ramping down of the temperature in the cycle. In some embodiments according to the present disclosure, the cycles can be slowed down such as to allow better detection/resolution during such partial melting curve analysis. In some embodiments in order to maintain a relatively fast cycle, the temperature within a cycle can be controlled to quickly go to the expected temperatures of the melt curve (e.g. partial melt curve) where various amplicons show a signature (e.g. melting temperature as per design of each of the different amplicons) and pass through other temperature points rapidly. Such controlling of the temperature within the cycle can be performed during either a ramping up or a ramping down of the temperature, with better results obtained dependent on the heating method used. FIG. 6B shows a modified cycle of the cycle represented in FIG. 6A whereby the temperature of the cycle corresponding to the transition between the denaturation and the annealing phases is controlled such as to allow for the partial melt curve analysis at the vicinity of the two melting temperature points (TM1) and (TM2). In the embodiment depicted by FIG. 6B, the transition region between the two temperature levels associated to the denaturation and annealing phases is segmented into regions at the vicinity of and including each distinct melting temperature point and segments devoid of a melting point, such as the sum of the segments contain the entire transition region. As depicted by FIG. 6B, segments containing a melting point provide a lower slope corresponding to a lower temperature change of rate such as to allow for better melt curve analysis, whereas segments devoid of a melting point have a larger slope such as to quickly pass through a temperature transition and minimize duration of the cycle. Although in the exemplary case depicted by FIG. 6B the partial melting curve analysis is performed during a ramping down of the temperature profile of the cycle, according to other embodiments of the present disclosure the partial melting curve analysis can be performed during a ramping up of the temperature profile of the cycle as depicted for example in FIGs. 7A and 7B. In some cases, the partial melting curve can be performed only during some (e.g. not all) of the cycles of the amplification process. In a case where the thermal characteristics of the system (e.g. including qPCR fluid) are such that the ramp rate need not be reduced but still a signature is obtained, the cycle can proceed at normal ramp without slowing down near (TM1) or (TM2).

[0042] In some embodiments partial melting curve analysis in some of the cycles can be skipped such as to reduce total reaction time (e.g. sum of all cycles times) and then quantify based on

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threshold only. Normally the Ct value is desired to be quantified. In some embodiments looking for a melt signature (e.g. a specific change in shape/slope of the detected fluorescence versus temperature at a melting temperature point) for an amplicon can be stopped as soon as its signature becomes detectable. In some embodiments it may be desirable to run a final melting curve analysis in order to verify the reaction and cross check against the observed amplicons (e.g. no unexpected amplicons generated).

A partial melting curve analysis algorithm is as follows:

Start cycling
For each cycle
- If fluorescence is above a threshold (signals start of an amplification)
- Run melting curve for undetected targets (or look for expected signature instead of going slowly through all temperatures)
- If a target is detected, mark it and optionally exclude analyzing it in next cycles (no need to ramp slowly across this melting temperature for next cycles)
- If all targets are detected, break
End for
Optionally verify using melting curve at end

[0043] In some cases it can be desirable to run full melting curve analysis in between cycles of an amplification reaction, at the expense of the extra time required to acquire the melting curve. However, since the melting temperature of each amplicon is known in advance, one can measure fluorescence (e.g. emitted intensity) at particular temperatures (e.g. at the vicinity of a known melting temperature of an amplicon) and skip temperature ranges which do not contain a melting temperature of an amplicon, instead of recording (e.g. measuring fluorescence) at more temperatures as is typically done in a melting curve analysis. By tracking the peaks in observed fluorescence for each of the known melting temperatures (e.g. associated to an amplicon) for each run melting curve, one can detect the presence of an amplicon (e.g. at a concentration). Such a method of accelerating the melting curve analysis can save overall time (e.g. for detection/quantification of amplicons) while quantification can be obtained for many amplicons

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with different melting temperatures. Also the amplitude information, obtained via the sum amplitude curve, can be used to detect two amplicons with the same or very close melting temperatures. For example, fluorescence increasing at double the rate (e.g. compared to an amplification rate of a single amplicon during an exponential amplification phase) or similar, is indicative of two amplicons being amplified instead of one.

[0044] According to a further embodiment of the present disclosure, an algorithm for an accelerated melting curve analysis is presented below. Such algorithm can be used in cases where it is desired to find out the concentration of an amplicon or amplicons (e.g. corresponding C_Ts) within, for example, a multiplexed reaction. It is assumed that each reaction will go through exponential phase, or in other words that the reactions are proper:

Start cycling
For each cycle
- *Measure fluorescence at temperatures related to each amplicon*
- *If a target is detected, mark it and optionally exclude analyzing it in next cycles (do not ramp slowly across the melting temperature of a detected target for next cycles)*
- *If all targets are detected, break*

End for
Optionally verify using melting curve at end

Same algorithm presented above can be used in the case of multiple amplicons with similar melting temperatures.

[0045] Non-specific probes, such as for example intercalating dyes, can be used to qualitatively find out which amplicon has amplified by using, for example, a melting curve analysis. Non-specific probes can be used to make an assay with targets which need quantification and targets which do not need quantification (e.g. only need to find out their presence and not concentration). Sequence specific probes can be used to measure quantification (e.g. C_T values). In a two channel (e.g. detection) instrument, one channel can be used to perform qualitative

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analysis of a multiplexed assay using non-specific probes, whereas a second channel can be used to perform a quantification of specific targets of the assay using sequence specific probes. Such a configuration thus provides two degrees of freedom (e.g. color and melting temperature) in multiplex assays.

