not have the fluorophore or the quencher. In some embodiments, the competitive probes have capped 5' and 3' ends to prevent enzymatic extension. In some embodiments, the competitive probes have a 5' end lacking a phosphate group. In some embodiments, the competitive probes have a 3' end lacking a hydroxyl group. In general, competitive probes behave identically to the specific target probes in binding to the amplified target sequence, and in the case of TaqMan style assay, the bound probes are digested by the 5'→3' exonuclease activity of Taq polymerase. However, because the competitive probes do not contain a fluorophore, no signal is generated.

[024] Disclosed herein are methods and compositions for using the disclosed non-fluorescing, competitive binding probes to adjust the signal intensity for a single gene by ratiometric titration of the competitive binding probe. In some examples, these methods are performed using a TaqMan probe. Figure 1 diagrams three different probes mixtures with differing proportions of competitive probes and signal probes. In some embodiments, the titration of the intensity allows

for control of intensity level ratio-metric coding and the ability to adjust the total probe concentration of a given target without affecting the intensity level coding.

[025] In some embodiments, during the probe annealing portion of the PCR reaction the competitive probes will bind to the amplified target at a rate proportional to the ratio of competitive probes to the total probes both labeled and unlabeled in the reaction yielding a fluorescent signal proportional to the ratio of labeled probes to the total probes.

[026] In some embodiments, the competitive probes are un-extendable probes. For example, the competitive probes are unable to initiate DNA synthesis by the Taq polymerase by employing a variety of 3' modifications including but not limited to the phosphorylation or the addition of a quenching molecule.

[027] In some embodiments, different purification methods and probe sequences are used to yield dramatically different fluorescence intensities. In some examples, differences in the quality of purification leads to more or less unlabeled product (similar to a competitive probe) reducing the fluorescence of a preparation. In some embodiments, the oligo sequence makeup of a probe affects the fluorescence, particularly guanine bases proximal to the fluorophore which can act as a quencher reducing the intensity of fluorescence. In some embodiments, the competitive probes are used to adjust the signal intensity to be consistent between preparations for more consistent lot to lot performance. In some embodiments, competitive probes are used if a dye's intensity is overloading the detector on a particular real-time PCR instrument.

[028] In some embodiments, competitive probes are used to engineer the optical characteristic of a PCR curve without altering its chemical characteristic. In some examples, this is achieved because competitive probes competitively interact with other sequences, along with their TaqMan probe equivalents. In these examples, this competitive interaction allows for the unique capability to fine tune the signal to noise of a PCR reaction without changing the chemical performance of the PCR itself.

[029] Disclosed herein are methods and compositions for using competitive primers for multiplex quantitation by normalizing the efficiency of multiplexed reactions. In some embodiments, the herein disclosed methods are used to drive the PCR reaction into producing more of one strand than the other which can lead to an earlier plateau phase of the PCR reaction.

[030] In some embodiments, competitive probes are used to replace a portion of priming oligonucleotides in a PCR reaction. In many of these examples, the competitive probes are rendered un-extendable by 3' phosphorylation or other methodology. In these cases, the competitive primer will compete with the extendable primer leading to a quantifiable fraction of template strands to fail to replicate during the extension phase of the PCR. One advantage of

these embodiments is that it allows the adjustment of amplification efficiency of either one or both of the replication strands.

[031] In some embodiments, the herein disclosed competitive probes are used in TaqMan style PCR. In some embodiments, the herein disclosed competitive probes are used in traditional PCR.

[032] Disclosed herein are methods for identifying a target by detecting a corresponding signal, such as a fluorescent signal. In some examples, multiple targets can be detected in a single experiment, wherein the corresponding signal from each is the same. For example, multiple targets can be identified, where each target has a corresponding fluorescent signal, wherein the signal generating molecule, such as a fluorophore, is the same among each of the different targets to be detected.

[033] Using methods disclosed herein, multiple targets can be detected using the same fluorophore by providing sets of target-binding molecules, such as probes, wherein each set of molecules comprises a unique ratio of competitive probes to signal probes. Signal probes can comprise a signal generating molecules, such as a fluorophore. Competitive probes often do not contain the signal generating molecule. In these cases, the total number of probes within each set of probes is the same, which the ratio of competitive probes to signal probes is unique for each target to be detected. This allows for the same level or concentration of total probes to be used for each target while still being able to distinguish each individual target.

[034] Disclosed herein are methods and compositions for detecting multiple target sequences with the same fluorophore. Also disclosed herein are methods and compositions for detecting multiple target specific reporter signals in a single reaction using fluorophore baring oligonucleotide probes in combination with non-fluorophore baring oligonucleotide probes. In some examples, each target sequence is detected using a set of oligonucleotide probes. Each oligonucleotide set can comprise two populations of oligonucleotide probes, each comprising the same nucleic acid sequence. In these examples, one population of oligonucleotides comprises a reporter fluorophore while the second population of oligonucleotides does not comprise a fluorophore. In some embodiments, up to five sets of oligonucleotide probes are used to detect up to five target sequences in a single reaction. In many of these examples, the fluorophore of the up to five sets of probes is the same. In some aspects, within each set of oligonucleotide probes the ratio of fluorophore baring and non-fluorophore baring oligonucleotides is set to a predetermined ratio. In some embodiments, the ratio within each set of oligonucleotides is different for each of the up to five sets of oligonucleotides. In these examples, the different ratios allow the reporter signal for each of the up to five targets to be distinguished from one another within a single reaction. In these examples, the total

concentration of probes in each set of probes is the same, while the ratio of the two populations of probes within the probe set are different between each of the sets of probes. A fluorophore bearing probe can be a signal probe. A non-fluorophore bearing probe can be a competitive probe.

[035] In some embodiments, up to 100 sets of oligonucleotide probes are used to detect up to 100 target sequences in a single reaction. For example, there can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100 sets of oligonucleotide probes used to detect 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100 target sequences in a single reaction. In many of these examples, the fluorophore of the up to 100 sets of probes is the same. In some aspects, within each set of oligonucleotide probes the ratio of fluorophore bearing and non-fluorophore bearing oligonucleotides is set to a predetermined ratio. In some embodiments, the ratio within each set of oligonucleotides is different for each of the up to 100 sets of oligonucleotides. In these examples, the different ratios allow the reporter signal for each of the up to 100 targets to be distinguished from one another within a single reaction. In these examples, the total concentration of probes in each set of probes is the same, while the ratio of the two populations of probes within the probe set are different between each of the sets of probes. A fluorophore bearing probe can be a signal probe. A non-fluorophore bearing probe can be a competitive probe.

[036] In some embodiments greater than 100 oligonucleotide probes are used to detect greater than 100 target sequences in a single reaction. In some embodiments, greater than 100 oligonucleotide probes are used to detect up to 100 target sequences in a single reaction. In some embodiments, up to 100 oligonucleotide probes are used to detect greater than 100 target sequences in a single reaction.

[037] Disclosed herein are methods and compositions for using an unlabeled analog to the corresponding labeled entity that generates signal for detection in the presence of a particular analyte in any system. In some examples, the system is an ELISA (enzyme-linked immunosorbent assay), in which case the labeled entity is a labeled antibody. In these cases, the unlabeled analog is an unlabeled antibody. In some embodiments, the ratio of labeled and unlabeled antibodies within a target set are predetermined as discussed previously. In some embodiments, multiple targets can be detected in a single ELISA reaction using multiple target

sets of antibodies. In many of these examples, each set of target antibodies has a different ratio of labeled and unlabeled antibodies which allow the independent target reporting signal to be distinguished from the others. In these examples, the total concentration of antibodies in each set of antibodies is the same, while the ratio of the two populations of antibodies within the antibodies set are different between each of the sets of antibodies. A fluorophore bearing antibody can be a signal antibody. A non-fluorophore bearing antibody can be a competitive antibody.

[038] In some embodiments, the total number of molecules in a reaction remains constant despite that the ratio of functional to competitive entities changes between different sets. In some aspects, the total number of molecules refers to the proteins, nucleic acids, solution volume, other entities required for the reaction to take place. In some aspects the reaction is a qPCR, ELISA, PCR, or sequencing reaction. In some aspects, the entities are oligonucleotides, proteins, antibodies, qPCR probes, aptamers, nucleic acid or other appropriate molecule. In some aspects function al comprises performing its intended purpose, such as PCR extension or emitting of fluorescence signal. In some aspects, competitive comprises binding to the target molecule, whether that be a protein, nucleic acid, or another molecule, but not being able to perform the function of the function molecule, such as extend during PCR or emit a fluorescence signal when excited.

Antibody labeling

[039] ELISA is a standard analytical technique for identifying proteins using fluorescence-labelled antibody markers. Without being bound by theory, in a sandwich ELISA, two antibodies, a primary antibody and a fluorescently labeled secondary antibody, are used to bind and identify a protein of interest (antigen). In general, the two antibodies bind to distinct regions of the protein. The primary antibody is adhered to a surface or a reaction vessel, with its binding domain exposed to allow for protein capture. When a sample is introduced, the protein of interest will diffuse through the chamber and favorably bind to the primary antibody. After a wash procedure, the fluorescently labeled secondary antibody is introduced. This fluorescently labeled secondary antibody binds to a distinct epitope of the fixed antigen. After washing the excess, unbound secondary antibody, the sample is interrogated. The presence and intensity of the fluorescence signal correlates with the concentration of antigen that has been captured. However, the binding dynamics of the antibodies are not readily engineerable. Unlike nucleic acids, antibodies cannot be inorganically assembled and even simple changes to the antibody structure result in dramatic changes in binding efficiencies. This critically limits the efficiency of antibody multiplexing for ELISA.

