probes which only emit fluorescence signals when they are bound to the target nucleic acid or in certain cases also by means of fluorescent dyes that bind to double-stranded DNA. A defined signal threshold is determined for all reactions to be analysed and the number of cycles Cp required to reach this threshold value is determined for the target nucleic acid as well as for the reference nucleic acids such as the standard or housekeeping gene. The absolute or relative copy numbers of the target molecule can be determined on the basis of the Cp values obtained for the target nucleic acid and the reference nucleic acid.

The fluorescence emitted by a sample is separated by a set of dichroic mirrors and filters into different wavelengths that can be recorded in one of the three detection channels (530 / 640 / 710 nm). This allows detection of the double-stranded DNA-binding dye SybrGreenI, mono color detection of the TaqMan Probe format and dual color detection of the Hybridization Probe(HybProbe) format. Details of the Lightcycler system are disclosed in WO 97/46707, WO 97/46712 and WO 98/46714, The complete contents of these applications are herewith incorporated by reference.

A very important feature of the LightCycler instrument is the color compensation software. In principle this software allows for accurate quantification and melting curve analysis by means of correcting spectral overlap of monitored fluorescent radiation in a temperature dependent manner. Technical details are disclosed in US 6,197,520.

Similar to the LightCycler system, the Corbett Rotor-Gene Real time PCR Thermocycler (www.corbettresearch.com) is a 4 channel multiplexing system comprising 4 different LEDs as excitation sources and corresponding photodiodes as fluorescent detection units. Thus, although this instrument hardware at least theoretically has the capacity of performing multiplex experiments with up to four differently labeled hybridization probes within one reaction vessel, no respective successful application protocol has been published so far.

Another real time PCR instrument ist the Biorad iQ Multi-color Real time PCR detection system (Cat. No: 170-8740), which allows for a fluorophore excitation and emission from 400 nm to 700 nm. The system is based on a conventional multiwell heating block for thermocycling, a tungsten lamp as an excitation source, a filter wheel for providing appropriate excitation wavelengths, a second filter wheel

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for selecting appropriate emission wavelengths and a CCD camera as a detection unit. The instrument has successfully been used in a multiplex assay for the detection of 4 different amplicons generated from targets with more or less equimolar concentrations, using four differently labeled TaqMan probes in the same reaction vessel (Pedersen, S., Bioradiations 107 (2001) 10-11).

In a further approach to increase multiplexing capacities, US 6,369,893 discloses a real time PCR thermocycling instrument comprising a first optics assembly with at least two light sources and a second optics assembly comprising at least two detectors for detecting and discriminating light of different emission wavelengths. In particular, a specific embodiment of different 4 LEDs as light sources and 4 different photodiodes as detectors is disclosed. Thus, the instrument disclosed in 6,369,893 in principle can be used for real time PCR detection with a broad selection of different fluorescent dyes which are known in the art. Yet, US 6,369,893 does neither anticipate nor suggest any approach on how a multiplex experiment comprising multiple different probes each labeled with a different fluorescent entity needs to be designed.

Real time PCR detection formates

In general, there exist different formates for real time detection of amplified DNA, of which the following are well known and commonly used in the art:

a) **DNA binding dye formate**

Since the amount of double stranded amplification product usually exceeds the amount of nucleic acid originally present in the sample to be analyzed, double-stranded DNA specific dyes may be used, which upon excitation with an appropriate wavelength show enhanced fluorescence only if they are bound to double-stranded DNA. Preferably, only those dyes may be used which like SybrGreenI I, for example, do not affect the efficiency of the PCR reaction.

All other fomates known in the art require the design of a fluorescent labeled Hybridization Probe which only emits fluorescence upon binding to its target nucleic acid.

b) TaqMan probe

A single-stranded Hybridization Probe is labeled with two components. When the first component is excited with light of a suitable wavelength, the absorbed energy is transferred to the second component, the so-called quencher, according to the principle of fluorescence resonance energy transfer. During the annealing step of the PCR reaction, the hybridization probe binds to the target DNA and is degraded by the 5'-3' exonuclease activity of the Taq Polymerase during the subsequent elongation phase. As a result the excited fluorescent component and the quencher are spatially separated from one another and thus a fluorescence emission of the first component can be measured (US5,538,848).

c) Molecular Beacons

These hybridization probes are also labeled with a first component and with a quencher, the labels preferably being located at both ends of the probe. As a result of the secondary structure of the probe, both components are in spatial vicinity in solution. After hybridization to the target nucleic acids both components are separated from one another such that after excitation with light of a suitable wavelength the fluorescence emission of the first component can be measured (US 5,118,801).

d) Single Label Probe (SLP) Format

This detection format consists of a single oligonucleotide labeled with a single fluorescent dye at either the 5'- or 3'-end (WO 02/14555). Two different designs can be used for oligo labeling: G-Quenching Probes and Nitroindole-Dequenching probes.

In the G-Quenching embodiment, the fluorescent dye is attached to a C at oligo 5'- or 3'-end. Fluorescence decreases significantly when the probe is hybridized to the target, in case two G's are located on the target strand opposite to C and in position 1 aside of complementary oligonucleotide probe.

In the Nitroindole Dequenching embodiment, the fluorescent dye is attached to Nitroindole at the 5'- or 3'-end of the oligonucleotide. Nitroindole somehow

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decreases the fluorescent signaling of the free probe. Fluorescence increases when the probe is hybridized to the target DNA due to a dequenching effect.

e) FRET hybridization probes

The FRET Hybridization Probe test format is especially useful for all kinds of homogenous hybridization assays (Matthews, J.A., and Kricka, L.J. , Analytical Biochemistry 169 (1988) 1-25. It is characterized by a pair of two single-stranded hybridization probes which are used simultaneously and are complementary to adjacent sites of the same strand of the amplified target nucleic acid. Both probes are labeled with different fluorescent components. When excited with light of a suitable wavelength, a first component transfers the absorbed energy to the second component according to the principle of fluorescence resonance energy transfer such that a fluorescence emission of the second component can be measured when both hybridization probes bind to adjacent positions of the target molecule to be detected.

When annealed to the target sequence, the hybridization probes must sit very close to each other, in a head to tail arrangement. Usually, the gap between the labeled 3' end of the first probe and the labeled 5' end or the second probe is as small as possible, i.e.1-5 bases. This allows for a close vicinity of the FRET donor compound and the FRET acceptor compound, which is typically 10-100 Angstroem.

Alternatively to monitoring the increase in fluorescence of the FRET acceptor component, it is also possible to monitor fluorescence decrease of the FRET donor component as a quantitative measurement of hybridization event.

In particular, the FRET Hybridization Probe format may be used in real time PCR, in order to detect the amplified target DNA. Among all detection formats known in the art of real time PCR, the FRET-Hybridization Probeformat has been proven to be highly sensitive, exact and reliable (WO 97/46707; WO 97/46712; WO 97/46714). Yet, the design of appropriate FRET Hybridization Probe sequences may sometimes be limited by the special characteristics of the target nucleic acid sequence to be detected.

As an alternative to the usage of two FRET hybridization probes, it is also possible to use a fluorescent-labeled primer and only one labeled oligonucleotide probe (Bernard, P. S., et al., Analytical Biochemistry 255 (1998) 101-107). In this regard, it may be chosen arbitrarily, whether the primer is labeled with the FRET donor or the FRET acceptor compound.

Besides PCR and real time PCR, FRET hybridization probes are used for melting curve analysis. In such an assay, the target nucleic acid is amplified first in a typical PCR reaction with suitable amplification primers. The hybridization probes may already be present during the amplification reaction or added subsequently. After completion of the PCR-reaction, the temperature of the sample is constitutively increased, and fluorescence is detected as long as the hybridization probe was bound to the target DNA. At melting temperature, the hybridization probes are released from their target, and the fluorescent signal is decreasing immediately down to the background level. This decrease is monitored with an appropriate fluorescence versus temperature-time plot such that a first derivative value can be determined, at which the maximum of fluorescence decrease is observed.

There exist many different pairs of fluorescent dyes known in the art which according to the invention are principally capable of acting together as a FRET donor/FRET acceptor pair. Yet, prior to the present invention, no functional example has been disclosed, characterized in that 4 different FRET pairs have succesfully been used in a multiplex detection assay. Among other reasons, this may be due to lack of approriate instrumentation and, moreover, due to fact that the functionality of the FRET process of a specific FRET pair is interfered by other fluorecent compounds which are present in the same reaction mixture.

As discussed above, there exist different real time detection thermocycler instruments having a maximum of 4 detector channels for multiplex/multicolor detection. Yet, the utility of all of these instruments for multiplex detection up to now has been very limited due to the fact that attempts to establish real time multicolor multiplex assays with several (at least more than two) differently labeled probes with sufficient sensitivity and specificity have not been successful so far.

Thus, it was the object of the present invention to provide an improved system which allows for an optimized and at the same time flexible design of

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multiplex/multicolor detection experiments. In one aspect the problem to be solved relates to improvements in the design of approriate hybridization probes. In another aspect, the problem to be solved relates to an improvement of instrumentation.

Brief description of the invention:

Thus, the new invention is directed to a real time PCR system with an extension of applicable detection formats and multicolor features. The system comprises specific reagent mixtures for multiplex/multicolor detection as well as an improved real time PCR instrument with an optical detection system comprising multiple detection units. The optical system is designed to provide excitation sources and detection channels for multiple detection formats, for example: SybrGreenI, multiplex FRET-Hybridization Probes, and multiplex TaqMan probes.

More precisely, the invention is directed to a system for performing multi-color real time PCR, comprising a real time PCR instrument and a specific composition or reaction mixture for performing multiplex PCR:

The composition or reaction mixture of the present invention comprises at least 3, preferably 4-5 and most preferably exactly 4 pairs of FRET hybridization probes. Each pair of said hybridization probes consists of a FRET donor probe carrying a FRET donor moiety and a FRET acceptor probe carrying a FRET acceptor moiety having an emission maximum between 550 and 710 nm.

In one embodiment at least 3, preferably at least 4 and most preferably exactly 4 FRET donor moieties are identical. Most preferably, all FRET donor moieties are identical. In other words, if all FRET donor moieties of the different FRET pairs are identical, they can be excited with the same excitation source.

In another embodiment of the invention at least 3, preferably at least 4 and most preferably exactly 4 FRET donor moieties are Fluorescein. Most preferably, all FRET donor moieties are Fluorescein.

In a specific embodiment, at least one additional FRET donor moiety is selected from a group consisiting of Atto425 and WI343.

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In another specific embodiment, which is not at all mutual exclusive to those embodiments disclosed above, one FRET acceptor moiety is selected from a group consisting of LC-Red 705 , Cy5.5, JA286 and a sulfonated cyanine dye as disclosed in US 6,027,709, formula 1.

As a second FRET acceptor moiety Cy5 may be selected.

As a third FRET acceptor moiety, LC-Red 640 may be used.

As a fourth FRET acceptor moiety, the LCRed derivative LC-Red 610 is chosen.

In a particular embodiment, the fifth FRET acceptor moiety is selected from a group consisting of Rh6G and TAMRA.

The instrument part of the system is characterized in that said real time PCR instrument comprises

- at least 1 light source, preferably an LED
- at least 4 and preferably 5-6 fluorescent detector entities, each of said entities having central detection wavelengths which are distinct from each other by at least 25 and preferably at least 30 nm

characterized in that said detector entities are capable of

- simultaneously detecting maximum fluorescene emission of at least 3, preferably 4 and most preferably 5 differently labeled FRET Hybridization Probe pairs,
- simultaneously detecting maximum fluorescence emission of at least 2 differently labeled TaqMan hybridization probes, and
  - detecting maximum fluorescence emission of SybrGreenI
  - means for heating and cooling

- multiple reaction vessels for containing a reaction mixture.

In a prefered embodiment of the invention the instrument part of the system is characterized in that said real time PCR instrument comprises exactly one light source.

In this context it is understood that although the instrument is being capable of detecting fluorescence emission of the FRET format, the TaqMan format and the SybrGreen format, the term „simultaneously" means that within the same thermcycling protocol (i. e. within one run of the instrument), either at least 3 differently labeled FRET Hybridization Probes or at least 2 differently TaqMan probes or SybrGreenI are detected.

Preferably, the instrument comprises at least 24, more preferably 32 and most preferably 48 of said reaction vessels.

Also preferably, said centrally detected wavelengths are selected from a group of range of wavelengths, said group consisting of 520-540 nm, 545-565 nm, 570-590 nm, 600-620 nm, 630-650 nm, 660-680 nm, and 700-720 nm.

Also preferably, each reaction vessel is characterized in that fluorescence excitation by said fluorescent light sources and fluorescence monitoring by said fluorescent detector entities are obtained along the same axis of said reaction vessel.

It has been proven to be particular advantageous, if said light sources and said detector entities are located in separate housings.

It has also been proven to be particular advantageous, if said means for heating and cooling are means of forced liquid or forced gas, preferentially means of forced air.

It has further been proven to be particular advantageous, if the reaction vessels are fixed in a rotating carousel.

In a last aspect, the invention is also directed to a corresponding method for amplyfying and detecting multiple target DNA sequences comprising

a) providing a reaction mixture as disclosed above,

b) subjecting said reaction mixture to a thermoclyling protiocol such that amplification of said multiple target sequences can take place

c) monitoring hybridization of each of said pairs of FRET hybridization probes at least once after a plurality of amplification cycles.

Hybridization may also be monitored at least once in a temperature dependent manner in order to perform a melting curve analysis.

Detailed description of the invention:

A) **Instrument**

In a first aspect, a system according to the invention comprises an instrument suitable for real time PCR. Optionally, the instrument is also suited for melting curve analysis, i. e. monitoring temperature dependent binding of a DNA binding entity such as a hybridization probe or a ds DNA binding dye.

The instrument basically consists of an optical part and a means for thermocycling which can subject multiple amplification solutions in their respective reaction vessels to a process of repeated thermocycling such that a polymerase chain reaction (PCR) can take place.

Preferably, the instrument part of the system is characterized in that said real time PCR instrument comprises

- at least 1 light source, preferably a light emitting diode
- at least 4 and preferably 5-6 fluorescent detector entities, each of said entities having central detection wavelengths which are distinct from each other by at least 25 and preferably at least 30 nm

characterized in that said detector entities are capable of

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- simultaneously detecting maximum fluorescene emission of at least 3, preferably 4 and most preferably 5 differently labeled FRET Hybridization Probe pairs,

- simultaneously detecting maximum fluorescence emission of at least 2 differently labeled TaqMan hybridization probes, and

- detecting maximum fluorescence emission of SybrGreenI

- means for heating and cooling, and

- multiple reaction vessels for containing a reaction mixture.

Most preferably, the instrument part of the system is characterized in that said real time PCR instrument comprises exactly one light source.

The means for thermocycling can be chosen arbitrarily. For example, metal block cyclers which are well known in the art may be used. Yet it is advantageous if the means for thermocycling provide means for active heating as well as independent means for active cooling. Moreover it has also been proven to be particular advantageous, if said means for heating and cooling are means of forced liquid or forced gas. In a specific embodiments said means use forced air.

It is also prefered, if said means for thermocycling are capable of performing rapid thermocycling like it is the case in the LightCycler technology (WO 97/46712). As it can be deduced from WO 97/46712, this technology is based on a forced air heating characterized in that ambient air is heated by a heating coil and forced air cooling characterized in that ambient air is provided.

A further improvement with respect to rapid thermocycling is the usage of capillaries as reaction vessels due to there comparatively high surface/volume ratio. Capillaries with an outer diameter of less than 5 mm, preferably of less than 3,2 mm and most preferably of less than 1,6 mm have been proven by the inventors to be particular advantageous, since they allow for reaction mixtures having total volumes between 10 and 100 µl which still can be subjected to a rapid thermocycling protocol with a reaction time of less than 2 minutes per cycle.

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Moreover, each reaction vessel needs to be fabricated at least partially or completely of an optically clear material such as glas or plastic.

Positioning of each reaction vessel (capillary) in a monitoring position is preferably characterized in that fluorescence excitation and monitoring are performed along the same axis of said reaction vessel. Such a principle enhances signalling due the phemomenon of total internal reflectance.

It has further been proven to be particular advantageous, if the reaction vessels are fixed in a rotating carousel in order to generate uniform thermocycling profiles throughout the multiple reaction vessels.

The optical part of the instrument basically consists of an excitation unit with one or more appropriate light sources in order to excite the fluorescent compound(s) which are present in the reaction solutions and a detection unit for detecting fluorescence emission from said reaction vessels.

According to WO 97/46712, it is possible to construct an optical system wherein the excitation unit and the detection unit are placed in a common housing, provided that an appropriate number of dichroidic mirrors is used. Yet, in the context of the present invention, it turned out to be advantageous, if the excitiation unit comprising the light sources and the detection unit comprising the detector entities are located in separate housings with the result that the improved real time PCR instrument comprises two separate optical units.

For the excitation unit, either monochromatic excitation or polychromatic excitation may be used according to the invention. For monochromatic excitation, either a monochromatic LED (light emitting diode) or a monochromatic laser may be used. It is also possible to use instruments with two or multiple LEDs or monochromatic lasers but only using one at a time in an individual measurement. For polychromatic excitation, a white LED, a Halogen lamp or a Xenon lamp may be used. In this case either a filter wheel or other means for moving appropriate filters into and out of the excitation beam need to be installed in the instrument such that light with different selective excitation wavelengths can be provided.

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The optical features of the excitation unit according to the invention are selected according to different parameters that have to be considered:

First, the shortest excitation wavelength should be longer than 360 nm and preferably longer than 400 nm, since short wavelength radiation has compartively low transmission rates through conventionally used optical material which is used for real time PCR reaction vessels such as glas or plastic. In addition, excitation with higher wavelengths for example in the range of 400-500 nm avoids interference of intrinsic fluorescence from the material of the reaction vessels which might disturb monitoring of the reaction.

In a prefered embodiment of the invention, the excitation unit consists of only one light source, because according to the present invention even with a single light source it is possible to perform a multiplex detection in real time PCR. Moreover, a multiplex detection in real time PCR is even possible with a single, monochromatic light source.

In one embodiment, the excitation unit comprises a blue LED emitting at 470 nm. Fig. 1 shows the excitation spectra for different fluorescent dyes which may be used according to the present invention. Among these dyes, Fluorescein as a frequently used FRET donor compound or TaqMan reporter dye known by a person skilled in the art can be excitated with a single light emitting diode having an excitation wavelength of 470 nm, whereas frequently used FRET acceptor dyes are only excited marginally with 470 nm LED.

In addition, a second UV-LED emitting in between 400 and 430 nm, preferably at 415 nm may be used optionally for exciting other FRET donor compounds having a shorter excitation maximum as compared to Fluorescein.

More precisely, the blue 470 nm LED is capable of exciting Fluorescein, which is used in the HybProbe format as donor dye in combination with 4 different acceptor (reporter) dyes. On the other hand, typical FRET acceptor dyes such as LC-Red-640 or Cy5 are only excited marginally by the 470 nm LED. The blue LED may also excite the reporter dyes FAM and HEX or VIC in dual color TaqMan assays. In addition, Fluorescein is used as reporter dye in the Single Label Probe (SLP) format.

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Moreover, the blue LED is used to excite SybrGreenI for non probe based detection of amplification products.

The optional UV LED may be used in the HybProbe format to excite a short wavelenth donor dye in combination with approriate reporter dyes that emit fluorescence at comparatively short wavelengths. Moreover, a third LED emitting at 580 nm may be included.

For the detection unit, different modes of detection which are known in the art can be used according to the invention. For example, it is well known in the art to use several photodiodes characterized in that each photodiode is capable of detecting fluorescent light of a specific wavelength. In one aspect of the invention, a detection system having exactly five or six channels using photodiodes as detectors is provided. As it will be shown, such embodiments already enable for a simultaneous 4 color FRET Hybridization Probe detection.

The optical features of the detection unit according to the invention are selected according to the following different parameters:

First of all, the longest emission wavelength that should be detected is around less than 800 nm, and preferably less than 730 nm, since detection of higher wavelength signals is affected by a possible background of putative infrared radiation by the heating part of the instrument. Moreover, long wavelength infrared dyes which are known in the art have a suboptimal quantum yield, are less stable and thus more difficult to couple to a desired hybridization probe.

As a result, the different detection channels should have central detection maximae between 500 and 800 nm and preferably between 520 and 730 nm. In this context, fig. 2 shows the emission spectra for different fluorescent dyes which may be used according to the present invention.

Within the range of 500 and 800 nm, the detection channnels can be defined exactly by means of seleting appropriate filters which the light beam has to pass prior to entering the respective photodiode. For appropriate selection, the following parameters have been identified by the inventors to be considered:

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First, the central wavelength detection maximum of each channel should be chosen dependent on the maxima of the emission spectra of those fluorescent dyes that shall be detected predominantly. As a rule of thumb, for appropriate detection sensitivity, the maximum of the fluorescent emission from the dye to be detected should not differ more than +/-10nm from the centrally detected wavelength of each channel.

Moreover, all detection channels should be able to detect discrete emissions, i. e. the spectral overlap should be minimized as far as possible. In this regard, it is advantageous, if the half band widths of each channel is 40 nm or less. More advantageously, the half band widths of each channel is 20 nm or less.

Preferably, crosstalk of the fluorophores to be detected in neighboring channnels is less than 70%, more preferably less than 50% und most prefered less than 30%. Another important criterium in this regard is the blocking value, which is indicative for the maximum intensity that is detected outside the specified transmission range of the channel (filter). Prefably, this value is less than 10^{-3}.

Based on these criteria, it has been proven to be advantageous, if the centrally detected wavelenghts of each of the 5-6 channels according to the invention are separated from each other by more than 25 nm and preferably even more than 30 nm.

In one embodiment, said centrally detected wavelengths are selected from a group of range of wavelengths, said group consisiting of 520-540 nm, 545-565 nm, 570-590 nm, 600-620 nm, 630-650 nm, 660-680 nm, and 700-720 nm.

Moreover, in one specific embodiment consisting of 6 detector channels said channels having centrally detected wavelengths at around 530, 580, 610, 640, 670, and 710 nm +/- 5 nm or 530, 555, 610, 640, 670, and 710 nm +/- 5 nm or preferably +/-2nm.

In another specific embodiment consisting of 5 detector channels said channels have centrally detected wavelengths at around 530, 610, 640, 670, and 710 nm +/-5nm or preferably +/-2nm.

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In a third specific embodiment consisting of 4 detector channels said channels have centrally detected wavelenghts at around 610, 640, 670, and 710 nm +/- 5 nm or preferably +/-2nm.

As an example, fig. 3 shows the 6 detection channels of a prefered embodiment and the emission spectra for different fluorescent dyes which may be used according to the present invention. In this embodiment, the six channels are separated by at least 25 nm spectral distance to minimize the crosstalk from each reporter dye into neighboring detection channels. The detection unit is capable of detecting SybrGreenI and the TaqMan reporter dye Fluorescein/FAM in the channel having its central wavelength detection maximum around 530 nm. In addition, the system is preferentially capable of detecting HEX/VIC in a second channel around 560 nm. Further long wavelength detection channels are capable of detecting HybProbe acceptor dyes and optionally long wavelength SLP reporter dyes.

The excitation and detection unit are connected by a multiple-leg fiber bundle. Emitted light from the reaction vessels (e.g. the glas capillaries) is homogeneously distributed using a lightpipe and is transmitted into six glas fiber bundles. These bundles of 50 µm single glas fibers transmit the light into each of the six detection channels.

This set up of excition unit and detection unit located in separate housings provides two advantages compared to the optical unit as disclosed in WO 97/46712: Homogeneous distribution of emitted light into all six detection channels and mechanical decoupling of the excitation and detection unit. This enables highly precise positioning of the excitation unit towards the reaction vessels to become monitored (e. g. capillary tips) without moving the detection unit. Moreover, the number of necessary dichrodic mirrors is minimized. An example of such a set up is shown in fig. 4, which discloses a possible embodiment of the invention. As it can be seen, excitation and detection unit are located in different housings.

In addition, the system according to the invention comprises a color compensation tool as disclosed in detail in US 6,197,520 which is incorporated into this application in its entirety by reference. The corresponding software enables for measuring fluorescence throughout a range of temperatures and correcting for temperature dependent spectral overlap of the 6 detector channels disclosed above.

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In principle this is achieved by

a) providing calibrator solutions for each fluorescent entity which is incorporated in the multiplex experiment to become performed

b) monitoring temperature dependent fluorescene of each of said solutions in each of the detector channels in order to determine the temperature dependent amount of crosstalk between different channels, and

c) generating a color compensation data set containing correction values.

B) **Test formats and selection of dyes**

In the context of the present invention, the term „multiplex PCR" is understood as a PCR reaction characterized in that 2 or more different amplification products are generated by means of using 2 or more pairs of amplication primers in the same PCR reaction.

Also in the context of the present invention, the term „multicolor real time PCR" is defined as an real time PCR assay characterized in that 1 or more different amplification products generated either in a multiplex PCR or in a monoplex PCR (using only 1 pair of amplification primers) are detected by differently labeled hybridization probes.

A major aspect of the present invention is based on usage of differently labeled hybridization reagents, each reagent comprising a pair of FRET hybridization probes comprising a pair of two fluorescent dyes which interact with each other on the principle of fluorescence resonance energy transfer (FRET). According to the invention, appropriate dye pairs can be selected for the labeling of a hybridization reagent which may be used for detection in at least three or four and preferably as much detection channels as possible.

More precisely, such a hybridization reagent is composed of two adjacently hybridizing oligonucleotides, appropriately labelled such that together they can act according to the FRET-HybProbe detection format as disclosed in WO 97/46707, WO 97/46712, and WO 97/46714). In many cases, it is sufficient if the

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hybridization reagent consists of a single oligonucleotide or in case of the FRET hybprobe format, of a pair of oligonucleotides acting together as a donor probe and an acceptor probe. Yet, in other cases there may exist many other sequence variants in the target sequences which need to be detected. Thus it may be impossible to detect the sequences of all members by just using just one pair of FRET oligonucleotide hybridization probes.

For those cases, a hybridization reagent may consist of 1, 2 or more hybridization probes, which are similar and bind to homologous sequences, but differ from each other by 1, 2, 3 or more mononucleotide-, dinucleotide- or trinucleotide exchanges, deletions or additions. In case of the hybridization reagent being a pair of FRET hybridization probes, said hybridization reagent may consist of 1, 2, 3, or more FRET donor oligonucleotide probes and/or 1, 2, 3, or more acceptor oligonucleotide probes. In this case all donor probes may be similar, but differ from each other by mononucleotide-, dinucleotide- or trinucleotide exchanges, deletions or additions. As well, all acceptor probes may be similar, differ from each other by mononucleotide-, dinucleotide- or trinucleotide exchanges, deletions or additions.

In addition it is also within the scope of the present invention, if multi-color FRET detection is performed wherein one member of said pair of labeled hybridization probes is replaced by an appropriately labeled primer as disclosed in Bernard, P. S., et al., Analytical Biochemistry 255 (1998) 101-107. Moreover, it is also within the scope of the present invention, if instead of an increase in fluorescenct emission from a respective FRET acceptor compound, the decrease in fluorescent emission of one, several, or all FRET donor moieties is monitored as being indicative for the presence of the target nucleic acid to be detected.

In addition to the various possibilites for performing multi-color detection based on the FRET principle, the system of the invention also allows for the performance of other detection formates such as detection of the target nucleic acid by means of SybrGreenI, multiplex detection using differentially labeled Molecular Beacons, Single Labeled Probes or dual color TaqMan detection. As it known in the art, dual color TaqMan assays may be based on the use of Cy 5 (Amersham) as Quencher compounds in combination with FAM as a first standard reporter dye and either HEX or alternatively VIC as a second reporter compound.

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In principle, there exist multiple possibilities of fluorescent dye combinations, which together may act as a FRET pair (Review: Resonance Energy Transfer Editors: Meer, B Wieb van der et al., VCH Publishers INC., 1994). However, based on the instrumental preconditions it is not trivial for person skilled in the art to develop a multi-color test which combines multiple FRET hybridization reagents that do not interfere with each other in such a way that sensitivity and/or specificity of a respective real time PCR experiment are affected.

Regarding the selection of approriate pairs of FRET hybridization probes for example, the dyes used for FRET donors and FRET acceptors can not be chosen arnbitrarily but need to fulfill at least the following requirements:

- sufficient solubility and capability of being coupled to an oligonkleotide
- stability of the dyes under PCR thermocycling conditions
- sufficient and reproducible emission intensity and quantum yield
- low temperature dependence of emission intensity
- essentially no spectral shifts under different chemical conditions
- essentially no spectral shifts when coupled to different oligonucleotide sequences
- discrete spectral emission maxima
- availability of a suitable partner dye for performing a FRET process

As a consequence, one important aspect of the system according to the invention is focussed on identification of multiple Fluorescence Resonance Energy Transfer (FRET) dye pairs usable in the HybProbe format for as many detection channels as possible. Excitation and emission spectra of dyes FRET dye pairs that enable a 4- or 5-plex multi-color detection set up are already shown in Fig. 1-3. Alternatives wil be be described below.

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In this regard, the following table summarizes various FRET acceptor dyes and TaqMan reporter dyes which have succesfully been used so far in the indicated detection channels performing either a 4-color FRET Hybridization Probe assay or a dual color TaqMen asssay:

Table 1

Channel	FRET Acceptor dye	TaqMan Reporter dye
530 (also detection of SybrGreenI)	-	Fluorescein (Fam) Coumarine
555	Rh6G Tamra	Hex Vic Tamra
580		Tamra ROX
610	LCRed610	TexasRed
640	LCRed640	
670	Cy5 Bodipi 655 Alexa 647	
710	Ja286 Cy5.5 LCRed705	

Yet, for several of the different reporter entities indicated above, different FRET donor moieties (Donor) and TaqMan quencher compounds (Q) respectively have to be used depending on the excitation wavelength which is available due to the presence of either one, two or three different LEDs. Therefore, different possibilities of appropriate FRET pairs or TaqMan combinations are summarized in the following more comprehensive table:

Table 2

	LED	530	555	580	610	640	670	710
FRET:								
Donor: Atto 425 WI 343	410	-	Rh6G TAMRA	-	LCRed 610	LCRed 640	Cy5 Bodipy655 Alexa647	JA286 LCRed705 Cy5.5
Donor: Fluores-cein	470	-	-	-	LCRed 610	LCRed 640	Cy5 Bodipy655 Alexa647	JA286 LCRed705 Cy5.5
Donor: LCRed 640	590	-	-	-	-	-	Cy5 Bodipy655 Alexa647	JA286 LC705 Cy5.5
TaqMan:								
Q: Arbi-trary	410	Cou ma-rine	-	-	-	-	-	-
Q: Tamra	470	Fam	Hex Vic	-	-	-	-	-
Q: Cy5	470	Fam	Hex Vic TAMRA	TAMRA Rox	-	-	-	-
Q: BHQ	470	Fam	Hex Vic TAMRA	TAMRA Rox	-	-	-	-
Q: Cy5	590	-	-	-	Texas-Red	-	-	-
Q: BHQ	590	-	-	-	Texas-Red	-	-	-

The dyes mentioned in the tables above are available as follows:

LC-Red-705 and LC Red-640 are available from Roche Applied Science (Cat. No. 2 015 161 and 2 157 594)

LC-Red 610 is synthesized according to standard protocols using a fluorescent dye as disclosed in US 5,750,409, compound II

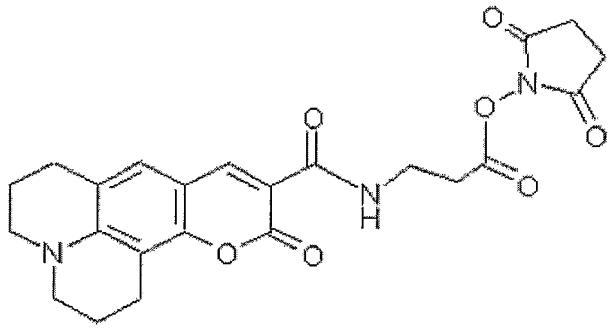
JA 286 is disclosed in EP 0 747 447, example 1. For this dye, it is advantageous, if this dye is connected to the hybridizing oligonucleotide by a long non hybridizing spacer moiety of five nucleotide residues.

Cy-5 NHS-ester is available from Amersham, (Cat. No. PA 15100) and Cyc 5.5 Phosphoramidite is also available from Amersham (Cat. No 271799 01).

Bodipy 650/665 NHS-Ester and Alexa 647 NHS Ester are available from Molecular Probes (Cat. Nos D1 000 1 and A 2 000 6).

Atto 425-NHS ester is available from Attotec (Cat. No. AD 425-3).

WI 343 is a derivative of Coumarin 343 (Aldrich, Cat. No. 55 804 654), which is coupled with a linker (beta alanin) and transformed to the succinimidyl ester according to standard methods:



Wi 343

In addition, all TaqMan dyes are available from different suppliers known by a person skilled in the art, for example Molecular probes.

The BlackHole Quencher (BHQ) is available from Biosearch Technologies (Cat. No. BHQ 1-3).

Appropriate labeling of oligonucleotide probes may be performed by conventional methods known by a person skilled in the art. In particular, labeling may be practiced using either fluorescent compounds that comprise an activated NHS-ester or fluorescent compounds linked to a Phosphoramidite, such that labeling can be

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performed during a de-novo oligonucleotide synthesis itself. Furthermore, a Fluorescein-labeled probe may be prepared by using a Fluorescein Controlled Pore Glass particle as a solid support (Roche Applied Science Cat. No. 3 138 178) for oligonucleotide synthesis.

C) Methods and Kits

In a further aspect, the invention is directed to methods of performing multiplex PCR by means of using all different embodiments of the systems, instruments and compositions disclosed above.

In particular, the present invention is directed to a method for amplifying and detecting multiple target DNA sequences comprising

- providing a composition or reaction mixture according to the invention,
- subjecting said reaction mixture to a thermocyling protocol such that amplification of said multiple target sequences can take place, and
- monitoring hybridization of each of said pairs of FRET hybridization probes at least once after a plurality of amplification cycles.

In a specific embodiment, hybridization is monitored at least once in a temperature dependent manner.

The composition or reaction mixture predominantly comprises at least 3, preferably 4-5, and most preferably exactly 4 pairs of FRET hybridization probes, each pair of hybridization probes consisting of a FRET donor probe carrying a FRET donor moiety and a FRET acceptor probe carrying a FRET acceptor moiety having an emission maximum between 550 and 710 nm.

In addition, such a composition or reaction mixture according to the invention may comprise one or several or preferably all compounds and reagents selected from the following list:

- Buffer, applicable for a polymerase chain reaction

- Deoxynucleoside triphosphates
- Template dependent DNA polymerase, preferably thermostable,
- at least one pair or several pairs of amplification primers

In yet another aspect, the invention is directed to a kit comprising at least a first set of amplification primers and at least three, four, or five pairs of FRET hybridization probes.

In addition, such a kit according to the invention may comprise one or several other compounds and reagents selected from the following list:

- Buffer, applicable for a polymerase chain reaction
- Deoxynucleoside triphosphates
- Template dependent DNA polymerase, preferably thermostable,
- At least one or multiple pairs of amplification primers

Such a kit may also comprise an internal control DNA, which can be amplified and detected using the same primers and probes as used for the detection of any target nucleic acid. Each of the components disclosed above may be stored in a single storage vessel. Yet, any combination of components for storage within the same vessel is possible as well.

D) **Best mode of the invention:**

The best mode of the invention known by the inventors at the date of filing the application is as follows:

In this prefered embodiment, a system according to the invention provides a photometer with one or two excitation sources and six detection channels. A blue (470 nm) and optionally an UV (410 nm) light-emitting diode are combined with

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detection channels having central detection wavelengths at 530, 555, 610, 640, 670 and 710 nm.

The photometer of the improved real time PCR instrument comprises two separate optical units. As disclosed in fig. 4, excitation and detection unit are located in different housings.

(I) An **excitation unit** with one or two LED's: A blue LED emitting at 470 nm and optionally a violet LED emitting at 415 nm. The blue LED is capable of exciting SybrGreenI and Fluorescein. Fluorescein is used in the HybProbe format as donor dye in combination with 4 different acceptor (reporter) dyes: Red 610, Red 640, Cy 5, Red 705. Furthermore the blue LED excites the reporter dyes FAM and HEX or VIC in dual color TaqMan assays. In addition, fluorescein is used as reporter dye in the Single Label Probe (SLP) format. The optional violet LED may used in the HybProbe format to excite a short wavelenth donor dye (Atto 425)) in combination with a reporter dye at 555 nm (Rh6G).

(II) A **detection unit** with six channels using photodiodes as detectors. The six channels are separated by at least 25 nm spectral distance to minimize the crosstalk from each reporter dye into neighboring detection channels. The detection unit is capable of detecting SybrGreenI and fluorescein/FAM in the 530 nm channel. In addition, the system is capable of detecting HEX/VIC and RH6G a in the 555 nm channel. The long wavelegth detection channels (610, 640, 670, 710 nm) are capable of detecting HybProbe acceptor dyes and optionally for long wavelength SLP reporter dyes.

The excitation and detection unit are connected by a six-leg fiber bundle. Emitted light from the glas capillaries is homogeneously distributed using a lightpipe and is transmitted into six glas fiber bundles. These bundles of 50 µm single glas fibers transmit the light into each of the six detection channels. This setup provides two advantages compared to the LC 1.2 optical unit: Homogeneous distribution of emitted light into all six detection channels and mechanical decoupling of the excitation and detection unit. This enables highly precise positioning of the excitation unit towards the capillary tips without moving the detection unit. An effective color compensation function allows simultaneous detection of multiple targets in different channels.

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Combined with these hardware and software features, multicolor capabilities of the system in order to perform Multiplex FRET Hybridization Probedetection have been established. In addition, the system of according to the invention allows for other non FRET-Hybprobe detection formats.

Based on the Hybridization Probe detection format, the system enables at least 4-color multiplexing of real-time quantification and melting curve analysis. Taking a melting curve resolution of six melting peaks per channel into consideration, up to 24 different PCR products are discernable using the photometer according to the invention. To enable this, new previously unknown FRET-acceptor dyes have been developed for detection channels with central emission wavelengths at 610 and 670 nm. In addition, the optional 410 nm LED provides an option for using a short wavelength donor dye in combination with an acceptor dye emitting at 555 nm thus providing a fifth HybProbe detection channel. Excitation and emission spectra of specifically useful dyes are shown in Fig. 1 and 2.

Using fluorescein as FRET donor dye two new FRET acceptor dyes could be identified being detectable in the 610 nm channel (Red 610) and in the 670 nm channel (Cy 5) respectively. Both dyes show high signal dynamics when used in the HybProbe FRET format. In addition, the spectral properties of the dyes fit perfectly into the photometer disclosed above thus minimizing the crosstalk into heterologous detection channels. Using a flexible multiple-channel color compensation algorithm it is shown that specific detection of all 4 FRET acceptor dyes (including the already existing dyes LC Red 640 and LC Red 705) from a single reaction is feasible over a temperature range from 40°C to 95°C.

When trying to identify a fifth FRET acceptor dye for the 555 nm channel it turned out that good FRET signal dynamics is inhibited by two effects:

(I) The fluorescein donor dye shows a high crosstalk into the 555 nm channel thus masking increasing signals of a potential FRET acceptor dye during the PCR process.

(II) A FRET acceptor dye emitting at 555 nm is itself directly excited by the 470 nm LED thus increasing the background fluorescence significantly and consequently lowering the detection sensitivity.

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A solution to these drawbacks was found by using a 410 nm UV LED for excitation of a short wavelength donor dye (Atto 425) in combination with a suitable 555 nm acceptor dye (Rh6G). In this combination, crosstalk from the donor into the 555 nm detection channel and direct excitation of the FRET acceptor were minimized and sufficient signal dynamics was achieved after color compensation.

Furthermore, it turned out that the short wavelength donor Atto 425 can even be used in combination with all long wavelength acceptor dyes. Unexpectedly, FRET signal dynamics of long wavelength acceptors in combination with Atto 425 are comparable to signal dynamics when used in combination with fluorescein. This observation provides an option to extend multi color features to a 5-channel format using just one donor dye.

Using the TaqMan detection format, dual color applications based on standard TaqMan reporter dyes FAM (detected at 530 nm) and HEX or VIC (detected at 560 nm) can be performed without major modifications of the TaqMan chemistry known in the art. The obtained detection sensitivity is comparable to the COBAS TaqMan instrument (Roche Molecular Systems).

Furthermore, the Single Labeled Probes (SLP's) format for melting curve analysis (WO 02/14555) is applicable within a system according to the invention. This format is based on quenching or, respectively, de-quenching of the probe-label after hybridization to its target sequence. The format requires just one probe terminally labeled with a single dye. Compared to HybProbe or TaqMan formats, SLP's thus enable a significantly less expensive setup of assays for integrated analysis of Single Nucleotide Polymorphisms (SNP's). By reporting melting temperatures, different alleles of an SLP can unambigiously be distiguished in a single reaction. Multiplexing options are provided by using different reporter dyes.

Possibilities for Real time multicolor PCR detection in the above disclosed instrument are summarized in the following table:

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Table 3

Assay Format	Detection Channels (Reporter Dyes)
SYBR Green I	530 nm (SYBR Green I)
HybProbes	610 nm (LC RED 610) 640 nm (LC RED 640) 670 nm (LC RED 670) 710 nm (LC RED 705)
Hydrolysis Probes (TaqMan Probes)	530 nm (FAM) 555 nm (VIC, HEX)
SimpleProbes	530 nm (Fluorescein)

The following examples, references, sequence listing and figures are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

Description of the Figures

Figure 1 Excitation specra of FRET donor and acceptor dyes for the present invention

Figure 2 Emission spectra of FRET donor and acceptor dyes for the present invention

Figure 3 Emission spectra of FRET donor and acceptor dyes and detection channels for the present invention

Figure 4 Photometer setup

Figure 5 4 color real time PCR quantification experiment of example 1

Figure 6 Primer/probe design of example 2

Figure 7 4-color Real time PCR melting curve experiment of example 2

Figure 8 Dual color TaqMan Real time PCR quantification experiment of example 3

Example 1

Quantitative real time PCR of Factor V DNA using different pairs of FRET hybridization probes labeled with different fluorescent compounds

For amplification of a Factor V DNA fragment, 4 different 100µl real time PCR reaction mixtures were set up as follows:

10^6 copies of a plasmid containing Factor V gene

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3 mM $MgCl_2$

500 nM each primers

200 nM FRET 3'Hybridization Probeaccording to Seq. A

200 nM FRET 5'Hybridization Probeaccording to Seq. B1, B2, B3 or B4, respectively.

In addition, PCR components from the LightCycler DNA Hyb Probes Kit (Roche Applied Science, Cat. No. 2158825) were used.

Primers and probes were used as follows:

Primer FactorV, forward:

5'-GAG AGA CAT CGC CTC TGG GCT A-3' (22-mer) (Seq. ID. No:1)

Primer FactorV, reverse:

5'-TGT TAT CAC ACT GGT GCT AA-3' (20-mer) (Seq. ID. No: 2)

A: 3'Fluorescein labeled Hybridization Probe

5'-AAT ACC TGT ATT CCT CGC CTG TC-3' (23-mer) (SEq. Id. No: 3)

B1: 5' Red610 Hybridization Probe

5'-AGG GAT CTG CTC TTA CAG ATT AGA AGT AGT CCT ATT-3' (36-mer)

B2: 5' Red640 Hybridization Probe

5'-AGG GAT CTG CTC TTA CAG ATT AGA AGT AGT CCT ATT-3' (36-mer)

B3: 5' Cy5 Hybridization Probe

5'-AGG GAT CTG CTC TTA CAG ATT AGA AGT AGT CCT ATT-3' (36-mer)

B4: 5' Red705 Hybridization Probe

5'-AGG GAT CTG CTC TTA CAG ATT AGA AGT AGT CCT ATT-3' (36-mer)

(B1-B4: Seq.Id. NO: 4)

Amplification was performed in a LightCycler instrument disclosed above as best mode of the invention according to the following thermocycling protocol:

	T[°C]	t[sec]	Ramp-rate[°C/sec]	Acquisition	Cycles
Denaturation	95	30	20.0	none	1
Amplification	95	10	20.0	none	
	55	20	20.0	single	45
	72	20	20.0	none	

Real time monitoring was performed with and without a color compensation algorithm and using the 2nd derivative threshold method over 45 cycles by measuring the fluorescence signals in a detection channel and using arithmetic background correction for normalization of initial fluorescence background intensities.

Results are shown in fig. 5a-5d. As can be seen in the figures, specific and quantitative amplification signals of all used FRET acceptor dyes could be obtained after color compensation in the corresponding 610, 640, 670 and 710 nm detection channels. Moreover, it is shown that the spectral overlap of the different dyes in the neighboring channels did not affect specific and quantitatve signalling when appropriate color compensation is performed.

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Example 2

4-color melting curve analysis

A 479 bp fragment of a specific plasmid containig a fragment of the human NAT-2 gene encoding N-Acetyl-Transsferase isozyme was amplified with specific primers and detected by fluorescence, using 4 different specific pairs of FRET Hybridization Probes. Whereas three probes were labeled at the 3'-end with Fluorescein, four detection probes were labeled 5' either with Light- Cycler-Red 610, 640, 705 or Cy5 and modified at the 3'-end by phosphorylation. A schematic drawing of this experimental set up is shown in fig. 6. As it can be deduced from the figure, there were four differently labeled FRET acceptor probes, but only three detection sites. This resulted in a competition of annealing of two detection probes.

Assay conditions were basically the same as disclosed in example 1 with the alteration that 3x106 copies of target DNA were used.

Primer forward:

5'-TGC CTT GCA TTT TCT GCT T-3' (19mer) (Seq. Id. No: 5)

Primer reverse:

5'-GAG TTG GGT GAT ACA TAC A-3' (19mer) (Seq. Id. No: 6)

3'-Fluorescein HybProbe 1:

5'-GAA ATT CTT TGT TTG TAA TAT ACT GCT CTC TC-Fluos-3' (32mer) (Seq. Id. No:7)

5'-Red610 HybProbe 1a:

5'-Red610-TGA TTT GGT CCA CGT ACC-3' (18mer) (Seq. Id. No:8)

5'-Red640 HybProbe 1b:

5'-Red640-TGA TTT GGT CCA AGT ACC C-3' (19mer) (Seq. Id. No:9)

3'-Fluorescein HybProbe 2:

5'-GTT GGA GAC GTC TGC AGG TAT GTA TTC ATA GAC TCA A-Fluos-3' (37mer)
(Seq. Id. No:10)

5'-Red705 HybProbe 2:

5'-Red705-ATC TTC AAT TGT TCG AGG TT-3' (20mer)
(Seq. Id. No:11)

3'-Fluorescein HybProbe 3:

5'-ATT TCC TTG GGG AGA AAT CTC GTG CCC A-Fluos-3' (28mer)
(Seq. Id. No:12)

5'-RedCy5 HybProbe 3:

5'-Cy5-ACC TGG TGA TGA ATC CCT TAC TAT TTA GAA TAA GGA AC-3' (37mer)
(Seq. Id. No:13)

Amplification and subsequent melting curve analysis was performed in a LightCycler instrument disclosed above as best mode of the invention according to the following thermocycling protocol:

	T[°C]	t[sec]	Ramp-rate[°C/sec]	Acquisition	Cycles
Denaturation	95	600	20.0	none	1
Amplification	95	10	20.0	none	
	55	10	20.0	single	45
	72	20	3.0	none	
Melting Curve	95	60	20.0	none	
	40	60	20.0	none	1

	80	0	0.1	cont	
Cooling	40	30	20.0	none	1

Results are shown in fig. 7. As it can be deduced from the figure, 4 color melting curve analysis using Hybridization Probes with the 4 different acceptor dyes could be performed using an approriate color compensation method. Hybridization Probe specific melting peaks could then be detected without any substantial crosstalk effects.

Example 3

Dual Color TaqMan Detection

A LightCycler instrument according to the best mode of the invention provided all means for sensitive detection of dual color TaqMan assays. The established TaqMan dye FAM and HEXs were well excited with the 470 nm LED and after using the instrument's color compensation function could differentially be identified in the 530 nm and 560 nm detection channels with high sensitivity. Transfer of dual color TaqMan assay protocols to the instrument according to the invention was simply performed by supplementing established assay conditions with BSA.

Different examples of successful dual color amplification and detection are shown in fig. 8. Fig. 8a shows that in mono-color experiments using either FAM or HEX, a detection sensitivity of about 1copy/µl for dual color TaqMan assays was obtained. (Only negative controls did not result in a significant increase in fluorescence after the PCR reaction). Fig,. 8b shows a dual color experiment, wherein a Hex labeled TaqMan probe was always used to detect 500 copies of a first target DNA and in the same reaction a FAM labeled TaqMan probe was used to detect different amounts (10^0-10^6 copies) of a second target DNA.

The dynamic range (being indicative for the relative difference in concentrations of the two targets to become detected) was about 10^3.

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WO 02/14555

WO 97/46707

WO 97/46712

WO 98/46714

Patent Claims

1. A composition or reaction mixture for performing multi-color real time PCR comprising at least 3, preferably 4-5 most preferably exactly 4 pairs of FRET hybridization probes, each pair of hybridization probes consisting of a FRET donor probe carrying a FRET donor moiety and a FRET acceptor probe carrying a FRET acceptor moiety having an emission maximum between 550 and 710 nm.

2. A composition or reaction mixture according to claim 1, wherein at least 3, preferably at least 4 and most preferably exactly 4 FRET donor moieties are identical.

3. A composition or reaction mixture according to claim 1, wherein all FRET donor moieties are identical.

4. A composition or reaction mixture according to claim 1, wherein at least 3, preferably at least 4 and most preferably exactly 4 FRET donor moieties are Fluorescein.

5. A composition or reaction mixture according to claim 1, wherein all FRET donor moieties are Fluorescein.

6. A composition or reaction mixture according to claims 1-5, wherein at least one additional FRET donor moiety is selected from a group consisiting of Atto425 and WI343.

7. A composition or reaction mixture according to claims 1-6, wherein one FRET acceptor moiety is selected from a group consisting of LC-Red 705, Cy5.5, and JA286.

8. A composition or reaction mixture according to claims 1-7, wherein at least one, two or three FRET acceptor moieties are selected from a group consisting of Cy5, LC-Red 640, and LC-Red 610

9. A composition or reaction mixture according to claims 1-8,

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wherein one FRET acceptor moiety is selected from a group consisting of Rh6G and TAMRA.

10. A system for performing multi-color real time PCR, comprising

- a real time PCR instrument, and
- a composition or reaction mixture according to claims 1-9.

11. A system according to claim 10, characterized in that said real time PCR instrument comprises

- at least 1 light source, preferably an LED
- at least 4 and preferably 5-6 fluorescent detector entities, each of said entities having central detection wavelengths which are distinct from each other by at least 25 and preferably at least 30 nm

characterized in that said detector entities are capable of

- simultaneously detecting maximum fluorescene emission of at least 3, preferably 4 and most preferably 5 differently labeled FRET Hybridization Probe pairs,
- simultaneously detecting maximum fluorescence emission of at least 2 differently labeled TaqMan hybridization probes, and
- detecting maximum fluorescence emission of SybrGreenI

- means for heating and cooling
- multiple reaction vessels for containing a reaction mixture.

12. A system according to claim 11, characterized in that said real time PCR instrument comprises exactly one light source.

13. A method for amplifying and detecting multiple target DNA sequences comprising

a) providing a composition or reaction mixture according to claims 1-9,

b) subjecting said reaction mixture to a thermocycling protocol such that amplification of said multiple target sequences can take place,

c) monitoring hybridization of each of said pairs of FRET hybridization probes at least once after a plurality of amplification cycles.

14. A method according to claim 13, wherein hybridization is monitored at least once in a temperature dependent manner.

15. A real time PCR instrument comprising

- at least 1 light source, preferably an LED
- 5-6 fluorescent detector entities, each of said entities having central detection wavelengths which are distinct from each other by at least 25 and preferably at least 30 nm,

characterized in that said detector entities are capable of

- simultaneously detecting maximum fluorescene emission of at least 3, preferably 4 and most preferably 5 differently labeled FRET Hybridization Probe pairs,
- simultaneously detecting maximum fluorescence emission of at least 2 differently labeled TaqMan hybridization probes, and
- detecting maximum fluorescence emission of SybrGreenI

- means for heating and cooling
- multiple reaction vessels for containing a reaction mixture.

16. A real time PCR instrument according to claim 15 comprising exactly one light source.

17. An instrument according to claim 15-16, characterized in that said central detection wavelengths are selected from a group of range of wavelengths, said group conisiting of 520-540 nm, 545-565 nm, 570-590 nm, 600-620 nm, 630-650 nm, 660-680 nm, and 700-720 nm.

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Fig. 1

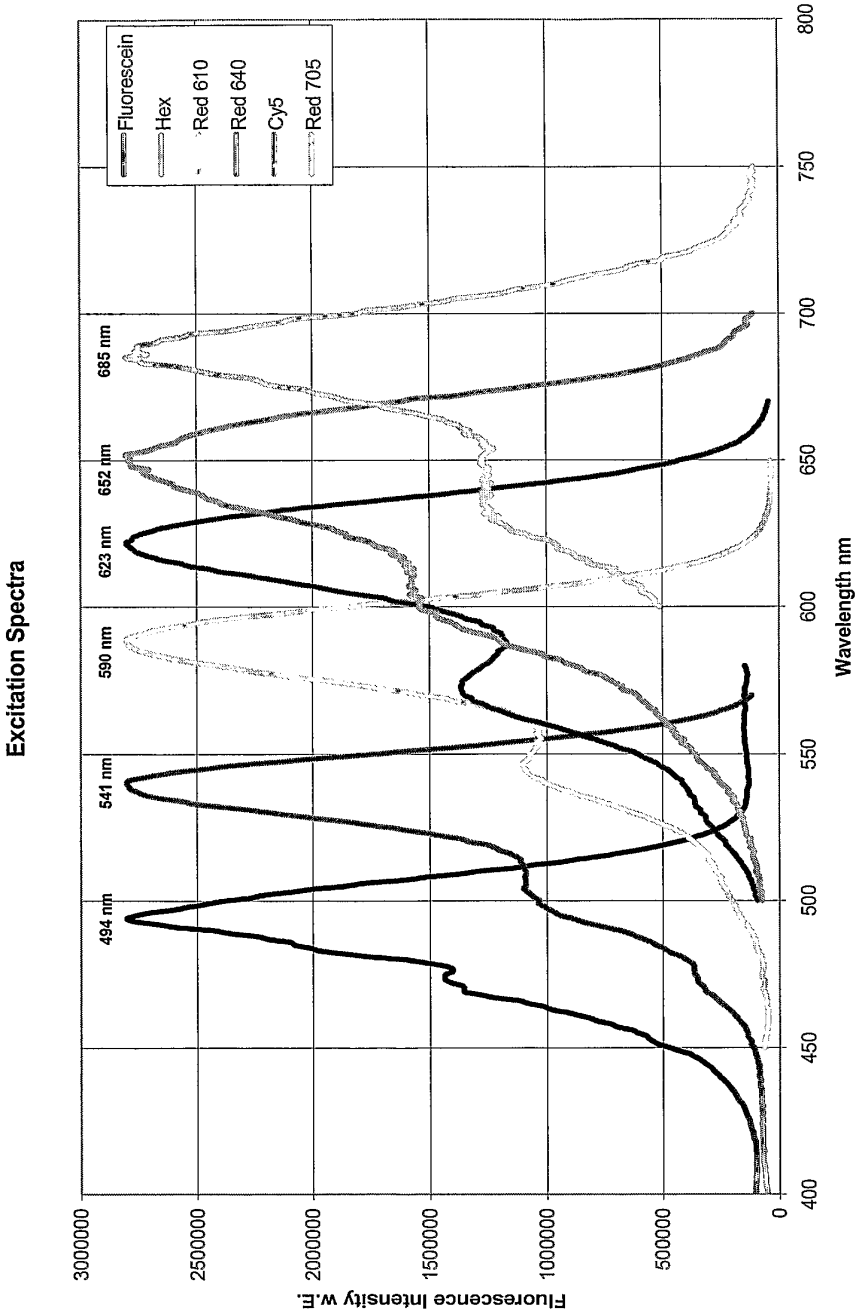

Excitation Spectra
Fluorescence Intensity w.E.
Wavelength nm
Fluorescein
Hex
Red 610
Red 640
Cy5
Red 705
494 nm
541 nm
590 nm
623 nm
652 nm
685 nm
3000000
2500000
2000000
1500000
1000000
500000
0
400
450
500
550
600
650
700
750
800

Fig. 2

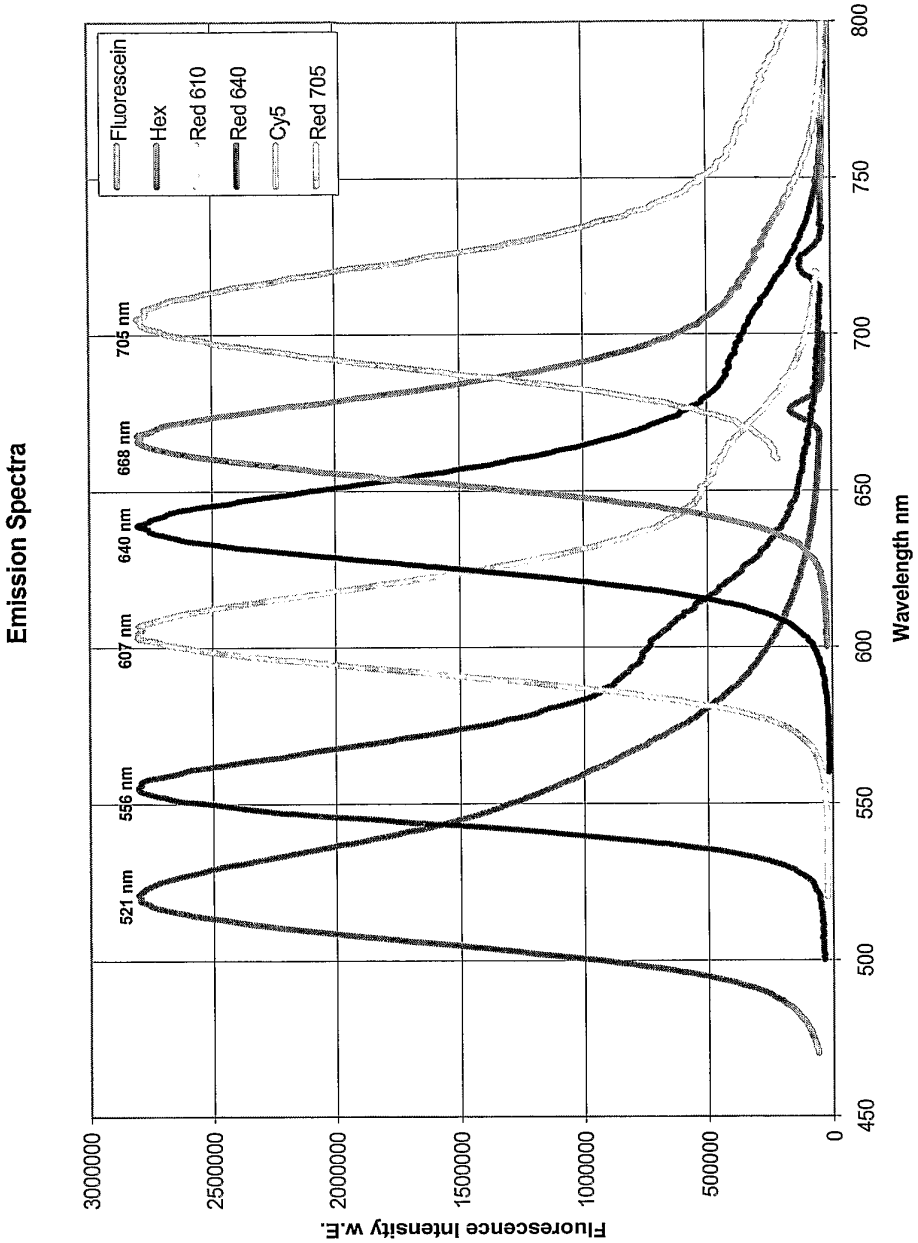

Emission Spectra
Fluorescein
Hex
Red 610
Red 640
Cy5
Red 705
521 nm
556 nm
607 nm
640 nm
668 nm
705 nm
Fluorescence Intensity w.E.
3000000
2500000
2000000
1500000
1000000
500000
0
450
500
550
600
650
700
750
800
Wavelength nm

Fig. 3

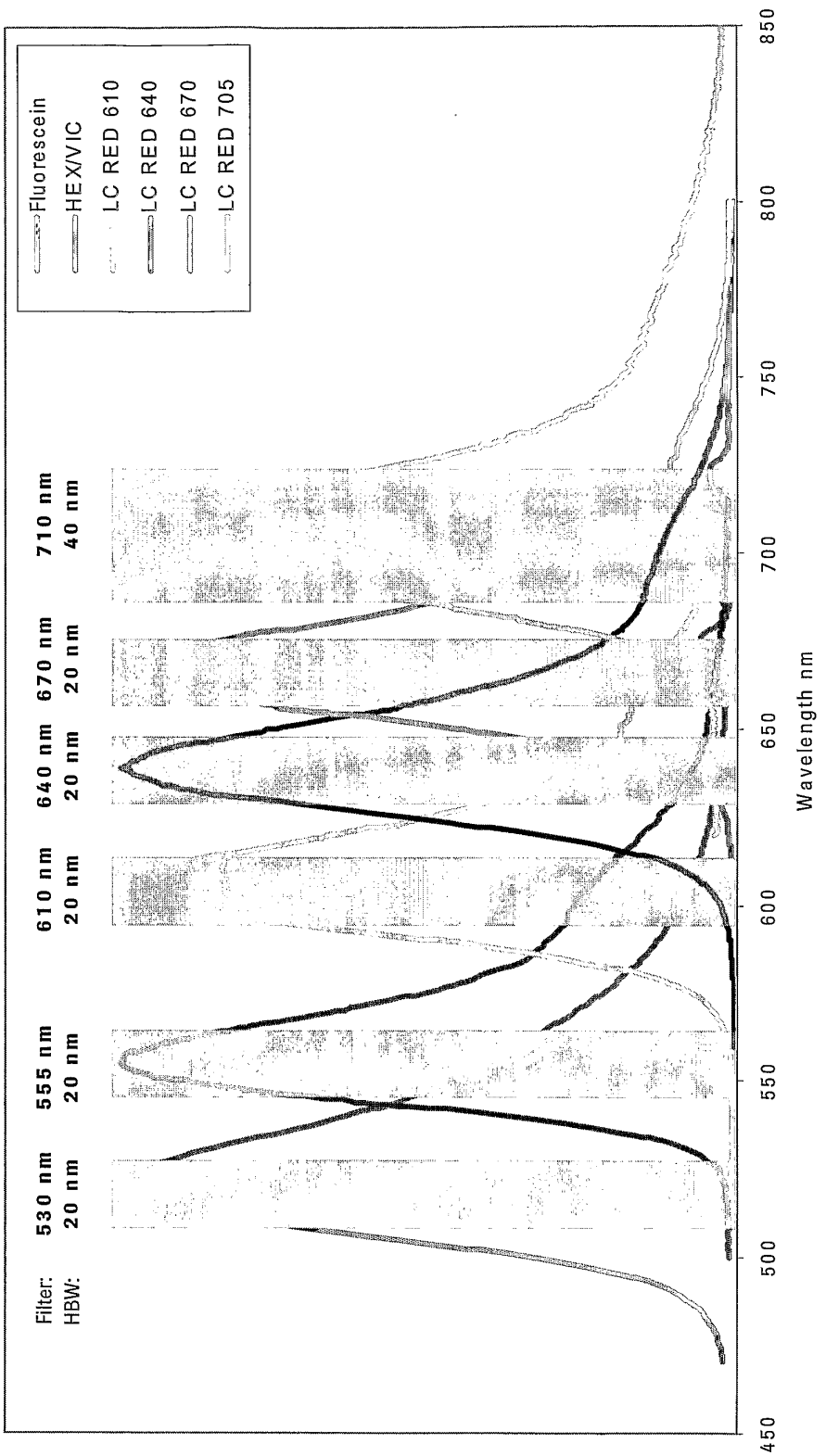
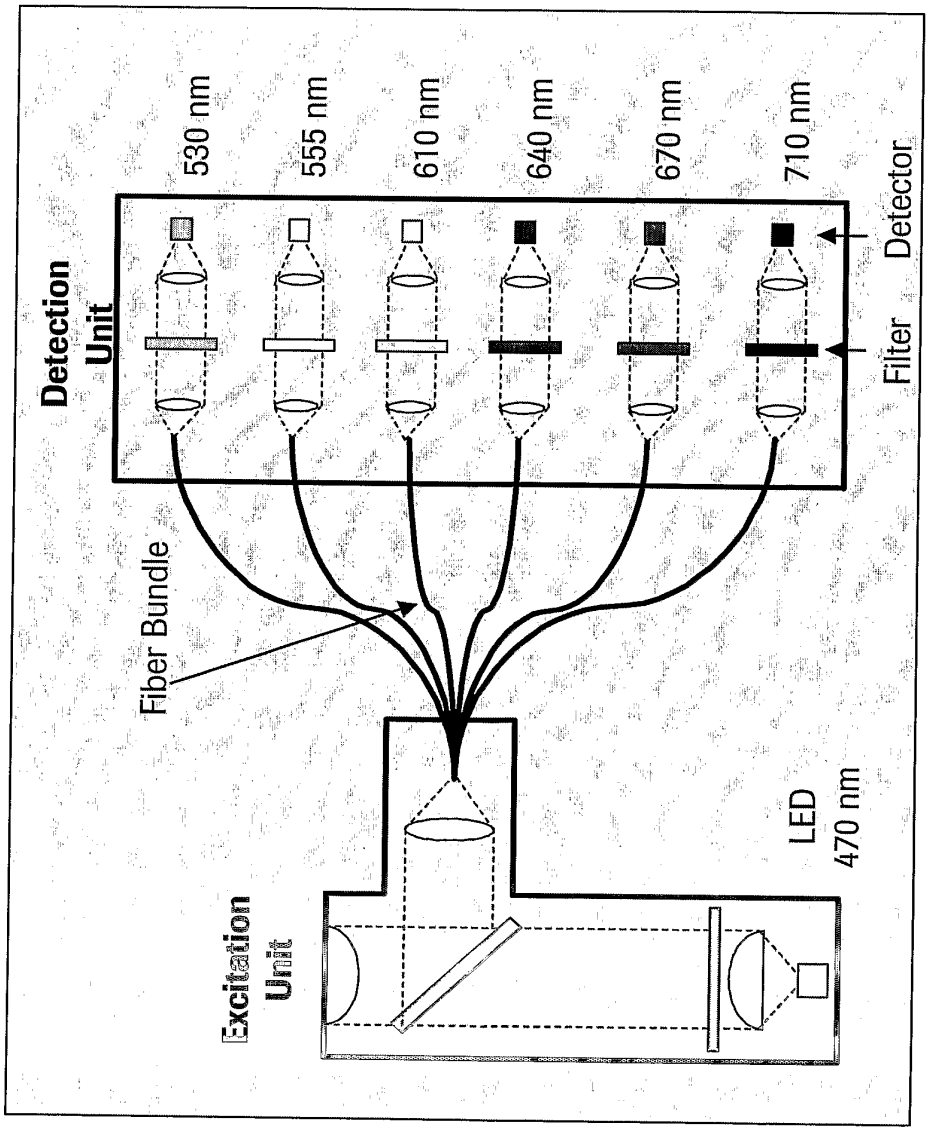

Fluorescein
HEX/VIC
LC RED 610
LC RED 640
LC RED 670
LC RED 705
Filter: 530 nm 555 nm 610 nm 640 nm 670 nm 710 nm
HBW: 20 nm 20 nm 20 nm 20 nm 20 nm 40 nm
450
500
550
600
650
700
750
800
850
Wavelength nm

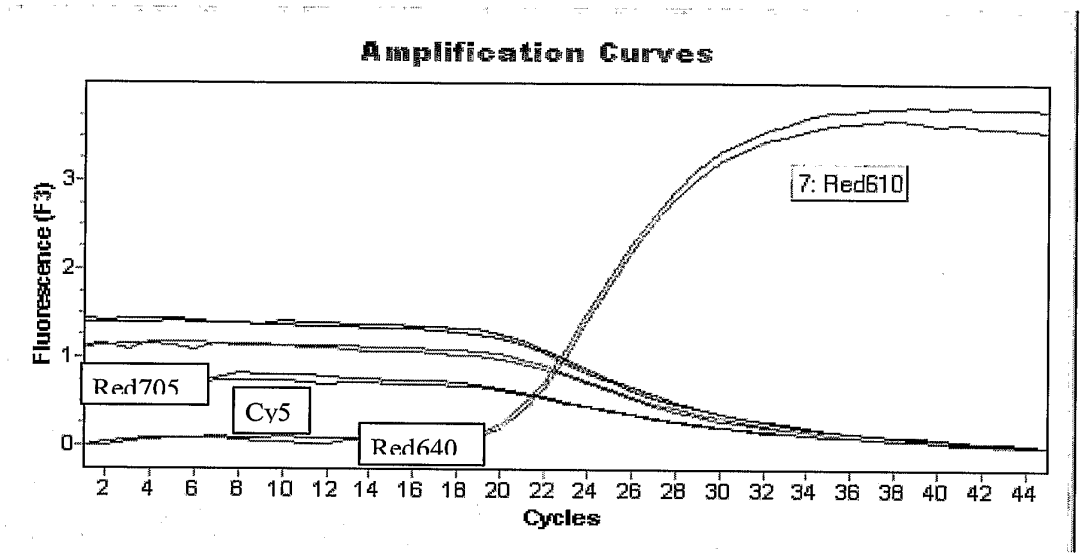
Fig. 4



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Fig. 5a

F3 = 610 nm
Without Color Compensation



With Color Compensation

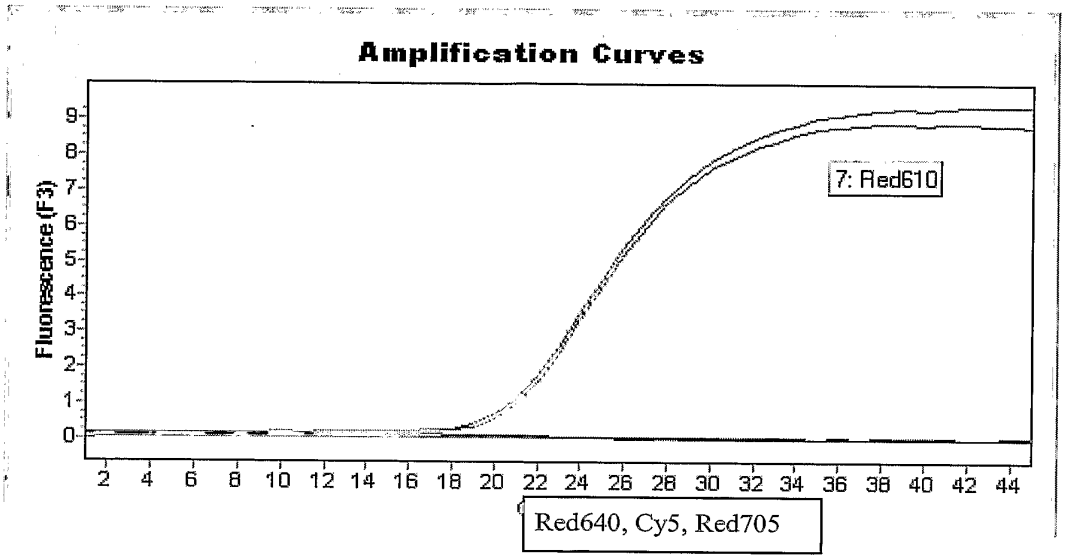
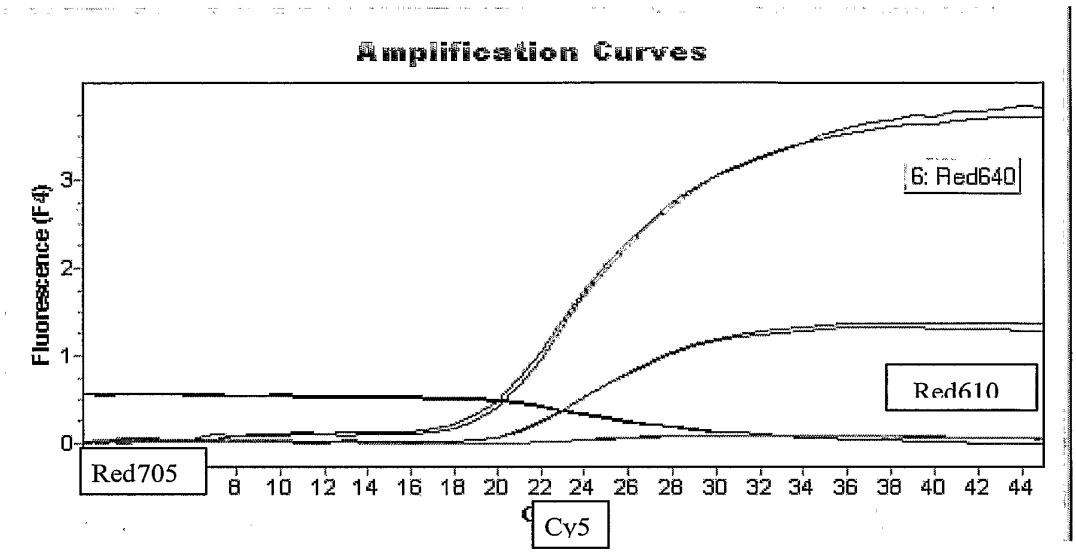


Fig. 5b

F4 = 640 nm
Without Color Compensation



With Color Compensation

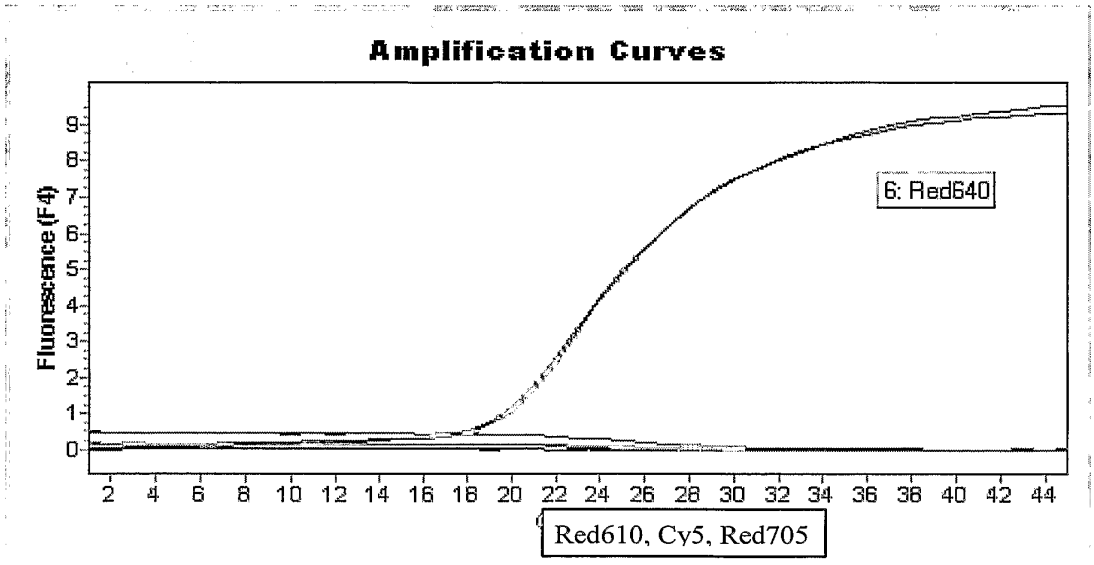
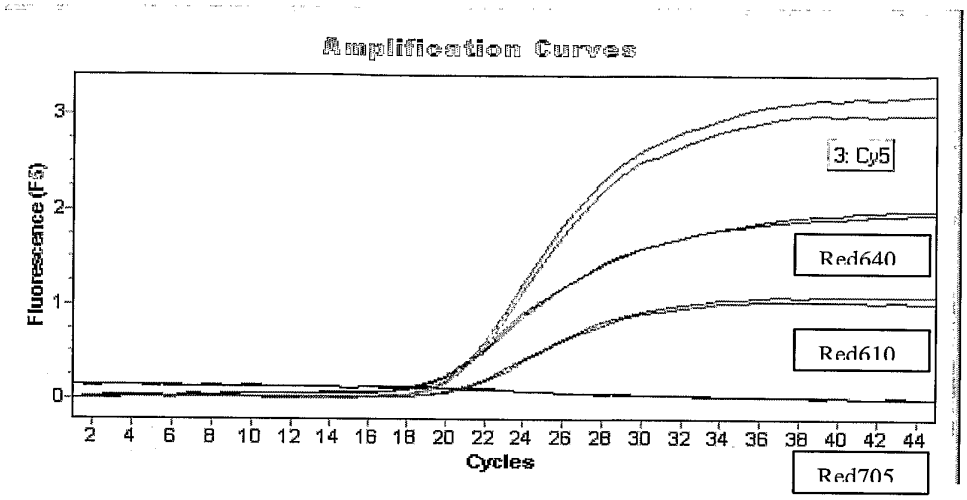