[0046] In the exemplary case of three amplicons, corresponding to a positive control, a negative control and a target, one can use both non-specific dyes and sequence specific hybridization probes (e.g. for all three corresponding amplicons) in a same reaction. According to an embodiment of the present disclosure, such multiplexed reaction can be analyzed, both quantitatively and qualitatively, using two excitation sources and only one detector. For example and as depicted by FIG. 8, if the non-specific dye is SYBR Green and the dye used for sequence specific detection is Alexa Fluor 610 then one can use a blue LED (e.g. 475nm/25 blue filter) to excite the green dye while the Alexa Fluor is not excited appreciably. This will give melting curve and qualitative answer to the amplification reaction.

[0047] Quantification can be determined by measuring the fluorescence during cycles, while a UV LED (e.g. 410nm/25 UV filter) provides the excitation wavelength, which does not excite the green dye appreciably. The advantage is that only one detector and emission filter is used thus possibly allowing more light capture. In this case the amplification of controls does not interfere with the florescence signal of the target amplicon.

[0048] According to further embodiments of the present disclosure, various mathematical transforms and functions used in signal processing (e.g. FFT, DFT, z-transform, etc...) can be used to compare, match or further analyze signals/amplitudes detected during a multiplexed reaction. Such transforms/functions can detect a phase difference between two shifted waveforms, such as slopes detected and/or assumed, and/or various derivatives of sum amplitude and/or melting curves. They can also help in normalizing the amplitudes and providing more insight into the behavior of the multiplexed reaction than by using only slopes of the observed signals. These transforms/functions can be applied to the entire and/or different parts of the observed/detected signals.

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[0049] According to a further embodiment of the present disclosure, teachings from the signal processing theory as well as the estimation theory can be used to further analyze the observed/detected signals during a multiplexed amplification reaction. In an exemplary embodiment according to the present disclosure, the estimation model given in FIG. 9 can be used to represent the amplification process.

In the estimation model depicted by FIG. 9:

$C_Tk = C_T$ of a corresponding amplification

Ak = amplitude of a corresponding amplification

Sk = slope/efficiency of a corresponding amplification

where k = (1,2,…) depending on the number of multiplexed amplicons.

By feeding an observed signal $\overline{y}$, from the multiplexed amplification reaction, to an estimator corresponding to the modeled process represented by a matrix $\overline{h}$, one can derive an estimation of the process parameter $\overline{x}$, which includes the main information of the reaction (e.g. C_T, amplitude, slope/efficiency).

[0050] One can use various estimators like ML (maximum likelihood), MAP (Maximum A Priori) and various others to estimate the values of parameters. The process ($\overline{h}$) can be modeled in various ways. It can be modeled recursively and a Kalman filter can be used to smooth out and or estimate the parameters. Various other control algorithms known to a person skilled in the art of signal processing and estimation theory can be used to analyze the observed/detected signals during the multiplexed reaction. As previously mentioned, such algorithms can be implemented in a combination of software/firmware to be used to upgrade an existing instrument and/or used in a new instrument configured to perform the multiplexed reaction (e.g. single channel detector) and/or configured to simply analyze off line data/signals detected/observed.

[0051] According to a further embodiment of the present disclosure, quality and concentration of a multiplexed reaction can be obtained by limiting the primers used in the reaction process such as to control the plateau levels of the various amplicons of the multiplexed reaction. Using such

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method therefore allows to find the C_T value of each amplicon and whether various amplicons amplified using only the amplification curves and without melting curve analysis at the end. By limiting the primers and control the number of cycles it takes to reach plateau phase for various amplicons then an estimate of the slope of fluorescence for each amplicon can be made. In some cases the peaks of derivative and double derivatives would be apart such as to allow easy quantification and detection. In overlapping cases of several C_T values , the prior slope knowledge obtained by limiting the amplicon specific primers can be used to resolve the amplicon amplified. For example, if a slope of the amplification is higher than an expected slope of any of the amplicon (e.g., in case of a same C_T value) then it can be estimated that two amplicons are amplified. Also the fluorescence value at the end of all amplifications can give a sum which can be used to find out how many amplicons did amplify, since plateau level of each amplicon is known per design. Mathematical analysis as described in prior paragraphs can further help to analyze the observed/detected amplitudes/slopes.

[0052] Therefore, in accordance with the present disclosure, systems and methods for multiplexing and quantification in PCR with reduced hardware and requirements are provided, which allow detection of different amplicons via a single detector and quantification of said amplicons using known mathematical analysis methods.

[0053] The person skilled in the art will be able to extend the teachings of the present disclosure to any type of process which can measure total or targeted amplicon concentration during an amplification, including but not limited to real-time quantitative polymerase chain reaction (qPCR) with fluorescence detection, electrochemical detection or any other real-time detection technique which can quantify the amount of DNA, whether total or specific, in a manner similar to what has already been discussed in the present disclosure. While the devices and methods have been described by means of specific embodiments and applications thereof, it is understood that numerous modifications and variations could be made thereto by those skilled in the art without departing from the spirit and scope of the disclosure. It is therefore to be understood that within the scope of the claims, the disclosure may be practiced otherwise than as specifically described herein

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[0054] A number of embodiments of the present inventive concept have been described. Nevertheless, it will be understood that various modifications may be made without departing from the scope of the inventive teachings.

[0055] Accordingly, it is to be understood that the inventive concept is not to be limited by the specific illustrated embodiments, but only by the scope of the appended claims. The description may provide examples of similar features as are recited in the claims, but it should not be assumed that such similar features are identical to those in the claims unless such identity is essential to comprehend the scope of the claim. In some instances the intended distinction between claim features and description features is underscored by using slightly different terminology.

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CLAIMS

1. A method comprising:
   detecting a plurality of different amplicons generated during a multiplexed amplification reaction, using a single channel detector generating a sum amplitude signal in correspondence of the plurality of different amplicons;
   quantifying the plurality of detected different amplicons, and
   verifying the plurality of detected different amplicons,
   wherein the quantifying and the verifying comprise using mathematical analysis of the generated sum amplitude signal.