[040] Disclosed herein are methods and compositions for using competitive, non-reporting probes for fluorescence reporting using antibodies. In some embodiments, the fluorescence

reporting is in the context of an ELISA assay. In some embodiments, a primary antibody is adhered to a surface or a reaction vessel and the protein of interest favorably binds to the primary antibody. In these cases, after a wash procedure, a fluorescently labeled secondary antibody is introduced and binds to a distinct epitope of the fixed antigen. In these cases, after a wash procedure, the sample is interrogated and the presence and intensity of the fluorescence signal correlates with the concentration of antigen that has been captured.

[041] In some embodiments, a set of secondary antibodies is introduced and binds to the fixed antigen. In some aspects, the antigen is fixed by being bound to a primary antibody, which is in turn adhered to a surface or a reaction vessel. In some aspects, the set of secondary antibodies comprises a labeled population and an unlabeled population. In some aspects, the secondary antibodies in the labeled and unlabeled populations are identical except for the presence or absence of the label. In some aspects, secondary antibodies being identical means they comprise the same protein sequence. In some aspects, identical means they bind to the same epitope. In some aspects, identical means they bind to the same antigen. In some embodiments, the label is a fluorescent label. In some embodiments, the ratio of labeled and unlabeled secondary antibodies within a set of secondary antibodies is set to a predetermined ratio. In some aspects, the ratio of labeled to unlabeled secondary antibodies correlated to a signal intensity. In some aspects, by measuring the signal intensity, the signal intensity can be correlated back to the ratio of labeled to unlabeled secondary antibodies.

[042] In some embodiments, the ratio of labeled to unlabeled secondary antibodies within a set of secondary antibodies is used to set the signal level for an ELISA immunoassay. In some aspects, the ratio is set without changing the sensitivity or the number of antigens captured. In some embodiments, the primary antibody determines capture sensitivity and sensitivity. In some embodiments, multiple distinct antigens can be detected in a single ELISA reaction using the methods disclosed herein. In some aspects, a set of secondary antibodies able to bind to each of the multiple antigens is used in the ELISA. In many cases, the same label is used to label the labeled secondary antibody within each of the multiple sets of secondary antibodies. In some aspects the ratio of labeled to unlabeled secondary antibodies is unique within each of the multiple sets of secondary antibodies. In many examples, the unique ratios correspond to unique signal intensities. In these and other examples, the unique signal intensities are measured and correlated back to the predetermined ratios, thereby identifying the set of secondary antibodies that generated the signal intensity. In this way, a specific antigen can be identified in an ELISA assay based on the unique signal generated by the unique ratio of labeled to unlabeled secondary antibodies within the unique set of secondary antibodies used to bind to the specific antigen. In many examples, these methods disclosed herein allow identification of multiple antigens despite

using the same label on the labeled secondary antibodies for each of the multiple antigens. This is achieved by using unique ratios of labeled to unlabeled secondary antibodies for each of the multiple antigens. The unique ratios correlate to unique signal intensities, thereby allowing identification of each of the multiple antigens.

Sequencing bias

[043] While genomic sequencing is poised to be the predominant clinical tool in the coming future, it has particularly susceptible to sample-preparation bias. Without being bound by theory, before nucleic acids are sequenced, they are enriched using highly multiplexed broad-binding PCR primers. However, this enrichment generates a large bias in the amplified targets. This bias results in ineffective sequencing. For applications such as cancer tissue genotyping and tumor heterogeneity assessment, this makes the technique inapplicable for many clinical use cases.

[044] Disclosed herein are methods and compositions for using competitive oligonucleotides to reduce sequencing bias. In some embodiments, sequencing bias is reduced by using a set of oligonucleotide primers during amplification. In some aspects, amplification comprises a polymerase chain reaction. In some embodiments, a set of primers comprises a population of functional primers and a population of competitive primers. In some aspects, functional primers comprise primers capable of annealing to a complementary sequence and being extended during a polymerase chain reaction. In some aspects, competitive primers comprise are identical in sequence to their corresponding functional primer, are able to bind to the same complementary sequence, but are not able to be extended during a polymerase chain reaction. In some embodiments, the ratio of functional and competitive primers is adjusted to alter the level of sequencing bias without changing the chemical interactivity of the primer sets themselves. In some embodiments, reducing or increasing the ratio of functional primers to competitive primers, increase or decrease the enrichment of DNA to be sequenced. In some aspects, this ratio adjustment is performed without affecting the enrichment of other portions of the genome. In some aspects, this ratio adjustment allows a fine control of PCR bias. In some aspects, this ratio adjustment allows for the compensation of the deficiencies of current PCR methods for sequencing prep.

[045] In some embodiments, the ratio of functional primers to competitive primers is increased. In some aspects, the ratio of functional primers to competitive primers is increased in order to increase the sequencing bias of a target sequence. In some aspects, altering sequencing bias comprises increasing the amplification level of the target sequence. In some aspects, increasing the amplification level of the target sequence leads to an increased level of the target sequence in the final sample to be sequenced.

[046] In some embodiments, the ratio of functional primers to competitive primers is decreased. In some aspects, the ratio of functional primers to competitive primers is decreased in order to decrease the sequencing bias of a target sequence. In some aspects, altering sequencing bias comprises decreasing the amplification level of the target sequence. In some aspects, decreasing the amplification level of the target sequence leads to an decreased level of the target sequence in the final sample to be sequenced.

[047] In some embodiments, competitive primers are not able to be extended. In some aspects, they are not able to be extended due to a competitive, capped, or otherwise modified end, such as those described and disclosed herein.

EXAMPLES

Example 1

[048] A Real-Time PCR reaction with an equimolar ratio of competitive probes and fluorogenic probes would yield approximately half the fluorescent signal at each reading as compared to a reaction with a similar amount of fluorogenic probes. Figure 2 shows the same TaqMan Assay with 4 differing proportions of fluorogenic probes to total probes. The 100% fluorescent probe reaction in yellow (solid top line) has an amplitude approximately 8 fold that of the assay in grey (long dashed line) which has 1 part fluorescent probes with 7 parts competitive probes. See Table 1 for the reaction make up and Table 2 for the PCR cycling parameters.

Table 1: Reaction components for Figure 2 reactions

Reaction Mix	Part #	12.5% fluorescent probe	25% fluorescent probe	50% fluorescent probe	100% fluorescent probe	No Template
ABI TaqMan Universal Master Mix	4304437	10 μ L	11 μ L	12 μ L	13 μ L	14 μ L
Ambion Nuclease Free Water	AM9937	4 μ L	4 μ L	4 μ L	4 μ L	4 μ L
Promega TE 1x (diluant)	V6231	6 μ L	6 μ L	6 μ L	6 μ L	6 μ L
Oligo (concentration in final reaction)						
Forward Primer 1	FBF2	200 nM	200 nM	200 nM	200 nM	200 nM
Reverse Primer 1	FBR2	200 nM	200 nM	200 nM	200 nM	200 nM
FAM Labeled Probe 1	FBP2-1	25 nM	25 nM	25 nM	25 nM	25 nM

Blunt Probe 1	FBP2-0	175 nM	175 nM	175 nM	175 nM	175 nM
Forward Primer 2	FAF1	200 nM	200 nM	200 nM	200 nM	200 nM
Reverse Primer 2	FAR1	200 nM	200 nM	200 nM	200 nM	200 nM
FAM Labeled Probe 2	FAP1-1	50 nM	50 nM	50 nM	50 nM	50 nM
Blunt Probe 2	FAP1-0	150 nM	150 nM	150 nM	150 nM	150 nM
Forward Primer 3	RBF1	200 nM	200 nM	200 nM	200 nM	200 nM
Reverse Primer 3	RBR1	200 nM	200 nM	200 nM	200 nM	200 nM
FAM Labeled Probe 3	RBP1-1	100 nM	100 nM	100 nM	100 nM	100 nM
Forward Primer 4	RBP1-0	100 nM	100 nM	100 nM	100 nM	100 nM
Reverse Primer 4	RAF2	200 nM	200 nM	200 nM	200 nM	200 nM
FAM Labeled Probe 4	RAR2	200 nM	200 nM	200 nM	200 nM	200 nM
Blunt Probe 4	RAP2-1	200 nM	200 nM	200 nM	200 nM	200 nM
template (copies per reaction)						
Template 1	FBT2	1000 copies	-	-	-	-
Template 2	FAT1	-	1000 copies	-	-	-
Template 3	RBT1	-	-	1000 copies	-	-
Template 4	RAT2	-	-	-	1000 copies	-
total volume [μ L]		20 μ L	20 μ L	20 μ L	20 μ L	20 μ L

Table 2: Temperature Schedule on ABI Vii7 for figure 2 reactions

Temperature Schedule:	Temperature [$^{\circ}$ C]	Time [s]	Data Collection
UNGase	50	120	N
Initial Denature	95	600	N
Number of Cycles	75		
Denature	95	15	N
Anneal	60	30	Y

Example 2

[049] A Real-Time PCR reaction is performed to detect three nucleic acid sequences of interest within a single reaction. In addition to the other components required for the RT-PCR reaction,

three sets of probes are added. One of each of the sets of probed corresponds to and is able to uniquely bind to one of the three transcripts of interest.

[050] Set 1 comprises 25% unlabeled competitive probes and 75% labeled probes, which are labeled with a green fluorophore and a quencher molecule. The probes within Set 1 are identical other than the presence of the fluorescent label and quencher molecule.