Fig. 5c

F5 = 670 nm
Without Color Compensation



With Color Compensation

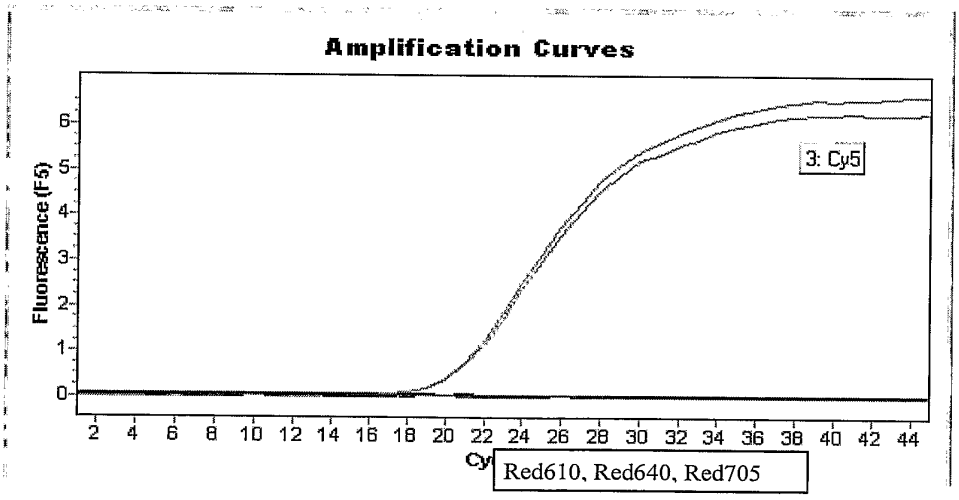
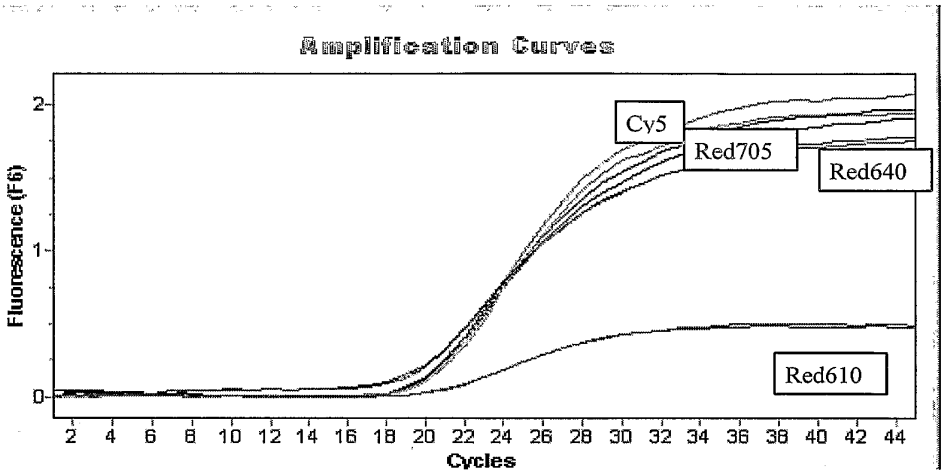


Fig. 5d

F6 = 710 nm
Without Color Compensation



With Color Compensation

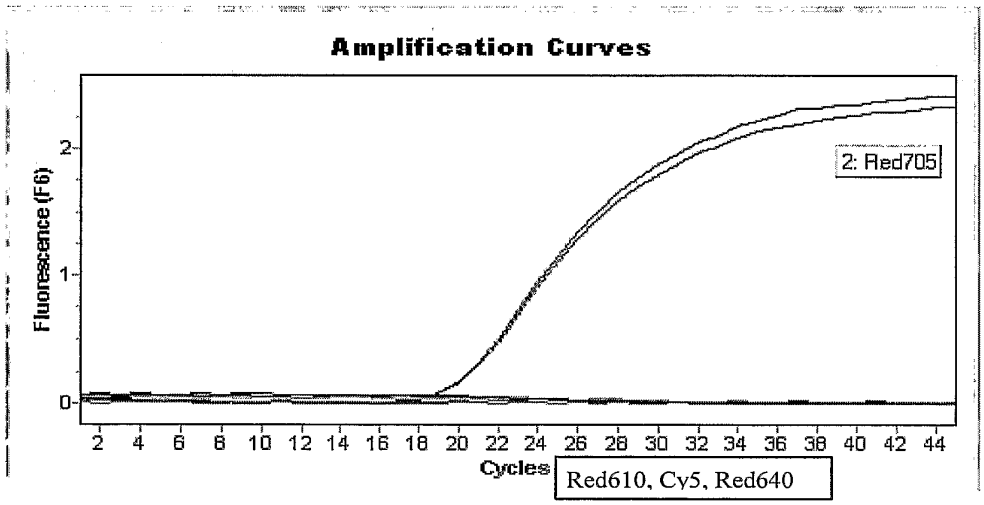


Fig. 6

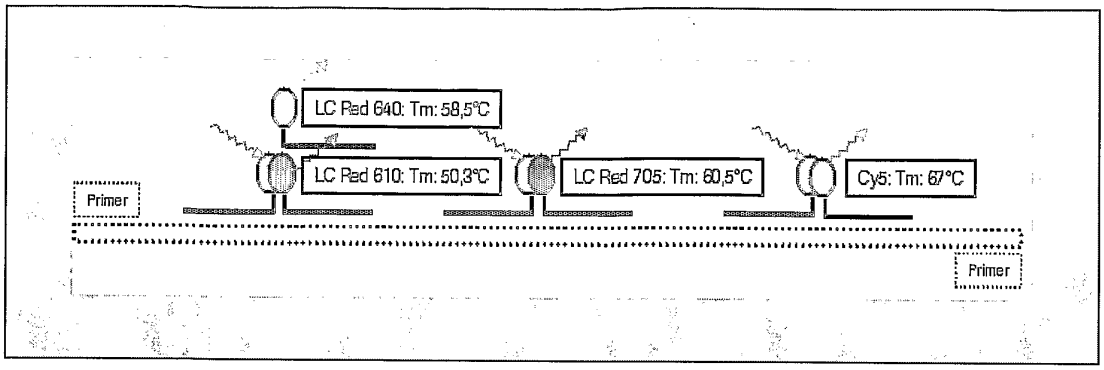
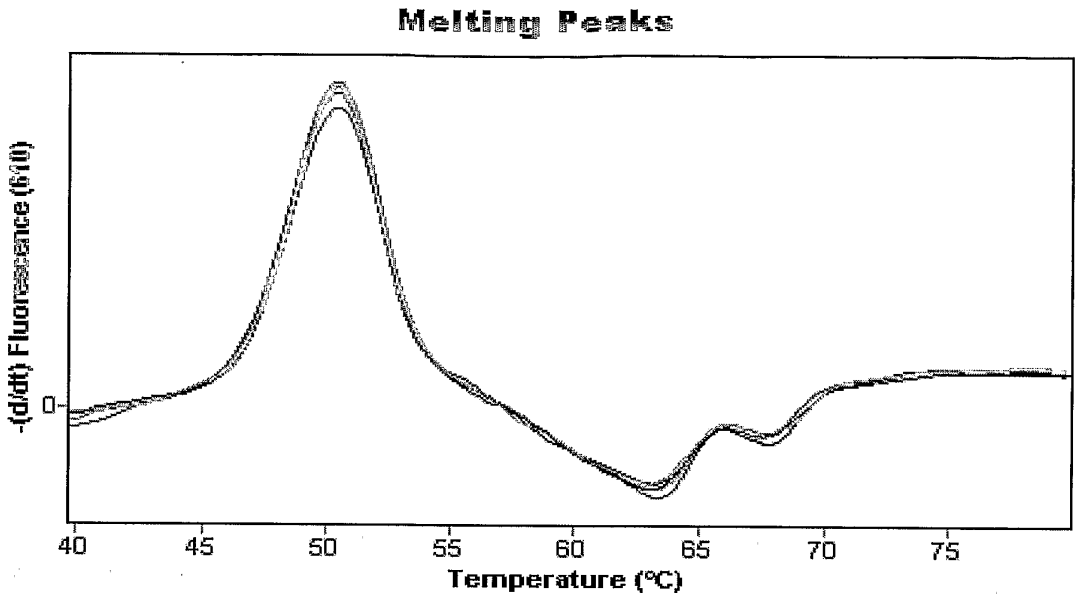
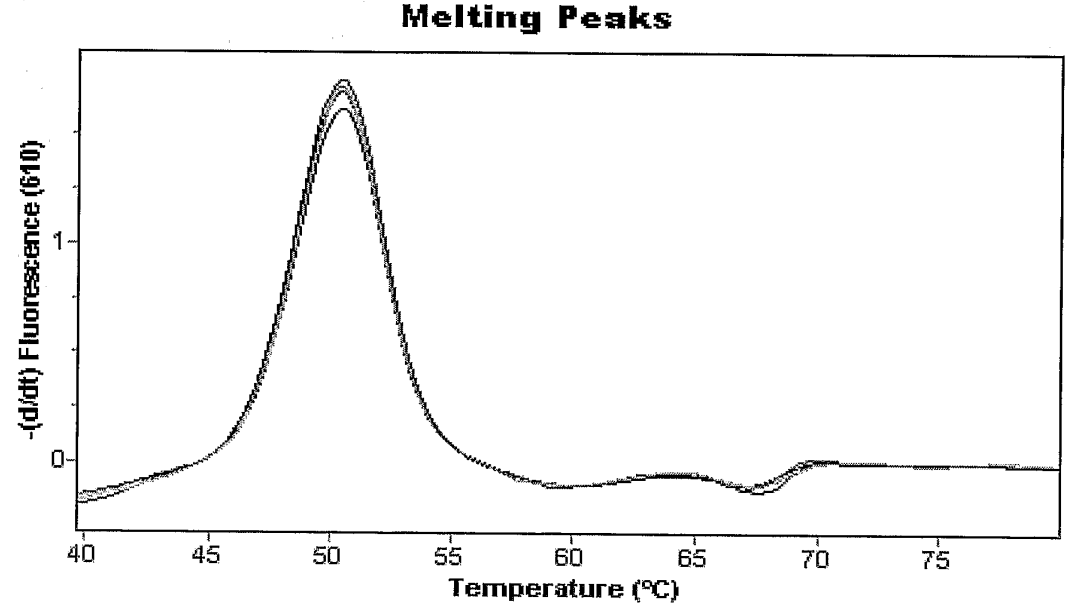

LC Red 640: Tm: 58,5°C
LC Red 610: Tm: 50,3°C
LC Red 705: Tm: 60,5°C
Cy5: Tm: 67°C
Primer
Primer

Fig. 7a

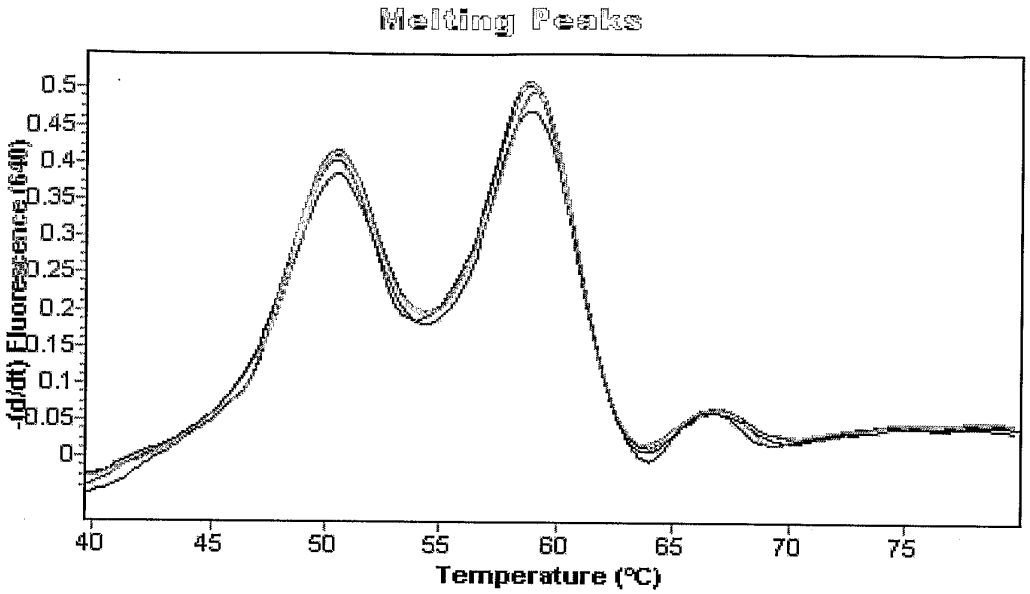


610 nm channel without color compensation

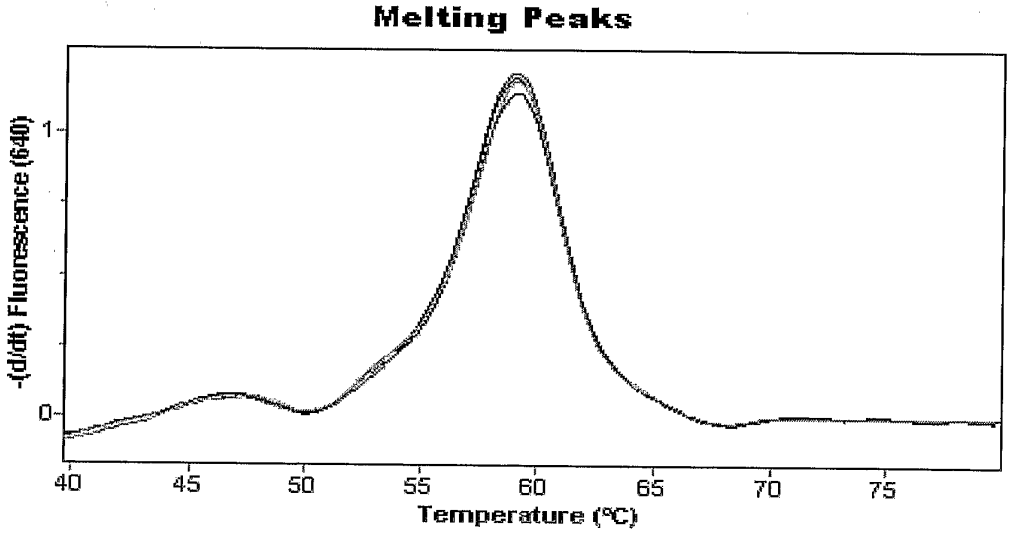


610 nm channel with color compensation

Fig. 7b

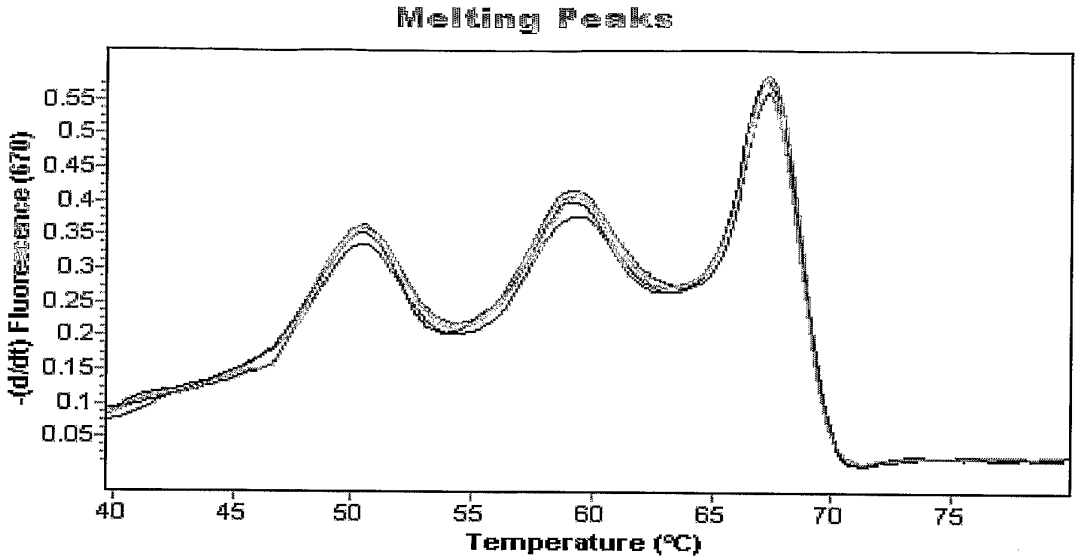


640 nm channel without color compensation

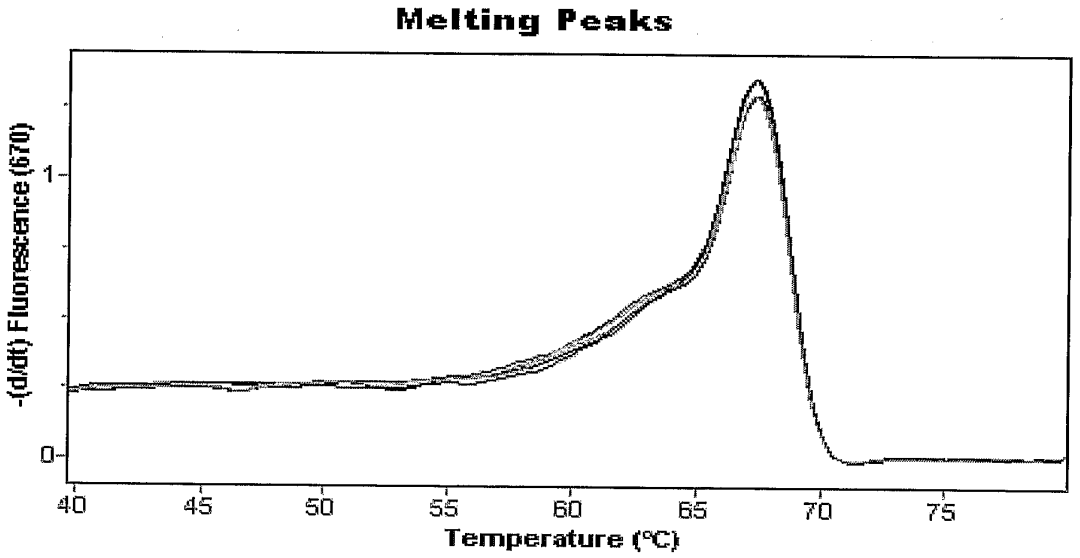


640 nm channel with color compensation

Fig. 7c

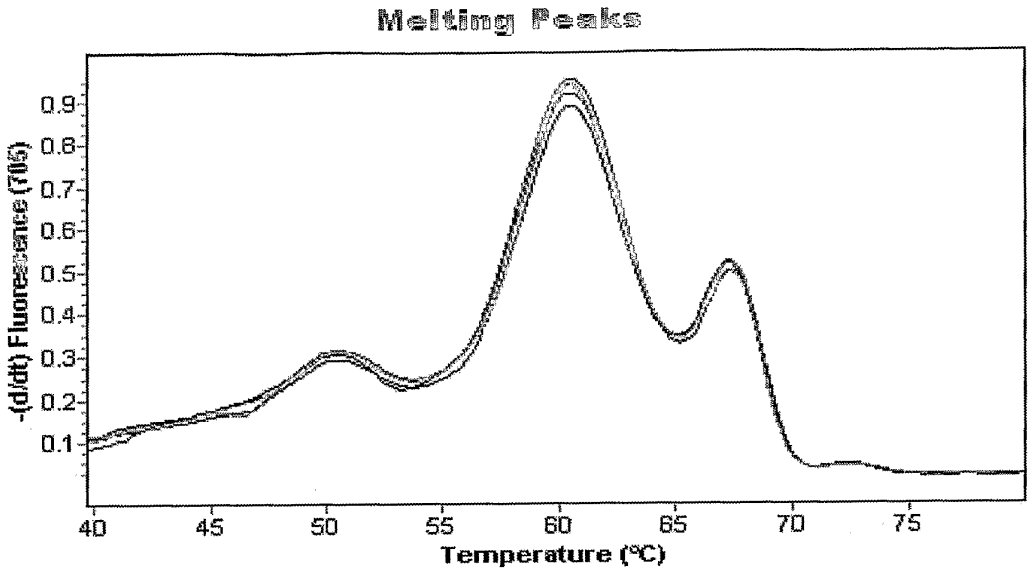


670 nm channel without color compensation

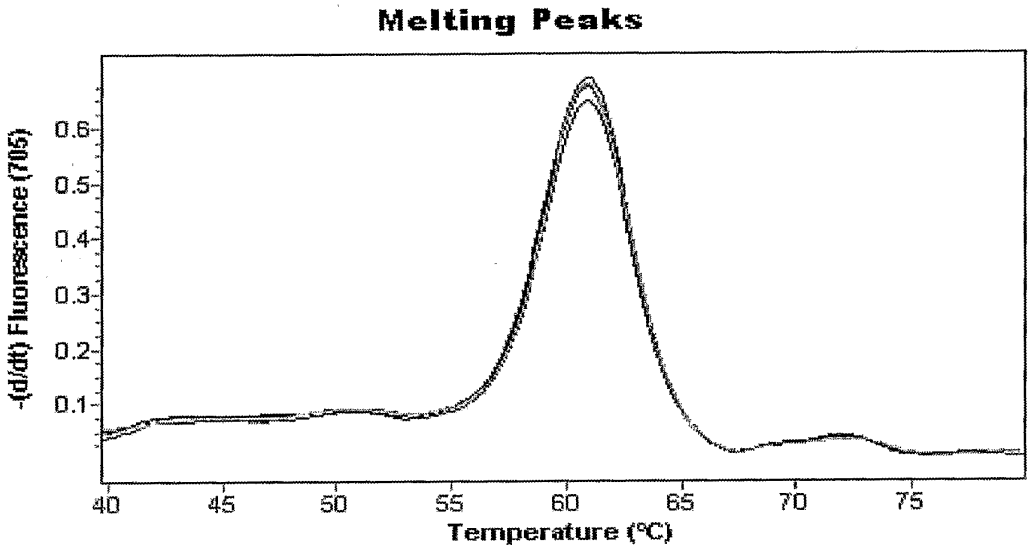


670 nm channel with color compensation

Fig. 7d



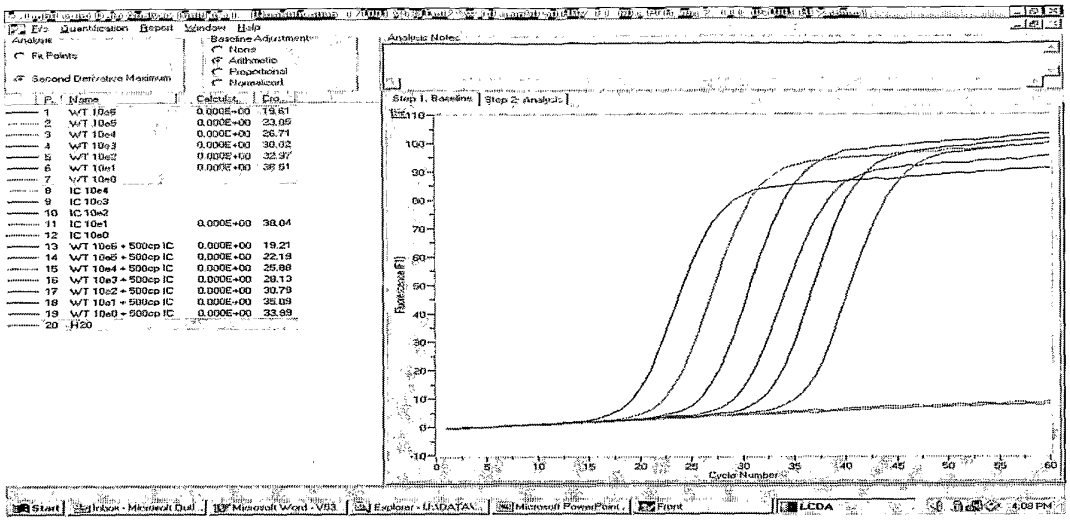
705 nm channel without color compensation



705 nm channel with color compensation

Fig. 8a

F1: 530nm (FAM)



F2: 560 nm (Hex)

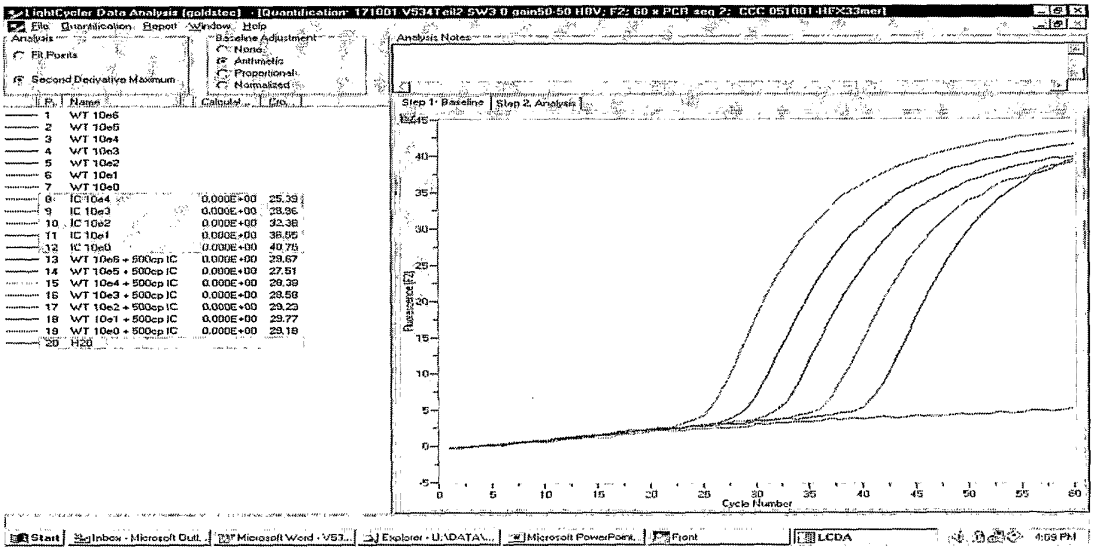
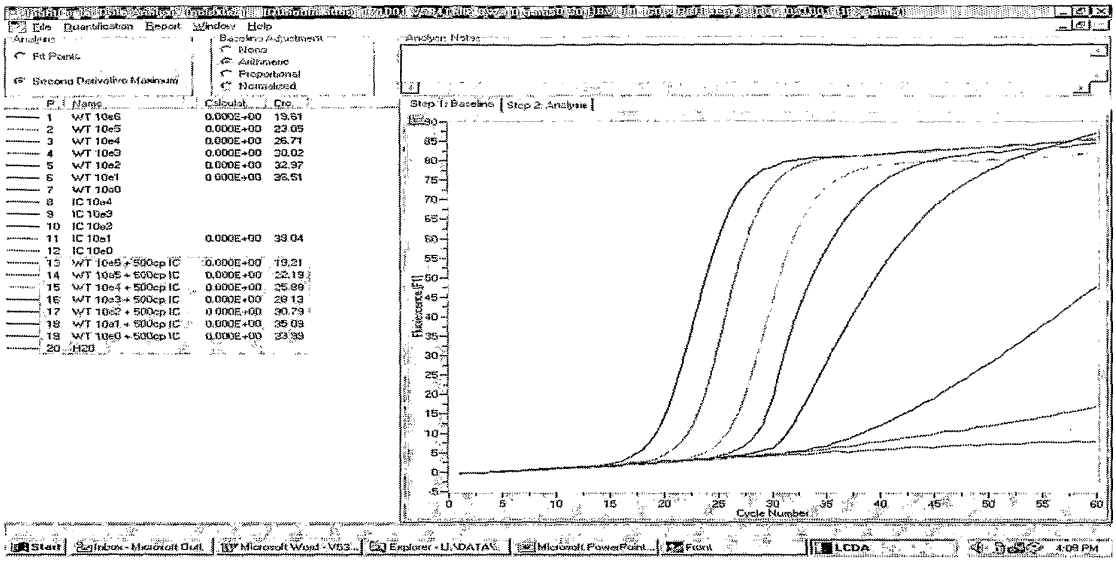


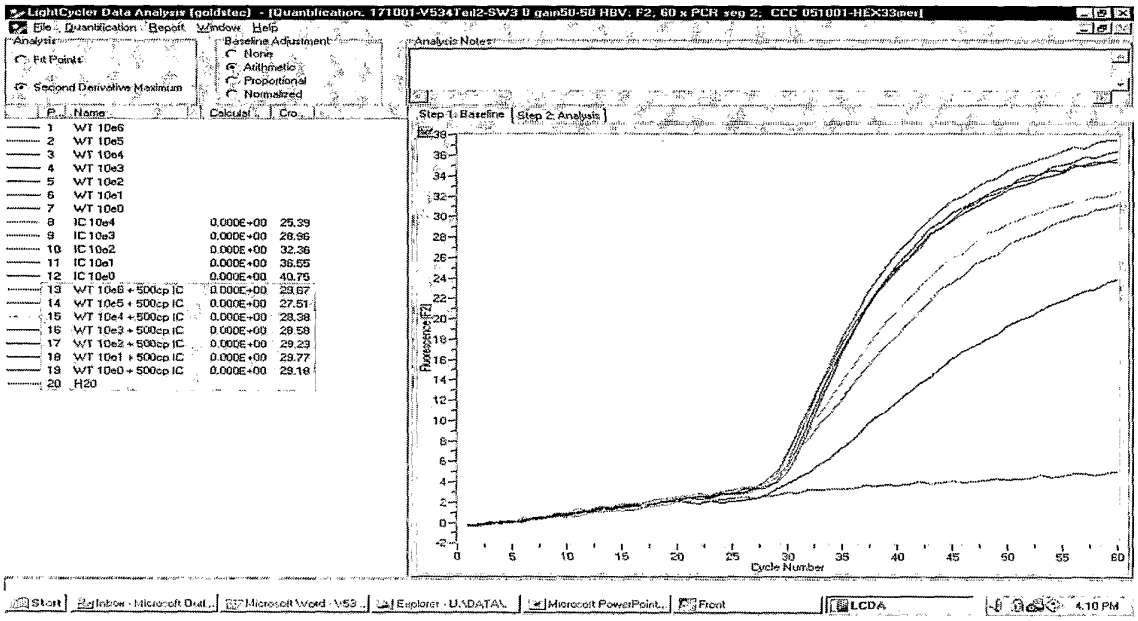
Fig. 8b

F1: 530nm (FAM)



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2	WT 10e5	0.000E+00	23.05	
3	WT 10e4	0.000E+00	26.71	
4	WT 10e3	0.000E+00	30.02	
5	WT 10e2	0.000E+00	32.97	
6	WT 10e1	0.000E+00	36.51	
7	WT 10e0			
8	IC 10e4			
9	IC 10e3			
10	IC 10e2			
11	IC 10e1	0.000E+00	39.04	
12	IC 10e0			
13	WT 10e6 + 500cp IC	0.000E+00	19.21	
14	WT 10e5 + 500cp IC	0.000E+00	22.19	
15	WT 10e4 + 500cp IC	0.000E+00	25.89	
16	WT 10e3 + 500cp IC	0.000E+00	29.13	
17	WT 10e2 + 500cp IC	0.000E+00	30.79	
18	WT 10e1 + 500cp IC	0.000E+00	35.09	
19	WT 10e0 + 500cp IC	0.000E+00	33.99	
20	H20			

F2: 560 nm (HEX)



1	WT 10e6			
2	WT 10e5			
3	WT 10e4			
4	WT 10e3			
5	WT 10e2			
6	WT 10e1			
7	WT 10e0			
8	IC 10e4	0.000E+00	25.39	
9	IC 10e3	0.000E+00	28.96	
10	IC 10e2	0.000E+00	32.36	
11	IC 10e1	0.000E+00	36.55	
12	IC 10e0	0.000E+00	40.75	
13	WT 10e6 + 500cp IC	0.000E+00	29.67	
14	WT 10e5 + 500cp IC	0.000E+00	27.51	
15	WT 10e4 + 500cp IC	0.000E+00	28.38	
16	WT 10e3 + 500cp IC	0.000E+00	29.58	
17	WT 10e2 + 500cp IC	0.000E+00	29.23	
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SEQUENCE LISTING

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F. Hoffmann-La Roche AG

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(54) **Title**: METHOD, INSTRUMENT AND COMPUTER PROGRAM PRODUCT FOR QUANTIFICATION OF PCR PRODUCTS

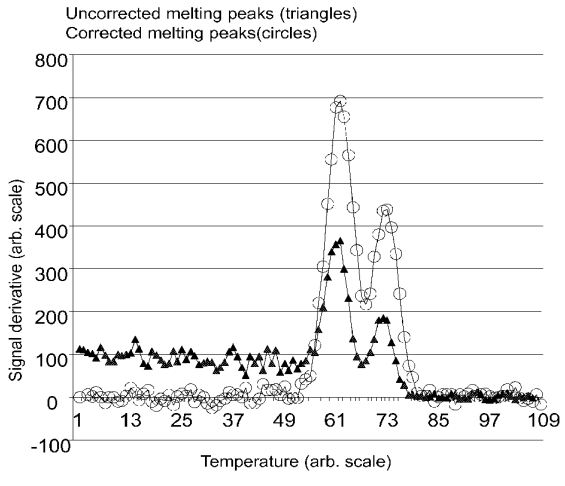


Fig. 2

(57) **Abstract**: This document relates to melting curve analysis of nucleic acids. The method according to first aspect of the invention comprises analyzing a nucleic acid melting curve measured from a sample, the melting curve comprising a sum signal of at least two nucleic acid melt signals and a background signal as a function of temperature. The method further comprises optimizing at least one constant in a temperature-dependent exponential correction function so as to minimize the variation of the nucleic acid melting curve at a temperature region where the target nucleic acids in the sample remain essentially double stranded, and generating a corrected nucleic acid melting curve representative of the nucleic acid melt signal by applying said exponential correction function over the region of the measured melting curve where the strands of the nucleic acids dissociate. According to further aspects, the invention relates to a curve fitting algorithm for precise estimation of melting peak areas and a mathematical transformation for linearization of calibration curve data to enhance the linear measuring range of a competitive PCR assay. The invention provides a powerful tool for analyzing PCR-amplified sample containing two or more different nucleic acids having similar but distinguishable melting temperature.

WO 2010/128206 A1

Method, instrument and computer program product for quantification of PCR products

FIELD OF THE INVENTION

The present invention relates to nucleic acid melting curve analysis. More specifically the embodiments of the present invention relate to the methods and systems for removing background signals and extracting specific signals in melting profiles of double stranded nucleic acids.

Melting curve analysis is typically performed in conjunction with polymerase chain reaction (PCR) for analysis of amplification products. The present invention thus also relates to instruments and software for melting curve analysis and real-time and competitive PCR techniques.

BACKGROUND OF THE INVENTION

Melting curve analysis is based on measurement of the temperature-dependent dissociation of double stranded nucleic acids, typically DNA, in a solution. The energy required for dissociation of the hydrogen bonds between the two strands is dependent on the length and base composition of the double stranded DNA molecules and on the complementarity of the opposing strands. The dissociation of the strands can be monitored using probes that change their fluorescent properties upon binding to nucleic acids, or fluorescent dyes that fluoresce when bound to double-stranded DNA but cease to fluoresce when DNA dissociates into single-stranded molecules. Thus, the amount of double stranded DNA present in a sample can be measured by optically monitoring the amount of fluorescence while increasing the temperature in a sample.

If the sample contains multiple groups of double stranded DNA molecules of different length or with a differing nucleotide composition, the result of the measurement will

comprise a sum of temperature-dependent fluorescent signals that are specific for each of the analyzed groups of DNA molecules. Thus, when measuring the PCR amplicons in a sample subsequent to PCR amplification of two or multiple templates, specific temperature-dependent fluorescent signals can be obtained for each of the specific PCR amplicons present in the sample.

In addition there is typically a decrease in fluorescent signal with an increasing temperature that occurs regardless of the temperature-specific dissociation of double stranded DNA molecules. This decrease in fluorescent signal can typically be observed over the whole temperature range, especially at temperatures where the DNA molecules in the sample remain essentially double stranded. This background signal is dependent on the amount of double stranded DNA present in the sample and it decreases exponentially as the temperature of the sample increases. The measured signal thus, comprises the specific decrease in fluorescent signal obtained from dissociation of double stranded DNA in the sample and of a temperature dependent exponentially decreasing background signal.

Real-time PCR involves amplification of cDNA or DNA templates and quantitative measurement of the amount of amplified DNA amplicons. In this technique the accumulation of PCR product in the reaction is monitored during each cycle of PCR amplification. The amount of template DNA present in a sample can be determined by comparing accumulation of reaction products in a sample to accumulation of reaction products in known standards. Additionally reaction efficiencies can be determined based on accumulation kinetics, but accuracy of such calculation is typically compromised by limited data points.Competitive PCR provides an alternative approach to control the variations in amplification efficiency between separate reaction vials. In this technique a known amount of a reference DNA template that differs in length or nucleotide composition from the wild type cDNA or DNA template, is included in the reaction and co-amplified with the wild type template in the same reaction vial. The relative amounts of wild type and reference PCR amplicons are determined subsequently to amplification. As, the amplification reaction efficiency influences both wild type and reference

templates equally, the amount of wild type template originally present in the sample can be determined from the relative amounts of wild type and reference PCR amplicons. In real-time PCR, quantitative measurement is performed during amplification, whereas in competitive PCR it is typically performed after completion of the amplification reaction.

Diagnostic tests based on RNA-expression analysis are faced with several technical challenges. A specific challenge has been the analysis of formalin-fixed paraffin-embedded (FFPE) tissue samples, in which the RNA molecules have been degraded into shorter fragments. Extraction and amplification of these short RNA fragments is technically demanding and the obtained signals are typically low. Genome-controlled RT-PCR has been used successfully for this purpose (*Stenman et al.*, *Clinical Chemistry 52:11 1988–1996 (2006)*). This technique is based on competitive PCR and enables amplification and quantitative measurement of short cDNA fragments, making it suitable for analysis of mRNA expression in FFPE samples. After competitive PCR amplification, the relative amounts of PCR amplicons originating from wild type cDNA and reference DNA templates are measured using melting curve analysis. In this technique the PCR amplicons generated from the wild type cDNA and reference DNA templates differ in nucleotide composition and differential detection is based on slight differences in the melting temperature. There are several specific benefits of this technique. Using genome-controlled RT-PCR it is possible to amplify exceptionally short cDNA fragments generated from mRNA of virtually any gene, as differentiation between expressed and genomic sequences is not dependent on intronic sequences. This is essential when analyzing FFPE samples. Co-amplification of a reference DNA template in every reaction regardless of the amount of wild type cDNA template present increases the reproducibility of the assay. The detection limit and imprecision of the assay can be controlled by varying the amount of the reference template, which is essential for diagnostic applications. Also, carryover of genomic DNA from the RNA extraction step does not cause false positive result following amplification. The requirements on the quantitative properties of the melting curve analysis in this type of assay are exceptionally high, as the relative levels of PCR products with close-to-equal melting temperatures are precisely quantified over a measuring range spanning several decades.

An instrument for rapid PCR amplification and detection of PCR products based on melting temperature is described in WO 1997/046712. The principle of melting curve analysis based on fluorescence measurements is extensively described in publications WO 2000/066777, WO 2004/038038 and WO 2006/121423. Extraction of qualitative and quantitative data from melting curve spectra typically involves measuring the fluorescent signal as a function of temperature to produce a raw melting curve. By calculating the rate of the change in signal as a function of temperature, a first derivative curve that displays dissociation of specific double stranded DNAs as specific "melting peaks", can be generated. A problem of this type of analysis is, however, the background signal, which affects the size and baseline of the separate melting peaks in the spectrum and complicates quantitative analysis especially when multiple peaks are present in the same spectra. In publication WO 2007/035806, an improvement of the analytical procedure is described in which the background signal is subtracted from the nucleic acid sample signal. In this technique an exponential algorithm is derived by interpolation from two separate slope values taken from points before and/or after the nucleic acid melting transition. This algorithm is then used to generate a corrected melting curve by subtracting the background from the raw melting curve data or from the first derivative melting curve spectra. This technique has been used primarily for genotyping applications, in which mutations in one or both alleles of a gene are detected. In these applications melting curve analysis has been used to detect dissociation of target-specific unlabelled probes from amplified DNA. Alternatively, whole amplicon melting curve analysis has been performed. In these types of applications, it is essential that the resolution of the assay is sufficient for separate detection of melting peaks occurring in the same spectrum.

Prior techniques for correcting the measured melting curve and for analyzing the melting peak areas, including those described in the abovementioned publications, are not capable of satisfactorily distinguishing between melting peaks which are situated very close to each other. In particular, improvements are needed for techniques for precise

quantification of the relative amounts of nucleic acids in the sample, in particular in RNA-expression analyses.

SUMMARY OF THE INVENTION

The aim of the present invention is to provide new techniques for accurate analysis of melting curve spectra. In particular, the aim of the present invention is to provide an improved method for relative quantification of two or more specific melting peaks present in a melting curve spectrum obtained from amplified DNA.

A further aim of the present invention is to improve instruments and software analysis for amplification and quantitative measurement of nucleic acids.

The invention has its specific focus on achieving techniques for accurate quantification of double-stranded DNA molecules, the length of which varies from 1000 to 30, in particular from 500 to 30 nucleotides. Specific advantages are obtained in studies involving molecules whose length is 100 or less, in particular 70 to 30 nucleotides.

The first aspect of the present invention is a signal-dependent method of transformation of raw melting curve data comprising the sum of the background signal and nucleic acid melt signal in order to render the data in an optimal form for final analysis. This is achieved by

- optimizing one or more constants in a temperature-dependent exponential correction function to render the decrease in the sum signal intensity close to zero at a temperature region of the raw melting curve where the target nucleic acids in the sample remain essentially double stranded, and
- generating a corrected nucleic acid melting curve representative of the nucleic acid melt signal using said correction function, in particular by multiplying the entire raw melting curve or its derivative, or a specific area of interest thereof, with said correction function. The absolute amount of correction is thus dependent on the fluorescent signal value at each given temperature point.

According to a preferred embodiment, the corrected melting curve is obtained directly by multiplying the measured melting curve by a correction function which is exponentially dependent on the temperature. That is, the correction function includes at least one term having an exponential behavior with respect to temperature. Thus, separate computation or subtraction of the background signal from the measured signal is not required or carried out. The corrected raw melting curve comprises the fluorescent signal related to the specific dissociation of the target nucleic acids in the sample.

According to one embodiment, the correction function $C(T)$ includes a correction term which is proportional to K^{BT}, where K is an exponential base, T is temperature and B is a scaling factor. In particular, the correction function can be of the mathematical form given in the detailed description below. The corrected melting curve $F_{corr}(T)$ is obtained as the product $C(T)F_{meas}(T)$, where F_{meas} is the measured melting curve.

According to one embodiment, the measured melting curve is pre-processed before applying the correction function to it in order to correct a potential offset in the general level of the curve or the correction function itself includes one or more factors correcting such offset (thus, for simplicity, the terms "melting curve" and F_{meas} can refer either to original or offset-corrected curve). This offset correction renders the area of the melting curve after the melting transition to zero or close to zero. Offset correction compensates for any systematic measurement errors and/or systematic (constant) fluorescence background that may be present.

Determination of the base K can be carried out by normalization of the melting curve at a region where no dissociation of target nucleic acids occurs. This can in practice be implemented by optimizing the correction factor (K), so as to render the change in the background signal close to zero over the non-dissociating region. For example, minimum sum or least squares method can be utilized for the optimization of K. After that, the K-based correction function dependent on temperature (or temperature difference with

respect to a predefined starting point) at each point is applied to all points of the melting curve or a specified portion thereof.

For obtaining a melting peak curve, the derivative, typically a negative derivative, of the corrected melting curve is calculated. It is appreciated by a person skilled in the art that an equivalent to the above-described correction method is a method where a corresponding correction is carried out to an already derived curve. The derivative of a product rule may be applied for obtaining a correction function suitable for this case. That is, the order of steps is not critical.

The above-described approach provided significant advantages over prior art. Exponential correction function as a multiplier of the measured melting curve produces a background-corrected melting curve having a steep melting region, the derivation of which further produces a melting peak curve where closely-situated peaks are narrow and well distinguishable. In addition, the correction takes into account that the measured signal level per molecule at a higher temperature is lower than at a lower temperature. As can be seen in the detailed description, the present method compensates for this phenomenon and causes the higher-temperature peak to be corrected upwards more than the lower-temperature peak, thus producing accurate data for further analysis.

Special advantages of the present invention are obtained in specific analytical applications. For example. in genotyping applications using high resolution melt protocols for detection of single nucleotide polymorphisms, the sequence difference between analyzed nucleic acids are minimal and the difference in melting temperature are typically less than 2 °C. In this type of application the distance between melting peaks is partially dictated by the natural nucleotide sequence of the target template. This results in partially overlapping peaks in subsequent melting curve spectra. When analyzing mRNA expression in a sample using a competitive PCR technique such as Genome-Controlled RT-PCR it is preferable that the difference in the nucleotide sequence of wild type and reference PCR amplicons is minimal in order to ensure close-to-identical amplification efficiency of the co-amplified templates. Generally, in this type of assay, competitively

amplified templates of less than 100 nucleotides in length, typically differing in nucleotide composition by 3-7 bases, which results in a 2-5 °C difference in melting temperature of the respective PCR amplicons are quantified. In this type of application the distance between the specific melting peaks can be adjusted to some extent. The size of the peaks, however, vary considerably depending on the original amounts of wild type and reference templates in the sample. Significant variations in peak size can cause overlapping of peaks even when the distance between adjacent peaks in a melting curve spectrum is greater than 3 °C. Regardless if the overlapping of adjacent peaks is caused by a small difference in melting temperature or by significant variations in peak size, such peaks can be efficiently and conveniently analyzed using the aspects of the present invention.

The second aspect of the present invention is a method for calculating the area of the separate melting peaks in a spectrum by a curve fitting algorithm to obtain a precise mathematical estimation of the areas of the independent, partially overlapping melting peaks in the spectrum. The peak areas based on curve fits are used instead of actual peak areas to calculate the relative amounts of PCR amplicons present in the sample subsequent to the amplification reaction.

The benefit of sequentially estimating peak areas by curve fitting, rather than from the actual peaks is that the areas of partially overlapping peaks in a spectrum can be precisely estimated. This is especially beneficial in situations where there is a overlapping of adjacent peaks due to considerable difference in areas of adjacent peaks and/or when the difference in melting temperature between separate amplicons is small. This results in accurate quantitative measurement of relative peak areas, which is fundamental for quantitative measurement of gene expression using competitive amplification techniques. Typically, in this type of assay, the melting temperatures of the specific PCR amplicons in the samples are known. This allows for pre-determining melting windows, where upper and lower temperature boundaries for the independent specific melting peaks are defined. This allows for automation of the assay analysis by automated detection and curve fit of multiple specific melting peaks present in the same spectra.

In competitive PCR techniques, the wild type template is co-amplified with one or multiple reference templates in the same reaction, with the same pair of PCR primers. The amplification of these templates is equally affected by variations in amplification efficiency between separate reaction vials. Thus, the amount of wild type template originally present in the sample prior to amplification can be determined from the ratios of wild type and reference PCR amplicons present after amplification. We have observed that a calibration curve consisting of serial samples with a linearly increasing amount of wild type template and a constant amount of reference template generated with this type of assay will have a sigmoid shape. In order to maximize the dynamic range of the assay it is beneficial to perform a mathematical transformation of the quantitative data that renders the calibration curve linear.

The third aspect of the present invention is a method for calculating the absolute amount of nucleic acid template molecules present in a sample prior to amplification. A calibration curve is generated for each of the analyzed genes by performing competitive amplification of samples with linearly increasing amounts of wildtype template and a constant amount of reference template or vice versa. Mathematical correction of the raw melting curves and estimation of the relative peak areas using a curve fitting algorithm can be performed as described above. A Logit-Log transformation of the obtained data is performed and a calibration curve is generated. There is a linear dependency between the *Logit (p)* value (*Log (p/(1-p))*) of the relative peak area estimates and the logarithm *Log R* of the relative amount of wild type and reference templates *R*. The Logit-Log transformation linearizes the calibration curve and thus significantly increases the dynamic range of the assay. After the transformation, the necessary parameters, that is, the linear regression slope and y-intercept values are calculated and stored in a database separately for each gene. When the absolute amount of reference template is known, the unknown amount of wild type template molecules present in a sample prior to PCR amplification, can be calculated from the relative melting peak areas of wildtype and reference amplicons, using the gene-specific slope and y-intercept values.

Logit-Log transformations have been extensively utilized for similar purposes in immunological assays. An example of this is presented in WO publication 2002/076343. We have observed that this type of mathematical transformation is applicable to assays based on PCR, in particular competitive PCR, despite the exponential nature of PCR amplification. In such assays a constant amout of a reference DNA template competes with a wild type template for DNA polymerase binding sites. This situation has proven to be analogous to an immunological assay, in which, a constant amount of labelled antigen competes with the antigen in the sample for antibody binding sites. It should be noted that the present idea of using the Logit-Log transformation in competitive PCR can be extended to other non-immunological PCR-employing applications for calculating amounts of template molecules, in particular in situations where two or more analytes compete for binding sites. This is the case with two templates and a polymerase in competitive PCR.

Each of the three main aspects of the present invention discussed above are independently beneficial for relative quantification of nucleic acids using melting curve analysis. These three aspects of the present invention can be used independently, or together in groups of two or three to gain maximal precision of the quantitative analysis. All three aspects of the present invention can be used in any melting curve assay using fluorescent or luminiscent detection in which the sample comprises two or more nucleic acid molecules with separate specific melting temperatures. Examples of such techniques are quantitative and/or competitive PCR techniques such as those described in publications*: Wang, A.M. et al., Proc. Natl. Acad. Sci. 86, 9717-9721 (1989), Becker-André, M. & Hahlbrock, K. Nucl. Acids Res. 17, 9437-9446 (1989), Gilliland, G. et al., Proc. Natl. Acad. Sci. 87, 2725-2729 (1990)* and *Stenman, J. et al., Clin. Chem. 52:11 1988–1996 (2006).* Additional examples are various genotyping applications, examples of which are given in WO 2007/035806 and techniques for quantitative analysis of differentially expressed alleles based on single nucleotide polymorphisms as described in *Kuokkanen M. et al., Gut 52, 647-652 (2003)* and techniques for quantitative analysis of differentially expressed highly homologous genes as described in *Stenman, J et al., Nat.*

Biotechnol. 17, 720-722 (1999). The first and second aspects, or portions thereof, can also be employed for samples containing only a single nucleic acid.

The invention can be used, for example, for the determination of relative amounts of mRNA transcripts in a cell or tissue sample. When using the Genome-Controlled RT-PCR technique for this purpose, sequence-modified cDNA templates are co-amplified competitively with one or several reference DNA templates using the same PCR primers. In this technique it is possible to use Genomic DNA (gDNA), synthesized or cloned DNA oligomers or polymers as a reference DNA template in order normalize for the differences in amplification reaction efficiency between separate reaction vials.

By using melting curve analysis to measure the relative amount of sequence-modified cDNA and reference DNA amplicons during PCR cycling or after amplification, the relative amounts of mRNA transcripts originally present in the sample can be determined. As cDNA and reference DNA templates are amplified using the same primers in the same reaction vials, the relative amount of the respective amplicons remain unchanged even when amplification reactions are run to the plateau phase. This can be utilized by running reactions to the plateau phase in order to bring the signal into the measuring range of the assay regardless of the amount of starting template present in the sample. This type of technique is presented extensively in WO publication 2005/116248, the essential contents of which are incorporated herein by reference.

Analogously, the invention can be used in an assay for determination of relative amounts of highly homologous mRNA or DNA templates naturally present in a sample by reverse transcription of mRNA templates and competitive co-amplification of cDNA and / or DNA templates and quantitative detection by melting curve analysis.

Next, embodiments and advantages of the invention are described with the aid of the attached drawings.

BRIEF DESCRIPTION OF DRAWINGS

Figs. 1a – 1d show the determination of a correction factor for the melting curve correction function according to one embodiment of the invention.

Fig. 2 shows exemplary melting peaks generated by calculating the negative derivative of the measured melting curve $-dF_{meas}/dT$ corrected melting curve $-dF_{corr}/dT$.

Figs. 3a – 3f illustrate the fitting of gauss functions to corrected melting peaks of two amplicons present in the sample.

Figs. 4a and 4b illustrate, respectively, a Log-Log plot of a standard curve and Logit-Log plot which linearizes the entire range of the standard curve (gene: dd3).

DETAILED DESCRIPTION OF EMBODIMENTS

As discussed above, the method according to first aspect of the invention comprises analyzing a nucleic acid melting curve measured from a sample, the melting curve comprising a sum signal of at least two nucleic acid melt signals and a background signal as a function of temperature. The method further comprises optimizing at least one constant in a temperature-dependent exponential correction function so as to minimize the variation of the nucleic acid melting curve at a temperature region where the target nucleic acids in the sample remain essentially double stranded, and generating a corrected nucleic acid melting curve representative of the nucleic acid melt signal by applying said exponential correction function over the region of the measured melting curve where the strands of the nucleic acids dissociate. According to further aspects, the invention relates to a curve fitting algorithm for precise estimation of melting peak areas and a mathematical transformation for linearization of calibration curve data to enhance the linear measuring range of a competitive PCR assay. In summary. the invention provides powerful tools for analyzing PCR-amplified sample containing two or more different nucleic acids having similar but distinguishable melting temperatures.

Definitions

The term ”PCR amplicon” means the PCR product of any PCR-amplifiable template molecule. In the context of competitive PCR, the term generally means the PCR product generated by amplification of a specific cDNA or DNA template. In techniques based on competitive PCR, separate amplicons are derived from wild type and reference templates that are co-amplified in the same reaction with the same primers.

The term ”wild type template” means a nucleic acid template that is being analyzed. Wild type cDNA templates are generated during reverse transcription of mRNA species present in the original sample, whereas, a wild type DNA templates occur naturally in the original sample. The amount of wild type template is usually unknown prior to analysis.

The term ”reference template” means a nucleic acid template that is included in each amplification reaction and serves to normalize for tube-to-tube variations in amplification efficiency. In the context of Genome-controlled RT-PCR, the reference DNA templates comprise genomic DNA contained in or added to the sample, or a pool of gene-specific templates such as cloned or synthesized DNA oligo- or polynucleotides for each of the analyzed genes. The wild type and reference templates in an individual amplification reaction are co-amplified in a competitive manner, using the same primers. Gene-specific synthetic or cloned reference templates can be concatenated by ligation or other means to form a single polynucleotide that serves as a universal reference template for several genes.

The term ”co-amplification” means simultaneous amplification of two or more templates. For example in competitive PCR, the co-amplified templates may be cDNA and/or DNA templates with differing nucleotide sequences within the generated amplicons. A pre-requisite for co-amplification as the term is used in this patent application is that templates have identical nucleotide sequences at the primer-binding sites used in the amplification reaction and that amplification of said templates is carried out in a competitive manner with the same primers and other reagents, in the same reaction vial.

Correction of measured melting curve

As briefly explained above, in the first aspect of the invention, the background fluorescence is estimated from the initial portion of the raw melting curve prior to the specific nucleic acid melting transition where the amplified nucleic acids remain essentially double-stranded. An exponential correction factor is calculated on the basis of the initial portion of the melting curve. The entire measured fluorescence curve is thereafter multiplied by a function of temperature in which said exponential correction factor is incorporated. This transformation renders the average derivative of the background signal close to zero. If the melting experiment includes several samples, as is typically the case, the transformation is performed separately for each independent sample taking into account the amount of signal present in each individual sample.

Thus, in general terms, the method according to one embodiment comprises the following steps:

- A correction factor K, which is the base in an exponential correction function, is optimized in order to render the change in background signal intensity close to zero over the range prior to the specific nucleic acid melting transition.
- The correction function is applied to every temperature point in the melting curve to generate a corrected melting curve.

Optimization of the correction factor K is performed using the data of the measured melting curve at a temperature range prior to the specific nucleic acid melting transition. The minimum range width required depends on the frequency of the temperature data points obtained, which is a functional feature of the specific instrument used. Basically, a slope can be formed using a minimum of two data points, but preferably three to five data points are used to obtain more reliable results. The range typically has a width of at least 1 °C, in particular at least 3 °C, and preferably at least 6 °C, In this fashion, the fluorescent signal used for estimation of background fluorescence is not affected by

melting of the specific nucleic acids in the sample. This ensures that the proportion of background fluorescence in the sample is correctly estimated.

The present method, renders the background fluorescence close to zero and enhances the dynamic range at the specific nucleic acid melting transition. We have observed that this type of correction gives a precise estimation of the sample-derived signal obtained from temperature-specific dissociation of the DNA strands of the PCR amplicons in the sample. When the correction is performed for two adjacent melting peaks with seemingly identical areas, the correction renders the area of a peak obtained at a higher melting temperature larger than that of a peak obtained at a lower melting temperature. This is due to the fact that the fluorescent signal per molecule of DNA is temperature-dependent.

According to a preferred embodiment, the correction obeys the formula

$$F_{corr}(T) = \frac{1+K^{dT}}{2} F_{meas}(T) \quad (1)$$

which can also be written in the following form:

$$F_{corr}(T) = \left(\frac{1+Ae^{BT}}{2}\right) F_{meas}(T) \quad (2)$$

where $F_{meas}(T)$ is the measured melting curve, $dT=T-T_0$ and constants A and B are defined as follows: $B = ln\ K$ and $A = e^{-BT_0}$.

Even more generally, the correction can be formulated as

$$F_{corr}(T) = (C_1 + C_2 e^{C_3 T}) F_{meas}(T) \quad (3)$$

where C_1, C_2 and C_3 are constants. The correction function can be calculated according to the above-described optimization principle for C_3 after fixing C_1 and C_2.

As appreciated by persons skilled in the art, the exponential correction term can be mathematically formulated in various ways.

The parameter T_0 can be relatively freely chosen from the range before the melting transition, as it only defines the starting point of correction and the correction equation is designed to yield $F_{corr}(T_0) = F_{meas}(T_0)$.

A typical melting curve spectrum is generated by calculating the negative derivative of the corrected raw melting curve $-dF/dT$. A benefit of the invention in comparison with earlier techniques is that the entire melting curve is corrected for the effect of background fluorescence which allows for precise measurement of multiple specific melting peaks over the entire range of the melting curve.

In practice, the fluorescence measurements are carried out at discrete steps and the measurement results stored in an array. The calculation of the correction factor can thus be performed by discrete mathematical methods by a computer program product designed for this purpose or in a spreadsheet program.

In the following, calculation of the corrected melting curve using the abovementioned formula is described in detail.

Figs. 1a – 1d illustrate the optimization of the correction factor K using the formula (1) above. The factor is gradually increased to find the optimal value, which normalizes the corrected curve at the initial part of the curve. Figs. 1a shows a situation where no correction is performed ($K = 1$). Fig. 1d shows an optimal correction ($K = 1.077$) which normalizes the non-dissociating region of the melting curve. This value is chosen for the K-value for correction of the whole curve using the same formula. Minimizing the sum of deviations of the curve from normal level can be implemented using first-order sums or e.g. least squares method.

It is also understood that other method for finding the optimal K-value can be used. These may include e.g. iterative or other computational methods.

Fig. 2 shows the first negative derivatives of an uncorrected and corrected melting curve. As can be seen from the data, the correction causes the relative heights of the peaks of different amplicons to change. In the uncorrected data, the ratio of peak heights is 184.5/365.5=0.50 and in the corrected data the ratio is 438.7/691.6=0.63. As the rise in temperature causes the measured signals to weaken, the correction can be found to rectify the data in this respect. Thus, the following calculation of the proportions of the amplicons in the sample is more accurate.

The analysis is preferably performed utilizing a computer program that is run in the central processing unit of a computer. The following preparational steps can be performed prior to the analysis:

- The temperature and fluorescence data are retrieved from the memory unit of the computer or directly from the instrument performing the sample melting and signal aquisition,
- the melting temperature range of the wild type and the reference amplicons and preferably the identification number or name of the analysis are retrieved from a database or recieved from the user, and
- appropriate start and end points are determined from the temperature and fluorescence data if these include excessive data points.

Calculating the proportions of amplicons

The relative amounts of amplicons in the sample can be calculated from the corrected melting curve using curve fittings representing the amounts of amplicons, rather than directly using the measured first derivative melting curve peaks.

In this method, a curve fit is performed for the first peak in the corrected first derivative melting curve spectrum in order to obtain a mathematical estimation of the area of the first melting peak. Subsequently the difference of the corrected first derivative melting

curve and said curve fit is calculated to obtain a residual corrected melting curve. A second curve fit is performed for the second peak in the residual melting curve to obtain a mathematical estimation of the area of the second melting peak. This procedure is repeated as many times as required in order to estimate the areas of each of the specific melting peaks in the spectrum. The areas of the curve fits for the separate melting peaks are used to determine the relative concentrations of PCR amplicons present in the sample. In competitive PCR such as the Genome-Controlled RT-PCR technique the relative concentrations of wild type and reference DNA amplicons reflect the amount of wild type cDNA and reference DNA originally present in the sample prior to amplification. In a modification of this technique two or multiple separate reference DNA templates added at different known concentrations to the sample can be co-amplified with the wild type cDNA template. In this manner a sample-specific calibration curve can be obtained from each individual amplification reaction, which further increases the quantitative precision of the analysis.

In the Genome-Controlled RT-PCR technique PCR amplicons are typically in the range 30-70 nucleotides in length. The melting temperature of reference DNA templates is typically modified by a nucleotide substitution without altering the length of the subsequent PCR amplicons. This results in a difference in the melting temperatures of wild type and reference DNA PCR amplicons of less than 5° C. When using competitive PCR and melting curve analysis for genotyping applications or for quantification of the expression level of differentially expressed alleles based on single nucleotide polymorphisms, the difference in melting temperature between the amplicons are typically less than 3° C. In addition, there is a degree of inherent temperature variation across the thermal block of many instruments currently being used for nucleic acid melting. As a result the melting peaks of a specific nucleic acid will appear at slightly different temperatures in the melting curve spectrum, depending on where the sample has been situated in the block.

Adjacent melting peaks that overlap cause significant skewing. In particular, a peak adjacent to a significantly larger peak often appears only as a shoulder on the larger peak.

In such a situation the estimation of actual peak areas is difficult and imprecise. The curve-fitting procedure of the present invention allows precise estimation of the relative amount of amplicons present in the sample, in particular when there is partial overlapping of adjacent peaks and/or when difference in peak areas is large.

The method of the present invention can be processed in a separate computer, which typically forms part of a quantitative PCR system, or in the central processing unit of the PCR cycling or melting curve instrument itself.

Example

This example illustrates the various aspects of the invention by describing the main steps of a melting curve analysis wherein the sample contains two nucleic acids having different melting temperatures.

a) Correction of the melting curve

Below is presented an example of calculation of the correction from two separate temperature points on the melting curve using the principles disclosed above. Values of a pre-specified temperature range where no dissociation of specific nucleic acids occur are used for generating an optimal correction of the entire melting curve. Herein, $T_0=59,3$ *(°C)*, corresponding to the starting temperature for recording of data in the measurement.

Table 1. Example of correction of a measured melting curve (at non-dissociating region)

point	Temp	F_{meas}	dT	(1+K^dT)/2	F_{corr}
1	59,3	**0,1536**			**0,1536**
12	62,2	0,1372			
13	62,5	**0,1355**	3,2	1,1328	**0,1535**
24	65,8	0,1175			
25	66,1	**0,1155**	6,8	1,3249	**0,1530**

Table 1 demonstrates that using the optimized correction factor K in the correction function (1+K^dT)/2 renders the corrected fluorescence values close-to-equal at temperatures below the melting transition of the target nucleic acids in the samples. After correction the raw (non-derived) melting curve will be horizontal, i.e. normalized at the non-dissociating region. The behaviour of the corrected curve at the dissociating region and after that is shown in the drawings.

b) Determination of melting peak areas

Measurement of the areas of the specific melting peaks can be carried out by

- determining the location of maximum rate of change in corrected signal strength, e.g. by finding the maximum value of the derivative for the first nucleic acid occurring in the melting curve (for this purpose pre-determined melting temperature ranges for the respective amplicons can be utilized),
- performing a curve fit for the first melting peak, for example by optimizing the width of a chosen curve form (i.e. gaussian) to fit the peak, i.e., until a minimum for the difference of the curve and the first melting peak (i.e., residual curve) is found (this is illustrated in Figs. 3a and 3b (showing a non-optimal fit) and Fig. 3c (showing an optimal fit)),
- when the width of the curve has been determined to fit the first nucleic acid melting peak, the difference of *the measured value – value of said curve* in each data point of the entire derivative melting curve is calculated to generate a residual melting curve (Fig. 3c),
- determining from the residual melting curve the the peak of the second nucleic acid by finding the maximum change of rate of the signal, e.g. by finding or the maximum value of the derivative for the second nucleic acid occurring in the melting curve (for this purpose pre-determined melting temperature ranges for the respective PCR amplicons can be utilized),
- performing a curve fit by optimizing the width of a curve (i.e. gaussian) to fit the peak of the second nucleic acid in the first derivative melting curve by using said

maximum value as the maximum for the curve (Figs. 3e (non-optimal fit) and 3f (optimal fit)),

- if more than two nucleic acids are present, repeating these steps are until a curve fit has been performed for each of the specific nucleic acid melting peaks in the sample,
- optionally, first derivative melting curves and/or the goodness of the curve fit and/or the calculated residual melting curves are graphically displayed and,
- calculating the areas of each of the fitted curves and using these areas to estimate the relative amounts of the respective amplicons present in the sample.

Automation of the curve fittings is generally possible using the above algorithm. However, the fittings can also be performed fully or partly manually, if desired.

c) Calculating the absolute amounts of nucleic acids in the sample

In one embodiment of the present invention a calibration curve is generated in order to enable quantification of the unknown absolute amount of wildtype template molecules present in the sample prior to amplification. This calibration curve is generated by performing amplification of samples with known wild type / reference template ratios at constant increments. A Logit-Log transformation where the logit of a number p between 0 and 1 is given by the formula $\text{logit}(p) = \log(p) - \log(1-p)$ is performed to linearize the sigmoidal calibration curve. The linear regression slope and y-intercept values of the linearized calibration curve are calculated and stored in a data base. These values are used to calculate the unknown absolute amount of wild type template present in the sample prior to amplification from the Logit-Log transformed ratio of wild type and reference amplicon-derived melting curve areas in a sample. This is achieved by:

- co-amplifying a linearly increasing amount of wild type template with a constant amount of reference template in serial samples. For this purpose synthetic or

cloned DNA oligo- or polynucleotides with the same nucleotide composition as the wildtype and reference templates can be used,

- performing melting curve analysis of the amplicons to obtain the ratio of cDNA and reference DNA-derived melting curve areas from each of the serial samples to generate a calibration curve (preferably, but not necessarily, the melting curve correction and/or melting peak area calculation according to other aspects of the invention are used),
- performing Logit-Log transformation of the relative curve areas in order to linearize the calibration curve, (Fig. 4a shows the sigmoidal calibration curve (gene: dd3) as a Log-Log graph, fig 4b shows the calibration curve after Logit-Log transformation)
- calculating the linear regression slope and y-intercept values from the calibration curve and storing these in a database, separately for each gene included in a subsequent analysis,
- using the gene-specific slope and y-intercept values to calculate the unknown absolute amount of wild type template molecules originally present in a sample, from the the sample-specific ratio of wild type and reference amplicon-derived melting curve areas

As can be seen from Fig. 4a, the Log-Log plot of the standard curve is typically sigmoidal, but the Logit-Log function, shown in Fig. 4b linearizes the entire range of the standard curve (gene: dd3). Thus, the linear dynamic range of the analysis is increased.

The basic mathematical principles of Logit-Log transformation, however, in the context of radioimmunological assay, is presented in *Rodbard D. et al, Mathematical analysis of kinetics of radioligand assays: improved sensitivity obtained by delayed addition of labeled ligand. J Clin Endocrinol Metab. 1971 Aug;33(2):343-55*.

The embodiments described above and shown in the attached drawings are given for illustrative purposes and are not intended to limit the scope of the present invention which is defined in the following claims.

Claims:

1. A method for analyzing a sample containing at least one nucleic acid using a nucleic acid melting curve measured from the sample, the melting curve comprising a sum signal of nucleic acid melt signal and a background signal as a function of temperature, the method comprising

- optimizing at least one constant in a temperature-dependent exponential correction function so as to minimize the variation of the nucleic acid melting curve at temperature region where the nucleic acids in the sample remain essentially double stranded, and
- generating a corrected nucleic acid melting curve representative of the nucleic acid melt signal by applying said exponential correction function over the region of the measured melting curve, or derivative thereof, where the strands of the nucleic acids dissociate.

2. The method according to claim 1, wherein the measured melting curve is multiplied with the exponential correction function in order to obtain said corrected melting curve.

3. The method according to claim 1 or 2, wherein the corrected nucleic acid melting curve is generated by multiplying the measured nucleic acid melting curve at the region of the measured melting curve where the strands of the nucleic acids dissociate by an exponential function including a term K^T, or a mathematical equivalent thereof, wherein K is a constant correction factor being optimized using data from the region of the melting curve where no dissociation of target nucleic acids occurs.

4. The method according to any of the preceding claims, wherein the correction obeys the formula

$$F_{corr}(T) = \frac{1+K^{dT}}{2} F_{meas}(T),$$

where $F_{corr}(T)$ is the corrected melting curve, $F_{meas}(T)$ is the measured melting curve, $dT=T-T_0$, where T_0 is a predefined starting temperature and K is a constant optimized

using the measured melting curve at the temperature region where the target nucleic acids in the sample remain essentially double stranded.

5. The method according to any of the preceding claims, wherein the optimization of the constant in the correction function is performed over a temperature range having a width of at least 1 °C, in particularly at least 3 °C, preferably at least 6 °C.

6. The method according to any of the preceding claims, wherein a derivative of the corrected melting curve is calculated in order to obtain a melting peak curve.

7. The method according to any of the preceding claims, wherein the nucleic acid melting curve is measured from a sample processed by PCR amplification, in particular competitive PCR amplification.

8. The method according to any of the preceding claims, wherein the sample contains two or more different nucleic acids having different melting temperatures.

9. The method according to claim 8, wherein the nucleic acid melting curve is measured from a Genome-Controlled RT-PCR sample containing a wild type cDNA and reference DNA nucleic acids.

10. The method according to claim 8 or 9, wherein the difference of the melting temperatures of the at least two different nucleic acids is less than 10 °C, in particular less than 5 °C, preferably 0.5 – 5 °C.

11. The method according to any of claims 8 - 10, wherein the length of each of the at least two nucleic acids is less than 500 nucleotides, advantageously less than 100 nucleotides, in particular 30 – 70 nucleotides.

12. The method according to any of claims 8 – 11, further comprising the steps of

- calculating a first derivative of the corrected melting curve in order to form a melting peak curve,
- determining the areas of at least two melting peaks of the melting peak curve,
- calculating the proportions of nucleic acids in the sample using said areas.

13. A system for analyzing melting properties of nucleic acids, comprising

- means for measuring a nucleic acid melting curve as a function of temperature, the melting curve comprising a sum signal of nucleic acid melt signal and a background signal,
- computing unit for analyzing the measured melting curve, and
- means for transferring the measured melting curve to said computing unit,

wherein the computing unit comprises

- means for optimizing at least one constant in a temperature-dependent exponential correction function so as to minimize the variation in the sum signal intensity at a temperature region where the nucleic acids in the sample remain essentially double stranded, and
- means for generating a corrected nucleic acid melting curve representative of the nucleic acid melt signal by applying said exponential correction function over the region of the measured melting curve, or derivative thereof, where the strands or the nucleic acids dissociate.

14. The system according to claim 13, wherein the computing unit is adapted to carry out the method according to any of claims 1 – 12.

15. A computer program product stored on a computer-readable medium for analyzing nucleic acid melting curves, the product being configured to

- read measured melting curve data including temperature values and corresponding signal values from a data file,
- optimize, based on said data, at least one constant in a temperature-dependent exponential correction function so as to minimize the variation in the sum signal

intensity at a temperature region where the nucleic acids in the sample remain essentially double stranded

- calculate a corrected nucleic acid melting curve using said exponential correction function over the region of the measured melting curve, or derivative thereof, where the strands or the nucleic acids dissociate.

16. The computer program product according to claim 15, configured to perform the method according to any of claims 1 – 12.

17. A method for determining the proportions of nucleic acids from a melting curve measured from a sample containing at least two nucleic acids, the method comprising

- correcting the measured melting curve by removing the effect of background signal so as to form a corrected melting curve reflecting melting of the target nucleic acids in the sample,
- calculating a first derivative of the corrected melting curve in order to form a melting peak curve,
- fitting mathematical functions, for example gaussian functions, to the melting peaks contained in the melting peak curve,
- estimating the areas of the melting peaks using said fitted mathematical functions, and
- determining the proportions of nucleic acids in the sample using said areas.

18. The method according to claim 17, wherein said fitting is performed by

- fitting a first mathematical function to a first peak found on the melting peak curve for estimating the first melting peak,
- calculating a residual curve by subtracting said first mathematical function from the melting peak curve,
- fitting a second mathematical function or the like to the residual curve for estimating the second melting peak, and

- optionally, repeating the previous steps for estimating further melting peaks that may be present.

19. The method according to claim 18, wherein the step of correcting the measured melting curve is carried out according to any of claims 1 - 12.

20. A method for determining the proportions of nucleic acids from a melting curve measured from a sample containing at least two nucleic acids, the method comprising

- correcting the measured melting curve by removing the effect of background signal so as to form a corrected melting curve reflecting real melting of the sample,
- calculating a first derivative of the corrected melting curve in order to form a melting peak curve,
- estimating the areas of the melting peaks, and
- determining the proportions of nucleic acids in the sample by Logit-Log transformation using said areas.

21. The method according to claim 20, comprising

- providing a calibration curve for quantification of the amounts of nucleic acids, the calibration curve being obtained by from samples with known nucleic acid ratios at at least two known concentrations,
- linearizing the calibration curve using Logit-Log transformation,
- calculating necessary parameters to describe the linearized calibration curve, and
- using said parameters and said estimated areas of the melting peaks to calculate the absolute amount of at least one of the nucleic acids in the sample prior to amplification.

22. The method according to claim 20 or 21, wherein the step of correcting the measured melting curve is carried out according to any of claims 1 - 12.

23. The method according to any of claims 20 – 22, wherein the proportions of nucleic acids are estimated according to any of claims 16 – 19.