2. The method of claim 1, wherein the sum amplitude signal is in correspondence of a sum of amplitude signals of each different amplicon of the plurality of different amplicons.

3. The method of claim 1, wherein the detecting is based on emitted wavelengths from sequence specific probes and/or sequence specific primers.

4. The method of claim 1, wherein the detecting is based on emitted wavelengths from fluorescent DNA binding dyes.

5. The method according to any one of claims 1-4, wherein the mathematical analysis is performed on a portion of the sum amplitude signal.

6. The method of claim 5, wherein the portion comprises slopes regions of the sum amplitude signal.

7. The method of claim 6, wherein the mathematical analysis of the slopes regions comprises analysis of the slopes variations.

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8. The method of claim 7, wherein the analysis of the slopes variations is used to detect a threshold cycle of an amplicon of the plurality of different amplicons.

9. The method of claim 8, wherein the slopes are dependent on different primer concentrations used for different amplicons of the plurality of different amplicons.

10. The method of claim 5, wherein the portion of the sum amplitude signal comprises flat regions of the sum amplitude signal.

11. The method of claim 10, wherein the mathematical analysis of the flat regions is used to identify an amplicon of the plurality of different amplicons.

12. The method of claim 11, wherein the flat regions are dependent on different primer concentrations used for different amplicons of the plurality of different amplicons.

13. The method according to any one of claims 1-12, wherein the mathematical analysis further comprises:

using signal processing techniques to separate an amplitude curve of an amplicon of the plurality of different amplicons from a sum amplitude curve in correspondence of the sum amplitude signal, and

deriving a value of a threshold cycle of the amplicon based on the amplitude curve.

14. The method of claim 13, wherein the signal processing techniques comprise one or more of: a) fast Fourier transform (FFT), b) discrete Fourier transform, and b) z-transform.

15. The method of claim 13 further comprising:

for each amplicon of the plurality of different amplicons, assuming a threshold cycle value based on prior mathematical analysis of the sum amplitude curve, and

based on the assuming, generating an assumed amplitude curve in correspondence of the each amplicon of the plurality of different amplicons,

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based on the generating, generating an assumed sum amplitude curve,

based on the generating, measuring a distance between the sum amplitude curve and the assumed sun amplitude curve,

repeating the assuming, generating, generating and measuring, and

based on the repeating, deriving for each amplicon of the plurality of different amplicons the value of the threshold cycle from the assumed value.

16. The method of claim 15, wherein the measuring of the distance is performed over a portion of the sum amplitude curve and the assumed sum amplitude curve.

17. The method of claim 16, wherein the portion comprises an exponential phase of the curves.

18. The method according to any one of claims 1-4, wherein the mathematical analysis further comprises using a model for modeling the multiplexed amplification reaction, the model being defined by a plurality of process parameters, and estimating the values of the process parameters based on an estimator associated to the model.

19. The method of claim 18, wherein a plurality of process parameters defining the model comprise: a) a threshold cycle, b) an amplitude, and c) a slope, of a corresponding amplification of the multiplexed amplification reaction.

20. The method of claim 18 or claim 19, wherein the estimator comprises one of: a) a maximum likelihood estimator, and b) a maximum a priori estimator.

21. The method according to any one of claims 18-20, wherein the model is modeled recursively using a Kalman filter.

22. The method according to any one of claims 1-5, 10, 13, 15 and 18, wherein the verifying further comprises:

running a melting curve analysis;

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based on the running, verifying a number of different amplicons of the plurality of different amplicons, and

based on the running, detecting presence of an amplicon of the plurality of different amplicons.

23. The method of claim 22, wherein the melting curve analysis further comprises:

defining a temperature range comprising a plurality of melt temperatures of the plurality of different amplicons;

segmenting the temperature range in temperature regions containing a melt temperature of the plurality of different amplicons and temperature regions devoid of a melt temperature of the plurality of different amplicons;

defining a slow temperature ramp rate and a fast temperature ramp rate;

associating the slow temperature ramp rate to the temperature regions containing a melt temperature and associating the fast temperature ramp rate to temperature regions devoid of a melt temperature;

based on the associating, ramping a melting curve analysis temperature over the temperature range, and

based on the ramping, analyzing during the slow ramp rate a melt curve in correspondence of the melting of the plurality of different amplicons.

24. The method of claim 23, wherein the quantifying further comprises:

waiting for a completion of a cycle of the multiplexed amplification reaction;

running a melting curve analysis;

based on the running, obtaining a quantity of an amplicon of the plurality of different amplicons, and

repeating the waiting, the running and the obtaining.

25. The method of claim 23, wherein the quantifying further comprises running one or more melting curve analyses during one or more corresponding cycles of the multiplexed amplification reaction.

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26. The method of claim 25, wherein the running of the one or more melting curve analyses is performed during a region of temperature transition between one of: a) a denaturation phase and an annealing phase of the one or more corresponding cycle, and b) an extension phase and a denaturation phase of the one or more corresponding cycles.

27. The method according to any one of claims 1-5, 10, 13, 15, 18 and 22, wherein the mathematical analysis further comprises taking into account a difference in amplitude between a first amplicon of the plurality of different amplicons and a second different amplicon of the plurality of amplicons, the difference in amplitude being based on a length difference between the first amplicon and the second amplicon.

28. The method according to any one of claims 1-5, 10, 13, 15, 18 and 22, wherein the mathematical analysis further comprises taking into account a difference between a first amplitude signal associated to a first amplicon of the plurality of different amplicons and a second amplitude signal associated to a second amplicon of the plurality of different amplicons, the difference being based on a difference in primer concentration used for the first and the second amplicons.