[051] Set 2 comprises 50% unlabeled competitive probes and 50% labeled probes, which are labeled with a green fluorophore and a quencher molecule. The probes within Set 2 are identical other than the presence of the fluorescent label and quencher molecule. The green fluorophore used in Set 2 is the same green fluorophore used in Set 1.

[052] Set 3 comprises 75% unlabeled competitive probes and 25% labeled probes, which are labeled with a green fluorophore and a quencher molecule. The probes within Set 3 are identical other than the presence of the fluorescent label and quencher molecule. The green fluorophore used in Set 3 is the same green fluorophore used in Set 1 and Set 2.

[053] The probes within each of Sets 1-3 are distinct from each other since they are selected for their ability to specifically bind to their corresponding nucleic acid target. However, the fluorophore used to label the labeled probes within each set is the same. Also, the ratio of unlabeled to labeled probes within each set are different and unique compared to the other sets.

[054] When the reaction first starts, no fluorescent signal is detected since the signals from each fluorophore are blocked by the quencher molecule. As the reaction proceeds, unlabeled and labeled probes within each set compete for binding to their complementary nucleic acid target sequence. Because the labeled and unlabeled probes within each set are basically identical, their binding and annealing kinetics are basically identical. When a labeled probe anneals, it is subsequently degraded by the 5' to 3' exonuclease activity of the polymerase. This degradation step separates the fluorophore and the quenching molecule, allowing for a fluorescence signal to be emitted upon excitation by the appropriate excitation source.

[055] An excitation source excites the green fluorophores and a signal is emitted from each of the released green fluorophores. Three distinct signal intensities are detected and measured. Each signal intensity correlated to a specific ratio. Because each set of probes comprised a unique ratio of labeled to unlabeled probes, a unique signal intensity can be correlated to that specific set of probes. Therefore, each unique signal intensity is used to identify one of each of the three nucleic acid targets.

Example 3

[056] Figures 3A and 3B demonstrate an example experiment exemplifying consistent leveling with competitive probes versus diluted probes.

[057] Ten RT-qPCR Master Mixes were made for a system containing three TaqMan™ assays in a single color channel for human rhinovirus, respiratory syncytial virus A (RSV A), and respiratory syncytial virus B (RSV B). All three assays utilized probes with the same fluorophore quencher combination. The total probe concentration of the mixtures was conserved for five of these reactions. Table 3 summarizes how the fluorescent and competitive probes were diluted to the same concentration. Table 4 summarizes how the primer sets were diluted to the same concentration. Table 5 displays the master mix composition for reactions containing both fluorescent and competitive probes. Table 5 displays the master mix composition for reactions containing fluorescent probes only with no competitive probes. Table 7 displays the concentration of the three templates included in each reaction.

[058] The fluorescent probe concentrations for two of the targets (RSV A and RSV B) were fixed but different. For the final target (human rhinovirus, RH), the fluorescent probe concentration was varied from 25nM. All reactions contained the same enzyme formulation and primer concentrations, as shown in Tables 5 and 6.

[059] The qPCR protocol for each of the 10 master mixes were tested with known single-stranded DNA templates at DNA templates with a fixed concentration of 10,000 copies per reaction (Table 7). The qPCR reactions were performed on a standard qPCR instrument with the cycling parameters shown in Table 8. The resulting data is depicted in Figure 3A and Figure 3B.

Table 3. Oligo mix

Oligo mix	Oligo	μM	Oligo volume to make 10x stock	TE Buffer	Total Volume
RH 100% Fluorescent	RHP1-1	101.1	2.47	997.53	1000.0
RH 100% Competitive	RHP1-0	95.2	2.63	997.37	1000.0
RSV B 100% Fluorescent	RBP1-1	101.1	2.24	997.76	1000.0
RSV A 25% Fluorescent	RAP2-1	95.9	0.50	999.50	1000.0
RSV A 75% Competitive	RAP2-0	93.8	2.00	998.00	1000.0

Table 4. 6plex primer mix

Primer set	Material	μM	Desired μM in 20X stock	Oligo volume to make 10x stock (μL)
Set 1	cdcFAUniF1	93.1	0.4	17.19
	cdcFAUniR1	99.2	0.4	16.13
Set 2	ccFAH1_2009F2	117	0.4	13.68
	ccFAH1_2009R2	114	0.4	14.04
Set 3	cdcFAH3F3	114.6	0.4	13.96
	cdcFAH3R3	97.23	0.4	16.46
Set 4	RHF1	97.7	0.4	16.38
	RHR1	96.7	0.4	16.55
Set 5	RAF2	102.2	0.4	15.66
	RAR2	94.7	0.4	16.90
Set 6	RBF1	94.2	0.4	16.99
	RBR1	94.7	0.4	16.90
Set 7	RBF1	94.2	0.4	42.46
	RBR1	94.7	0.4	42.24
	Promega V6231, TE	n/a	n/a	9.2

Table 5. Reaction Master Mix – with competitive probes

Material	100 FP	80 FP + 20 CP	60 FP + 40 CP	40 FP + 60 CP	20 FP + 80 CP
TaqPath ABI A28626	60 μL	60	60	60	60
20X 6plex primer mix	12	12	12	12	12
10X RH 100% Fluorescent	24	19.2	14.4	9.6	4.8
10X RH Competitive	0	4.8	9.6	14.4	19.2
10X RSV B 100% Fluorescent	24	24	24	24	24
10X RSV A 25% Fluorescent	24	24	24	24	24
10X RSV A Competitive	24	24	24	24	24
water RxBiosciences	12	12	12	12	12

Table 6. Reaction Master Mix – without competitive probes

Material	100 FP - BP	80 FP	60 FP	40 FP	20 FP
TaqPath ABI A28626	60 μ L	60	60	60	60
20X 6plex primer mix	12	12	12	12	12
10X RH 100% Fluorescent	24	19.2	14.4	9.6	4.8
10X RSV B 100% Fluorescent	24	24	24	24	24
10X RSV A 25% Fluorescent	24	24	24	24	24
water RxBiosciences	12	12	12	12	12
TE Promega V6231	24	28.8	33.6	38.4	43.2

Table 7. Templates

Name	Concentration
RHT1	2E3 copies/ μ L
RAT2	2E3 copies/ μ L
RBT1	2E3 copies/ μ L
NTC TE	NA (buffer only)

Table 8

Cycling Parameters

Step	time(seconds)	temperature($^{\circ}$ C)	
1	120	25	
2	600	53	
3	120	95	
4	3	95	
5	30	60	detection
	repeat step 4 & 5 90 cycles		

Example 4

[060] The following example experiments and resulting data displayed in Figure 4A and Figure 4B demonstrate the ability to deterministically generate a 3plex assay with the same

reporting fluorophore and quencher combination with the binary separation based upon initial 100% fluorescent probe data.

[061] To generate the initial fluorescence data a RT-qPCR Master Mix was made containing three TaqMan™ assays for human rhinovirus (Target A), respiratory syncytial virus A (Target B) and respiratory syncytial virus B (Target C). No non-fluorescent, competitive probes were used in these assays. All three assays were labeled with the same fluorophore quencher combination. The primer concentration was selected to be in excess of the total probe concentration. The same concentration of fluorogenic probe, and therefore total probe, was used for each target.

[062] Table 9 displays the composition of the oligo master mix, which contained the indicated fluorescent probes and no competitive probes. Table 10 displays the composition of the primer master mix. Table 11 displays the master mix composition for reactions containing only 100% fluorescent probes and no competitive probes. Table 12 displays the concentration of the three templates included in each reaction. Table 13 displays the cycling parameters used for the RT-qPCR reaction.

Table 9. 25X fluorescence probe Oligo Mix

Oligo Mix	Oligo	Stock Conc, μM	Conc. of 1X, μM	Vol, μL
Target A RH 100% Fluorescent	RHP1-1	79.9	0.025	3.13
Target B RSV A 100% Fluorescent	RAP2-1	95.9	0.025	2.61
Target C RSV B 100% Fluorescent	RBP1-1	101.1	0.025	2.47
	Promega V6231, TE	n/a	n/a	391.79
	Total			400.00

Table 10. 25X Primer Mix

Primer set	Name	Stock Conc, μM	Conc of 1X, μM	Vol, μL
Target A set	RHF1	97.7	0.1	10.24
	RHR1	96.7	0.1	10.34
Target B set	RAF2	102.2	0.1	9.78
	RAR2	94.7	0.1	10.56
Target C set	RBF1	94.2	0.1	10.62
	RBR1	94.7	0.1	10.56
	Promega V6231, TE	n/a	n/a	337.90
	Total			400.00

Table 11. Reaction Master Mix

Name	1 rxn	24 rxns
4x TaqPath with MP	5 μL	120
25X Primer mix	0.8	19.2
25X Fluorescent probe oligo mix	0.8	19.2
water	8.4	201.6

Table 12. Templates

Target	Name	Conc
Target A	RHT1	2E3 copies/μL
Target B	RAT2	2E3 copies/μL
Target C	RBT1	2E3 copies/μL
No template control	NTC TE	NA

Table 13. Cycling parameters

Step	time(seconds)	temperature(°C)	
1	120	25	
2	600	53	
3	120	95	
4	3	95	
5	30	60	detection
	repeat step 4 & 5 90 cycles		

[063] The qPCR protocol for each of the 10 master mixes were tested with known single-stranded DNA templates at DNA templates with a fixed concentration of 10,000 copies per reaction (Table 12). The qPCR reactions were performed on a standard qPCR instrument with the parameters displayed in Table 13. The results from this experiment are displayed in Table 14 and in Figure 4A. Six replicates were performed along with a no template control (NTC).