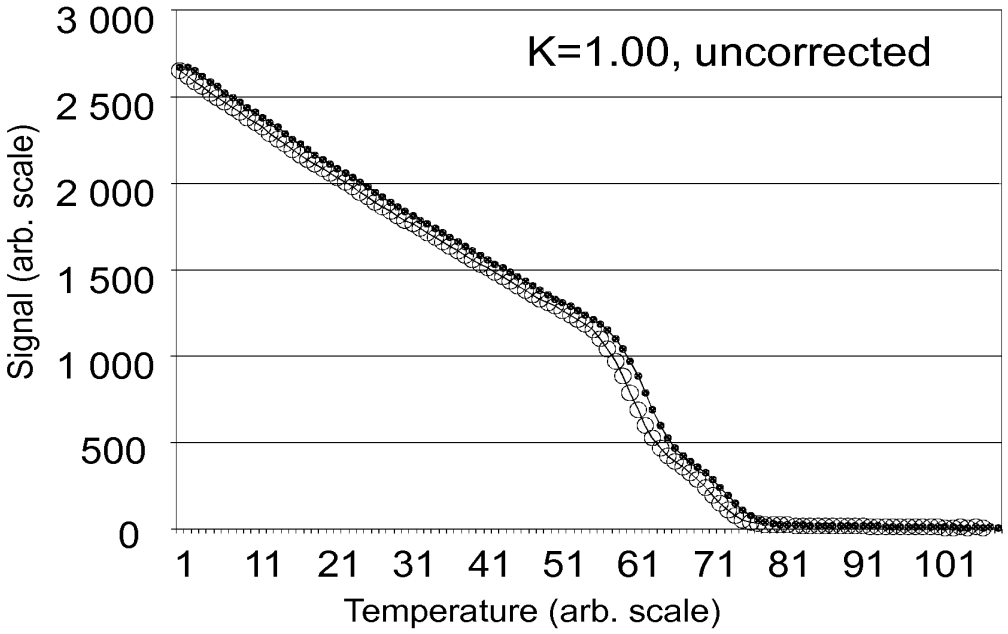

K=1.00, uncorrected
Signal (arb. scale)
3 000
2 500
2 000
1 500
1 000
500
0
1 11 21 31 41 51 61 71 81 91 101
Temperature (arb. scale)

Fig. 1a

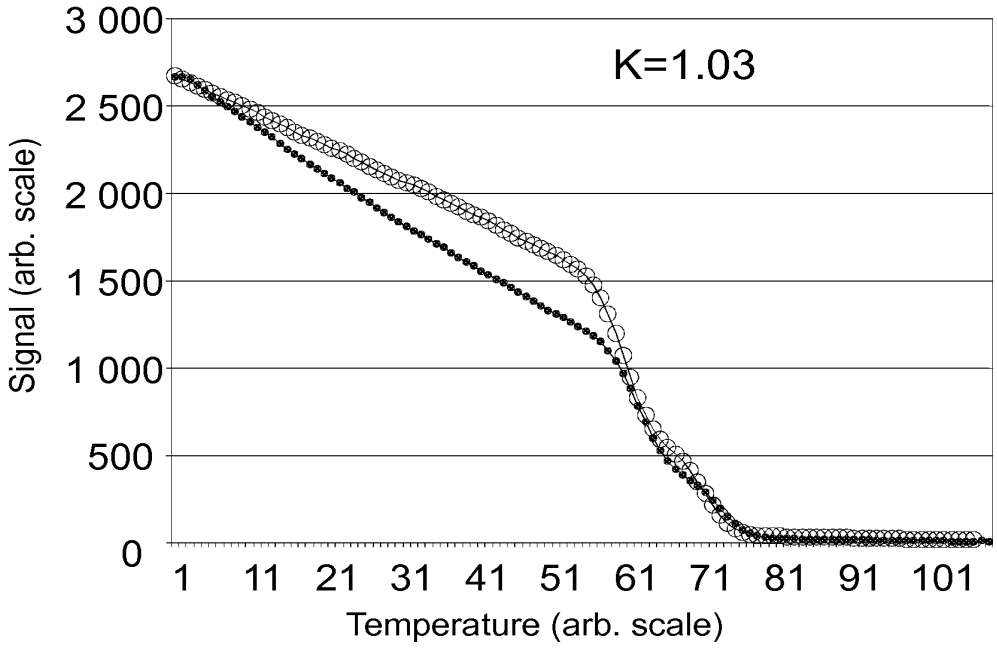

K=1.03
Signal (arb. scale)
3 000
2 500
2 000
1 500
1 000
500
0
1 11 21 31 41 51 61 71 81 91 101
Temperature (arb. scale)

Fig. 1b

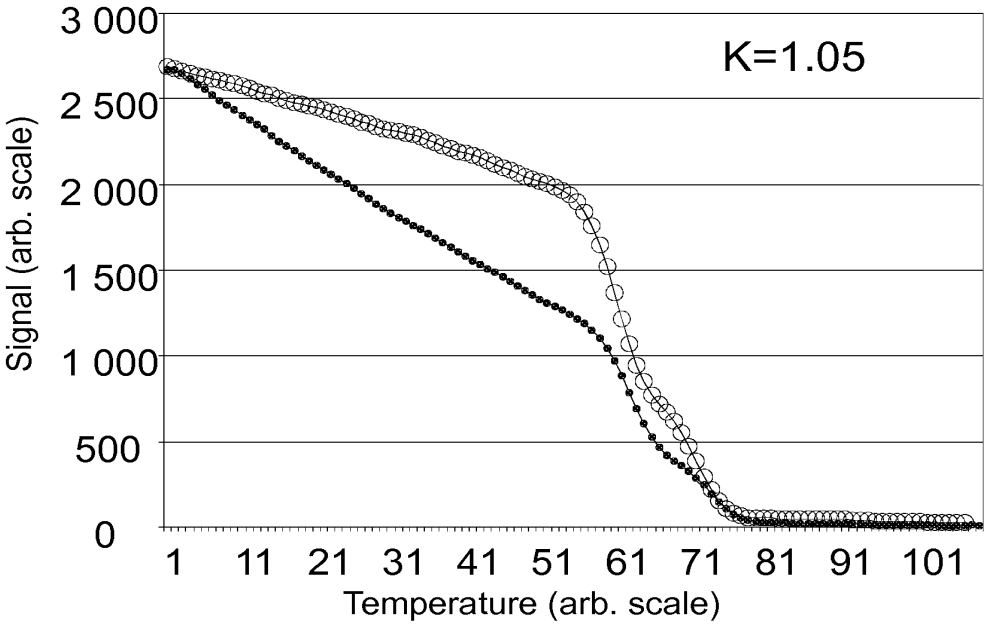

K=1.05
3 000
2 500
2 000
1 500
1 000
500
0
Signal (arb. scale)
1 11 21 31 41 51 61 71 81 91 101
Temperature (arb. scale)

Fig. 1c

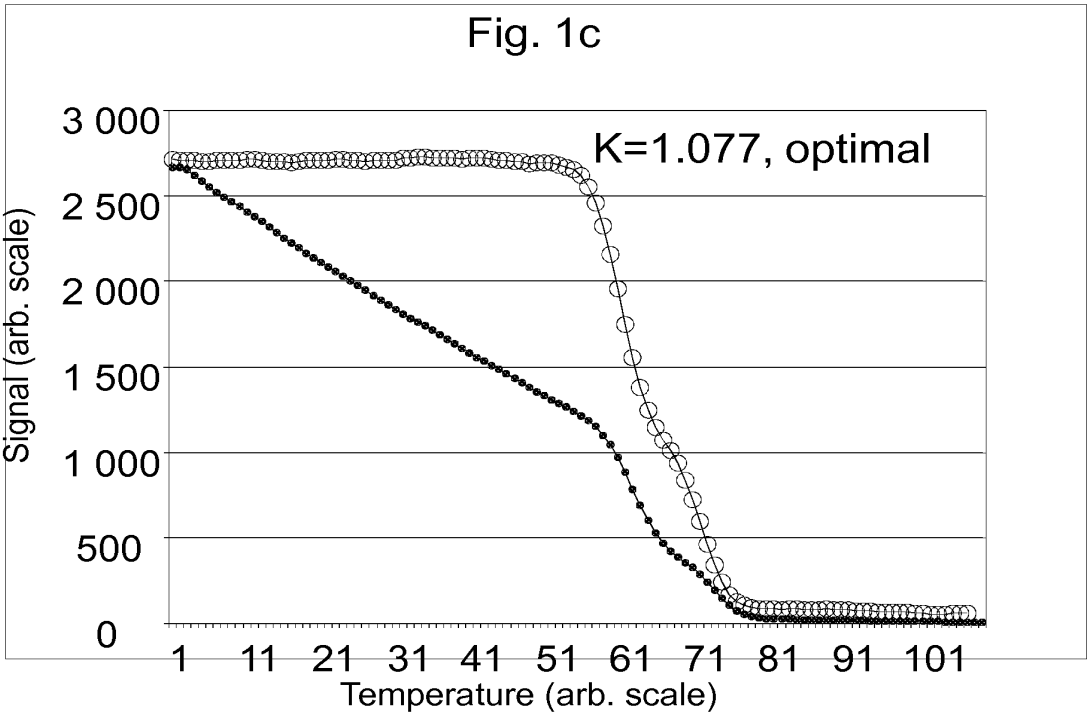

K=1.077, optimal
3 000
2 500
2 000
1 500
1 000
500
0
Signal (arb. scale)
1 11 21 31 41 51 61 71 81 91 101
Temperature (arb. scale)

Fig. 1d

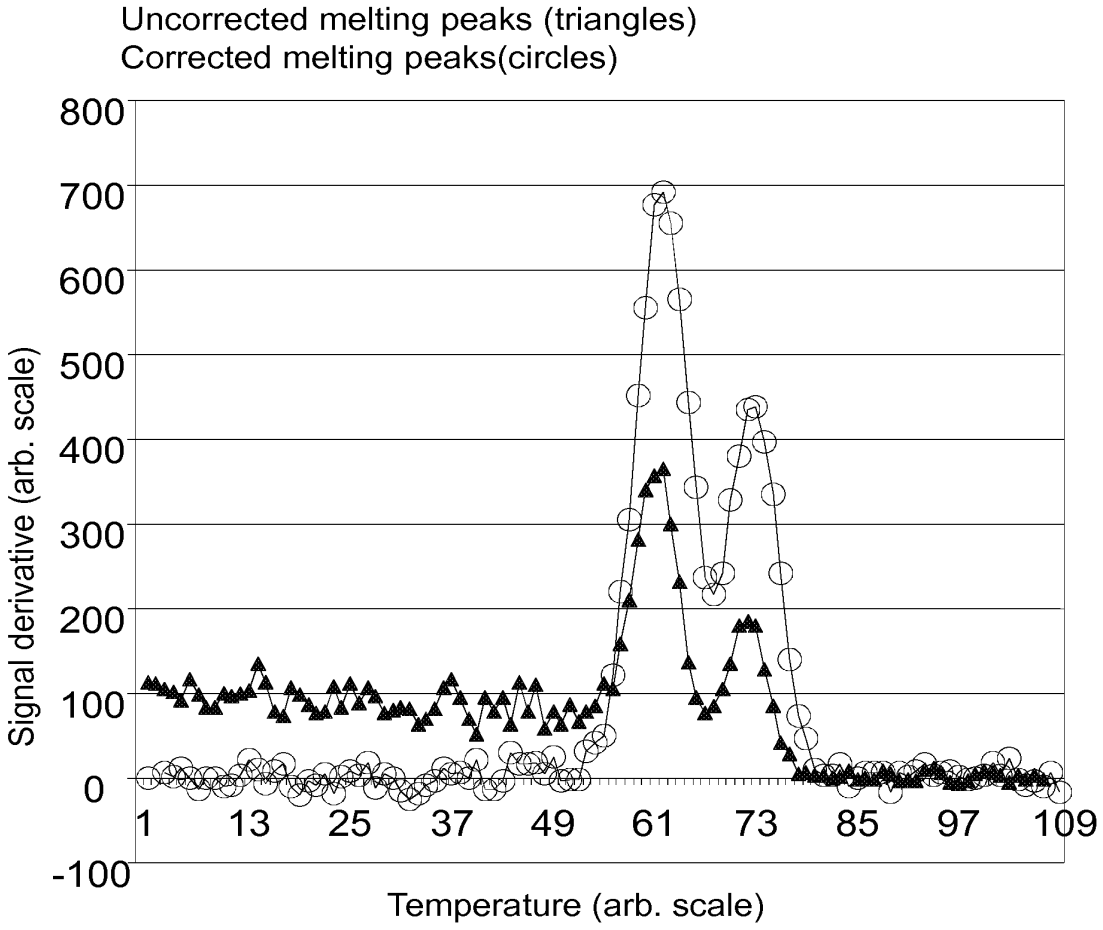

Uncorrected melting peaks (triangles)
Corrected melting peaks(circles)
800
700
600
500
400
300
200
100
0
-100
Signal derivative (arb. scale)
1
13
25
37
49
61
73
85
97
109
Temperature (arb. scale)

Fig. 2

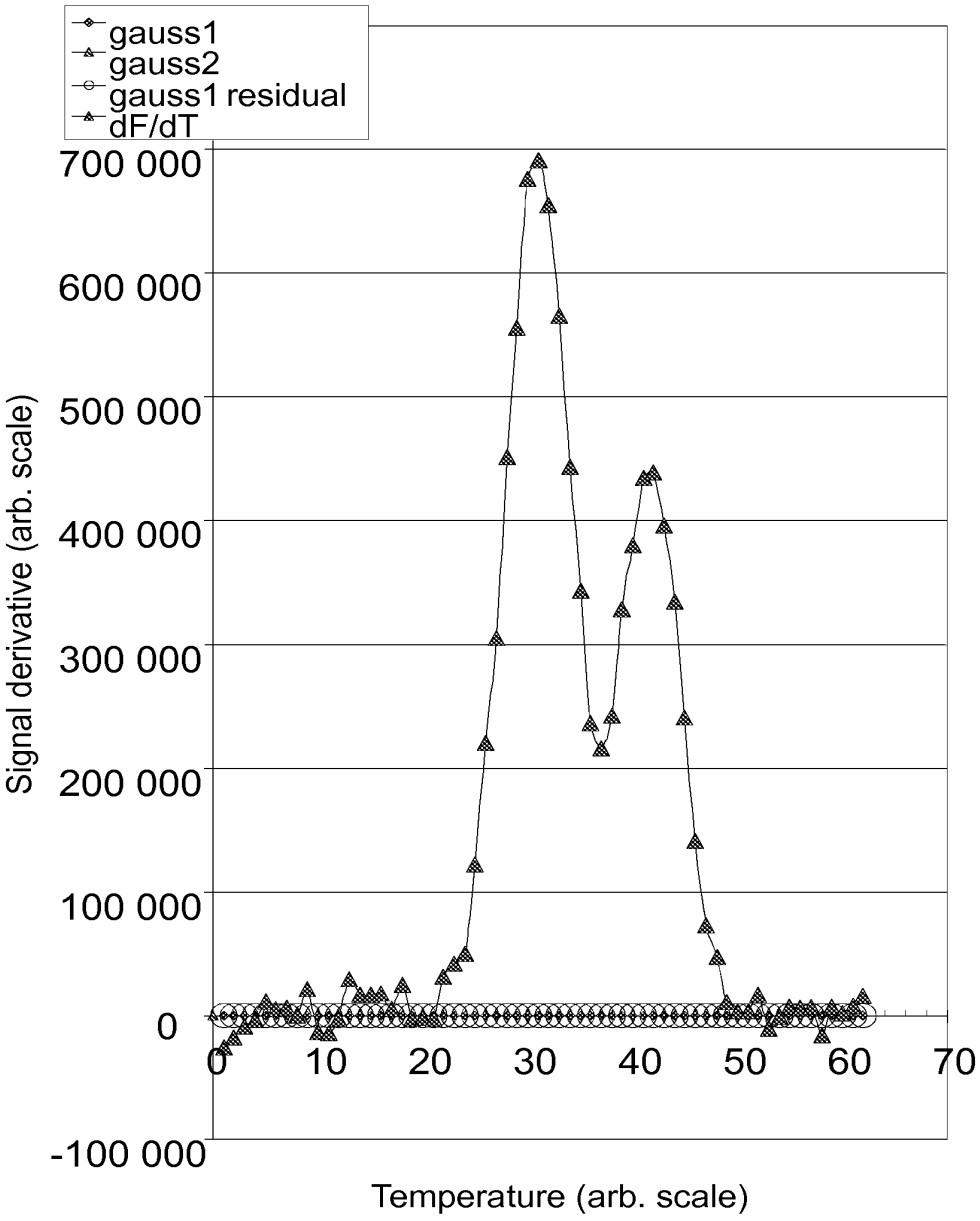

gauss1
gauss2
gauss1 residual
dF/dT
700 000
600 000
500 000
400 000
300 000
200 000
100 000
0
-100 000
Signal derivative (arb. scale)
0
10
20
30
40
50
60
70
Temperature (arb. scale)

Fig. 3a

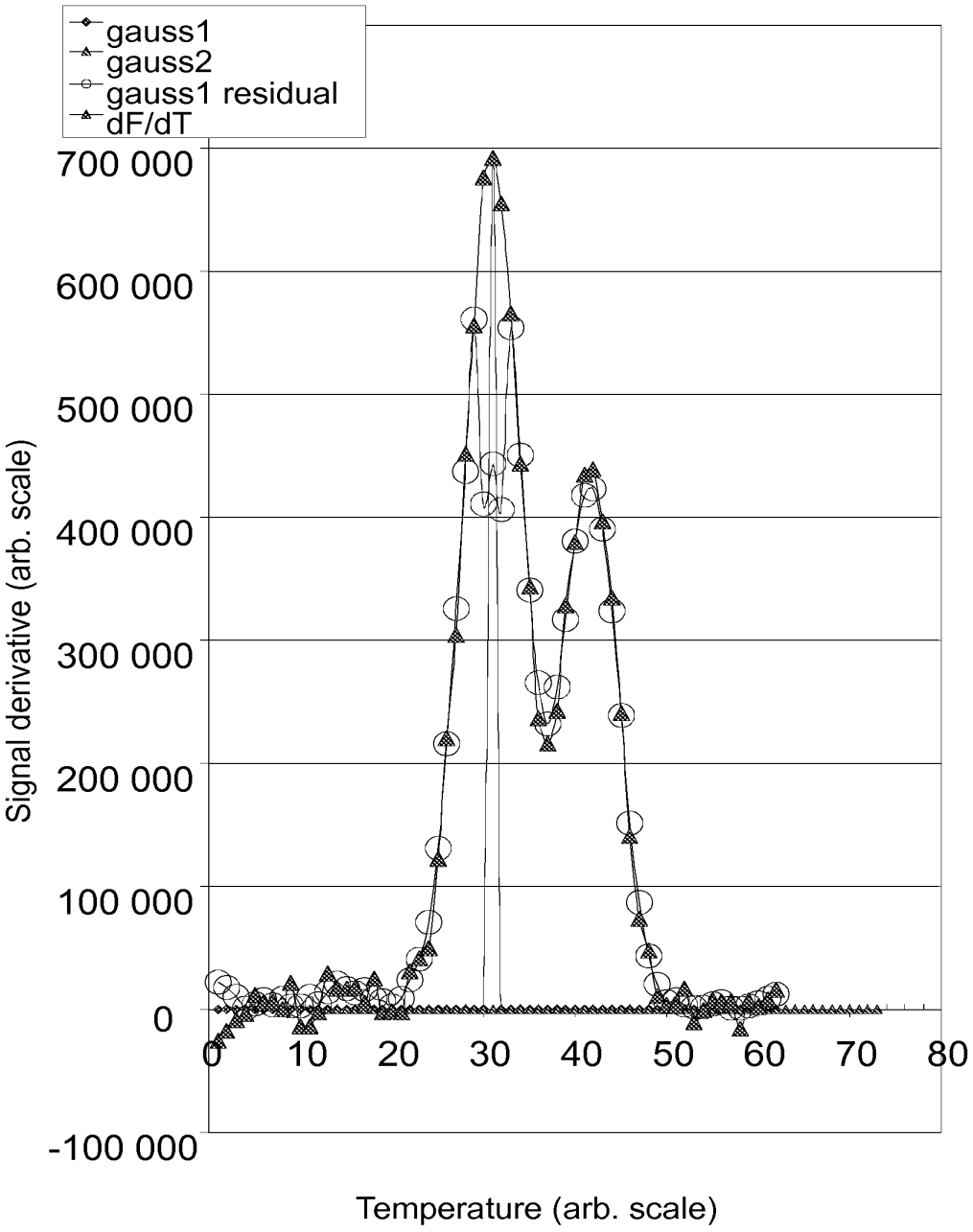

gauss1
gauss2
gauss1 residual
dF/dT
700 000
600 000
500 000
400 000
300 000
200 000
100 000
0
-100 000
Signal derivative (arb. scale)
0
10
20
30
40
50
60
70
80
Temperature (arb. scale)

Fig. 3b

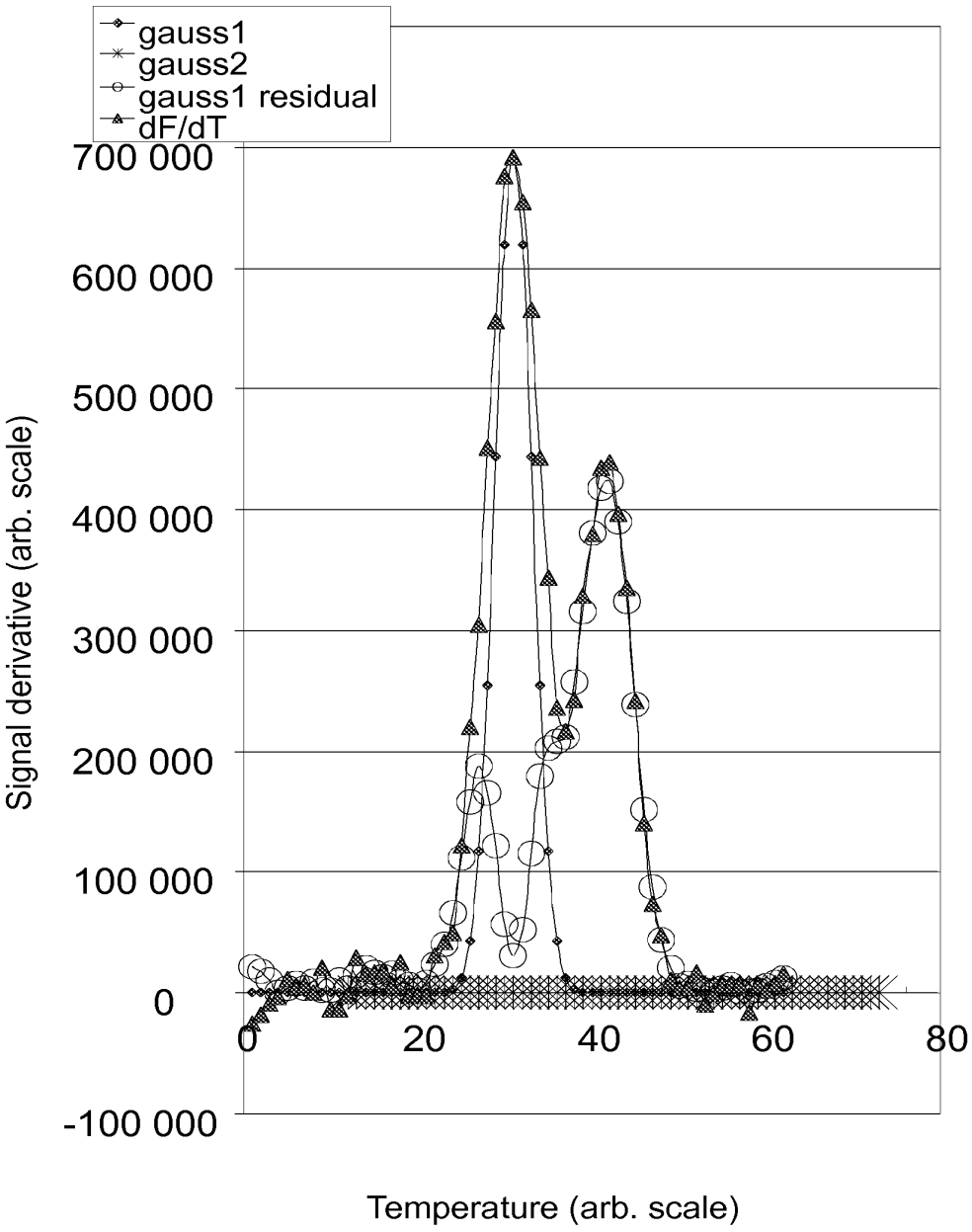

gauss1
gauss2
gauss1 residual
dF/dT
700 000
600 000
500 000
400 000
300 000
200 000
100 000
0
-100 000
Signal derivative (arb. scale)
0
20
40
60
80
Temperature (arb. scale)

Fig. 3c

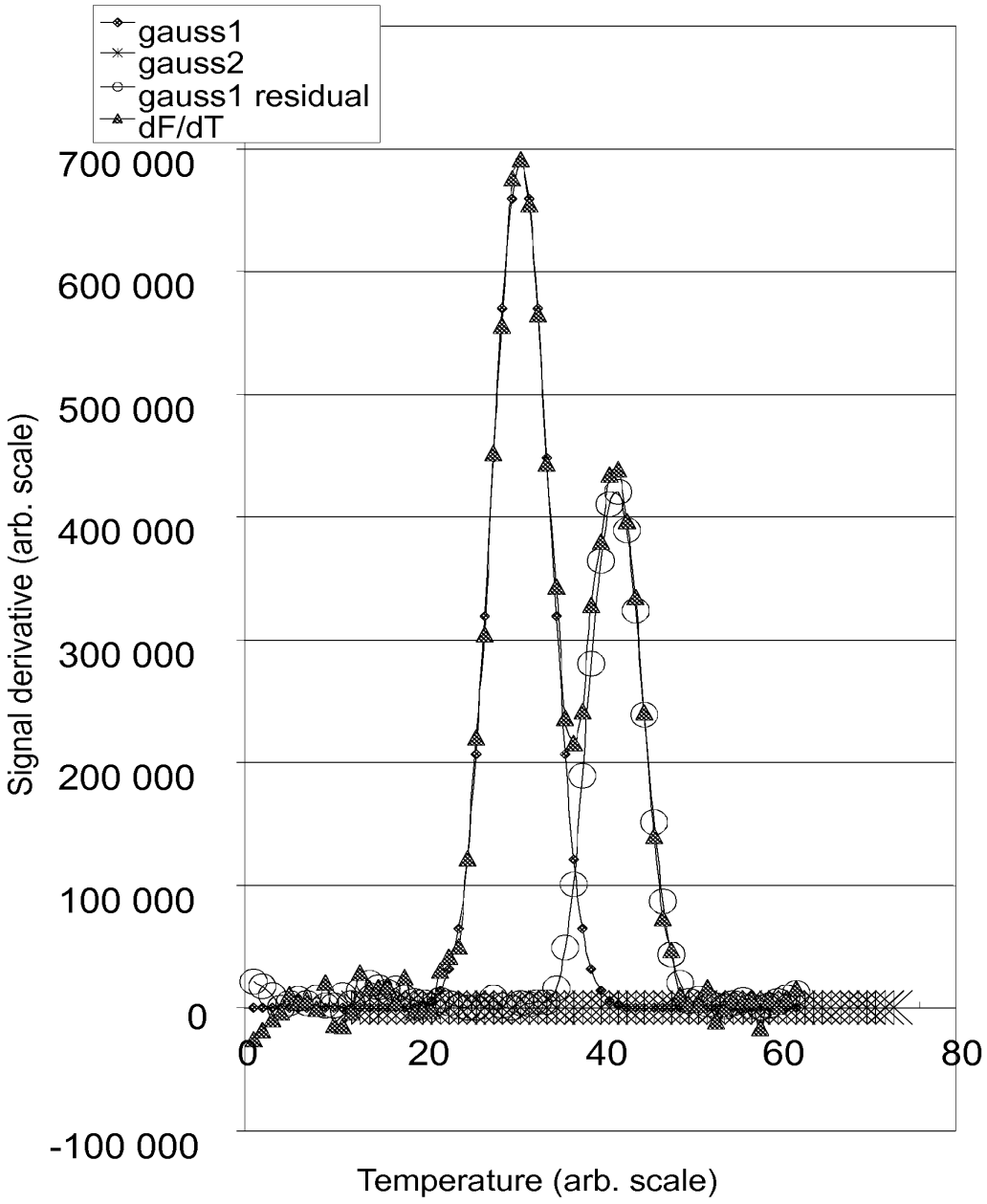

gauss1
gauss2
gauss1 residual
dF/dT
700 000
600 000
500 000
400 000
300 000
200 000
100 000
0
-100 000
Signal derivative (arb. scale)
0
20
40
60
80
Temperature (arb. scale)

Fig. 3d

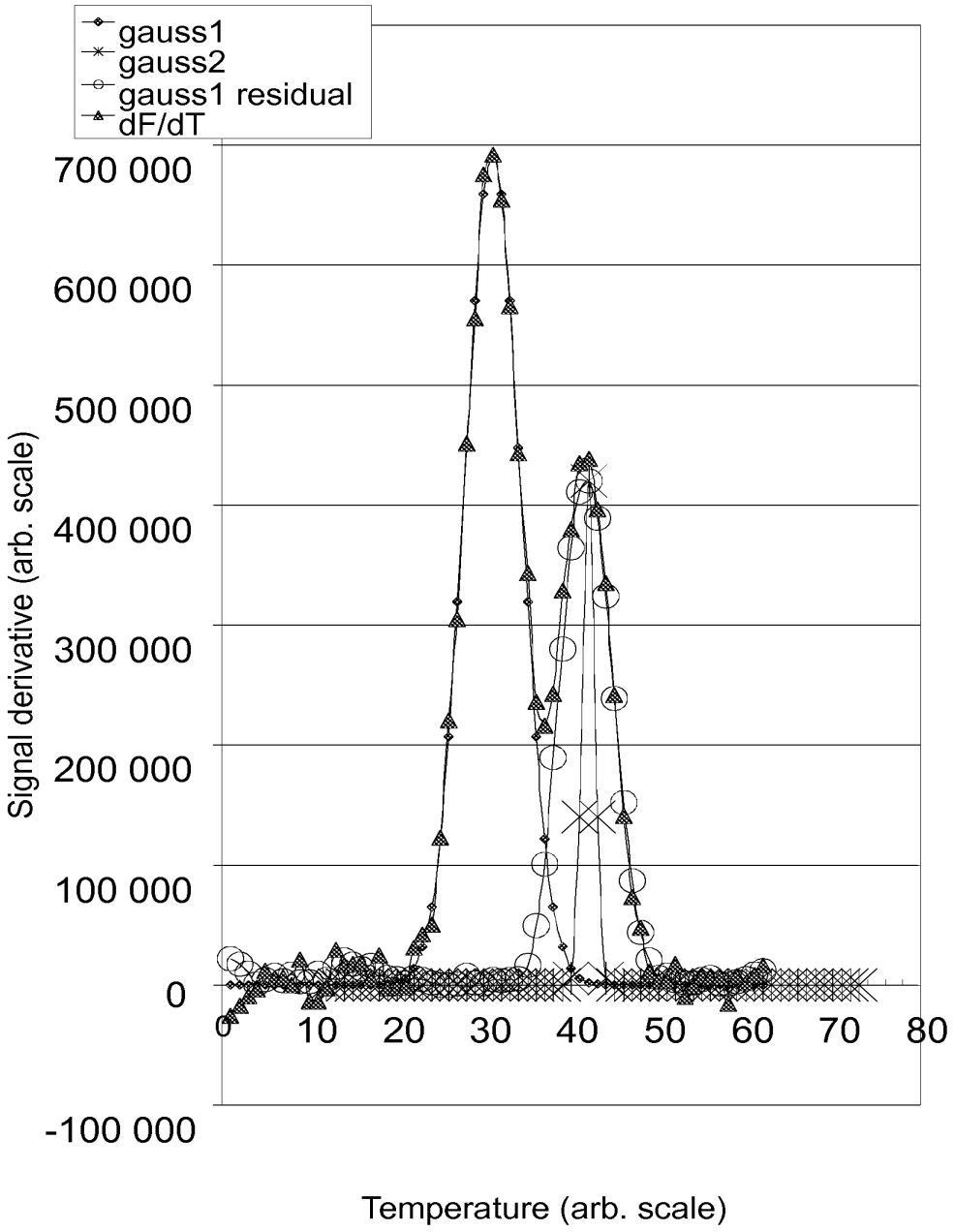

gauss1
gauss2
gauss1 residual
dF/dT
700 000
600 000
500 000
400 000
300 000
200 000
100 000
0
-100 000
Signal derivative (arb. scale)
0
10
20
30
40
50
60
70
80
Temperature (arb. scale)

Fig. 3e

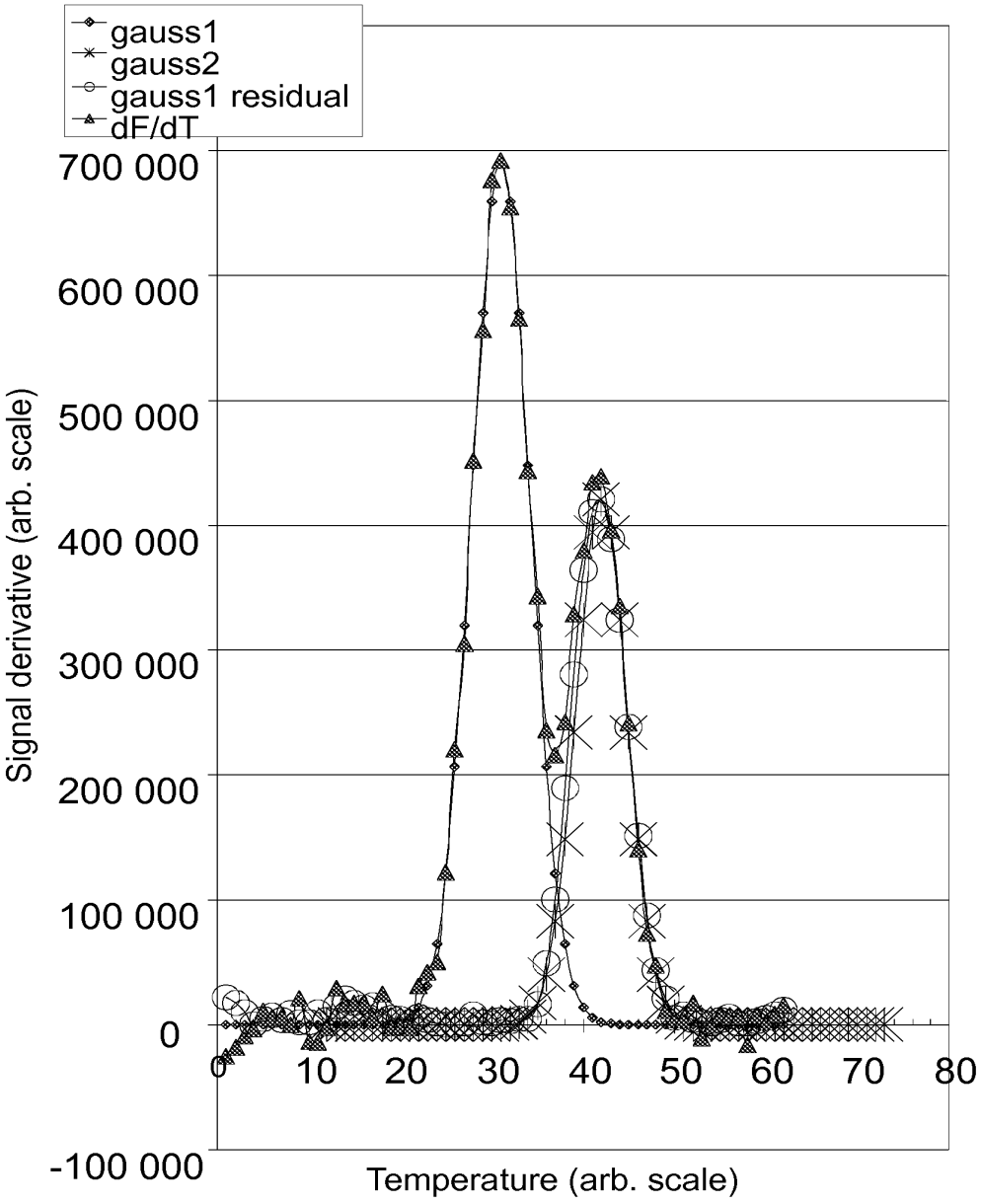

gauss1
gauss2
gauss1 residual
dF/dT
700 000
600 000
500 000
400 000
300 000
200 000
100 000
0
-100 000
Signal derivative (arb. scale)
0
10
20
30
40
50
60
70
80
Temperature (arb. scale)

Fig. 3f

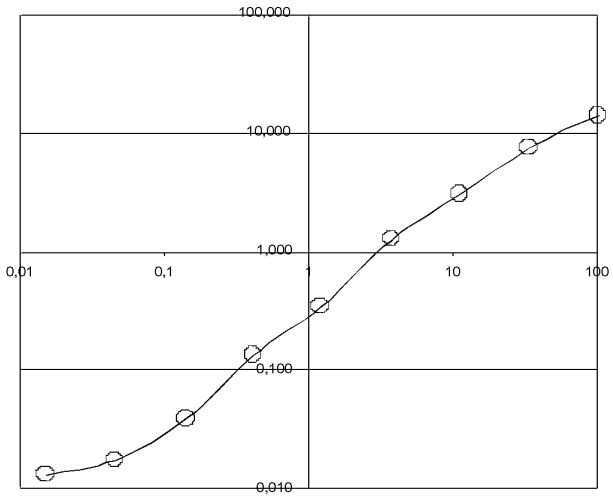


Fig. 4a

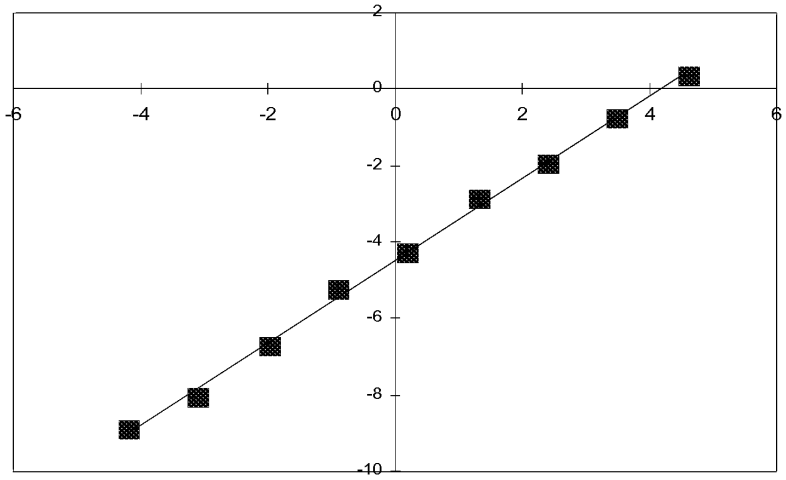


Fig. 4b

INTERNATIONAL SEARCH REPORT

International application No
PCT/FI2010/050360

A. CLASSIFICATION OF SUBJECT MATTER
INV. G06F19/00
ADD. C12Q1/68

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)
G06F

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2007/035806 A2 (UNIV UTAH RES FOUND [US]; PALAIS ROBERT ANDREW [US]; WITTWER CARL THOM) 29 March 2007 (2007-03-29) cited in the application * abstract page 5, paragraph 1 - page 7, paragraph 2 page 9, paragraph 2 - page 12, last paragraph figures 1A,1B claim 1 ----- -/--	1-23

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

[X] 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 document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 July 2010	04/08/2010

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

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/FI2010/050360

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WILHELM ET AL: "SoFAR: Software for Fully Automatic Evaluation of Real-Time PCR Data" BIOTECHNIQUES, INFORMA LIFE SCIENCES PUBLISHING, WESTBOROUGH, MA, US, vol. 34, no. 2, 1 January 2003 (2003-01-01), pages 324-332, XP003024150 ISSN: 0736-6205 the whole document	1-23

Form PCT/ISA/210 (continuation of second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/FI2010/050360

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2007035806 A2	29-03-2007	AU 2006292252 A1	29-03-2007
		CA 2623268 A1	29-03-2007
		EP 1927064 A2	04-06-2008
		JP 2009508530 T	05-03-2009
		US 2009222503 A1	03-09-2009

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61/703,093 19 September 2012 (19.09.2012) US

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

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

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

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

WO 2013/116780 A1

(54) **Title**: SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

(57) **Abstract**: This disclosure provides methods, systems, compositions, and kits for the multiplexed detection of a plurality of analytes in a sample. In some examples, this disclosure provides methods, systems, compositions, and kits wherein multiple analytes may be detected in a single sample volume by acquiring a cumulative measurement or measurements of at least one quantifiable component of a signal. In some cases, additional components of a signal, or additional signals (or components thereof) are also quantified. Each signal or component of a signal may be used to construct a coding scheme which can then be used to determine the presence or absence of any analyte.

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SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS

CROSS-REFERENCE

[0001] This application claims the benefit of U.S. provisional applications 61/594,480, filed February 3, 2012 and 61/703,093, filed September 19, 2012, each of which is incorporated herein by reference in its entirety.

BACKGROUND

[0002] Multiplexed reactions offer significant advantages over traditional uniplex reactions, including performance of parallel reactions on the same sample, use of the same chamber to perform multiple reactions, and the ability to extract rich information from a sample in a fast and efficient manner. However, to achieve these benefits, multiplexed assays generally require complex reporting mechanisms, namely spectrally resolved fluorescence or chemiluminescence (e.g., PCR, ELISA), spatially resolved signals (e.g., microarrays, gel electrophoresis), temporally resolved signals (e.g., capillary electrophoresis), or combinations thereof (e.g., Sanger sequencing). There is a need for multiplexed reactions that can be carried out in a single solution.

SUMMARY OF THE INVENTION

[0003] This disclosure provides methods, compositions, systems, and kits for the multiplexed detection of analytes. In some cases, this disclosure provides assays that are capable of unambiguously detecting the presence or absence of each of at least 7 analytes, in any combination of presence or absence, in a single sample volume without immobilization, separation, mass spectrometry, or melting curve analysis. In some examples, each of the analytes is encoded as a value of one (or at least one) component of a signal.

[0004] In some examples, an assay provided in this disclosure is capable of unambiguously detecting the presence or absence of M analytes, in any combination of presence or absence, where $M = \log_2 (F + 1)$ and F is the maximum cumulative value of one component of a signal (e.g., an intensity) when all the analytes are present.

[0005] In some cases, each analyte is encoded as at least one first value in a first component of a signal (e.g., at least one intensity or range of intensities) and at least one second value in a second component of a signal (e.g., at least one wavelength or range of wavelengths). In some examples, each analyte is encoded as a first value in a first component of a signal at each of a plurality of

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second values in a second component of a signal (e.g., a signal intensity or range of signal intensities at each of a plurality of wavelengths or ranges of wavelengths).

[0006] In some examples, an assay provided herein is capable of unambiguously detecting the presence or absence of M analytes, in any combination of presence or absence, where $M = C * \log_2 (F + 1)$, where C is the number of the second values used to encode the analytes and F is the maximum cumulative value of the first component of the signal, for any second value, when all of the analytes are present.

[0007] In some cases, an assay provided in this disclosure is capable of unambiguously detecting the presence or absence of M analytes, in any combination of presence or absence, where $M = (P * T) + 1$, where P is the number of codes per tier in a coding scheme and T is the number of tiers. In some cases, the number of tiers $T = \log_4 (F + 1)$, and F is the maximum cumulative value of a first component of a signal, for any second value, when all of the analytes are present.

[0008] In some examples, the first value is an intensity or range of intensities. In some cases, a coding scheme comprises at least three intensities or ranges of intensities.

[0009] In some cases, the second value is a wavelength or range of wavelengths. In some examples, the coding scheme comprises at least five wavelengths or ranges of wavelengths.

[0010] In some examples, the signal is an electromagnetic signal. In some cases, the electromagnetic signal is a fluorescence emission signal. In some examples, the intensity of the fluorescence emission signal is measured at at least four wavelengths or ranges of wavelengths.

[0011] In some cases, an assay provided herein is performed with reagents that are lyophilized prior to use.

[0012] In some cases, a detecting step is performed with reagents comprising hybridization probes. In some examples, the number of the hybridization probes is greater than the number of analytes. In some cases, the set of hybridization probes comprise one or more hybridization probes specific for different analytes and comprising an identical fluorophore or combination of fluorophores. In some examples, a sample is contacted with at least 18 of said hybridization probes. In some examples, a detecting step is performed with reagents comprising at least one pair of primers. In some cases, at least one pair of primers are capable of amplifying a region complementary to at least three of said hybridization probes.

[0013] In some examples, the signal that is measured is generated during a polymerase chain reaction. In some cases, the polymerase chain reaction is selected from the group consisting of an end-point polymerase chain reaction, a real-time polymerase chain reaction, a digital polymerase chain reaction, and combinations thereof.

[0014] In some examples, at least one cumulative measurement is performed on a solution.

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[0015] In some examples, at least one analyte is encoded by at least one additional value (i.e., at least two values together), wherein the at least one additional value is selected from the group consisting of a value from at least one additional component of a signal, a value from at least one component of a different signal, and combinations thereof. For example, the at least one additional value may be selected from the group consisting of a fluorescence emission intensity, a fluorescence emission wavelength, a Förster resonance energy transfer (FRET) emission intensity, a FRET emission wavelength, an electrochemical signal, a chemiluminescence wavelength, a chemiluminescence intensity, a fluorescence bleaching rate, a chemiluminescence bleaching rate, and combinations thereof.

[0016] In some cases, a chromatogram is constructed. The chromatogram may be constructed by plotting all possible combinations of first values and second values for positive control samples for each analyte.

[0017] In some examples, at least one of the analytes comprises a polynucleotide. In some cases, the polynucleotide is from a source selected from the group consisting of an animal, a plant, a bacteria, a fungus, a parasite, and a virus. In some examples, the polynucleotide is from a source selected from the group consisting of human immunodeficiency virus, herpes simplex virus, human papilloma virus, Plasmodium, Mycobacterium, dengue virus, hepatitis virus, and influenza virus. In some cases, the polynucleotide is selected from the group consisting of human immunodeficiency virus polyprotease, human immunodeficiency virus p17, human papilloma virus E6, and human papilloma virus E7.

[0018] In some cases, a sample is selected from the group consisting of a clinical sample, a food sample, an environmental sample, a pharmaceutical sample, and a sample from a consumer product.

[0019] In some examples, information concerning the presence or absence of an analyte is transmitted through a computer network.

[0020] In some cases, information concerning the presence or absence of an analyte is provided to a physician. In some examples, a clinical decision is made based on such information.

[0021] In some examples, at least one step of a method provided herein is performed using instructions on a computer readable medium. In some cases, the instructions are located on a remote server. In some examples, the instructions are located on a thermal cycler. In some cases, the instructions are located on a computer in communication with a thermal cycler.

[0022] In some cases, at least one of the analytes is a positive control analyte.

[0023] In some examples, the coding scheme is non-degenerate. In some cases, the coding scheme is designed to be non-degenerate. In some examples, the coding scheme is made non-degenerate by enumerating every legitimate result that can be obtained from the coding scheme, identifying each

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legitimate result that is degenerate, and eliminating at least one potential analyte code from the coding scheme to eliminate degeneracy.

[0024] In some cases, an assay provided herein is an end-point assay. In some examples an assay provided in this disclosure is ended at a threshold number of cycles set by the limit of detection of an instrument.

[0025] In certain examples, an assay provided herein is a liquid phase assay.

[0026] In some cases, an assay provided herein is quantitative.

[0027] In some cases, this disclosure provides a method of detecting the presence or absence of each analyte of a plurality of analytes, comprising: (a) encoding each of said analytes as a first value of a signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said first value, wherein said encoding is performed in a manner that eliminates degeneracy; (b) providing a sample comprising, or potentially comprising, at least one of said analytes; (c) contacting said sample with analyte-specific reagents that generate said first value, as represented in said coding scheme, when each of said analytes is present; (d) cumulatively measuring said first values of said signal within said sample, thereby providing a cumulative measurement; and (e) determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme.

[0028] In some examples, the method described in the preceding paragraph is capable of unambiguously detecting the presence or absence of M analytes, in any combination of presence or absence, where $M = \log_2 (F + 1)$ and F is the maximum cumulative value of the first values when all of the analytes are present. In some cases, the first value is an intensity or range of intensities. In some examples, the first value has a minimum value in the coding scheme that is selected from the group consisting of at least 1, at least 2, at least 4, at least 8, at least 16, at least 32, and at least 64. In some cases, the first value is incremented in the coding scheme by an amount equal to the cumulative maximum of said preceding first values plus one. In other cases, the first value is incremented in the coding scheme by an amount greater than the cumulative maximum of the preceding first values plus one.

[0029] In some cases, this disclosure provides a method of detecting the presence or absence of each analyte of a plurality of analytes, comprising: (a) encoding each of said analytes as at least one first value and at least one second value, wherein said first value is a value from a first component of a signal and said second value is a value from a second component of said signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said at least one first value and said at least one second value, wherein said encoding is performed in a manner that reduces or eliminates degeneracy; (b) providing a sample comprising, or potentially comprising,

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at least one of said analytes; (c) contacting said sample with analyte-specific reagents that generate said at least one first value and at least one said second value, as represented in said coding scheme, when each of said analytes is present; (d) cumulatively measuring said first values and said second values within said sample, thereby providing a cumulative measurement; and (e) determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme, wherein, when degeneracy is eliminated, said method is capable of unambiguously detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said reagents generates only one second value.

[0030] In some cases, each of the analytes of the method described in the preceding paragraph is encoded as a first value in a first component of the signal at each of a plurality of second values in a second component of the signal. In some cases, when degeneracy is eliminated, this method is capable of unambiguously detecting the presence or absence of each of at least seven analytes in a single volume using four second values. In some examples, when degeneracy is eliminated, the coding scheme comprises T non-degenerate tiers, wherein $T = \log_4 (F + 1)$ and F is the maximum cumulative value of the first component of the signal, for any second value, when all of the analytes are present. In some cases, when degeneracy is eliminated, the method is capable of unambiguously detecting the presence or absence of M analytes, in any combination of presence or absence, where $M = (P * T) + 1$ and P is the number of codes per tier. In some examples, when degeneracy is eliminated, the method is capable of unambiguously detecting the presence or absence of M analytes, in any combination of presence or absence, where $M = C * \log_2 (F + 1)$, C is the number of second values in the coding scheme, and F is the maximum cumulative value of the first component of the signal, for any second value, when all of the analytes are present.

[0031] This disclosure also provides non-degenerate coding schemes capable of unambiguously encoding the presence or absence of each of at least 7 analytes, in any combination of presence or absence, in a single sample volume without immobilization, separation, mass spectrometry, or melting curve analysis.

[0032] In some examples, a non-degenerate coding scheme of this disclosure is generated by a method comprising: (a) generating a code for each potential analyte, wherein each potential analyte is encoded by at least one value of at least one component of a signal; (b) enumerating every legitimate cumulative result for all possible combinations of presence or absence of each analyte; (c) identifying each legitimate result that is degenerate; and (d) eliminating at least one code to eliminate degeneracy. In some cases, the coding scheme may be expanded by adding additional codes that are at least one unit greater than the sum of all previous codes in the at least one component of said

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signal. In some cases a coding scheme is generated by a method of mathematical iteration that guarantees non-degeneracy by construction.

[0033] In some cases, this disclosure provides systems for detecting the presence or absence of each analyte of a plurality of analytes, comprising: (a) encoding each of said analytes as a first value of a signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said first value, wherein said encoding is performed in a manner that eliminates degeneracy; (b) providing a sample comprising, or potentially comprising, at least one of said analytes; (c) contacting said sample with analyte-specific reagents that generate said first value, as represented in said coding scheme, when each of said analytes is present; (d) cumulatively measuring said first values of said signal within said sample, thereby providing a cumulative measurement; and (e) determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme.

[0034] In some cases, this disclosure provides systems for detecting the presence or absence of each analyte of a plurality of analytes, comprising: (a) encoding each of said analytes as at least one first value and at least one second value, wherein said first value is a value from a first component of a signal and said second value is a value from a second component of said signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said at least one first value and said at least one second value, wherein said encoding is performed in a manner that reduces or eliminates degeneracy; (b) providing a sample comprising, or potentially comprising, at least one of said analytes; (c) contacting said sample with analyte-specific reagents that generate said at least one first value and said at least one second value, as represented in said coding scheme, when each of said analytes is present; (d) cumulatively measuring said first values and said second values within said sample, thereby providing a cumulative measurement; and (e) determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme, wherein, when degeneracy is eliminated, said system is capable of unambiguously detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said reagents generates only one second value.

[0035] In some cases, this disclosure provides methods of detecting the presence or absence of each analyte of a plurality of analytes for a third party, comprising: (a) obtaining the identity of each of said analytes from a party; (b) encoding each of said analytes as a first value of a signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said first value, wherein said encoding is performed in a manner that eliminates degeneracy; (c) providing said party with analyte-specific reagents that generate said first value, as represented in said coding scheme, when each of said analytes is present, said party: (i) contacting a sample

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comprising, or potentially comprising, at least one of said analytes with said reagents; and (ii) cumulatively measuring said first values within said sample, thereby providing a cumulative measurement; and (d) obtaining said cumulative measurement from said party; (e) determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme; and (f) providing said party with information about the presence or absence of each of said analytes.

[0036] In some cases, this disclosure provides methods of detecting the presence or absence of each analyte of a plurality of analytes for a third party, comprising: (a) obtaining the identity of each of said analytes from a party; (b) encoding each of said analytes as at least one first value and at least one second value, wherein said first value is a value from a first component of a signal and said second value is a value from a second component of said signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said at least one first value and said at least one second value, wherein said encoding is performed in a manner that reduces or eliminates degeneracy; (c) providing said party with analyte-specific reagents that generate said at least one first value and said at least one second value, as represented in said coding scheme, when each of said analytes is present, said party: (i) contacting a sample comprising, or potentially comprising, at least one of said analytes with said reagents; and (ii) cumulatively measuring said first values and said second values within said sample, thereby providing a cumulative measurement; and (d) obtaining said cumulative measurement from said party; (e) determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme; and (f) providing said party with information about the presence or absence of each of said analytes.

[0037] In some cases, this disclosure provides compositions for detecting the presence or absence of each analyte of a plurality of analytes, comprising analyte-specific reagents, each reagent generating a signal comprising a first value, wherein said reagents are capable of unambiguously detecting the presence or absence of each of at least seven analytes in a single sample volume, in any combination of presence or absence, without immobilization, separation, mass spectrometry, or melting curve analysis.

[0038] In some cases, this disclosure provides compositions for detecting the presence or absence of each analyte of a plurality of analytes, comprising analyte-specific reagents, each reagent generating a signal comprising at least one first value that is a value from a first component of said signal and at least one second value that is a value from a second component of said signal, wherein said reagents are capable of detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said reagents generates only one second value.

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[0039] In some cases, this disclosure provides kits for detecting the presence or absence of each analyte of a plurality of analytes, comprising analyte-specific reagents, packaging, and instructions, each reagent generating a signal comprising a first value, wherein said kit is capable of unambiguously detecting the presence or absence of each of at least seven analytes in a single sample volume, in any combination of presence or absence, without immobilization, separation, mass spectrometry, or melting curve analysis.

[0040] In some examples, this disclosure provides kits for detecting the presence or absence of each analyte of a plurality of analytes, comprising analyte-specific reagents, packaging, and instructions, each reagent generating a signal comprising at least one first value that is a value from a first component of said signal and at least one second value that is a value from a second component of said signal, wherein said kit is capable of detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said reagents generates only one second value.

[0041] In some cases, this disclosure provides kits for detecting the presence or absence of each analyte of a plurality of analytes, comprising a kit body (601), clamping slots arranged in the kit body for placing bottles, a bottle comprising probes for the detection of an analyte (602), a bottle comprising primers for amplification (603), and a bottle comprising reagents for amplification (604), wherein said kit is capable of unambiguously detecting the presence or absence of each of at least seven analytes in a single sample volume, in any combination of presence or absence, without immobilization, separation, mass spectrometry, or melting curve analysis.

[0042] In some examples, this disclosure provides kits for detecting the presence or absence of each analyte of a plurality of analytes, a kit body (601), clamping slots arranged in the kit body for placing bottles, a bottle comprising probes for the detection of an analyte (602), a bottle comprising primers for amplification (603), and a bottle comprising reagents for amplification (604), each probe generating a signal comprising at least one first value that is a value from a first component of said signal and at least one second value that is a value from a second component of said signal, wherein said kit is capable of detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said probes generates only one second value.

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INCORPORATION BY REFERENCE

[0043] All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

[0044] The novel features of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

[0045] **Fig. 1** shows a comparison between a traditional encoding method of detecting four analytes with four colors and an encoding method of the invention able to detect 16 or more sequences with four colors. Colors are indicated by B, G, Y, and R, which indicate blue, green, yellow, and red, respectively.

[0046] **Fig. 2** shows a schematic representation of detection of six analytes (including control) with four colors. The analytes are detected using hybridization probes attached to a fluorophore (B, G, Y, R; blue, green, yellow, and red, respectively) and a quencher (oval with "X"). The control sequence and the five other sequences (Dengue Fever = dengue virus; Tuberculosis = *Mycobacterium tuberculosis*; P17 = HIV p17; Malaria = *Plasmodium falciparum;* and Herpes = herpes simplex virus 2) are all detected using a probe labeled with a blue fluorophore. The non-control analytes are each detected using 1-3 additional probes. For example, the dengue virus analyte is detected using three additional probes with green, yellow, and red fluorophores; the herpes simplex virus 2 analyte is detected using one additional probe with a red fluorophore; and so on.

[0047] **Fig. 3** shows chromatograms of experimental results obtained as described in Example 2.

[0048] **Fig. 4** shows an exemplary embodiment of the invention in which a computer is used to perform one or more steps of the methods provided herein.

[0049] **Fig. 5** shows a schematic of two different methods of detecting wavelength and intensity of a light signal, such as a fluorescence emission signal.

[0050] **Fig. 6** shows a schematic of exemplary kits.

[0051] **Fig. 7** shows chromatograms of experimental results obtained as described in Example 3.

[0052] **Fig. 8** shows a schematic of a legitimate result (top) and an illegitimate result (bottom).

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DETAILED DESCRIPTION OF THE INVENTION

[0053] Fluorescence detection has been a preferred technique for multiplexed assays because of several desirable features, including compatibility with biochemical assays, the relatively small size of fluorescent labels, simple means of conjugation to molecules of interest, affordability, low toxicity, stability, robustness, detectability with inexpensive optics, and an ability to be combined with spatial arrays. However, standard fluorophores have wide emission spectra. Therefore, in order to avoid spectral overlap (i.e., to preserve spectral resolution) only a relatively small number of colors (e.g., 4 to 6) are typically used simultaneously in multiplexed fluorescent assays.

[0054] The traditional encoding method for multiplexed fluorescent assays has been to encode each analyte with a single color; i.e., M=N, where M is the number of analytes that can be detected and N is the number of spectrally resolved fluorescent probes. Whenever higher factors of multiplexing are required (i.e., M>N), fluorescence is generally combined with other techniques, such as aliquoting, spatial arraying, or sequential processing. These additional processing steps are labor-intensive and frequently require relatively expensive and complex optical and mechanical systems (e.g., spectrometers, mechanized microscopy stages, microfluidics, droplet generators, scanners, and the like). Such systems are often impractical to deploy in certain settings, particularly point-of care and low-resource settings. Thus, there is a significant need for multiplexed encoding and decoding methods that can provide an inexpensive means of multiplexing while avoiding the use of expensive additional processing steps.

[0055] This disclosure provides methods, systems, compositions, and kits for the detection of multiple analytes in a sample. Analytes are detected based on the encoding, analysis, and decoding methods presented herein. In some examples, each analyte to be detected is encoded as a value of a signal (e.g., intensity), where the values are assigned so that the results of the assay unambiguously indicate the presence or absence of the analytes being assayed. In other examples, each analyte to be detected is encoded as a value in each of at least two components of a signal (e.g., intensity and wavelength). The at least two components of a signal may be orthogonal. Similarly, as described more fully elsewhere in this disclosure, multiple orthogonal signals may be used, such as a combination of a fluorescent signal and an electrochemical signal. The analyte may be any suitable analyte, such as a polynucleotide, a protein, a small molecule, a lipid, a carbohydrate, or mixtures thereof. The signal may be any suitable signal such as an electromagnetic signal, a light signal, a fluorescence emission signal, an electrochemical signal, a chemiluminescent signal, and combinations thereof. The at least two components of the signal may be any suitable two components, such as an amplitude and a frequency or an intensity and a wavelength.

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[0056] After encoding of the analytes, a sample is provided wherein the sample comprises or may comprise at least one of the encoded analytes. The sample is contacted with an analyte-specific reagent or reagents that generate a particular signal, as specified for each analyte in the coding scheme, in the presence of an analyte. A reagent may be any suitable reagent that is capable of generating such a signal in the presence of its corresponding analyte, for example, an oligonucleotide probe attached to a fluorophore and a quencher (e.g., a TAQMAN probe). If the reagent is an oligonucleotide probe attached to a fluorophore and a quencher, a nucleic acid amplification may be performed to generate the signal.

[0057] After addition of the reagent(s), the signal is quantified. In some cases, this quantification is performed by measuring one component of the signal (e.g., fluorescence intensity) and determining the presence and absence of certain analytes based on the values used to encode the presence of each analyte and the cumulative value of the signal.

[0058] In some cases, at least two components of a signal (e.g., intensity and wavelength) are cumulatively measured for the sample. This measurement can be performed, for example, by measuring the intensity at a particular wavelength or the intensity within a particular range of wavelengths. The presence or absence of an analyte may then be determined based on the values of each of the at least two components of the signal and the values used to encode the presence of the analyte (i.e., those values in the coding scheme).

[0059] The encoding may be performed in a manner that reduces or eliminates the number of possible degenerate (e.g., ambiguous) results that can be obtained by the method. As described elsewhere in this specification, the full coding capability of a particular coding scheme may be enumerated, and certain potential analyte codes may be eliminated from the coding scheme to reduce or eliminate degeneracy. Similarly, a coding scheme may be designed to be non-degenerate, so that a reduction or elimination of degeneracy is not necessary. A decoding matrix may be constructed to translate cumulative signal measurements (e.g., intensities or intensities at particular wavelengths) into the presence or absence of certain analytes, corresponding to the constituent signals of the cumulative signal measurement.

I. Definitions

[0060] The terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

[0061] As used herein, the singular forms "a," "an," and "the" are intended to include the plural forms as well, unless the context clearly indicates otherwise. Furthermore, to the extent that the terms "including," "includes," "having," "has," "with," "such as," or variants thereof, are used in

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either the specification and/or the claims, such terms are not limiting and are intended to be inclusive in a manner similar to the term "comprising".

[0062] The term "about," as used herein, generally refers to a range that is 15% greater than or less than a stated numerical value within the context of the particular usage. For example, "about 10" would include a range from 8.5 to 11.5.

[0063] The terms "dimension" and "component," as used herein when referring to a signal, generally refer to an aspect of the signal that may be quantified. For example, if a signal is generated by a fluorescent molecule, it may be quantified in terms of its wavelength (e.g., a first dimension or component) and its intensity (e.g., a second dimension or component).

[0064] The term "decoding," as used herein, generally refers to a method of determining which analytes are present based on the cumulative signal and a coding scheme or decoding matrix that enables the conversion of a cumulative signal to information concerning the presence or absence of one or more analytes.

[0065] The term "encoding," as used herein, generally refers to the process of representing an analyte using a code comprising values of a signal, such as intensity, or values in each of at least two components of a signal or signals, such as wavelength and intensity.

[0066] The terms "oligonucleotide probe attached to a fluorophore and a quencher" and "TAQMAN probe" generally refer to hydrolysis probes used to detect the presence of an analyte in a polynucleotide amplification assay. These probes comprise an oligonucleotide probe attached to a fluorophore and a quencher. So long as the quencher and the fluorophore are in proximity, the quencher quenches the fluorescence emitted by the fluorophore upon excitation by a light source. The sequence of the oligonucleotide probe is designed to be complementary to a polynucleotide sequence present in an analyte, and therefore capable of hybridizing to the polynucleotide sequence present in the analyte. Hybridization of the oligonucleotide probe is performed in a nucleic acid amplification reaction comprising primers (e.g., a polymerase chain reaction). Upon extension of the primers by a DNA polymerase, the 5' to 3' exonuclease activity of the polymerase degrades the probe, releasing the fluorophore and the quencher into the medium. The proximity between the fluorophore and the quencher is broken and the signal from the fluorophore is no longer quenched. Thus, the amount of fluorescence detected is a function of the amount of analyte present. If no analyte is present, the probe will not hybridize to an analyte, and the fluorophore and quencher will remain in close proximity. Little or no signal will be produced.

[0067] The term "orthogonal," as used herein, generally refers to at least two components of a signal (e.g., wavelength and intensity), or at least two different signals (e.g., fluorescence emission and electrochemical signal), that can be varied independently or approximately independently. For

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example, wavelength and intensity are considered orthogonal or approximately orthogonal when fluorescent molecules are used. Among other factors, the wavelength of fluorescence emission will depend on the composition of the fluorescent molecule and the intensity of the fluorescence will depend on the amount of molecule present. Although wavelength and intensity are examples of two components of a signal that can be varied approximately independently, the methods described herein are not limited to components that can be varied independently or approximately independently. Components of a signal, or signals, that vary non-independently may also be used, so long as the components or signals are characterized well enough to enable encoding, measurement, and decoding. For example, if the variance in one component or signal affects the variance in another component or signal, the two components or signals may still be used so long as the relationship between the variances is understood.

[0068] The terms "polynucleotide," "oligonucleotide," or "nucleic acid," as used herein, are used herein to refer to biological molecules comprising a plurality of nucleotides. Exemplary polynucleotides include deoxyribonucleic acids, ribonucleic acids, and synthetic analogues thereof, including peptide nucleic acids.

[0069] The term "probe," as used herein, generally refers to a reagent capable of generating a signal in the presence of a particular analyte. A probe generally has at least two portions: a portion capable of specifically recognizing an analyte, or a portion thereof, and a portion capable of generating a signal in the presence of an analyte, or a portion thereof. A probe may be an oligonucleotide probe attached to a fluorophore and a quencher, as described above and elsewhere in this disclosure. A probe may also be any reagent that generates a signal in the presence of an analyte, such as an antibody that detects an analyte, with a fluorescent label that emits or is quenched upon binding of the antibody to an analyte. Any suitable probe may be used with the methods presented in this disclosure, so long as the probe generates a quantifiable signal in the presence of an analyte. For example, the analyte-specific portion of a probe may be coupled to an enzyme that, in the presence of an analyte, converts an uncharged substrate into a charged product, thereby increasing the electrical conductivity in the medium over time. In this case, different analytes may be encoded by coupling the analyte-specific portion of the probe (e.g., hybridization probe or antibody) to an enzyme at different ratios. The resulting rate of increased conductivity in the medium will be cumulative for all analytes present in the medium. Encoding analytes according to the methods provided herein enables conversion of the conductivity measurements into unambiguous (i.e., non-degenerate) results providing information about the presence or absence of particular analytes. Similarly, a probe may comprise an enzyme producing a chemiluminescent product from a substrate. The amount of chemiluminescence may then be used to encode the presence of particular analytes.

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II. Encoding and Decoding Methods

A. Traditional Fluorescent Encoding and Decoding Method

[0070] A commonly used method of determining the presence of an analyte uses four spectrally resolved fluorescent molecules to indicate the presence or absence of four analytes. An example of this method is presented on the left-hand side of **Fig. 1**. The left-hand side of **Fig. 1** shows an encoding method where four analytes (Seq 1, Seq 2, Seq 3, and Seq 4) are each encoded by a single color (blue, green, yellow, and red, respectively). The color represents a fluorophore attached to an oligonucleotide probe that also comprises a quencher. In the system shown on the left-hand side of **Fig. 1**, there are four different oligonucleotide probes, each comprising a single fluorophore (blue, green, yellow, or red) and a quencher. The presence or absence of an analyte is determined based on the presence or absence of a signal in a particular color.

[0071] The chart on the left-hand side of **Fig. 1** shows intensity versus color for a hypothetical sample containing two analytes: Seq 1 and Seq 3. The presence of these analytes is determined based on the measurement of a blue signal (corresponding to Seq 1) and a yellow signal (corresponding to Seq 3). The absence of Seq 2 and Seq 4 is indicated by the absence of a blue and red signal.

[0072] Table 1 shows a translation of this coding scheme into a binary format. Each analyte is encoded as a value in each of two components of a fluorescent signal: (1) color (also known as wavelength; or range of wavelengths) and (2) intensity (indicated by the numbers within the table). For example, A (e.g., Seq 1) has a color of blue and an intensity of 1; B (e.g., Seq 2) has a color of green and an intensity of 1; and so on. The intensity of the signal within each color range may be quantified as described herein, for example by measuring the intensity of the signal within a particular wavelength range determined by a band pass filter. A result of 1000 indicates that only analyte A is present; a result of 1100 indicates that analytes A and B are present; and so on.

Table 1. Traditional encoding of four analytes with four probes, each probe having a single color, and one probe per analyte.

	Blue	Green	Yellow	Red
A	1	0	0	0
B	0	1	0	0
C	0	0	1	0
D	0	0	0	1

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B. Encoding Methods Using More Than One Color Per Analyte

[0073] The traditional method described above suffers from the fact that it is limited by the number of spectrally resolvable fluorophores. More specifically, the number of detectable analytes is equal to the number of spectrally resolvable fluorophores. Therefore, the number of analytes may only be increased by increasing the number of spectrally resolvable fluorophores.

[0074] This disclosure provides methods that overcome this limitation. More specifically, in some cases, by utilizing at least two components of a signal during encoding, the methods described herein may be used to detect more than one analyte per fluorophore. For example, using the method provided herein 1.5, 1.6, 1.7, 1.8, 1.9, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 analytes may be detected per fluorophore. In some cases, the methods provided herein may be used to detect at least 1.5, 1.6, 1.7, 1.8, 1.9, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 analytes may be detected per fluorophore. In some cases, the methods provided herein may be used to detect 1.5-2, 2-4, 1.5-4, 4-6, 2-6, 6-10 analytes per fluorophore.

[0075] In some cases, the methods provided in this disclosure may include the use of a control color. The control color may be attached to one or more probes binding a positive control analyte, and each analyte to be detected, in a sample. If the same sequence occurs in the positive control analyte and each analyte to be detected, a single control probe may be used. If the same sequence does not occur in the positive control analyte and each analyte to be detected, different probes may be used, but each probe may still be attached to the control color.

[0076] For example, building on the traditional methods described above, one color (e.g., blue) may be used to encode the presence of a control analyte that is always present in the sample. The control analyte may be added to the sample, or may be inherently present in the sample. The remaining colors (e.g., green, yellow, red) may be used to encode the presence of additional analytes. Table 2 shows an example of one such method.

Table 2. Encoding of four analytes, including control, with four colors and up to two colors per analyte.

	Blue	Green	Yellow	Red
Ctrl. (p)	1	0	0	0
A	1	0	0	1
B	1	0	1	0
C	1	1	0	0

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[0077] In Table 2, the presence of the control (p) is indicated by a result of 1000. The presence of the control and analyte A is indicated by a result of 2001. The presence of the control and all three other analytes is indicated by a result of 4111. The control color (blue) provides an indication that the assay is functioning properly. The intensity of the blue color reports the number of analytes that are present in a test sample. Of course, any color may be used as the control color. One of skill in the art will recognize that certain practical considerations might make it preferable to use one color over another as the control color. For example, if one color is better detected in a particular optical system (or system of fluorophores), it might be practically preferable to use that color as a control color.

[0078] The coding scheme shown in Table 2 encodes each analyte using one control color and one additional color (up to 2 probes per analyte). However, as shown below, the number of analytes that can be encoded increases when up to 4 colors are used per analyte. Table 3 shows an exemplary coding scheme where each analyte is encoded by up to 4 colors. In the scheme shown in Table 3, the control color (blue) is generated in the presence of the control sequence and each of the other seven analytes (A-G). The other seven analytes are each encoded by the presence of one to three additional colors. The colors may be contained on different probes (e.g., oligonucleotide probes attached to a fluorophore and a quencher) or on the same probe (which can have multiple fluorophores and multiple quenchers).

Table 3. Encoding of eight analytes (including control) with four colors and up to four colors per analyte.

	Blue	Green	Yellow	Red
Ctrl. (p)	1	0	0	0
A	1	0	0	1
B	1	0	1	0
C	1	1	0	0
D	1	0	1	1
E	1	1	0	1
F	1	1	1	0
G	1	1	1	1

[0079] In Table 3, the presence of the control and all seven other analytes is indicated by a result of 8444. Three analytes (A, B, and C) are encoded by two colors. Three analytes (D, E, and F) are encoded by three colors, and one analyte (G) is encoded by four colors. The presence of the control (p) is indicated by a result of 1000. The presence of the control and analyte A is indicated by a result of 2001. The presence of the control and analyte G is indicated by a result of 2111. These results express the cumulative intensity of the signal in each color. For example, the result 2111 has a 2X

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signal intensity in the blue channel, while the result 1000 has only a 1X signal intensity in the blue channel.

[0080] Using Table 3, each of the possible cumulative assay results can be enumerated, in terms of color (blue, green, yellow, red) and intensity (0-8). Table 4 shows a "decoding matrix" generated by enumerating each of the possible cumulative assay results based on the encoding method presented in Table 3 and providing the corresponding decoded result of each assay in terms of the analyte(s) present in the sample. A similar decoding matrix may be generated for any coding scheme described herein, by enumerating each of the possible cumulative assay results based on the coding scheme and providing the corresponding decoded result of each assay in terms of the analyte(s) present in (or absent from) the sample. In some cases, as described below, one or more analytes may be removed from the coding scheme in order to reduce or eliminate degeneracy.

Table 4. Decoding matrix for encoding method presented in Table 3.

Cumulative Assay Result				
Blue	**Green**	**Yellow**	**Red**	**Analyte(s) Present**
1	0	0	0	p
2	0	0	1	pA
2	0	1	0	pB
2	1	0	0	pC
2	0	1	1	pD
2	1	0	1	pE
2	1	1	0	pF
2	1	1	1	pG
3	0	1	1	pAB
3	1	0	1	pAC
3	0	1	2	pAD
3	1	0	2	pAE
3	1	1	1	pAF, pCD, pBE
3	1	1	2	pAG, pDE
3	1	1	0	pBC
3	0	2	1	pBD
3	1	2	0	pBF
3	1	2	1	pBG, pDF
3	2	0	1	pCE
3	2	1	0	pCF
3	2	1	1	pCG, pEF
3	1	2	2	pDG
3	2	1	2	pEG
3	2	2	1	pFG
4	1	1	1	pACB
4	0	2	2	pABD
4	2	2	0	pBCF
4	2	0	2	pACE
4	1	1	2	pABE, pACD

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4	1	2	1	pABF, pBCD
4	2	1	1	pACF, pBCE
4	1	2	2	pADF, pABG, pBDE
4	2	1	2	pACG, pCDE, pAEF
4	2	2	1	pBCG, pBEF, pCDF
4	2	2	2	pCDG, pAFG, pBEG, pDEF
4	1	1	3	pADE
4	1	3	1	pBDF
4	3	1	1	pCEF
4	1	2	3	pADG
4	3	2	1	pCFG
4	2	1	3	pAEG
4	2	3	1	pBFG
4	1	3	2	pBDG
4	3	1	2	pCEG
4	2	2	3	pDEG
4	3	2	2	pEFG
5	1	2	2	pABCD
5	2	1	2	pABCE
5	2	2	1	pABCF
5	2	2	2	pABCG, pABEF, pBCDE, pACDF
5	1	2	3	pABDE
5	2	1	3	pACDE
5	1	3	2	pABDF
5	2	1	3	pBCDF
5	3	1	2	pACEF
5	3	2	1	pBCEF
5	2	2	3	pABEG, pACDG, pADEF
5	2	3	2	pABFG, pBCDG, pBDEF
5	3	2	2	pACFG, pBCEG, pCDEF
5	1	3	3	pABDG
5	3	1	3	pACEG
5	3	3	1	pBCFG
5	2	3	3	pADFG, pBDEG
5	3	3	2	pBEFG, pDEFG
5	3	2	3	pCDEG, pAEFG
5	2	2	4	pADEG
5	2	4	2	pBDFG
5	4	2	2	pCEFG
5	3	3	3	pDEFG
6	2	2	3	pABCDE
6	2	3	2	pABCDF
6	3	2	2	pABCEF
6	2	3	3	pABCDG, pABDEF
6	3	2	3	pABCEG, pACDEF
6	3	3	2	pABCFG, pBCDEF
6	4	3	2	pBCEFG
6	4	2	3	pACEFG
6	3	3	3	pABEFG, pACDFG, pBCDEG

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6	3	4	2	pBCDFG
6	2	4	3	pABDFG
6	3	2	4	pACDEG
6	2	3	4	pABDEG
6	4	3	3	pCDEFG
6	3	4	3	pBDEFG
6	3	3	4	pADEFG
7	4	4	3	pBCDEFG
7	4	3	4	pACDEFG
7	3	4	4	pABDEFG
7	4	3	3	pABCEFG
7	3	4	3	pABCDFG
7	3	3	4	pABCDEG
7	3	3	3	pABCDEF
8	4	4	4	pABCDEFG

[0081] The decoding matrix provided in Table 4 is constructed using two assumptions. First, the decoding matrix assumes that the positive control (p) always produces a positive outcome. Second, the decoding matrix assumes that, within each color, the intensity is additive and scales in the same way with changing probe concentration, regardless of which probe the signal may come from. This essentially means that the signals are additive and digital. The conditions underlying these assumptions may be met by properly preparing the assay. If a fluorescent signal is used, the intensity need only be approximately additive and digital, as demonstrated in the Examples provided herein.

[0082] Table 4 allows the conversion of a cumulative measurement of intensity in four ranges of fluorescent wavelengths (i.e., signal intensity within each color range), into the corresponding analytes present in the sample. For example, a result of 4321 indicates that p, C, F, and G are present and the other analytes are not present. This result is referred to as a "legitimate" result, because it is present in the decoding matrix. By contrast, a result of 4000, while possible to measure, does not occur in the decoding matrix. More specifically, the result of 4000 cannot be achieved by adding any combination of control and analyte codes from Table 3. This result is referred to as an "illegitimate" result. An illegitimate result may indicate that the assay malfunctioned. Thus, the control (p) and the decoding matrix provide a means of verifying that the assay is functioning properly.

[0083] Table 4 is exhaustive for any combination of fluorophores generating four resolvable emission spectra (e.g., colors). The term "rank" is used to describe the number of detected analytes, including the control. In the example provided above, if the assay functions properly, the rank is equal to the value of the blue signal. For example, a rank of 8 indicates that the control and all seven other analytes (A-G) are present. A rank of 2 indicates that the control and only one analyte are

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present. The lowest rank is a rank of 1, which indicates that only the control is present. The number of possibilities at each rank can be enumerated. For example, continuing to refer to the encoding and decoding method described in Tables 3-4, there are 7 possibilities for rank 2. The number of possibilities at rank 3 can be calculated as a combination of 7 take 2, or $7!/(5! * 2!) = 21$ possibilities. More generally, the number of possibilities for a combination of N take K is $N!/((N-K)! * K!)$. Analogously, at ranks 4, 5, 6, 7, and 8, the number of possibilities is 35, 35, 21, 7, and 1, respectively. Referring to Table 4 shows that the table agrees with the theoretical prediction. Thus, Table 4 is an exhaustive decoding matrix for the encoding method provided in Table 3.

C. Reducing or Eliminating Degeneracy

[0084] The terms "degenerate" and "degeneracy," as used herein, generally describe a situation where a legitimate result is not definitive, because it can indicate more than one possibility in terms of the presence or absence of an analyte. For example, with reference to Table 4, result 5233 is degenerate because it can be decoded as either pADFG or pBDEG. Similarly, result 4222 is degenerate because it can be decoded as any of the following: pCDG, pAFG, pBEG, pDEF. By contrast, result 3110 can only indicate pBC and thus is not degenerate.

[0085] This disclosure provides methods of reducing or eliminating degeneracy, thereby increasing the confidence with which an analyte is detected. In one embodiment, degeneracy is eliminated by a method comprising (i) encoding each potential analyte to be detected as a value of a signal and, optionally, as a value in each of at least two components of a signal; (ii) enumerating every legitimate result that can be obtained from the coding scheme; (iii) identifying each legitimate result that is degenerate; and (iv) eliminating at least one potential analyte (or potential analyte code) from the coding scheme, wherein eliminating the at least one potential analyte reduces or eliminates degeneracy. For example, with reference to the coding scheme described in Table 3 and the decoding matrix described in Table 4 (enumerating every legitimate result), eliminating any two of analytes D, E, and F eliminates the degeneracy. Eliminating any one of analytes D, E, and F would not eliminate the degeneracy, but would reduce it.

[0086] With continued reference to the coding scheme described in Table 3, eliminating any two of analytes D, E, and F from the coding scheme results in a scheme where six analytes (including control) can be analyzed, with no degeneracy, using only 4 colors. By contrast, conventional methods of multiplexing would allow for only the reporting of 3 analytes and 1 control using 4 colors. Therefore, the number of analytes that can be analyzed is nearly doubled by using the methods provided herein.

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[0087] **Fig. 2** shows an exemplary embodiment of the invention in which 4 colors are used to detect five analytes and a control. The analytes are nucleic acids from dengue virus ("Dengue Fever"), *Mycobacterium tuberculosis* ("Tuberculosis"), human immunodeficiency virus (HIV) p17 (P17), *Plasmodium* ("Malaria"), and herpes simplex ("Herpes"). The "colors" in this example are fluorophores attached to oligonucleotide probes that also comprise a quencher. The oligonucleotide probes will generally be different for different analytes, whereas the color is the same. For example, all probes designated as Probe 1 have a blue color but generally will have a different oligonucleotide sequence. Of course, probes designated as Probe 1 could also have the same oligonucleotides sequence, if the complementary sequence was present in each of the analytes. The mechanism of detection with these probes is described elsewhere in this disclosure. Probe 1 (blue) hybridizes to all six analytes, including control. Probe 2 (green) hybridizes to analytes from dengue virus, *Mycobacterium*, and HIV p17. Probe 3 (yellow) hybridizes to analytes from dengue fever and *Plasmodium*. Probe 4 hybridizes to analytes from dengue virus, *Mycobacterium*, and herpes simplex. The coding scheme defined by these probes is illustrated in Table 5. With reference to Table 3, potential analytes D (1011) and F (1110) have been eliminated from the coding scheme. Therefore, the coding scheme presented in Table 5 is non-degenerate and each legitimate result from the assay corresponds to the presence or absence of a unique combination of analytes in a sample.

Table 5. Coding scheme for detection of dengue virus, *Mycobacterium*, HIV, *Plasmodium*, and herpes simplex, as exemplified in Fig. 2.