29. The method according to any one of claims 1-5, 10, 13, 15, 18, 22, 27 and 28, wherein the detecting further comprises:

using a filter configured to detect a wider range of wavelengths;

based on the using, detecting different wavelengths associated to different amplicons of the plurality of different amplicons, and

based on the detecting, generating the sum amplitude signal.

30. The method according to any one of claims 1-5, 10, 13, 15, 18, 27 and 28, wherein the quantifying further comprises:

running a melting curve analysis;

based on the running, deriving a relative amplitude of an amplicon of the plurality of different amplicons;

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associating the relative amplitude to a threshold point derived from a sum melt curve associated to the sum amplitude signal, and

based on the associating, determining the threshold point of the amplicon, wherein the threshold point quantifies the amplicon.

31. A method for further multiplexing a single channel detector multiplexed amplification reaction, the method comprising:

providing a plurality of excitation sources;

activating one of the plurality of excitation sources;

based on the activating, exciting one or more fluorophores in correspondence of one or more different amplicons from a plurality of different amplicons;

based on the exciting, performing the detecting, the quantifying and the verifying of the one or more different amplicons in accordance to the method according to any one of claims 1-5, 10, 13, 15, 18, 22, 25, 27 and 28;

deactivating the one excitation source; and

repeating the activating, the exciting, the performing, and the deactivating for each remaining excitation source of the plurality of excitation sources, wherein the repeating detects, quantifies and verifies the plurality of different amplicons.

32. The method according to any one of claims 1-5, 10, 13, 15, 18, 22, 25, 27, 28 and 31, wherein the detecting further comprises:

using a filter configured to detect a wider range of wavelengths;

based on the using, detecting different wavelengths associated to different amplicons of the one or more different amplicons, and

based on the detecting, generating the sum amplitude signal.

33. A processor-based hardware analyzer for analyzing a multiplexed amplification reaction, wherein the processor-based hardware analyzer is configured to quantify and verify the multiplexed amplification reaction based on a provided digital representation of a sum amplitude signal in correspondence of a plurality of different amplicons of the multiplexed amplification reaction.

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34. The processor-based analyzer of claim 33, wherein the quantification and verification is performed using mathematical analysis of the digital representation of the sum amplitude signal, the mathematical analysis being implemented in software and/or firmware code and executed on the processor-based analyzer.

35. The processor-based analyzer of claim 34, wherein the mathematical analysis further comprises signal processing techniques adapted to separate an amplitude curve of an amplicon of the plurality of different amplicons from the digital representation of the sum amplitude signal, and adapted to derive a value of a threshold cycle of the amplicon based on the amplitude curve.

36. The processor-based analyzer according to any one of claims 33-35, wherein the processor-based analyzer is further configured to derive a relative amplitude of an amplicon of the plurality of different amplicons based on a provided sum melt curve.

37. An apparatus for performing a multiplexed amplification reaction, the apparatus comprising:

a single channel detector configured to detect a sum amplitude signal in correspondence of a plurality of different amplicons of the multiplexed amplification reaction and generate a digital representation of said signal; and

the processor-based analyzer of any one of claims 33-35, configured to receive the digital representation of the sum amplitude signal.

38. The apparatus of claim 37 further comprising a plurality of excitation sources.

39. The apparatus of claim 37 or claim 38, further comprising a wider bandwidth filter adapted to detect a wider range of wavelengths.