Table 14. Data from RT-qPCR with no competitive probes

Target	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6	NTC
Target A	0.4016	0.4028	0.3967	0.4018	0.4057	0.4056	0.0631
Target B	0.5291	0.5235	0.4415	0.5147	0.5324	0.5331	NA
Target C	0.4535	0.4415	0.4449	0.4445	0.4438	0.4381	0.0600

Table 15. Fluorescence score for experiment without competitive probes

Target	Name	Score
Target A	RH T1	0.4023
Target B	RSV A T2	0.5263
Target C	RSV B T1	0.4441

[064] To calculate the desired ratio of fluorogenic probe to competitive probe for a leveled mix, the median fluorescence level from the 6 replicates was determined from the data from the fluorescent probe only experiment (Table 15). Target C was set to the highest intensity level and left at 100% of the initial concentration of fluorogenic probe. The levels for Target A and Target B were engineered to be distinct by replacing a proportion of the fluorescent probe with a non-fluorescent, competitive probe. The total amount of probe (fluorescent plus non-fluorescent, competitive) was the same for every target and identical to the total probe concentrations for the experiments shown in Figure 4A.

[065] Table 16 displays the composition of the oligo master mix, which contained the indicated fluorescent probes and competitive probes. The primer master mix and reaction master mix used was the same as those displayed in Table 10 and Table 11 respectively. The templates and cycling parameters used were the same as those displayed in Tables 12 and Table 13 respectively.

[066] Six replicates along with a no template control (NTC) were performed. The resulting data is displayed in Table 17 and Figure 4B. The fluorescence score calculated for each target is displayed in Table 18.

Table 16. Fluorescent and Competitive probe oligo mix

Probe	Name	Stock Conc, μ M	Conc of 1X, μ M	Vol, μ L
Target A - Fluorescent	RHP1-1	53.7	0.0125	4.67
Target A - Competitive	RHP1-0	90.2	0.0125	2.76
Target B - Fluorescent	RAP2-1	9.59	0.0044	9.11
Target B - Competitive	RAP2-0	93.8	0.0206	4.40
Target C - Fluorescent	RBP1-1	101.1	0.0250	4.95
	Promega V6231, TE	n/a	n/a	774.11
	Total			800.00

Table 17. Data from RT-qPCR with competitive probes

Target	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	Replicate 6	NTC
Target A	0.2154	0.2121	0.2072	0.2129	0.2142	0.2161	0.0369
Target B	0.1192	0.1174	0.1145	0.1169	0.1129	0.1214	0.0359
Target C	0.4323	0.4247	0.4195	0.4190	0.4217	0.4243	0.0374

Table 18. Fluorescence score for experiment with competitive probes

Target	Name	Score
Target A	RH T1	0.2135
Target B	RSV A T2	0.1171
Target C	RSV B T1	0.4230

Example 5

[067] An ELISA assay is performed to detect three proteins of interest within a single reaction vessel. The sets of primary antibodies are adhered to a surface of a reaction vessel. One of each

sets is able to uniquely bind to one of each of the three proteins of interest. A sample comprising the three proteins of interest is added to the reaction vessel and each favorably binds to the corresponding primary antibody. A wash procedure is performed to removed unbound sample. Next, three sets of secondary antibodies are added to the reaction vessel, one of the sets corresponding to each of the three proteins of interest.

[068] Set 1 comprises 25% unlabeled competitive secondary antibodies and 75% labeled secondary antibodies, which are labeled with a green fluorophore. The secondary antibodies in Set 1 are identical other than the presence of the fluorescent label.

[069] Set 2 comprises 50% unlabeled competitive secondary antibodies and 50% labeled secondary antibodies, which are labeled with a green fluorophore. The secondary antibodies in Set 2 are identical other than the presence of the fluorescent label. The green fluorophore used in Set 2 is the same green fluorophore used in Set 1.

[070] Set 3 comprises 75% unlabeled competitive secondary antibodies and 25% labeled secondary antibodies, which are labeled with a green fluorophore. The secondary antibodies in Set 3 are identical other than the presence of the fluorescent label. The green fluorophore used in Set 3 is the same green fluorophore used in Set 1 and Set 2.

[071] The secondary antibodies within each of Sets 1-3 are distinct from each other since they are selected for their ability to specifically bind to their corresponding protein of interest. However, the fluorophore used to label the labeled secondary antibodies within each set is the same. Also, the ratio of unlabeled to labeled secondary antibodies within each set are different and unique compared to the other sets.

[072] The three sets of secondary antibodies are added to the reaction vessel. Labeled and unlabeled secondary antibodies from each set compete for binding to their corresponding protein epitope. Because the labeled and unlabeled secondary antibodies within each set are identical other than the presence of the fluorophore, their binding kinetics are basically the same. After the binding step, a wash procedure is performed to remove unbound secondary antibody.

[073] An excitation source excites the green fluorophores and a signal is emitted from each of the bound labeled secondary antibodies. Three distinct signal intensities are detected and measured. Each signal intensity correlated to a specific ratio. Because each set of secondary antibodies comprised a unique ratio of labeled to unlabeled secondary antibodies, a unique signal intensity can be correlated to that specific set of secondary antibodies. Therefore, each unique signal intensity is used to identify one of each of the three proteins of interest.

Example 6

[074] mRNA is isolated from a sample following an experiment and processed for sequencing. The mRNA sample reverse transcribed to cDNA and then PCR amplified in order to prepare the

sequencing library. Sequencing is performed to detect a target of interest and to determine how the target level has changed due to the conditions of the experiment compared to a control gene. Unfortunately, the target is from a very highly expressed gene and is over-represented in the sequencing library compared to the control gene. This makes it difficult to accurately calculate the impact of the experimental conditions on the expression level of the gene.

[075] The frustrated scientists go back to the original cDNA sample and re-amplify it by PCR. However, this time, they include a different set of target specific primers in the PCR reaction mix. The set of target primers comprises 25% functional primers capable of being extended during PCR, and 75% of competitive primers which cannot be extended due to a capped end.

[076] The resulting sequencing results has a lower level of the first transcript, which allows for a more accurate calculation of the transcription level caused by the conditions used in the original experiment.

CLAIMS

What is claimed is:

1. Method of identifying a first target nucleic acid comprising,
 - providing a sample comprising the first target nucleic acid,
 - providing a first set of paired oligonucleotides with complementarity to the first target nucleic acid, said first set of paired oligonucleotides comprising a first ratio of (a) first competitive oligonucleotides to (b) first signal oligonucleotides comprising a first signal tag,
 - wherein the first competitive oligonucleotides compete with the first signal oligonucleotides for binding to the first target nucleic acid,
 - amplifying the first target nucleic acid with the polymerase chain reaction, thereby degrading the first signal oligonucleotide and permitting generation of a first signal,
 - generating the first signal,
 - measuring intensity of the first signal, and
 - correlating the intensity of the first signal to the first ratio, thereby identifying the first target nucleic acid.
2. The method of claim 1, wherein the sample comprises a second target nucleic acid.
3. The method of claim 2, further comprising identifying the second target nucleic acid by:
 - providing a second set of paired oligonucleotides with complementarity to the second target nucleic acid, said second set of paired oligonucleotides comprising a second ratio of (a) second competitive oligonucleotides to (b) second signal oligonucleotides comprising a second signal tag,
 - wherein the second competitive oligonucleotides compete with the second signal oligonucleotides for binding to the second target nucleic acid,
 - amplifying the second target sequence with the polymerase chain reaction, thereby degrading the second signal oligonucleotide and permitting generation of a second signal,
 - generating the second signal,
 - measuring intensity of the second signal, and
 - correlating the intensity of the second signal to the second ratio, thereby identifying the second target nucleic acid.
4. The method of claim 3, wherein the first signal oligonucleotides and the second signal oligonucleotides comprise the same signal tag.

5. The method of any of claims 3-4, wherein the first target nucleic acid and the second target nucleic acid are identified from differing signal intensities generated during the same polymerase chain reaction.
6. The method of any of claims 3-5, wherein the first signal intensity is different from the second signal intensity.
7. The method of claim 1, wherein the paired oligonucleotides comprise qPCR probes.
8. The method of any of claims 1-7, wherein the signal oligonucleotides comprise a signal tag and a signal quenching tag.
9. The method of claim 8, wherein the signal tag comprises a fluorophore.
10. The method of and of claims 8-9, wherein the signal quenching tag blocks the generation of the signal corresponding with the signal tag until the signal quenching tag is separated from the signal tag by oligonucleotide degradation during the polymerase chain reaction, thereby permitting the signal to be generated.
11. The method of any of claims 1-10, wherein generating the signal comprises exciting the signal tag.
12. The method of claim 11, wherein exciting comprises directing at the signal tag at least one of illumination, electromagnetic radiation, and energy emitted from an energy source.
13. The method of any of claims 1-12 wherein the competitive oligonucleotides do not comprise a signal tag.
14. The method of any of claims 1-13, wherein the competitive oligonucleotides comprise at least one modified end.
15. The method of claim 14, wherein the modified end prevents enzymatic extension during amplification.
16. The method of claim 15, wherein the modified end comprises a 3' end lacking a hydroxyl group.
17. The method of claim 15, wherein the modified end comprises a 5' end lacking a phosphate group.
18. The method of claim 15, wherein the modified end comprises a 5' capped end.
19. The method of claim 15, wherein the modified end comprises a 3' capped end.
20. The method of any of claims 1-19, wherein the ratio of signal oligonucleotides to competitive oligonucleotides or the ratio of competitive oligonucleotides to signal oligonucleotides is about 1:1.
21. The method of any of claims 1-19, wherein the ratio of signal oligonucleotides to competitive oligonucleotides or the ratio of competitive oligonucleotides to signal oligonucleotides is about 1:2.