	Blue	Green	Yellow	Red
Ctrl. (p)	1	0	0	0
Herpes Simplex	1	0	0	1
Plasmodium	1	0	1	0
HIV	1	1	0	0
Mycobacterium	1	1	0	1
Dengue Virus	1	1	1	1

[0088] The methods for encoding and decoding presented above, including the methods for eliminating degeneracy, are all equally applicable to encoding methods using additional colors. For example, Table 3 could be extended by including additional colors and additional intensities (described further below). The decoding matrix is then generated as described above, enumerating every legitimate result that can be obtained from the coding scheme. A decoding matrix analogous to the decoding matrix provided in Table 4 may be constructed for any coding scheme described in this disclosure. The legitimate results that are degenerate are then identified and at least one

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potential analyte code is eliminated from the coding scheme to reduce or eliminate degeneracy. The method of eliminating degeneracy may be carried out using software on a computer readable medium, or hardware configured to carry out the method (e.g., a microchip).

[0089] Although degeneracy can be reduced or eliminated by the methods described above, and elsewhere in this disclosure, this disclosure also provides coding schemes that are non-degenerate by design. For example, after elimination of degeneracy in the coding scheme described in Table 3, the coding scheme may be extended indefinitely in a non-degenerate manner where the non-degeneracy is by design (see, e.g., the coding scheme exemplified in Table 6, described more fully below). Similarly, this disclosure provides coding schemes that are completely non-degenerate by design and therefore do not require any reduction or elimination of degeneracy (see, e.g., the coding scheme exemplified in Table 8, described more fully below). Thus, the coding schemes provided in this disclosure may have reduced or eliminated degeneracy, or be non-degenerate by design.

D. Encoding Methods Using More Than One Color and More Than One Intensity

[0090] The encoding method described above (e.g., Tables 3-5) may be further extended by allowing analytes to be encoded by an intensity greater than 1. For example, each of the analytes encoded in the coding scheme provided in Table 5 is encoded by a fluorescence intensity of either 1 or 0. Allowing higher values for the signal intensity in at least one color further increases the number of analytes that can be encoded by any of the methods provided in this disclosure. In some examples, these higher intensity values may be assigned any color except for the control color, in order to maintain the analyte counting capability of the control color.

[0091] Table 6 shows the first three tiers of an exemplary coding scheme that utilizes four colors and multiple intensities. Tier 1 of Table 6 is a reproduction of Table 3, showing the encoding of seven analytes and a control with four colors. As described above, any two of potential analytes D, E, and F may be eliminated from the coding scheme in order to produce a non-degenerate coding scheme. Tier 1 of Table 6 indicates that potential analytes D (1011) and F (1110) have been eliminated from the coding scheme to eliminate degeneracy. Therefore, the coding scheme presented in Tier 1 of Table 6 is capable of determining the presence of five analytes and one control using four colors, as described above.

[0092] The coding scheme of Tier 1 of Table 6 may be expanded to a second tier (Tier 2) by allowing the intensity in any of the colors to increase. As described above, the intensity of the control color may be maintained at 1, in order to preserve the sequence counting capability of the control. Increasing the intensity of any of the remaining three colors will yield codes 100Y, 10Y0, 1Y00, 10YY, 1Y0Y, 1YY0, and 1YYY, where $Y > 1$. The minimal value of Y for a new tier of

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encoding is equal to the cumulative maximum value from the prior tier(s) plus 1. As described below, a value greater than 1 could also be used, to maximize the differences between the intensities.

[0093] Thus, in this context, the term "tier" is generally used to describe a set of codes that fully utilize the coding capability provided by a particular number of first values (e.g., intensities) and second values (e.g., colors), without degeneracy. For example, Tier 1 of Table 6 fully utilizes the coding capability provided by four colors with up to one intensity in each color, without degeneracy. As shown in Table 6, Tier 1, this results in six encoded analytes, including the control. To introduce a second tier, the minimum value of Y (described above) may be incremented to equal the cumulative maximum result from the prior tier(s) plus one (or more than one). In the example provided in Table 6, the intensity of the blue (control) color is maintained as one, to preserve the sequence counting capability in this color. Thus, Tier 2 consists of five non-degenerate encoding possibilities obtained by incrementing the intensities in the green, yellow, and red channels to equal the cumulative maximum results in each of these channels from Tier 1, plus one. All possibilities of these codes may then be enumerated for Tier 2, and codes resulting in degeneracy may be eliminated, or Tier 2 may be made non-degenerate by design, using the information used to eliminate the corresponding codes from Tier 1. Further coding capacity may then be achieved by adding a third tier, or further tiers, which are constructed according to analogous methods. A coding scheme may have an infinite number of tiers, for example 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100 or more tiers.

[0094] More specifically, with reference to Table 6, Tier 2, analyte H is encoded by 1004. The value of the control color is maintained as 1. The value of the red color is equal to the cumulative maximum result from the prior tier (3) plus 1, or 4. Similarly, analytes I and J are encoded by 1030 and 1400, respectively. Combinations of these codes are used to encode the remaining four analytes (K-N), as was done for analytes D-G in Tier 1. This completes Tier 2. In Tier 2, the inclusion of potential analytes K (1034) and M (1430) results in degeneracy. Therefore, these analytes have been eliminated from the coding scheme, to eliminate degeneracy.

[0095] Continuing to refer to Table 6, a third tier (Tier 3) is constructed using the same principles described above. Analyte O is encoded by 1-0-0-16. The value of the control color is still maintained as 1. The value of the red color is equal to the cumulative maximum result from the prior tiers (15) plus 1, or 16. Similarly, analytes P and Q are encoded by 1-0-9-0 and 1-16-0-0, respectively. Combinations of these codes are used to encode the remaining four analytes (R-U), as was done for analytes D-G in Tier 1 and analytes K-N in Tier 2. In Tier 3, the inclusion of potential

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analytes R (1-0-9-16) and T (1-16-9-0) results in degeneracy. Therefore, these analytes have been eliminated from the coding scheme, to eliminate degeneracy.

[0096] The three-tier coding scheme shown in Table 6 shows the encoding of 15 analytes and one control using four colors. This coding scheme may be indefinitely extended, by adding more intensities to generate additional tiers and/or adding more colors, to generate additional coding capability within the tiers. The methods of reducing or eliminating degeneracy, as described in this disclosure, may be used with the addition of each intensity and/or color, to reduce or eliminate degenerate results.

[0097] More generally, the coding scheme depicted in Table 6 is a non-degenerate, infinite extension of the coding scheme depicted in Tables 3-5. The maximum intensity of the cumulative measurement at the first tier is 6. The maximum intensity of the cumulative measurement at the second tier is 15. The maximum intensity of the cumulative measurement at the third tier is 63, and so on. Given a maximum cumulative intensity value (F), the maximum number of tiers (T) available in this coding scheme is $T = \log_4 (F + 1)$. The coding scheme depicted in Table 6 provides five non-degenerate codes per tier (P). Thus, the maximum number of codes $M = 5 * \log_4 (F + 1)$, or $M = P * T$, where P is the number of non-degenerate codes per tier and T is the number of tiers. For example, given $F = 63$, the maximum number of codes (i.e., analytes) is 15 for 5 non-degenerate codes per tier. This formula does not include the 1000 code, which is reserved for a positive control in Table 6. To include the control in the total number of analytes, one would simply add one, to provide the formula $M = (P * T) + 1$.

[0098] The methods provided in this disclosure may be used to expand this coding scheme infinitely. For example, by utilizing combinations of different intensities (i.e., first values) and colors (i.e., second values) one can encode any number of analytes (M) by varying the number of non-degenerate codes per tier (P) and the number of tiers (T). For example, the number of non-degenerate codes per tier is 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100, or more. Similarly, the number of tiers may be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100, or more.

Table 6. Encoding of 15 analytes and one control using four colors and multiple intensities.

Tier	Analyte	B	G	Y	R	Comments
1	Ctrl. (p)	1	0	0	0	
	A	1	0	0	1	
	B	1	0	1	0	

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	C	1	1	0	0	
	D	1	0	1	1	Eliminated from coding scheme to eliminate degeneracy.
	E	1	1	0	1	
	F	1	1	1	0	Eliminated from coding scheme to eliminate degeneracy.
	G	1	1	1	1	
Cumulative Maximum Result		*6*	*3*	*2*	*3*	
2	**H**	1	0	0	4	
	I	1	0	3	0	
	J	1	4	0	0	
	K	1	0	3	4	Eliminated from coding scheme to eliminate degeneracy.
	L	1	4	0	4	
	M	1	4	3	0	Eliminated from coding scheme to eliminate degeneracy.
	N	1	4	3	4	
Cumulative Maximum Result		*11*	*15*	*8*	*15*	
3	**O**	1	0	0	16	
	P	1	0	9	0	
	Q	1	16	0	0	
	R	1	0	9	16	Eliminated from coding scheme to eliminate degeneracy.
	S	1	16	0	16	
	T	1	16	9	0	Eliminated from coding scheme to eliminate degeneracy.
	U	1	16	9	16	
Cumulative Maximum Result						
Cumulative Maximum Result for Three Non-Degenerate Tiers		***16***	***63***	***26***	***63***	

[0099] The right-hand side of **Fig. 1** shows one exemplary embodiment of the method described above. More specifically, the right-hand side of **Fig. 1** shows an encoding method where nine or more analytes (Seq 1 - Seq 9, etc.) are each encoded by at least two colors, with varying intensities within each color. The color represents a fluorophore attached to an oligonucleotide probe that also comprises a quencher. The system can be designed so that each probe is labeled with single

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fluorophore, or each probe is labeled with more than one fluorophore. For example, the code for analyte H, in Table 6, is 1004. The intensity of 4 in the red channel may be achieved by either using an H-specific probe comprising 4 red fluorophores, or by using 4 H-specific probes each comprising a single red fluorophore. Of course, any combination of probes and fluorophores producing a result of 4 in the red channel would be equally appropriate, such as 2 probes with 2 red fluorophores each, and 1 probe with 1 red fluorophore and 1 probe with 3 red fluorophores, or simply one probe with one red fluorophore but present at 4x amount in the reaction mixture.

[00100] The coding scheme depicted on the right-hand side of **Fig. 1** may be represented as in Table 7. The result of the analysis shown on the right-hand side of **Fig. 1** is 4112, or an intensity of 4 in the blue channel, 1 in the green channel, 1 in the yellow channel, and 2 in the red channel. Using a decoding matrix constructed as described herein, this result is decoded to indicate the presence of Seq 1, Seq 3, Seq 4, and Seq 5.

Table 7. Coding scheme illustrated on the right-hand side of Fig. 1.

Tier	Analyte	B	G	Y	R	Comments
1	**Seq 1**	1	0	0	0	
	Seq 2	1	1	0	0	
	Seq 3	1	0	1	0	
	Seq 4	1	0	0	1	
	Seq 5	1	1	0	1	
	Seq 6	1	1	1	1	
	~~Eliminated Potential Seq~~	~~1~~	~~0~~	~~1~~	~~1~~	Eliminated from coding scheme to eliminate degeneracy.
	~~Eliminated Potential Seq~~	~~1~~	~~1~~	~~1~~	~~0~~	Eliminated from coding scheme to eliminate degeneracy.
Cumulative Maximum Result		*6*	*3*	*2*	*3*	
2	**Seq 7**	1	4	0	0	
	Seq 8	1	0	3	0	
	Seq 9	1	0	0	4	
Etc. - i.e. Continue as provided in Table 6.						

E. Encoding Methods Using One Color and One Intensity Per Analyte but Different Intensities Among Analytes

[00101] In some methods provided herein, each analyte is encoded by a single color and intensity combination. For example, in a four color system, the first four analytes may be encoded

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by 1000, 2000, 4000, and 8000. The next four analytes may be encoded by 0100, 0200, 0400, and 0800. Analytes 9-12 and 13-16 would be assigned analogously, as shown in Table 8.

[00102] Like the encoding method described in Table 6, this coding scheme is theoretically infinite, non-degenerate by construction, and only limited by the bandwidth of the instrument used to measure the signal. However, this coding scheme enables more analytes to be quantified per unit of bandwidth than the encoding method described in Table 6. The reason is that the available multiplicity of signal is used with maximal efficiency, as each level of intensity is utilized in the coding (i.e. there are no gaps in the chromatogram; see below for description of chromatograms). Table 8 shows one embodiment of this method, illustrating four tiers of encoding based on four colors and intensities 1, 2, 4, and 8. The coding scheme is non-degenerate, and the result if all 16 analytes are present is 15-15-15-15. This encoding method is more efficient, in terms of bandwidth utilization, than the encoding methods presented above. However, this method does not have the proofreading capability of the first scheme, as all the results decode to legitimate outcomes in the absence of gaps in the chromatogram.

Table 8. Example of encoding method using one color and one intensity per analyte, but different intensities among analytes.

Tier	Analyte	B	G	Y	R
1	**A**	1	0	0	0
	B	2	0	0	0
	C	4	0	0	0
	D	8	0	0	0
2	**E**	0	1	0	0
	F	0	2	0	0
	G	0	4	0	0
	H	0	8	0	0
3	**I**	0	0	1	0
	J	0	0	2	0
	K	0	0	4	0
	L	0	0	8	0
4	**M**	0	0	0	1
	N	0	0	0	2
	O	0	0	0	4
	P	0	0	0	8
		15	***15***	***15***	***15***

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[00103] The method presented above, and exemplified in Table 8, may be extended by introducing additional colors and/or intensities. For example, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100, or more colors may be used. Additional intensity levels, such as 16, 32, 64, 128, 255, 512, 1024, 2048, 4096, 8192, 16384, 32768, 65536, and so on may also be used. The method is generalizable and may be used to encode an infinite number of analytes. Each analyte is represented by a code in a single color, wherein the value of the code in that single color equal to the sum of all previous values plus one. For example, if the first code contains a 1 in a particular color, the next codes are 2, 4, 8, 16, 32, 64, 128 and so on. Other progressions are possible, but will not be as efficient in terms of usage of the bandwidth. However, one of ordinary skill in the art will recognize that less efficient use of bandwidth may be desirable when one wishes to maximize separation between values. Similarly, the value 1 could be excluded from the coding scheme, for example to maximize the difference in the intensity between the first encoded value and instrumental noise. For example, in some cases a coding scheme may begin with a value of 2, which would provide a progression of 2, 4, 8, 16, 32, 64, 128, and so on for the analyte codes, while the progression of cumulative results would be 2, 4, 6, 8, 10, 12, 14, 16, and so on. This approach generates uniform gaps in the possible cumulative results, allowing for higher tolerance to noise in comparison to an analyte code progression of 1, 2, 4, 8, 16, 32, and so on, and its corresponding cumulative result progression of 1, 2, 3, 4, 5, 6, 7, 8, and so on. In this example, the increased robustness in the measurement comes at a cost of a decrease in the number of available codes within a fixed bandwidth of multiplicity. For example, if only 63 multiplicity states can be measured reliably, the coding scheme starting with 1 offers 7 codes per color whereas the coding scheme starting with 2 offers 6 codes per color. If 4 colors are available, the former will offer 28 codes, while the latter will offer 24 codes. In summary, both the starting signal intensity and the progression (i.e., difference between intensity values) may be scaled in order to maximize the ability to distinguish over instrumental noise and maximize the differences between the intensity values, thereby enhancing the ability to distinguish between distinct experimental outcomes.

[00104] The coding scheme illustrated in Table 8 is non-degenerate by design. Although the coding scheme illustrated in Table 8 uses both intensity and color to encode each of the 16 analytes, each of the analytes could also be encoded by simply utilizing intensity. For example, given the 16 analytes provided in Table 8 (A-P), a single color coding scheme encoding all 16 analytes may assign the values 1, 2, 4, 8, 16, 32, 64, 128, 256, 512, 1024, 2048, 4096, 8192, and 16384 to analytes A, B, C, D, E, F, G, H, I, J, K, L, M, N, O, and P, respectively. Such a coding scheme is non-degenerate by design and the cumulative intensity result can be unambiguously decoded to indicate

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the presence or absence of analytes A-P. This coding scheme is capable of detecting the presence or absence of M analytes, where $M = \log_2 (F + 1)$ and F is the maximum cumulative value of a signal (e.g., signal intensity). By adding a second component to this signal (e.g., color, as depicted in Table 8), the capacity of this coding scheme can be increased to $M = C * \log_2 (F + 1)$, where C is the number of colors used in said coding scheme.

[00105] As described throughout this specification, any suitable value may be used for C or F. For example, C may be at least about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100, or more. F may be at least about 2, 4, 8, 16, 32, 64, 128, 255, 512, 1024, 2048, 4096, 8192, 16384, 32768, 65536, 131072, 262144, 524288, 1048576, and so on. As described elsewhere in this disclosure, both the initial starting value of F and the steps in its progression may also be varied.

III. Analytes

[00106] An analyte may be any suitable analyte that may be analyzed using the methods and compositions of the invention, where the analyte is capable of interacting with a reagent in order to generate a signal with at least two components that can be measured. An analyte may be naturally-occurring or synthetic. An analyte may be present in a sample obtained using any methods known in the art. In some cases, a sample may be processed before analyzing it for an analyte. The methods and compositions presented in this disclosure may be used in solution phase assays, without the need for particles (such as beads) or a solid support.

[00107] In some cases, an analyte may be a polynucleotide, such as DNA, RNA, peptide nucleic acids, and any hybrid thereof, where the polynucleotide contains any combination of deoxyribo- and/or ribo-nucleotides. Polynucleotides may be single stranded or double stranded, or contain portions of both double stranded or single stranded sequence. Polynucleotides may contain any combination of nucleotides or bases, including, for example, uracil, adenine, thymine, cytosine, guanine, inosine, xanthine, hypoxanthine, isocytosine, isoguanine and any nucleotide derivative thereof. As used herein, the term "nucleotide" may include nucleotides and nucleosides, as well as nucleoside and nucleotide analogs, and modified nucleotides, including both synthetic and naturally occurring species. Polynucleotides may be any suitable polynucleotide for which one or more reagents (or probes) as described herein may be produced, including but not limited to cDNA, mitochondrial DNA (mtDNA), messenger RNA (mRNA), ribosomal RNA (rRNA), transfer RNA (tRNA), nuclear RNA (nRNA), small interfering RNA (siRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), small Cajal body-specific RNA (scaRNA), microRNA (miRNA), double

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stranded (dsRNA), ribozyme, riboswitch or viral RNA. Polynucleotides may be contained within any suitable vector, such as a plasmid, cosmid, fragment, chromosome, or genome.

[00108] Genomic DNA may be obtained from naturally occurring or genetically modified organisms or from artificially or synthetically created genomes. Analytes comprising genomic DNA may be obtained from any source and using any methods known in the art. For example, genomic DNA may be isolated with or without amplification. Amplification may include PCR amplification, multiple displacement amplification (MDA), rolling circle amplification and other amplification methods. Genomic DNA may also be obtained by cloning or recombinant methods, such as those involving plasmids and artificial chromosomes or other conventional methods (see Sambrook and Russell, *Molecular Cloning: A Laboratory Manual.*, cited *supra.*) Polynucleotides may be isolated using other methods known in the art, for example as disclosed in *Genome Analysis: A Laboratory Manual Series* (Vols. I-IV) or *Molecular Cloning: A Laboratory Manual*. If the isolated polynucleotide is an mRNA, it may be reverse transcribed into cDNA using conventional techniques, as described in Sambrook and Russell, *Molecular Cloning: A Laboratory Manual.*, cited *supra.*

[00109] An analyte may be a protein, polypeptide, lipid, carbohydrate, sugar, small molecule, or any other suitable molecule that can be detected with the methods and compositions provided herein. An analyte may be an enzyme or other protein. An analyte may be a drug or metabolite (e.g., anti-cancer drug, chemotherapeutic drug, anti-viral drug, antibiotic drug, or biologic). An analyte may be any molecule, such as a co-factor, receptor, receptor ligand, hormone, cytokine, blood factor, antigen, steroid, or antibody.

[00110] An analyte may be any molecule from any pathogen, such as a virus, bacteria, parasite, fungus, or prion (e.g., PrP^{Sc}). Examples of viruses include those from the families Adenoviridae, Flaviviridae, Hepadnaviridae, Herpesviridae, Orthomyxoviridae, Papovaviridae, Paramyxoviridae, Picornaviridae, Polyomavirus, Retroviridae, Rhabdoviridae, and Togaviridae. Specific examples of viruses include adenovirus, astrovirus, bocavirus, BK virus, coxsackievirus, cytomegalovirus, dengue virus, Ebola virus, enterovirus, Epstein-Barr virus, feline leukemia virus, hepatitis virus, hepatitis A virus, hepatitis B virus, hepatitis C virus, hepatitis D virus, hepatitis E virus, herpes simplex virus (HSV), HSV type 1, HSV type 2, human immunodeficiency virus (HIV), HIV type 1, HIV type 2, human papilloma virus (HPV), HPV type 1, HPV type 2, HPV type 3, HPV type 4, HPV type 6, HPV type 10, HPV type 11, HPV type 16, HPV type 18, HPV type 26, HPV type 27, HPV type 28, HPV type 29, HPV type 30, HPV type 31, HPV type 33, HPV type 34, HPV type 35, HPV type 39, HPV type 40, HPV type 41, HPV type 42, HPV type 43, HPV type 44, HPV type 45, HPV type 49, HPV type 51, HPV type 52, HPV type 54, HPV type 55, HPV type 56, HPV type 57, HPV type 58, HPV type 59, HPV type 68, HPV type 69, influenza virus, JC virus, Marburg

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virus, measles virus, mumps virus, Norwalk virus, parovirus, polio virus, rabies virus, respiratory syncytial virus, retrovirus, rhinovirus, rotavirus, Rubella virus, smallpox virus, vaccinia virus, West Nile virus, and yellow fever virus.

[00111] Examples of bacteria include those from the genuses *Bordetella, Borrelia, Brucella, Campylobacter*, *Chlamydia, Clostridium, Corynebacterium, Enterococcus, Escherichia, Francisella, Haemophilus, Helicobacter, Legionella, Leptospira, Listeria, Mycobacterium*, *Mycoplasma, Neisseria, Pseudomonas*, *Rickettsia, Salmonella*, *Shigella*, *Staphylococcus, Streptococcus*, *Treponema, Vibrio, and Yersinia.* Specific examples of bacteria include *Bordetella par apertussis, Bordetella pertussis, Borrelia burgdorferi, Brucella abortus, Brucella canis, Brucella melitensis, Brucella suis, Campylobacter jejuni, Chlamydia pneumoniae, Chlamydia psittaci, Chlamydia trachomatix, Clostridium botulinum, Clostridium difficile, Clostridium perfringens, Clostridium tetani, Corynebacterium diphtheriae, Enterococcus faecalis, Enterococcus faecium, Escherichia coli*, *Francisella tularensis, Haemophilus influenzae, Helicobacter pylori, Legionella pneumophila, Leptospira interrogans, Listeria monocytogenes, Mycobacterium leprae, Mycobacterium tuberculosis, Mycobacterium ulcerans, Mycoplasma pneumoniae, Neisseria gonorrhoeae, Neisseria meningitidis, Pseudomonas aeruginosa, Rickettsia rickettsii, Salmonella choleraesuis, Salmonella dublin, Salmonella enteritidis, Salmonella typhi, Salmonella typhimurium, Shigella sonnei, Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus saprophyticus, Streptococcus agalactiae, Streptococcus pneumoniae, Streptococcus pyogenes, Treponema pallidum, Vibrio cholerae, Yersinia pestis, and Yersinia enterocolitica.*

[00112] Examples of parasites include those from the genuses *Acanthamoeba, Babesia, Balamuthia, Balantidium, Blasocystis, Cryptosporidium, Dientamoeba, Entamoeba, Giardia, Isospora, Leishmania, Naegleria, Pediculus, Plasmodium, Rhinosporidium, Sarcocystis, Schistosoma, Toxoplasma, Trichomonas,* and *Trypanosoma.* Specific examples of parasites include *Babesia divergens, Babesia bigemina, Babesia equi, Babesia microfti, Babesia duncani, Balamuthia mandrillaris, Balantidium coli, Dientamoeba fragilis, Entamoeba histolytica, Giardia lamblia, Isospora belli, Naegleria fowleri, Pediculus humanus, Plasmodium falciparum, Plasmodium knowlesi, Plasmodium malariae, Plasmodium ovale, Plasmodium vivax, Rhinosporidium seeberi, Sarcocystis bovihominis, Sarcocystis suihominis, Schistosoma mansoni, Toxoplasma gondii, Trichomonas vaginalis, Trypanosoma brucei,* and *Trypansoma cruzi.*

[00113] Examples of fungi include those from the genuses *Apophysomyces, Aspergillus, Blastomyces, Candida, Cladosporium, Coddidioides, Cryptococcos, Exserohilum, Fusarium, Histoplasma, Pichia, Pneumocystis, Saccharomyces, Sporothrix, Stachybotrys,* and *Trichophyton.* Specific examples of fungi include *Aspergillus fumigatus, Aspergillus flavus, Aspergillus clavatus,*

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Blastomyces dermatitidis, Candida albicans, Coccidioides immitis, Crytptococcus neoformans, Exserohilum rostratum, Fusarium verticillioides, Histoplasma capsulatum, Pneumocystis jirovecii, Sporothrix schenckii, Stachybotrys chartarum, and *Trichophyton mentagrophytes.*

[00114] In some cases, the methods provided in this disclosure may be used to detect any one of the analytes described above, or elsewhere in the specification. In some cases the methods provided in this disclosure may be used to detect panels of the analytes described above, or elsewhere in the specification. For example, a panel may comprise an analyte selected from the group consisting of any 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 60, 70, 80, 90, 100 or more analytes described above or elsewhere in the specification.

[00115] An analyte may be obtained from any suitable location, including from organisms, whole cells, cell preparations and cell-free compositions from any organism, tissue, cell, or environment. Analytes may be obtained from environmental samples, biopsies, aspirates, formalin fixed embedded tissues, air, agricultural samples, soil samples, petroleum samples, water samples, or dust samples. In some instances, an analyte may be obtained from bodily fluids which may include blood, urine, feces, serum, lymph, saliva, mucosal secretions, perspiration, central nervous system fluid, vaginal fluid, or semen. Analytes may also be obtained from manufactured products, such as cosmetics, foods, personal care products, and the like. Analytes may be the products of experimental manipulation including, recombinant cloning, polynucleotide amplification, polymerase chain reaction (PCR) amplification, purification methods (such as purification of genomic DNA or RNA), and synthesis reactions.

[00116] More than one type of analyte may be detected in each multiplexed assay. For example, a polynucleotide, a protein, a polypeptide, a lipid, a carbohydrate, a sugar, a small molecule, or any other suitable molecule may be detected simultaneously in the same multiplexed assay with the use of suitable reagents. Any combination of analytes may be detected at the same time.

[00117] Detection of an analyte may be useful for any suitable application, including research, clinical, diagnostic, prognostic, forensic, and monitoring applications. Exemplary applications include detection of hereditary diseases, identification of genetic fingerprints, diagnosis of infectious diseases, cloning of genes, paternity testing, criminal identification, phylogeny, anti-bioterrorism, environmental surveillance, and DNA computing. For example, an analyte may be indicative of a disease or condition. An analyte may be used to make a treatment decision, or to assess the state of a disease. The presence of an analyte may indicate an infection with a particular pathogen, or any other disease, such as cancer, autoimmune disease, cardiorespiratory disease, liver disease, digestive

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disease, and so on. The methods provided herein may thus be used to make a diagnosis and to make a clinical decision based on that diagnosis. For example, a result that indicates the presence of a bacterial polynucleotide in a sample taken from a subject may lead to the treatment of the subject with an antibiotic.

[00118] In some cases the methods and compositions of the invention may be used to detect at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, or 1000 analytes. In some cases the methods and compositions of the invention may be used to detect 7-50, 8-40, 9-30, 10-20, 10-15, 8-12, or 7-12 analytes.

[00119] In some cases, this disclosure provides assays that are capable of unambiguously detecting the presence or absence of each of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, or 1000 analytes, in any combination of presence or absence, in a single sample volume without immobilization, separation, mass spectrometry, or melting curve analysis. In some cases, this disclosure provides assays that are capable of unambiguously detecting the presence or absence of each of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, or 1000 analytes, in any combination of presence or absence, in a single sample volume without immobilization, separation, mass spectrometry, or melting curve analysis. In some cases, this disclosure provides assays that are capable of unambiguously detecting the presence or absence of less than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, or 1000 analytes, in any combination of presence or absence, in a single sample volume without immobilization, separation, mass spectrometry, or melting curve analysis.

IV. Signals

[00120] The methods presented in this disclosure may be used with any quantifiable signal. In some cases, this disclosure provides coding schemes and methods to encode an infinite number of targets without degeneracy, using a single component of a signal (e.g., intensity). For example, as

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described above and in Example 3, a coding scheme may rely on a multiplicity of signal intensity without consideration of color. Although fluorescent probes have been used to illustrate this principle, the coding scheme is equally applicable to any other method providing a quantifiable signal, including an electrochemical signal and a chemiluminescent signal, as described elsewhere in this disclosure.

[00121] The methods presented in this disclosure may also utilize the measurement of a signal in at least two dimensions, also referred to as the measurement of at least two components of a signal. In comparison to the coding scheme described in the paragraph above, which relies on, for example, signal intensity to differentiate between analytes, utilization of at least two components of a signal (e.g., color and intensity) allows the generation of more unique codes per unit of signal intensity bandwidth. When at least two components of a signal are utilized, a cumulative measurement of the at least two components may be obtained for a single sample volume. For example in the coding scheme described in Table 5, the presence of each analyte results in a particular intensity (one component of the signal) in each of the four colors (a second component of a signal). The combination of these constituent signals leads to a cumulative signal that may be measured by measuring an intensity at each wavelength or range of wavelengths. The corresponding coding scheme or decoding matrix may then be used to convert the cumulative measurement into a determination of the presence or absence of an analyte.

[00122] In some cases, a quantifiable signal comprises a waveform that has both a frequency (wavelength) and an amplitude (intensity). A signal may be an electromagnetic signal. An electromagnetic signal may be a sound, a radio signal, a microwave signal, an infrared signal, a visible light signal, an ultraviolet light signal, an x-ray signal, or a gamma-ray signal. In some cases, an electromagnetic signal may be a fluorescent signal, for example a fluorescence emission spectrum that may be characterized in terms of wavelength and intensity.

[00123] In certain portions of this disclosure, the signal is described and exemplified in terms of a fluorescent signal. This is not meant to be limiting, and one of ordinary skill in the art will readily recognize that the principles applicable to the measurement of a fluorescent signal are also applicable to other signals. For example, like fluorescent signals, any of the electromagnetic signals described above may also be characterized in terms of a wavelength and an intensity. The wavelength of a fluorescent signal may also be described in terms of color. The color may be determined based on measuring intensity at a particular wavelength or range of wavelengths, for example by determining a distribution of fluorescent intensity at different wavelengths and/or by utilizing a band pass filter to determine the fluorescence intensity within a particular range of wavelengths. Such band pass filters are commonly employed in a variety of laboratory

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instrumentation, including quantitative PCR machines. Intensity may be measured with a photodetector. A range of wavelengths may be referred to as a "channel."

[00124] In some cases, the methods provided in this disclosure may be used with any signal where the cumulative signal scales with the constituent signals of the same color, frequency, absorption band, and so on. However, the cumulative signal need not be digital or scale linearly with the number and intensity of the constituent signals. For example, if the physical principle of measurement is absorption, the cumulative attenuation is a product of constituent attenuations while the constituent concentrations are additive, due to the exponential nature of the Beer-Lambert law. The logarithm of the cumulative attenuation will then scale linearly with constituent concentrations in each absorption band (the equivalent of color, if fluorescent detection is used). The methods of the invention are therefore applicable. The methods of the invention may also be used with chemiluminescent signals and electrochemical signals.

[00125] The number of signals, and the number of dimensions or components measured, may also be expanded beyond the numbers shown in the exemplary embodiments of the invention, leading to an expansion in multiplexing capability. The exemplary embodiments provided in this disclosure utilize coding schemes constructed utilizing a fluorescent signal with one or two components measured: wavelength and/or intensity. The number of analytes that can be encoded can be increased by increasing the number of wavelengths and/or intensities. The number of analytes that can be encoded can also be increased by increasing the number of signals, for example by combining a fluorescent signal with an electrochemical signal or a FRET signal (fluorescence resonance energy transfer).

[00126] In some cases, more than two components of a signal may be measured. For example, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 18, 20 or more components of a signal may be measured. At least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 18, or 20 components of a signal may be measured. At least 2, but fewer than 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 18, or 20 components of a signal may be measured. In some cases, 2-3, 2-4, 2-5, 2-6, 3-5, 3-6, 3-8, or 5-10 components of a signal may be measured. These additional components may include kinetic components, such as a rate of signal decay and rate of photobleaching.

[00127] If a fluorescent signal is employed, the number of analytes that can be encoded may be further expanded by utilizing additional fluorophores. For example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more fluorophores may be used. In some cases, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 fluorophores

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

[00128] Generally, the number of analytes that can be encoded may be further expanded by utilizing additional first values (e.g., intensities) that are values or ranges of values from a first component of a signal. For example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more first values that are values or ranges of values from a first component of a signal may be used. In some cases, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more first values that are values or ranges of values from a first component of a signal may be used. In some cases, fewer than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 first values that are values or ranges of values from a first component of a signal may be used. In some cases, 4-20, 4-6, 4-7, 4-8, 4-9, 4-10, 5-6, 5-7, 5-8, 5-9, 5-10, 10-15, or 10-20 first values that are values or ranges of values from a first component of a signal may be used.

[00129] In instances in which intensity is a component of a signal that is quantified, such as when a fluorescent signal is utilized, the presence of an analyte may be encoded using a variety of intensities or ranges of intensities. For example, a coding scheme may utilize 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more intensities or ranges of intensities. A coding scheme may utilize at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more intensities or ranges of intensities. A coding scheme may utilize fewer than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 intensities or ranges of intensities. In some cases, a coding scheme may utilize 2-20, 2-3, 2-4, 2-5, 2-6, 2-7, 2-8, 2-9, 2-10, 3-4, 3-5, 3-6, 3-7, 3-8, 3-9, 3-10, 4-5, 4-6, 4-7, 4-8, 4-9, 4-10, 5-6, 5-7, 5-8, 5-9, or 5-10 intensities or ranges of intensities.

[00130] The number of analytes that can be encoded may be further expanded by utilizing additional second values (e.g., wavelengths) that are values or ranges of values from a second component of a signal. For example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47,

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48, 49, 50, or more second values that are values or ranges of values from a second component of a signal may be used. In some cases, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more second values that are values or ranges of values from a second component of a signal may be used. In some cases, fewer than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 second values that are values or ranges of values from a second component of a signal may be used. In some cases, 4-20, 4-6, 4-7, 4-8, 4-9, 4-10, 5-6, 5-7, 5-8, 5-9, 5-10, 10-15, or 10-20 second values that are values or ranges of values from a second component of a signal may be used.

[00131] In instances in which wavelength is a component of a signal that is quantified, such as when a fluorescent signal is utilized, the presence of an analyte may be encoded using a variety of wavelengths or ranges of wavelengths. For example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more wavelengths or ranges of wavelengths may be used. In some cases, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more wavelengths or ranges of wavelengths may be used. In some cases, fewer than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 wavelengths or ranges of wavelengths may be used. In some cases, 4-20, 4-6, 4-7, 4-8, 4-9, 4-10, 5-6, 5-7, 5-8, 5-9, 5-10, 10-15, or 10-20 wavelengths or ranges of wavelengths may be used.

[00132] In some cases, when degeneracy is eliminated, the methods of the invention are capable of detecting the presence or absence of at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more analytes in a single volume when each reagent used to generate a signal in the volume generates only one second value (e.g., each reagent, emits light at only one wavelength).

[00133] In other cases, when degeneracy is eliminated, the methods of the invention are capable of detecting the presence or absence of at least 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more analytes in a single volume using a total of four second values in that volume (e.g., a total of four wavelengths or ranges or wavelengths, which might be implemented by using four spectrally resolvable fluorophores).

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[00134] As described throughout the specification, the assay provided herein utilizes cumulative measurements on a sample. A cumulative measurement may be, for example, a single measurement of intensity values, or a measurement of intensity values at one or more wavelengths or ranges of wavelengths. A plurality of cumulative measurements may be obtained. For example, an intensity may be measured at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more wavelengths or ranges of wavelengths. An intensity may be measured at at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more wavelengths or ranges of wavelengths. An intensity may be measured at less than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 wavelengths or ranges of wavelengths.

[00135] More generally a cumulative measurement may be obtained for any quantifiable component of a signal, and for any quantifiable component of a signal at another quantifiable component of a signal. For example, at least a first component of a signal may be measured at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more second components of a signal. At lest a first component of a signal may be measured at at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more second components of a signal. At least a first component of a signal may be measured at less than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 second components of a signal.

[00136] As is apparent from this disclosure, each analyte to be detected can be encoded as a code utilizing any number of suitable components of a signal or any number of signals. For example, each analyte to be detected can be encoded in 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more components of a signal or signals. In some cases, each analyte to be detected can be encoded in at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more components of a signal or signals. In some cases, each analyte to be detected can be encoded in fewer than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 components of a signal or signals. In some cases, 1-4, 4-20, 4-6, 4-7, 4-8, 4-9, 4-10,

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5-6, 5-7, 5-8, 5-9, 5-10, 10-15, or 10-20 components of signal or signals may be used to encode each analyte. Each analyte in a coding scheme may be encoded by the same number of components of a signal, or signals, or different numbers of components of a signal, or signals.

A. Probes, Primers, Fluorophores, and Quenchers

[00137] Some of the methods provided in this disclosure utilize a reagent that generates a signal in the presence of an analyte. Any suitable reagent may be used with the invention. Generally, a reagent will have an analyte-specific component and a component that generates a signal in the presence of the analyte. In some cases, these reagents are referred to as probes. The probes may be hybridization probes. The hybridization probes may be an oligonucleotide probe attached to a fluorophore and a quencher (e.g., a TAQMAN probe).

[00138] The methods of the invention may use one or more reagents or probes to detect the presence or absence of each analyte. For example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more or probes may be used to detect the presence or absence of each analyte. In some cases, at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 probes may be used to detect the presence or absence of each analyte. In some cases, fewer than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, , 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 probes may be used to detect the presence or absence of each analyte. In some cases, the number of probes used to detect the presence or absence of each analyte is 1-10, 2-9, 2-8, 2-7, 2-6, 2-5, 2-4, 1-3, or 1-4.

[00139] In some cases, a sample is contacted with 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, or more probes to detect the presence or absence of all analytes. In some cases, a sample is contacted with at least 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more probes to detect the presence or absence of all analytes. In some cases, a sample is contacted with fewer than 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 probes to detect the presence or absence of all analytes. In some cases, the number of probes that a sample is contacted with to detect the presence or absence of all analytes is 4-50, 4-40, 4-30, 4-20, 5-15, 5-10, or 3-10.

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[00140] As described above, oligonucleotide probes attached to a fluorophore and a quencher may be used to detect the presence of an analyte in a polynucleotide amplification assay. So long as the quencher and the fluorophore are in proximity, the quencher quenches the fluorescence emitted by the fluorophore upon excitation by a light source. The sequence of the oligonucleotide probe is designed to be complementary to a polynucleotide sequence present in an analyte, and therefore capable of hybridizing to the polynucleotide sequence present in the analyte. Hybridization of the oligonucleotide probe may be performed in a nucleic acid amplification reaction comprising primers (e.g., a polymerase chain reaction). Upon extension of the primers by a DNA polymerase, the 5' to 3' exonuclease activity of the polymerase degrades the probe, releasing the fluorophore and the quencher into the medium. The proximity between the fluorophore and the quencher is broken and the signal from the fluorophore is no longer quenched. Thus, the amount of fluorescence detected is a function of the amount of analyte present. If no analyte is present, the probe will not hybridize to an analyte, and the fluorophore and quencher will remain in close proximity. Little or no signal will be produced.

[00141] Oligonucleotide probes may have one or a plurality of fluorophores and quenchers per probe. For example, in some embodiments an oligonucleotide probe may comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more fluorophores. An oligonucleotide probe may comprise at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 fluorophores. An oligonucleotide probe may comprise fewer than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 fluorophores.

[00142] An oligonucleotide probe may comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more quenchers. An oligonucleotide probe may comprise at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 quenchers. An oligonucleotide probe may comprise fewer than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 quenchers.

[00143] Attachment of probes and quenchers to a probe may be performed in the same reaction or in serial reactions. A series of reactions may be performed to label probes with at least

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one fluorophore and the reaction products may be mixed to generate a mixture of probes with different fluorophores.

[00144] If an oligonucleotide probe comprises two or more fluorophores, these fluorophores may be arranged to allow Förster (Fluorescence) resonance energy transfer (FRET) to occur between the fluorophores. Briefly, FRET is a mechanism of energy transfer between fluorophores. Using FRET-based probes allows one excitation source to generate two different fluorescence emission signals by excitation of a single fluorophore. For example, the methods described herein may used paired probes, where one probe in the pair is attached to a fluorophore and a quencher and a second probe is attached to two fluorophores (in close enough proximity for FRET to occur) and a quencher. Any combination of fluorophores and quenchers that provide for FRET may be used. Such an approach doubles the number of fluorescent probes that may be used with an excitation source, because a single excitation source can be used to produce two signals: one from the probe with one fluorophore and one from the probe with two fluorophores in close enough proximity to provide for FRET. For example, using the encoding method described in Table 8, the number of unambiguously detectable analytes could be increased from 16 to 32 by pairing each of the probes in Table 8 with a corresponding FRET probe.

[00145] The primers may be specific for a particular analyte and capable of amplifying a region complementary to a probe. In some cases, the number of pairs of primers used is equivalent to the number of probes. In other cases, the number of probes used may exceed the number of primer pairs used. In still other cases, the number of primer pairs used may exceed the number of probes used. In some cases a sample to be analyzed is contacted with 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more pairs of primers. In some cases a sample to be analyzed is contacted with at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more pairs of primers. In some cases a sample to be analyzed is contacted with fewer than 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 pairs of primers. In some cases, the number of pairs of primers is 2-10, 3-15, 4-20, 3-10, 4-10, 5-10, 6-8, or 6-10.

[00146] The primers may amplify regions of a polynucleotide in which different numbers of hybridization probes hybridize. For example, at least one pair of primers may amplify a region complementary to at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 hybridization probes. In some cases, all of said pairs of primers may amplify a region

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complementary to at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 hybridization probes.

[00147] In one example, a first probe may be labeled with the fluorophore 5'-fluorescein amidite (5'-FAM) and the quencher black hole quencher 1 (BHQ-1). A second probe may be labeled with 5'-FAM in close proximity (e.g., attached to) to cyanine 5.5 (Cy5.5) and the quencher black hole quencher 3 (BHQ-3). Upon digestion of the probe, via the nuclease activity of the polymerase, two fluorescent signals are generated from a single excitation wavelength (e.g., 470 nm). The fluorophore from the first probe will fluoresce at about 520 nm. The fluorophores on the second probe undergo FRET. The donor fluorophore, FAM is excited by the excitation light source (e.g., 470 nm) and transfers its energy to the acceptor fluorophore, Cy5.5. The acceptor fluorophore emits at about 705 nm. These fluorophores and quenchers are merely exemplary. Any fluorophores that can undergo FRET and any quenchers that can quench fluorescence are suitable for use with the invention. Methods for producing FRET-based probes are described in Jothikumar et al., BioTechniques, 2009, 46(7):519-524.

[00148] In some examples, a single hybridization probe may be used for each analyte. In order to utilize multi-color encoding (e.g., as shown in Table 3), each probe may be labeled with a plurality of fluorophores at pre-determined ratios. The ratio may be determined so that a positive control signal provides the same intensity as other positive control signals of the same intensity, in the same color. This approach reduces the number of probes that must be synthesized and may be less expensive to deploy than methods utilizing multiple probes. Moreover, in the case of hybridization probes, it is easier to fit one hybridization probe to an analyte sequence than multiple probes.

[00149] Although many aspects of the invention are exemplified using nucleic acid-based probes, one of ordinary skill in the art will readily recognize that other forms of probes would work equally well with the invention described in this disclosure. For example, a binding molecule specific to an analyte could be used as a probe. Non-limiting exemplary binding molecules include an antibody recognizing an analyte, and generating a signal in the presence of an analyte.

[00150] In embodiments of the invention that utilize fluorescent labels, any suitable fluorescent label may be used. Exemplary fluorescent labels suitable for use with the invention include rhodamine, rhodol, fluorescein, thiofluorescein, aminofluorescein, carboxyfluorescein, chlorofluorescein, methylfluorescein, sulfofluorescein, aminorhodol, carboxyrhodol, chlororhodol, methylrhodol, sulforhodol; aminorhodamine, carboxyrhodamine, chlororhodamine, methylrhodamine, sulforhodamine, and thiorhodamine, cyanine, indocarbocyanine, oxacarbocyanine,

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thiacarbocyanine, merocyanine, cyanine 2, cyanine 3, cyanine 3.5, cyanine 5, cyanine 5.5, cyanine 7, oxadiazole derivatives, pyridyloxazole, nitrobenzoxadiazole, benzoxadiazole, pyrene derivatives, cascade blue, oxazine derivatives, Nile red, Nile blue, cresyl violet, oxazine 170, acridine derivatives, proflavin, acridine orange, acridine yellow, arylmethine derivatives, auramine, crystal violet, malachite green, tetrapyrrole derivatives, porphin, phtalocyanine, and bilirubin. Exemplary quenchers include black hole quenchers, such as BHQ-0, BHQ-1, BHQ-2, BHQ-3; ATTO quenchers, such as ATTO 540Q, ATTO580Q, and ATTO612Q; and the like.

[00151] The fluorophores that may be used with the invention are not limited to any of the fluorophores described herein. For example, fluorophores with improved properties are continually developed, and these fluorophores could readily be used with the methods provided in this disclosure. Such improved fluorophores include quantum dots, which may emit energy at different wavelengths after being excited at a single wavelength. The advantage of using such fluorophores is that only a single excitation source is needed, but many different signals may be quantified, for example in terms of color and intensity. Moreover, fluorophores with narrow emission spectra would be particularly useful with the method described herein, as such fluorophores could be included in multiplex assays with minimal or no overlap between their emission spectra, thereby offering many more "colors" and boosting significantly the overall number of coded analytes.

[00152] In some cases, one or more reagents are lyophilized. Any suitable reagent may be lyophilized. For example, probes, primers, enzymes, antibodies, or any other reagent used for detection may be lyophilized. In some cases, a sample comprising an analyte may also be lyophilized. Lyophilization may be useful, for example, when distributing reagents and/or samples in developing regions where access to cold storage is expensive, not readily available, or not reliably available. In one example, lyophilized reagents comprise any probe or analyte-specific reagent described in this disclosure or otherwise suitable for use with the invention. In another example, lyophilized reagents comprise PCR primers. In yet another example, lyophilized reagents comprise reagents suitable for performing a PCR reaction.

V. Analytical Techniques and Instrumentation

[00153] The methods provided herein are suitable for use with a variety of detection methods. For example, the methods may be applied using an analytical technique that measures the wavelength and intensity of a fluorescent signal. This may be accomplished by measuring the intensity of a signal across a spectrum of wavelengths, or by using band pass filters that restrict the passage of certain wavelengths of light, thereby allowing only light of certain wavelengths to reach a photodetector. Many real-time PCR and quantitative PCR instruments comprise an excitation light

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source and band pass filters that enable the detection of fluorescent signals in four colors (e.g., blue, green, yellow, and red). Therefore, the methods of the invention can be readily applied using instruments widely used in the art. Importantly, the methods and compositions provided herein may be used to detect multiple analytes by obtaining a cumulative measurement on a single solution. No separation is necessary. The invention does not require the use of beads or a solid phase. Of course, one of ordinary skill in the art would understand that the invention could be used with separation, beads, or a solid phase, if desired.

[00154] **Fig. 5** shows two examples of instrumental configurations that may be used to obtain cumulative measurements useful for the methods of the invention. With reference to **Fig. 5**, an instrument with a detector configured to detect both wavelength and intensity (e.g., a fluorometer) is shown in **501.** With reference to **501**, at least one excitation light source **502** is directed into a chamber **503** containing analytes and reagents that generate a signal in the presence of the analytes **504.** As described elsewhere, the analyte may be a nucleic acid and the reagents generating the signal may be hybridization probes comprising a fluorophore and a quencher. If the analyte is present, an emission signal **505** is generated. In the configuration shown in **501**, the wavelength and intensity of this emission signal are measured across a spectrum by a detector capable of generating a fluorescence emission spectrum **506.**

[00155] The wavelength and intensity may also be determined using a combination of a photodetector and band pass filters. This configuration is used in several thermal cyclers known in the art. With reference to **Fig. 5**, an instrument with band pass filters and a photodetector is depicted in **507.** With reference to **507**, at least one excitation light source **508** is directed into a chamber **509** containing analytes and reagents that generate a signal in the presence of the analytes **510.** As described elsewhere, the analyte may be a nucleic acid and the reagents generating the signal may be hybridization probes comprising a fluorophore and a quencher. If the analyte is present, an emission signal **511** is generated. In the configuration shown in **507**, band pass filters **512, 513,** and **514** are used to restrict the passage of light to light within certain ranges of wavelengths. For example band pass filter **512** may restrict the passage of light to light within wavelength range 1. Band pass filter **513** may restrict the passage of light to light within wavelength range 2. Band pass filter **514** may restrict the passage of light to light within wavelength range 3. Any number of band pass filters (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, or more) may be used with the invention. In addition, the positions of the band pass filters in **507** are not meant to be limiting. In some cases, emitted light may be passed through more than one band pass filter at any time. In other cases, the band pass filters may be configured so that all emitted light passes through only one band pass filter at a time. After passage through a band pass

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filter, filtered light **515, 516,** and **517** is detected by a photodetector. For example, filtered light **515** may be light within wavelength range 1, filtered light **516** may be light within wavelength range 2, and filtered light **517** may be light within wavelength range 3. The intensity of each of the filtered lights **515, 516,** and **517** may then be quantified by a photodetector **518.**

[00156] The methods described in this disclosure are compatible with a variety of amplification methods, including quantitative PCR (qPCR) methods, end point PCR methods, reverse transcriptase PCR, and digital PCR methods. Digital PCR methods (e.g., DROPLET DIGITAL (BIORAD) and DYNAMIC ARRAY (FLUIDIGM)) produce highly sensitive quantification of polynucleotide copy numbers. The methods provided herein can be easily integrated into these systems to significantly expand their throughput by allowing multiplexing in a droplet or a dynamic array.

[00157] Similarly, the methods described in this disclosure may be applied in a real-time PCR assay. For example, real-time data may be fully recorded as the PCR runs to completion. The end-point values may then be decoded to indicate the presence or absence of analytes. The individual cycle thresholds (Ct) values may be analyzed for each color by detecting the maxima of the second derivative of the real-time curve in that color. As a particular analyte is amplified, fluorophores are released from its corresponding probes in the same ratios as the color multiplicities in the coding of that sequence. Hence, e.g. a code of 1100 will have the same Ct in blue and green, while a code of 1400 will have its green Ct precede its blue Ct by 2 cycles. This additional data enables determination of the identity and starting quantity of each analyte.

[00158] The methods described herein may also be used directly on a tissue sample. For example, a tissue sample may be obtained and photolithographic methods may be used to build wells directly onto the tissue sample. The tissue sample may be fixed prior to building the wells. The tissue sample may then be analyzed by dispensing appropriate reagents into the wells in the tissue sample. The encoding and decoding methods described in this disclosure may be used for the multiplexed detection of analytes within the wells etched into the tissue. Each well may correspond to a single cell or a few cells, providing excellent spatial resolution when analyzing different portions of a tissue for an analyte. This method may be used to detect analytes in different areas of the same tissue.

[00159] In some cases, instruments may be modified or constructed, for example, to provide additional excitation light sources, at multiple wavelength, and/or to provide additional band pass filters or a capability of determining a complete spectrum. Including additional excitation sources would allow for the excitation of a larger variety of fluorophores. Including additional band pass filters, or modifying or constructing an instrument capable of determining an entire spectrum allows

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detection of a wider variety of emissions from fluorophores. These techniques can be used to increase the number of fluorophores that can be used with the methods described in this disclosure and, accordingly, to increase the number of analytes that be simultaneously detected.

[00160] In some cases, the methods described in this disclosure utilize end-point PCR methods. However, in some cases a result may be obtained before the end-point. This may be advantageous when, for example, results are needed as soon as possible. For example, in one case calibration experiments are performed in which different starting concentrations of an analyte are analyzed to determine the cycle number at which the signal becomes saturated, as a function of the starting amount of the analyte. Using this data, a computer monitoring a PCR reaction in real-time can be programmed to search for saturation up to the maximal cycle number according to the limit of detection (LOD) of the particular system. If there is no amplification of an analyte (e.g., other than a positive control, if present), by the maximal cycle number, the result for that analyte is negative. If there is amplification of the analyte by the maximal cycle number, the result for that analyte is positive and the saturation intensity in each color may be used to decode the result using the coding scheme. In both cases, there would be no need to run the PCR reaction beyond the number of cycles set by the LOD for that instrument. Although PCR has been used to illustrate this quantitative method, one of ordinary skill in the art will readily recognize that similar principles could be applied in any catalytic system where the starting amount of an analyte limits the rate of reaction, and the rate of reaction can be measured and reported by an evolution in a signal intensity over time.

VI. Compositions and Kits

[00161] This disclosure also provides compositions and kits for use with the methods described herein. The compositions may comprise any component, reaction mixture and/or intermediate described herein, as well as any combination. For example, the disclosure provides detection reagents for use with the methods provided herein. Any suitable detection reagents may be provided, including hybridization probes labeled with a fluorophore and a quencher and primers, as described elsewhere in the specification.

[00162] In some cases, compositions comprise reagents for the detection of at least seven analytes using four fluorophores. In some cases, compositions comprise reagents for the detection of at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 analytes using four fluorophores. In some cases, compositions comprise reagents for the detection of at least 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 analytes using five fluorophores. In some

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cases, compositions comprise reagents for the detection of at least 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 analytes using six fluorophores. In some cases, compositions comprise reagents for the detection of at least 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 analytes using seven fluorophores.

[00163] In some cases the compositions comprise primers. The compositions may comprise at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 pairs of primers.

[00164] The invention also provides kits for carrying out the methods of the invention. Accordingly, a variety of kits are provided in suitable packaging. The kits may be used for any one or more of the uses described herein, and, accordingly, may contain instructions for detecting the presence or absence of each analyte of a plurality of analytes. A kit may comprise a coding scheme, or a decoding matrix, to assist the user in converting a cumulative measurement to a result indicating the presence or absence of each of a plurality of analytes. A kit may be a diagnostic kit, for example, a diagnostic kit suitable for the detection of any analyte, including the analytes recited herein. A kit may contain any of the compositions provided in this disclosure, including those recited above.

[00165] **Fig. 6** shows schematics of exemplary kits of the invention. With reference to **Fig. 6**, a kit comprising a kit body **601** is shown that contains reagents for the detection of analytes (e.g., polynucleotide probes comprising a fluorophore and a quencher) **602** and primers for the amplification of a nucleic acid **603.** The kit may also contain any other reagents, such as reagents suitable for performing an amplification reaction **604.** In the embodiment depicted in **601**, the reagents for the detection of analytes and the primers are each contained in a single volume (e.g., tube or a bottle). However, these reagents may also be provided in separate volumes, as depicted the kit body shown in **605.** The kit body **605** contains seven separate volumes. Three volumes (**606, 607,** and **608**) contain reagents for the detection of analytes (or mixtures of such reagents) and three volumes (**609, 610,** and **611**) contain primers (or mixtures of primers). The kit may also contain reagents suitable for performing an amplification reaction **604.**

[00166] **Fig. 6** is provided for illustrative purposes only. Any number of reagents for the detection of analytes (e.g., probes) and any number of primers may be included in a single tube or bottle, as appropriate for the application. For example, in some embodiments, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more reagents for the detection of analytes may be included in a single bottle. In other cases, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16,

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17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more primers for amplification may be included in a single bottle or tube. In some cases, primers and probes may be provided in the same bottle or tube. For example, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more probes may be provided per pair of primers. In other cases, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more primers may be provided per pair of probes. The kits may contain 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50 or more bottles or tubes. Control analytes, and their associated detection reagents and primers, may also be included in the kit. These may be included in a separate bottle or tube or, for example, included within the bottle or tube providing reagents for an amplification reaction **604**. As described elsewhere in the specification, any reagent in the kits may be lyophilized.

VII. Systems and Software

[00167] The methods of this disclosure may also be performed as part of a system. For example, one or more steps of the methods described herein may be performed by software contained on a computer readable medium. The software may be used to encode analytes, check for degeneracy, reduce or eliminate degeneracy, and/or produce a decoding matrix. The software may also be used to convert cumulative measurements made on an instrument (e.g., a PCR machine) into information concerning the presence or absence of an analyte. For example, with reference to **Fig. 4**, a computer **401** may be connected to an input device **403**, a display **402**, and a printer **404**. The computer may execute one or more of the steps of any of the methods described in this disclosure, including, for example, encoding methods **410**, analysis methods **420**, and decoding methods **430.** A computer may reduce or eliminate degeneracy, for example as part of the encoding methods **410.** Similarly, a computer may generate a coding schme that is non-degenerate by design using, for example, mathematical iteration. A computer may also be connected to a network **405**. The network may be a local network and/or the Internet. The network may be used to perform the methods of the invention on multiple computers in different locations. For example, one computer may perform the encoding, another computer may perform the measuring, and yet another computer may perform the decoding. A computer may perform any or all of these methods. The network may be used to transmit information obtained using the methods described in this disclosure throughout the world, for example across a border.

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[00168] In one example, the software is embedded in the instrument making the measurement, such as a thermal cycler. In another example, the software is installed on a computer attached to the instrument making the measurement, such as a computer attached to a thermal cycler. In another example, the software is in the "cloud" -- i.e., on a computer that another computer may communicate with through a network.

[00169] Each of the elements described above may be connected to a controller that communicates with each of the elements and coordinates their actions. For example, a controller may initiate the encoding of a series of analytes into values of color and intensity. As described throughout this disclosure, the controller can execute software to reduce or eliminate degeneracy by removing one or more potential analyte codes from the encoding matrix. The controller may then automatically order probes corresponding to this code from a manufacturer of probes. Alternatively, the controller may operate an oligonucleotide synthesis instrument that can synthesize the appropriate probes. After analysis of the sample on the instrument (which may also be automated), the controller may process the results of the analysis using the decoding matrix. The controller may then provide these results to a user.

VIII. Services

[00170] The methods provided herein may also be performed as a service. For example, a service provider may obtain the identity of a plurality of analytes that a customer wishes to analyze. The service provider may then encode each analyte to be detected by any of the methods described herein and provide appropriate reagents to the customer for the assay. The customer may perform the assay and provide the results to the service provider for decoding. The service provider may then provide the decoded results to the customer. The customer may also encode analytes, generate probes, and/or decode results by interacting with software installed locally (at the customer's location) or remotely (e.g., on a server reachable through a network). Exemplary customers include clinical laboratories, physicians, manufacturers of food and consumer products, industrial manufacturers (e.g., petroleum companies) and the like. A customer or party may be any suitable customer or party with a need or desire to use the methods, systems, compositions, and kits of the invention.

EXAMPLES

Example 1: General Materials & Methods

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1. General Reagents

[00171] TE Buffer, pH7 (LIFE TECHNOLOGIES, Carlsbad, CA); UltraPure RNAse-free Water (LIFE TECHNOLOGIES, Carlsbad, CA); Taq 5x Master Mix (FISHER SCIENTIFIC COMPANY, Tustin, CA).

2. DNA Sequences, Primers, and Probes

[00172] Five nucleic acids from organisms of clinical relevance were chosen for the exemplary study: (1) human immunodeficiency virus 1 (HIV-1); (2) *Plasmodium falciparum* (Malaria); (3) herpes simplex virus-2 (HSV-2); (4) *Mycobacterium tuberculosis* (TB); (5) and dengue virus type 3 (dengue fever).

[00173] Two regions of diagnostic relevance from the HIV-1 genome, p17 and poly-protease, were selected from the Los Alamos National Laboratory HIV-1 reference sequence. A diagnostic sequence from the *Plasmodium falciparum* ChR7 gene was obtained from the UCSC *Plasmodium falciparum* Genome Browser. A sequence for Herpes Simplex Virus-2 was synthesized from the sequence obtained from the European Molecular Biology Library. Similarly a diagnostic sequence for the rpoB gene in *Mycobacterium tuberculosis* was synthesized from a sequence obtained from the European Molecular Biology Library. A PCR diagnostic sequence for Dengue Virus Type 3 was obtained from the National Institute of Health genetic sequence database.

[00174] Oligonucleotides were synthesized by INTEGRATED DNA TECHNOLOGIES (IDT). Probes and primer pairs were chosen for each analyte using OLIGOANALYZER, from IDT. Sense probes containing a fluorophore at the 5' end and a quencher at the 3' end were synthesized for all analytes. All oligonucleotides were lyophilized and reconstituted in TE buffer before use. Tables 9 - 14 show sequence information, target sequence, primers, and probes for each of the six analytes.

Table 9. HIV-1 polyprotease sequence information.

Sequence Information	HIV-1 Poly protease 198mer synthesized from bases 2253 – 2550
Source	HIV-1 Reference Sequence, Los Alamos National Laboratory
Target sequence (3' to 5')	GGAAGCTCTATTAGATACAGGAGCAGATGATACAGTATTAGAAGAAATGA GTTTGCCAGGAAGATGGAAACCAAAAATGATAGGGGGAATTGGAGGTTTT ATCAAAGTAAGACAGTATGATCAGATACTCATAGAAATCTGTGGACATAA AGCTATAGGTACAGTATTAGTAGGACCTACACCTGTCAACATAATTGG
Forward Primer (3' to 5')	GGAAGCTCTATTAGATACAGGAGCAG

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Reverse Primer (3' to 5')	CCAATTATGTTGACAGGTGTAGGTCC
Probe 1 (3' to 5')	/**56-FAM**/TGAGTTTGCCAGGAAGATGGAAACCA/3BHQ_1/

Table 10. HIV p17 sequence information.

Sequence Name	HIV-1 P17 199mer synthesized from bases 790 – 1186
Source	HIV-1 Reference Sequence, Los Alamos National Laboratory
Target sequence (3' to 5')	CAGCTACAACCATCCCTTCAGACAGGATCAGAAGAACTTAGATCATTATA TAATACAGTAGCAACCCTCTATTGTGTGCATCAAAGGATAGAGATAAAAG ACACCAAGGAAGCTTTAGACAAGATAGAGGAAGAGCAAAACAAAAGTAAG AAAAAAGCACAGCAAGCAGCAGCTGACACAGGACACAGCAATCAGGTCA
Forward Primer (3' to 5')	CAGCTACAACCATCCCTTCAGACA
Reverse Primer (3' to 5')	TGACCTGATTGCTGTGTCCTGTGT
Probe 1 (3' to 5')	/**56-FAM**/AGCAACCCTCTATTGTGTGCATCAAAGG/3BHQ_1
Probe 2 (3' to 5')	/**5Cy3**/AAAGCACAGCAAGCAGCAGCTGA/3BHQ_2/

Table 11. *Plasmodium* ChR7 sequence information.

Sequence Name	*Plasmodium* ChR7 199mer synthesized from bases 1139138 - 1141223
Source	UCSC Plasmodium falciparum Genome Browser Gateway
Target sequence (3' to 5')	GCCTAACATGGCTATGACGGGTAACGGGGAATTAGAGTTCGATTCCGGAG AGGGAGCCTGAGAAATAGCTACCACATCTAAGGAAGGCAGCAGGCGCGTA AATTACCCAATTCTAAAGAAGAGAGGTAGTGACAAGAAATAACAATGCAA GGCCAATTTAAAACCTTCCCAGAGTAACAATTGGAGGGCAAGTCTGGTG
Forward Primer (3' to 5')	GCCTAACATGGCTATGACGGGTAA
Reverse Primer (3' to 5')	CACCAGACTTGCCCTCCAATTGTT
Probe 1 (3' to 5')	/**56-FAM**/ATTCCGGAGAGGGAGCCTGAGAAATA/3BHQ_1/
Probe 2 (3' to 5')	/**56-ROXN**/AAGGAAGGCAGCAGGCGCGTAAATTA/3BHQ_2/

Table 12. Herpes simplex virus 2 sequence information.

Sequence Name	HSV-2 193mer synthesized from HSV-2 genome
Source	EMBL Bank AJ303204

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Target sequence (3' to 5')	TCAGCCCATCCTCCTTCGGCAGTATGGAGGGTGTCGCGGCGGCGAGCCGC CGTCCCCAAAGACGTGCGGGTCGTACACGTACACGTACCAGGGCGGCGGG CCTCCGACCCGGTACGCTCTCGTAAATGCTTCCCTGCTGGTGCCGATCTG GGACCGCGCCGCGGAGACATTCGAGTACCAGATCGAACTCGG
Forward Primer (3' to 5')	TCAGCCCATCCTCCTTCGGCAGTAT
Reverse Primer (3' to 5')	CCGAGTTCGATCTGGTACTCGAATGT
Probe 1 (3' to 5')	/**56-FAM**/AAAGACGTGCGGGTCGTACACGTACA/3BHQ_1/
Probe 2 (3' to 5')	/**5Cy5**/TAAATGCTTCCCTGCTGGTGCCGAT/3IAbRQSp/

Table 13. *Mycobacterium tuberculosis* rpoB sequence information

Sequence Name	Mycobacterium Tuberculosis rpoB 200mer synthesized from Mycobacterium tuberculosis rpoB genome
Source	EMBL Bank GQ395623
Target sequence (3' to 5')	GAGTGCAAAGACAAGGACATGACGTACGCGGCCCCGCTGTTCGTCACGGC CGAGTTCATCAACAACAACACCGGCGAGATCAAGAGCCAGACGGTGTTCA TGGGTGACTTCCCGATGATGACCGAGAAGGGCACCTTCATCATCAACGGC ACCGAGCGCGTCGTGGTCAGCCAGCTGGTCCGCTCGCCCGGTGTGTACTT
Forward Primer (3' to 5')	GAGTGCAAAGACAAGGACATGACG
Reverse Primer (3' to 5')	AAGTACACACCGGGCGAGC
Probe 1 (3' to 5')	/**56-FAM**/CGCTGTTCGTCACGGCCGAGTTCAT/3BHQ_1/
Probe 2 (3' to 5')	/**5Cy3**/AGATCAAGAGCCAGACGGTGTTCATG/3BHQ_2/
Probe 3 (3' to 5')	/**5Cy5**/AAGGGCACCTTCATCATCAACGGCA/3IAbRQSp/

Table 14. Dengue virus type 3 sequence information.

Sequence Name	Dengue Virus Type 3 200mer synthesized from Dengue Virus Type 3 genome
Source	GenBank M93130
Target sequence (3' to 5')	ATGCCAACTGTGATTGAGCACTTAGAAAGACTACAAAGGAAACATGGAGG AATGCTTGTGAGAAATCCACTCTCACGAAACTCCACGCACGAAATGTATT GGATATCCAATGGTACAGGCAACATCGTCTCATTCACAATGACACACAGG AGACCCACCATAGAGAAAGATGTGGATTTAGGAGCAGGAACCCGACATGT
Forward Primer (3' to 5')	ATGCCAACTGTGATTGAGCACT

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Reverse Primer (3' to 5')	ACATGTCGGGTTCCTGCTCCTAAA
Probe 1 (3' to 5')	/**56-FAM**/ACAAAGGAAACATGGAGGAATGCTTGTGA/3BHQ_1/
Probe 2 (3' to 5')	/**5Cy3**/ACTCTCACGAAACTCCACGCACGAAA/3BHQ_2/
Probe 3 (3' to 5')	/**56-ROXN**/ACAATGACACACAGGAGACCCACCAT/3BHQ_2/
Probe 4 (3' to 5')	/**5Cy5**/GGATATCCAATGGTACAGGCAACATCGT/3IAbRQSp/

3. Polymerase Chain Reactions

[00175] All PCR reactions were performed in a ROCHE 480 LIGHTCYCLER instrument. Forty-five thermal cycles were performed, with a 60 second hot-start at 95°C. The cycling conditions for denaturation, annealing, and extension were 45 seconds, 50 seconds, and 60 seconds at 95°C, 65°C, and 70°C, respectively. Each experiment was run in quintuplicate, with a reaction volume of 15 μL. Fluorescence measurements were obtained in the following channels after every annealing step: 483 nm to 533 nm (FAM), 523 nm to 568 nm (Cy3), 558 nm to 610 nm (ROX), and 615 nm to 670 nm (Cy5). Fluorescence measurements were also obtained after the hot-start and the end of the 45 thermal cycles. The change in the fluorescence intensity between these two measurements (after hot-start and at the end of the 45 cycles) determined the end-point fluorescent signal.

Example 2: Simultaneous Detection of Polynucleotide Markers from Pathogens Based on an Encoding Method Using More Than One Color Per Analyte

[00176] This example describes the simultaneous detection of up to four polynucleotides selected from HIV (2 polynucleotides), HSV-2, *Mycobacterium*, *Plasmodium*, and dengue virus. Fourteen PCR reactions were assembled, using the reagents described in Table 15.

Table 15. Reagents for 14 PCR reactions.

Reaction No.	Type of Reagent	Reagent	Concentration	Volume
1		UltraPure Water	-	34 μL
		Taq 5x Master Mix	-	42 μL
	Template	Poly Protease Template	17 nM	2 μL
	Primers	Poly Protease FWD Primer	1 nM	2 μL
		Protease RVS Primer	1 nM	2 μL
	Probe	Poly Protease Probe 1	1 nM	2 μL
2		UltraPure Water	-	28 μL
		Taq 5x Master Mix	-	42 μL
	Templates	Malaria Template	15 nM	2 μL

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		Herpes Template	17 nM	2 µL
		TB Template	16 nM	2 µL
		Dengue Virus Template	17 nM	2 µL
	Primers	Poly Protease FWD Primer	1 nM	2 µL
		Protease RVS Primer	1 nM	2 µL
	Probe	Poly Protease Probe 1	1 nM	2 µL
3		UltraPure Water	-	32 µL
		Taq 5x Master Mix	-	42 µL
	Template	Malaria Template	15 nM	2 µL
	Primers	Malaria FWD Primer	1 µM	2 µL
		Malaria RVS Primer	1 µM	2 µL
	Probes	Malaria Probe 1	1 µM	2 µL
		Malaria Probe 2	1 µM	2 µL
4		UltraPure Water	-	26 µL
		Taq 5x Master Mix	-	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Herpes Template	17 nM	2 µL
		TB Template	16 nM	2 µL
		Dengue Virus Template	17 nM	2 µL
	Primers	Malaria FWD Primer	1 µM	2 µL
		Malaria RVS Primer	1 µM	2 µL
	Probes	Malaria Probe 1	1 µM	2 µL
		Malaria Probe 2	1 µM	2 µL
5		UltraPure Water	-	32 µL
		Taq 5x Master Mix	-	42 µL
	Template	Herpes Template	17 nM	2 µL
	Primers	Herpes FWD Primer	1 µM	2 µL
		Herpes RVS Primer	1 µM	2 µL
	Probes	Herpes Probe 1	1 µM	2 µL
		Herpes Probe 2	1 µM	2 µL
6		UltraPure Water	-	26 µL
		Taq 5x Master Mix	-	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Malaria Template	15 nM	2 µL
		TB Template	16 nM	2 µL
		Dengue Virus Template	17 nM	2 µL
	Primers	Herpes FWD Primer	1 µM	2 µL
		Herpes RVS Primer	1 µM	2 µL
	Probes	Herpes Probe 1	1 µM	2 µL
		Herpes Probe 2	1 µM	2 µL
7		UltraPure Water	-	30 µL
		Taq 5x Master Mix	-	42 µL
	Template	TB Template	16 nM	2 µL
	Primers	TB FWD Primer	1 µM	2 µL
		TB RVS Primer	1 µM	2 µL
	Probes	TB Probe 1	1 µM	2 µL
		TB Probe 2	1 µM	2 µL
		TB Probe 3	1 µM	2 µL
8		UltraPure Water	-	30 µL

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		Taq 5x Master Mix	-	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Malaria Template	15 nM	2 µL
		Herpes Template	17 nM	2 µL
		Dengue Virus Template	17 nM	2 µL
	Primers	TB FWD Primer	1 µM	2 µL
		TB RVS Primer	1 µM	2 µL
	Probes	TB Probe 1	1 µM	2 µL
		TB Probe 2	1 µM	2 µL
		TB Probe 3	1 µM	2 µL
9		UltraPure Water	-	28 µL
		Taq 5x Master Mix	-	42 µL
	Template	Dengue Virus Template	17 nM	2 µL
	Primers	Dengue Virus FWD Primer	1 µM	2 µL
		Dengue Virus RVS Primer	1 µM	2 µL
	Probes	Dengue Virus Probe 1	1 µM	2 µL
		Dengue Virus Probe 2	1 µM	2 µL
		Dengue Virus Probe 3	1 µM	2 µL
		Dengue Virus Probe 4	1 µM	2 µL
10		UltraPure Water	-	22 µL
		Taq 5x Master Mix	-	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Malaria Template	15 nM	2 µL
		Herpes Template	17 nM	2 µL
		TB Template	16 nM	2 µL
	Primers	Dengue Virus FWD Primer	1 µM	2 µL
		Dengue Virus RVS Primer	1 µM	2 µL
	Probes	Dengue Virus Probe 1	1 µM	2 µL
		Dengue Virus Probe 2	1 µM	2 µL
		Dengue Virus Probe 3	1 µM	2 µL
		Dengue Virus Probe 4	1 µM	2 µL
11		Taq 5x Master Mix	-	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Malaria Template	15 nM	2 µL
		Herpes Template	17 nM	2 µL
		Dengue Virus Template	17 nM	2 µL
	Primers	Poly Protease FWD Primer	1 µM	2 µL
		Poly Protease RVS Primer	1 µM	2 µL
		Malaria FWD Primer	1 µM	2 µL
		Malaria RVS Primer	1 µM	2 µL
		Herpes FWD Primer	1 µM	2 µL
		Herpes RVS Primer	1 µM	2 µL
		Dengue Virus FWD Primer	1 µM	2 µL
		Dengue Virus RVS Primer	1 µM	2 µL
	Probes	Poly Protease Probe 1	1 µM	2 µL
		Malaria Probe 1	1 µM	2 µL
		Malaria Probe 2	1 µM	2 µL
		Herpes Probe 1	1 µM	2 µL
		Herpes Probe 2	1 µM	2 µL

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		Dengue Virus Probe 1	1 µM	2 µL
		Dengue Virus Probe 2	1 µM	2 µL
		Dengue Virus Probe 3	1 µM	2 µL
		Dengue Virus Probe 4	1 µM	2 µL
12		UltraPure Water	-	2 µL
		Taq 5x Master Mix	-	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Malaria Template	15 nM	2 µL
		Herpes Template	17 nM	2 µL
		TB Template	16 nM	2 µL
	Primers	Poly Protease FWD Primer	1 µM	2 µL
		Poly Protease FWD Primer	1 µM	2 µL
		Malaria FWD Primer	1 µM	2 µL
		Malaria RVS Primer	1 µM	2 µL
		Herpes FWD Primer	1 µM	2 µL
		Herpes RVS Primer	1 µM	2 µL
		TB FWD Primer	1 µM	2 µL
		TB RVS Primer	1 µM	2 µL
	Probes	Poly Protease Probe 1	1 µM	2 µL
		Malaria Probe 1	1 µM	2 µL
		Malaria Probe 2	1 µM	2 µL
		Herpes Probe 1	1 µM	2 µL
		Herpes Probe 2	1 µM	2 µL
		TB Probe 1	1 µM	2 µL
		TB Probe 2	1 µM	2 µL
		TB Probe 3	1 µM	2 µL
13		UltraPure Water	-	12 µL
		Taq 5x Master Mix	-	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Herpes Template	17 nM	2 µL
		TB Template	16 nM	2 µL
	Primers	Poly Protease FWD Primer	1 µM	2 µL
		Poly Protease FWD Primer	1 µM	2 µL
		Herpes FWD Primer	1 µM	2 µL
		Herpes RVS Primer	1 µM	2 µL
		TB FWD Primer	1 µM	2 µL
		TB RVS Primer	1 µM	2 µL
	Probes	Poly Protease Probe 1	1 µM	2 µL
		Herpes Probe 1	1 µM	2 µL
		Herpes Probe 2	1 µM	2 µL
		TB Probe 1	1 µM	2 µL
		TB Probe 2	1 µM	2 µL
		TB Probe 3	1 µM	2 µL
14		UltraPure Water	-	10 µL
		Taq 5x Master Mix	-	42 µL
	Templates	Poly Protease Template	17 nM	2 µL
		Malaria Template	15 nM	2 µL
		Dengue Virus Template	17 nM	2 µL
	Primers	Poly Protease FWD Primer	1 µM	2 µL

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		Poly Protease FWD Primer	1 μM	2 μL
		Malaria FWD Primer	1 μM	2 μL
		Malaria RVS Primer	1 μM	2 μL
		Dengue Virus FWD Primer	1 μM	2 μL
		Dengue Virus RVS Primer	1 μM	2 μL
	Probes	Poly Protease Probe 1	1 μM	2 μL
		Malaria Probe 1	1 μM	2 μL
		Malaria Probe 2	1 μM	2 μL
		Dengue Virus Probe 1	1 μM	2 μL
		Dengue Virus Probe 2	1 μM	2 μL
		Dengue Virus Probe 3	1 μM	2 μL
		Dengue Virus Probe 4	1 μM	2 μL

[00177] Referring to Table 15, reactions 1, 3, 5, 7, and 9 (containing HIV polyprotease, *Plasmodium*, HSV, *Mycobacterium*, and dengue analytes, respectively) were positive controls that provided baseline fluorescence intensity for each polynucleotide analytes alone, in the absence of other analytes. The change in fluorescence intensity in each color was used to provide the expected cumulative intensity level for each color in the chromatogram **(Fig. 3)**, as described more fully below. Reactions 2, 4, 6, 8, and 10 were negative controls used to determine the extent of non-specific amplification. In these reactions, the primers provided were not specific for the analytes provided.

[00178] Experiments 11, 12, 13, and 14 were multiplex detection experiments. Encoding was performed as indicated in Table 16. The measured fluorescence intensity for each assay, in each color, was plotted as black circles in the chromatogram of **Fig. 3.**

Table 16. Coding scheme for detection of pathogens.