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1/12

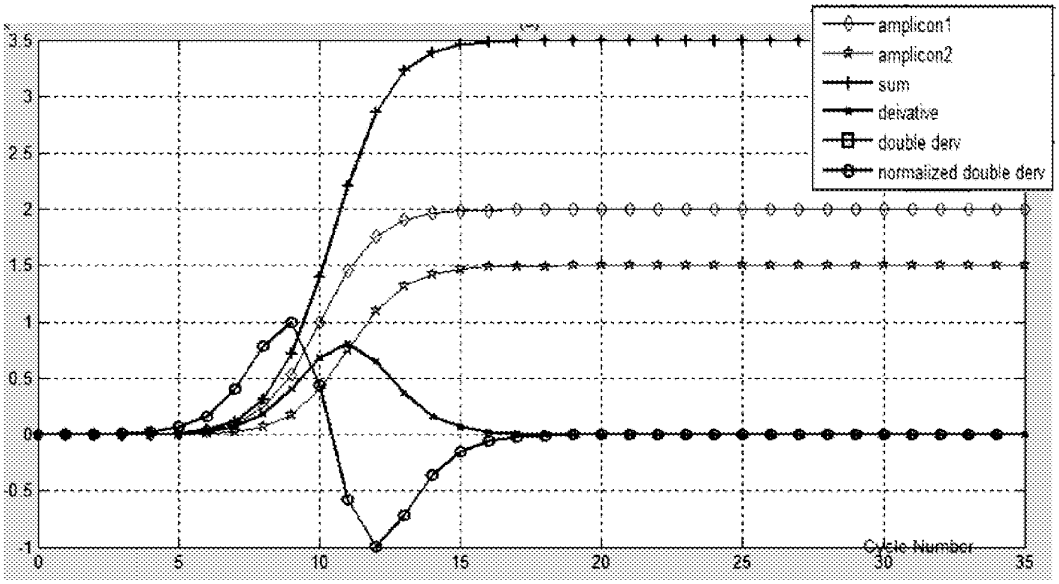


FIG. 1A

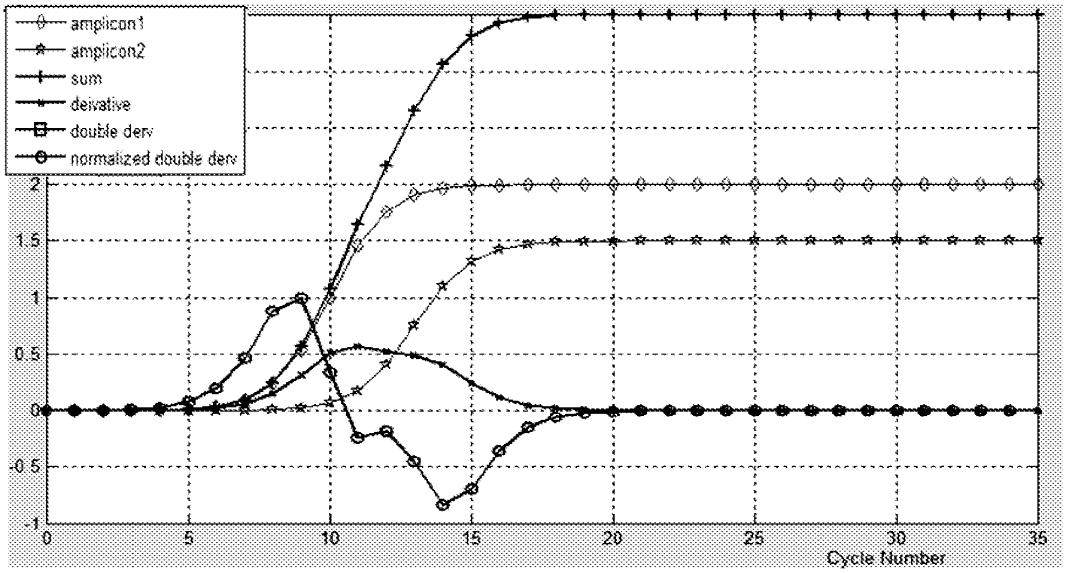


FIG. 1B

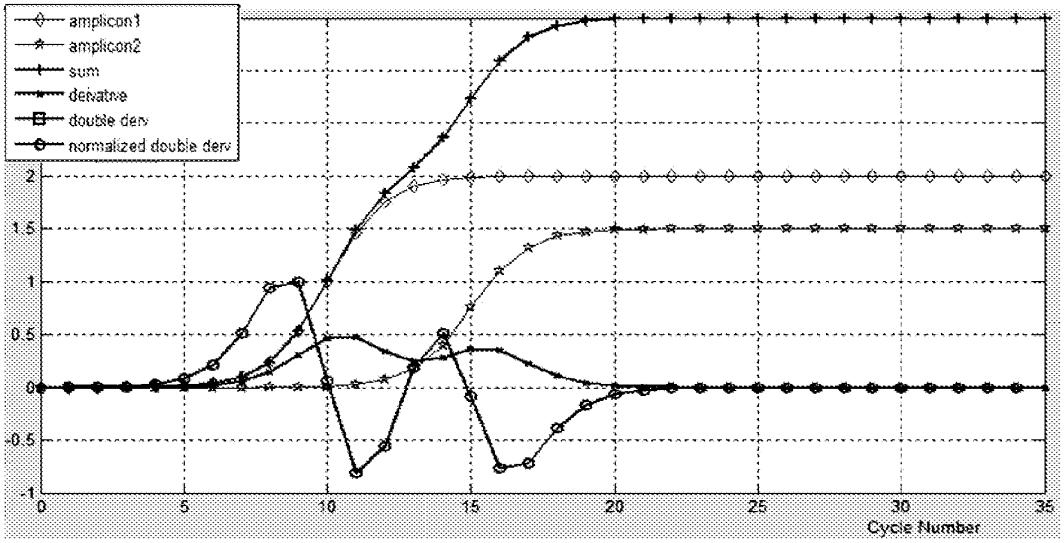

amplicon1
amplicon2
sum
derivative
double derv
normalized double derv
2
1.5
1
0.5
0
-0.5
-1
0
5
10
15
20
25
30
35
Cycle Number

FIG. 1C

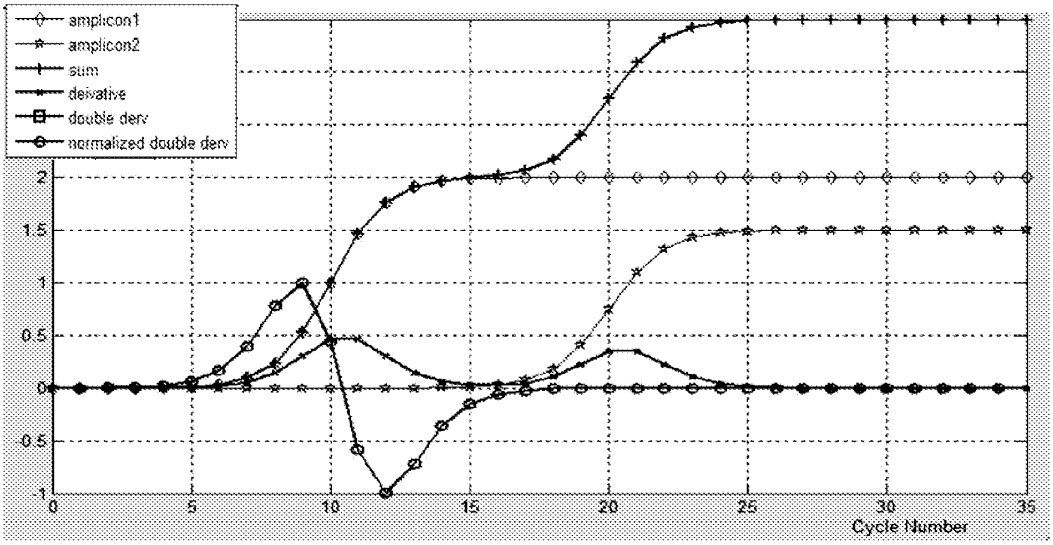

amplicon1
amplicon2
sum
derivative
double derv
normalized double derv
2
1.5
1
0.5
0
-0.5
-1
0
5
10
15
20
25
30
35
Cycle Number