22. The method of any of claims 1-19, wherein the ratio of signal oligonucleotides to competitive oligonucleotides or the ratio of competitive oligonucleotides to signal oligonucleotides is about 1:3.
23. The method of any of claims 1-19, wherein the ratio of signal oligonucleotides to competitive oligonucleotides or the ratio of competitive oligonucleotides to signal oligonucleotides is about 1:4.
24. The method of any of claims 1-19, wherein the ratio of signal oligonucleotides to competitive oligonucleotides or the ratio of competitive oligonucleotides to signal oligonucleotides is about 1:5.
25. The method of any of claims 1-19, wherein the ratio of signal oligonucleotides to competitive oligonucleotides or the ratio of competitive oligonucleotides to signal oligonucleotides is about 1:6.
26. The method of any of claims 1-19, wherein the ratio of signal oligonucleotides to competitive oligonucleotides or the ratio of competitive oligonucleotides to signal oligonucleotides is about 1:7.
27. The method of any of claims 1-19, wherein the ratio of signal oligonucleotides to competitive oligonucleotides or the ratio of competitive oligonucleotides to signal oligonucleotides is about 1:8.
28. The method of any of claims 1-19, wherein the ratio of signal oligonucleotides to competitive oligonucleotides or the ratio of competitive oligonucleotides to signal oligonucleotides is about 1:9.
29. The method of any of claims 1-19, wherein the ratio of signal oligonucleotides to competitive oligonucleotides or the ratio of competitive oligonucleotides to signal oligonucleotides is about 1:10.
30. The method of any of claims 1-19, wherein the ratio of signal oligonucleotides to competitive oligonucleotides or the ratio of competitive oligonucleotides to signal oligonucleotides is about 1:16.
31. The method of any of claims 1-19, wherein the ratio of signal oligonucleotides to competitive oligonucleotides or the ratio of competitive oligonucleotides to signal oligonucleotides is about 1:20.
32. The method of any of claims 1-19, wherein the paired oligonucleotides comprise only signal oligonucleotides or only competitive oligonucleotides.
33. The method of any of the claims 1-32, wherein the total concentration of the first set of paired oligonucleotides is the same as the total concentration of the second set of paired oligonucleotides.

34. The method of any of claims 1-33, further comprising identifying up to thirty target nucleic acid sequences using up to thirty sets of paired oligonucleotides,
- wherein each set of paired oligonucleotides possesses complementarity to one of the up to thirty target nucleic acid sequences,
- wherein each of the up to thirty sets of paired oligonucleotides comprises a unique ratio of (a) competitive oligonucleotides to (b) signal oligonucleotides comprising a signal tag,
- wherein the competitive oligonucleotides within each set compete with the signal oligonucleotides within the same set for binding to a corresponding target nucleic acid sequence,
- amplifying the up to thirty target nucleic acid sequences with the polymerase chain reaction, thereby degrading the signal oligonucleotides and permitting generation of up to thirty signals,
- generating up to thirty signals,
- measuring the intensities of the up to thirty signals, and
- correlating the intensities of the up to thirty signals to the up to thirty ratios,
- thereby identifying the up to thirty target nucleic acid sequences.
35. The method of claim 34, wherein the signal tag of the signal oligonucleotides within each of the up to thirty sets of paired oligonucleotides are the same.
36. The method of any of claims 34-35, wherein the up to thirty target nucleic acid sequences are identified from signal intensities generated during the same polymerase chain reaction.
37. The method of any of claims 34-36, wherein each of the up to thirty ratios differs from every other of the up to thirty ratios.
38. The method of any of claims 34-37, wherein each of the up to thirty signal intensities differs from every other of the up to thirty signal intensities.
39. The method of any of claims 1-38, wherein the polymerase chain reaction is carried out by use of a nucleic acid polymerase having 5' to 3' exonuclease activity, an exo-polymerase, or a molecular beacon.
40. The method of any of claims 34-39, wherein the up to five signals are measured in a single fluorescence channel.
41. The method of any of the claims 34-40, wherein the total concentration of each of the up to five sets of paired oligonucleotides is the same.
42. The method of any of claims 1-41, wherein a total number of molecules in the reaction remains constant

43. A composition comprising:
- a first set of paired oligonucleotides with complementarity to a first target nucleic acid,
 - said first set of paired oligonucleotides comprising a first ratio of (a) competitive oligonucleotides to (b) signal oligonucleotides comprising a signal tag,
 - wherein the competitive oligonucleotides are configured to compete with the signal oligonucleotides for binding to the first target nucleic acid during amplification of the first target nucleic acid with the polymerase chain reaction,
 - wherein the signal oligonucleotides are adapted to generate a first signal having a first intensity when degraded by a nucleic acid polymerase during the polymerase chain reaction and excited by an energy source,
 - and wherein the first intensity correlates with the first ratio.
44. Method of identifying a first target molecule comprising,
- providing a sample comprising the first target molecule,
 - providing a first set of paired capture molecules that bind to the first target molecule, said first set of paired capture molecules comprising a first ratio of (a) first competitive capture molecules to (b) first signal capture molecules comprising a signal tag,
 - wherein the competitive capture molecules compete with the signal capture molecules for binding to the first target molecule,
 - generating the first signal,
 - measuring intensity of the first signal, and
 - correlating the intensity of the first signal to the first ratio, thereby identifying the first target molecule.
45. The method of claim 44, wherein the sample comprises a second target molecule.
46. The method of claim 45, further comprising identifying the second target molecule by:
- providing a second set of paired capture molecules that binds to the second target molecule, said second set of paired capture molecules comprising a second ratio of (a) second competitive capture molecules to (b) second signal capture molecules comprising the signal tag,
 - wherein the second competitive capture molecules compete with the second signal capture molecules for binding to the second target molecule,
 - generating the second signal,
 - measuring intensity of the second signal, and

correlating the intensity of the second signal to the second ratio, thereby identifying the second target molecule.

47. The method of claim 46, wherein the first signal capture molecules and the second signal capture molecules comprise the same signal tag.
48. The method of any of claims 45-47, wherein the first target capture molecules and the second target capture molecules are identified from differing signal intensities generated.
49. The method of any of claims 45-48, wherein the first signal intensity is different from the second signal intensity.
50. The method of any of claims 44-49, wherein the signal tag comprises a fluorophore.
51. The method of any of claims 44-50, wherein generating the signal comprises exciting the signal tag.
52. The method of claim 51, wherein exciting comprises directing at the signal tag at least one of illumination, electromagnetic radiation, and energy emitted from an energy source.
53. The method of any of claims 44-52 wherein the competitive capture molecules do not comprise a signaling tag.
54. The method of any of claims 44-53, wherein the ratio of signal capture molecules to competitive capture molecules or the ratio of competitive capture molecules to signal capture molecules is about 1:1.
55. The method of any of claims 44-53, wherein the ratio of signal capture molecules to competitive capture molecules or the ratio of competitive capture molecules to signal capture molecules is about 1:2.
56. The method of any of claims 44-53, wherein the ratio of signal capture molecules to competitive capture molecules or the ratio of competitive capture molecules to signal capture molecules is about 1:3.
57. The method of any of claims 44-53, wherein the ratio of signal capture molecules to competitive capture molecules or the ratio of competitive capture molecules to signal capture molecules is about 1:4.
58. The method of any of claims 44-53, wherein the ratio of signal capture molecules to competitive capture molecules or the ratio of competitive capture molecules to signal capture molecules is about 1:5.
59. The method of any of claims 44-53, wherein the ratio of signal capture molecules to competitive capture molecules or the ratio of competitive capture molecules to signal capture molecules is about 1:6.

60. The method of any of claims 44-53, wherein the ratio of signal capture molecules to competitive capture molecules or the ratio of competitive capture molecules to signal capture molecules is about 1:7.
61. The method of any of claims 44-53, wherein the ratio of signal capture molecules to competitive capture molecules or the ratio of competitive capture molecules to signal capture molecules is about 1:8.
62. The method of any of claims 44-53, wherein the ratio of signal capture molecules to competitive capture molecules or the ratio of competitive capture molecules to signal capture molecules is about 1:9.
63. The method of any of claims 44-53, wherein the ratio of signal capture molecules to competitive capture molecules or the ratio of competitive capture molecules to signal capture molecules is about 1:10.
64. The method of any of claims 44-53, wherein the ratio of signal capture molecules to competitive capture molecules or the ratio of competitive capture molecules to signal capture molecules is about 1:16.
65. The method of any of claims 44-53, wherein the ratio of signal capture molecules to competitive capture molecules or the ratio of competitive capture molecules to signal capture molecules is about 1:20.
66. The method of any of claims 44-53, wherein the first paired capture molecules comprise only signal capture molecules or only competitive capture molecules.
67. The method of any of claims 44-66, further comprising identifying up to one hundred target molecules using up to one hundred sets of paired capture molecules,
wherein each set of paired capture molecules that binds to one of the up to one hundred target molecules,
wherein each of the up to one hundred sets of paired capture molecules comprises a unique ratio of (a) competitive capture molecules to (b) signal capture molecules comprising a signal tag,
wherein the competitive capture molecules within each set compete with the signal capture molecules within the same set for binding to a corresponding target molecule,
generating up to one hundred signals,
measuring the intensities of the up to one hundred signals, and
correlating the intensities of the up to one hundred signals to the up to one hundred ratios, thereby identifying the up to one hundred target molecules.