	FAM	**Cy3**	**ROX**	**Cy5**
HIV poly-protease	1	0	0	0
HIV p17	1	1	0	0
Plasmodium	1	0	1	0
HSV	1	0	0	1
Mycobacterium	1	1	0	1
Dengue Virus	1	1	1	1

[00179] To construct the chromatogram, the signals of the positive controls (reactions 1, 3, 5, 7, and 9) were used to assemble every possible combination of present sequences, in each color. The cumulative signals were plotted in a diagram, called a "chromatogram" (**Fig. 3**). Cumulative signals corresponding to all possible combinations of the same rank in the same color were organized into their own "band." Doing this for all four colors produced a level and band structure, against which the experimental results of each combination (reactions 11, 12, 13, and 14) could be evaluated. **Fig.**

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3 shows three replicates of a chromatogram constructed using the positive control samples, with experimental results 4112, 3121, and 3102 superimposed on the chromatogram. The experimental results are indicated by black dots.

[00180] The intensity value for each color in reactions 11-14 was measure in arbitrary fluorescence units (AFU), plotted on the chromatogram, and then assigned to a band based on two criteria. First, if an experimental result was within a band, then the result was assigned to the multiplicity of the band (e.g., the Cy3 value of 1 in the left-most chromatogram of **Fig. 3**). Second, if an experimental result was between two bands, a two-step analysis was employed. If the experimental result was between two legitimate results, the experimental result was assigned to the closest band. (e.g., the Cy5 value of 2 in the right-most chromatogram of **Fig. 3**). If the experimental result was between a legitimate result and an illegitimate result, the result was assigned to the band with the legitimate result (e.g., the FAM value of 3 in the middle chromatogram of **Fig. 3**). **Fig. 8** schematically shows a legitimate result of 4112 (top) indicating the presence of HIV polyprotease, *Plasmodium*, herpes simplex, and *Mycobacterium*; and an illegitimate result of 4110 (bottom), which cannot be decoded and indicates a malfunction in the assay.

[00181] Using these criteria, the output of Experiment 12 was designated 4112, which was the correct answer. The output of Experiment 14 could have been designated as 2121 or 3121, but 2121 is illegitimate, so the result must be 3121, which is the correct answer. The output of Experiment 13 could be designated as 3102 or 3101, both of which are legitimate, but the result is clearly closer to 2 than 1 in the Cy5 channel, so 3102, the correct answer, was designated as the result. These results provide experimental validation of the methods of the provided herein.

[00182] The results indicate a trend of slightly lower combined signal than what would be expected from a simple summation of positive-control signals. This may be because the combination of signals is not perfectly linear. This may be a result of additional effects for which the chromatogram construction does not account for at present. These effects may be related to the absolute concentration of quenchers. The higher the absolute concentration of quenchers, the larger the percentage of reaction volume they quench, so that the same percentage of released fluorophores fails to contribute as much as expected to the cumulative signal. One solution to this problem is to use low concentrations of probes, so that the percentile loss in signal is negligible, regardless of how much quencher is released.

[00183] **Fig. 3** shows that each rank containing multiple combinations has a relatively wide band. The width of the band is ultimately the difference between the highest and lowest cumulative signals for the positive control samples. If all positive controls in the particular color produced exactly the same fluorescence signal, then the width of each band would be zero. Since instead those

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signals are somewhat different, the resulting bands are relatively wide. However, there is a simple means to tighten the bands. Instead of loading all probes at the same concentration, as was done for the experiment above, the concentration of each probe can be adjusted so that each of the resulting positive-control signals is the same as the others in the same color. This approach would significantly tighten the bands, making it easier to determine the multiplicity of a signal for a sample containing analytes.

Example 3: Simultaneous Detection of Polynucleotide Markers from Pathogens Based on an Encoding Method Using One Color Per Analyte and Different Intensities

[00184] This example describes the simultaneous detection of up to three polynucleotides selected from dengue virus, HIV p17, and HIV polyprotease. The coding scheme was as depicted in Table 11. As shown in Table 17, each analyte was encoded as a single value of fluorescence intensity in a single color. In this case, as described elsewhere in this disclosure, the coding scheme is non-degenerate by design. This is achieved by assigning an intensity value to each analyte that is equal to the cumulative intensity values for the prior analytes, plus one. Of course, as described elsewhere in the specification, the initial assigned value can be greater than one, for example to better distinguish over noise. Similarly, the values assigned to each analyte can be greater than the cumulative intensity values for the prior analytes plus one, for example, to increase the separation between the intensities and enable more simple assignment of intensities.

Table 17. Coding scheme for detection of dengue virus, HIV polyprotease, and HIV p17.

	FAM
Dengue Virus	1
HIV p17	2
HIV polyprotease	4

[00185] The same reagents (e.g., templates, primers, and probes) used in Example 2 were used here. The fluorescence signals generated by the respective probes in positive-control end-point PCR reactions were measured and used to calculate the probe concentrations that would produce 1x, 2x, and 4x signal intensities. Experiments were then set up to detect the seven non-null combinations of analyte occurrences. Tables 18-24 provide the PCR reaction components for each of these mixtures.

Table 18. Binary FAM Experiment 1 Cocktail: HIV Polyprotease, HIV p17, and dengue virus

Reagents	Concentration	Volume Added
UltraPure Water	-	50μL

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Taq 5x Master Mix	-	25μL
Templates	Concentration	Volume Added
Poly Protease Template	17nM	4μL
P17 Template	15nM	4μL
Dengue Virus Template	17nM	4μL
Primers	Concentration	Volume Added
Poly Protease FWD Primer	1μM	4μL
Poly Protease RVS Primer	1μM	4μL
P17 FWD Primer	1μM	4μL
P17 RVS Primer	1μM	4μL
Dengue Virus FWD Primer	1μM	4μL
Dengue Virus RVS Primer	1μM	4μL
Probes	Concentration	Volume Added
Poly Protease Probe 1	800nM	4μL
P17 Probe 1	400nM	4μL
Dengue Virus Probe 1	200nM	4μL

Table 19. Binary FAM Experiment 2 Cocktail: dengue virus and HIV p17

Reagents	Concentration	Volume Added
UltraPure Water	-	54μL
Taq 5x Master Mix	-	25μL
Templates	Concentration	Volume Added
Poly Protease Template	17nM	-
P17 Template	15nM	4μL
Dengue Virus Template	17nM	4μL
Primers	Concentration	Volume Added
Poly Protease FWD Primer	1μM	4μL
Poly Protease RVS Primer	1μM	4μL
P17 FWD Primer	1μM	4μL
P17 RVS Primer	1μM	4μL
Dengue Virus FWD Primer	1μM	4μL
Dengue Virus RVS Primer	1μM	4μL
Probes	Concentration	Volume Added

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Poly Protease Probe 1	800nM	4μL
P17 Probe 1	400nM	4μL
Dengue Virus Probe 1	200nM	4μL

Table 20. Binary FAM Experiment 3 Cocktail: dengue virus and HIV polyprotease

Reagents	Concentration	Volume Added
UltraPure Water	-	54μL
Taq 5x Master Mix	-	25μL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	4μL
P17 Template	15nM	-
Dengue Virus Template	17nM	4μL
Primers	**Concentration**	**Volume Added**
Poly Protease FWD Primer	1μM	4μL
Poly Protease RVS Primer	1μM	4μL
P17 FWD Primer	1μM	4μL
P17 RVS Primer	1μM	4μL
Dengue Virus FWD Primer	1μM	4μL
Dengue Virus RVS Primer	1μM	4μL
Probes	**Concentration**	**Volume Added**
Poly Protease Probe 1	800nM	4μL
P17 Probe 1	400nM	4μL
Dengue Virus Probe 1	200nM	4μL

Table 21. Binary FAM Experiment 4 Cocktail: HIV polyprotease and HIV p17

Reagents	Concentration	Volume Added
UltraPure Water	-	54μL
Taq 5x Master Mix	-	25μL
Templates	**Concentration**	**Volume Added**
Poly Protease Template	17nM	4μL
P17 Template	15nM	4μL
Dengue Virus Template	17nM	-
Primers	**Concentration**	**Volume Added**

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Poly Protease FWD Primer	1μM	4μL
Poly Protease RVS Primer	1μM	4μL
P17 FWD Primer	1μM	4μL
P17 RVS Primer	1μM	4μL
Dengue Virus FWD Primer	1μM	4μL
Dengue Virus RVS Primer	1μM	4μL
Probes	Concentration	Volume Added
Poly Protease Probe 1	800nM	4μL
P17 Probe 1	400nM	4μL
Dengue Virus Probe 1	200nM	4μL

Table 22. Binary FAM Experiment 5 Cocktail: dengue virus

Reagents	Concentration	Volume Added
UltraPure Water	-	58μL
Taq 5x Master Mix	-	25μL
Templates	Concentration	Volume Added
Poly Protease Template	17nM	-
P17 Template	15nM	-
Dengue Virus Template	17nM	4μL
Primers	Concentration	Volume Added
Poly Protease FWD Primer	1μM	4μL
Poly Protease RVS Primer	1μM	4μL
P17 FWD Primer	1μM	4μL
P17 RVS Primer	1μM	4μL
Dengue Virus FWD Primer	1μM	4μL
Dengue Virus RVS Primer	1μM	4μL
Probes	Concentration	Volume Added
Poly Protease Probe 1	800nM	4μL
P17 Probe 1	400nM	4μL
Dengue Virus Probe 1	200nM	4μL

Table 23. Binary FAM Experiment 6 Cocktail: HIV p17

Reagents	Concentration	Volume Added

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UltraPure Water	-	58µL
Taq 5x Master Mix	-	25µL
Templates	Concentration	Volume Added
Poly Protease Template	17nM	-
P17 Template	15nM	4µL
Dengue Virus Template	17nM	-
Primers	Concentration	Volume Added
Poly Protease FWD Primer	1µM	4µL
Poly Protease RVS Primer	1µM	4µL
P17 FWD Primer	1µM	4µL
P17 RVS Primer	1µM	4µL
Dengue Virus FWD Primer	1µM	4µL
Dengue Virus RVS Primer	1µM	4µL
Probes	Concentration	Volume Added
Poly Protease Probe 1	800nM	4µL
P17 Probe 1	400nM	4µL
Dengue Virus Probe 1	200nM	4µL

Table 24. Binary FAM Experiment 7 Cocktail: HIV polyprotease

Reagents	Concentration	Volume Added
UltraPure Water	-	58µL
Taq 5x Master Mix	-	25µL
Templates	Concentration	Volume Added
Poly Protease Template	17nM	4µL
P17 Template	15nM	-
Dengue Virus Template	17nM	-
Primers	Concentration	Volume Added
Poly Protease FWD Primer	1µM	4µL
Poly Protease RVS Primer	1µM	4µL
P17 FWD Primer	1µM	4µL
P17 RVS Primer	1µM	4µL
Dengue Virus FWD Primer	1µM	4µL
Dengue Virus RVS Primer	1µM	4µL

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Probes	Concentration	Volume Added
Poly Protease Probe 1	800nM	4μL
P17 Probe 1	400nM	4μL
Dengue Virus Probe 1	200nM	4μL

[00186] The total fluorescence signal of each experiment was plotted on a chromatogram **(Fig. 7)** that was constructed as described above. The width of the bands around each positive control measurement is the propagated uncertainty of the 1X measurement. That uncertainty was equated to the standard deviation of the fluorescence signals of the last five PCR cycles in saturation.

[00187] **Fig. 7** shows the results of this experiment. The three right-most results show the measured intensities for dengue virus alone (DEN), HIV p17 alone (P17), and HIV polyprotease (TPP). The assigned intensities for each of these analytes were as indicated in Table 11, and **Fig. 7** confirms that the three analytes yielded the expected intensity results when alone (see DEN, P17, and TPP). At the end of the assay, essentially all of the probes have been hydrolyzed, releasing essentially all of the fluorophores, which emit a signal. A fixed amount of a fluorophore produces a fixed and predictable amount of signal. Therefore a target that is present should generally produce a fixed amount of signal, as determined by the amount of probe added to the reaction volume.

[00188] As expected, the intensity values for the combinations of analytes are equivalent to the sum of the assigned intensities. Since this coding scheme is designed to be non-degenerate, each result can be unambiguously decoded into the presence or absence of a particular analyte. For example, starting from the left side of **Fig. 7**, a result of 7 indicates the presence of all three analytes. A result of 3 indicates the presence of dengue virus and HIV p17 analytes, and the absence of HIV polyprotease. A result of 5 indicates the presence of dengue virus and HIV polyprotease, and the absence of HIV p17. Finally a result of 6 indicates the presence of HIV p17 and HIV polyprotease, and the absence of dengue virus. This coding scheme can be extended to encode additional analytes, as described elsewhere in this disclosure.

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CLAIMS

WHAT IS CLAIMED IS:

1. An assay that is capable of unambiguously detecting the presence or absence of each of at least 7 analytes, in any combination of presence or absence, in a single sample volume without immobilization, separation, mass spectrometry, or melting curve analysis.
2. The assay of claim 1, wherein each of said analytes is encoded as a value of one component of a signal.
3. The method of claim 2, wherein said method is capable of unambiguously detecting the presence or absence of M analytes, in any combination of presence or absence, where $M = \log_2 (F + 1)$ and F is the maximum cumulative value of said one component of said signal when all of said analytes are present.
4. The assay of claim 1, wherein each of said analytes is encoded as at least one first value in a first component of said signal and at least one second value in a second component of said signal.
5. The assay of claim 4, wherein each of said analytes is encoded as a first value in a first component of said signal at each of a plurality of second values in a second component of said signal.
6. The method of claim 4, wherein said method is capable of unambiguously detecting the presence or absence of M analytes, in any combination of presence or absence, where $M = C * \log_2 (F + 1)$, where C is the number of said second values used to encode said analytes and F is the maximum cumulative value of said first component of said signal, for any second value, when all of said analytes are present.
7. The method of claim 4, wherein said method is capable of unambiguously detecting the presence or absence of M analytes, in any combination of presence or absence, where $M = (P * T) + 1$, where P is the number of codes per tier in a coding scheme and T is the number of tiers.
8. The method of claim 7, wherein the number of tiers $T = \log_4 (F + 1)$, and F is the maximum cumulative value of said first component of said signal, for any second value, when all of said analytes are present.
9. The method of claim 4, wherein said first value is an intensity or range of intensities.
10. The method of claim 9, wherein said coding scheme comprises at least three intensities or ranges of intensities.
11. The method of claim 4, wherein said second value is a wavelength or range of wavelengths.
12. The method of claim 11, wherein said coding scheme comprises at least five wavelengths or ranges of wavelengths.

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13. The method of claim 1, wherein said signal is an electromagnetic signal.

14. The method of claim 13, wherein said electromagnetic signal is a fluorescence emission signal.

15. The method of claim 14, wherein intensity of said fluorescence emission signal is measured at at least four wavelengths or ranges of wavelengths.

16. The method of claim 1, wherein said detecting is performed with reagents that are lyophilized prior to use.

17. The method of claim 1, wherein said detecting is performed with reagents comprising hybridization probes.

18. The method of claim 17, wherein the number of said hybridization probes is greater than the number of said analytes.

19. The method of claim 17, wherein said hybridization probes comprise one or more hybridization probes specific for different analytes and comprising an identical fluorophore or combination of fluorophores.

20. The method of claim 17, wherein said sample is contacted with at least 18 of said hybridization probes.

21. The method of claim 17, wherein said reagents further comprise at least one pair of primers capable of amplifying a region complementary to at least three of said hybridization probes.

22. The method of claim 21, wherein said signal is generated during a polymerase chain reaction.

23. The method of claim 22, wherein said polymerase chain reaction is selected from the group consisting of an end-point polymerase chain reaction, a real-time polymerase chain reaction, and a digital polymerase chain reaction.

24. The method of claim 1, wherein said cumulative measurement is performed on a solution.

25. The method of claim 1, wherein at least one of said analytes is encoded by at least one additional value, wherein said at least one additional value is selected from the group consisting of a value from at least one additional component of said signal, a value from at least one component of an additional signal, and combinations thereof.

26. The method of claim 25, wherein said at least one additional value is selected from the group consisting of a fluorescence emission intensity, a fluorescence emission wavelength, a Förster resonance energy transfer (FRET) emission intensity, a FRET emission wavelength, an electrochemical signal, a chemiluminescence wavelength, a chemiluminescence intensity, a fluorescence bleaching rate, a chemiluminescence bleaching rate, and combinations thereof.

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27. The method of claim 1, further comprising constructing a chromatogram.

28. The method of claim 27, wherein said chromatogram is constructed by plotting all possible combinations of said first values and said second values for positive control samples.

29. The method of claim 1, wherein at least one of said analytes comprises a polynucleotide.

30. The method of claim 29, wherein said polynucleotide is from a source selected from the group consisting of an animal, a plant, a bacteria, a fungus, a parasite, and a virus.

31. The method of claim 23, wherein said polynucleotide is from a source selected from the group consisting of human immunodeficiency virus, herpes simplex virus, human papilloma virus, Plasmodium, Mycobacterium, dengue virus, hepatitis virus, and influenza virus.

32. The method of claim 31, wherein said polynucleotide is selected from the group consisting of human immunodeficiency virus polyprotease, human immunodeficiency virus p17, human papilloma virus E6, and human papilloma virus E7.

33. The method of claim 1, wherein said sample is selected from the group consisting of a clinical sample, a food sample, an environmental sample, a pharmaceutical sample, and a sample from a consumer product.

34. The method of claim 1, further comprising transmitting information concerning the presence or absence of said analyte through a computer network.

35. The method of claim 1, further comprising providing information concerning the presence or absence of said analyte to a physician.

36. The method of claim 35, further comprising making a clinical decision based on said information.

37. The method of claim 1, wherein at least one step of said method is performed using instructions on a computer readable medium.

38. The method of claim 37, wherein said instructions are located on a remote server.

39. The method of claim 37, wherein said instructions are located on a thermal cycler.

40. The method of claim 37, wherein said instructions are located on a computer in communication with a thermal cycler.

41. The method of claim 1, wherein at least one of said analytes is a positive control analyte.

42. The method of claim 1, wherein said coding scheme is non-degenerate.

43. The method of claim 42, wherein said coding scheme is designed to be non-degenerate.

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44. The method of claim 42, wherein said coding scheme is made non-degenerate by enumerating every legitimate result that can be obtained from said coding scheme, identifying each legitimate result that is degenerate, and eliminating at least one potential analyte code from said coding scheme to eliminate degeneracy.
45. The method of claim 1, wherein said assay is an end-point assay.
46. The method of claim 1, wherein said assay is ended at a threshold number of cycles set by the limit of detection of an instrument.
47. The method of claim 1, wherein said assay is a liquid phase assay.
48. The method of claim 1, wherein said assay is quantitative.
49. A method of detecting the presence or absence of each analyte of a plurality of analytes, comprising:
    a. encoding each of said analytes as a first value of a signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said first value, wherein said encoding is performed in a manner that eliminates degeneracy;
    b. providing a sample comprising, or potentially comprising, at least one of said analytes;
    c. contacting said sample with analyte-specific reagents that generate said first value, as represented in said coding scheme, when each of said analytes is present;
    d. cumulatively measuring said first values of said signal within said sample, thereby providing a cumulative measurement; and
    e. determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme.
50. The method of claim 49, wherein said method is capable of unambiguously detecting the presence or absence of M analytes, in any combination of presence or absence, where $M = \log_2 (F + 1)$ and F is the maximum cumulative value of said first values when all of said analytes are present.
51. The method of claim 49, wherein said first value is an intensity or range of intensities.
52. The method of claim 49, wherein said first value has a minimum value in said coding scheme that is selected from the group consisting of at least 1, at least 2, at least 4, at least 8, at least 16, at least 32, and at least 64.
53. The method of claim 49, wherein said first value is incremented in said coding scheme by an amount equal to the cumulative maximum of said preceding first values plus one.
54. The method of claim 49, wherein said first value is incremented in said coding scheme by an amount greater than the cumulative maximum of said preceding first values plus one.

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55. A method of detecting the presence or absence of each analyte of a plurality of analytes, comprising:

    a. encoding each of said analytes as at least one first value and at least one second value, wherein said first value is a value from a first component of a signal and said second value is a value from a second component of said signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said at least one first value and said at least one second value, wherein said encoding is performed in a manner that reduces or eliminates degeneracy;

    b. providing a sample comprising, or potentially comprising, at least one of said analytes;

    c. contacting said sample with analyte-specific reagents that generate said at least one first value and at least one said second value, as represented in said coding scheme, when each of said analytes is present;

    d. cumulatively measuring said first values and said second values within said sample, thereby providing a cumulative measurement; and

    e. determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme,

    wherein, when degeneracy is eliminated, said method is capable of unambiguously detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said reagents generates only one second value.

56. The method of claim 55, wherein each of said analytes is encoded as a first value in a first component of said signal at each of a plurality of second values in a second component of said signal.

57. The method of claim 55, wherein, when degeneracy is eliminated, said method is capable of unambiguously detecting the presence or absence of each of at least seven analytes in a single volume using four second values.

58. The method of claim 55, wherein, when degeneracy is eliminated, said coding scheme comprises T non-degenerate tiers, wherein $T = \log_4 (F + 1)$ and F is the maximum cumulative value of said first component of said signal, for any second value, when all of said analytes are present.

59. The method of claim 56, wherein, when degeneracy is eliminated, said method is capable of unambiguously detecting the presence or absence of M analytes, in any combination of presence or absence, where $M = (P * T) + 1$ and P is the number of codes per tier.

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60. The method of claim 55, wherein, when degeneracy is eliminated, said method is capable of unambiguously detecting the presence or absence of M analytes, in any combination of presence or absence, where $M = C * \log_2 (F + 1)$, C is the number of second values in said coding scheme, and F is the maximum cumulative value of said first component of said signal, for any second value, when all of said analytes are present.

61. A non-degenerate coding scheme capable of unambiguously encoding the presence or absence of each of at least 7 analytes, in any combination of presence or absence, in a single sample volume without immobilization, separation, or melting curve analysis.

62. The non-degenerate coding scheme of claim 61, wherein said coding scheme is generated by a method comprising:

    a. generating a code for each potential analyte, wherein each potential analyte is encoded by at least one value of at least one component of a signal;

    b. enumerating every legitimate cumulative result for all possible combinations of presence or absence of each analyte;

    c. identifying each legitimate result that is degenerate; and

    d. eliminating at least one code to eliminate degeneracy.

63. The method of claim 62, further comprising expanding said coding scheme by adding additional codes that are at least one unit greater than the sum of all previous codes in said at least one component of said signal.

64. The method of claim 61, wherein said coding scheme is generated by a method of mathematical iteration that guarantees non-degeneracy by construction.

65. A system for detecting the presence or absence of each analyte of a plurality of analytes, comprising:

    a. encoding each of said analytes as a first value of a signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said first value, wherein said encoding is performed in a manner that eliminates degeneracy;
    b. providing a sample comprising, or potentially comprising, at least one of said analytes;
    c. contacting said sample with analyte-specific reagents that generate said first value, as represented in said coding scheme, when each of said analytes is present;
    d. cumulatively measuring said first values of said signal within said sample, thereby providing a cumulative measurement; and
    e. determining whether each of said analytes is present or absent based on said cumulative

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measurement and said coding scheme.

66. A system for detecting the presence or absence of each analyte of a plurality of analytes, comprising:

    a. encoding each of said analytes as at least one first value and at least one second value, wherein said first value is a value from a first component of a signal and said second value is a value from a second component of said signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said at least one first value and said at least one second value, wherein said encoding is performed in a manner that reduces or eliminates degeneracy;

    b. providing a sample comprising, or potentially comprising, at least one of said analytes;

    c. contacting said sample with analyte-specific reagents that generate said at least one first value and said at least one second value, as represented in said coding scheme, when each of said analytes is present;

    d. cumulatively measuring said first values and said second values within said sample, thereby providing a cumulative measurement; and

    e. determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme,

    wherein, when degeneracy is eliminated, said system is capable of unambiguously detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said reagents generates only one second value.

67. A method of detecting the presence or absence of each analyte of a plurality of analytes, comprising:

    a. obtaining the identity of each of said analytes from a party;

    b. encoding each of said analytes as a first value of a signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said first value, wherein said encoding is performed in a manner that eliminates degeneracy;

    c. providing said party with analyte-specific reagents that generate said first value, as represented in said coding scheme, when each of said analytes is present, said party:

        i. contacting a sample comprising, or potentially comprising, at least one of said analytes with said reagents; and

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ii. cumulatively measuring said first values within said sample, thereby providing a cumulative measurement; and

d. obtaining said cumulative measurement from said party;

e. determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme; and

f. providing said party with information about the presence or absence of each of said analytes.

68. A method of detecting the presence or absence of each analyte of a plurality of analytes, comprising:

g. obtaining the identity of each of said analytes from a party;

h. encoding each of said analytes as at least one first value and at least one second value, wherein said first value is a value from a first component of a signal and said second value is a value from a second component of said signal, thereby generating a coding scheme, wherein each of said analytes is represented in said coding scheme by said at least one first value and said at least one second value, wherein said encoding is performed in a manner that reduces or eliminates degeneracy;

i. providing said party with analyte-specific reagents that generate said at least one first value and said at least one second value, as represented in said coding scheme, when each of said analytes is present, said party:

i. contacting a sample comprising, or potentially comprising, at least one of said analytes with said reagents; and

ii. cumulatively measuring said first values and said second values within said sample, thereby providing a cumulative measurement; and

j. obtaining said cumulative measurement from said party;

k. determining whether each of said analytes is present or absent based on said cumulative measurement and said coding scheme; and

l. providing said party with information about the presence or absence of each of said analytes.

69. A composition for detecting the presence or absence of each analyte of a plurality of analytes, comprising analyte-specific reagents, each reagent generating a signal comprising a first value, wherein said reagents are capable of unambiguously detecting the presence or absence of each of

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at least seven analytes in a single sample volume, in any combination of presence or absence, without immobilization, separation, or melting curve analysis.

70. A composition for detecting the presence or absence of each analyte of a plurality of analytes, comprising analyte-specific reagents, each reagent generating a signal comprising at least one first value that is a value from a first component of said signal and at least one second value that is a value from a second component of said signal, wherein said reagents are capable of detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said reagents generates only one second value.

71. A kit for detecting the presence or absence of each analyte of a plurality of analytes, comprising analyte-specific reagents, packaging, and instructions, each reagent generating a signal comprising a first value, wherein said kit is capable of unambiguously detecting the presence or absence of each of at least seven analytes in a single sample volume, in any combination of presence or absence, without immobilization, separation, or melting curve analysis.

72. A kit for detecting the presence or absence of each analyte of a plurality of analytes, comprising analyte-specific reagents, packaging, and instructions, each reagent generating a signal comprising at least one first value that is a value from a first component of said signal and at least one second value that is a value from a second component of said signal, wherein said kit is capable of detecting the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said reagents generates only one second value.

73. A kit for detecting the presence or absence of each analyte of a plurality of analytes, comprising a kit body (601), clamping slots arranged in the kit body for placing bottles, a bottle comprising probes for the detection of an analyte (602), a bottle comprising primers for amplification (603), and a bottle comprising reagents for amplification (604), wherein said kit is capable of unambiguously detecting the presence or absence of each of at least seven analytes in a single sample volume, in any combination of presence or absence, without immobilization, separation, or melting curve analysis.

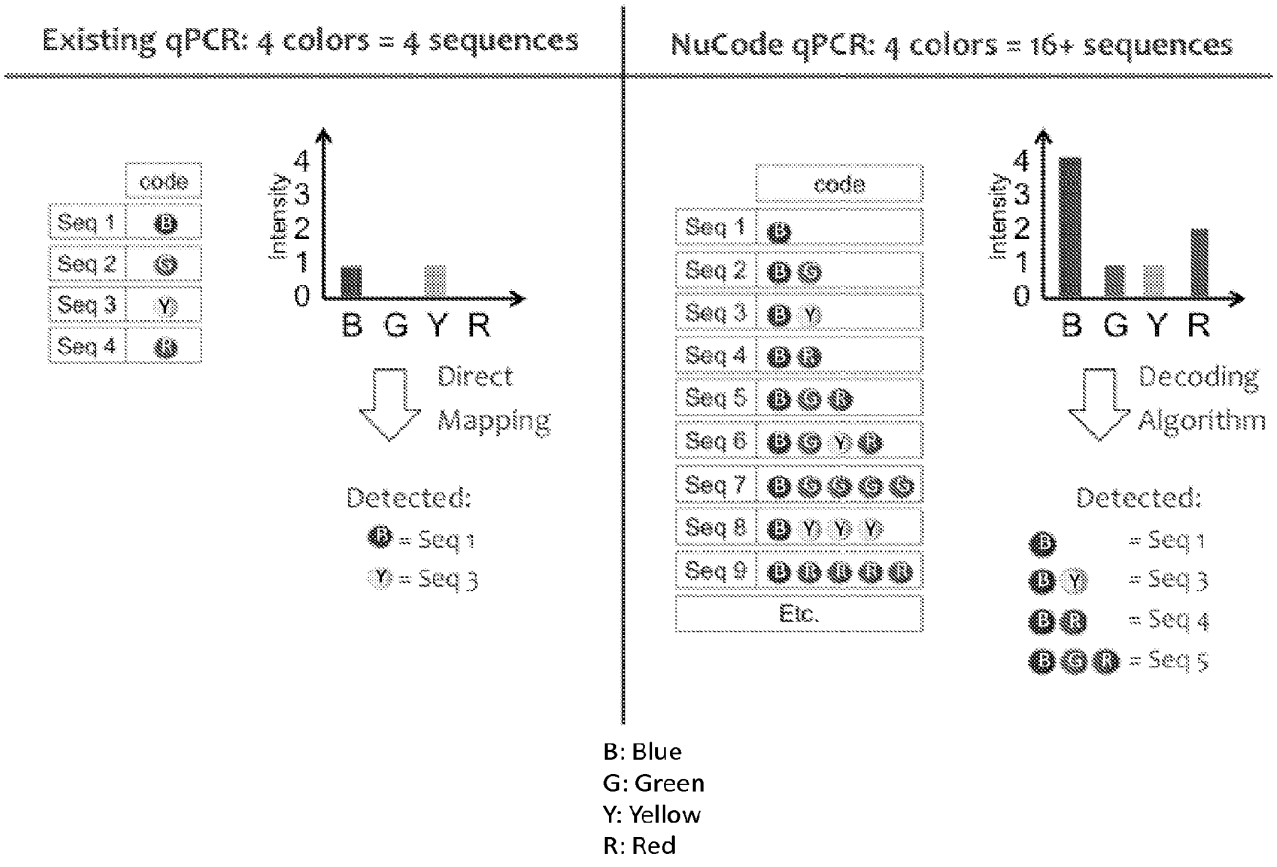
74. A kit for detecting the presence or absence of each analyte of a plurality of analytes, a kit body (601), clamping slots arranged in the kit body for placing bottles, a bottle comprising probes for the detection of an analyte (602), a bottle comprising primers for amplification (603), and a bottle comprising reagents for amplification (604), each probe generating a signal comprising at least one first value that is a value from a first component of said signal and at least one second value that is a value from a second component of said signal, wherein said kit is capable of detecting

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the presence or absence of each of at least six analytes in a single volume, in any combination of presence or absence, when each of said probes generates only one second value.

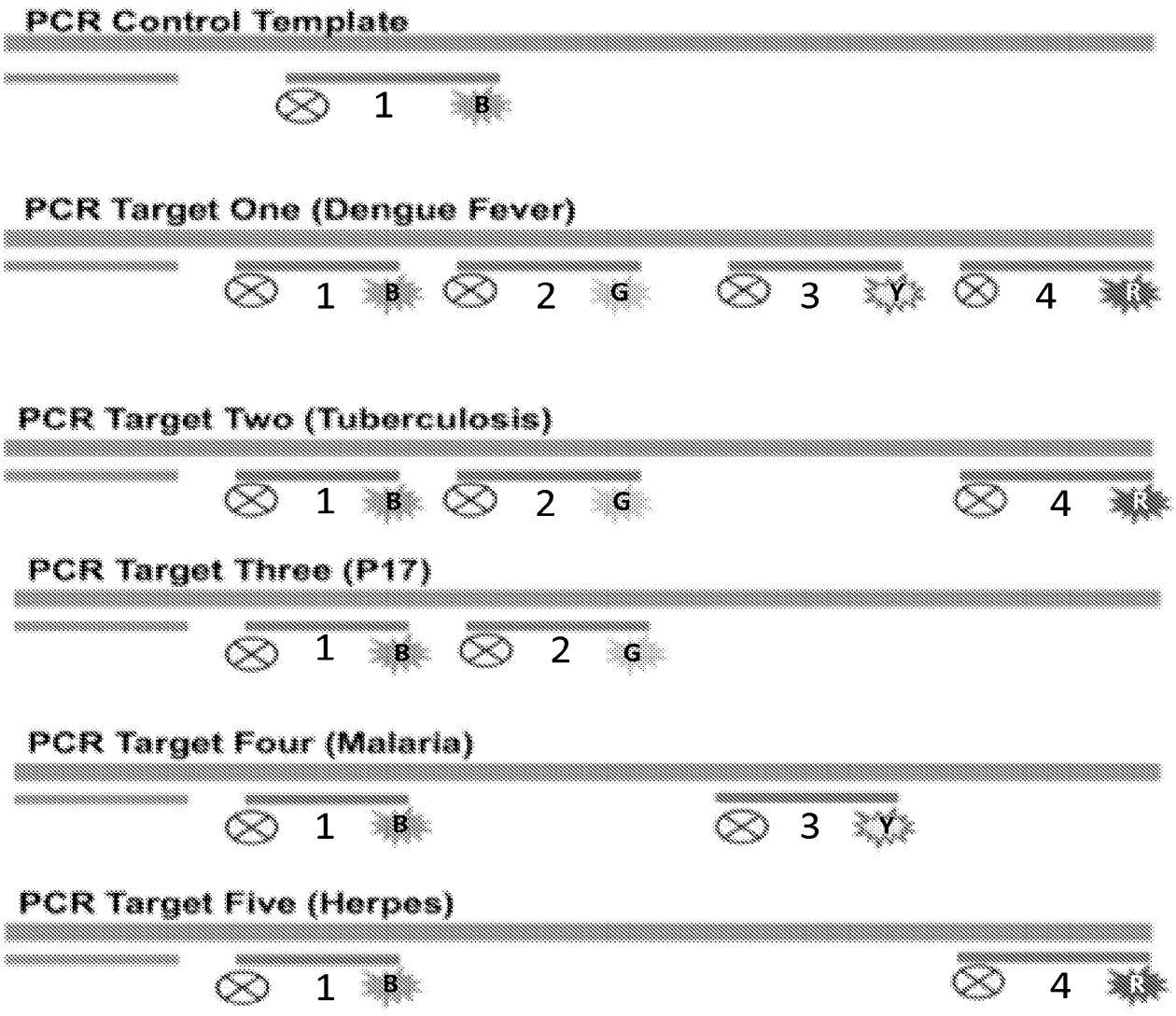
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Fig. 1



B: Blue
G: Green
Y: Yellow
R: Red

Fig. 2



B: Blue
G: Green
Y: Yellow
R: Red

Fig. 3

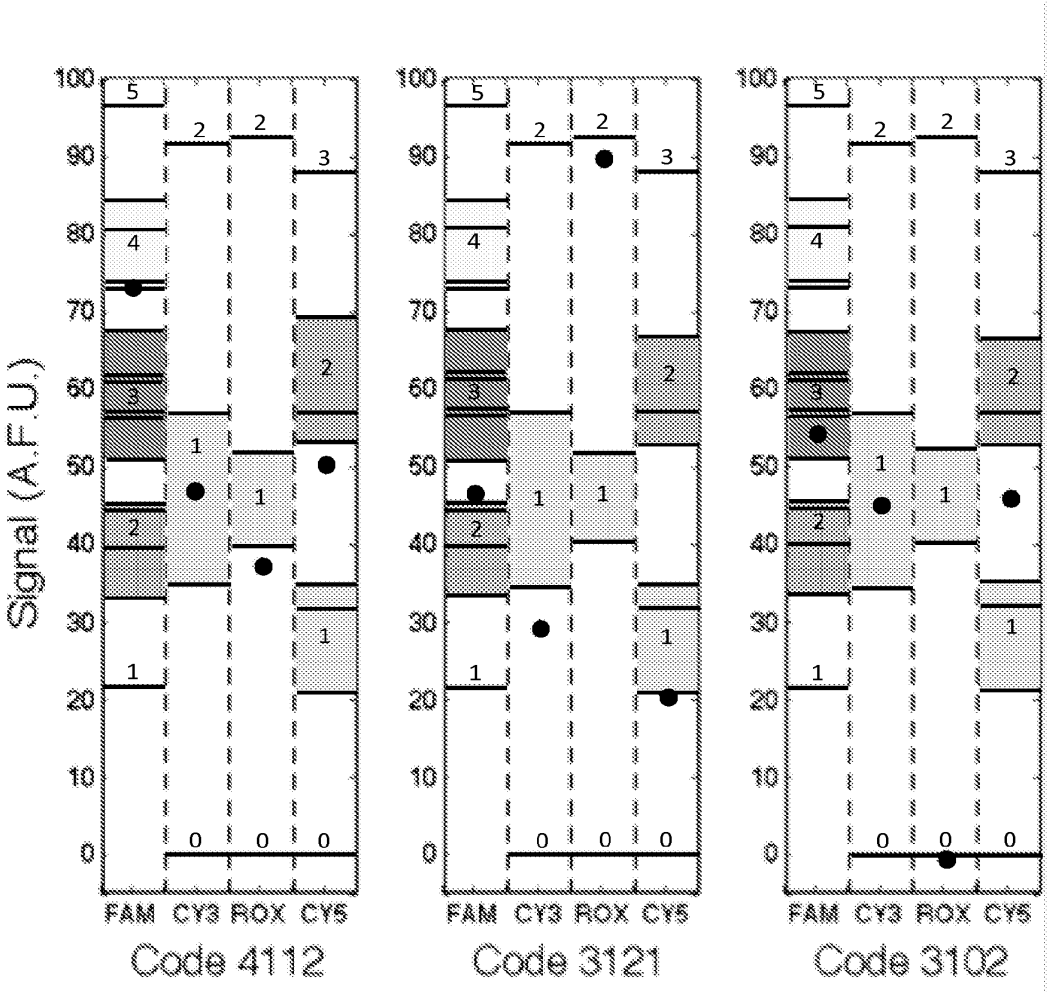


Fig. 4

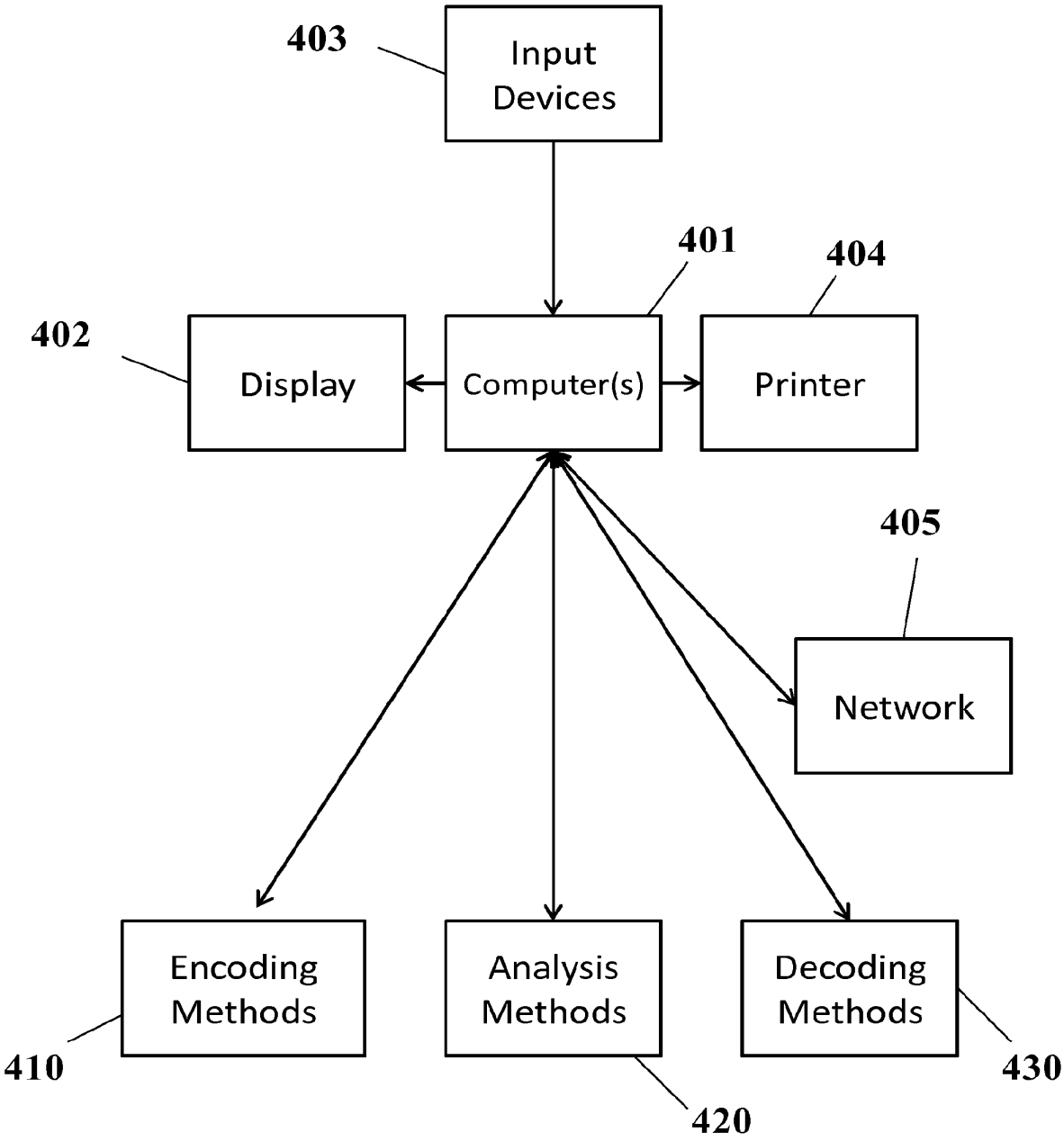

403
Input Devices
401
404
402
Display
Computer(s)
Printer
405
Network
Encoding Methods
Analysis Methods
Decoding Methods
410
420
430

Fig. 5

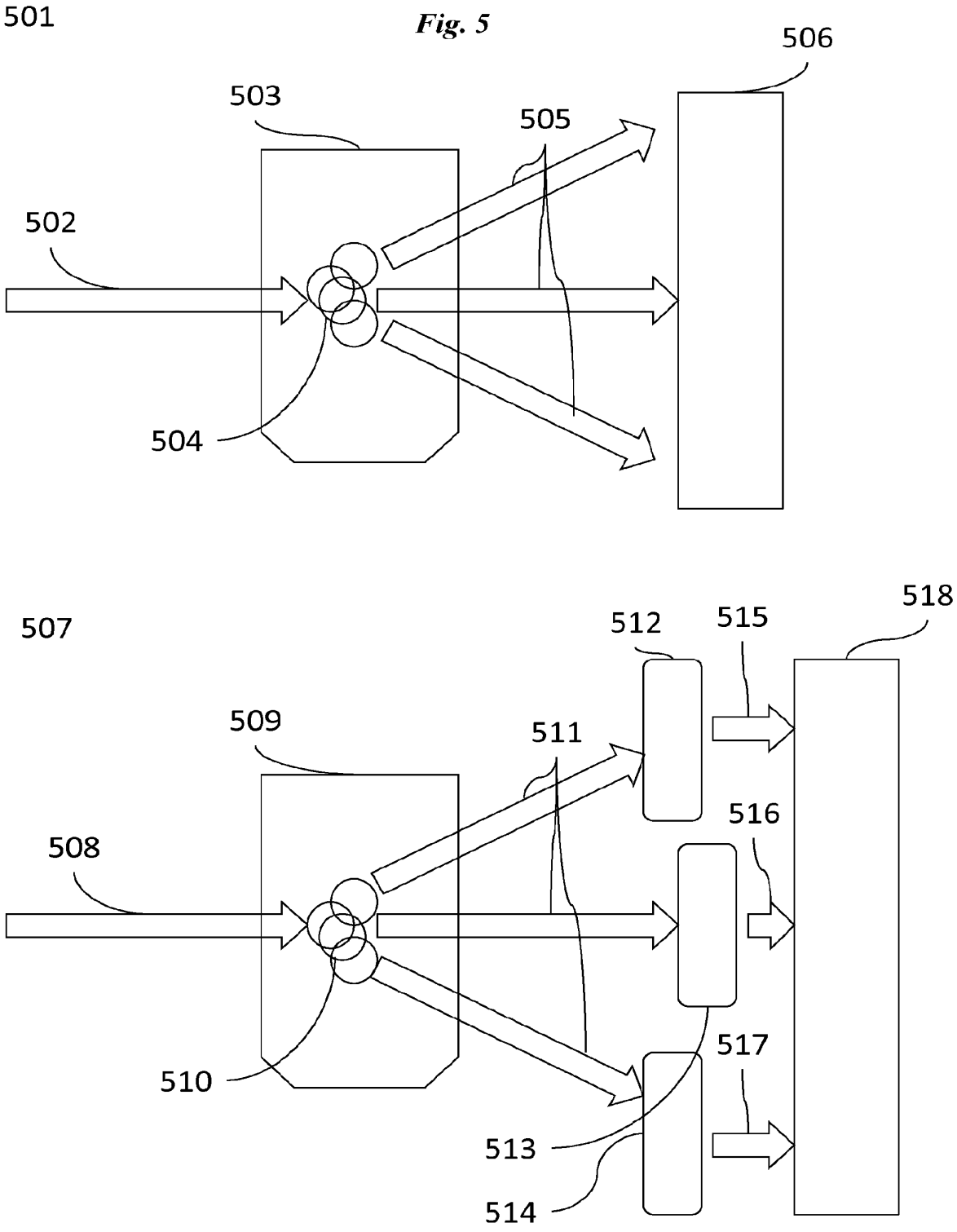
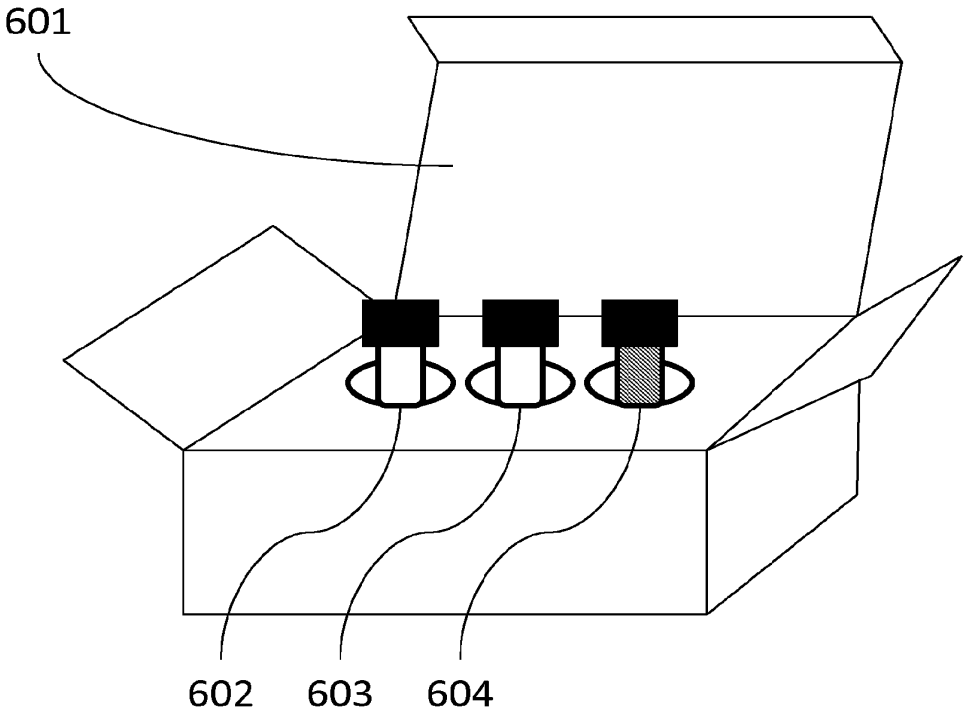

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Fig. 6



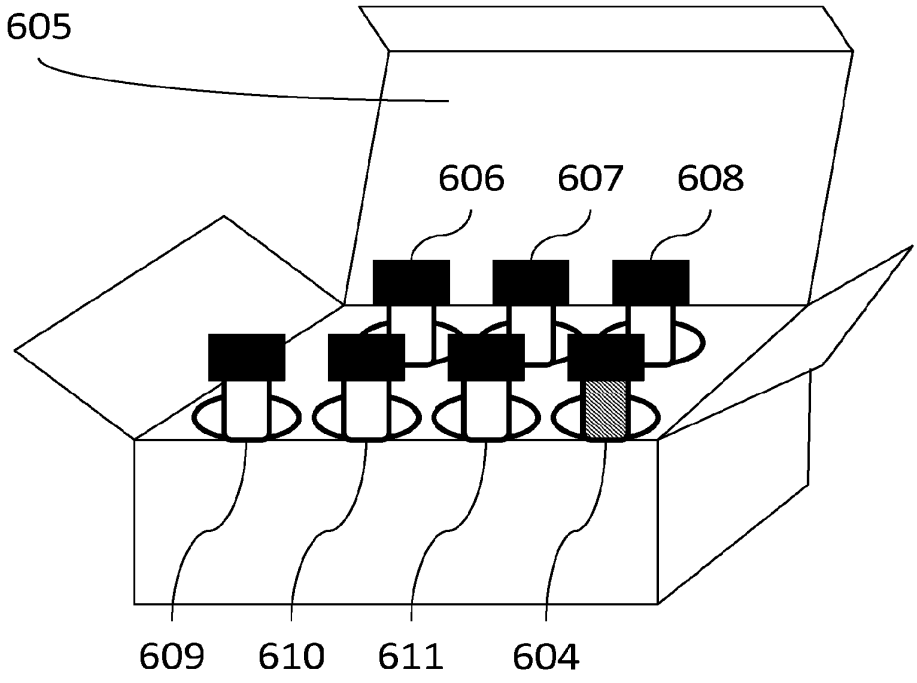


Fig. 7

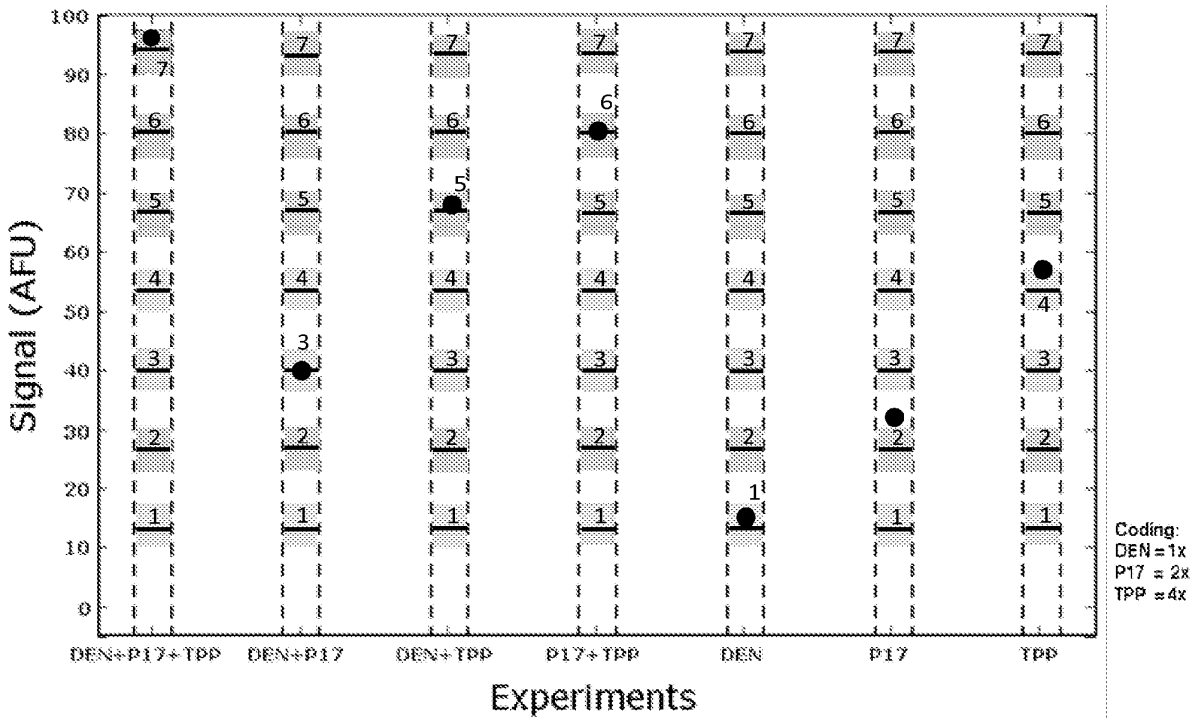
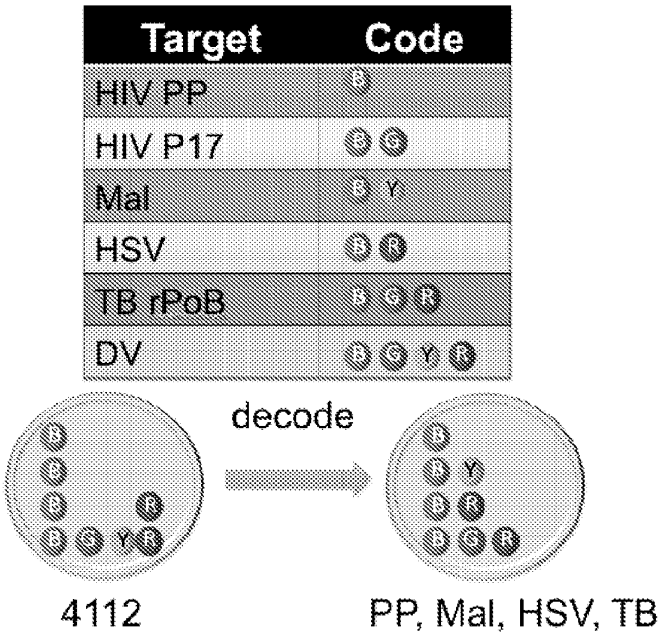
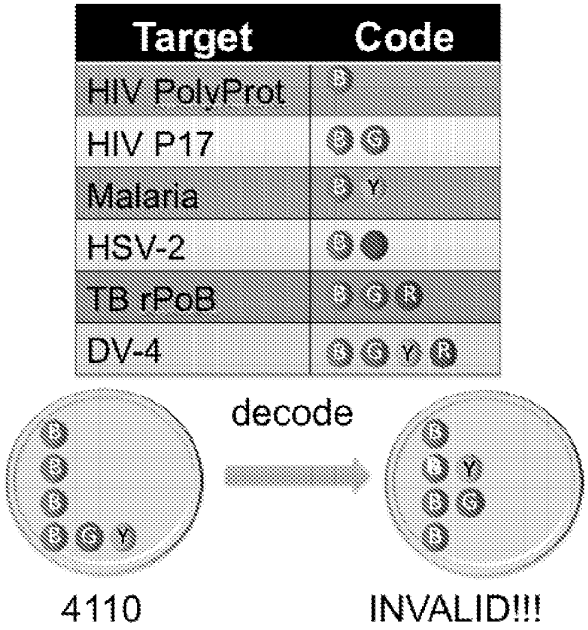

Signal (AFU)
Experiments
DEN+P17+TPP
DEN+P17
DEN+TPP
P17+TPP
DEN
P17
TPP
Coding:
DEN = 1x
P17 = 2x
TPP = 4x

Fig. 8


Target
Code
HIV PP
HIV P17
Mal
HSV
TB rPoB
DV
decode
4112
PP, Mal, HSV, TB


Target
Code
HIV PolyProt
HIV P17
Malaria
HSV-2
TB rPoB
DV-4
decode
4110
INVALID!!!

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 13/24509

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12Q 1/68 (2013.01)
USPC - 435/6.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
USPC 435/6.1
IPC C12Q 1/68 (2013.01)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC 435/6.11; 435/6.19 (Text search - see search terms below)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatBase, Google Scholar Search Terms: signal, encoding, decoding, multiplexed, biochemical, assays

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2010/0267064 A1 (KARTALOV et al) 21 October 2010 (21.10.2010) entire document	1-74
A	US 2005/0260640 A1 (ANDERSEN et al) 24 November 2005 (24.11.2005) entire document	1-74
A	US 2007/0231824 A1 (CHEE et al) 04 October 2007 (04.10.2007) entire document	1-74
A	US 2002/0022273 A1 (EMPEDOCLES et al) 21 February 2002 (21.02.2002) entire document	1-74
A	US 2010/0210472 A1 (EMPEDOCLES et al) 19 August 2010 (19.08.2010) entire document	1-74
A	US 2008/0069733 A1 (MALTEZOS et al) 20 March 2008 (20.03.2008) entire document	1-74
A	US 2009/0062129 A1 (McKERNAN et al) 05 March 2009 (05.03.2009) entire document	1-74
A	US 2011/0237459 A1 (NOVA et al) 29 September 2011 (29.09.2011) entire document	1-74
A	US 2005/0214753 A1 (SCHULTZ et al) 29 September 2005 (29.09.2005) entire document	1-74
A	US 2005/0164264 A1 (SHIPWASH) 28 July 2005 (28.07.2005) entire document	1-74

☐ Further documents are listed in the continuation of Box C. ☐

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

Date of the actual completion of the international search	Date of mailing of the international search report
22 March 2013 (22.03.2013)	12 APR 2013
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

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

Declarations under Rule 4.17:

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

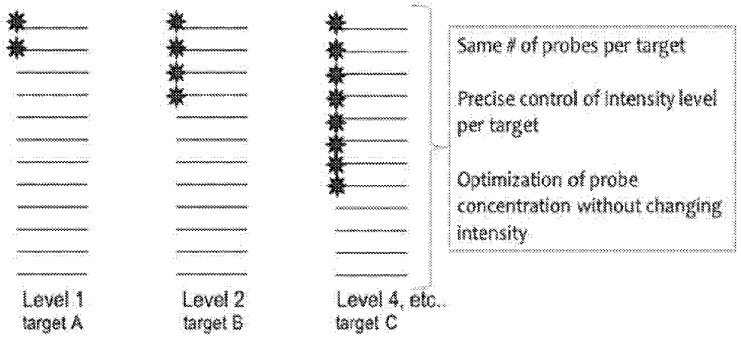
— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

Published:

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

(54) Title: COMPETITIVE PROBES FOR ENGINEERING SIGNAL GENERATION

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.

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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.

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[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

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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

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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

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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

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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

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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.

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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

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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 ratiometic 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

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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

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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

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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 baring probe can be a signal probe. A non-fluorophore baring 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 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 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 baring probe can be a signal probe. A non-fluorophore baring 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

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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 baring antibody can be a signal antibody. A non-fluorophore baring 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

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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

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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.

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[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 µL	11 µL	12 µL	13 µL	14 µL
Ambion Nuclease Free Water	AM9937	4 µL	4 µL	4 µL	4 µL	4 µL
Promega TE 1x (diluant)	V6231	6 µL	6 µL	6 µL	6 µL	6 µL
Oligo (concentation 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

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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 [μL]		20 μL	20 μL	20 μL	20 μL	20 μL

Table 2: Temperature Schedule on ABI Viia7 for figure 2 reactions

Temperature Schedule:	Temperature [°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,

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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.

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[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

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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

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Table 6. Reaction Master Mix – without competitive probes

Material	100 FP - BP	80 FP	60 FP	40 FP	20 FP
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 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/µL
RAT2	2E3 copies/µL
RBT1	2E3 copies/µL
NTC TE	NA (buffer only)

Table 8

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		

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

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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

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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

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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 completive 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-fluorscent, competitive) was the same for every target and identical to the total probe concentrations for the experiments shown in Figure 4A.

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[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, µM	Conc of 1X, µM	Vol, µL
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

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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

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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.

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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.

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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.

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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.

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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

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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

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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.

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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.

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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.

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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.

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99. A composition comprising:

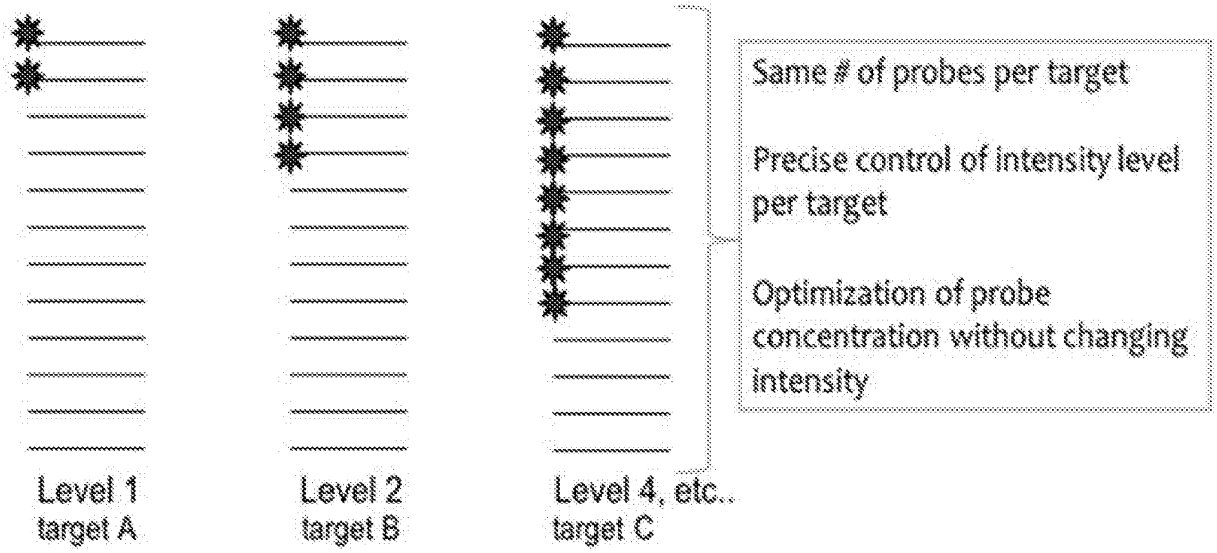
a set of paired oligonucleotides with complementarity to a target nucleic acid,

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.

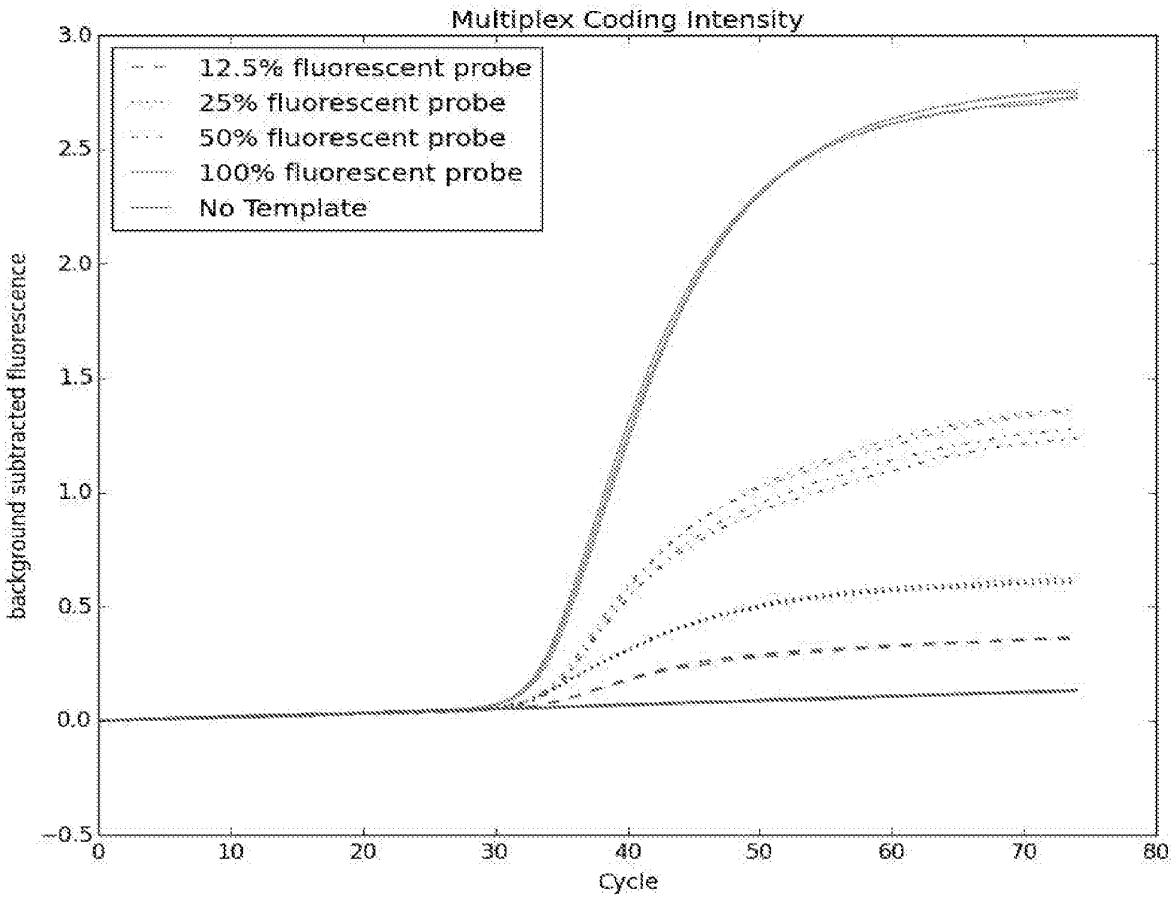
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FIG. 1



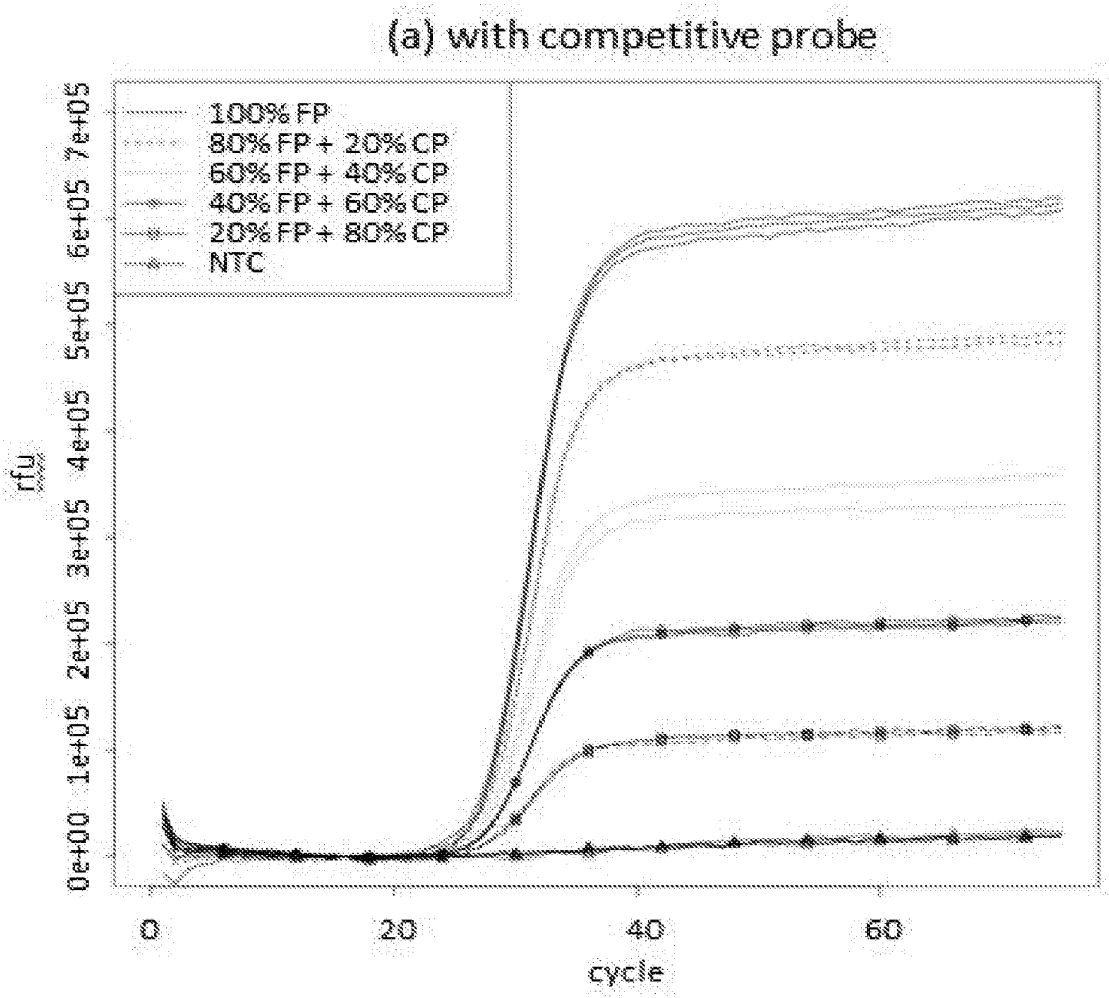
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FIG. 2



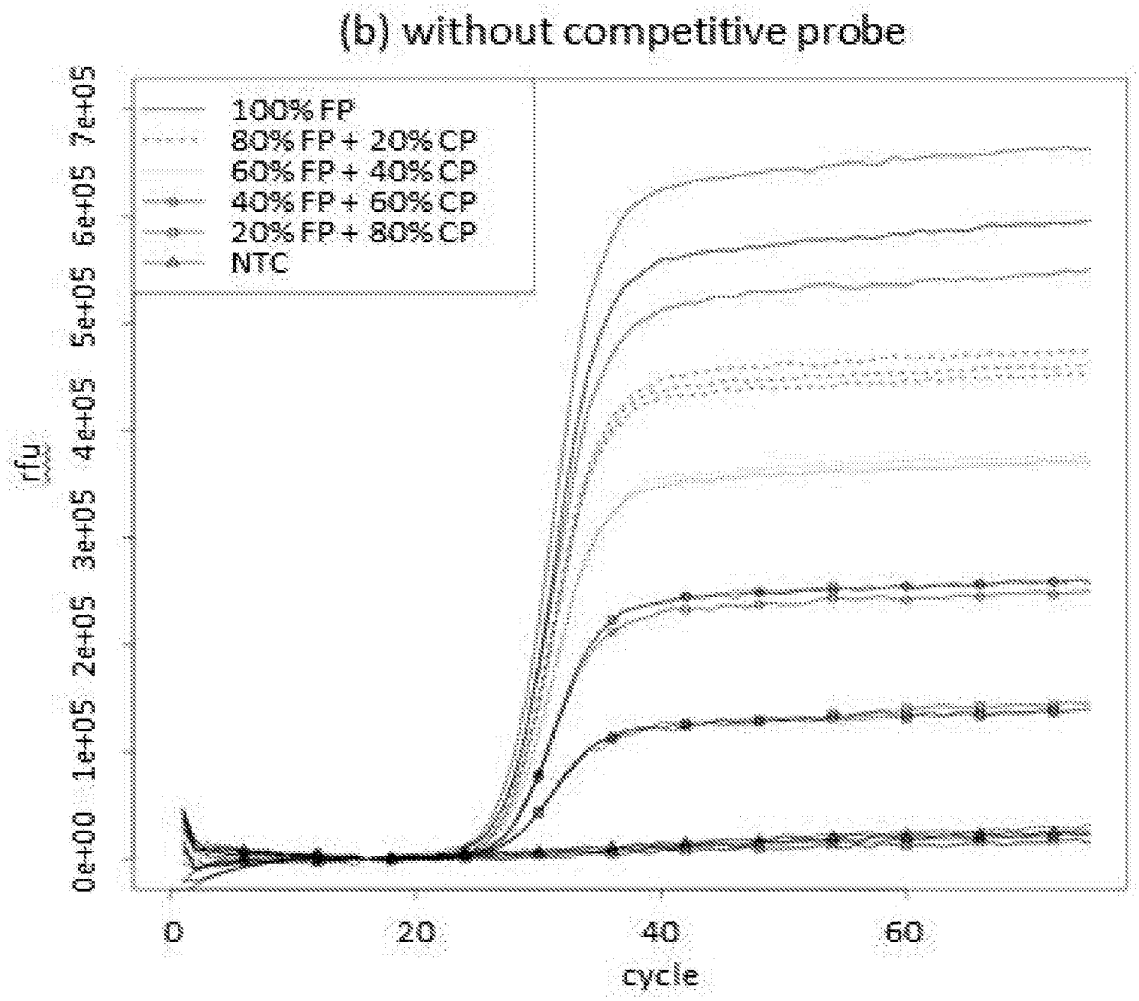
IPR2024-01178
California Institute of Technology - Ex. 2003 Page 605

FIG. 3A



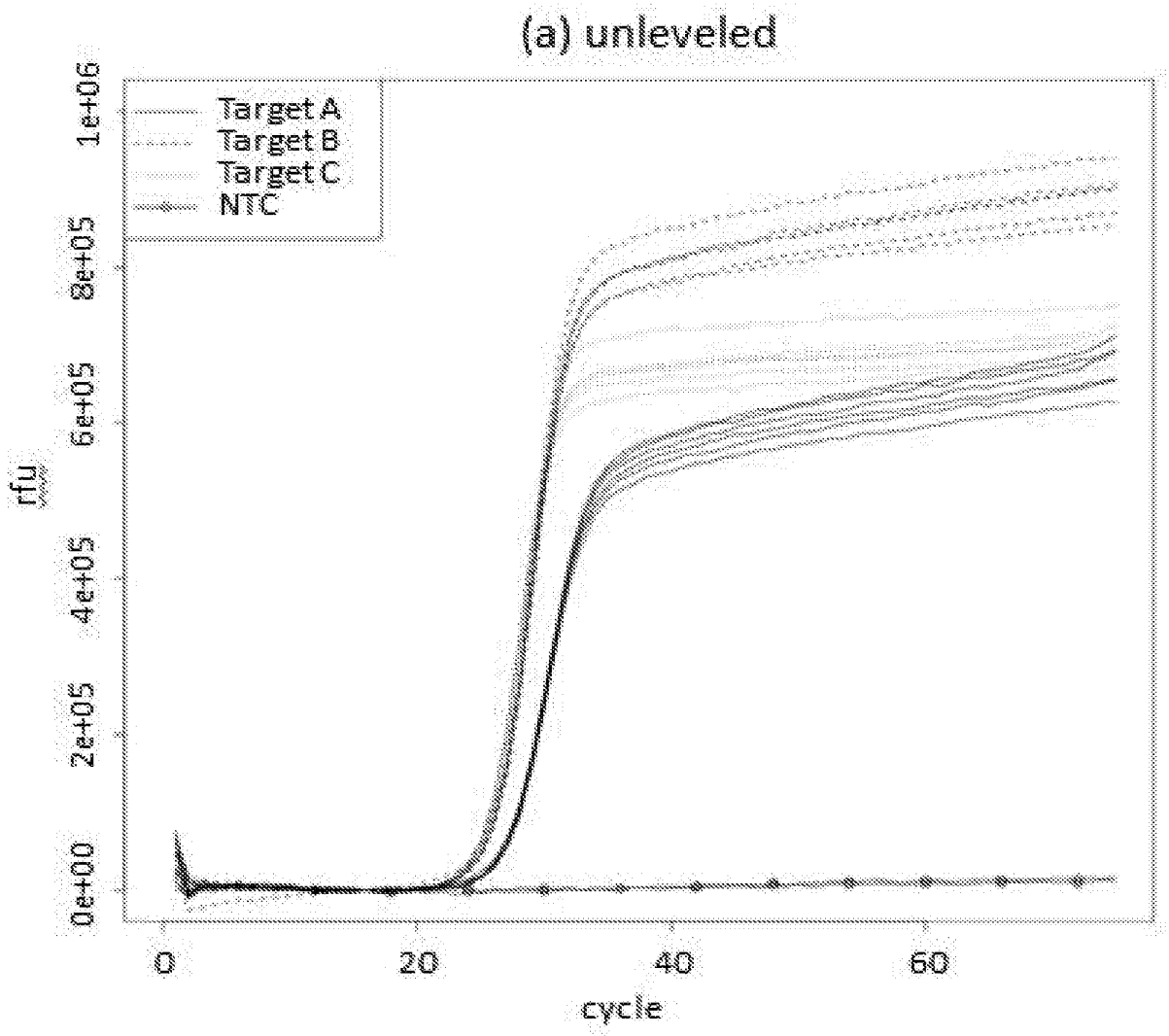
IPR2024-01178
California Institute of Technology - Ex. 2003 Page 606

FIG. 3B



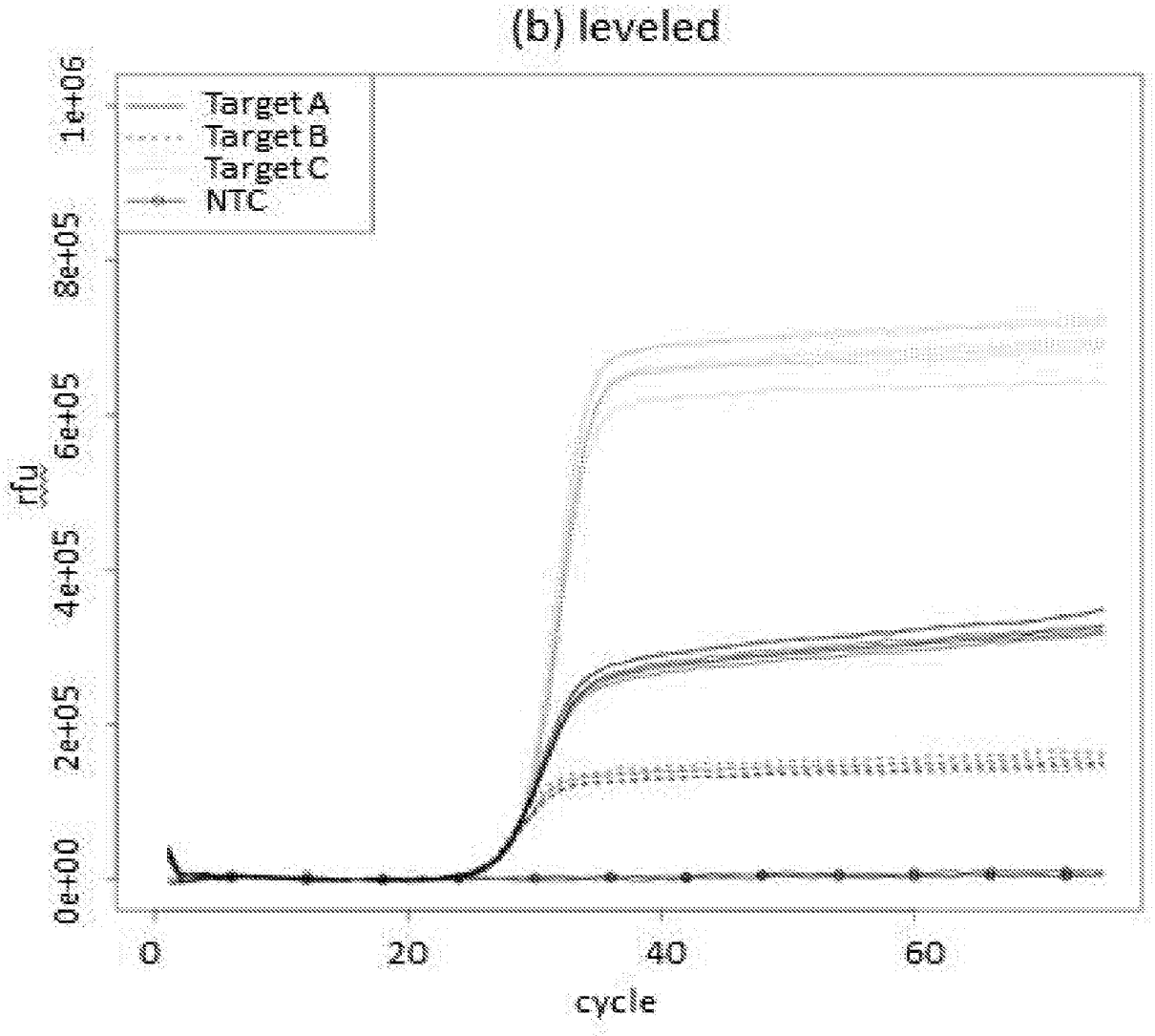
IPR2024-01178
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FIG. 4A



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FIG. 4B



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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	Date of mailing of the international search report
3 June 2017 (03.06.2017)	22 JUN 2017
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

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

ChromaCode / 44158-706.601
VN/ KSS/ LO6/ CGD/ LKIM/ ASE/ ANM

JUN 26 2017

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

To: Lee Alan Johnson
Wilson Sonsini Goodrich & Rosati
650 Page Mill Road
Palo Alto, California 94304
United States of America

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT AND THE WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY, OR THE DECLARATION

(PCT Rule 44.1)

Applicant's or agent's file reference: 44158-706601	Date of mailing (day/month/year): 22 JUN 2017
	FOR FURTHER ACTION See paragraphs 1 and 4 below
International application No: PCT/US17/24933	International filing date (day/month/year): 30 March 2017 (30.03.2017)
Applicant: CHROMACODE INC.	