FIG. 1D

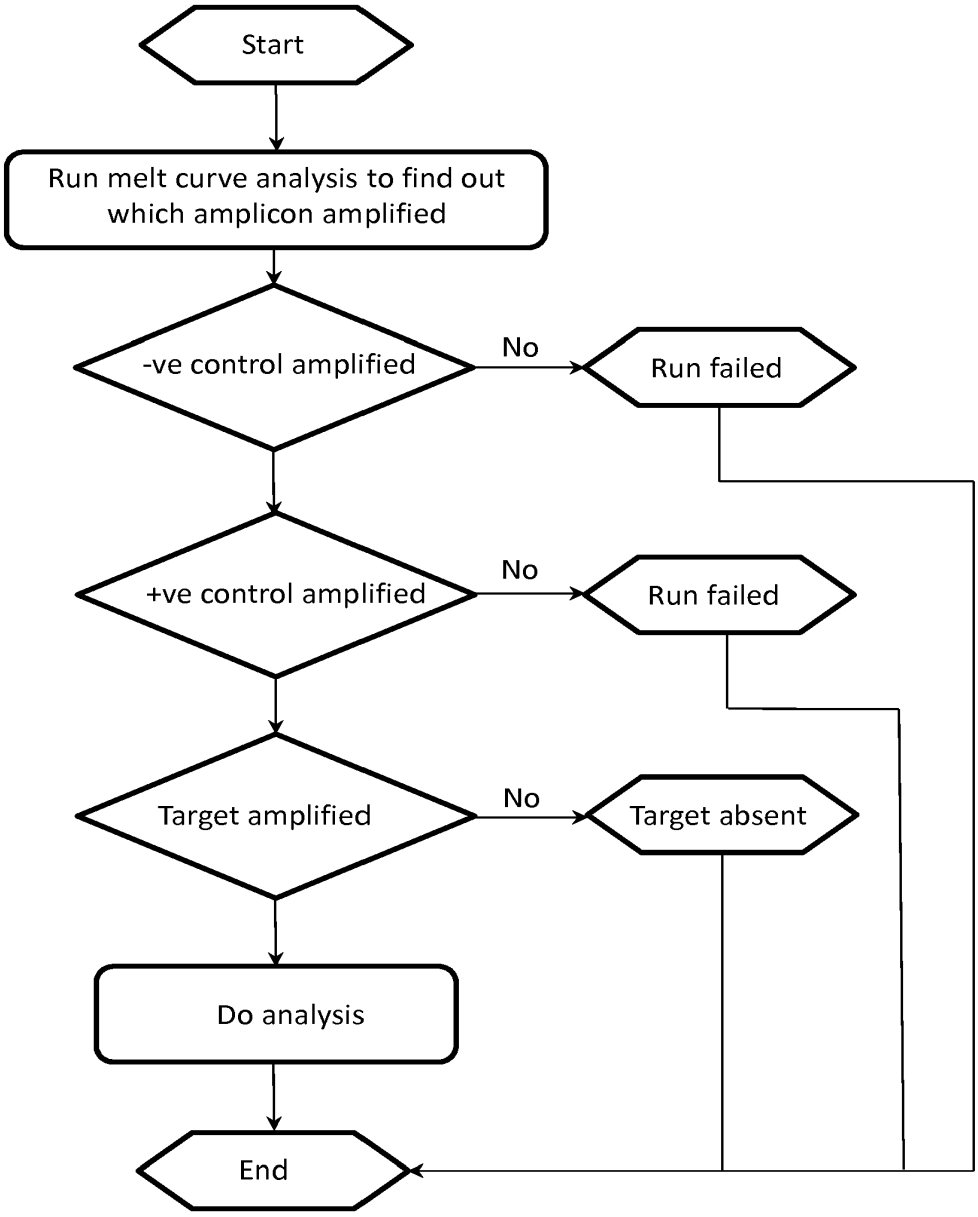

Start
Run melt curve analysis to find out which amplicon amplified
-ve control amplified
No
Run failed
+ve control amplified
No
Run failed
Target amplified
No
Target absent
Do analysis
End