68. The method of claim 67, wherein the signal tag of the signal capture molecules within each of the up to one hundred sets of capture molecules are the same.
69. The method of any of claims 67-68, wherein the up to one hundred target molecules are identified from signal intensities generated at the same time.
70. The method of any of claims 67-69, wherein each of the up to one hundred ratios differs from every other of the up to one hundred ratios.
71. The method of any of claims 67-70, wherein each of the up to one hundred signal intensities differs from every other of the up to one hundred signal intensities.
72. The method of any of claims 67-71, wherein the up to one hundred signals are measured in a single fluorescence channel.
73. The method of any of claims 44-72, wherein the target molecule is fixed to a surface.
74. The method of any of claims 44-73, wherein the capture molecule is fixed to a surface.
75. The method of any of claims 44-74, wherein the target molecule comprises a protein or a functional fragment thereof.
76. The method of any of claims 44-74, wherein the target molecule comprises an antigen.
77. The method of any of claims 44-74, wherein the target molecule comprises an aptamer.
78. The method of any of claims 44-74, wherein the target molecule comprises a nucleic acid.
79. The method of any of claims 44-78, wherein the capture molecule comprises a protein a protein or a functional fragment thereof.
80. The method of any of claims 44-78, wherein the capture molecule comprises an antibody a protein or a functional fragment thereof.
81. The method of any of claims 44-78, wherein the capture molecule comprises an aptamer.
82. The method of any of claims 44-81, wherein a total number of molecules in the reaction remains constant.
83. A composition comprising:
a first set of paired capture molecules with complementarity to a first target molecule,
said first set of paired capture molecules comprising a first ratio of (a) competitive capture molecules to (b) signal capture molecules comprising a signal tag, wherein the competitive capture molecules are configured to compete with the signal capture molecules for binding to the target molecule,
wherein the signal capture molecules are adapted to generate a first signal having a first intensity when excited by an energy source,
and wherein the first intensity correlates with the first ratio.

84. The composition of claim 83, wherein the target molecule comprises a protein or a functional fragment thereof.
85. The composition of claim 83, wherein the target molecule comprises a antigen.
86. The composition of claim 83, wherein the target molecule comprises a aptamer.
87. The composition of claim 83, wherein the target molecule comprises a nucleic acid.
88. The composition of any of claims 83-87, wherein the capture molecule comprises a protein or a functional fragment thereof.
89. The composition of any of claims 83-87, wherein the capture molecule comprises an antibody or a functional fragment thereof.
90. The composition of any of claims 83-87, wherein the capture molecule comprises an aptamer.
91. Method of altering amplification level of a target nucleic acid comprising,
providing a sample comprising the target nucleic acid,
providing a set of paired oligonucleotides with complementarity to the target nucleic acid, said set of paired oligonucleotides comprising a ratio of (a) competitive oligonucleotides to (b) functional oligonucleotides,
wherein the competitive oligonucleotides compete with the functional oligonucleotides for binding to the target nucleic acid and are not extendible by a nucleic acid polymerase, wherein the functional oligonucleotides are extendible by a nucleic acid polymerase as primers for amplifying the target nucleic acid by the polymerase chain reaction (PCR),
and
amplifying the target nucleic acid with PCR.
92. The method of claim 91, wherein the competitive oligonucleotides comprise at least one modified end.
93. The method of claim 92, wherein the modified end prevents enzymatic extension during amplification.
94. The method of claim 93, wherein the modified end comprises a 3' end lacking a hydroxyl group.
95. The method of claim 93, wherein the modified end comprises a 5' end lacking a phosphate group.
96. The method of claim 93, wherein the modified end comprises a 5' capped end.
97. The method of claim 93, wherein the modified end comprises a 3' capped end.
98. The method of any of claims 91-97, wherein a total number of molecules in the reaction remains constant.

99. A composition comprising:

a set of paired oligonucleotides with complementarity to a target nucleic acid,
said set of paired oligonucleotides comprising a ratio of (a) competitive
oligonucleotides to (b) functional oligonucleotides,

wherein the competitive oligonucleotides are configured to compete with the
functional oligonucleotides for binding to the nucleic acid target during amplification of
the first target nucleic acid with the polymerase chain reaction and are not extendible by
a nucleic acid polymerase.