1. ☒ The applicant is hereby notified that the international search report and the written opinion of the International Searching Authority have been established and are transmitted herewith.

Filing of amendments and statement under Article 19:
The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46).

When? The time limit for filing such amendments is normally two months from the date of transmittal of the international search report.

How? Directly to the International Bureau of WIPO preferably through ePCT or on paper to: 34, chemin des Colombettes 1211 Geneva 20, Switzerland, Facsimile No.: +41 22 338 82 70

For more detailed instructions, see *PCT Applicant's Guide*, International Phase, paragraphs 9.004 – 9.011.

2. ☐ The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect and the written opinion of the International Searching Authority are transmitted herewith.

3. ☐ **With regard to any protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:
   - ☐ the protest together with the decision thereon has been transmitted to the International Bureau together with any request to forward the texts of both the protest and the decision thereon to the designated Offices
   - ☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made

4. **Reminders**

The applicant may **submit comments on an informal basis on the written opinion of the International Searching Authority** to the International Bureau. These comments will be made available to the public after international publication. The International Bureau will send a copy of such comments to all designated Offices unless an international preliminary examination report has been or is to be established.

Shortly after the expiration of **18 months from the priority date, the international application will be published** by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau before the completion of the technical preparations for international publication (Rules 90*bis*.1 and 90*bis*.3).

Within **19 months** from the priority date, but only in respect of some designated Offices, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until **30 months** from the priority date (in some Offices even later); otherwise, the applicant must, **within 20 months** from the priority date, perform the prescribed acts for **entry into the national phase** before those designated Offices. In respect of other designated Offices, the time limit of **30 months** (or later) will apply even if no demand is filed within 19 months. For details about the applicable time limits, Office by Office, see www.wipo.int/pct/en/texts/time_limits.html and the *PCT Applicant's Guide*, National Chapters.

Within **19 months from the priority date, the applicant may request that a supplementary international search be carried out** by a different International Searching Authority that offers this service (Rule 45*bis*.1). The procedure for requesting supplementary international search is described in the *PCT Applicant's Guide*, International Phase, paragraphs 8.006-8.032.

Name and mailing address of the ISA/US: Mail Stop PCT, Attn. ISA/US, Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450	Authorized officer: Shane Thomas
Facsimile No. 571-273-8300	Telephone No. PCT Helpdesk: 571-272-4300, PCT OSP: 571-272-7774

Form PCT/ISA/220 (July 2014)

VISOR PATENT DOCKET
S: FOREIGN: ✓
DOCKETED: 6/26/17 BY:
ACTION: Search report 8/22/17
DUE DATES: written opinion 2/1/18
No related US case

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 44158-706601	FOR FURTHER ACTION	see Form PCT/ISA/220 as well as, where applicable, item 5 below.
International application No. PCT/US17/24933	International filing date *(day/month/year)* 30 March 2017 (30.03.2017)	(Earliest) Priority Date *(day/month/year)* 01 April 2016 (01.04.2016)
Applicant CHROMACODE INC.		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 3 sheets.

☐ It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language**, the international search was carried out on the basis of:

☒ the international application in the language in which it was filed.

☐ a translation of the international application into _____ which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).

b. ☐ This international search report has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43.6*bis*(a)).

c. ☐ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, see Box No. I.

2. ☒ **Certain claims were found unsearchable** (see Box No. II)

3. ☐ **Unity of invention is lacking** (see Box No. III).

4. With regard to the **title**,

☒ the text is approved as submitted by the applicant.

☐ the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

☒ the text is approved as submitted by the applicant.

☐ the text has been established, according to Rule 38.2, by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. With regard to the **drawings**,

a. the figure of the **drawings** to be published with the abstract is Figure No. 1

☒ as suggested by the applicant.

☐ as selected by this Authority, because the applicant failed to suggest a figure.

☐ as selected by this Authority, because this figure better characterizes the invention.

b. ☐ none of the figures is to be published with the abstract.

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California Institute of Technology - Ex. 2003 Page 613

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US17/24633

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.

IPR2024-01178
California Institute of Technology - Ex. 2003 Page 614

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], [0092]	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 PY, et al.) 16 February 2012; paragraphs [0030]-[0034], [0064]	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	Date of mailing of the international search report
3 June 2017 (03.06.2017)	22 JUN 2017
Name and mailing address of the ISA/	Authorized officer
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

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

PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To: Lee Alan Johnson
Wilson Sonsini Goodrich & Rosati
650 Page Mill Road
Palo Alto, California 94304
United States of America

PCT

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43*bis*.1)

Date of mailing *(day/month/year)* 22 JUN 2017

Applicant's or agent's file reference
44158-706601

FOR FURTHER ACTION
See paragraph 2 below

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US17/24933	30 March 2017 (30.03.2017)	01 April 2016 (01.04.2016)

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

Applicant CHROMACODE INC.

1. This opinion contains indications relating to the following items.

- ☒ Box No. I Basis of the opinion
- ☐ Box No. II Priority
- ☒ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the international application
- ☐ Box No. VIII Certain observations on the international application

2. FURTHER ACTION

If a demand for international preliminary examination is made, this opinion will be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1*bis*(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

Name and mailing address of the ISA/US	Date of completion of this opinion	Authorized officer
Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	03 June 2017 (03.06.2017)	Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/237 (cover sheet) (January 2015)

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US17/24933

Box No. I Basis of this opinion

1. With regard to the **language**, this opinion has been established on the basis of:

☒ the international application in the language in which it was filed.

☐ a translation of the international application into _____ which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).

2. ☐ This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43*bis*.1(a)).

3. ☐ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, this opinion has been established on the basis of a sequence listing:

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

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

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

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

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

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

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

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

5. Additional comments:

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WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US17/24933

Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 6-42 and 49-82

because:

☐ the said international application, or the said claims Nos. _____ relate to the following subject matter which does not require an international search *(specify)*:

☒ the description, claims or drawings *(indicate particular elements below)* or said claims Nos. 6-42 and 49-82 are so unclear that no meaningful opinion could be formed *(specify)*:

because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

☐ the claims, or said claims Nos. _____ are so inadequately supported by the description that no meaningful opinion could be formed *(specify)*:

☒ no international search report has been established for said claims Nos. 6-42 and 49-82

☐ a meaningful opinion could not be formed without the sequence listing: the applicant did not, within the prescribed time limit:

☐ furnish a sequence listing in the form of an Annex C/ST.25 text file, and such listing was not available to the International Searching Authority in the form and manner acceptable to it; or the sequence listing furnished did not comply with the standard provided for in Annex C of the Administrative Instructions.

☐ furnish a sequence listing on paper or in the form of an image file complying with the standard provided for in Annex C of the Administrative Instructions, and such listing was not available to the International Searching Authority in the form and manner acceptable to it; or the sequence listing furnished did not comply with the standard provided for in Annex C of the Administrative Instructions.

☐ pay the required late furnishing fee for the furnishing of a sequence listing in response to an invitation under Rule 13*ter*.1(a) or (b).

☐ See Supplemental Box for further details.

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California Institute of Technology - Ex. 2003 Page 618

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US17/24933

Box No. V **Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement**

1. Statement

Novelty (N)	Claims	***see box below****	YES
	Claims	NONE	NO
Inventive step (IS)	Claims	***see box below***	YES
	Claims	NONE	NO
Industrial applicability (IA)	Claims	***see box below***	YES
	Claims	NONE	NO

2. Citations and explanations:

Novelty (N): YES; 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

Inventive step (IS): YES; 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

Industrial applicability (IA): 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
****

Claims 1-4, 5/3-4, 43-47, 48/45-47, 83-87, 88/83-87, 89/83-87, 90/83-87, 90/83-87, 91-97, 98/91-97, and 99 meet the criteria set out in PCT Article 33(2)-(3).

Claim 1 meets the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest 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.

WO 2006/079049 A2 to Third Wave Technologies, Inc. (hereinafter 'Wave') discloses method of identifying a first target nucleic acid (method of detection of a target nucleic acids (first); page 2, lines 10-14;; comprising, providing a sample comprising the first target nucleic acid (providing a sample with target nucleic acids (first); page 2, lines 10-14), providing a first set of paired oligonucleotides with complementarity to the first target nucleic acid (first and second probe oligonucleotides that occupy probe hybridization site on the target nucleic acid (first); page 2, lines 23-31), said first set of paired oligonucleotides comprising a first ratio (ratio of paired oligonucleotides, first probe oligonucleotides to second probe oligonucleotides, page 3, lines 13-20; page 6, lines 28-30) of (a) first oligonucleotides (first probe oligonucleotides; page 2, lines 23-31) to (b) first signal oligonucleotides comprising a first signal tag (first oligonucleotides have a first label; page 5, lines 5-8), wherein the first competitive oligonucleotides compete with the first signal oligonucleotides for binding to the first target nucleic acid (first probe oligonucleotides and second probe oligonucleotides are configured to bind the target nucleic acid with same affinity and identical sequences (competitive); page 15, lines 24-31; page 16, lines 6-8), amplifying the first target nucleic acid with the polymerase chain reaction (amplifying the first target nucleic acid with TAQMAN assay (PCR); page 6, lines 18-28; page 7, lines 14-19), thereby degrading the first signal oligonucleotide and permitting generation of a first signal (cleavage (degrading) of first probe oligonucleotide containing a label and allowing generation of a signal (first); page 7, lines 14-25) , generating the first signal (generating a first signal; page 18, lines 14-16), measuring intensity of the first signal (measuring the strength of the signal; page 18, lines 22-28), and thereby identifying the first target nucleic acid (identifying the target nucleic acid; page 7, lines 14-19), but Wave does not disclose (a) first competitive oligonucleotides, and correlating the intensity of the first signal to the first ratio.

US 2009/0042736 A1 to Blair, et al. (hereinafter 'Blair') discloses method of identifying a first target nucleic acid (method of identifying a target nucleic acid; paragraph [0018]) comprising, providing a sample comprising the first target nucleic acid (one target nucleic acid is present in a given sample; paragraphs [0035], [0041]), providing a first set of paired oligonucleotides with complementarity to the first target nucleic acid (providing a set (first) of paired nucleic acid probes that can hybridize (complementarity) to target (first) nucleic acids; paragraphs [0041]-[0042]), said first set of paired oligonucleotides comprising a first ratio (set of paired probe and competitive labeled probe nucleic acids (first set), would inherently have a ratio; paragraphs [0041]-[0042]) of (a) first competitive oligonucleotides to (competitive probe nucleic acid; paragraphs [0041]-[0042]) (b) first signal oligonucleotides comprising a first signal tag (labeled competitor nucleic acids (first signal); paragraphs [0041]-[0042]), wherein the first competitive oligonucleotides compete with the first signal oligonucleotides for binding to the first target nucleic acid (probe nucleic acids (first) competes with labeled competitor nucleic acids; paragraphs [0041]-[0042]), amplifying the first target nucleic acid with the polymerase chain reaction (amplifying the first target nucleic acid with a polymerase chain reaction; paragraph [0072]), but Blair does not disclose 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.

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Supplemental Box

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US 2014/0213471 A1 to California Institute of Technology (hereinafter 'CalTech') discloses method of identifying a first target nucleic acid (method of detecting at least one polynucleotide analyte (target); paragraph [0003]) comprising, providing a sample comprising the first target nucleic acid (providing a sample comprising a first polynucleotide analyte (target); paragraph [0003]), providing a first set of paired oligonucleotides with complementarity to the first target nucleic acid (providing a set of first primer and a first oligonucleotide specific (complementarity) for binding a first polynucleotide analyte (target; paragraph [0003]), said first set of paired oligonucleotides comprising a first ratio (set of paired probe labeled nucleic acids (first set), would inherently have a ratio; paragraph [0003]) of (b) first signal oligonucleotides comprising a first signal tag (first primer oligonucleotide comprises a first signal tag; paragraph [0011]), amplifying the first target nucleic acid with the polymerase chain reaction (amplifying the first polynucleotide analyte using a polymerase chain reaction; paragraph [0010]), generating the first signal (generating a first signal; paragraph [0011]), measuring intensity of the first signal (measuring a first signal; paragraph [0011]), thereby identifying the first target nucleic acid (identifying the first polynucleotide analyte (target); paragraph [0011]), but Caltech does not disclose (a) first competitive oligonucleotides to, wherein the first competitive oligonucleotides compete with the first signal oligonucleotides for binding to the first target nucleic acid, thereby degrading the first signal oligonucleotide and permitting generation of a first signal, and correlating the intensity of the first signal to the first ratio.
US 2012/0040349 A1 to Lode, et al. (hereinafter 'Lode') discloses method of identifying a first target nucleic acid (method of detecting target nucleic acids; paragraphs [0031]) comprising, providing a sample comprising the first target nucleic acid (providing a sample with target nucleic acids; paragraph [0032]), providing a first set of paired oligonucleotides with complementarity to the first target nucleic acid (providing a set (first) of paired oligonucleotides that are complementary to the target nucleic acids (first); paragraph [0032]), said first set of paired oligonucleotides comprising: a first ratio of said first set of paired oligonucleotides comprising a first ratio (set (first) of paired oligonucleotides would inherently have a ratio; paragraph [0032]) of (a) first oligonucleotides (quencher probe oligonucleotide; paragraph [0032]-[0033]) to (b) first signal oligonucleotides comprising a first signal tag (label probe oligonucleotide for target nucleic acids (first); paragraphs [0032]-[0033]), amplifying the first target nucleic acid with the polymerase chain reaction (amplifying the target nucleic acids (first) by polymerase chain reaction; paragraph [0034]), but Lode does not disclose (a) first competitive oligonucleotides, wherein the first competitive oligonucleotides compete with the first signal oligonucleotides for binding to the first target nucleic acid, 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.
Wave, Blair, Caltech, and Lode, fail to disclose either solely or in combination the method of correlating the intensity of the first signal to the first ratio. Wave discloses ratios of a first probe oligonucleotide with a label and a second probe oligonucleotide with a label, pair of oligonucleotides, that are complimentary to a target nucleic acids, amplifying the target nucleic acids, detecting the cleavage (degradation) of first and second probes (page 5, lines 23-30; page 7, lines 14-19; page 15, lines 25-31, page 16, lines 1-3), but Wave does not disclose correlating the intensity of the first signal to the first ratio. Caltech discloses method of detecting a plurality of polynucleotide analytes using oligonucleotides with attached chromophore in polymerization techniques and measuring signals to detect presence or absence of analytes in samples (paragraph [0011], [0045], [0082]), but Caltech does not disclose correlating the intensity of the first signal to the first ratio. Further there is no motivation to combine the references of record without the advantage of permissible hindsight.

Claim 2-4 and 5/3-4 meet the criteria set out in PCT Article 33(2)-(3), based on their dependency on positive claim 1.

Claim 43 meets the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest 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.
Wave discloses a composition (composition; page 2, lines 15-17) comprising: a first set of paired oligonucleotides with complementarity to a first target nucleic acid (first and second probe oligonucleotides that occupy probe hybridization site on the target nucleic acid (first); page 2, lines 23-31), said first set of paired oligonucleotides comprising a first ratio (ratio of paired oligonucleotides, first probe oligonucleotides to second probe oligonucleotides, page 3, lines 13-20; page 6, lines 28-30) of (a) oligonucleotides (first probe oligonucleotides; page 2, lines 23-31) to (b) signal oligonucleotides comprising a signal tag (first oligonucleotides have a first label; page 5, lines 5-8), 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 (cleavage (degrading) of first probe oligonucleotide containing a label by a polymerase chain reaction and allowing generation of a signal (first); page 7, lines 14-25) and excited by an energy source (generating a first detectable signal in FRET (exited by an energy source); page 18, lines 14-16), but Wave does not disclose (a) competitive oligonucleotides, 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, and wherein the first intensity correlates with the first ratio.
Blair discloses a composition (composition; paragraph [0016]) comprising: a first set of paired oligonucleotides with complementarity to a first target nucleic acid (providing a set of paired probe nucleic acids and labeled probe nucleic acids that competitive to target nucleic acids; paragraph [0041]-[0042]), said first set of paired oligonucleotides comprising a first ratio (set of paired probe and competitive labeled probe nucleic acids, would inherently be in a ratio; paragraphs [0042]-[0043]) of (a) competitive oligonucleotides to (competitive probe nucleic acid; paragraphs [0041]-[0042]) (b) signal oligonucleotides comprising a signal tag (labeled competitor nucleic acids (first signal); paragraphs [0041]-[0042]), wherein the competitive oligonucleotides are configured to compete with the signal oligonucleotides for binding to the first target nucleic acid (probe nucleic acids (first) competes with labeled competitor nucleic acids (first); paragraphs [0041]-[0042], [0072]), but Blair does not disclose 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.

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Caltech discloses a composition (compositions; paragraph [0003]) comprising: a first set of paired oligonucleotides with complementarity to a first target nucleic acid (providing a set of first primer and a first oligonucleotide specific (complementarity) for binding a first polynucleotide analyte (target; paragraph [0003]), said first set of paired oligonucleotides comprising a first ratio (set of paired probe labeled nucleic acids (first set) would inherently have a ratio; paragraph [0003]) of (b) signal oligonucleotides comprising a signal tag (first primer oligonucleotide comprises a first signal tag; paragraph [0011]), but Caltech does not disclose (a) competitive oligonucleotides to, 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.

Lode discloses a composition (composition; paragraph [0054]) comprising: a first set of paired oligonucleotides with complementarity to a first target nucleic acid (providing a set (first) of paired oligonucleotides that are complementary to the target nucleic acids (first); paragraph [0032]), said first set of paired oligonucleotides comprising a first ratio (set (first) of paired oligonucleotides would inherently have a ratio; paragraph [0032]) of (a) oligonucleotides (quencher probe oligonucleotide; paragraph [0032]-[0033]) to (b) signal oligonucleotides comprising a signal tag (label probe oligonucleotide for target nucleic acids (first); paragraphs [0032]-[0033]), but Lode does not disclose (a) competitive oligonucleotides, 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.

Wave, Blair, Caltech, and Lode, fail to disclose either solely or in combination a composition 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. Wave discloses ratios of a first probe oligonucleotide with a label and a second probe oligonucleotide with a label, pair of oligonucleotides, that are complimentary to a target nucleic acids, amplifying the target nucleic acids, detecting the cleavage (degradation) of first and second probes (page 5, lines 23-30; page 7, lines 14-19, page 15, lines 25-31; page 16, lines 1-3), but Wave does not disclose 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 correlating the intensity of the first signal to the first ratio. Caltech discloses method of detecting a plurality of polynucleotide analytes using oligonucleotides with attached chromophore in polymerization techniques and measuring signals to detect presence or absence of analytes in samples (paragraph [0011], [0045], [0082]), but Caltech does not disclose 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 correlating the intensity of the first signal to the first ratio. Further there is no motivation to combine the references of record without the advantage of permissible hindsight.

Claim 44 meets the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest 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.

Wave discloses method of identifying a first target molecule (method of detection of a target nucleic acids (first target molecule); page 2, lines 10-14);comprising, providing a sample comprising the first target molecule (providing a sample with target nucleic acids (first target molecule); page 2, lines 10-14), providing a first set of paired capture molecules that bind to the first target molecule (first and second probe oligonucleotides (capture molecules) that occupy probe hybridization site on the target nucleic acid (first target molecule); page 2, lines 23-31), said first set of paired capture molecules comprising a first ratio (ratio of paired oligonucleotides (capture molecules), first probe oligonucleotides to second probe oligonucleotides; page 3, lines 13-29, page 6, lines 28-30) of (a) first capture molecules (first probe oligonucleotides; page 2, lines 23-31) to (b) first signal capture molecules comprising a signal tag (first probe oligonucleotides (capture molecules) have a first label; page 8, lines 5-8), generating the first signal (generating a first signal, page 16, lines 14-16), measuring intensity of the first signal (measuring the strength of the signal; page 18, lines 22-26), and thereby identifying the first target molecule (identifying the target nucleic acid; page 7, lines 14-19), but Wave does not disclose (a) first competitive capture molecules, wherein the competitive capture molecules compete with the signal capture molecules for binding to the first target molecule, and correlating the intensity of the first signal to the first ratio.

Blair discloses method of identifying a first target molecule (method of identifying a target nucleic acid (first target molecule; paragraph [0018]) comprising, providing a sample comprising the first target molecule (one target nucleic acid (first target molecule) is present in a given sample; paragraphs [0035], [0041]), providing a first set of paired capture molecules that bind to the first target molecule (providing a set (first) of paired nucleic acid probes (capture molecules) that can hybridize (complementarity) to target (first) nucleic acids; paragraphs [0041]-[0042]), said first set of paired capture molecules comprising a first ratio (set of paired probe and competitive labeled probe nucleic acids (first set paired capture molecules), would inherently have a ratio; paragraphs [0041]-[0042]) of (a) first competitive capture molecules (competitive probe nucleic acid; paragraphs [0041]-[0042]) to (b) first signal capture molecules comprising a signal tag (labeled competitor nucleic acids (first signal); paragraphs [0041]-[0042]), wherein the competitive capture molecules compete with the signal capture molecules for binding to the first target molecule (probe nucleic acids (capture molecule) competes with labeled competitor nucleic acids (labeled capture molecule); paragraphs [0041]-[0042]), but Blair does not disclose 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.

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Supplemental Box

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Caltech discloses method of identifying a first target molecule (method of detecting at least one polynucleotide analyte (first target molecule); paragraph [0003]) comprising, providing a sample comprising the first target molecule (providing a sample comprising a first polynucleotide analyte (first target molecule); paragraph [0003]), providing a first set of paired capture molecules that bind to the first target molecule (providing a set of first primer and a first oligonucleotide specific (complementarity) for binding a first polynucleotide analyte (first target molecule); paragraph [0003]), said first set of paired capture molecules comprising a first ratio of (set of paired probe labeled nucleic acids (first set), would inherently have a ratio; paragraph [0003]), to (b) first signal capture molecules comprising a signal tag (first primer oligonucleotide (first capture molecules) comprises a signal tag; paragraph [0011]), generating the first signal (generating a first signal; paragraph [0011]), measuring intensity of the first signal (measuring a first signal; paragraph [0011]), and thereby identifying the first target molecule (identifying the first polynucleotide analyte (target); paragraph [0011]), but Caltech does not disclose (a) first competitive capture molecules, wherein the competitive capture molecules compete with the signal capture molecules for binding to the first target molecule, and correlating the intensity of the first signal to the first ratio.
Lode discloses method of identifying a first target molecule (method of detecting target nucleic acids (first target molecule); paragraphs [0031]) comprising, providing a sample comprising the first target molecule (providing a sample with target nucleic acids (first target molecule); paragraph [0032]), providing a first set of paired capture molecules that bind to the first target molecule providing a set (first) of paired oligonucleotides (capture molecule) that are complementary to the target nucleic acids (first target molecule); paragraph [0032]), said first set of paired capture molecules comprising a first ratio (set (first) of paired oligonucleotides (capture molecules) would inherently have a ratio; paragraph [0032]) of (a) first capture molecules (quencher probe oligonucleotide; paragraph [0032]-[0033]) to (b) first signal capture molecules comprising a signal tag (label probe oligonucleotide for target nucleic acids (first); paragraphs [0032]-[0033]), but Lode does not disclose (a) first competitive capture molecules, 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.
Wave, Blair, Caltech, and Lode, fail to disclose either solely or in combination the method of correlating the intensity of the first signal to the first ratio. Wave discloses ratios of a first probe oligonucleotide (capture molecule) with a label and a second probe oligonucleotide (capture molecule) with a label, pair of oligonucleotides, that are complimentary to a target nucleic acids (target molecule), amplifying the target nucleic acids, detecting the cleavage (degradation) of first and second probes (page 5, lines 23-30; page 7, lines 14-19; page 15, lines 25-31; page 16, lines 1-3), but Wave does not disclose wherein the competitive capture molecules compete with the signal capture molecules for binding to the first target molecule and correlating the intensity of the first signal to the first ratio. Caltech discloses method of detecting a plurality of polynucleotide analytes using oligonucleotides with attached chromophore in polymerization techniques and measuring signals to detect presence or absence of analytes in samples (paragraph [0011], [0045], [0082]), but Caltech does not disclose wherein the competitive capture molecules compete with the signal capture molecules for binding to the first target molecule and correlating the intensity of the first signal to the first ratio. Further there is no motivation to combine the references of record without the advantage of permissible hindsight

Claim 45-47 and 48/45-47 meet the criteria set out in PCT Article 33(2)-(3), based on their dependency on positive claim 44.

Claim 83 meets the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest 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.
Wave discloses a composition (composition; page 2, lines 15-17) comprising: a first set of paired capture molecules with complementarity to a first target molecule (first and second probe oligonucleotides (capture molecules) that occupy probe hybridization site (complimentary) on the target nucleic acid (first target molecule); page 2, lines 23-31), said first set of paired capture molecules comprising a first ratio (ratio of paired oligonucleotides (capture molecules), first probe oligonucleotides to second probe oligonucleotides; page 3, lines 13-20; page 6, lines 28-30) of (a) capture molecules (first probe oligonucleotides (capture molecules); page 2, lines 23-31) to (b) signal capture molecules comprising a signal tag (first oligonucleotides (capture molecules) have a first label; page 5, lines 5-8), but Wave does not disclose (a) competitive capture molecules, 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.
Blair discloses a composition (composition; paragraph [0016]) comprising: a first set of paired capture molecules with complementarity to a first target molecule (providing a set of paired probe nucleic acids and labeled probe nucleic acids that competitive to target nucleic acids (target molecule); paragraph [0041]-[0042]), said first set of paired capture molecules comprising a first ratio (set of paired probe and competitive labeled probe nucleic acids (capture molecules), would inherently be in a ratio; paragraphs [0042]-[0043]) of (a) competitive capture molecules (competitive probe nucleic acid (capture molecules); paragraphs [0041]-[0042]) to (b) signal capture molecules comprising a signal tag (labeled competitor nucleic acids (signal capture molecules); paragraphs [0041]-[0042]), wherein the competitive capture molecules are configured to compete with the signal capture molecules for binding to the target molecule (probe nucleic acids (capture molecules) competes with labeled competitor nucleic acids (signal capture molecules) for binding to target nucleic acids (target molecule); paragraphs [0041]-[0042], [0072]), but Blair does not disclose 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.

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Supplemental Box

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Caltech discloses a composition (compositions; paragraph [0003]) comprising: a first set of paired capture molecules with complementarity to a first target molecule (providing a set of first primer and a first oligonucleotide (capture molecules) specific (complementarity) for binding a first polynucleotide analyte (first target molecule); paragraph [0003]), said first set of paired capture molecules comprising a first ratio (set of paired probe labeled nucleic acids (first set of paired capture molecules) would inherently have a ratio; paragraph [0003]) of (b) signal capture molecules comprising a signal tag (first primer oligonucleotide comprises a first signal tag (signal capture molecules; paragraph [0011]), but Caltech does not disclose (a) competitive capture molecule, 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.
Lode discloses a composition (composition; paragraph [0054]) comprising: a first set of paired capture molecules with complementarity to a first target molecule (providing a set (first) of paired oligonucleotides (capture molecules) that are complementary to the target nucleic acids (first target molecule); paragraph [0032]), said first set of paired capture molecules comprising a first ratio (set (first) of paired oligonucleotides (capture molecules) would inherently have a ratio; paragraph [0032]) of (a) competitive capture molecules (quencher probe oligonucleotide; paragraph [0032]-[0033]) to (b) signal capture molecules comprising a signal tag (label probe oligonucleotide (signal capture molecule); paragraphs [0032]-[0033]), but Lode does not disclose (a) competitive capture molecules, 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.
Wave, Blair, Caltech, and Lode, fail to disclose either solely or in combination 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. Wave discloses ratios of a first probe oligonucleotide (capture molecules) with a label and a second probe oligonucleotide with a label, pair of oligonucleotides, that are complimentary to a target nucleic acids (target molecules), amplifying the target nucleic acids, detecting the cleavage (degradation) of first and second probes (page 5, lines 23-30; page 7, lines 14-19; page 15, lines 25-31; page 16, lines 1-3), but Wave does not disclose 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. Caltech discloses method of detecting a plurality of polynucleotide analytes using oligonucleotides with attached chromophore in polymerization techniques and measuring signals to detect presence or absence of analytes in samples (paragraph [0011], [0045], [0062]), but Caltech does not disclose 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. Further there is no motivation to combine the references of record without the advantage of permissible hindsight.

Claim 84-87, 88/83-87, 89/83-87, and 90/83-87 meet the criteria set out in PCT Article 33(2)-(3), based on their dependency on positive claim 83.

Claim 91 meets the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly 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
Wave discloses method of altering amplification level of a target nucleic acid (method of altering amplification level of target nucleic acid; page 6, lines 24-30) comprising, providing a sample comprising the target nucleic acid (providing a sample with target nucleic acids (first); page 2, lines 10-14),, providing a set of paired oligonucleotides with complementarity to the target nucleic acid (first and second probe oligonucleotides that occupy probe hybridization site on the target nucleic acid; page 2, lines 23-31), said set of paired oligonucleotides comprising a ratio (ratio of paired oligonucleotides, first probe oligonucleotides to second probe oligonucleotides; page 3, lines 13-20; page 6, lines 28-30) of (a) competitive oligonucleotides to (b) functional oligonucleotides (oligonucleotides having a label (functional); page 5, lines 5-8), but Wave does not disclose 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.
Blair discloses method of altering amplification level of a target nucleic acid (method of altering amplification of target nucleic acids; paragraphs [0072], [0074]) comprising, providing a sample comprising the target nucleic acid (one target nucleic acid is present in a given sample; paragraphs [0035], [0041]), providing a set of paired oligonucleotides with complementarity to the target nucleic acid (providing a set (first) of paired nucleic acid probes that can hybridize (complementarity) to target (first) nucleic acids; paragraphs [0041]-[0042]), said set of paired oligonucleotides comprising a ratio (set of paired probe and competitive labeled probe nucleic acids (first set) would inherently have a ratio; paragraphs [0041]-[0042]) of (a) competitive oligonucleotides (competitive probe nucleic acid; paragraphs [0041]-[0042]) to (b) functional oligonucleotides (labeled competitor nucleic acids (functional); paragraphs [0041]-[0042]), but Blair does not disclose 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.

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WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US17/24933

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Caltech discloses method of altering amplification level of a target nucleic acid (method of amplification of polynucleotide analyte (target nucleic acid); paragraph [0003]) comprising, providing a sample comprising the target nucleic acid (providing a sample comprising a first polynucleotide analyte (first target molecule); paragraph [0003]), providing a set of paired oligonucleotides with complementarity to the target nucleic acid (providing a set of first primer and a first oligonucleotide specific (complementarity) for binding a first polynucleotide analyte (first target molecule); paragraph [0003]), said set of paired oligonucleotides comprising a ratio (set of paired probe labeled nucleic acids (first set), would inherently have a ratio; paragraph [0003]) of (a) competitive oligonucleotides to (b) functional oligonucleotides (nucleotide with signal (functional oligonucleotide; paragraph [0011]), but Caltech does not disclose (a) competitive 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.
Lode discloses method of altering amplification level of a target nucleic acid (method for amplification of detected nucleic acids; paragraph [0030]) comprising, providing a sample comprising the target nucleic acid (providing a sample with target nucleic acids; paragraph [0032]), providing a set of paired oligonucleotides with complementarity to the target nucleic acid (providing a set (first) of paired oligonucleotides that are complementary to the target nucleic acids (first); paragraph [0032]), said set of paired oligonucleotides comprising a ratio (set (first) of paired oligonucleotides would inherently have a ratio: paragraph [0032]) of (a) competitive oligonucleotides (quencher probe oligonucleotide; paragraph [0032]-[0033]) to (b) functional oligonucleotides (label probe oligonucleotide (functional); paragraphs [0032]-[0033]), but Lode does not discloses 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.
Wave, Blair, Caltech, and Lode, fail to disclose either solely or in combination the method 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. Wave discloses ratios of a first probe oligonucleotide with a label and a second probe oligonucleotice with a label (functional), pair of oligonucleotides, that are complimentary to a target nucleic acids, amplifying the target nucleic acids, detecting the cleavage (degradation) of first and second probes (page 5, lines 23-30; page 7, lines 14-19; page 15, lines 25-31; page 16, lines 1-3), but Wave does not disclose 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. Caltech discloses method of detecting a plurality of polynucleotide analytes using oligonucleotides with attached chromophore in polymerization techniques and measuring signals to detect presence or absence of analytes in samples (paragraph [0011], [0045], [0052]), but Caltech does not disclose 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. Further there is no motivation to combine the references of record without the advantage of permissible hindsight.

Claim 92-97 and 98/91-97 meet the criteria set out in PCT Article 33(2)-(3), based on their dependency on positive claim 91.

Claim 98 meets the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly 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.
Wave discloses a composition (composition: page 2, lines 15-17) comprising: a set of paired oligonucleotides with complementarity to a target nucleic acid (first and second probe oligonucleotides that occupy probe hybridization site on the target nucleic acid (first); page 2, lines 23-31), said set of paired oligonucleotides comprising a ratio (ratio of paired oligonucleotides, first probe oligonucleotides to second probe oligonucleotides; page 3, lines 13-20; page 6, lines 28-30) of (b) functional oligonucleotides (first oligonucleotides have a first label; page 5, lines 5-8), but Wave does not disclose (a) competitive 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.
Blair discloses a composition (composition; paragraph [0016]) comprising: a set of paired oligonucleotides with complementarity to a target nucleic acid molecule (providing a set of paired probe nucleic acids and labeled probe nucleic acids that are competitive to target nucleic acids (target molecule); paragraph [0041]-[0042]), said set of paired oligonucleotides comprising a ratio of (set of paired probe and competitive labeled probe nucleic acids (capture molecules), would inherently be in a ratio; paragraphs [0042]-[0043]) (a) competitive oligonucleotides (competitive probe nucleic acid (capture molecules); paragraphs [0041]-[0042]) to (b) functional oligonucleotides (labeled competitor nucleic acids (functional oligonucleotides); paragraphs [0041]-[0042]), wherein the competitive oligonucleotides are configured to compete with the functional oligonucleotides (probe nucleic acids (capture molecules) competes with labeled competitor nucleic acids (signal capture molecules) for binding to target nucleic acids (target molecule); paragraphs [0041]-[0042], [0072]), but Blair does not disclose 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.

-***-Continued Within the Next Supplemental Box-***-.

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California Institute of Technology - Ex. 2003 Page 624

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US17/24933

Supplemental Box

In case the space in any of the preceding boxes is not sufficient.
Continuation of:

-***-Continued from Previous Supplemental Box-***-

Caltech discloses a composition (compositions; paragraph [0003]) comprising: a set of paired oligonucleotides with complementarity to a target nucleic acid (providing a set of first primer and a first oligonucleotide (capture molecules) specific (complementarity) for binding a first polynucleotide analyte (first target molecule); paragraph [0003]), said set of paired oligonucleotides comprising a ratio (set of paired probe labeled nucleic acids (first set of paired capture molecules) would inherently have a ratio; paragraph [0003]) of (b) functional oligonucleotides (primer oligonucleotide (functional; paragraph [0011]), but Caltech does not disclose (a) competitive olignucleotide, 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.
Lode discloses a composition (composition; paragraph [0054]) comprising: a set of paired oligonucleotides with complementarity to a target nucleic acid (providing a set of paired oligonucleotides that are complementary to the target nucleic acids); paragraph [0032]), said set of paired oligonucleotides comprising a ratio (set (first) of paired oligonucleotides (capture molecules) would inherently have a ratio; paragraph [0032]) of (a) competitive oligonucleotides (quencher probe oligonucleotide; paragraph [0032]-[0033]) to (b) functional oligonucleotides (label probe oligonucleotide (functional oligonucleotides); paragraphs [0032]-[0033]), but Lode does not disclose 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.
Wave, Blair, Caltech, and Lode, fail to disclose either solely or in combination the composition 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. Wave discloses ratios of a first probe oligonucleotide with a label and a second probe oligonucleotide with a label (functional), pair of oligonucleotides, that are complimentary to a target nucleic acids, amplifying the target nucleic acids, detecting the cleavage (degradation) of first and second probes (page 5, lines 23-30; page 7, lines 14-19; page 15, lines 25-31; page 16, lines 1-3), but Wave does not disclose 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.
Caltech discloses method of detecting a plurality of polynucleotide analytes using oligonucleotides with attached chromophore in polymerization techniques and measuring signals to detect presence or absence of analytes in samples (paragraph [0011], [0045], [0082]), but Caltech does not disclose 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.

Claims 1-4, 43-47, 46/45-47, 83-87, 88/83-87, 89/83-87, 90/83-87, 91-97, 98/91-97, and 99 have industrial applicability as defined by PCT Article 33(4) because the subject matter can be made or used in industry.

PCT Recordation of Search History

Case/PCT Application Number: PCT/US17/24933

CLIN Number/Technical Field of PCT Application: 008 Chemistry

Dates During Which the Search was Conducted: 25 May – 03 June 2017

Date of Completion of Recordation of Search History Form: 03 June 2017

Research Analyst Initials: TFW

Search Approval Official (SAO) Initials: DGF

Field of Search/Classification Information:

IPC(8) Classifications: A61K 31/7088, 31/711, 31/713; C07H 21/00, 21/04; C12Q 1/68; G01N 21/64, 33/532, 33/533, 33/542 (2017.01)

CPC Classifications: A61K 31/7088, 31/711, 31/713; C07H 21/00, 21/04; C12Q 1/68, 1/686, 1/6806, 1/6851, 2561/101, 2561/113; G01N 21/64, 33/532, 33/533, 33/542

Databases Searched (Patent and Non-Patent Literature (NPL), Including Sub-Databases and Files Searched) and Search Terms Used:

PatSeer (US, EP, WO, JP, DE, GB, CN, FR, KR, ES, AU, IN, CA, Other Countries (INPADOC), RU, AT, CH, TH, BR, PH), EBSCO, Google Scholar, PubMed;

target nucleic acid, PCR, qPCR, competitive oligonucleotides, competitive probes, dimming probes, non-fluorescing probes, target probes, signal oligonucleotides, fluorescent probe, fluorophore, functional oligonucleotides,

Database Search String Recordation, Including Dates of Searches:

Patent Database Search Strategy/Results:

PatSeer (US, EP, WO, JP, DE, GB, CN, FR, KR, ES, AU, IN, CA, Other Countries (INPADOC), RU, AT, CH, TH, BR, PH):

ID	Search String	Records	Date
L36	TACD:((DNA OR nucleic*_acid* OR RNA OR deoxyribonucl*_acid* OR DNAs OR "DNA" OR ribonucl*_acid* OR "DNAs" OR "RNA" OR RNAs OR "RNAs") AND ((ampli*) W10 (DNA OR nucleic*_acid* OR RNA OR deoxyribonucl*_acid* OR DNAs OR "DNA" OR ribonucl*_acid* OR "DNAs" OR "RNA" OR RNAs OR "RNAs")) AND (competit*_probe* OR competit*_oligonucleotid* OR competit*_captur*_molecul*) AND ((function*_oligonucleotid* OR signal*_oligonucleotid* OR fluoresc*_probe* OR fluoresc*_oligonucleotid*) w10 (extend*)))	97	3-Jun-17
L35	TACD:((DNA OR nucleic*_acid* OR RNA OR deoxyribonucl*_acid* OR DNAs OR "DNA" OR ribonucl*_acid* OR "DNAs" OR "RNA" OR RNAs OR "RNAs") AND ((ampli*) W10 (DNA OR nucleic*_acid* OR RNA OR deoxyribonucl*_acid* OR DNAs OR "DNA" OR ribonucl*_acid* OR "DNAs" OR "RNA" OR RNAs OR "RNAs")) AND (competit*_probe* OR competit*_oligonucleotid* OR competit*_captur*_molecul*) AND (function*_oligonucleotid* OR signal*_oligonucleotid* OR fluoresc*_probe* OR fluoresc*_oligonucleotid*))	824	3-Jun-17
L34	TAC:((DNA OR nucleic*_acid* OR RNA OR deoxyribonucl*_acid* OR DNAs OR "DNA" OR ribonucl*_acid* OR "DNAs" OR "RNA" OR RNAs OR "RNAs") AND ((ampli*) W10 (DNA OR nucleic*_acid* OR RNA OR deoxyribonucl*_acid* OR DNAs OR "DNA" OR ribonucl*_acid* OR "DNAs" OR "RNA" OR RNAs OR "RNAs")) AND (competit*_probe* OR competit*_oligonucleotid* OR competit*_captur*_molecul*) AND (function*_oligonucleotid* OR signal*_oligonucleotid* OR fluoresc*_probe* OR fluoresc*_oligonucleotid*))	2	3-Jun-17
L33	TA:((DNA OR nucleic*_acid* OR RNA OR deoxyribonucl*_acid* OR DNAs OR "DNA" OR ribonucl*_acid* OR "DNAs" OR "RNA" OR RNAs OR "RNAs") AND ((ampli*) W10 (DNA OR nucleic*_acid* OR RNA OR deoxyribonucl*_acid* OR DNAs OR "DNA" OR ribonucl*_acid* OR "DNAs" OR "RNA" OR RNAs OR "RNAs")) AND (competit*_probe* OR competit*_oligonucleotid* OR competit*_captur*_molecul*) AND (function*_oligonucleotid* OR signal*_oligonucleotid* OR fluoresc*_probe* OR fluoresc*_oligonucleotid*))	0	3-Jun-17
L32	TACD: ((amplific*) w10 ("nucleic acid" or oligonucleotides)) and ((paired or set) w5 (oligonucleotides or "nucleic acids" or probes)) and (competitive w5 (oligonucleotides or "nucleic acids" or probes)) and (functional w5 (oligonucleotides or "nucleic acids" or probes)) and ("polymerase chain reaction" or PCR or qPCR) and (extend* w10 polymerase)	197	1-Jun-17
L31	TACD: ((amplific*) w10 ("nucleic acid" or oligonucleotides)) and ((paired or set) w5 (oligonucleotides or "nucleic acids" or probes)) and (competitive w5 (oligonucleotides or "nucleic acids" or probes)) and (functional w5 (oligonucleotides or "nucleic acids" or probes)) and ("polymerase chain reaction" or PCR or qPCR)	352	1-Jun-17
L30	TACD: ((amplific*) w10 ("nucleic acid" or oligonucleotides)) and ((paired or set) w5 (oligonucleotides or "nucleic acids" or probes)) and (competitive w5 (oligonucleotides or "nucleic acids" or probes)) and (functional w5 (oligonucleotides or "nucleic acids" or probes))	356	1-Jun-17

L29	TACD: ((amplific*_level) w10 ("nucleic acid" or oligonucleotides)) and ((paired or set) w5 (oligonucleotides or "nucleic acids" or probes)) and (competitive w5 (oligonucleotides or "nucleic acids" or probes)) and (functional w5 (oligonucleotides or "nucleic acids" or probes))	0	1-Jun-17
L28	TAC: ((amplific*_level) w10 ("nucleic acid" or oligonucleotides)) and ((paired or set) w5 (oligonucleotides or "nucleic acids" or probes)) and (competitive w5 (oligonucleotides or "nucleic acids" or probes)) and (functional w5 (oligonucleotides or "nucleic acids" or probes))	0	1-Jun-17
L27	TA: ((amplific*_level) w10 ("nucleic acid" or oligonucleotides)) and ((paired or set) w5 (oligonucleotides or "nucleic acids" or probes)) and (competitive w5 (oligonucleotides or "nucleic acids" or probes)) and (functional w5 (oligonucleotides or "nucleic acids" or probes))	0	1-Jun-17
L26	TA:(target*_nucleic*_acid* or target_molecule or target_DNA or target_gene or target_polynucleotide) AND TACD: ((paired w3 (primers or oligonucleotides or "capture molecules")) and ((competit*_oligonucleotid* or competit*_polynucleotide OR competit*_probe* or Competit*_primer or capture_molecul*) AND (signal*_oligonucleotid* OR fluoresc*_oligonucleotid* OR fluoresc*_probe* or functional_oligonucleotid* or functional_primer or signal_primer or fluresc*_primer or (signal w5 capture_molecule)) and (((signal or first) w5 intensity) or amplif*)))	113	30-May-17
L25	TAC:(target*_nucleic*_acid* or target_molecule or target_DNA or target_gene) AND TAC: ((competit*_oligonucleotid* OR competit*_probe* or Competit*_primer or capture_molecul*) AND (signal*_oligonucleotid* OR fluoresc*_oligonucleotid* OR fluoresc*_probe* or functional_oligonucleotid* or functional_primer or signal_primer or fluresc*_primer or (signal w5 capture_molecule)) and (((signal or first) w5 intensity) or amplif*))	26	30-May-17
L24	TA:(target w5 (oligonucleotide or "Nucleic acid" or DNA)) AND TACD: ((competitive w5 (oligonucleotide or probe)) and (signal w5 (oligonucleotide or probe)) and (signal w5 intensity))	195	27-May-17
L23	TAC:(oligonucleotide or "Nucleic acid" or DNA) AND TACD: ((competitive w5 (oligonucleotide or probe)) and (signal w5 (oligonucleotide or probe)) and (signal w5 intensity))	917	27-May-17
L22	TAC:(oligonucleotide or "Nucleic acid" or DNA) AND TACD: (competitive w5 (oligonucleotide or probe))	4336	27-May-17
L21	TAC:(oligonucleotide or "Nucleic acid" or DNA) AND TACD: ("multi_analyte" w5 coding)	0	27-May-17
L20	TAC:((target or detect*) w5 ("Nucleic acid" or DNA)) AND TACD: ("multi_analyte" w5 coding)	0	27-May-17
L19	TAC:((target or detect*) w5 ("Nucleic acid" or DNA)) AND TACD:((competitive w5 (oligonucleotide or probes)) and ((signal or fluorscen*) w5 (probe or oligonucleotide))) AND CPC:("C12Q2561/101" or "C12Q2561/113")	76	27-May-17
L18	TAC:((target or detect*) w5 ("Nucleic acid" or DNA)) AND CPC:("C12Q2561/101" or "C12Q2561/113")	1925	27-May-17
L17	CPC:("C12Q2561/101" or "C12Q2561/113")	3090	27-May-17
L16	TAC:((target or detect*) w5 ("Nucleic acid" or DNA)) AND TACD:(("real_time" or "multi_plex" or multiplex) w5 (pcr or qpcr or "polymerase chain reaction") and (competitive w5 (oligonucleotide or probes)) and ((signal or fluorscen*) w5 (probe or oligonucleotide))) AND CPC:(("A61K31/7088" or "A61K31/711" or "A61K31/713" or "C07H21/00" or "C07H21/04") and ("C12Q1/68" or "C12Q1/686" or "C12Q1/6806" or "C12Q1/6851" or "G01N21/64" or "G01N33/532" or "G01N33/533" or "G01N33/542"))	10	27-May-17
L15	CPC:(("A61K31/7088" or "A61K31/711" or "A61K31/713" or "C07H21/00" or "C07H21/04") and ("C12Q1/68" or "C12Q1/686" or "C12Q1/6806" or	4579	27-May-17

	"C12Q1/6851" or "G01N21/64" or "G01N33/532" or "G01N33/533" or "G01N33/542"))		
L14	TAC:((target or detect*) w5 ("Nucleic acid" or DNA)) AND TACD:(("real_time" or "multi_plex" or multiplex) w5 (pcr or qpcr or "polymerase chain reaction") and (competitive w5 (oligonucleotide or probes)) and ((signal or fluorscen*) w5 (probe or oligonucleotide))) AND IC:(("A61K31/7088" or "A61K31/711" or "A61K31/713" or "C07H21/00" or "C07H21/04") and ("C12Q1/68" or "G01N21/64" or "G01N33/532" or "G01N33/533" or "G01N33/542"))	109	27-May-17
L13	TAC:((target or detect*) w5 ("Nucleic acid" or DNA)) AND TACD:(("real_time" or "multi_plex" or multiplex) w5 (pcr or qpcr or "polymerase chain reaction") and ((competitive or signal) w5 (oligonucleotide or probes))) AND IC:(("A61K31/7088" or "A61K31/711" or "A61K31/713" or "C07H21/00" or "C07H21/04") and ("C12Q1/68" or "G01N21/64" or "G01N33/532" or "G01N33/533" or "G01N33/542"))	2071	27-May-17
L12	TAC:((target or detect*) w5 ("Nucleic acid" or DNA)) AND TACD:(("real_time" or "multi_plex" or multiplex) w5 (pcr or qpcr or "polymerase chain reaction")) AND IC:(("A61K31/7088" or "A61K31/711" or "A61K31/713" or "C07H21/00" or "C07H21/04") and ("C12Q1/68" or "G01N21/64" or "G01N33/532" or "G01N33/533" or "G01N33/542"))	4904	27-May-17
L11	TAC:((target or detect*) w5 ("Nucleic acid" or DNA)) AND IC:(("A61K31/7088" or "A61K31/711" or "A61K31/713" or "C07H21/00" or "C07H21/04") and ("C12Q1/68" or "G01N21/64" or "G01N33/532" or "G01N33/533" or "G01N33/542"))	27321	27-May-17
L10	IC:(("A61K31/7088" or "A61K31/711" or "A61K31/713" or "C07H21/00" or "C07H21/04") and ("C12Q1/68" or "G01N21/64" or "G01N33/532" or "G01N33/533" or "G01N33/542"))	104578	27-May-17
L9	TA: (target w5 ("nucleic acids" or DNA)) and TACD: ((competitive w5 (probe or oligonucleotide or hybridization)) and ((detectable or signal or reporter) w5 (oligonucleotide or probe)) and (PCR or qPCR or "polymerase chain reaction") and ((signal or flourescence) w5 intensity))	74	27-May-17
L8	TA: (target w5 ("nucleic acids" or DNA)) and TAC: ((competitive w5 (probe or oligonucleotide or hybridization)) and ((detectable or signal or reporter) w5 (oligonucleotide or probe)) and (PCR or qPCR or "polymerase chain reaction") and ((signal or flourescence) w5 intensity))	0	27-May-17
L7	TACD:((target*_nucleic*_acid* or target_molecule) AND ((competit*_oligonucleotid* OR competit*_probe*) AND (signal*_oligonucleotid* OR fluoresc*_oligonucleotid* OR fluoresc*_probe*)) AND (pcr OR qpcr OR polymerase_chain_reaction) and (signal w5 intensi*))	369	27-May-17
L6	TACD:((target*_nucleic*_acid* or target_molecule) AND ((competit*_oligonucleotid* OR competit*_probe*) AND (signal*_oligonucleotid* OR fluoresc*_oligonucleotid* OR fluoresc*_probe*)) AND (pcr OR qpcr OR polymerase_chain_reaction))	762	27-May-17
L5	TAC:((target*_nucleic*_acid* or target_molecule) AND ((competit*_oligonucleotid* OR competit*_probe*) AND (signal*_oligonucleotid* OR fluoresc*_oligonucleotid* OR fluoresc*_probe*)) AND (pcr OR qpcr OR polymerase_chain_reaction))	2	27-May-17
L4	TA:((target*_nucleic*_acid* or target_molecule) AND ((competit*_oligonucleotid* OR competit*_probe*) AND (signal*_oligonucleotid* OR fluoresc*_oligonucleotid* OR fluoresc*_probe*)) AND (pcr OR qpcr OR polymerase_chain_reaction))	0	27-May-17
L3	ASN:(chromacode)	0	27-May-17
L2	TACD:("multi-analyte" or ((competitive or signal) w5 (probes or oligonucleotides))) AND INV:((Jacky w3 Lucien) or (Rajagopal w3 Aditya) or (Menge w3 Karen) or (Gosch w3 Gregory))	29	27-May-17

L1	INV:((Jacky w3 Lucien) or (Rajagopal w3 Aditya) or (Menge w3 Karen) or (Gosch w3 Gregory))	152	27-May-17

Non-Patent Literature (NPL) Search Strategy/Results:

Google Scholar; 25 May 2017 (25.05.2017)

Search Terms	Results
"target nucleic acids") AND (("competitive oligonucleotides" OR "competitive probes") AND ("signal oligonucleotides" OR "fluorescent oligonucleotides" OR "fluorescent probes")) AND (PCR OR qPCR OR "polymerase chain reaction")	3
Identification target nucleic acids competitive oligonucleotides signal oligonucleotides PCR	48,900
Identification multiple target nucleic acids "fluorescence intensity" oligonucleotides PCR	27,700
multi-plex "real-time PCR" "competitive hybridization" probes ratio-metric coding of signal intensity	2,560
qPCR multi-analyte coding techniques	827
qPCR multi-analyte coding techniques ratiometric "signal intensity"	4

PubMed; 25 May 2017 (25.05.2017)

Search Terms	Results
((((PCR) AND real-time) AND multiplex)) AND signal oligonucleotides	15
((PCR) AND Real-time) AND competitive hybridzation	57
(identification target nucleic acids) AND competitive oligonucleotides	21
(((Jacky[Author]) OR Rajagopal A[Author]) OR Menge K[Author]) OR Gosch G[Author]	47

EBSCO; 01 June 2017 (01.06.2017)

Search ID#	Search Terms	Results
S8	AB (("nucleic acids" or oligonucleotide) N5 (detection or identification or target)) AND ((paired or set) N5 (oligonucleotides or "nucleic acids" or "capture molecules)) AND signal n5 intensity	119
S7	AB (("nucleic acids" or oligonucleotide) N5 (detection or identification or target)) AND ((paired or set) N5 (oligonucleotides or "nucleic acids" or "capture molecules))	779
S6	(("nucleic acids" or oligonucleotide) N5 (detection or identification or target)) AND ((paired or set) N5 (oligonucleotides or "nucleic acids" or "capture molecules))	117,761
S5	amplification N10 (target nucleic acid) AND (paired N5 (oligonucleotides	568

	or "nucleic acids")) AND (functional n5 (oligonucleotides or "nucleic acids"))	
S4	(Target N5 ("nucleic acids" or DNA or molecules)) AND (competitive N5 (oligonucleotides or "capture molecules" or antibodies)) AND polymerase chain reaction AND (ratio N10 (label or oligonucleotides)) AND signal N5 intensity	413
S3	(Target N5 ("nucleic acids" or DNA or molecules)) AND (competitive N5 (oligonucleotides or "capture molecules" or antibodies)) AND polymerase chain reaction AND (ratio N10 (label or oligonucleotides))	2,214
S2	(Target N5 ("nucleic acids" or DNA or molecules)) AND (competitive N5 (oligonucleotides or "capture molecules" or antibodies)) AND polymerase chain reaction	6,356
S1	(Target N5 ("nucleic acids" or DNA or molecules)) AND (competitive N5 (oligonucleotides or "capture molecules" or antibodies))	20,756

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

To: LEE JOHNSON
WILSON SONSINI GOODRICH & ROSATI
650 PAGE MILL ROAD
PALO ALTO, CA 94304-1050

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL SEARCH REPORT AND THE WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY, OR THE DECLARATION

(PCT Rule 44.1)

	Date of mailing *(day/month/year)* 16 NOV 2017
Applicant's or agent's file reference 38075-729601	**FOR FURTHER ACTION** See paragraphs 1 and 4 below
International application No. PCT/US 17/37682	International filing date *(day/month/year)* 15 June 2017 (15.06.2017)
Applicant CALIFORNIA INSTITUTE OF TECHNOLOGY	

1. ☒ The applicant is hereby notified that the international search report and the written opinion of the International Searching Authority have been established and are transmitted herewith.

   Filing of amendments and statement under Article 19:
   The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46):

   When? The time limit for filing such amendments is normally two months from the date of transmittal of the international search report.

   How? Directly to the International Bureau of WIPO preferably through ePCT or on paper to, 34 chemin des Colombettes 1211 Geneva 20, Switzerland, Facsimile No.: +41 22 338 82 70

   For more detailed instructions, see *PCT Applicant's Guide*, International Phase, paragraphs 9.004 – 9.011.

2. ☐ The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect and the written opinion of the International Searching Authority are transmitted herewith.

3. ☐ **With regard to any protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:
   ☐ the protest together with the decision thereon has been transmitted to the International Bureau together with any request to forward the texts of both the protest and the decision thereon to the designated Offices.
   ☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. **Reminders**

   The applicant may **submit comments on an informal basis on the written opinion of the International Searching Authority** to the International Bureau. These comments will be made available to the public after international publication. The International Bureau will send a copy of such comments to all designated Offices unless an international preliminary examination report has been or is to be established.

   Shortly after the expiration of **18 months from the priority date, the international application will be published** by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau before the completion of the technical preparations for international publication (Rules 90*bis*.1 and 90*bis*.3).

   Within **19 months** from the priority date, but only in respect of some designated Offices, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase **until 30 months** from the priority date (in some Offices even later); otherwise, the applicant must, **within 20 months** from the priority date, perform the prescribed acts for **entry into the national phase** before those designated Offices. In respect of other designated Offices, the time limit of **30 months** (or later) will apply even if no demand is filed within 19 months. For details about the applicable time limits, Office by Office, see www.wipo.int/pct/en/texts/time_limits.html and the *PCT Applicant's Guide*, National Chapters.

   Within **22 months from the priority date, the applicant may request that a supplementary international search be carried out** by a different International Searching Authority that offers this service (Rule 45*bis*.1). The procedure for requesting supplementary international search is described in the *PCT Applicant's Guide*, International Phase, paragraphs 8.006-8.032.

Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Lee W. Young PCT Helpdesk: 571-272-4300 Telephone No. PCT OSP: 571-272-7774

Form PCT/ISA/220 (July 2014)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Form PCT/ISA/220 as well as, where applicable, item 5 below.
38075-729601		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/US 17/37682	15 June 2017 (15.06.2017)	17 June 2016 (17.06.2016)
Applicant CALIFORNIA INSTITUTE OF TECHNOLOGY		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 5 sheets.

☐ It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language**, the international search was carried out on the basis of:

☒ the international application in the language in which it was filed.

☐ a translation of the international application into _____ which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).

b. ☐ This international search report has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43.6*bis*(a)).

c. ☐ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, see Box No. I.

2. ☐ **Certain claims were found unsearchable** (see Box No. II).

3. ☒ **Unity of invention is lacking** (see Box No. III).

4. With regard to the **title**,

☒ the text is approved as submitted by the applicant.

☐ the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

☒ the text is approved as submitted by the applicant.

☐ the text has been established, according to Rule 38.2, by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. With regard to the **drawings**,

a. the figure of the **drawings** to be published with the abstract is Figure No. 6

☒ as suggested by the applicant.

☐ as selected by this Authority, because the applicant failed to suggest a figure.

☐ as selected by this Authority, because this figure better characterizes the invention.

b. ☐ none of the figures is to be published with the abstract.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 17/37682

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

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

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

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

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

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

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

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

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

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

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

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

Remark on Protest

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

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

☐ No protest accompanied the payment of additional search fees.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 17/37682

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

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History Document

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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

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

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

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

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

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 17/37682

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

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

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

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

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

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

Special Technical Features

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

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

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

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

Common Technical Features

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

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

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

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

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

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

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

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

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

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

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

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

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

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PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To: LEE JOHNSON
WILSON SONSINI GOODRICH & ROSATI
650 PAGE MILL ROAD
PALO ALTO, CA 94304-1050

PCT

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43*bis*.1)

Date of mailing *(day/month/year)* 16 NOV 2017

Applicant's or agent's file reference
38075-729601

FOR FURTHER ACTION
See paragraph 2 below

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/US 17/37682	15 June 2017 (15.06.2017)	17 June 2016 (17.06.2016)

International Patent Classification (IPC) or both national classification and IPC
IPC(8) - C12Q 1/68, C12P 19/34 (2017.01)
CPC - C12Q 1/6844, C12N 15/10

Applicant CALIFORNIA INSTITUTE OF TECHNOLOGY

1. This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☐ Box No. II Priority
- ☐ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☒ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the international application
- ☐ Box No. VIII Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1*bis*(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

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

Form PCT/ISA/237 (cover sheet) (January 2015)

WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US 17/37682

Box No. I Basis of this opinion

1. With regard to the **language**, this opinion has been established on the basis of:

   ☒ the international application in the language in which it was filed.

   ☐ a translation of the international application into _____ which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b)).

2. ☐ This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43*bis*.1(a)).

3. ☐ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, this opinion has been established on the basis of a sequence listing:

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

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

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

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

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

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

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

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

5. Additional comments:

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WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US 17/37682

Box No. IV Lack of unity of invention

1. ☒ In response to the invitation (Form PCT/ISA/206) to pay additional fees the applicant has, within the applicable time limit:

☐ paid additional fees.

☐ paid additional fees under protest and, where applicable, the protest fee.

☐ paid additional fees under protest but the applicable protest fee was not paid.

☒ not paid additional fees.

2. ☐ This Authority found that the requirement of unity of invention is not complied with and chose not to invite the applicant to pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rule 13.1, 13.2 and 13.3 is

☐ complied with.

☒ not complied with for the following reasons:

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

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

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

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

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

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

Special Technical Features

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

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

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

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

----------Please see continuation in first supplemental box----------

4. Consequently, this opinion has been established in respect of the following parts of the international application:

☐ all parts.

☒ the parts relating to claims Nos. 1-6

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WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US 17/37682

Box No. V **Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

1. Statement

Novelty (N)	Claims	1-6	YES
	Claims	None	NO
Inventive step (IS)	Claims	None	YES
	Claims	1-6	NO
Industrial applicability (IA)	Claims	1-6	YES
	Claims	None	NO

2. Citations and explanations:

Claims 1-6 lack an inventive step under PCT Article 33(3) as being obvious over US 2007/0072211 A1 to Newton et al., (hereinafter 'Newton').

Regarding Claim 1, Newton teaches a method of detecting the presence or absence of a target nucleic acid analyte in a sample (Claim 1 - 'A method for identifying one or more nucleic acid targets in a sample,'), the method comprising:
a) performing an amplification reaction on the sample (Claim 1 - 'the method comprising: a) performing an asymmetric kinetic PCR in a reaction mixture');
b) measuring a signal generated during the amplification reaction and generating a kinetic signature from the measured signal (Claim 1 - 'b) monitoring one or more growth curves from the kinetic PCR'); and
c) comparing the generated kinetic signature to a reference signature and determining whether the generated kinetic signature corresponds with the reference signature, thereby detecting the presence or absence of the target nucleic acid analyte in the sample (Claim 1 - 'e) correlating the melting curve or annealing curve of step d) to a melting or annealing curve of a completely complementary probe of one or more known nucleic acid targets, thus, identifying the one or more nucleic acid targets in the sample.'). Newton does not state expressly that the growth curve from kinetic PCR is a kinetic signature. However, since Newton teaches the kinetic PCR is aimed at differentiating between closely related nucleic acid molecules (para [0007] - 'For example, many diseases and infections are caused by a number of, often related, pathogens. While the disease symptoms may present as similar, it can be of utmost importance to determine the actual causative pathogen in order to present an effective treatment. Not only is this true in terms of differentiation between different infectious species, but is also true, and can be even more difficult to resolve, when trying to discriminate between closely related agents, e.g., different strains of a pathogen such as the subtypes of hepatitis C virus (HCV).'), it would have been obvious to one of ordinary skill in the art that the growth curve from the kinetic PCR of Newton represents a kinetic signature enabling specific identification of a nucleic acid target.

Regarding Claim 2, Newton teaches the method of claim 1, wherein the kinetic signature comprises a signal selected from an electromagnetic signal, a wavelength, and a fluorescence emission signal (Claim 1 - 'c) monitoring one or more fluorescent signals generated from the one or more labeled hybridization probes thereby producing a melting curve or annealing curve').

Regarding Claim 3, Newton teaches the method of claim 1, wherein the kinetic signature is plotted as a curve (Claim 1 - 'd) monitoring one or more fluorescent signals generated from the one or more labeled hybridization probes thereby producing a melting curve or annealing curve').

Regarding Claim 4, Newton teaches the method of claim 1 wherein the reference signature corresponds with a kinetic signature generated by amplification of the target nucleic acid analyte under one or more members of a set of constrained amplification parameters, the set of constrained amplification parameters comprising any combination of: (i) a range of forward and/or reverse primer concentrations; (ii) a range of polymerase concentrations; (iii) polymerase type; (iv) a range of nucleotide concentrations; (v) chromophore concentration; and (vi) chromophore type; (vii) a number of thermocycles; (viii) rate of thermocycling; (ix) one or more thermocycle stage temperatures; and (x) one or more thermocycle stage time lengths; (xi) a range of target-specific molecular probes; (xii) primers associated with one or more targetspecific probes; and (xiii) a concentration of one or more enzyme cofactors (Claim 1 - 'e) correlating the melting curve or annealing curve of step d) to a melting or annealing curve of a completely complementary probe of one or more known nucleic acid targets'; Claim 3 - 'The method of claim 1, wherein the asymmetric kinetic PCR comprises a first primer and at least a second primer, wherein the amount of the first primer is greater than the amount of the second primer and wherein the hybridization probe is present in an equal or greater amount than the second primer.').

---------Please see continuation in second supplemental box----------

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WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/US 17/37682

Supplemental Box

In case the space in any of the preceding boxes is not sufficient.
Continuation of:
Box No. IV.3. Lack of unity of invention

Common Technical Features

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

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

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

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

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

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

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

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

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

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

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

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Supplemental Box

In case the space in any of the preceding boxes is not sufficient.
Continuation of:
Box No V2 Citations and Explanations

Regarding Claim 5, Newton teaches the method of claim 4, wherein the amplification reaction is carried out within one or more of the constrained amplification parameters (Claim 3 - 'The method of claim 1, wherein the asymmetric kinetic PCR comprises a first primer and at least a second primer, wherein the amount of the first primer is greater than the amount of the second primer and wherein the hybridization probe is present in an equal or greater amount than the second primer.').

Regarding Claim 6, Newton teaches the method of claim 3, wherein the at least one of an exponential region, a linear region, and a plateau region of the curve occurs over at least 4 thermocycles or over at least 8 thermocycles (para [0068] - 'FIG. 3 provides growth curves generated during an asymmetric kPCR'; and data shown in Figure 3). Newton does not state expressly at least 4 thermocycles or over at least 8 thermocycles. However, data shown in Figure 3 illustrates at least 4 thermocycles or over at least 8 thermocycles.

Claims 1-6 have industrial applicability as defined by PCT Article 33(4) because the subject matter can be made or used in industry.

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California Institute of Technology - Ex. 2003 Page 643

SEARCH HISTORY

Application Number	PCT/US 17/37682
Search Conducted By	AEM
Search Approved By	PP

CPC/IPC Classifications Searched	IPC(8) - C12 Q1/68, C12P 19/34 (2017.01) CPC - C12Q 1/6844, C12N 15/10
Date Conducted	22 August 2017 (22.08.2017)

Documentation Searched	Keyword limited; search terms below
Search Terms Used	Amplification, kinetic PCR or polymerase chain reaction, plot or curve, primer, electromagnetic or wavelength or fluorescence, reference or control or standard, primer set, 2 forward primers, 2 reverse primers, nested primer, limiting primer, annealing temperature, thermocycling or temperature cycle
Date Conducted	22 August 2017 (22.08.2017)

Electronic Database Searched	Patbase
Files Searched	Full-text: AU BE BR CA CH CN DE DK EP ES FI FR GB IN JP KR SE TH TW US WO Bibliographic: (Europe) AT BA BE BG CH CS CY CZ DD DK EE ES FI GE GR HR HU IE IS IT LT LU LV MC MD MT NL NO PL PT RO RS SE SI SK SM TR UA YU (Asia) EA GC HK ID IL IN KZ MN MY PH RU SG SU TH TJ TW UZ VN (North America) CA CR CU DO GT HN MX NI PA SV TT (South America) AR BR CL CO EC PE UY (Australasia) AU NZ (Africa) AP DZ EG KE MA MW OA ZA ZM ZW
Date Conducted	22 August 2017 (22.08.2017)
Search Logic: Classification based search	

Search 1: IC=(C12Q1/68 OR C12P19/34) (Results 128654)
Search 2: CPC=(C12Q1/6844 OR C12N15/10 OR C12Q1/68 OR C12Q1/686 OR C12Q1/6853 OR G01N21/6486 OR G01N2021/6439) (Results 14465)
Search 3: 1 OR 2 (Results 131488)
Search 4: 3 AND FT=(KINETIC W1 PCR) (Results 509)
Search 5: 4 AND FT=(LIMIT* W1 PRIMER%) (Results 9)
Search 6: 4 AND FT=(CONSTRAINED NEAR (PCR OR AMPLIFICATION OR PARAMETER%)) (Results 0)
Search 7: 3 AND FT=(CONSTRAINED NEAR (PCR OR AMPLIFICATION OR PARAMETER%)) (Results 79)
Search 8: 7 AND FT=(KINETIC NEAR PCR) (Results 0)
Search 9: 7 AND FT=(ASYMMETRIC NEAR (PCR OR AMPLIFICATION)) (Results 6)
Search 10: 4 AND FT=(NESTED NEAR PCR) (Results 159)
Search 11: 10 AND FT=(TWO NEAR (REVERSE OR FORWARD)) (Results 6)
Search 12: 11 NOT (5 OR 9) (Results 6)
Search 13: 4 AND FT=(PCR NEAR SIGNATURE) (Results 3)

Keyword only search

Search 14: FT=(KINETIC W1 PCR) (Results 639)
Search 15: 14 AND FT=(LIMIT* W1 PRIMER%) (Results 9)
Search 16: 15 NOT (5 OR 9 OR 13) (Results 0)
Search 17: 14 AND FT=(CONSTRAINED NEAR (PCR OR AMPLIFICATION OR PARAMETER%)) (Results 0)
Search 18: FT=(CONSTRAINED NEAR (PCR OR AMPLIFICATION OR PARAMETER%)) (Results 2434)
Search 19: 18 AND FT=(KINETIC NEAR PCR) (Results 0)
Search 20: 18 AND FT=(ASYMMETRIC NEAR (PCR OR AMPLIFICATION)) (Results 6)
Search 21: 20 NOT (5 OR 9 OR 13) (Results 0)
Search 22: FT=(NESTED NEAR PCR) (Results 9272)
Search 23: 22 AND FT=(TWO NEAR (REVERSE OR FORWARD)) (Results 579)
Search 24: 23 AND FT=(KINETIC W1 PCR) (Results 7)
Search 25: FT=(PCR NEAR SIGNATURE) (Results 604)
Search 26: 25 AND FT=(ASYMMETRIC NEAR (PCR OR AMPLIFICATION)) (Results 10)
Search 27: 26 NOT (5 OR 9 OR 13) (Results 9)

Forward and backward search

Search 28: PN=(US2007072211 OR US2010233686 OR US8367325) (Results 3)
Search 29: CTF 28 OR CTB 28 (Results 194)
Search 30: 29 AND FT=(KINETIC W1 PCR) (Results 6)
Search 31: 30 NOT (5 OR 9 OR 13) (Results 2)

Electronic Database Searched	Google
Files Searched	Patents
Date Conducted	22 August 2017 (22.08.2017)

Search Logic:

kinetic PCR

About 67,500 results (0.34 seconds)

kinetic PCR signature limiting primer

About 12,700 results (0.34 seconds)

constrained PCR amplification parameters

About 36,900 results (0.38 seconds)

constrained PCR amplification parameters kinetic PCR

About 16,100 results (0.61 seconds)

constrained PCR amplification kinetic PCR limiting primer

About 16,400 results (0.52 seconds)

constrained nested PCR amplification kinetic PCR limiting primer

About 2,650 results (0.52 seconds)

constrained nested PCR amplification kinetic PCR signature limiting primer

About 554 results (0.47 seconds)

kinetic PCR with 2 reverse primers

About 65,000 results (0.93 seconds)

kinetic nested PCR with 2 reverse primers

About 9,020 results (0.48 seconds)

asymmetric kinetic nested PCR with 2 reverse primers

About 1,820 results (0.52 seconds)

asymmetric kinetic nested PCR with 2 forward primers

About 1,290 results (0.58 seconds)

Electronic Database Searched	Google
Files Searched	Scholar
Date Conducted	22 August 2017 (22.08.2017)

Search Logic:

kinetic PCR
About 466,000 results (0.11 sec)

kinetic PCR signature limiting primer
About 21,700 results (0.07 sec)

constrained PCR amplification parameters
About 45,900 results (0.09 sec)

constrained PCR amplification parameters kinetic PCR
About 21,400 results (0.09 sec)

constrained PCR amplification kinetic PCR limiting primer
About 18,800 results (0.11 sec)

constrained nested PCR amplification kinetic PCR limiting primer
About 4,580 results (0.15 sec)

constrained nested PCR amplification kinetic PCR signature limiting primer
About 1,450 results (0.07 sec)

kinetic pcr with 2 reverse primers
About 235,000 results (0.09 sec)

kinetic nested pcr with 2 reverse primers
About 21,800 results (0.15 sec)

asymmetric kinetic nested pcr with 2 reverse primers
About 3,340 results (0.06 sec)

asymmetric kinetic nested pcr with 2 forward primers
About 2,050 results (0.08 sec)

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Supplemental Search

Electronic Database Searched	PatBase
Files Searched	Full-text: AU BE BR CA CH CN DE DK EP ES FI FR GB IN JP KR SE TH TW US WO Bibliographic: (Europe) AT BA BE BG CH CS CY CZ DD DK EE ES FI GE GR HR HU IE IS IT LT LU LV MC MD MT NL NO PL PT RO RS SE SI SK SM TR UA YU (Asia) EA GC HK ID IL IN KZ MN MY PH RU SG SU TH TJ TW UZ VN (North America) CA CR CU DO GT HN MX NI PA SV TT (South America) AR BR CL CO EC PE UY (Australasia) AU NZ (Africa) AP DZ EG KE MA MW OA ZA ZM ZW
Date Conducted	30 October 2017
Search Logic:	
1) IC=(C12Q1/68 or C12P19/34) (130470) 2) CPC=(C12Q1/6844 or C12N15/10 or C12Q1/68 or C12Q1/686 or C12Q1/6853 or G01N21/6486 or G01N2021/6439) (15934) 3) 1 or 2 (133809) 4) (PCR or polymerase chain reaction) and amplif* (168197) 5) 4 and kinetic (16544) 6) 5 and (plot or curve) (9932) 7) TAC=(detect* w5 (nucleic acid or DNA or RNA or nucleotide or oligonucleotide or polynucleotide)) (31528) 8) 7 and 6 (1344) 9) 3 and 8 (1120) 10) kinetic signature (29) 11) 10 and 8 (6) 12) 3 and 11 (6)	

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventor:	KARTALOV; Emil P.	Group Art Unit:	2877
Serial Number:	15/914,356	Examiner:	Unassigned
Filing or 371 (c) Date:	2018-03-07	**CONFIRMATION NO:**	**2457**
Title:	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS		

FILED ELECTRONICALLY ON: March 22, 2018

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

INFORMATION DISCLOSURE STATEMENT UNDER 37 CFR § 1.97

Commissioner for Patents:

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

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

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

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

IPR2024-01178
California Institute of Technology - Ex. 2003 Page 649

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

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

-- OR --

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

-- OR --

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

-- OR --

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

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

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

-- OR --

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

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

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

-- AND --

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

D. ☐ *37 CFR §1.97 (e).* Statement.

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

-- AND/OR --

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

-- AND/OR --

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

IPR2024-01178
California Institute of Technology - Ex. 2003 Page 650

E. ☐ *Statement Under 37 C.F.R. §1.704(d).* Each item of information contained in the information disclosure statement was first cited in any communication from a patent office in a counterpart foreign or international application or from the Office or is a communication that was issued by a patent office in a counterpart foreign or international application or by the Office that was received by an individual designated in § 1.56(c) not more than thirty (30) days prior to the filing of this information disclosure statement. This statement is made pursuant to the requirements of 37 C.F.R. §1.704(d) to avoid reduction of the period of adjustment of the patent term for Applicant(s) delay.

F. ☒ *37 CFR §1.98 (a) (2).* The content of the Information Disclosure Statement is as follows:

☐ Copies of each of the references listed on the attached Form PTO/SB/08 are enclosed herewith.