FIG. 2

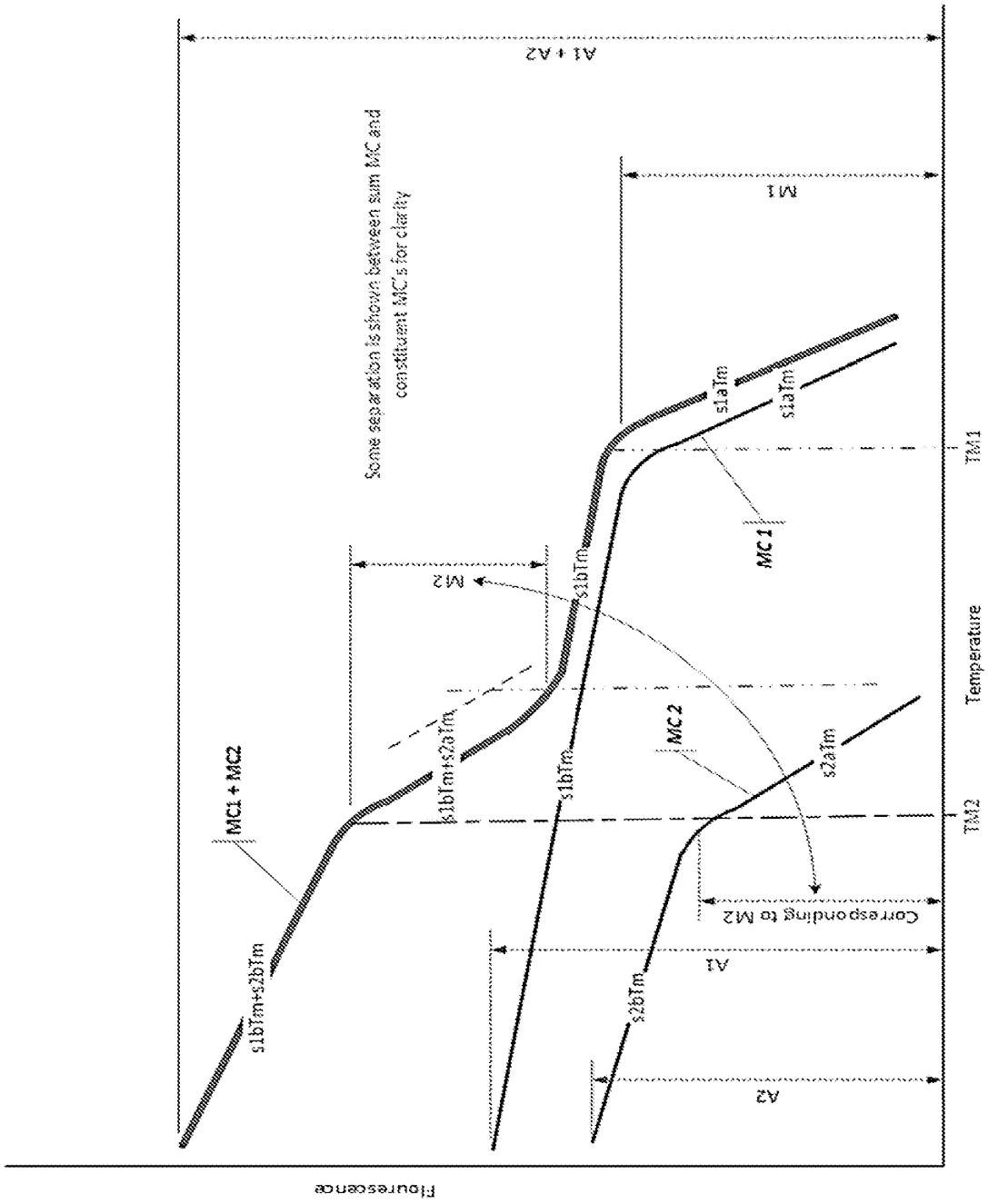

A1 + A2
Some separation is shown between sum MC and constituent MCs for clarity
M1
s1aTm
s1aTm
TM1
MC1
M2
s1bTm
MC1 + MC2
s1bTm+s2aTm
MC 2
s2aTm
s1bTm
TM2
Temperature
Corresponding to M2
A1
s1bTm+s2bTm
s2bTm
A2
Fluorescence