FIG. 1

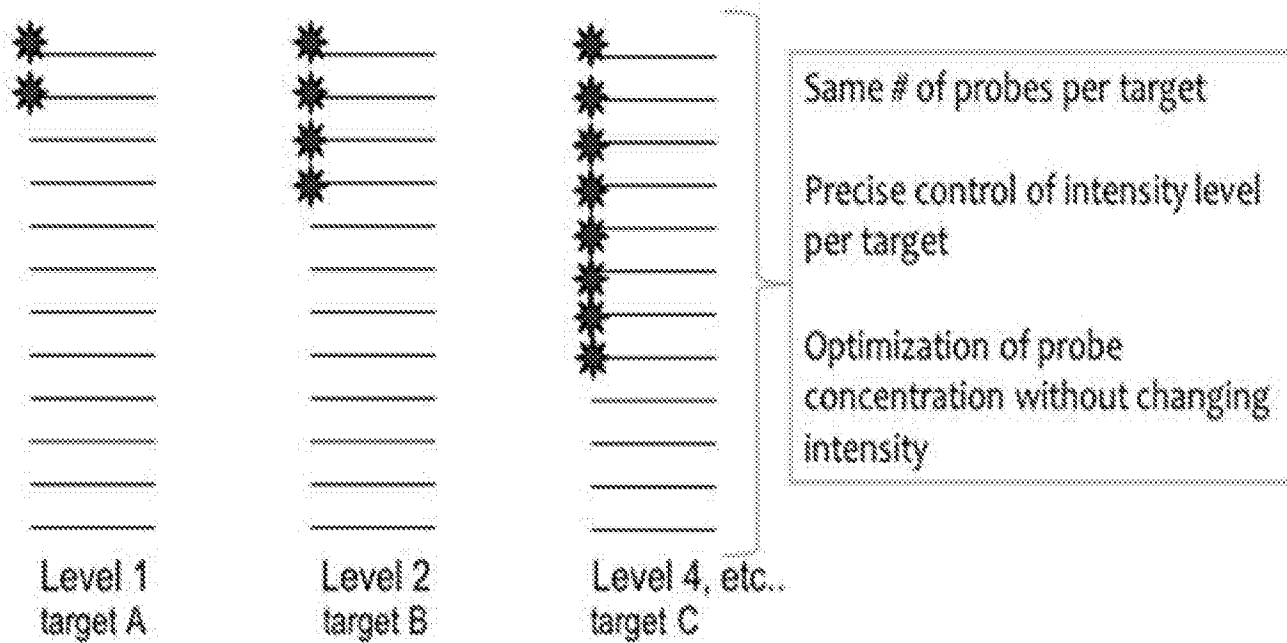


FIG. 2

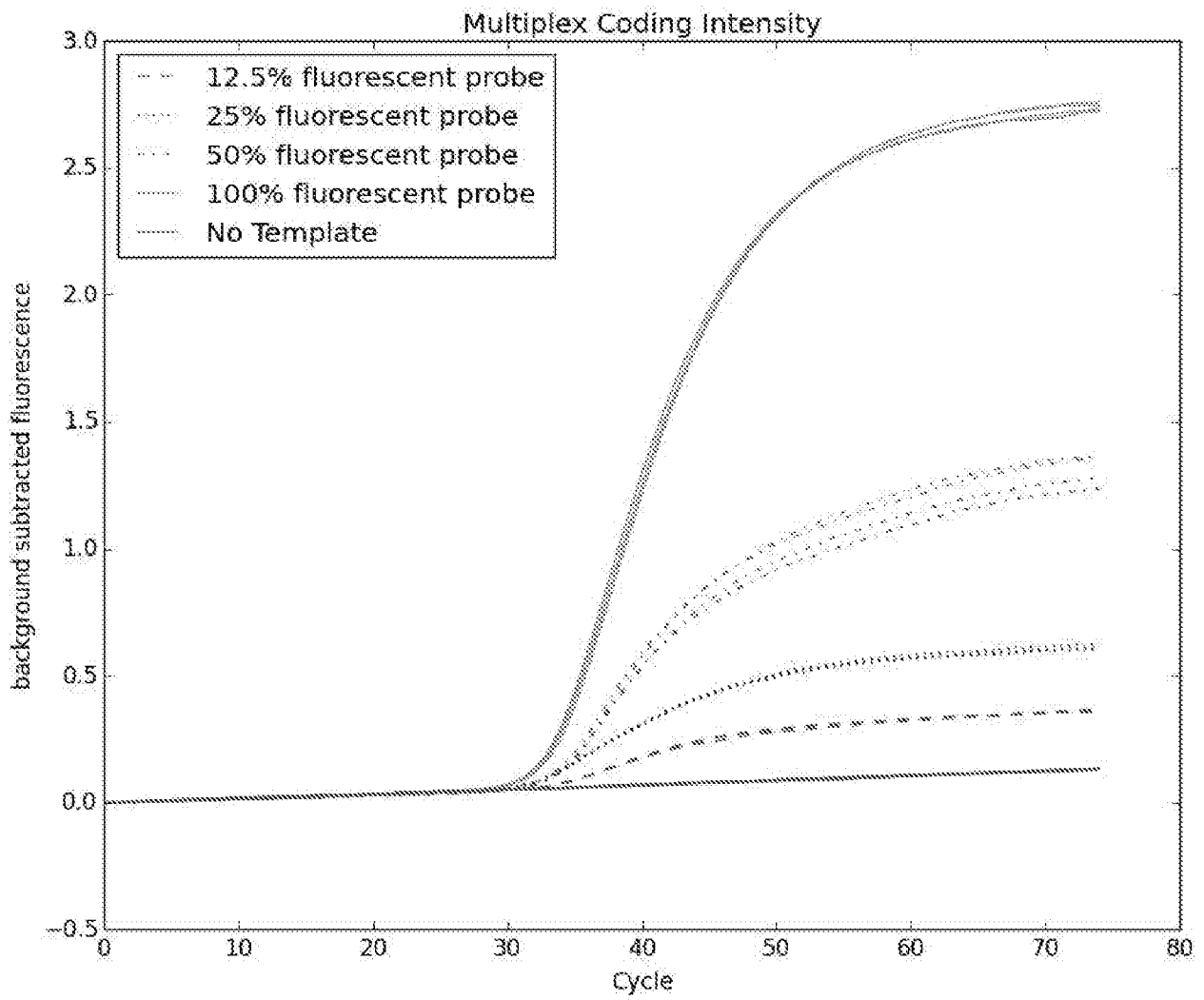


FIG. 3A

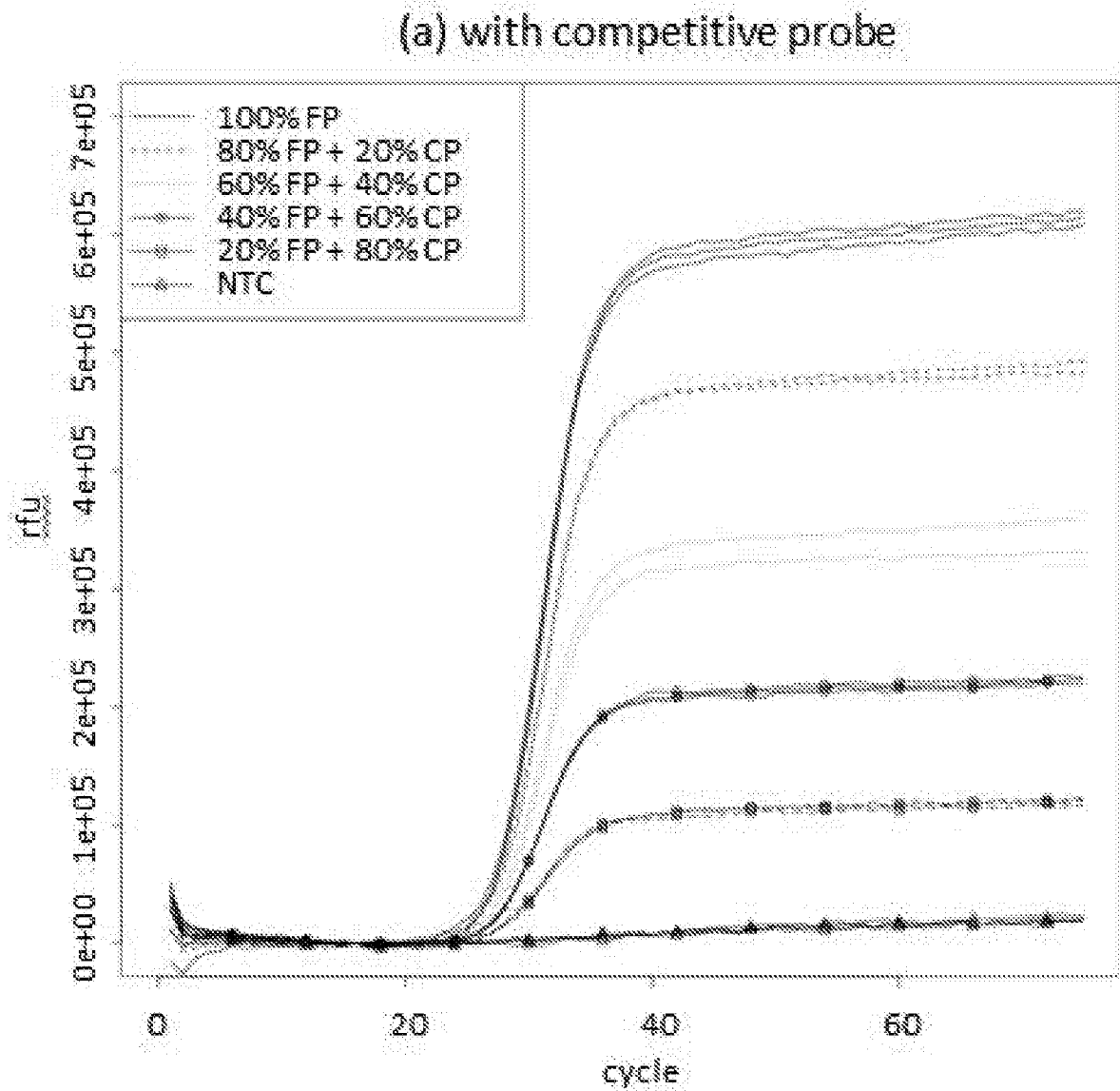


FIG. 3B

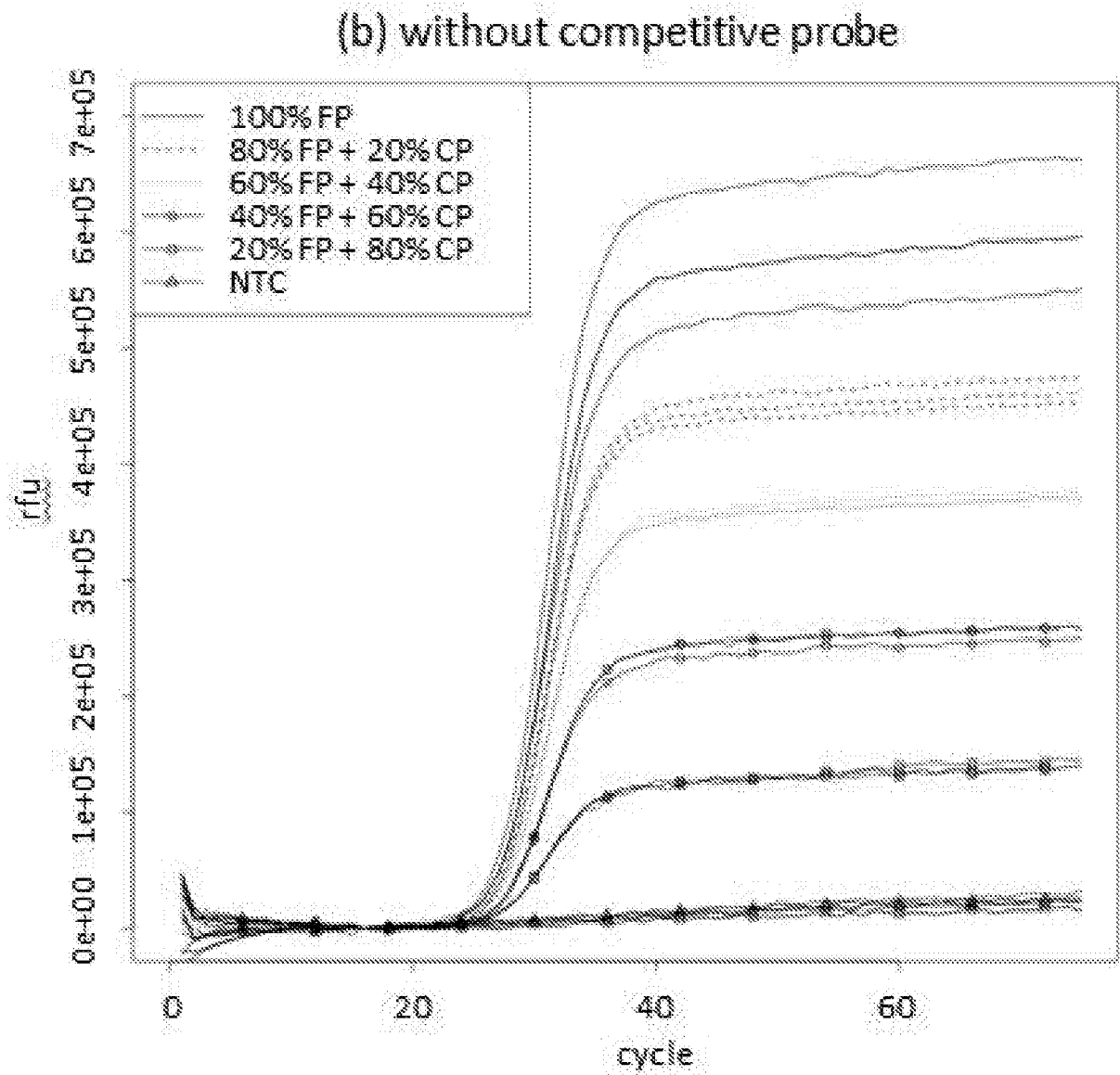


FIG. 4A

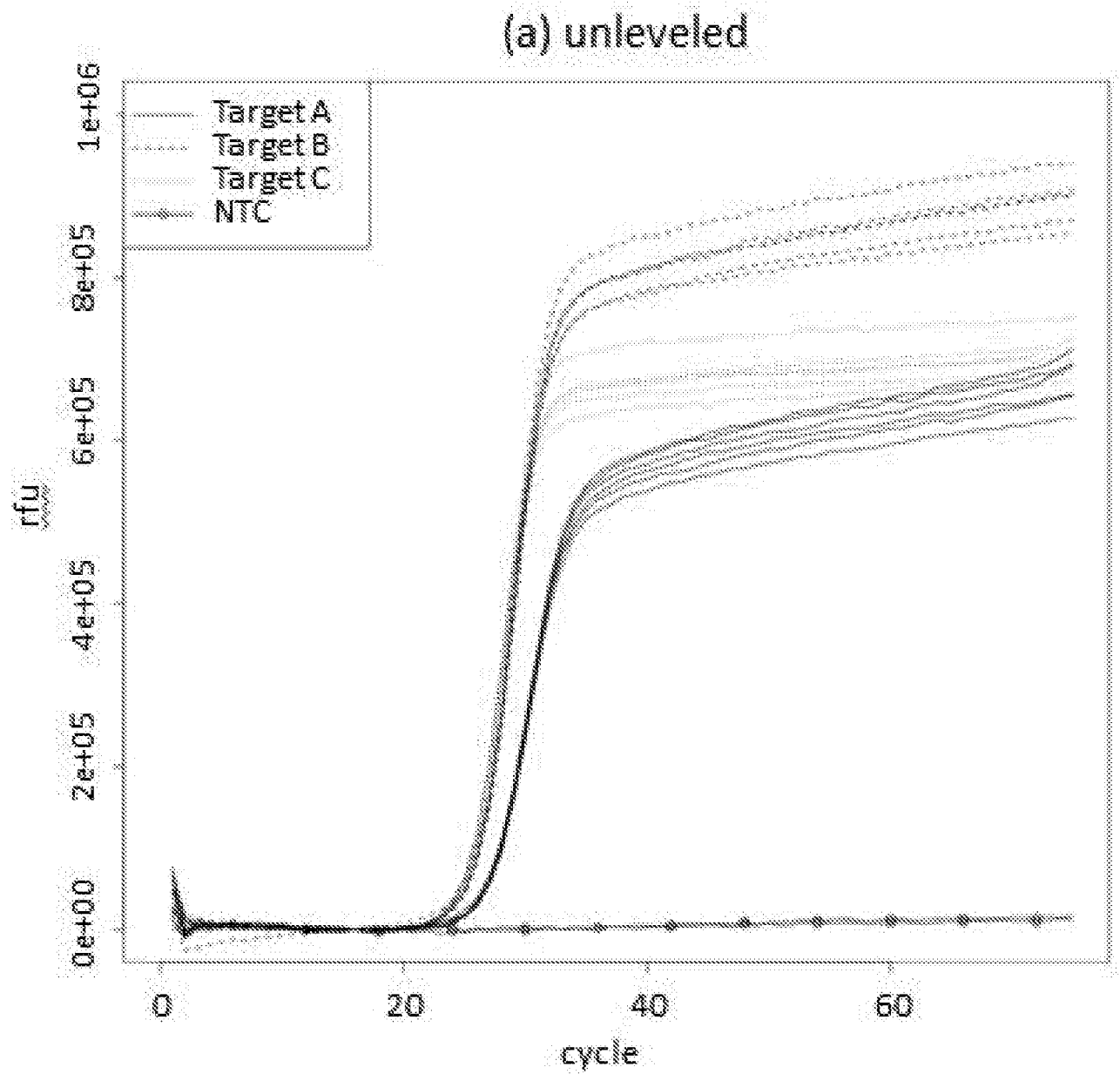
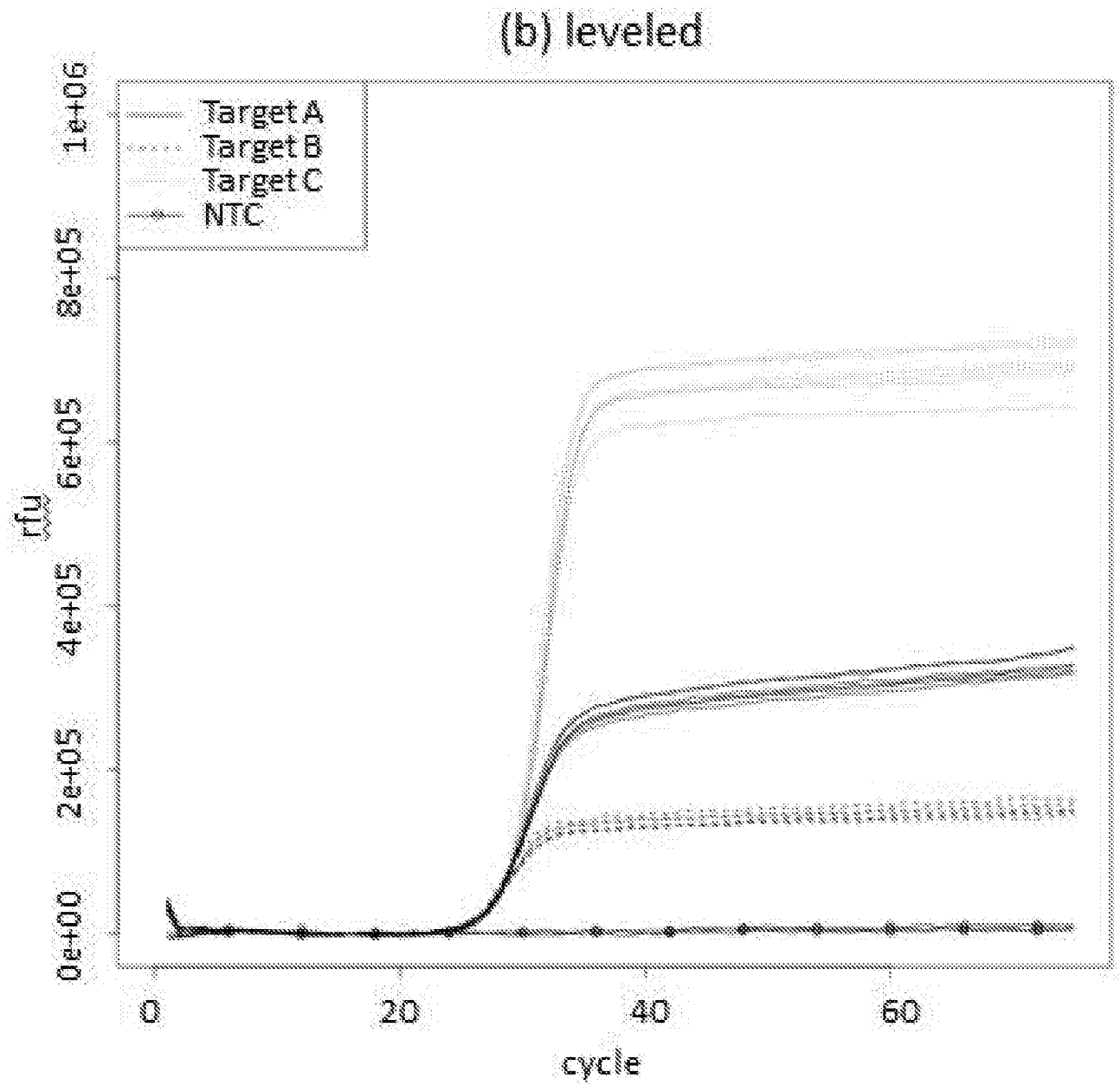


FIG. 4B



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US17/24933

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

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

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

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

3. ☒ Claims Nos.: 6-42 and 49-82
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.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US17/24933

A. CLASSIFICATION OF SUBJECT MATTER

IPC - C07H 21/00, 21/04; C12Q 1/68; G01N 21/64, 33/533, 33/542 (2017.01)

CPC - C07H 21/00, 21/04; C12Q 1/68, 1/686, 1/6806, 1/6851, 2561/101, 2561/113; G01N 21/64, 33/533, 33/542

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.