-- OR --

☒ Copies of U.S. Patent Documents (issued patents and patent publications) listed on the attached Form PTO/SB/08 are not enclosed.

-- AND/OR --

☒ Copies of Foreign Patent Documents and/or Non Patent Literature Documents listed on the attached Form PTO/SB/08 are enclosed in accordance with 37 CFR §1.98 (a)(2).

-- AND/OR --

☐ Copies of pending unpublished U.S. patent applications are enclosed in accordance with 37 CFR §1.98 (a) (2) (iii).

G. ☒ *37 CFR §1.98(a)(3).* The Information Disclosure Statement includes non-English patents and/or references.

☐ Pursuant to 37 CFR §1.98(a)(3)(i), a concise explanation of the relevance of each patent, publication or other information provided that is not in English is provided herewith.

☐ Pursuant to MPEP 609(B), an English language copy of a foreign search report is submitted herewith to satisfy the requirement for a concise explanation where non-English language information is cited in the search report.

-- OR --

☐ A concise explanation of the relevance of each patent, publication or other information provided that is not in English is as follows: _____

☒ Pursuant to 37 CFR §1.98(a) (3) (ii), a copy of a translation, or a portion thereof, of the non-English language reference(s) is provided herewith.

H. ☐ *37 CFR §1.98(d).* Copies of patents, publications and pending U.S. patent applications, or other information specified in 37 C.F.R. § 1.98(a) are not provided herewith because:

☐ Pursuant to 37 CFR §1.98(d)(1) the information was previously submitted in an Information Disclosure Statement, or cited by examiner for another application under which this application claims priority for an earlier effective filing date under 35 U.S.C. 120.

Application in which the information was submitted: _____

Information Disclosure Statement(s) filed on: _____

AND

☐ The information disclosure statement submitted in the earlier application complied with paragraphs (a) through (c) of 37 CFR §1.98.

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California Institute of Technology - Ex. 2003 Page 651

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

Respectfully submitted,

WILSON SONSINI GOODRICH & ROSATI

Dated: March 22, 2018

By: /Kyle Bruner/
Kyle Bruner, Patent Agent
Register No.: 75,716

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

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 15

Complete if Known	
Application Number	15914356
Filing Date	03-07-2018
First Named Inventor	KARTALOV; Emil P.
Art Unit	2877
Examiner Name	
Attorney Docket Number	38075-716.302

U. S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	001	US-5079351	01-07-1992	SNINSKY; John J. et al.	
	002	US-5538848	07-23-1996	LIVAK; Kenneth J. et al.	
	003	US-5618711	04-08-1997	GELFAND; David H. et al.	
	004	US-5677152	10-14-1997	BIRCH; David Edward et al.	
	005	US-5723591	03-03-1998	LIVAK; Kenneth J. et al.	
	006	US-5773258	06-30-1998	BIRCH; David Edward et al.	
	007	US-5789224	08-04-1998	GELFAND; David H. et al.	
	008	US-5804375	09-08-1998	GELFAND; David H. et al.	
	009	US-5876930	03-02-1999	LIVAK; Kenneth J. et al.	
	010	US-5928862	07-27-1999	MORRISON; Larry E.	
	011	US-5948360	09-07-1999	RAO; Prabhakar P. et al.	
	012	US-5981180	11-09-1999	CHANDLER; Van S. et al.	
	013	US-5994056	11-30-1999	HIGUCHI; Russell G.	
	014	US-6030787	02-29-2000	LIVAK; Kenneth J. et al.	
	015	US-6127155	10-03-2000	GELFAND; David H. et al.	
	016	US-6171785	01-09-2001	HIGUCHI; Russell G.	
	017	US-6258569	07-10-2001	LIVAK; Kenneth J. et al.	
	018	US-6534266	03-18-2003	SINGER; Robert H.	
	019	US-6548259	04-15-2003	WARD; David C. et al.	
	020	US-6642062	11-04-2003	KAUVAR; Lawrence M. et al.	
	021	US-7101663	09-05-2006	GODFREY; Tony E. et al.	

Examiner Signature		Date Considered	

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

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 2 of 15

Complete if Known	
Application Number	15914356
Filing Date	03-07-2018
First Named Inventor	KARTALOV; Emil P.
Art Unit	2877
Examiner Name	
Attorney Docket Number	38075-716.302

U. S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	022	US-7141377	11-28-2006	GELFAND; David H. et al.	
	023	US-7348141	03-25-2008	FRENCH; David John et al.	
	024	US-7385043	06-10-2008	KRAMER; Fred R.	
	025	US-7410764	08-12-2008	GOCKE; Christopher D. et al.	
	026	US-7413708	08-19-2008	MAYRAND; Paul E.	
	027	US-7473767	01-06-2009	DIMITROV; Krassen	
	028	US-7575864	08-18-2009	BEDZYK; Laura A. et al.	
	029	US-7667024	02-23-2010	MAO; Fei et al.	
	030	US-7767423	08-03-2010	KOPRESKI; Michael S. et al.	
	031	US-7771949	08-10-2010	KRAMER; Fred R.	
	032	US-7919237	04-05-2011	DIMITROV; Krassen et al.	
	033	US-7919244	04-05-2011	MADEJON; Seiz Antonio et al.	
	034	US-7941279	05-10-2011	HWANG; Jenq-Neng et al.	
	035	US-8148512	04-03-2012	DIMITROV; Krassen et al.	
	036	US-9458497	10-04-2016	HASSIBI; Arjang et al.	
	037	US-20020022273	02-21-2002	EMPEDOCLES; Stephen A. et al.	
	038	US-20030148280	08-07-2003	HARRIS; Barbara J. et al.	
	039	US-20030148544	08-07-2003	NIE; Shuming et al.	
	040	US-20050064435	03-24-2005	SU; Xing et al.	
	041	US-20050164264	07-28-2005	SHIPWASH; Edward	

Examiner Signature		Date Considered	

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

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

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Sheet 3 of 15

Complete if Known	
Application Number	15914356
Filing Date	03-07-2018
First Named Inventor	KARTALOV; Emil P.
Art Unit	2877
Examiner Name	
Attorney Docket Number	38075-716.302

U. S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	042	US-20050214753	09-29-2005	SHULTZ; John W. et al.	
	043	US-20050260640	11-24-2005	ANDERSEN; Mark R. et al.	
	044	US-20060216708	09-28-2006	VENEMA; Fokke	
	045	US-20070178485	08-02-2007	EL-DEIRY; Wafik S. et al.	
	046	US-20070231824	10-04-2007	CHEE; Mark S. et al.	
	047	US-20080050737	02-28-2008	ARIELI; Boaz et al.	
	048	US-20080069733	03-20-2008	MALTEZOS; George et al.	
	049	US-20090048785	02-19-2009	KATZIR; Nir et al.	
	050	US-20090062129	03-05-2009	MCKERNAN; Kevin et al.	
	051	US-20100015607	01-21-2010	GEISS; Gary K. et al.	
	052	US-20100047924	02-25-2010	WEBSTER; Philippa J. et al.	
	053	US-20100112710	05-06-2010	GEISS; Gary K. et al.	
	054	US-20100159447	06-24-2010	LI; Bin et al.	
	055	US-20100210472	08-19-2010	EMPEDOCLES; Stephen A. et al.	
	056	US-20100248257	09-30-2010	JACOBSEN; Kivin et al.	
	057	US-20100261026	10-14-2010	FERREE; Sean M. et al.	
	058	US-20100267064	10-21-2010	KARTALOV; Emil P. et al.	
	059	US-20100273173	10-28-2010	HIRAI; Mitsuharu et al.	
	060	US-20110104684	05-05-2011	HOOPER; Dennis G.	
	061	US-20110151459	06-23-2011	ROTHMANN; Thomas et al.	
	062	US-20110207623	08-25-2011	DIMITROV; Krassen et al.	

Examiner Signature		Date Considered	

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

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 4 of 15

Complete if Known	
Application Number	15914356
Filing Date	03-07-2018
First Named Inventor	KARTALOV; Emil P.
Art Unit	2877
Examiner Name	
Attorney Docket Number	38075-716.302

U. S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	063	US-20110237459	09-29-2011	NOVA; Michael P. et al.	
	064	US-20120003646	01-05-2012	JOO; Sunghae A. et al.	
	065	US-20120045756	02-23-2012	ROTHMANN; Thomas et al.	
	066	US-20120077195	03-29-2012	LI; Qingge et al.	
	067	US-20120122704	05-17-2012	ATCHLEY; Daniel H. et al.	
	068	US-20120141995	06-07-2012	LI; Qingge et al.	
	069	US-20120171677	07-05-2012	LUDOWISE; Peter D.	
	070	US-20120184017	07-19-2012	CHATTERJEE; Deb K.	
	071	US-20120190030	07-26-2012	CHUN; Jong Yoon et al.	
	072	US-20120196283	08-02-2012	BABIEL; Reiner et al.	
	073	US-20120252017	10-04-2012	REED; Michael W. et al.	
	074	US-20120258457	10-11-2012	JAROSCH; Florian et al.	
	075	US-20130116780	05-09-2013	MILLER; Eran et al.	

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	001	EP-1448581-B1	11-12-2008	INST SYSTEMS BIOLOGY [US]		☐
	002	EP-1963531-B1	09-21-2011	NANOSTRING TECHNOLOGIES INC [US], et al.		☐

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

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Sheet 5 of 15

Complete if Known	
Application Number	15914356
Filing Date	03-07-2018
First Named Inventor	KARTALOV; Emil P.
Art Unit	2877
Examiner Name	
Attorney Docket Number	38075-716.302

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	003	WO-02056014-A2	07-18-2002	GLAXO GROUP LTD [GB], et al.		☐
	004	WO-02056014-A3	10-09-2003	GLAXO GROUP LTD [GB], et al.		☐
	005	WO-2007076128-A2	07-05-2007	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	006	WO-2007076128-A3	11-29-2007	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	007	WO-2007076129-A2	07-05-2007	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	008	WO-2007076129-A3	03-06-2008	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	009	WO-2007076132-A2	07-05-2007	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	010	WO-2007076132-A3	09-07-2007	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	011	WO-2007139766-A2	12-06-2007	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	012	WO-2007139766-A3	12-11-2008	NANOSTRING TECHNOLOGIES INC [US], et al.		☐

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	013	WO-2008124847-A2	10-16-2008	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	014	WO-2008124847-A3	02-19-2009	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	015	WO-2010019826-A1	02-18-2010	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	016	WO-2011047087-A2	04-21-2011	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	017	WO-2011047087-A3	08-18-2011	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	018	WO-2011100541-A2	08-18-2011	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	019	WO-2011100541-A3	01-05-2012	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	020	WO-2011116088-A2	09-22-2011	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	021	WO-2011116088-A3	02-02-2012	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	022	WO-2012058638-A2	05-03-2012	HARVARD COLLEGE [US], et al.		☐

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	023	WO-2012058638-A3	12-20-2012	HARVARD COLLEGE [US], et al.		☐
	024	WO-2012106428-A2	08-09-2012	UNIV FLORIDA [US], et al.		☐
	025	WO-2012135340-A2	10-04-2012	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	026	WO-2012135340-A3	12-20-2012	NANOSTRING TECHNOLOGIES INC [US], et al.		☐
	027	WO-9919515-A1	04-22-1999	LUMINEX CORP [US]		☐
	028	WO-9952708-A1	10-21-1999	LUMINEX CORP [US]		☐

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Attorney Docket Number	38075-716.302

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	001	BEIGE, et al. Clinical evaluation of a Mycobacterium tuberculosis PCR assay. J Clin Microbiol. 1995 Jan;33(1):90-5.	☐
	002	CHAMBERLAIN, et al. Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification. Nucleic Acids Res. 1988 Dec 9;16(23):11141-56.	☐
	003	CHEN, et al. A Homogeneous, ligase-mediated DNA diagnostic test. Genome Research, 1998, volume 8, pages 549-556.	☐
	004	CHONG, et al. Single-tube multiplex-PCR screen for common deletional determinants of alpha-thalassemia. Blood. 2000 Jan 1;95(1):360-2.	☐
	005	Chromatogram, 2011, 2 pages. Dorland's illustrated medical dictionary. Retrieved online on 22 January 2014 from «http://www.credoreference.com».	☐
	006	CHUN, et al. Dual priming oligonucleotide system for the multiplex detection of respiratory viruses and SNP genotyping of CYP2C19 gene. Nucleic Acids Res. 2007;35(6):e40. Epub 2007 Feb 7.	☐
	007	CRAIG, et al. Ordering of cosmid clones covering Herpes simplex virus type I (HSV-I) genome: a test case for fingerprinting by hybridisation. Nucleic Acids Research, 1990, volume 18, pages 2653-2660.	☐
	008	DOS SANTOS, et al. A simple one-step real-time RT-PCR for diagnosis of dengue virus infection. J Med Virol. 2008 Aug;80(8):1426-33. doi: 10.1002/jmv.21203.	☐
	009	EL-HAJJ, et al. Detection of rifampin resistance in Mycobacterium tuberculosis in a single tube with molecular beacons. J Clin Microbiol. 2001 Nov;39(11):4131-7.	☐

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	010	EMBL-Bank: AJ303204. http://www.ebi.ac.uk/Tools/dbfetch/emblfetch?db=embl&id=AJ303204&format=default&style=default&Retrieve=Retrieve. Accessed February 2012.	☐
	011	EMBL-Bank: GQ395623. http://www.ebi.ac.uk/Tools/dbfetch/emblfetch?db=embl&id=GQ395623&format=default&style=default&Retrieve=Retrieve. Accessed February 2012.	☐
	012	FODOR, et al. Multiplexed biochemical assays with biological chips. Nature. 1993 Aug 5;364(6437):555-6.	☐
	013	FORTINA, et al. Digital mRNA profiling. Nat Biotechnol. 2008 Mar;26(3):293-4. doi: 10.1038/nbt0308-293.	☐
	014	GenBank: M93130.1. Dengue type 3 virus complete genome RNA, complete cds. http://www.ncbi.nlm.nih.gov/nuccore/M93130. Accessed February 2012.	☐
	015	HAN, et al. Quantum-dot-tagged microbeads for multiplexed optical coding of biomolecules. Nat Biotechnol. 2001 Jul;19(7):631-5.	☐
	016	HARTMAN, et al. Development of a novel internal positive control for Taqman based assays. Mol Cell Probes. 2005 Feb;19(1):51-9. Epub 2004 Dec 10.	☐
	017	HEIDARI, et al. Detection of Plasmodium falciparum Directly from Blood Samples Using the Polymerase Chain Reaction. Journal of Sciences, Islamic Republic of Iran. 2005 16(1):21-24.	☐
	018	HENEGARIU, et al. Multiplex PCR: critical parameters and step-by-step protocol. Biotechniques. 1997 Sep;23(3):504-11.	☐
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	020	HOREJSH, et al. A molecular beacon, bead-based assay for the detection of nucleic acids by flow cytometry. Nucleic Acids Res. 2005 Jan 19;33(2):e13.	☐
	021	HUANG, et al. Identification of 8 foodborne pathogens by multicolor combinational probe coding technology in a single real-time PCR. Clin Chem. 2007 Oct;53(10):1741-8. Epub 2007 Aug 10.	☐
	022	HUANG, et al. Multicolor combinatorial probe coding for real-time PCR. PLoS One. 2011 Jan 14;6(1):e16033. doi: 10.1371/journal.pone.0016033.	☐
	023	International search report and written opinion dated 04/12/2013 for PCT/US2013/024509.	☐
	024	JOTHIKUMAR, et al. Design of FRET-TaqMan probes for multiplex real-time PCR using an internal positive control. Biotechniques. 2009 Jun;46(7):519-24. doi: 10.2144/000113127.	☐
	025	KUHN, et al. Hybridization of DNA and PNA molecular beacons to single-stranded and double-stranded DNA targets. J Am Chem Soc. 2002 Feb 13;124(6):1097-103.	☐
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	027	LEE, et al. Novel multiplex PCR using dual-priming oligonucleotides for detection and discrimination of the Mycobacterium tuberculosis complex and M. bovis BCG. J Clin Microbiol. 2010 Dec;48(12):4612-4. doi: 10.1128/JCM.00872-10. Epub 2010 Oct 13.	☐
	028	LEE, et al. Seven-color, homogeneous detection of six PCR products. Biotechniques. 1999 Aug;27(2):342-9.	☐
	029	LIEW, et al. Validating a custom multiplex ELISA against individual commercial immunoassays using clinical samples. Biotechniques. 2007 Mar;42(3):327-8, 330-3.	☐

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	030	LIVAK, et al. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. PCR Methods Appl. 1995 Jun;4(6):357-62.	☐
	031	MORRISON, et al. Two-color ratio-coding of chromosome targets in fluorescence in situ hybridization: quantitative analysis and reproducibility. Cytometry. 1997 Apr 1;27(4):314-26.	☐
	032	NOORDHOEK, et al. Sensitivity and specificity of PCR for detection of Mycobacterium tuberculosis: a blind comparison study among seven laboratories. J Clin Microbiol. 1994 Feb;32(2):277-84.	☐
	033	OLIVEIRA, et al. Multiplex PCR strategy for rapid identification of structural types and variants of the mec element in methicillin-resistant Staphylococcus aureus. Antimicrob Agents Chemother. 2002 Jul;46(7):2155-61.	☐
	034	OU, et al. DNA amplification for direct detection of HIV-1 in DNA of peripheral blood mononuclear cells. Science. 1988 Jan 15;239(4837):295-7.	☐
	035	PATON, et al. Detection and characterization of Shiga toxigenic Escherichia coli by using multiplex PCR assays for stx1, stx2, eaeA, enterohemorrhagic E. coli hlyA, rfbO111, and rfbO157. J Clin Microbiol. 1998 Feb;36(2):598-602.	☐
	036	PATTERSON, et al. Detection of HIV-1 DNA and messenger RNA in individual cells by PCR-driven in situ hybridization and flow cytometry. Science. 1993 May 14;260(5110):976-9.	☐
	037	PETERSEN, et al. Short PNA molecular beacons for real-time PCR allelic discrimination of single nucleotide polymorphisms. Mol Cell Probes. 2004 Apr;18(2):117-22.	☐
	038	Plasmodium falciparum (Plasmodium falciparum) Genome Browser Gateway. http://microbes.ucsc.edu/cgi-bin/hgGateway?hgsid=612764&clade=eukaryota-protista&org=0&db=0. Accessed February 2012.	☐

Examiner Signature		Date Considered	

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

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

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U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Substitute for form 1449/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 12 of 15

Complete if Known	
Application Number	15914356
Filing Date	03-07-2018
First Named Inventor	KARTALOV; Emil P.
Art Unit	2877
Examiner Name	
Attorney Docket Number	38075-716.302

NON-PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author(in CAPITAL LETTERS),title of the article(when appropriate), title of the item (book,magazine,journal,serial,symposium,catalog,etc.),date,page(s),volume-issue number(s),publisher, city and/or country where published.	T[2]
	039	PTAK, et al. Inhibition of human immunodeficiency virus type 1 replication in human cells by Debio-025, a novel cyclophilin binding agent. Antimicrob Agents Chemother. 2008 Apr;52(4):1302-17. doi: 10.1128/AAC.01324-07. Epub 2008 Jan 22.	☐
	040	ROSENSTRAUS, et al. An internal control for routine diagnostic PCR: design, properties, and effect on clinical performance. J Clin Microbiol. 1998 Jan;36(1):191-7.	☐
	041	ROTH, et al. Feasibility and efficacy of routine PCR screening of blood donations for hepatitis C virus, hepatitis B virus, and HIV-1 in a blood-bank setting. Lancet. 1999 Jan 30;353(9150):359-63.	☐
	042	SANGER, et al. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci U S A. 1977 Dec;74(12):5463-7.	☐
	043	SPEICHER, et al. Karyotyping human chromosomes by combinatorial multi-fluor FISH. Nat Genet. 1996 Apr;12(4):368-75.	☐
	044	TIRASOPHON, et al. A novel detection of a single Plasmodium falciparum in infected blood. Biochem Biophys Res Commun. 1991 Feb 28;175(1):179-84.	☐
	045	TYAGI, et al. Multicolor molecular beacons for allele discrimination. Nat Biotechnol. 1998 Jan;16(1):49-53.	☐
	046	TYAGI, et al. Wavelength-shifting molecular beacons. Nat Biotechnol. 2000 Nov;18(11):1191-6.	☐
	047	URDEA, et al. Requirements for high impact diagnostics in the developing world. Nature. 2006 Nov 23;444 Suppl 1:73-9.	☐
	048	VET, et al. Multiplex detection of four pathogenic retroviruses using molecular beacons. Proc Natl Acad Sci U S A. 1999 May 25;96(11):6394-9.	☐

Examiner Signature		Date Considered	

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

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

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Substitute for form 1449/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 13 of 15

Complete if Known	
Application Number	15914356
Filing Date	03-07-2018
First Named Inventor	KARTALOV; Emil P.
Art Unit	2877
Examiner Name	
Attorney Docket Number	38075-716.302

NON-PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author(in CAPITAL LETTERS),title of the article(when appropriate), title of the item (book,magazine,journal,serial,symposium,catalog,etc.),date,page(s),volume-issue number(s),publisher, city and/or country where published.	T[2]
	049	WANG, et al. Locked nucleic acid molecular beacons. J Am Chem Soc. 2005 Nov 16;127(45):15664-5.	☐
	050	WATERS, et al. Microchip device for cell lysis, multiplex PCR amplification, and electrophoretic sizing. Anal Chem. 1998 Jan 1;70(1):158-62.	☐
	051	WEIDMANN, et al. Rapid detection of herpes simplex virus and varicella-zoster virus infections by real-time PCR. J Clin Microbiol. 2003 Apr;41(4):1565-8.	☐
	052	WIESE, et al. Simultaneous multianalyte ELISA performed on a microarray platform. Clin Chem. 2001 Aug;47(8):1451-7.	☐
	053	ZHANG, et al. Novel Multiplex PCR Assay for Characterization and Concomitant Subtyping of Staphylococcal Cassette Chromosome mec Types I to V in Methicillin-Resistant Staphylococcus aureus. J Clin Microbiol. 2005 Oct;43(10):5026-33.	☐

Examiner Signature		Date Considered	

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

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

Approved for use through 07/31/2016. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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Substitute for form 1449/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 14 of 15

Complete if Known	
Application Number	15914356
Filing Date	03-07-2018
First Named Inventor	KARTALOV; Emil P.
Art Unit	2877
Examiner Name	
Attorney Docket Number	38075-716.302

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

☐ That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

☐ See attached certification statement.

☐ Fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Kyle Bruner/	Date (YYYY-MM-DD)	2018-03-22
Name/Print	Kyle Bruner	Registration Number	75,716

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

Privacy Act Statement

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

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

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

Electronic Acknowledgement Receipt

EFS ID:	32134908
Application Number:	15914356
International Application Number:	
Confirmation Number:	2457
Title of Invention:	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS
First Named Inventor/Applicant Name:	Emil P. Kartalov
Customer Number:	21971
Filer:	J Kyle Bruner/Judy Wan
Filer Authorized By:	J Kyle Bruner
Attorney Docket Number:	38075-716.302
Receipt Date:	22-MAR-2018
Filing Date:	
Time Stamp:	19:53:29
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		IDS-38075-716-302-03-22-18-1.pdf	433451 6bc9bee2a8f67b3341bfec7fa96582db48927177	yes	19

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Transmittal Letter	1	4
	Information Disclosure Statement (IDS) Form (SB08)	5	19
Warnings:			
Information:			
	Total Files Size (in bytes):	433451	

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

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.
National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.
New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventor:	KARTALOV; Emil P.	Group Art Unit:	2877
Serial Number:	15/914,356	Examiner:	Unassigned
Filing or 371 (c) Date:	2018-03-07	**CONFIRMATION NO:**	**2457**
Title:	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS		

FILED ELECTRONICALLY ON: March 22, 2018

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

INFORMATION DISCLOSURE STATEMENT UNDER 37 CFR § 1.97

Commissioner for Patents:

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

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

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

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

IPR2024-01178
California Institute of Technology - Ex. 2003 Page 670

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

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

-- OR --

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

-- OR --

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

-- OR --

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

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

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

-- OR --

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

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

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

-- AND --

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

D. ☐ *37 CFR §1.97 (e).* Statement.

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

-- AND/OR --

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

-- AND/OR --

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

E. ☐ *Statement Under 37 C.F.R. §1.704(d).* Each item of information contained in the information disclosure statement was first cited in any communication from a patent office in a counterpart foreign or international application or from the Office or is a communication that was issued by a patent office in a counterpart foreign or international application or by the Office that was received by an individual designated in § 1.56(c) not more than thirty (30) days prior to the filing of this information disclosure statement. This statement is made pursuant to the requirements of 37 C.F.R. §1.704(d) to avoid reduction of the period of adjustment of the patent term for Applicant(s) delay.

F. ☒ *37 CFR §1.98 (a) (2).* The content of the Information Disclosure Statement is as follows:

☐ Copies of each of the references listed on the attached Form PTO/SB/08 are enclosed herewith.

-- OR --

☒ Copies of U.S. Patent Documents (issued patents and patent publications) listed on the attached Form PTO/SB/08 are not enclosed.

-- AND/OR --

☐ Copies of Foreign Patent Documents and/or Non Patent Literature Documents listed on the attached Form PTO/SB/08 are enclosed in accordance with 37 CFR §1.98 (a)(2).

-- AND/OR --

☐ Copies of pending unpublished U.S. patent applications are enclosed in accordance with 37 CFR §1.98 (a) (2) (iii).

G. ☐ *37 CFR §1.98(a)(3).* The Information Disclosure Statement includes non-English patents and/or references.

☐ Pursuant to 37 CFR §1.98(a)(3)(i), a concise explanation of the relevance of each patent, publication or other information provided that is not in English is provided herewith.

☐ Pursuant to MPEP 609(B), an English language copy of a foreign search report is submitted herewith to satisfy the requirement for a concise explanation where non-English language information is cited in the search report.

-- OR --

☐ A concise explanation of the relevance of each patent, publication or other information provided that is not in English is as follows: _____

☐ Pursuant to 37 CFR §1.98(a) (3) (ii), a copy of a translation, or a portion thereof, of the non-English language reference(s) is provided herewith.

H. ☒ *37 CFR §1.98(d).* Copies of patents, publications and pending U.S. patent applications, or other information specified in 37 C.F.R. § 1.98(a) are not provided herewith because:

☐ Pursuant to 37 CFR §1.98(d)(1) the information was previously submitted in an Information Disclosure Statement, or cited by examiner for another application under which this application claims priority for an earlier effective filing date under 35 U.S.C. 120.

Application in which the information was submitted: 13/756,760

Information Disclosure Statement(s) filed on: 06/14/2013

AND

☒ The information disclosure statement submitted in the earlier application complied with paragraphs (a) through (c) of 37 CFR §1.98.

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

Respectfully submitted,

WILSON SONSINI GOODRICH & ROSATI

Dated: March 22, 2018

By: /Kyle Bruner/
Kyle Bruner, Patent Agent
Register No.: 75,716

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

IPR2024-01178
California Institute of Technology - Ex. 2003 Page 673

Electronically filed on: March 22, 2018 PATENT

Attorney Docket No.: 38075-716.302

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventors: Emil Kartlov, *et al.*	Group Art Unit: 2877
Serial Number: 15/914,356	Examiner: Unassigned
Filing Date: March 7, 2018	**CONFIRMATION NO: 2457**
Title: SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS	

PRELIMINARY AMENDMENT

Commissioner for Patents
P.O. Box 1450
Alexandria, Virgina 22313

Dear Commissioner:

Prior to an examination on the merits of the above-identified application, please amend the application as follows:

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

Remarks begin on page **6** of this paper.

IPR2024-01178
California Institute of Technology - Ex. 2003 Page 674

AMENDMENTS IN THE SPECIFICATION

Please insert the following paragraphs on page 1, after paragraph [0001]:

SEQUENCE LISTING

[0001.1] The instant application contains a Sequence Listing which has been submitted in ASCII format via EFS-Web and is hereby incorporated by reference in its entirety. Said ASCII copy, created on August 20, 2014, is named 38075-716-301-Seqlist.txt and is 9,260 bytes in size.

STATEMENT AS TO FEDERALLY SPONSERED RESEARCH

[0001.5] This invention was made with the support of the United States government under Grant No. 1144469 awarded by the National Science Foundation. The government has certain rights in the invention.

Please delete paragraph [00174] and replace it with the following paragraph:

[00174] Oligonucleotides were synthesized by INTEGRATED DNA TECHNOLOGIES (IDT). Probes and primer pairs were chosen for each analyte using OLIGOANALYZER, from IDT. Sense probes containing a fluorophore at the 5' end and a quencher at the 3' end were synthesized for all analytes. All oligonucleotides were lyophilized and reconstituted in TE buffer before use. Tables 9 - 14 show sequence information, target sequence, primers, and probes for each of the six analytes.

Table 9. HIV-1 polyprotease sequence information.

Sequence Information	HIV-1 Poly protease 198mer synthesized from bases 2253 – 2550
Source	HIV-1 Reference Sequence, Los Alamos National Laboratory
Target sequence (3' to 5')	GGAAGCTCTATTAGATACAGGAGCAGATGATACAGTATTAGAAGAAATGA GTTTGCCAGGAAGATGGAAACCAAAAATGATAGGGGGAATTGGAGGTTTT ATCAAAGTAAGACAGTATGATCAGATACTCATAGAAATCTGTGGACATAA AGCTATAGGTACAGTATTAGTAGGACCTACACCTGTCAACATAATTGG **(SEQ ID NO: 1)**
Forward Primer (3' to 5')	GGAAGCTCTATTAGATACAGGAGCAG **(SEQ ID NO: 2)**

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California Institute of Technology - Ex. 2003 Page 675

Reverse Primer (3' to 5')	CCAATTATGTTGACAGGTGTAGGTCC **(SEQ ID NO: 3)**
Probe 1 (3' to 5')	**/56-FAM**/TGAGTTTGCCAGGAAGATGGAAACCA/3BHQ_1/ **(SEQ ID NO: 4)**

Table 10. HIV p17 sequence information.

Sequence Name	HIV-1 P17 199mer synthesized from bases 790 – 1186
Source	HIV-1 Reference Sequence, Los Alamos National Laboratory
Target sequence (3' to 5')	CAGCTACAACCATCCCTTCAGACAGGATCAGAAGAACTTAGATCATTATA TAATACAGTAGCAACCCTCTATTGTGTGCATCAAAGGATAGAGATAAAAG ACACCAAGGAAGCTTTAGACAAGATAGAGGAAGAGCAAAACAAAAGTAAG AAAAAAGCACAGCAAGCAGCAGCTGACACAGGACACAGCAATCAGGTCA **(SEQ ID NO: 5)**
Forward Primer (3' to 5')	CAGCTACAACCATCCCTTCAGACA **(SEQ ID NO: 6)**
Reverse Primer (3' to 5')	TGACCTGATTGCTGTGTCCTGTGT **(SEQ ID NO: 7)**
Probe 1 (3' to 5')	**/56-FAM**/AGCAACCCTCTATTGTGTGCATCAAAGG/3BHQ_1 **(SEQ ID NO: 8)**
Probe 2 (3' to 5')	**/5Cy3**/AAAGCACAGCAAGCAGCAGCTGA/3BHQ_2/ **(SEQ ID NO: 9)**

Table 11. *Plasmodium* ChR7 sequence information.

Sequence Name	*Plasmodium* ChR7 199mer synthesized from bases 1139138 - 1141223
Source	UCSC Plasmodium falciparum Genome Browser Gateway
Target sequence (3' to 5')	GCCTAACATGGCTATGACGGGTAACGGGGAATTAGAGTTCGATTCCGGAG AGGGAGCCTGAGAAATAGCTACCACATCTAAGGAAGGCAGCAGGCGCGTA AATTACCCAATTCTAAAGAAGAGAGGTAGTGACAAGAAATAACAATGCAA GGCCAATTTAAAACCTTCCCAGAGTAACAATTGGAGGGCAAGTCTGGTG **(SEQ ID NO: 10)**
Forward Primer (3' to 5')	GCCTAACATGGCTATGACGGGTAA **(SEQ ID NO: 11)**

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Reverse Primer (3' to 5')	CACCAGACTTGCCCTCCAATTGTT **(SEQ ID NO: 12)**
Probe 1 (3' to 5')	/**56-FAM**/ATTCCGGAGAGGGAGCCTGAGAAATA/3BHQ_1/ **(SEQ ID NO: 13)**
Probe 2 (3' to 5')	/**56-ROXN**/AAGGAAGGCAGCAGGCGCGTAAATTA/3BHQ_2/ **(SEQ ID NO: 14)**

Table 12. Herpes simplex virus 2 sequence information.

Sequence Name	HSV-2 193mer synthesized from HSV-2 genome
Source	EMBL Bank AJ303204
Target sequence (3' to 5')	TCAGCCCATCCTCCTTCGGCAGTATGGAGGGTGTCGCGGCGGCGAGCCGC CGTCCCCAAAGACGTGCGGGTCGTACACGTACACGTACCAGGGCGGCGGG CCTCCGACCCGGTACGCTCTCGTAAATGCTTCCCTGCTGGTGCCGATCTG GGACCGCGCCGCGGAGACATTCGAGTACCAGATCGAACTCGG **(SEQ ID NO: 15)**
Forward Primer (3' to 5')	TCAGCCCATCCTCCTTCGGCAGTAT **(SEQ ID NO: 16)**
Reverse Primer (3' to 5')	CCGAGTTCGATCTGGTACTCGAATGT **(SEQ ID NO: 17)**
Probe 1 (3' to 5')	/**56-FAM**/AAAGACGTGCGGGTCGTACACGTACA/3BHQ_1/ **(SEQ ID NO: 18)**
Probe 2 (3' to 5')	/**5Cy5**/TAAATGCTTCCCTGCTGGTGCCGAT/3IAbRQSp/ **(SEQ ID NO: 19)**

Table 13. *Mycobacterium tuberculosis* rpoB sequence information

Sequence Name	Mycobacterium Tuberculosis rpoB 200mer synthesized from Mycobacterium tuberculosis rpoB genome
Source	EMBL Bank GQ395623
Target sequence (3' to 5')	GAGTGCAAAGACAAGGACATGACGTACGCGGCCCCGCTGTTCGTCACGGC CGAGTTCATCAACAACAACACCGGCGAGATCAAGAGCCAGACGGTGTTCA TGGGTGACTTCCCGATGATGACCGAGAAGGGCACCTTCATCATCAACGGC ACCGAGCGCGTCGTGGTCAGCCAGCTGGTCCGCTCGCCCGGTGTGTACTT **(SEQ ID NO: 20)**

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Forward Primer (3' to 5')	GAGTGCAAAGACAAGGACATGACG **(SEQ ID NO: 21)**
Reverse Primer (3' to 5')	AAGTACACACCGGGCGAGC **(SEQ ID NO: 22)**
Probe 1 (3' to 5')	/**56-FAM**/CGCTGTTCGTCACGGCCGAGTTCAT/3BHQ_1/ **(SEQ ID NO: 23)**
Probe 2 (3' to 5')	/**5Cy3**/AGATCAAGAGCCAGACGGTGTTCATG/3BHQ_2/ **(SEQ ID NO: 24)**
Probe 3 (3' to 5')	/**5Cy5**/AAGGGCACCTTCATCATCAACGGCA/3IAbRQSp/ **(SEQ ID NO: 25)**

Table 14. Dengue virus type 3 sequence information.

Sequence Name	Dengue Virus Type 3 200mer synthesized from Dengue Virus Type 3 genome
Source	GenBank M93130
Target sequence (3' to 5')	ATGCCAACTGTGATTGAGCACTTAGAAAGACTACAAAGGAAACATGGAGG AATGCTTGTGAGAAATCCACTCTCACGAAACTCCACGCACGAAATGTATT GGATATCCAATGGTACAGGCAACATCGTCTCATTCACAATGACACACAGG AGACCCACCATAGAGAAAGATGTGGATTTAGGAGCAGGAACCCGACATGT **(SEQ ID NO: 26)**
Forward Primer (3' to 5')	ATGCCAACTGTGATTGAGCACT **(SEQ ID NO: 27)**
Reverse Primer (3' to 5')	ACATGTCGGGTTCCTGCTCCTAAA **(SEQ ID NO: 28)**
Probe 1 (3' to 5')	/**56-FAM**/ACAAAGGAAACATGGAGGAATGCTTGTGA/3BHQ_1/ **(SEQ ID NO: 29)**
Probe 2 (3' to 5')	/**5Cy3**/ACTCTCACGAAACTCCACGCACGAAA/3BHQ_2/ **(SEQ ID NO: 30)**
Probe 3 (3' to 5')	/**56-ROXN**/ACAATGACACACAGGAGACCCACCAT/3BHQ_2/ **(SEQ ID NO: 31)**
Probe 4 (3' to 5')	/**5Cy5**/GGATATCCAATGGTACAGGCAACATCGT/3IAbRQSp/ **(SEQ ID NO: 32)**

3. Polymerase Chain Reactions

REMARKS

Examination and favorable consideration is respectfully requested in view of the present amendment.

CONCLUSION

Applicants respectfully request that the Examiner advance the application to issuance. The Commissioner is authorized to charge any additional fees which may be required, including petition fees and extension of time fees, to Deposit Account No. 23-2415 (Docket No. 38075-716.302).

Respectfully submitted,

WILSON SONSINI GOODRICH & ROSATI

Date: March 22, 2018

By: /Kyle Bruner/
Kyle Bruner, Reg. No. 75,716

650 Page Mill Road
Palo Alto, California 94304
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SEQUENCE LISTING

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

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PTO/SB/93 (11-14)
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REQUEST FOR TRANSFER OF A COMPUTER READABLE FORM UNDER 37 CFR 1.821(e)

Application No.:	15/914,356	First Named Inventor:	Emil P. Kartalov
Filing Date:	March 7, 2018	Practitioner Docket No.:	38075-716.302
Title of the Invention:	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS		

The sequence information in the paper copy or PDF file of the Sequence Listing submitted in this application in accordance with 37 CFR 1.821(c),

- ☐ as part of the originally-filed specification of this application
- ☒ as a separate amendment of the specification filed
  - ☒ herewith
  - ☐ on _____

is identical to the sequence information in the

- ☒ only computer readable form
- ☐ last filed computer readable form
- ☐ other computer readable form, which was filed on _____

in application number 14/451,876, which was filed on August 5, 2014.

This computer readable form was compliant with 37 CFR 1.821-1.825, and applicant hereby requests that it be used as the computer readable form for the present application, in accordance with 37 CFR 1.821(e).

The above referenced paper copy or PDF file of the Sequence Listing contains no new matter.

A sequence listing text file submitted via EFS-Web that complies with the requirements of 37 CFR 1.824(a) (2)-(6) and (b) (i.e., is a compliant sequence listing ASCII text file), serves as both the paper copy required by 37 CFR 1.821(c) and the CRF required by 37 CFR 1.821(e). If a user submits a compliant sequence listing ASCII text file via EFS-Web, the U.S. Patent and Trademark Office will not carry out a request to use a compliant computer readable "Sequence Listing" that is already on file for another application pursuant to 37 CFR 1.821(e) but will use the sequence listing submitted with the application as originally filed via EFS-Web.

It is understood that upon the transfer of a copy of the computer readable form to this application, the U.S. Patent and Trademark Office will update the copy of the computer readable form to reflect the application number and filing date for this application.

Signature /Kyle Bruner/	Date March 22, 2018
Name (Print/Typed) Kyle Bruner	Practitioner Registration Number 75,716

Note: *This form must be signed in accordance with 37 CFR 1.33. See 37 CFR 1.4(d) for signature requirements and certifications. Submit multiple forms if more than one signature is required.**

☒ *Total of 1 forms are submitted.

This collection of information is required by 35 U.S.C. 22 and 37 CFR 1.821-1.825. The information is required to obtain or retain a benefit by the public, which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.11 and 1.14. This collection is estimated to take 6 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS.

Electronic Acknowledgement Receipt

EFS ID:	32131926
Application Number:	15914356
International Application Number:	
Confirmation Number:	2457
Title of Invention:	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS
First Named Inventor/Applicant Name:	Emil P. Kartalov
Customer Number:	21971
Filer:	J Kyle Bruner/Katherine Lopez
Filer Authorized By:	J Kyle Bruner
Attorney Docket Number:	38075-716.302
Receipt Date:	22-MAR-2018
Filing Date:	
Time Stamp:	16:30:07
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		CIT_38075_716_302_Signed_Prelim_Amd.pdf	142790 217b0c6414fb21d4039ac877f7a93f1ae1200d46	yes	6

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	Preliminary Amendment		1	1	
	Specification		2	5	
	Applicant Arguments/Remarks Made in an Amendment		6	6	
Warnings:					
Information:					
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Information:					
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If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

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

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

New International Application Filed with the USPTO as a Receiving Office

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

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 4

Complete if Known	
Application Number	15914356
Filing Date	03-07-2018
First Named Inventor	KARTALOV; Emil P.
Art Unit	2877
Examiner Name	Unassigned
Attorney Docket Number	38075-716.302

U. S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	001	US-9366632	06-14-2016	LINK; Darren Roy et al.	
	002	US-9441266	09-13-2016	LARSON; Jonathan William et al.	

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3]–Number[4]–Kind Code[5] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear	T[6]
		None				

Examiner Signature		Date Considered	

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

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 2 of 4

Complete if Known	
Application Number	15914356
Filing Date	03-07-2018
First Named Inventor	KARTALOV; Emil P.
Art Unit	2877
Examiner Name	Unassigned
Attorney Docket Number	38075-716.302

NON-PATENT LITERATURE DOCUMENTS			
Examiner Initials*	Cite No.[1]	Include name of the author(in CAPITAL LETTERS),title of the article(when appropriate), title of the item (book,magazine,journal,serial,symposium,catalog,etc.),date,page(s),volume-issue number(s),publisher, city and/or country where published.	T[2]
		None	

Examiner Signature		Date Considered	

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

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U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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Substitute for form 1449/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet	3	of	4

Complete if Known	
Application Number	15914356
Filing Date	03-07-2018
First Named Inventor	KARTALOV; Emil P.
Art Unit	2877
Examiner Name	Unassigned
Attorney Docket Number	38075-716.302

CERTIFICATION STATEMENT

Please see 37 CFR 1.97 and 1.98 to make the appropriate selection(s):

☐ That each item of information contained in the information disclosure statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(1).

OR

☐ That no item of information contained in the information disclosure statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing the certification after making reasonable inquiry, no item of information contained in the information disclosure statement was known to any individual designated in 37 CFR 1.56(c) more than three months prior to the filing of the information disclosure statement. See 37 CFR 1.97(e)(2).

☐ See attached certification statement.

☐ Fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Kyle Bruner/	Date (YYYY-MM-DD)	2018-04-26
Name/Print	Kyle Bruner	Registration Number	75,716

This collection of information is required by 37 CFR 1.97 and 1.98. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR1.14. This collection is estimated to take 1 hour to complete, including gathering, preparing and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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

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

Electronic Acknowledgement Receipt

EFS ID:	32460170
Application Number:	15914356
International Application Number:	
Confirmation Number:	2457
Title of Invention:	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS
First Named Inventor/Applicant Name:	Emil P. Kartalov
Customer Number:	21971
Filer:	Lee Alan Johnson/Judy Wan
Filer Authorized By:	Lee Alan Johnson
Attorney Docket Number:	38075-716.302
Receipt Date:	26-APR-2018
Filing Date:	
Time Stamp:	19:42:19
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		IDS-38075-716-302-04-26-18.pdf	283732 fd12d5c62ce3b591e6db2d945da440dbdffbc8d	yes	8

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Transmittal Letter	1	4
	Information Disclosure Statement (IDS) Form (SB08)	5	8
Warnings:			
Information:			
	Total Files Size (in bytes):	283732	

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

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.
National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.
New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventor:	KARTALOV; Emil P.	Group Art Unit:	2877
Serial Number:	15/914,356	Examiner:	Unassigned
Filing or 371 (c) Date:	2018-03-07	**CONFIRMATION NO:**	**2457**
Title:	SIGNAL ENCODING AND DECODING IN MULTIPLEXED BIOCHEMICAL ASSAYS		

FILED ELECTRONICALLY ON: April 26, 2018

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

INFORMATION DISCLOSURE STATEMENT UNDER 37 CFR § 1.97

Commissioner for Patents:

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

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

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

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

IPR2024-01178
California Institute of Technology - Ex. 2003 Page 698

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

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

-- OR --

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

-- OR --

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

-- OR --

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

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

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

-- OR --

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

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

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

-- AND --

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

D. ☐ *37 CFR §1.97 (e).* Statement.

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

-- AND/OR --

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

-- AND/OR --

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

IPR2024-01178
California Institute of Technology - Ex. 2003 Page 699

E. ☐ *Statement Under 37 C.F.R. §1.704(d).* Each item of information contained in the information disclosure statement was first cited in any communication from a patent office in a counterpart foreign or international application or from the Office or is a communication that was issued by a patent office in a counterpart foreign or international application or by the Office that was received by an individual designated in § 1.56(c) not more than thirty (30) days prior to the filing of this information disclosure statement. This statement is made pursuant to the requirements of 37 C.F.R. §1.704(d) to avoid reduction of the period of adjustment of the patent term for Applicant(s) delay.

F. ☒ *37 CFR §1.98 (a) (2).* The content of the Information Disclosure Statement is as follows:

☐ Copies of each of the references listed on the attached Form PTO/SB/08 are enclosed herewith.

-- OR --

☒ Copies of U.S. Patent Documents (issued patents and patent publications) listed on the attached Form PTO/SB/08 are not enclosed.

-- AND/OR --

☐ Copies of Foreign Patent Documents and/or Non Patent Literature Documents listed on the attached Form PTO/SB/08 are enclosed in accordance with 37 CFR §1.98 (a)(2).

-- AND/OR --

☐ Copies of pending unpublished U.S. patent applications are enclosed in accordance with 37 CFR §1.98 (a) (2) (iii).

G. ☐ *37 CFR §1.98(a)(3).* The Information Disclosure Statement includes non-English patents and/or references.

☐ Pursuant to 37 CFR §1.98(a)(3)(i), a concise explanation of the relevance of each patent, publication or other information provided that is not in English is provided herewith.

☐ Pursuant to MPEP 609(B), an English language copy of a foreign search report is submitted herewith to satisfy the requirement for a concise explanation where non-English language information is cited in the search report.

-- OR --

☐ A concise explanation of the relevance of each patent, publication or other information provided that is not in English is as follows: _____

☐ Pursuant to 37 CFR §1.98(a) (3) (ii), a copy of a translation, or a portion thereof, of the non-English language reference(s) is provided herewith.

H. ☐ *37 CFR §1.98(d).* Copies of patents, publications and pending U.S. patent applications, or other information specified in 37 C.F.R. § 1.98(a) are not provided herewith because:

☐ Pursuant to 37 CFR §1.98(d)(1) the information was previously submitted in an Information Disclosure Statement, or cited by examiner for another application under which this application claims priority for an earlier effective filing date under 35 U.S.C. 120.

Application in which the information was submitted: _____

Information Disclosure Statement(s) filed on: _____

AND

☐ The information disclosure statement submitted in the earlier application complied with paragraphs (a) through (c) of 37 CFR §1.98.

IPR2024-01178
California Institute of Technology - Ex. 2003 Page 700

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

Respectfully submitted,

WILSON SONSINI GOODRICH & ROSATI

Dated: April 26, 2018

By: /Kyle Bruner/
Kyle Bruner, Patent Agent
Register No.: 75,716

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

IPR2024-01178
California Institute of Technology - Ex. 2003 Page 701

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Substitute for form 1449/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 4

Complete if Known	
Application Number	15914356
Filing Date	03-07-2018
First Named Inventor	KARTALOV; Emil P.
Art Unit	2877
Examiner Name	Not Yet Assigned
Attorney Docket Number	38075-716.302

U. S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	001	US-5882856	03-16-1999	SHUBER; Anthony P.	
	002	US-20090042735	02-12-2009	BLAIR; Steven M. et al.	
	003	US-20120040349	02-16-2012	VON; Lode Piia et al.	
	004	US-20140213471	07-31-2014	RAJAGOPAL; Aditya et al.	
	005	US-20150275295	10-01-2015	WANG; Linfa et al.	
	006	US-20180057864	03-01-2018	JACKY; Lucian A.E.	

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3]-Number[4]-Kind Code[5] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear	T[6]
	001	WO-2006079049-A2	07-27-2006	THIRD WAVE TECH INC [US], et al.		☐
	002	WO-2017218777-A1	12-21-2017	CALIFORNIA INST OF TECHN [US]		☐

Examiner Signature		Date Considered	

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

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Sheet 2 of 4

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First Named Inventor	KARTALOV; Emil P.
Art Unit	2877
Examiner Name	Not Yet Assigned
Attorney Docket Number	38075-716.302

NON-PATENT LITERATURE DOCUMENTS			
Examiner Initials*	Cite No.[1]	Include name of the author(in CAPITAL LETTERS),title of the article(when appropriate), title of the item (book,magazine,journal,serial,symposium,catalog,etc.),date,page(s),volume-issue number(s),publisher, city and/or country where published.	T[2]
	001	EVANS et al. Digital PCR for Noninvasive Detection of Aneuploidy: Power Analysis Equations for Feasibility. Fetal Diagn. Ther. 31:244-247(2012).	☐
	002	U.S. Serial No. 15/623,974 Notice of Allowance dated July 3, 2018.	☐
	003	HUDECOVA, I. et al. Maternal Plasma Fetal DNA Fractions in Pregnancies with Low and High Risks for Fetal Chromosomal Aneuploidies. PLoS ONE 9(2): e88484..	☐
	004	U.S. Application No. 15/677,772 Final Office Action Mailed June 7, 2018.	☐
	005	U.S. Application No. 15/677,772 Non-Final Office Action Mailed February 8, 2018.	☐
	006	U.S. Application No. 15/892,245 Pre-interview First Interview Office Action Mailed May 11, 2018.	☐
	007	U.S. Serial No. 14/451,876 Notice of Allowance dated July 11, 2018.	☐
	008	U.S. Serial No. 14/451,876 Notice of Allowance dated June 14, 2018.	☐
	009	YANG, L. et al. A novel universal real-time PCR system using the attached universal duplex probes for quantitative analysis of nucleic acids. BMC Molecular Biology, 9:54 (1-13) June 4, 2008.	☐
	010	ZHANG, et al. A novel real-time quantitative PCR method using attached universal template probe. Nucleic Acids Res. 2003 Oct 15;31(20):e123(pp. 1-8).	☐

Examiner Signature		Date Considered	

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☐ See attached certification statement.

☐ Fee set forth in 37 CFR 1.17 (p) has been submitted herewith.

☒ A certification statement is not submitted herewith.

SIGNATURE

A signature of the applicant or representative is required in accordance with CFR 1.33, 10.18. Please see CFR 1.4(d) for the form of the signature.

Signature	/Kyle Bruner/	Date (YYYY-MM-DD)	2018-08-14
Name/Print	Kyle Bruner	Registration Number	75,716

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1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
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5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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

INFORMATION DISCLOSURE STATEMENT UNDER 37 CFR § 1.97

Commissioner for Patents:

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

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

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

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

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E. ☐ *Statement Under 37 C.F.R. §1.704(d).* Each item of information contained in the information disclosure statement was first cited in any communication from a patent office in a counterpart foreign or international application or from the Office or is a communication that was issued by a patent office in a counterpart foreign or international application or by the Office that was received by an individual designated in § 1.56(c) not more than thirty (30) days prior to the filing of this information disclosure statement. This statement is made pursuant to the requirements of 37 C.F.R. §1.704(d) to avoid reduction of the period of adjustment of the patent term for Applicant(s) delay.

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Application in which the information was submitted: _____

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AND

☐ The information disclosure statement submitted in the earlier application complied with paragraphs (a) through (c) of 37 CFR §1.98.

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

Respectfully submitted,

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Dated: August 14, 2018

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(54) Title: METHODS AND COMPOSITIONS FOR INCREASED DYNAMIC RANGE DETECTION OF NUCLEIC ACID MOLECULES

(57) Abstract: The present invention provides systems, methods and kits for increasing the dynamic range of detection of a target nucleic acid in a sample. In particular, the present invention provides methods and kits for increasing the dynamic range of detection of a target nucleic acid in a sample through the use of one or more probe oligonucleotides (e.g., analyte-specific probe oligonucleotides).

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COMPOSITIONS AND METHODS FOR INCREASED DYNAMIC RANGE DETECTION OF NUCLEIC ACIDS

The present invention claims priority to U.S. Patent Application Serial Number 60/645,696, filed January 21, 2005, the disclosure of which is herein incorporated by reference in its entirety.

FIELD OF THE INVENTION

The present invention provides systems, methods and kits for increasing the dynamic range of detection of a target nucleic acid in a sample. In particular, the present invention provides methods and kits for increasing the dynamic range of detection of a target nucleic acid in a sample through the use of one or more probe oligonucleotides.

BACKGROUND

All nucleic acid detection systems that rely on amplification of either the target being detected or the signal being generated inherently possess a dynamic range that limits their usefulness. At low concentrations of the target being detected, the signal generated is too low to detect or to low to be scored above background levels, and therefore is below the limit of detection, i.e., outside the dynamic range of the detection system. By contrast, at very high levels of the target being generated, the components of the detection system are exhausted such that the signal is said to be saturated, i.e. addition of still more target results in no increase in signal. In these cases, the quantity of target is said to be above the limit of detection, i.e. outside the dynamic range of the detection system.

In the real-world case of detection systems being used to detect targets from biological specimens, the range of target present in the sample being detected can be quite large, and is often either below or above the limit of detection of the system in use. Therefore, previous attempts to cover larger ranges of target concentration have required the generation of more than one detection system, to be used separately, that are optimized for a given dynamic range. Because the quantity of target nucleic acid in the specimen is by definition an unknown quantity, this very frequently requires the use of

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multiple detection systems sequentially to finally use the appropriate detection system that possesses the appropriate dynamic range for the specimen under examination.

As such, a single detection system with a broader dynamic range, if it was available, would significantly reduce costs, decrease labor time, and decrease expenditure of the specimen being examined. Even more, a method of increasing the dynamic range of an existing detection system would greatly aid the field of detection of targets within biological specimens generally.

SUMMARY OF THE INVENTION

The present invention provides systems, methods and kits for increasing the dynamic range of detection of a target nucleic acid in a sample. In particular, the present invention provides systems, methods and kits for increasing the dynamic range of detection of a target nucleic acid in a sample through the use of one or more probe oligonucleotides (e.g., analyte-specific probe oligonucleotides).

For example, in some embodiments, the present invention provides compositions, kits, and methods of quantitating nucleic acid targets (e.g., viral pathogens) using multiple probes that bind to a target nucleic acid at different strengths. In some embodiments, groups of probes are used in which each probe exhibits different binding affinities to the target sequence (e.g., by altering complementarity, length, concentration, additives, etc.). The use of multiple probes with different properties allows for an increase in the dynamic range of detection assays. In some embodiments, the multiple probes are used in invasive cleavage assays.

Accordingly, in some embodiments, the present invention provides a method for detecting the presence of, absence of, or amount of a target nucleic acid in a sample, comprising: incubating a sample suspected of containing a target nucleic acid with a plurality of first probe oligonucleotides and a plurality of second probe oligonucleotides, wherein each of the first and second probe oligonucleotides comprises an analyte specific region, wherein the plurality of second probe oligonucleotides are configured to occupy a probe hybridization site on the target nucleic acid at a different frequency than the plurality of first probe oligonucleotides; and measuring hybridization of the first and said second probe oligonucleotides over time, thereby measuring the amount of the target

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nucleic acid. In some embodiments a plurality of third, fourth, fifth, etc. probe oligonucleotides are used. These additional oligonucleotides may be configured to bind to the same analyte-specific region of a target nucleic acid or may bind to different analyte-specific regions of the same or different target nucleic acids (e.g., the third and fourth probes are configured to hybridize to a second analyte-specific region of the same target nucleic acid such that the third probe occupies the hybridization site at a different frequency than the fourth probe).

In some embodiments, the analyte specific regions of the first probe oligonucleotides are completely complementary to the target nucleic acid. In some embodiments, the analyte specific regions of the second probe oligonucleotides are partially complementary to the target nucleic acid (e.g., contain a single mismatch). In some embodiments, the second probe oligonucleotide is shorter in length than the first probe oligonucleotide (e.g., by one, two, three, or four or more nucleotides). In some embodiments, the second probe oligonucleotides are present at at least a 5 fold, and preferably at least a 10 fold lower concentration than the first probe oligonucleotides. In some embodiments, the second probe oligonucleotides are present at at least a 20 fold (e.g., 100 fold, 500 fold, 1000 fold, 10,000 fold, etc. lower concentration than the first probe oligonucleotide). Where three or more probes of different concentrations are used, each probe may be separated by at at least 5 fold (10 fold, 20 fold, 100 fold, etc.) concentration from one another (e.g., a third probe 10000 fold more than a first probe and a second probe 100 fold more than a first probe). In some embodiments, one of the mixtures comprises an agent known to increase or decrease hybridization efficiency (e.g., a charge tag, minor groove binding agent, or an intercalating agent). In other embodiments, one of the probes comprises one or more modified bases (e.g., amino T, indole, or nitropyrrole). In some embodiments, the analyte specific region of second probe oligonucleotide is shorter than the analyte specific region of the first probe oligonucleotide (e.g., by one or more nucleotides). In other embodiments, the analyte specific region of the second probe oligonucleotide comprises increased secondary structure relative to the analyte specific region of the first probe oligonucleotide. In certain embodiments, the first probe oligonucleotides further comprise a non-analyte specific region, wherein the non-analyte specific region comprises one or more

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nucleotides that are not complementary to the target nucleic acid. In some embodiments, each of the second probe oligonucleotides further comprises a non-analyte specific region, wherein the non-analyte specific region comprises one or more nucleotides that are not complementary to the target nucleic acid. In some embodiments, incubating the sample with the second probe oligonucleotides comprises incubating the sample with competitor oligonucleotides, wherein the competitor oligonucleotides each comprise a region that is complementary to the non-analyte specific regions of the second probe oligonucleotides. The present invention is not limited by the nature of the competitor. The competitor may be a second target nucleic acid or a different region of the first oligonucleotide where, for example, hybridization of the non-analyte specific region of the second probe to the competitor does not generate a detectable event or generates a detectable event that is distinguishable from the detectable event generated by the first and/or second probes hybridizing to the analyte-specific region.

In some embodiments, incubating the sample with the second probe oligonucleotides comprises incubating the sample with competitor oligonucleotides, wherein the competitor oligonucleotides each comprise a region that is complementary to the non-analyte specific regions of the second probe oligonucleotides. In certain embodiments, one of the mixtures comprises altered reaction conditions that alter hybridization efficiency of a probe (e.g., altered pH, buffer, ionic strength or additional compositions (e.g., crowding agents)).

In some embodiments, the sample is a sample from an animal (e.g., a human) comprising blood, serum, stool, urine, or lymph known to or suspected of comprising a target nucleic acid (e.g., a virus or a bacterium). In some embodiments, the sample comprises a purified sample of nucleic acid (e.g., total DNA or RNA from a tissue, fluid or cell; genomic DNA; etc.). In some embodiments, the target nucleic acid is from a virus (e.g., human immunodeficiency virus (HIV) and other retroviruses, hepatitis C virus (HCV), hepatitis B virus (HBV), hepatitis A virus (HAV), human cytomegalovirus, (CMV), herpes simplex virus (HSV), Epstein bar virus (EBV), varicella zoster virus (VZV), human papilloma virus (HPV), bacteriophages (e.g., phage lambda), influenzaviruses, adenoviruses, or lentiviruses) or a bacterium (e.g., *Chlamydia sp.*, *N. gonorrhea*, or group B *streptococcus*). In other embodiments, the sample is from a plant.

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For example, in some embodiments, the plant is infected with or suspected of being infected with a pathogenic microorganism (e.g., a fungus, a virus, or a bacteria).

In some embodiments, the methods of incubating the sample with the first and second probe oligonucleotides occur in the same reaction vessel (e.g., the first and second probe oligonucleotides are mixed in solution in the same reaction vessel). In some embodiments, the first an second probe oligonucleotides comprise labels. In some embodiments, the first and second labels are different from each other. In some embodiments, the first and second labels are the same label. In some embodiments, measuring the hybridization of the first and second probe oligonucleotides comprises performing an invasive cleavage structure type assay (e.g., an INVADER assay). In some such embodiments, the probes are unlabeled, but comprise a flap sequence that is removed from the probe upon cleavage during the invasive cleavage assay. In some embodiments, the removed flaps are configured to hybridize to a FRET cassette to trigger a detection reaction. In some embodiments, the first and second probes report to the same FRET cassette (e.g., the first and second probe generate identical flaps upon cleavage in the primary invasive cleavage reaction). In other embodiments, determining the amount of the target nucleic acid comprises performing a detection assay including, but not limited to, a hybridization assay, any real-time amplification assay that involves hybridization, a TAQMAN assay, SNP-IT assay, a Southern blot, a ligase assay, a microarray assay, a FULLVELOCITY assay, a cycling probe assay, NASBA, branched DNA assay, TMA, methods employing molecular beacons, capillary electrophoresis detection methods, microfluidic detection methods, and the like.

In other embodiments, the present invention provides a method for detecting the presence of, absence of, or amount of a target nucleic acid in a sample, comprising: providing a sample containing or suspected of containing a target nucleic acid; a first probe oligonucleotide comprising an analyte specific region and a first label, wherein the analyte specific region of the first probe oligonucleotide is completely complementary to the target nucleic acid; and a second probe oligonucleotide comprising an analyte specific region and a second label, wherein the analyte specific region of the second probe oligonucleotide is partially complementary to the target nucleic acid; and exposing the

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sample to the first and second probe oligonucleotides; and, in some embodiments, determining the amount of the target nucleic acid in the sample.

The present invention further provides a kit comprising reagents and, in some embodiments, instructions, for performing the detection assays of the present invention. For example, in some embodiments, the present invention provide a kit for detecting the presence of, absence of, or quantitation of target nucleic acids in a sample, comprising: a plurality of first probe oligonucleotides comprising a first analyte specific region and, optionally, a first label, and a plurality of second probe oligonucleotides comprising a second analyte specific region and, optionally, a second label, wherein the second probe oligonucleotides are configured to occupy a probe hybridization site on the target nucleic acid at a different frequency than the first mixture of probe oligonucleotides; and reagents for performing an INVADER assay using the first and second probe oligonucleotides. In some embodiments, the analyte specific regions of the first probe oligonucleotides are completely complementary to the target nucleic acid. In other embodiments, the analyte specific regions of the second probe oligonucleotides are partially complementary to the target nucleic acid (e.g., contain one or more mismatches with the target nucleic acid). In still further embodiments, the second probe oligonucleotides are present at a lower concentration than the first probe oligonucleotides. In some embodiments, the kit further comprises instructions for using the kit for performing a nucleic acid detection assay. In some embodiments, the kit comprises reagents and/or instructions for use of the methods of the present invention with a one or more different detection assay technologies (e.g., an invasive cleavage assay (e.g., INVADER assay), a TAQMAN assay, SNP-IT assay, etc.)).

In some embodiments, the present invention provides methods for detecting a target nucleic acid, comprising: a) amplifying a target nucleic acid at two different levels of amplification to generate amplification products; b) hybridizing the amplification products to a first probe and second probe, wherein the first probe hybridizes to the amplification products at a different frequency than the second probe. In certain embodiments, the second probe is present at a 10-fold lower concentration than the first probe. In other embodiments, the at least two probes bind to the same sequence.

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In additional embodiments, the present invention provides methods for detecting a target nucleic acid in a plurality of samples over a broad dynamic range, comprising: exposing a first sample having less than 10^3 copies of target nucleic acid and a second sample having greater than 10^5 copies of target nucleic acid to a set of reagents under conditions such that the target nucleic acid in the first and second samples is detected, wherein method comprises exposing each of the first and second samples to a first probe and a second probe, wherein the second probe hybridizes to the target nucleic acids at a different frequency than the first probe. In particular embodiments, the target nucleic acid in the first and second samples is quantitated. In further embodiments, the second probe is present at a 10-fold lower concentration than the first probe. In some embodiments, the target nucleic acids are treated under two or more different amplification conditions prior to detection. In other embodiments, the method is conducted without any amplification of the target nucleic acid.

In some embodiments, the present invention provides methods for detecting a target nucleic acid, comprising: a) amplifying a target nucleic acid to generate amplification products; b) contacting the amplification products with first and second probes, wherein the second probe hybridizes to the amplification products at a different frequency that the first probe; c) cleaving the first and second probes; and d) detecting the cleavage of the first and second probes.

In other embodiments, the present invention provides kits comprising: a polymerase, a 5' nuclease, and two probes configured to hybridize to an analyte-specific region of a target nucleic acid, wherein the second probe hybridizes to the analyte-specific region at a different frequency than the first probe oligonucleotide, and wherein the first and second probes are configured to both directly or indirectly generate a detectable signal in the presence of the target nucleic acid. In some embodiments, the first and second probes generate the same type of detectable signal. In certain embodiments, the first and second probes each comprise a flap sequence that is complementary to a FRET cassette. In other embodiments, the flap of the first probe is identical to the flap of the second probe.

In some embodiments, the present invention provides methods for detecting a target nucleic acid in a sample comprising; a) contacting a sample suspected of

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containing a target nucleic acid with amplification reagents such that, if the target nucleic acid is present: i) a first region of the target nucleic acid is either not amplified, or is amplified at a first level to generate plurality of first product sequences; and ii) a second region of the target nucleic acid is amplified at a second level to generate a plurality of second product sequences, wherein the second level of amplification is greater than the first level of amplification (e.g. such that the second product sequences are present at a level of at least 10-fold ... 100-fold ... 1000-fold ... 10,000-fold ... or 100,000-fold higher concentration after amplification that the target nucleic acid, or first product sequences if produced); and b) incubating the sample with a plurality of first and second probe oligonucleotides, wherein: i) the first and second probe oligonucleotides hybridize to the first region of the target nucleic acid, and the first product sequences if produced, at different frequencies, or ii) the first and second probe oligonucleotides hybridize to the second product sequences at a different frequency; and c) measuring hybridization of the first and second probe oligonucleotides thereby detecting the target nucleic acid in the sample. In particular embodiments, the second product sequences are present at a level between 100-fold and 100,000 fold higher concentration after amplification than the target nucleic acid, or first product sequences if produced. In certain embodiments, the target nucleic acid is micro-RNA.

In certain embodiments, the present invention provides methods for detecting a target nucleic acid in a plurality of samples over a broad dynamic range, comprising: exposing a first sample having less than 10^3 copies of target nucleic acid and a second sample having greater than 10^5 copies of target nucleic acid to a set of reagents under conditions such that the target nucleic acid in the first and second samples is detected, wherein the method comprises exposing each of the first and second samples to a first probe and a second probe, wherein the second probe hybridize to the target at different frequencies.

In particular embodiments, the present invention provides methods for detecting a target nucleic acid, comprising: a) linearly amplifying a first region of the target nucleic acid to generate linearly amplified amplification products; b) exponentially amplifying a second region of the target nucleic acid to generate exponentially amplified amplification products; c) hybridizing the linearly amplified amplification products with a first set of

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probes and the exponentially amplified amplification products with a second set of probes, wherein either the first or the second set of probes comprises a first plurality of probes that hybridize to amplified target nucleic acid and a second plurality of probes that hybridize to amplified target nucleic acid at a different frequency than the first plurality of probes. In certain embodiments, both the first set and the second set of probes comprises a first plurality of probes that hybridize to amplified target nucleic acid and a second plurality of probes that hybridize to amplified target nucleic acid at a different frequency than the first plurality of probes.

In some embodiments, the present invention provides methods for detecting a target nucleic acid, comprising: a) amplifying a target nucleic acid both linearly and exponentially to generate amplification products; b) hybridizing the amplification products to at least two probes, wherein the first probe hybridizes to amplified target nucleic acid at a different frequency than the second probe. In certain embodiments, the first and second probes both hybridize to the same probe binding site on the target nucleic acid.

In certain embodiments, the present invention provides methods for detecting a target nucleic acid in a sample comprising; a) contacting a sample suspected of containing a target nucleic acid with amplification reagents such that, if the target nucleic acid is present: i) a first region of the target nucleic acid comprising a first probe hybridization site is either not amplified, or is amplified at a first level to generate plurality of first product sequences that comprise the first probe hybridization site; and ii) a second region of the target nucleic acid is amplified at a second level to generate a plurality of second product sequences that comprise a second probe hybridization site, wherein the second level of amplification is greater than the first level of amplification (e.g. such that the second product sequences are present at a level of at least 10-fold ... 100-fold ... 1000-fold ... 10,000-fold ... or 100,000-fold higher concentration after amplification that the target nucleic acid, or first product sequences if produced); and b) incubating the sample with a plurality of first and second probe oligonucleotides, wherein: i) the first and second probe oligonucleotides occupy the first probe hybridization site on the first region of the target nucleic acid, and the first product sequences if produced, at different frequencies, or ii) the first and second probe

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oligonucleotides occupy the second probe hybridization site on the second product sequences at a different frequency; and c) measuring hybridization of the first and second probe oligonucleotides thereby detecting the target nucleic acid in the sample. In particular embodiments, the second product sequences are present at a level between 100-fold and 100,000 fold higher concentration after amplification than the target nucleic acid, or first product sequences if produced.

In other embodiments, the methods further comprise incubating the sample with a third probe oligonucleotide that occupies the first probe hybridization site on the first region of the target nucleic acid, and the first product sequences if produced, at a first frequency, and measuring the hybridization of the third probe oligonucleotide. In some embodiments, the methods further comprise incubating the sample with a fourth probe oligonucleotide that occupies the first probe hybridization site on the first region of the target nucleic acid, and the first product sequences if produced, at a second frequency, wherein the second frequency is different from the first frequency, and measuring the hybridization of the fourth probe oligonucleotide.

In certain embodiments, the methods further comprise incubating the sample with a third probe oligonucleotide that occupies the second probe hybridization site on the second product sequences at a first frequency, and measuring the hybridization of the third probe oligonucleotide. In particular embodiments, the methods further comprise incubating the sample with a fourth probe oligonucleotide that occupies the second probe hybridization site on the second product sequences at a second frequency, wherein the second frequency is different from the first frequency, and measuring the hybridization of the fourth probe oligonucleotide.

In some embodiments, the first level of amplification is achieved by linear amplification, and the second level is achieved is achieved with logarithmic amplification (e.g., polymerase chain reaction). In further embodiments, the first level of amplification is achieved with compromised amplification (e.g. using inefficient primers and/or inefficient polymerases). In other embodiments, the second level of amplification is at least 10-fold greater than no amplification or the first level of amplification. In certain embodiments, the target nucleic acid is micro-RNA.

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In some embodiments, the present invention provides methods for detecting a target nucleic acid in a sample, comprising; a) contacting a sample suspected of containing a target nucleic acid with amplification reagents such that, if the target nucleic acid is present: i) a first region of the target nucleic acid is amplified non-logarithmically to generate a plurality of non-logarithmically amplified sequences that comprise a first probe hybridization site, and ii) a second region of the target nucleic acid is amplified logarithmically to generate a plurality of logarithmically amplified sequences that comprise a second probe hybridization site; b) incubating the sample with a plurality of first probe oligonucleotides, a plurality of second probe oligonucleotides, and a plurality of third probe oligonucleotides, wherein each of the first, second, and third probe oligonucleotides comprises an analyte specific region, wherein the plurality of second probe oligonucleotides are configured to occupy the second probe hybridization site on the logarithmically amplified sequences at a different frequency than the plurality of first probe oligonucleotides, and wherein the third probe oligonucleotides are configured to occupy the first probe hybridization site on the non-logarithmically amplified sequences at a first frequency; and c) measuring hybridization of the first, second, and third probe oligonucleotides, thereby detecting the target nucleic acid in the sample. In other embodiments, the target nucleic acid is initially present in the sample in an amount between about 10^1 and about 10^8 molecules (e.g. the dynamic range of the methods extend over at least about seven orders of magnitude). In certain embodiments, the target nucleic acid is micro-RNA.

In certain embodiments, the measuring detects the amount of the target nucleic acid in the sample. In other embodiments, the measuring is conduced over time. In further embodiments, the plurality of logarithmically amplified sequences do not contain the first probe hybridization site.

In particular embodiments, the analyte specific regions of the first probe oligonucleotides are completely complementary to the second probe hybridization site of the second product sequence (e.g. logarithmically amplified sequences). In other embodiments, the analyte specific regions of the second probe oligonucleotides are partially complementary to the second probe hybridization site of the second product sequences (e.g., logarithmically amplified sequences).

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In certain embodiments, the methods further comprise incubating the sample with a plurality of fourth probe oligonucleotides comprising an analyte specific region, wherein the fourth probe oligonucleotides are configured to occupy the first probe hybridization site on the first product sequences (e.g., non-logarithmically amplified sequences) at a second frequency which is different from the first frequency of the third probe oligonucleotides. In further embodiments, the target nucleic acid is initially present in the sample in an amount between about 10^1 and about 10^{10} molecules (e.g. the dynamic range of the methods extend over at least about nine orders of magnitude).

In further embodiments, the analyte specific regions of the third probe oligonucleotides are completely complementary to the first probe hybridization site of the first product sequences (e.g., non-logarithmically amplified sequences). In other embodiments, the analyte specific regions of the third probe oligonucleotides are partially complementary to the first probe hybridization site of the first product sequences (e.g, non-logarithmically amplified sequences). In additional embodiments, the analyte specific regions of the third oligonucleotides are identical to the analyte specific regions of the fourth oligonucleotides.

In some embodiments, the second probe oligonucleotides are present in at least a 5-fold lower concentration than the first probe oligonucleotides (e.g. 5-fold, 6-fold, 7-fold, 8-fold, or 9-fold lower concentration). In certain embodiments, the second probe oligonucleotides are present in at least a 10-fold lower concentration than the first probe oligonucleotides (e.g. 10-fold ... 15-fold ... 25-fold ... 50-fold ...75-fold ... or 95-fold lower concentration, or any range between 10-fold and 100-fold). In particular embodiments, the second probe oligonucleotides are present in at least a 100-fold lower concentration than the first probe oligonucleotides (e.g. 100-fold ... 125-fold ... 150-fold ... 250-fold ... 500-fold ... 750-fold ... or 900-fold lower concentration, or any range between 100-fold and 1000-fold). In further embodiments, the second probe oligonucleotides are present in at least a 1000-fold lower concentration than the first probe oligonucleotides (e.g., 1000-fold ... 1100-fold ... 1300-fold ... 1500-fold ... 10,000-fold ... 15,000-fold ... 25,000-fold ... 100,000-fold ... 500,000-fold ... or 1,000,000-fold, or any range between 1000-fold and 1,000,000-fold).

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In some embodiments, the third probe oligonucleotides are present in at least a 5-fold lower concentration than the fourth probe oligonucleotides (e.g. 5-fold, 6-fold, 7-fold, 8-fold, or 9-fold lower concentration). In certain embodiments, the third probe oligonucleotides are present in at least a 10-fold lower concentration than the fourth probe oligonucleotides (e.g. 10-fold ... 15-fold ... 25-fold ... 50-fold ...75-fold ... or 95-fold lower concentration, or any range between 10-fold and 100-fold). In particular embodiments, the third probe oligonucleotides are present in at least a 100-fold lower concentration than the fourth probe oligonucleotides (e.g. 100-fold ... 125-fold ... 150-fold ... 250-fold ... 500-fold ... 750-fold ... or 900-fold lower concentration, or any range between 100-fold and 1000-fold). In further embodiments, the third probe oligonucleotides are present in at least a 1000-fold lower concentration than the fourth probe oligonucleotides (e.g., 1000-fold ... 1100-fold ... 1300-fold ... 1500-fold ... 10,000-fold ... 15,000-fold ... 25,000-fold ... 100,000-fold ... 500,000-fold ... or 1,000,000-fold, or any range between 1000-fold and 1,000,000-fold).

In certain embodiments, the target nucleic acid is initially present in the sample in an amount between about 10^1 and about 10^3 molecules, and the amount of the target nucleic acid is determined by the measuring hybridization of the first probe oligonucleotides. In other embodiments, the target nucleic acid is initially present in the sample in an amount between about 10^3 and about 10^6 molecules, and the amount of the target nucleic acid is determined by the measuring hybridization of the second probe oligonucleotides. In some embodiments, the target nucleic acid is initially present in the sample in an amount between about 10^6 and about 10^8 molecules, and the amount of the target nucleic acid is determined by the measuring hybridization of the third probe oligonucleotides.

In certain embodiments, the method is conducted on two samples, wherein the target nucleic acid is initially present in one sample in an amount less than 10^3 and initially present in a second sample in an amount greater than 10^5. In other embodiments, the method is conducted on two samples, wherein the target nucleic acid is initially present in one sample in an amount less than 10^2 and initially present in a second sample in an amount greater than 10^6. In further embodiments, the method is conducted on two samples, wherein the target nucleic acid is initially present in one sample in an amount

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less than 10^4 and initially present in a second sample in an amount greater than 10^7, or greater than 10^8, or greater than 10^9.

In particular embodiments, the plurality of first product sequences (e.g, non-logarithmically amplified sequences) further comprise the second probe hybridization site. In other embodiments, the plurality of second product sequences (e.g., non-logarithmically amplified sequences) do not contain the second probe hybridization site. In certain embodiments, the non-logarithmic amplification of the first region comprises single-stranded PCR or compromised PCR.

In some embodiments, the first probe oligonucleotides further comprise a non-analyte specific region, wherein the non-analyte specific region comprises one or more nucleotides that are not complementary to the second product sequences (e.g, logarithmically amplified sequences). In other embodiments, the second probe oligonucleotides further comprise a non-analyte specific region, wherein the non-analyte specific region comprises one or more nucleotides that are not complementary to the second product sequences (e.g., logarithmically amplified sequences). In other embodiments, the third probe oligonucleotides further comprise a non-analyte specific region, wherein the non-analyte specific region comprises one or more nucleotides that are not complementary to the first product sequences (e.g, non-logarithmically amplified sequences). In further embodiments, the fourth probe oligonucleotides further comprise a non-analyte specific region, wherein the non-analyte specific region comprises one or more nucleotides that are not complementary to the first product sequences (e.g, non-logarithmically amplified sequences).

In certain embodiments, the analyte specific region of second probe oligonucleotide is shorter than the analyte specific region of the first probe oligonucleotide. In other embodiments, the analyte specific region of the fourth probe oligonucleotide is shorter than the analyte specific region of the third probe oligonucleotide.

In some embodiments, the first probe oligonucleotides comprise first labels and wherein the second probe oligonucleotides comprise second labels. In other embodiments, the third probe oligonucleotides comprise third labels and the fourth probe oligonucleotides comprise fourth labels. In particular embodiments, at least one of the

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first, second, or third oligonucleotides is unlabeled. In additional embodiments, the first, second, and third probe oligonucleotides are unlabeled. In some embodiments, the fourth probe oligonucleotides are un-labeled. In certain embodiments, the fourth probe oligonucleotides comprises a label. In other embodiments, the first, the second, and the third labels are different from each other or are the same as each other. In certain embodiments, the amplification reagents comprise first and second primers, and a polymerase.

In some embodiments, the first and second probe oligonucleotides further comprise a non-analyte specific region configured to not hybridize to the second probe hybridization site of the second product sequences (e.g, logarithmically amplified sequences), wherein the non-analyte specific region is 5' of the analyte specific region. In certain embodiments, the first and second probe oligonucleotides form an invasive cleavage structure with an upstream oligonucleotide, wherein the upstream oligonucleotide comprise a 5' portion and a 3' portion, wherein the 5' portion is configured to hybridize to a region contiguous with the second probe hybridization site on the second product sequences (e.g., logarithmically amplified sequences), and wherein the 3' portion is configured to not hybridize to the second product sequences (e.g., logarithmically amplified sequences). In other embodiments, the methods further comprise incubating the sample with a plurality of additional probe oligonucleotides comprising an analyte specific region, wherein the additional probe oligonucleotide is configured to occupy the second probe hybridization site on the second product sequences (e.g., logarithmically amplified sequences) at a frequency different that the first and second probe oligonucleotides.

In particular embodiments, the present invention provides methods for detecting an amount of a target nucleic acid in a sample, comprising; a) incubating a sample suspected of containing a target nucleic acid with a plurality of first probe oligonucleotides and a plurality of second probe oligonucleotides, wherein each of the first and the second probe oligonucleotides comprises an analyte specific region, wherein the plurality of second probe oligonucleotides are configured to occupy a probe hybridization site on the target nucleic acid with the same affinity as the plurality of first probe oligonucleotides, and wherein the plurality of second probe oligonucleotides are

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present in at least 5-fold lower concentration than the first probe oligonucleotides; and b) measuring hybridization of the first and the second probe oligonucleotides over time, thereby detecting the amount of the target nucleic acid. In some embodiments, the first probe oligonucleotides further comprise a first non-analyte specific region, and the second probe oligonucleotides further comprise a second non-analyte specific region which is not identical to the first non-analyte specific region. In other embodiments, the analyte specific regions of the first and second oligonucleotides have an identical sequence.

In additional embodiments, the present invention provides methods for detecting an amount of a target nucleic acid in a sample, comprising; a) incubating a sample suspected of containing a target nucleic acid with a plurality of un-labeled first probe oligonucleotides and a plurality of un-labeled second probe oligonucleotides, wherein each of the first and the second probe oligonucleotides comprises an analyte specific region, wherein the plurality of second probe oligonucleotides are configured to occupy a probe hybridization site on the target nucleic acid at a different frequency than the plurality of first probe oligonucleotides; and b) measuring hybridization of the first and the second probe oligonucleotides over time, thereby detecting the amount of the target nucleic acid.

In further embodiments, the present invention provides methods for detecting an initial amount of a target nucleic acid in a sample without amplifying initial amount of the target nucleic acid, comprising; a) incubating a sample initially containing 300 copies or less of a target nucleic acid with a plurality of first probe oligonucleotides and a plurality of second probe oligonucleotides, wherein each of the first and the second probe oligonucleotides comprises an analyte specific region, wherein the plurality of second probe oligonucleotides are configured to occupy a probe hybridization site on the target nucleic acid at a different frequency than the plurality of first probe oligonucleotides; b) measuring hybridization of the first and the second probe oligonucleotides over time, thereby measuring the amount of the target nucleic acid, wherein the 300 copies or less of the target nucleic acid are not amplified prior to the measuring step. In particular embodiments, the 300 copies or less is between 100 and 300 copies or between 100 and 200 copies.

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In some embodiments, the present invention provides methods for detecting an amount of a target nucleic acid in a sample, comprising; a) contacting a sample suspected of containing target nucleic acid with amplification reagents such that, if the target nucleic acid is present, a region of the target nucleic acid containing a probe hybridization site is amplified to generate a plurality of amplified sequences, b) incubating the sample with a plurality of first probe oligonucleotides and a plurality of second probe oligonucleotides, wherein each of the first and the second probe oligonucleotides comprises an analyte specific region, wherein the plurality of second probe oligonucleotides are configured to occupy the ~~a~~ probe hybridization site on the amplified sequence at a different frequency than the plurality of first probe oligonucleotides; c) measuring hybridization of the first and the second probe oligonucleotides over time, thereby measuring the amount of the target nucleic acid, wherein the measuring is possible when the target nucleic acid is initially present in the sample in an amount between about 1 molecule and about 10^7 molecules.

In certain embodiments, the incubating and measuring steps are conducted in a single vessel. In other embodiments, the contacting, incubating, and measuring steps are conducted in a single vessel. In further embodiments, the analyte specific regions of the first oligonucleotides are identical to the analyte specific regions of the second oligonucleotides. In some embodiments, the analyte specific regions of the second probe oligonucleotides contain a single mismatch with the logarithmically amplified sequences.

In some embodiments, the second or fourth probe oligonucleotides contain a charge tag. In other embodiments, the second or fourth probe oligonucleotide contains at least one modified nucleotide. In further embodiments, the second probe oligonucleotide has a lower or higher affinity for the second probe hybridization site than the first probe oligonucleotide. In particular embodiments, the second probe oligonucleotide has a lower or higher Tm with the second probe hybridization site than the first probe oligonucleotide. In additional embodiments, the fourth probe oligonucleotide has a lower or higher affinity for the first probe hybridization site than the third probe oligonucleotide. In other embodiments, the fourth probe oligonucleotide has a lower or higher Tm with the first probe hybridization site than the third probe oligonucleotide.

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In some embodiments, the measuring hybridization of the first, second, and/or third, and/or fourth probe oligonucleotides comprises performing a hybridization assay. In particular embodiments, the hybridization assay is selected from the group consisting of a TAQMAN assay, SNP-IT assay, an invasive cleavage assay, a Southern blot, and a microarray assay. In further embodiments, the invasive cleavage assay in an INVADER assay.

In certain embodiments, the present invention provides methods for genotyping a polymorphic locus in a target nucleic acid in a sample, comprising; a) contacting a sample suspected of containing the target nucleic acid with amplification reagents such that, if the target nucleic acid is present, a region of the target nucleic acid containing the polymorphic locus is amplified to generate a plurality of amplified sequences, wherein the amplification is conducted until saturation; b) incubating the sample with a plurality of first probe oligonucleotides and a plurality of second probe oligonucleotides, wherein each of the first probe oligonucleotides comprises: i) a first analyte specific region configured for detecting a first allele at the polymorphic locus, and ii) a label capable of generating a detectable signal or a cleavable portion configured to cause a detectable signal to be generated, and wherein the second probe oligonucleotides comprise: i) a second analyte specific region configured for detecting a second allele at the polymorphic locus, ii) a label capable of generating a detectable signal or a cleavable portion configured to cause a detectable signal to be generated, wherein the plurality of second probe oligonucleotides are configured to occupy a probe hybridization site on the amplified sequences at a different frequency than the plurality of first probe oligonucleotides, and wherein the type of detectable signal from the first and second probe oligonucleotides is the same; c) measuring the strength of the detectable signal generated, thereby determining the presence of the first allele, the second allele, or both the first and second alleles in the target nucleic acid. In certain embodiments, the polymorphic locus is a single nucleotide polymorphism. In other embodiments, the polymorphic locus is a repeat sequence.

In some embodiments, the present invention provides methods for detecting a target nucleic acid in a sample, comprising; a) incubating a sample suspected of containing a target nucleic acid with a plurality of first and second probe

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oligonucleotides, a plurality of upstream oligonucleotides, and a cleavage agent, wherein each of the first probe oligonucleotides comprise: i) a first analyte specific region configured to hybridize to a probe hybridization site on the target nucleic acid, and ii) a first non-analyte specific region configured to not hybridize to the target nucleic acid, wherein the first non-analyte specific region is 5' of the first analyte specific region, and wherein each of the second probe oligonucleotides comprises i) a second analyte specific region configured to hybridize to the probe hybridization site on the target nucleic acid, and ii) a second non-analyte specific region configured to not hybridize to the target nucleic acid, wherein the second non-analyte specific region is not identical to the first non-analyte specific region, and wherein the plurality of second probe oligonucleotides are configured to occupy the probe hybridization site on the target nucleic acid at a different frequency than the plurality of first probe oligonucleotides; wherein the incubating is under conditions such that invasive cleavage structures are formed resulting in the cleavage of both the first and second probe oligonucleotides by the cleavage agent to generate: i) first non-target cleavage products comprising the first non-analyte specific region, and ii) second non-target cleavage products comprising the second non-analyte specific region; and b) measuring hybridization of the first and the second probe oligonucleotides by detecting a signal generated by the first and second non-target cleavage products, thereby detecting the target nucleic acid. In some embodiments, the amount of the target is detected.

In certain embodiments, the present invention provides methods for detecting a target nucleic acid in a sample, comprising; a) incubating a sample suspected of containing a target nucleic acid with a plurality of first and second probe oligonucleotides, a plurality of first upstream oligonucleotides, a plurality of second upstream oligonucleotides, and a cleavage agent, wherein each of the first probe oligonucleotides comprise: i) a first analyte specific region configured to hybridize to a first probe hybridization site on the target nucleic acid, and ii) a first non-analyte specific region configured to not hybridize to the target nucleic acid, wherein the first non-analyte specific region is 5' of the first analyte specific region, and wherein each of the second probe oligonucleotides comprises i) a second analyte specific region configured to hybridize to a second probe hybridization site on the target nucleic acid, wherein the

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second probe hybridization site is not the same as the first probe hybridization site, and ii) a second non-analyte specific region configured to not hybridize to the target nucleic acid, wherein the second non-analyte specific region is not identical to the first non-analyte specific region, and wherein the plurality of second probe oligonucleotides are present in at least 5-fold lower concentration than the first probe oligonucleotides; wherein the incubating is under conditions such that invasive cleavage structures are formed resulting in the cleavage of both the first and second probe oligonucleotides by the cleavage agent to generate: i) first non-target cleavage products comprising the first non-analyte specific region, and ii) second non-target cleavage products comprising the second non-analyte specific region; and b) measuring hybridization of the first and the second probe oligonucleotides over time by detecting a signal generated by the first and second non-target cleavage products, thereby measuring the amount of the target nucleic acid. In certain embodiments, the target nucleic acid is micro-RNA. In some embodiments, the amount of the target is detected.

In certain embodiments, the second probe oligonucleotides are present in at least a 10-fold, 100-fold, or 1000-fold, lower concentration than the first probe oligonucleotides. In further embodiments, the signal generated by the first and second non-target cleavage products is the same. In other embodiments, the signal generated by the first and second non-target cleavage products is different. In some embodiments, the upstream oligonucleotides comprise a 5' portion and a 3' portion, wherein the 5' portion is configured to hybridize to a region contiguous with the probe hybridization site on the target nucleic acid, and wherein the 3' portion is configured to not hybridize to the target nucleic acid. In further embodiments, the methods further comprise incubating the sample with first and second labeled sequences, wherein the first labeled sequence is configured to generate a first detectable signal when hybridized to the first non-target cleavage product, and wherein the second labeled sequence is configured to generate a second detectable signal when hybridized to the second non-target cleavage product. In particular embodiments, the first and second detectable signals are the same. In additional embodiments, the first and second labeled sequences comprise FRET cassettes. In other embodiments, the plurality of upstream oligonucleotides are generated in the

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sample (e.g., by a polymerase). In some embodiments, the upstream oligonucleotides are supplied pre-synthesized.

In certain embodiments, the present invention provides kits for quantitation of target nucleic acids in a sample, comprising: a) a plurality of first probe oligonucleotides, wherein each of the first probe oligonucleotides comprises a first analyte specific region, wherein the first probe oligonucleotides are un-labeled, or comprise a label, b) a plurality of second probe oligonucleotides, wherein each of the second probe oligonucleotides comprises a second analyte specific region, wherein the second probe oligonucleotides are un-labeled, or comprise a label, wherein the plurality of second probe oligonucleotides are configured to occupy a probe hybridization site on the target nucleic acids at a different frequency than the plurality of first probe oligonucleotides; and c) reagents for performing an INVADER assay using the pluralities of the first and second probe oligonucleotides.

In certain embodiments, the present invention provides kits or compositions comprising: i) a plurality of first oligonucleoitdes, and ii) a plurality of second probe oligonucleotides, wherein the first probe oligonucleotides comprise a first 5' region and a first 3' region, and the second probe oligonucleotides comprises a second 5' region and a second 3' region, wherein both of the first and second probe oligonucleotides will form an invasive cleavage structure in the presence of the same upstream oligonucleotide and target sequence, and will both be cleaved by the same cleavage agent to form a first 5' region product and a second 5' region product, wherein the second 5' region product is not identical to the first 5' region product. In some embodiments, the kit or composition further comprises iii) first and second labeled sequences, wherein the first labeled sequence is configured to generate a first detectable signal when hybridized to the first 5' region product, and wherein the second labeled sequence is configured to generate a second detectable signal when hybridized to the second 5' region product. In some embodiments, the kits further comprise the target sequence as a control.

In particular embodiments, the first and second probe oligonucleotides are provides in a first vessel. In further embodiments, the , and kit further comprises a second vessel containing a polymerase and FEN enzyme. In additional embodiments, the kit further comprises a third vessel containing a buffer.

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In some embodiments, the first 3' region and the second 3' region have the identical sequence. In other embodiments, the first 3' region and the second 3' region do not have identical sequences. In particular embodiments, the second probe oligonucleotides are present in at least a 5 fold lower concentration than the first probe oligonucleotides. In other embodiments, the second probe oligonucleotides are present in at least a 10 fold ... 100-fold ... 1000-fold ... 10,000-fold ... or 500,000 lower concentration than the first probe oligonucleotides. In some embodiments, the first and second probe oligonucleotides are un-labeled.

In further embodiments, the kits or compositions further comprise a third probe oligonucleotide comprising a third 5' region and a third 3' region, wherein the third probe oligonucleotide will not form an invasive cleavage structure with the target and the upstream oligonucleotide that is cleavable by the cleavage agent. In some embodiments, the first and second detectable signals are the same or they are different.

In some embodiments, the present invention provides kits comprising i) a plurality of un-labeled first probe oligonucleotides and ii) a plurality of un-labled second probe oligonucleotides, wherein the first and second probe oligonucleotides comprises an analyte specific region, wherein the plurality of second probe oligonucleotides are configured to occupy a probe hybridization site on a target nucleic acid at a different frequency than the plurality of first probe oligonucleotides. In further embodiments, the kits further comprise a polymerase and/or a FEN enzyme. In other embodiments, the kits further comprise a buffer.

In some embodiments, the present invention provides kits comprising; a) a first vessel comprising a plurality of first probe oligonucleotides (e.g., unlabeled) and a plurality of second probe oligonucleotides (e.g., unlabeled), wherein the first and second probe oligonucleotides comprises an analyte specific region, wherein the plurality of second probe oligonucleotides are configured to occupy a probe hybridization site on a target nucleic acid at a different frequency than the plurality of first probe oligonucleotides; b) a second vessel comprising a polymerase and/or a FEN enzyme, and c) a third vessel comprising a buffer. In certain embodiments, the kits further comprise d) a control target sequence comprising the probe hybridization site.

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In particular embodiments, the present invention provides kits and compositions comprising; a) a plurality of first and second probe oligonucleotides, wherein the first probe oligonucleotides comprise a first 5' region and a first 3' region, and the second probe oligonucleotides comprises a second 5' region and a second 3' region, wherein both of the first and second probe oligonucleotides will form an invasive cleavage structure in the presence of the same upstream oligonucleotide and target sequence, and will both be cleaved by the same cleavage agent to form a first 5' region product and a second 5' region product, wherein the second 5' region product is identical to the first 5' region product, and wherein the first 3' region is not identical to the second 3' region, and b) first labeled sequences, wherein the first labeled sequence is configured to generate a detectable signal when hybridized to the first or second 5' region product.

Other embodiments of the invention are described in the Detailed Description of the Invention and the Examples.

DEFINITIONS

To facilitate an understanding of the present invention, a number of terms and phrases are defined below:

As used herein, the term "dynamic range" refers to the quantitative range of usefulness in a detection assay (e.g., a nucleic acid detection assay). For example, the dynamic range of a viral detection assay is the range between the smallest number of viral particles (e.g., copy number) and the largest number of viral particles that the assay can distinguish between.

As used herein, the terms "subject" and "patient" refer to any organisms including plants, microorganisms and animals (e.g., mammals such as dogs, cats, livestock, and humans).

The term "primer" refers to an oligonucleotide that is capable of acting as a point of initiation of synthesis when placed under conditions in which primer extension is initiated. An oligonucleotide "primer" may occur naturally, as in a purified restriction digest or may be produced synthetically.

The term "cleavage structure" as used herein, refers to a structure that is formed by the interaction of at least one probe oligonucleotide and a target nucleic acid, forming

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a structure comprising a duplex, the resulting structure being cleavable by a cleavage means, including but not limited to an enzyme. The cleavage structure is a substrate for specific cleavage by the cleavage means in contrast to a nucleic acid molecule that is a substrate for non-specific cleavage by agents such as phosphodiesterases, which cleave nucleic acid molecules without regard to secondary structure (*i.e.*, no formation of a duplexed structure is required).

The term "invasive cleavage structure" as used herein refers to a cleavage structure comprising i) a target nucleic acid, ii) an upstream nucleic acid (*e.g.*, an INVADER oligonucleotide), and iii) a downstream nucleic acid (*e.g.*, a probe), where the upstream and downstream nucleic acids anneal to contiguous regions of the target nucleic acid, and where an overlap forms between the upstream nucleic acid and duplex formed between the downstream nucleic acid and the target nucleic acid. An overlap occurs where one or more bases from the upstream and downstream nucleic acids occupy the same position with respect to a target nucleic acid base, whether or not the overlapping base(s) of the upstream nucleic acid are complementary with the target nucleic acid, and whether or not those bases are natural bases or non-natural bases. In some embodiments, the 3' portion of the upstream nucleic acid that overlaps with the downstream duplex is a non-base chemical moiety such as an aromatic ring structure, *e.g.*, as disclosed, for example, in U.S. Patent No. 6,090,543, incorporated herein by reference in its entirety. In some embodiments, one or more of the nucleic acids may be attached to each other, *e.g.*, through a covalent linkage such as nucleic acid stem-loop, or through a non-nucleic acid chemical linkage (*e.g.*, a multi-carbon chain).

The term "cleavage means" or "cleavage agent" as used herein refers to any means that is capable of cleaving a cleavage structure, including but not limited to enzymes. "Structure-specific nucleases" or "structure-specific enzymes" are enzymes that recognize specific secondary structures in a nucleic molecule and cleave these structures. The cleavage means of the invention cleave a nucleic acid molecule in response to the formation of cleavage structures; it is not necessary that the cleavage means cleave the cleavage structure at any particular location within the cleavage structure.

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The cleavage means may include nuclease activity provided from a variety of sources including the CLEAVASE enzymes, the FEN-1 endonucleases (including RAD2 and XPG proteins), *Taq* DNA polymerase and *E. coli* DNA polymerase I. The cleavage means may include enzymes having 5' nuclease activity (*e.g.*, Taq DNA polymerase (DNAP), *E. coli* DNA polymerase I). The cleavage means may also include modified DNA polymerases having 5' nuclease activity but lacking synthetic activity. Examples of cleavage means suitable for use in the method and kits of the present invention are provided in U.S. Patent Nos. 5,614,402; 5,795,763; 5,843,669; PCT Appln. Nos WO 98/23774; WO 02/070755A2; and WO0190337A2, each of which is herein incorporated by reference it its entirety.

The term "thermostable" when used in reference to an enzyme, such as a 5' nuclease, indicates that the enzyme is functional or active (i.e., can perform catalysis) at an elevated temperature, i.e., at about 55°C or higher. In some embodiments the enzyme is functional or active at an elevated temperature of 65°C or higher (e.g., 75°C, 85°C, 95°C, etc.).

The term "cleavage products" as used herein, refers to products generated by the reaction of a cleavage means with a cleavage structure (i.e., the treatment of a cleavage structure with a cleavage means).

"Amplification" is a special case of nucleic acid replication involving template specificity. It is to be contrasted with non-specific template replication (*i.e.*, replication that is template-dependent but not dependent on a specific template). Template specificity is here distinguished from fidelity of replication (*i.e.*, synthesis of the proper polynucleotide sequence) and nucleotide (ribo- or deoxyribo-) specificity. Template specificity is frequently described in terms of "target" specificity. Target sequences are "targets" in the sense that they are sought to be sorted out from other nucleic acid. Amplification techniques have been designed primarily for this sorting out.

Template specificity is achieved in most amplification techniques by the choice of enzyme. Amplification enzymes are enzymes that, under conditions they are used, will process only specific sequences of nucleic acid in a heterogeneous mixture of nucleic acid. For example, in the case of Qβ replicase, MDV-1 RNA is the specific template for the replicase (D.L. Kacian *et al.*, Proc. Natl. Acad. Sci. USA 69:3038 [1972]). Other

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nucleic acid will not be replicated by this amplification enzyme. Similarly, in the case of T7 RNA polymerase, this amplification enzyme has a stringent specificity for its own promoters (Chamberlin *et al.*, Nature 228:227 [1970]). In the case of T4 DNA ligase, the enzyme will not ligate the two oligonucleotides or polynucleotides, where there is a mismatch between the oligonucleotide or polynucleotide substrate and the template at the ligation junction (D.Y. Wu and R. B. Wallace, Genomics 4:560 [1989]). Finally, *Taq* and *Pfu* polymerases, by virtue of their ability to function at high temperature, are found to display high specificity for the sequences bounded and thus defined by the primers; the high temperature results in thermodynamic conditions that favor primer hybridization with the target sequences and not hybridization with non-target sequences (H.A. Erlich (ed.), *PCR Technology*, Stockton Press [1989]).

As used herein, the term "amplifiable nucleic acid" is used in reference to nucleic acids that may be amplified by any amplification method. It is contemplated that "amplifiable nucleic acid" will usually comprise "sample template."

As used herein, the term "sample template" refers to nucleic acid originating from a sample that is analyzed for the presence of "target." In contrast, "background template" is used in reference to nucleic acid other than sample template that may or may not be present in a sample. Background template is most often inadvertent. It may be the result of carryover, or it may be due to the presence of nucleic acid contaminants sought to be purified away from the sample. For example, nucleic acids from organisms other than those to be detected may be present as background in a test sample.

The term "analyte specific region" as used in reference to an oligonucleotide, such as a probe oligonucleotide or an INVADER oligonucleotide, refers to a region of an oligonucleotide selected to hybridize to a specific sequence in a target nucleic acid or set of target nucleic acids. In some embodiments, an analyte specific region may be completely complementary to the segment of a target nucleic acid to which it hybridizes, while in other embodiments, an analyte specific region may comprise one or more mismatches to the segment of a target nucleic acid to which it hybridizes. In yet other embodiments, an analyte specific region may comprise one or more base analogs, *e.g.*, compounds that have altered hydrogen bonding, or that do not hydrogen bond, to the bases in the target strand. In some embodiments, the entire sequence of an

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oligonucleotide is an analyte specific region, while in other embodiments an oligonucleotide comprises an analyte specific region and one or more regions not complementary the target sequence (*e.g.*, non-complementary flap regions).

The term "frequency" as used herein in reference to hybridization of nucleic acids refers to the probability that one particular nucleic acid (*e.g.*, a probe oligonucleotide) will be base-paired to a complementary nucleic acid (*e.g.*, a target nucleic acid) under particular hybridization conditions. The frequency of hybridization is influenced by many factors, including but not limited to the probability with which the complementary sequences will form a duplex under particular conditions (*e.g.*, likelihood of encounter and of successful duplex formation) and the stability of the duplex, once formed. Reaction conditions that increase the likelihood of initial duplex formation between a probe and a target (*e.g.*, increased concentration of one or both nucleic acids, absence of competitors such as other nucleic acids with sequences that can compete with a probe for binding to the target, or that can bind to the probe) can be said to increase the frequency of hybridization of between the probe and target (*i.e.*, increase the frequency with which the probe oligonucleotide will occupy, or hybridize to, the complementary target strand). Similarly, reaction conditions and probe features that increase the stability of a hybrid between an oligonucleotide and another nucleic acid strand (or that slow disassociation of the strands, *e.g.*, reduced reaction temperature, increased salt or divalent cation conditions, increased length of complementary regions, fewer mismatches, use of charged moieties favoring hybridization) can also be said to increase the frequency of hybridization of between the probe and target. Conversely, reaction conditions and probe features that decrease the likelihood of hybridization (*e.g.*, reduction in concentration of one or both nucleic acids, the presence of a competitor or other additive that reduces the effective concentration of a probe or target strand) or that reduce the stability and/or life time of hybrids that are formed (*e.g.*, increased reaction temperature, decreased salt or divalent cation conditions, decreased length of complementary regions, more mismatches, use of charged moieties disfavoring hybridization) are said to decrease the frequency of hybridization or occupation.

As used herein, the term "target," refers to a nucleic acid sequence or structure to be detected or characterized. Thus, the "target" is sought to be sorted out from other

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nucleic acid sequences. A "segment" is defined as a region of nucleic acid within the target sequence.

The term "substantially single-stranded" when used in reference to a nucleic acid substrate means that the substrate molecule exists primarily as a single strand of nucleic acid in contrast to a double-stranded substrate which exists as two strands of nucleic acid which are held together by inter-strand base pairing interactions.

As used herein, the phrase "non-amplified oligonucleotide detection assay" refers to a detection assay configured to detect the presence or absence of a particular target sequence (e.g. genomic DNA or viral DNA or RNA) that has not been amplified (e.g. by PCR), without creating copies of the target sequence. A "non-amplified oligonucleotide detection assay" may, for example, amplify a signal used to indicate the presence or absence of a particular polymorphism in a target sequence, so long as the target sequence is not copied.

The term "liberating" as used herein refers to the release of a nucleic acid fragment from a larger nucleic acid fragment, such as an oligonucleotide, by the action of, for example, a 5' nuclease such that the released fragment is no longer covalently attached to the remainder of the oligonucleotide.

The term "microorganism" as used herein means an organism too small to be observed with the unaided eye and includes, but is not limited to bacteria, virus, protozoans, fungi, and ciliates.

The term "microbial gene sequences" refers to gene sequences derived from a microorganism.

The term "bacteria" refers to any bacterial species including eubacterial and archaebacterial species.

The term "virus" refers to obligate, ultramicroscopic, intracellular parasites incapable of autonomous replication (i.e., replication requires the use of the host cell's machinery).

The term "multi-drug resistant" or multiple-drug resistant" refers to a microorganism that is resistant to more than one of the antibiotics or antimicrobial agents used in the treatment of said microorganism.

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The term "source of target nucleic acid" refers to any sample that contains nucleic acids (RNA or DNA). Particularly preferred sources of target nucleic acids are biological samples including, but not limited to blood, saliva, cerebral spinal fluid, pleural fluid, milk, lymph, sputum and semen.

A sample "suspected of containing" a first and a second target nucleic acid may contain either, both or neither target nucleic acid molecule.

The term "reactant" is used herein in its broadest sense. The reactant can comprise, for example, an enzymatic reactant, a chemical reactant or light (e.g., ultraviolet light, particularly short wavelength ultraviolet light is known to break oligonucleotide chains). Any agent capable of reacting with an oligonucleotide to either shorten (i.e., cleave) or elongate the oligonucleotide is encompassed within the term "reactant."

As used herein the term "portion" when in reference to a protein (as in "a portion of a given protein") refers to fragments of that protein. The fragments may range in size from four amino acid residues to the entire amino acid sequence minus one amino acid (e.g., 4, 5, 6, . . ., n-1).

The term "continuous strand of nucleic acid" as used herein is means a strand of nucleic acid that has a continuous, covalently linked, backbone structure, without nicks or other disruptions. The disposition of the base portion of each nucleotide, whether base-paired, single-stranded or mismatched, is not an element in the definition of a continuous strand. The backbone of the continuous strand is not limited to the ribose-phosphate or deoxyribose-phosphate compositions that are found in naturally occurring, unmodified nucleic acids. A nucleic acid of the present invention may comprise modifications in the structure of the backbone, including but not limited to phosphorothioate residues, phosphonate residues, 2' substituted ribose residues (*e.g.*, 2'-O-methyl ribose) and alternative sugar (*e.g.*, arabinose) containing residues.

The term "continuous duplex" as used herein refers to a region of double stranded nucleic acid in which there is no disruption in the progression of basepairs within the duplex (*i.e.*, the base pairs along the duplex are not distorted to accommodate a gap, bulge or mismatch with the confines of the region of continuous duplex). As used herein the term refers only to the arrangement of the basepairs within the duplex, without

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implication of continuity in the backbone portion of the nucleic acid strand. Duplex nucleic acids with uninterrupted basepairing, but with nicks in one or both strands are within the definition of a continuous duplex.

The term "duplex" refers to the state of nucleic acids in which the base portions of the nucleotides on one strand are bound through hydrogen bonding the their complementary bases arrayed on a second strand. The condition of being in a duplex form reflects on the state of the bases of a nucleic acid. By virtue of base pairing, the strands of nucleic acid also generally assume the tertiary structure of a double helix, having a major and a minor groove. The assumption of the helical form is implicit in the act of becoming duplexed.

The term "template" refers to a strand of nucleic acid on which a complementary copy is built from nucleoside triphosphates through the activity of a template-dependent nucleic acid polymerase. Within a duplex the template strand is, by convention, depicted and described as the "bottom" strand. Similarly, the non-template strand is often depicted and described as the "top" strand.

As used herein, the term "sample" is used in its broadest sense. For example, in some embodiments, it is meant to include a specimen or culture (e.g., microbiological culture), whereas in other embodiments, it is meant to include both biological and environmental samples (e.g., suspected of comprising a target sequence, gene or template). In some embodiments, a sample may include a specimen of synthetic origin.

The present invention is not limited by the type of biological sample used or analyzed. The present invention is useful with a variety of biological samples including, but are not limited to, tissue (e.g., organ (e.g., heart, liver, brain, lung, stomach, intestine, spleen, kidney, pancreas, and reproductive (e.g., ovaries) organs), glandular, skin, and muscle tissue), cell (e.g., blood cell (e.g., lymphocyte or erythrocyte), muscle cell, tumor cell, and skin cell), gas, bodily fluid (e.g., blood or portion thereof, serum, plasma, urine, semen, saliva, etc), or solid (e.g., stool) samples obtained from a human (e.g., adult, infant, or embryo) or animal (e.g., cattle, poultry, mouse, rat, dog, pig, cat, horse, and the like). In some embodiments, biological samples may be solid food and/or feed products and/or ingredients such as dairy items, vegetables, meat and meat by-products, and waste. Biological samples may be obtained from all of the various families of domestic animals,

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as well as feral or wild animals, including, but not limited to, such animals as ungulates, bear, fish, lagamorphs, rodents, etc.

Biological samples also include biopsies and tissue sections (e.g., biopsy or section of tumor, growth, rash, infection, or paraffin-embedded sections), medical or hospital samples (e.g., including, but not limited to, blood samples, saliva, buccal swab, cerebrospinal fluid, pleural fluid, milk, colostrum, lymph, sputum, vomitus, bile, semen, oocytes, cervical cells, amniotic fluid, urine, stool, hair and sweat), laboratory samples (e.g., subcellular fractions), and forensic samples (e.g., blood or tissue (e.g., spatter or residue), hair and skin cells containing nucleic acids), and archeological samples (e.g., fossilized organisms, tissue, or cells).

Environmental samples include, but are not limited to, environmental material such as surface matter, soil, water (e.g., freshwater or seawater), algae, lichens, geological samples, air containing materials containing nucleic acids, crystals, and industrial samples, as well as samples obtained from food and dairy processing instruments, apparatus, equipment, utensils, disposable and non-disposable items.

Other types of biological samples include bacteria (e.g., *Actinobacteria* (e.g., *Actinomyces*, *Arthrobacter*, *Corynebacterium* (e.g., *C. diphtheriae*)), *Mycobacterium* (e.g., *M. tuberculosis* and *M. leprae*), *Propionibacterium* (e.g., *P. acnes*), *Streptomyces*, *hlamydiae* (e.g., *C. trachomatis* and *C. pneumoniae*), *Cyanobacteria*, *Deinococcus* (e.g., *Thermus* (e.g., *T. aquaticus)*), *Firmicutes* (e.g., *Bacilli* (e.g., *B. anthracis*, *B. cereus*, *B. thuringiensis*, and *B. subtilis*)), *Listeria* (e.g., *L. monocytogenes*), *Staphylococcus* (e.g., *S. aureus*, *S. epidermidis*, and *S. haemolyticus*), *Fusobacteria*, *Proteobacteria* (e.g., *Rickettsiales*, *Sphingomonadales*, *Bordtella* (e.g., *B. pertussis*), *Neisserisales* (e.g., *N. gonorrhoeae* and *N. meningitidis*), *Enterobacteriales* (e.g., *Escherichia* (e.g., *E. coli*), *Klebsiella*, *Plesiomonas*, *Proteus*, *Salmonella*, *Shigella*, and *Yersinia*), *Legionellales*, *Pasteurellales* (e.g., *Haemophilus influenzae*), *Pseudomonas*, *Vibrio* (e.g., *V. cholerae* and *V. vulnificus*), *Campylobacterales* (e.g., *Campylobacteria* (e.g., *C. jejuni*), and *Helicobacter* (e.g., *H. pylori*)), and *Spirochaetes* (e.g., *Leptospira, B. bergdorferi*, and *T. pallidum*)); Archaea (e.g., *Halobacteria* and *Methanobacteria*); Eucarya (e.g., *Animalia* (e.g., *Annelidia*, *Arthropoda* (e.g., *Chelicerata*, *Myriapoda*, *Insecta*, and *Crustacea*), *Mollusca*, *Nematoda*,(e.g., *C. elegans*, and *T. spiralis*) and *Chordata* (e.g.,

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Actinopterygii, *Amphibia*, *Aves*, *Chondrichthyes*, *Reptilia*, and *Mammalia* (e.g., *Primates*, *Rodentia*, *Lagomorpha*, and *Carnivora*)))); Fungi (e.g., *Dermatophytes*, *Fusarium*, *Penicillum*, and *Saccharomyces*); *Plantae* (e.g., *Magnoliophyta* (e.g., *Magnoliopsida* and *Liliopsida*)), and *Protista* (e.g., *Apicomplexa* (e.g., *Cryptosporidium*, *Plasmodium* (e.g., *P. falciparum*, and *Toxoplasma*), and *Metamonada* (e.g., *G. lambia*))); and Viruses (e.g., dsDNA viruses (e.g., *Bacteriophage*, *Adenoviridae*, *Herpesviridiae*, *Papillomaviridae*, *Polyomaviridae*, and *Poxviridae*), ssDNA virues (e.g., *Parvoviridae*), dsRNA viruses (including *Reoviridae*), (+)ssRNA viruses (e.g., *Coronaviridae*, *Astroviridae*, *Bromoviridae*, *Comoviridae*, *Flaviviridae*, *Picornaviridae*, and *Togaviridae*), (-) ssRNA viruses (e.g., *Bornaviridae*, *Filoviridae*, *Paramyxoviridae*, *Rhabdoviridae*, *Bunyaviridae*, and *Orthomyxovirdiae*), ssRNA-reverse transcribing viruses (e.g., *Retroviridae*), and dsDNA-reverse transcribing viruses (e.g., *Hepadnaviridae* and *Caulomoviridae*)).

Sample may be prepared by any desired or suitable method. In some embodiments, nucleic acids are analyzed directly from bodily fluids or other samples using the methods described in U.S. Pat. Pub. Serial No. 20050186588, herein incorporated by reference in its entirety.

The above described examples are not, however, to be construed as limiting the sample (e.g., suspected of comprising a target sequence, gene or template (e.g., the presence or absence of which can be determined using the compositions and methods of the present invention)) types applicable to the present invention.

The terms "nucleic acid sequence" and "nucleic acid molecule" as used herein refer to an oligonucleotide, nucleotide or polynucleotide, and fragments or portions thereof. The terms encompasses sequences that include any of the known base analogs of DNA and RNA including, but not limited to, 4-acetylcytosine, 8-hydroxy-N6-methyladenosine, aziridinylcytosine, pseudoisocytosine, 5-(carboxyhydroxylmethyl) uracil, 5-fluorouracil, 5-bromouracil, 5-carboxymethylaminomethyl-2-thiouracil, 5-carboxymethylaminomethyluracil, dihydrouracil, inosine, N6-isopentenyladenine, 1-methyladenine, 1-methylpseudouracil, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-methyladenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine,

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5'-methoxycarbonylmethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, oxybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, N-uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, pseudouracil, queosine, 2-thiocytosine, and 2,6-diaminopurine.

A nucleic acid sequence or molecule may be DNA or RNA, of either genomic or synthetic origin, that may be single or double stranded, and represent the sense or antisense strand. Thus, nucleic acid sequence may be dsDNA, ssDNA, mixed ssDNA, mixed dsDNA, dsDNA made into ssDNA (e.g., through melting, denaturing, helicases, etc.), A-, B-, or Z- DNA, triple-stranded DNA, RNA, ssRNA, dsRNA, mixed ss and dsRNA, dsRNA made into ssRNA (e.g., via melting, denaturing, helicases, etc.), messenger RNA (mRNA), ribosomal RNA (rRNA), transfer RNA (tRNA), catalytic RNA, snRNA, or protein nucleic acid (PNA).

The present invention is not limited by the type or source of nucleic acid (e.g., sequence or molecule (e.g. target sequence and/or oligonucleotide)) utilized. For example, the nucleic acid sequence may be amplified or created sequence (e.g., amplification or creation of nucleic acid sequence via synthesis (e.g., polymerization (e.g., primer extension (e.g., RNA-DNA hybrid primer technology)) and reverse transcription (e.g., of RNA into DNA)) and/or amplification (e.g., polymerase chain reaction (PCR), rolling circle amplification (RCA), nucleic acid sequence based amplification (NASBA), transcription mediated amplification (TMA), ligase chain reaction (LCR), cycling probe technology, Q-beta replicase, strand displacement amplification (SDA), branched-DNA signal amplification (bDNA), hybrid capture, and helicase dependent amplification).

The terms "nucleotide" and "base" are used interchangeably when used in reference to a nucleic acid sequence, unless indicated otherwise herein.

The term "nucleotide analog" as used herein refers to modified or non-naturally occurring nucleotides including, but not limited to, analogs that have altered stacking interactions such as 7-deaza purines (i.e., 7-deaza-dATP and 7-deaza-dGTP); base analogs with alternative hydrogen bonding configurations (e.g., Iso-C and Iso-G and other non-standard base pairs described in U.S. Patent No. 6,001,983, herein incorporated

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by reference in its entirety); non-hydrogen bonding analogs (e.g., non-polar, aromatic nucleoside analogs such as 2,4-difluorotoluene, described by B.A. Schweitzer and E.T. Kool, J. Org. Chem., 1994, 59, 7238-7242; B.A. Schweitzer and E.T. Kool, J. Am. Chem. Soc., 1995, 117, 1863-1872, each of which is herein incorporate by reference in its entirety); "universal" bases such as 5-nitroindole and 3-nitropyrrole; and universal purines and pyrimidines (e.g., "K" and "P" nucleotides, respectively; See, e.g., P. Kong, et al., Nucleic Acids Res., 1989, 17, 10373-10383; P. Kong et al., Nucleic Acids Res., 1992, 20, 5149-5152). Still other nucleotide analogs include modified forms of deoxyribonucleotides as well as ribonucleotides. Various oligonucleotides of the present invention (e.g., a primary probe or INVADER oligo) may contain nucleotide analogs.

The term "oligonucleotide" as used herein is defined as a molecule comprising two or more nucleotides (e.g., deoxyribonucleotides or ribonucleotides), preferably at least 5 nucleotides, more preferably at least about 10-15 nucleotides and more preferably at least about 15 to 30 nucleotides, or longer (e.g., oligonucleotides are typically less than 200 residues long (e.g., between 15 and 100 nucleotides), however, as used herein, the term is also intended to encompass longer polynucleotide chains). The exact size will depend on many factors, which in turn depend on the ultimate function or use of the oligonucleotide. Oligonucleotides are often referred to by their length. For example a 24 residue oligonucleotide is referred to as a "24-mer". Oligonucleotides can form secondary and tertiary structures by self-hybridizing or by hybridizing to other polynucleotides. Such structures can include, but are not limited to, duplexes, hairpins, cruciforms, bends, and triplexes. Oligonucleotides may be generated in any manner, including chemical synthesis, DNA replication, reverse transcription, PCR, or a combination thereof. In some embodiments, oligonucleotides that form invasive cleavage structures are generated in a reaction (e.g., by extension of a primer in an enzymatic extension reaction).

Because mononucleotides are reacted to make oligonucleotides in a manner such that the 5' phosphate of one mononucleotide pentose ring is attached to the 3' oxygen of its neighbor in one direction via a phosphodiester linkage, an end of an oligonucleotide is referred to as the "5' end" if its 5' phosphate is not linked to the 3' oxygen of a mononucleotide pentose ring and as the "3' end" if its 3' oxygen is not linked to a 5'

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phosphate of a subsequent mononucleotide pentose ring. As used herein, a nucleic acid sequence, even if internal to a larger oligonucleotide, also may be said to have 5' and 3' ends. A first region along a nucleic acid strand is said to be upstream of another region if the 3' end of the first region is before the 5' end of the second region when moving along a strand of nucleic acid in a 5' to 3' direction.

When two different, non-overlapping oligonucleotides anneal to different regions of the same linear complementary nucleic acid sequence, and the 3' end of one oligonucleotide points towards the 5' end of the other, the former may be called the "upstream" oligonucleotide and the latter the "downstream" oligonucleotide. Similarly, when two overlapping oligonucleotides are hybridized to the same linear complementary nucleic acid sequence, with the first oligonucleotide positioned such that its 5' end is upstream of the 5' end of the second oligonucleotide, and the 3' end of the first oligonucleotide is upstream of the 3' end of the second oligonucleotide, the first oligonucleotide may be called the "upstream" oligonucleotide and the second oligonucleotide may be called the "downstream" oligonucleotide.

As used herein, the terms "complementary" or "complementarity" are used in reference to polynucleotides (e.g., a sequence of two or more nucleotides (e.g., an oligonucleotide or a target nucleic acid)) related by the base-pairing rules. For example, the sequence "5'-A-G-T-3'," is complementary to the sequence "3'-T-C-A-5'." Complementarity may be "partial," in which only some of the nucleic acid bases are matched according to the base pairing rules. Or, there may be "complete" or "total" complementarity between the nucleic acid bases. The degree of complementarity between nucleic acid strands has significant effects on the efficiency and strength of hybridization between nucleic acid strands. This is of particular importance in amplification reactions, as well as detection methods that depend upon the association of two or more nucleic acid strands. Either term may also be used in reference to individual nucleotides, especially within the context of polynucleotides. For example, a particular nucleotide within an oligonucleotide may be noted for its complementarity, or lack thereof, to a nucleotide within another nucleic acid sequence (e.g., a target sequence), in contrast or comparison to the complementarity between the rest of the oligonucleotide and the nucleic acid sequence.

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The complement of a nucleic acid sequence as used herein refers to an oligonucleotide which, when aligned with the nucleic acid sequence such that the 5' end of one sequence is paired with the 3' end of the other, is in "antiparallel association." Certain bases not commonly found in natural nucleic acids may be included in the nucleic acids of the present invention and include, for example, inosine and 7-deazaguanine. Complementarity need not be perfect; stable duplexes may contain mismatched base pairs or unmatched bases. Those skilled in the art of nucleic acid technology can determine duplex stability empirically considering a number of variables including, for example, the length of the oligonucleotide, base composition and sequence of the oligonucleotide, ionic strength and incidence of mismatched base pairs.

The term "homology" refers to a degree of complementarity. There may be partial homology or complete homology (i.e., identity). A partially homologous sequence is one that is less than 100% identical to another sequence. A partially complementary sequence that is "substantially homologous" is a nucleic acid molecule that at least partially inhibits a completely complementary nucleic acid molecule from hybridizing to a target nucleic acid. The inhibition of hybridization of the completely complementary sequence to the target sequence may be examined using a hybridization assay (e.g., Southern or Northern blot, solution hybridization and the like) under conditions of low stringency. A substantially homologous sequence or probe will compete for and inhibit the binding (e.g., the hybridization) of a completely homologous nucleic acid molecule to a target under conditions of low stringency. This is not to say that conditions of low stringency are such that non-specific binding is permitted (e.g., the low stringency conditions may be such that the binding of two sequences to one another be a specific (e.g., selective) interaction). The absence of non-specific binding may be tested by the use of a second target that is substantially non-complementary (e.g., less than about 30% identity); in the absence of non-specific binding the probe will not hybridize to the second non-complementary target.

When used in reference to a double-stranded nucleic acid sequence such as a cDNA or genomic clone, the term "substantially homologous" refers to any probe that can hybridize to either or both strands of the double-stranded nucleic acid sequence under conditions of low stringency as described above.

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When used in reference to a single-stranded nucleic acid sequence, the term "substantially homologous" refers to any probe that can hybridize (e.g., is complementary to) the single-stranded nucleic acid sequence under conditions of low stringency as described above.

The terms "target nucleic acid" and "target sequence," when used in reference to an invasive cleavage reaction, refer to a nucleic acid molecule containing a sequence that has at least partial complementarity with at least a first nucleic acid molecule (e.g. probe oligonucleotide) and may also have at least partial complementarity with a second nucleic acid molecule (e.g. INVADER oligonucleotide). Generally, the target nucleic acid (e.g., present within, isolated from, enriched from, or amplified from or within a sample (e.g., a biological or environmental sample)) is located within a target region and is identifiable via the successful formation of an invasive cleavage structure in combination with a first and second nucleic acid molecule (e.g., probe oligonucleotide and INVADER oligonucleotide) that is cleavable by a cleavage agent. Target nucleic acids from an organism are not limited to genomic DNA and RNA. Target nucleic acids from an organism may comprise any nucleic acid species, including but not limited to genomic DNAs and RNAs, messenger RNAs, structural RNAs, ribosomal and tRNAs, and small RNAs such as snRNAs, siRNAs and microRNAs miRNAs). See, *e.g.*, co-pending U.S. Patent Application Ser. No. 10/740,256, filed 12/18/03, which is incorporated herein by reference in its entirety.

As used herein, the term "probe oligonucleotide," when used in reference to an invasive cleavage reaction, refers to an oligonucleotide that interacts with a target nucleic acid to form a cleavage structure in the presence or absence of an INVADER oligonucleotide. When annealed to the target nucleic acid, the probe oligonucleotide and target form a cleavage structure and cleavage occurs within the probe oligonucleotide.

The term "INVADER oligonucleotide" refers to an oligonucleotide that hybridizes to a target nucleic acid at a location near the region of hybridization between a probe and the target nucleic acid, wherein the INVADER oligonucleotide comprises a portion (e.g., a chemical moiety, or nucleotide—whether complementary to that target or not) that overlaps with the region of hybridization between the probe and target. In some

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embodiments, the INVADER oligonucleotide contains sequences at its 3' end that are substantially the same as sequences located at the 5' end of a probe oligonucleotide.

The term "cassette," when used in reference to an invasive cleavage reaction, as used herein refers to an oligonucleotide or combination of oligonucleotides configured to generate a detectable signal in response to cleavage of a probe oligonucleotide in an INVADER assay. In preferred embodiments, the cassette hybridizes to an cleavage product from cleavage of the probe oligonucleotide to form a second invasive cleavage structure, such that the cassette can then be cleaved.

In some embodiments, the cassette is a single oligonucleotide comprising a hairpin portion (i.e., a region wherein one portion of the cassette oligonucleotide hybridizes to a second portion of the same oligonucleotide under reaction conditions, to form a duplex). In other embodiments, a cassette comprises at least two oligonucleotides comprising complementary portions that can form a duplex under reaction conditions. In preferred embodiments, the cassette comprises a label. In particularly preferred embodiments, the cassette comprises labeled moieties that produce a fluorescence resonance energy transfer (FRET) effect.

An oligonucleotide is said to be present in "excess" relative to another oligonucleotide (or target nucleic acid sequence) if that oligonucleotide is present at a higher molar concentration than the other oligonucleotide (or target nucleic acid sequence). When an oligonucleotide such as a probe oligonucleotide is present in a cleavage reaction in excess relative to the concentration of the complementary target nucleic acid sequence, the reaction may be used to indicate the amount of the target nucleic acid present. Typically, when present in excess, the probe oligonucleotide will be present in at least a 100-fold molar excess; typically at least 1 pmole of each probe oligonucleotide would be used when the target nucleic acid sequence was present at about 10 fmoles or less.

As used herein, the term "gene" refers to a nucleic acid (e.g., DNA) sequence that comprises coding sequences necessary for the production of a polypeptide, precursor, or RNA (e.g., rRNA, tRNA). The polypeptide can be encoded by a full length coding sequence or by any portion of the coding sequence so long as the desired activity or functional properties (e.g., enzymatic activity, ligand binding, signal transduction,

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immunogenicity, etc.) of the full-length or fragment are retained. The term also encompasses the coding region of a structural gene and the sequences located adjacent to the coding region on both the 5' and 3' ends for a distance of about 1 kb or more on either end such that the gene corresponds to the length of the full-length mRNA. Sequences located 5' of the coding region and present on the mRNA are referred to as 5' non-translated sequences. Sequences located 3' or downstream of the coding region and present on the mRNA are referred to as 3' non-translated sequences. The term "gene" encompasses both cDNA and genomic forms of a gene. A genomic form or clone of a gene contains the coding region interrupted with non-coding sequences termed "introns" or "intervening regions" or "intervening sequences." Introns are segments of a gene that are transcribed into nuclear RNA (e.g., hnRNA); introns may contain regulatory elements (e.g., enhancers). Introns are removed or "spliced out" from the nuclear or primary transcript; introns therefore are absent in the messenger RNA (mRNA) transcript. The mRNA functions during translation to specify the sequence or order of amino acids in a nascent polypeptide.

As used herein, the term "heterologous gene" refers to a gene that is not in its natural environment. For example, a heterologous gene includes a gene from one species introduced into another species (e.g., a viral or bacterial gene present within a human host (e.g., extrachromosomally or integrated into the host's DNA)). A heterologous gene also includes a gene native to an organism that has been altered in some way (e.g., mutated, added in multiple copies, linked to non-native regulatory sequences, etc). In some embodiments, a heterologous gene can be distinguished from endogenous genes in that the heterologous gene sequences are typically joined to DNA sequences that are not found naturally associated with the gene sequences in the chromosome or are associated with portions of the chromosome not found in nature (e.g., genes expressed in loci where the gene is not normally expressed).

As used herein, the term "gene expression" refers to the process of converting genetic information encoded in a gene into RNA (*e.g.*, mRNA, rRNA, tRNA, or snRNA) through "transcription" of the gene (e.g., via the enzymatic action of an RNA polymerase), and for protein encoding genes, into protein through "translation" of mRNA. Gene expression can be regulated at many stages in the process. "Up-

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regulation" or "activation" refers to regulation that increases the production of gene expression products (e.g., RNA or protein), while "down-regulation" or "repression" refers to regulation that decrease production. Molecules (e.g., transcription factors) that are involved in up-regulation or down-regulation are often called "activators" and "repressors," respectively.

In addition to containing introns, genomic forms of a gene may also include sequences located on both the 5' and 3' end of the sequences that are present on the RNA transcript. These sequences are referred to as "flanking" sequences or regions (e.g., these flanking sequences can be located 5' or 3' to the non-translated sequences present on the mRNA transcript). The 5' flanking region may contain regulatory sequences such as promoters and enhancers that control or influence the transcription of the gene. The 3' flanking region may contain sequences that direct the termination of transcription, post-transcriptional cleavage and polyadenylation.

The term "wild-type" refers to a gene or gene product isolated from a naturally occurring source. A wild-type gene is that which is most frequently observed in a population and is thus arbitrarily designed the "normal" or "wild-type" form of the gene. In contrast, the term "modified" or "mutant" refers to a gene or gene product that displays modifications in sequence and or functional properties (e.g., altered characteristics) when compared to the wild-type gene or gene product. It is noted that naturally occurring mutants can be isolated (e.g., identified by the fact that they have altered characteristics (e.g., altered nucleic acid sequences) when compared to the wild-type gene or gene product).

The term "isolated" when used in relation to a nucleic acid (e.g., "an isolated oligonucleotide" or "isolated polynucleotide" or "an isolated nucleic acid sequence") refers to a nucleic acid sequence that is separated from at least one component or contaminant with which it is ordinarily associated in its natural source. Thus, an isolated nucleic acid is present in a form or setting that is different from that in which it is found in nature. In contrast, non-isolated nucleic acids are nucleic acids such as DNA and RNA found in the state they exist in nature. For example, a given DNA sequence (e.g., a gene) is found on the host cell chromosome in proximity to neighboring genes; RNA sequences, such as a specific mRNA sequence encoding a specific protein, are found in

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the cell as a mixture with numerous other mRNAs that encode a multitude of proteins. However, isolated nucleic acid encoding a given protein includes, by way of example, such nucleic acid in cells ordinarily expressing the given protein where the nucleic acid is in a chromosomal location different from that of natural cells, or is otherwise flanked by a different nucleic acid sequence than that found in nature. The isolated nucleic acid, oligonucleotide, or polynucleotide may be present in single-stranded or double-stranded form. When an isolated nucleic acid, oligonucleotide or polynucleotide is to be utilized to express a protein, the oligonucleotide or polynucleotide will contain at a minimum the sense or coding strand (e.g., the oligonucleotide or polynucleotide may be single-stranded), but may contain both the sense and anti-sense strands (e.g., the oligonucleotide or polynucleotide may be double-stranded).

As used herein, the terms "purified" or "to purify" when used in reference to a sample (e.g., a molecule (e.g., a nucleic acid or amino acid sequence)) refers to removal (e.g., isolation and/or separation) of the sample from its natural environment. The term "substantially purified" refers to a sample (e.g., molecule (e.g. a nucleic acid or amino acid sequence) that has been removed (e.g., isolated and/or purified) from its natural environment and is at least 60% free, preferably 75% free, or most preferably 90% or more free from other components with which it is naturally associated. An "isolated polynucleotide" or "isolated oligonucleotide" may therefore be substantially purified if it is rendered free (e.g., 60%, 75% or more preferably 90% or more) from other components with which it is naturally associated.

The present invention is not limited to any particular means of purification (e.g., to generate purified or substantially purified molecules (e.g., nucleic acid sequences)). Indeed, a variety of purification techniques may be utilized including, but not limited to, centrifugation (e.g., isopycnic, rate-zonal, gradient, and differential centrifugation), electrophoresis (e.g., gel and capillary electrophoresis), gel filtration, matrix capture, charge capture, mass capture, antibody capture, magnetic separation, flow cytometry, and sequence-specific hybridization array capture.

As used herein, the term "hybridization" is used in reference to the pairing of complementary nucleic acids. Hybridization and the strength of hybridization (e.g., the strength of the association between the nucleic acids) is impacted by such factors as the

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degree of complementary between the nucleic acids, stringency of the conditions involved, the T_m of the formed hybrid, and the G:C ratio within the nucleic acids. A single molecule that contains pairing of complementary nucleic acids within its structure is said to be "self-hybridized."

As used herein, the term "T_m" is used in reference to the "melting temperature." The melting temperature is the temperature at which a population of double-stranded nucleic acid molecules becomes half dissociated into single strands. Several equations for calculating the T_m of nucleic acids are well known in the art. As indicated by standard references, a simple estimate of the T_m value may be calculated by the equation: $T_m = 81.5 + 0.41(\% G + C)$, when a nucleic acid is in aqueous solution at 1 M NaCl (See, e.g., Young and Anderson, (1985) in *Nucleic Acid Hybridisation: A Practical Approach* (Hames & Higgins, Eds.) pp 47-71, IRL Press, Oxford). Other computations for calculating T_m are known in the art and take structural and environmental, as well as sequence characteristics into account (See, e.g., Allawi, H.T. and SantaLucia, J., Jr. Biochemistry 36, 10581-94 (1997)).

As used herein, the term "INVADER assay reagents" refers to one or more reagents for detecting target sequences, said reagents comprising nucleic acid molecules capable of forming an invasive cleavage structure in the presence of the target sequence. In some embodiments, the INVADER assay reagents further comprise an agent for detecting the presence of an invasive cleavage structure (e.g., a cleavage agent). In some embodiments, the nucleic acid molecules comprise first and second oligonucleotides, said first oligonucleotide comprising a 5' portion complementary to a first region of the target nucleic acid and said second oligonucleotide comprising a 3' portion and a 5' portion, said 5' portion complementary to a second region of the target nucleic acid downstream of and contiguous to the first portion. In some embodiments, the 3' portion of the second oligonucleotide comprises a 3' terminal nucleotide not complementary to the target nucleic acid. In preferred embodiments, the 3' portion of the second oligonucleotide consists of a single nucleotide not complementary to the target nucleic acid. INVADER assay reagents may be found, for example, in U.S. Patent Nos. 5,846,717; 5,985,557; 5,994,069; 6,001,567; 6,913,881; and 6,090,543, WO 97/27214, WO 98/42873, U.S. Pat.

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Publ. Nos. 20050014163, 20050074788, 2005016596, 20050186588, 20040203035, 20040018489, and 20050164177; U.S. Pat. Appln. Ser. No. 11/266,723; and Lyamichev et al., Nat. Biotech., 17:292 (1999), Hall et al., PNAS, USA, 97:8272 (2000), each of which is herein incorporated by reference in its entirety for all purposes.

In some embodiments, INVADER assay reagents are configured to detect a target nucleic acid sequence comprising first and second non-contiguous single-stranded regions separated by an intervening region comprising a double-stranded region. In certain embodiments, the INVADER assay reagents comprise a bridging oligonucleotide capable of binding to said first and second non-contiguous single-stranded regions of a target nucleic acid sequence. In particularly preferred embodiments, either or both of said first and/or said second oligonucleotides of said INVADER assay reagents are bridging oligonucleotides.

In some embodiments, the INVADER assay reagents further comprise a solid support. For example, in some embodiments, the one or more oligonucleotides of the assay reagents (e.g., first and/or second oligonucleotide, whether bridging or non-bridging) is attached to said solid support. The one or more oligonucleotides of the assay reagents may be linked to the solid support directly or indirectly (e.g., via a spacer molecule (e.g., an oligonucleotide)). Exemplary solid phase invasive cleavage reactions are described in U.S. Pat. Pub. Nos. 20050164177 and 20030143585, herein incorporated by reference in their entireties.

As used herein, a "solid support" is any material that maintains its shape under assay conditions, and that can be separated from a liquid phase. The present invention is not limited by the type of solid support utilized. Indeed, a variety of solid supports are contemplated to be useful in the present invention including, but not limited to, a bead, planar surface, controlled pore glass (CPG), a wafer, glass, silicon, plastic, paramagnetic bead, magnetic bead, latex bead, superparamagnetic bead, plurality of beads, microfluidic chip, a silicon chip, a microscope slide, a microplate well, a silica gel, a polymeric membrane, a particle, a derivatized plastic film, a glass bead, cotton, a plastic bead, an alumina gel, a polysaccharide, polyvinylchloride, polypropylene, polyethylene, nylon, Sepharose, poly(acrylate), polystyrene, poly(acrylamide), polyol, agarose, agar, cellulose, dextran, starch, FICOLL, heparin, glycogen, amylopectin, mannan, inulin, nitrocellulose,

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diazocellulose or starch, polymeric microparticle, polymeric membrane, polymeric gel, glass slide, styrene, multi-well plate, column, microarray, latex, hydrogel, porous 3D hydrophilic polymer matrix (e.g., HYDROGEL, Packard Instrument Company, Meriden, Conn.), fiber optic bundles and beads (e.g., BEADARRAY (Illumina, San Diego, Calif.), described in U.S. Pat. App. 20050164177), small particles, membranes, frits, slides, micromachined chips, alkanethiol-gold layers, non-porous surfaces, addressable arrays, and polynucleotide-immobilizing media (e.g., described in U.S. Pat. App. 20050191660). In some embodiments, the solid support is coated with a binding layer or material (e.g., gold or streptavidin).

In some embodiments, the INVADER assay reagents further comprise a buffer solution. In some preferred embodiments, the buffer solution comprises a source of divalent cations (e.g., Mn^{2+} and/or Mg^{2+} ions). Individual ingredients (e.g., oligonucleotides, enzymes, buffers, target nucleic acids) that collectively make up INVADER assay reagents are termed "INVADER assay reagent components."

In some embodiments, the INVADER assay reagents further comprise a third oligonucleotide complementary to a third portion of the target nucleic acid upstream of the first portion of the first target nucleic acid (e.g., a stacker oligonucleotides). In yet other embodiments, the INVADER assay reagents further comprise a target nucleic acid. In some embodiments, the INVADER assay reagents further comprise a second target nucleic acid. In yet other embodiments, the INVADER assay reagents further comprise a third oligonucleotide comprising a 5' portion complementary to a first region of the second target nucleic acid. In some specific embodiments, the 3' portion of the third oligonucleotide is covalently linked to the second target nucleic acid. In other specific embodiments, the second target nucleic acid further comprises a 5' portion, wherein the 5' portion of the second target nucleic acid is the third oligonucleotide. In still other embodiments, the INVADER assay reagents further comprise an ARRESTOR molecule (e.g., ARRESTOR oligonucleotide).

In some embodiments one or more of the INVADER assay reagents may be provided in a predispensed format (e.g., premeasured for use in a step of the procedure without re-measurement or re-dispensing). In some embodiments, selected INVADER assay reagent components are mixed and predispensed together. In preferred

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embodiments, predispensed assay reagent components are predispensed and are provided in a reaction vessel (e.g., including, but not limited to, a reaction tube or a well (e.g., a microtiter plate)). In certain preferred embodiments, the INVADER assay reagents are provided in microfluidic devices such as those described in U.S. Pats., 6,627,159; 6,720,187; 6,734,401; and 6,814,935, as well as U.S. Pat. Pub. 2002/0064885, each of which is herein incorporated by reference in its entirety. In particularly preferred embodiments, predispensed INVADER assay reagent components are dried down (e.g., desiccated or lyophilized) in a reaction vessel.

In some embodiments, the INVADER assay reagents are provided as a kit. As used herein, the term "kit" refers to any delivery system for delivering materials. In the context of reaction assays, such delivery systems include systems that allow for the storage, transport, or delivery of reaction reagents (e.g., oligonucleotides, enzymes, etc. in the appropriate containers) and/or supporting materials (e.g., buffers, written instructions for performing the assay etc.) from one location to another. For example, kits include one or more enclosures (e.g., boxes) containing the relevant reaction reagents and/or supporting materials. As used herein, the term "fragmented kit" refers to delivery systems comprising two or more separate containers that each contains a subportion of the total kit components. The containers may be delivered to the intended recipient together or separately. For example, a first container may contain an enzyme for use in an assay, while a second container contains oligonucleotides. The term "fragmented kit" is intended to encompass kits containing Analyte specific reagents (ASR's) regulated under section 520(e) of the Federal Food, Drug, and Cosmetic Act, but are not limited thereto. Indeed, any delivery system comprising two or more separate containers that each contains a subportion of the total kit components are included in the term "fragmented kit." In contrast, a "combined kit" refers to a delivery system containing all of the components of a reaction assay in a single container (e.g., in a single box housing each of the desired components). The term "kit" includes both fragmented and combined kits.

In some embodiments, the present invention provides INVADER assay reagent kits comprising one or more of the components necessary for practicing the present invention. For example, the present invention provides kits for storing or delivering the

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enzymes and/or the reaction components necessary to practice an INVADER assay. The kit may include any and all components necessary or desired for assays including, but not limited to, the reagents themselves, buffers, control reagents (e.g., tissue samples, positive and negative control target oligonucleotides, etc.), solid supports, labels, written and/or pictorial instructions and product information, inhibitors, labeling and/or detection reagents, package environmental controls (e.g., ice, desiccants, etc.), and the like. In some embodiments, the kits provide a sub-set of the required components, wherein it is expected that the user will supply the remaining components. In some embodiments, the kits comprise two or more separate containers wherein each container houses a subset of the components to be delivered. For example, a first container (e.g., box) may contain an enzyme (e.g., structure specific cleavage enzyme in a suitable storage buffer and container), while a second box may contain oligonucleotides (e.g., INVADER oligonucleotides, probe oligonucleotides, control target oligonucleotides, etc.).

In some preferred embodiments, the INVADER assay reagents further comprise reagents for detecting a nucleic acid cleavage product. In some embodiments, one or more oligonucleotides in the INVADER assay reagents comprise a label. In some preferred embodiments, said first oligonucleotide comprises a label. In other preferred embodiments, said third oligonucleotide comprises a label. In particularly preferred embodiments, the reagents comprise a first and/or a third oligonucleotide labeled with moieties that produce a fluorescence resonance energy transfer (FRET) effect.

As used herein, the term "label" refers to any moiety (e.g., chemical species) that can be detected or can lead to a detectable response. In some preferred embodiments, detection of a label provides quantifiable information. Labels can be any known detectable moiety, such as, for example, a radioactive label (e.g., radionuclides), a ligand (e.g., biotin or avidin), a chromophore (e.g., a dye or particle that imparts a detectable color), a hapten (e.g., digoxgenin), a mass label, latex beads, metal particles, a paramagnetic label, a luminescent compound (e.g., bioluminescent, phosphorescent or chemiluminescent labels) or a fluorescent compound.

A label may be joined, directly or indirectly, to an oligonucleotide or other biological molecule. Direct labeling can occur through bonds or interactions that link the label to the oligonucleotide, including covalent bonds or non-covalent interactions such

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as hydrogen bonding, hydrophobic and ionic interactions, or through formation of chelates or coordination complexes. Indirect labeling can occur through use of a bridging moiety or "linker", such as an antibody or additional oligonucleotide(s), which is/are either directly or indirectly labeled.

Labels can be used alone or in combination with moieties that can suppress (e.g., quench), excite, or transfer (e.g., shift) emission spectra (e.g., fluorescence resonance energy transfer (FRET)) of a label (e.g., a luminescent label).

As used herein, the term "FRET" refers to fluorescence resonance energy transfer, a process in which moeities (e.g., fluorphores) transfer energy (e.g., among themselves, or, from a fluorophore to a non-fluorophore (e.g., a quencher molecule)). In some circumstances, FRET involves an excited donor fluorophore transferring energy to a lower-energy acceptor fluorophore via a short-range (e.g., about 10 nm or less) dipole-dipole interaction. In other circumstances, FRET involves a loss of fluorescence energy from a donor and an increase in fluorescence in an acceptor fluorophore. In still other forms of FRET, energy can be exchanged from an excited donor flurophore to a non-fluorescing molecule (e.g., a quenching molecule). FRET is known to those of skill in the art and has been described (See, e.g., Stryer et al., 1978, Ann. Rev. Biochem., 47:819; Selvin, 1995, Methods Enzymol., 246:300; Orpana, 2004 Biomol Eng 21, 45-50; Olivier, 2005 Mutant Res 573, 103-110, each of which is incorporated herein by reference in its entirety).

As used herein, the term "donor" refers to a moiety (e.g., a fluorophore) that absorbs at a first wavelength and emits at a second, longer wavelength. The term "acceptor" refers to a moiety such as a fluorophore, chromophore, or quencher and that is able to absorb some or most of the emitted energy from the donor when it is near the donor group (typically between 1-100 nm). An acceptor may have an absorption spectrum that overlaps the donor's emission spectrum. Generally, if the acceptor is a fluorophore, it then re-emits at a third, still longer wavelength; if it is a chromophore or quencher, it releases the energy absorbed from the donor without emitting a photon. In some preferred embodiments, alteration in energy levels of donor and/or acceptor moieties are detected (e.g., via measuring energy transfer (e.g., by detecting light emission) between or from donors and/or acceptor moieties). In some preferred

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embodiments, the emission spectrum of an acceptor moeity is distinct from the emission spectrum of a donor moiety such that emissions (e.g., of light and/or energy) from the moieties can be distinguished (e.g., spectrally resolved) from each other.

In some embodiments, a donor moiety is used in combination with multiple acceptor moieties. In a preferred embodiment, a donor moiety is used in combination with a non-fluorescing quencher moiety and with an acceptor moiety, such that when the donor moiety is close (e.g.. between 1-100 nm, or more preferably, between 1-25 nm, or even more preferably around 10 nm or less) to the quencher, its excitation is transferred to the quencher moiety rather than the acceptor moiety, and when the quencher moiety is removed (e.g., by cleavage of a probe), donor moiety excitation is transferred to an acceptor moiety. In some preferred embodiments, emission from the acceptor moiety is detected (e.g., using wavelength shifting molecular beacons) (See, e.g., Tyagi, et al., Nature Biotechnology 18:1191 (2000); Mhlanga and Malmberg, 2001 Methods 25, 463-471; Olivier, 2005 Mutant Res 573, 103-110, and U.S. Pat. App. 20030228703, each of which is incorporated herein by reference in its entirety).

Detection of labels or a detectable response (e.g., provided by the labels) can be measured using a multitude of techniques, systems and methods known in the art. For example, a label may be detected because the label provides detectable fluorescence (e.g., simple fluorescence, FRET, time-resolved fluorescence, fluorescence quenching, fluorescence polarization, etc.), radioactivity, chemiluminescence, electrochemiluminescence, RAMAN, colorimetry, gravimetry, hyrbridization (e.g., to a sequence in a hybridization protection assay), X-ray diffraction or absorption, magnetism, enzymatic activity, characteristics of mass or behavior affected by mass (e.g., MALDI time-of-flight mass spectrometry), and the like.

A label may be a charged moiety (positive or negative charge) or alternatively, may be charge neutral. Labels can include or consist of nucleic acid or protein sequence, so long as the sequence comprising the label is detectable. In some embodiments, the label is not nucleic acid or protein.

In some embodiments, a label comprises a particle for detection. For example, in some embodiments, the particle is a phosphor particle. An example of a phosphor particle includes, but is not limited to, an up-converting phosphor particle (See, e.g.,

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Ostermayer, Preparation and properties of infrared-to-visible conversion phosphors. Metall. Trans. 752, 747--755 (1971)). In some embodiments, rare earth-doped ceramic particles are used as phosphor particles. Phosphor particles may be detected by any suitable method, including but not limited to up-converting phosphor technology (UPT), in which up-converting phosphors transfer low energy infrared (IR) radiation to high-energy visible light. Although an understanding of the mechanism is not necessary to practice the present invention and the present invention is not limited to any particular mechanism of action, in some embodiments the UPT up-converts infrared light to visible light by multi-photon absorption and subsequent emission of dopant-dependant phosphorescence (See, e.g., U.S. Patent No. 6,399,397; van De Rijke, et al., Nature Biotechnol. 19(3):273-6 (2001); Corstjens, et al., IEE Proc. Nanobiotechnol. 152(2):64 (2005), each incorporated by reference herein in its entirety.

As used herein, the term "distinct" in reference to signals (e.g., of one or more labels) refers to signals that can be differentiated one from another, *e.g.*, by spectral properties such as fluorescence emission wavelength, color, absorbance, mass, size, fluorescence polarization properties, charge, etc., or by capability of interaction with another moiety, such as with a chemical reagent, an enzyme, an antibody, etc.

It will be apparent to one of skill in the art that there are a large number of methods (e.g., analytical procedures) that may be used to detect the presence or absence of a nucleic acid sequence (e.g., a gene (e.g., wild-type, mutant (e.g., comprising one or more variant nucleotides at one or more positions), heterologous, etc.)). Such methods include, but are not limited to, nucleic acid discrimination techniques, amplification reactions and/or a signal generating systems. Such methods include, but are not limited to, DNA sequencing, hybridization sequencing, protein truncation test, single-strand conformation polymorphism analysis (SSCP), denaturing gradient gel electrophoresis, temperature gradient gel electrophoresis, heteroduplex analysis, chemical mismatch cleavage, restriction enzyme digestion, and enzymatic mismatch cleavage, solid phase hybridization, dot blots, multiple allele specific diagnostic assays, reverse dot blots, oligonucleotide arrays (e.g., DNA chips), solution phase hybridization (e.g., TAQMAN (See, e.g., U.S. Pat. Nos. 5,210,015 and 5,487,972, each of which is herein incorporated by reference in its entirety) and molecular beacons (See, e.g., Tyagi et al. 1996 Nature

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Biotech, 14, 303 and Int. App. WO 95/13399, herein incorporated by reference), extension based amplification (e.g., amplification refractory mutation systems, amplification refractory mutation system linear extensions (See, e.g., EP 332435, herein incorporated by reference in its entirety), competitive oligonucleotide priming system (See, e.g., Gibbs et al., 1989 Nucleic Acids Research 17, 2347, herein incorporated by reference in its entirety), mini sequencing, restriction fragment length polymorphism, restriction site generating PCR, oligonucleotide ligation assay and many others described herein and elsewhere.

DESCRIPTION OF THE DRAWINGS

Figure 1 shows the dynamic range of detection of a first target using methods of the present invention.

Figure 2 shows the dynamic range of detection of a second target using methods of the present invention.

Figure 3 shows an overview of one exemplary embodiment of the INVADER assay.

Figure 4 shows an Excel graph showing results of an assay using two probe concentrations covering six orders of magnitude of target concentration, from 10 copies to 10,000,000. Data points represent: The resulting signal from 2 sets of probes and FRET cassettes in a combined reaction as described in Example 5 (Diamonds); the resulting signal from only the 1X probe (Squares); and the resulting signal from only the 1/150X probe (Triangles).

Figure 5 shows an Excel graph showing results of an assay using three probe concentrations covering six orders of magnitude of target concentration, from 10 copies to 10,000,000, as described in Example 6. Individual contributions of each of the three probe concentrations is shown with additional lines. Data points represent: The resulting signal from three probe concentrations (Squares); the 1X probe in isolation (Diamonds); the 1/125X probe in isolation (Squares); and the 1/1000X probe in isolation (Triangles).

Figure 6 shows an Excel graph showing the results of varying the length of incubation time of the invasive cleavage reaction, as described in Example 7. Data points represent: The resulting signal from 30 minutes incubation (Diamonds); 1 hour

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incubation (Squares); 2 hours incubation (Triangles); 4 hours incubation (Circles); and 8 hours incubation (Asterisks).

Figure 7 shows an Excel graph showing the detection of two different targets in simultaneous multiplex using the methods of the present invention as described in Example 8. CMV and EBV were simultaneously detected across over six orders of magnitude of dynamic range, in the same reaction vessel. In this experiment, CMV (Diamonds) and EBV (Squares) were detected in multiplex over a range from approximately 20 to 1,000,000 copies per reaction.

Figure 8 shows an Excel graph showing detection over nine orders of magnitude of target concentration, as described in Example 9. Combining single strand amplification with standard PCR, and detecting both products simultaneously with two sets of two probes broadened the dynamic range to at least nine orders of magnitude (10-10,000,000,000 copies of target detected by a single reaction setup). As shown, basic target detection with no single strand product (detecting only double stranded PCR product; Diamonds); basic reaction with the addition of a 1X probe to detect single strand product (Squares); the basic reaction with the addition of a 1/125X probe to detect single strand product (Triangles); and the basic reaction with the addition of both 1X and 1/125X probes to detect both PCR and single strand products.

Figure 9 shows an Excel graph showing the detection of RNA using multiple probe concentrations, as described in Example 10. As shown, RNA target detection with 1X probe alone (Diamonds); 1/125X probe alone (Squares), and of both 1X and 1/125X probes to detect RT-PCR products (Triangles).

DESCRIPTION OF THE INVENTION

The present invention provides systems, methods and kits for increasing the dynamic range of detection of a target nucleic acid in a sample. In particular, the present invention provides methods and kits for increasing the dynamic range of detection of a target nucleic acid in a sample through the use of one or more probe oligonucleotides (e.g., analyte-specific probe oligonucleotides).

In some embodiments, the present invention achieves greater dynamic range of detection through the use of differential levels of amplification of regions of a target

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nucleic acid (e.g., no amplification, linear amplification at one or more efficiencies, and/or exponential amplification at one or more efficiencies). In some embodiments, the present invention achieves greater dynamic range of detection through the use of probes with different hybridization properties to one or more analyte-specific regions of a target nucleic acid or target nucleic acids. In some embodiments, the present invention achieves greater dynamic range of detection through the use of different signal generation methods. In some embodiments, the present invention achieves greater dynamic range of detection through the use of different signal detection methods. In preferred embodiments, combinations of two or more of the methods are employed. For example, in some preferred embodiments, two or more probes (e.g., three, four, etc.) are contacted with first and second amplicons obtained via different levels of amplification. In some such embodiments, each probe generates the same type of signal so that one simply detects total signal generated by the reactions. The collective signal permits detection of target nucleic acid over a broad dynamic range. For example, experiments conducted during the development of the present invention have demonstrated the ability to detect target nucleic acid from samples differing in over eight logs of copy number of target nucleic acid originally present in the sample.

In certain embodiments, the present invention provides methodologies for expansion of the dynamic range of hybridization assays, such as serial invasive cleavage assays. In some embodiments, the upper limit of dynamic range may be expanded by the use of an additional probe that is present in the reaction at a lower concentration than another probe. In some embodiments, this additional probe will hybridize to the same region of the target. For invasive cleavage reactions, this probe may contain a different arm, or flap, sequence that is released after cleavage. In certain embodiments related to invasive cleavage assays, a second FRET cassette will also be added to the reaction with the appropriate sequence to detect those cleaved flaps from the additional probe. Generally the concentration of the second FRET cassette is about the same as the first FRET cassette. For example, in certain embodiments, if probe B is present in the reaction at 100-fold lower concentration than probe A, this will enable the detection of target nucleic acid when it is present at concentrations above the upper limit of detection of probe A. In this manner, each additional probe, present at 100-fold lower

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