A	WO 2006/079049 A2 (THIRD WAVE TECHNOLOGIES, INC.) 27 July 2006; page 2, lines 10-17 and 23-31; page 3, lines 13-20, page 5, lines 5-8 and 23-30; page 6, lines 18-30; page 7, lines 14-19; page 15, lines 24-31; page 16, lines 1-3 and 18-28	1-4, 5/3-4, 43-47, 48/45-47, 83-87, 88/83-87, 89/83-87, 90/83-87, 91-97, 98/91-97, 99
A	US 2009/0042735 A1 (BLAIR SM, et al.) 12 February 2009; paragraphs [0018], [0035], [0041]-[0043], [0072], [0074]	1-4, 5/1-3, 43-47, 48/45-47, 83-87, 88/83-87, 89/83-87, 90/83-87, 91-97, 98/91-97, 99
A	US 2014/0213471 A1 (CALIFORNIA INSTITUTE OF TECHNOLOGY) 31 July 2014; paragraphs [0003], [0010]-[0011], [0045], [0082]	1-4, 5/1-3, 43-47, 48/45-47, 83-87, 88/83-87, 89/83-87, 90/83-87, 91-97, 98/91-97, 99
A	US 2012/0040349 A1 (LODE PV, et al.) 16 February 2012; paragraphs [0030]-[0034], [0054]	1-4, 5/1-3, 43-47, 48/45-47, 83-87, 88/83-87, 89/83-87, 90/83-87, 91-97, 98/91-97, 99

☐ 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

3 June 2017 (03.06.2017)

Date of mailing of the international search report

22 JUN 2017

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