FIG 3

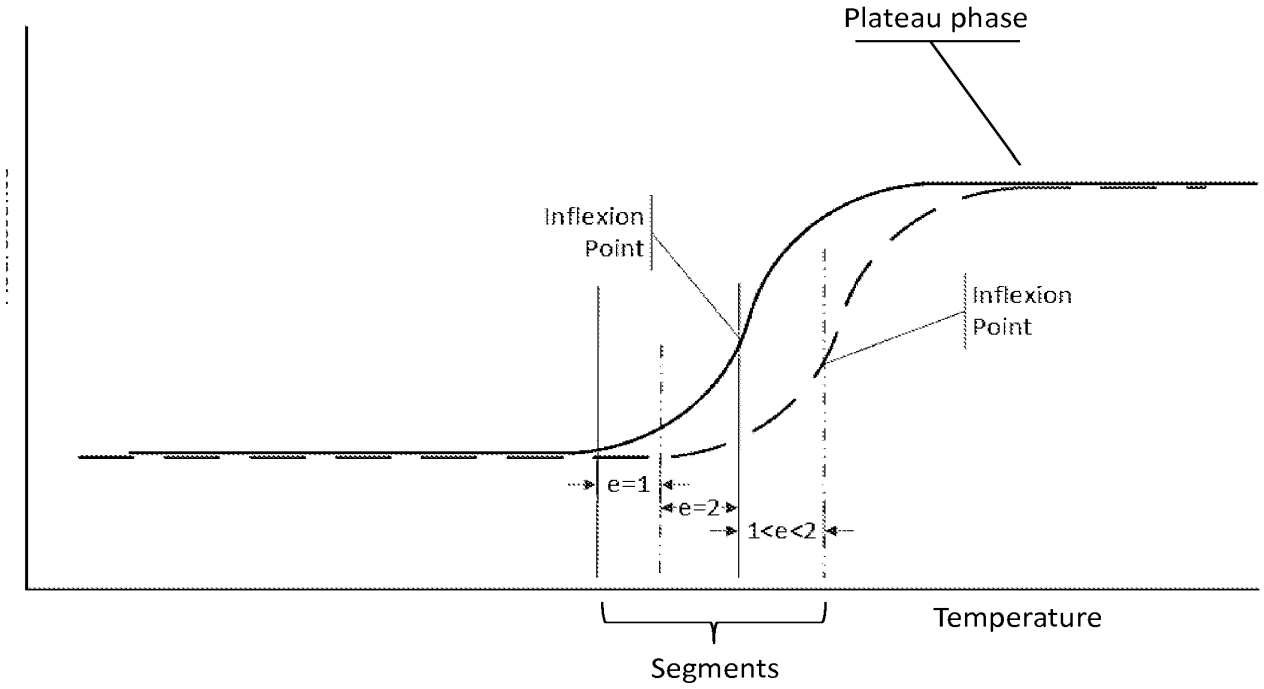

Plateau phase
Inflexion Point
Inflexion Point
e=1
e=2
1<e<2
Temperature
Segments

FIG. 4

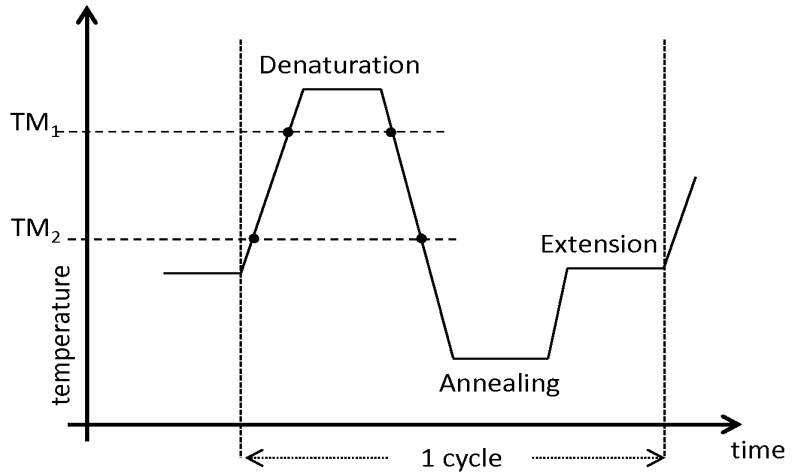

Denaturation
TM1
TM2
Extension
temperature
Annealing
1 cycle
time

FIG. 5A

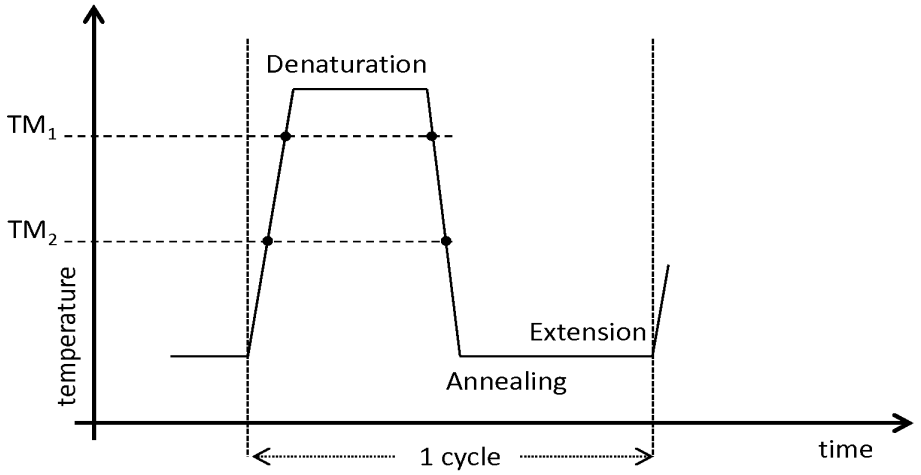

Denaturation
TM1
TM2
temperature
Extension
Annealing
1 cycle
time

FIG. 5B

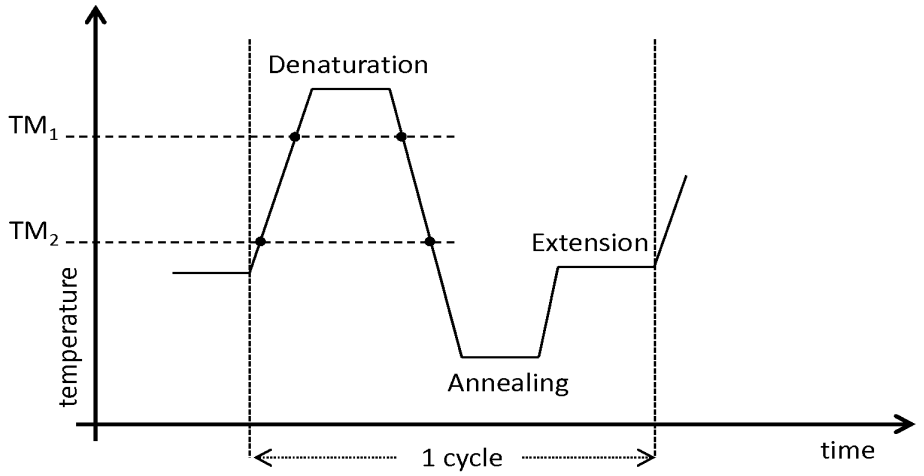

Denaturation
TM1
TM2
Extension
temperature
Annealing
1 cycle
time

FIG. 6A

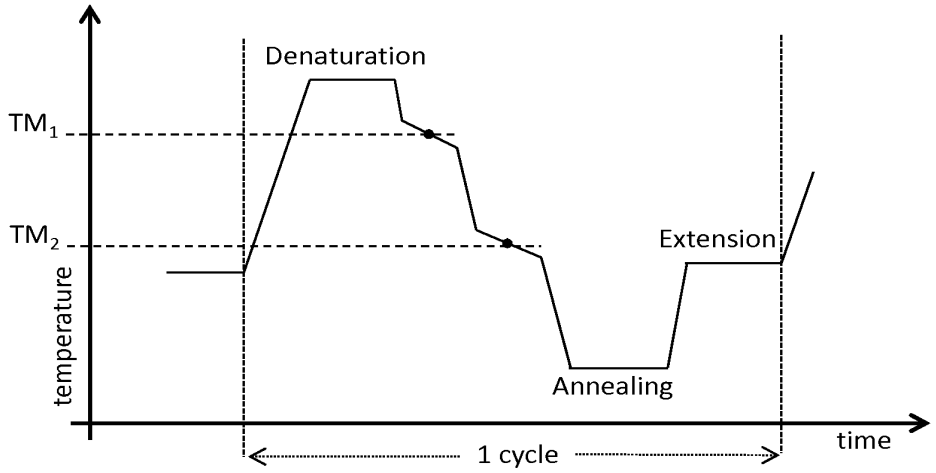

Denaturation
TM1
TM2
Extension
temperature
Annealing
1 cycle
time

FIG. 6B

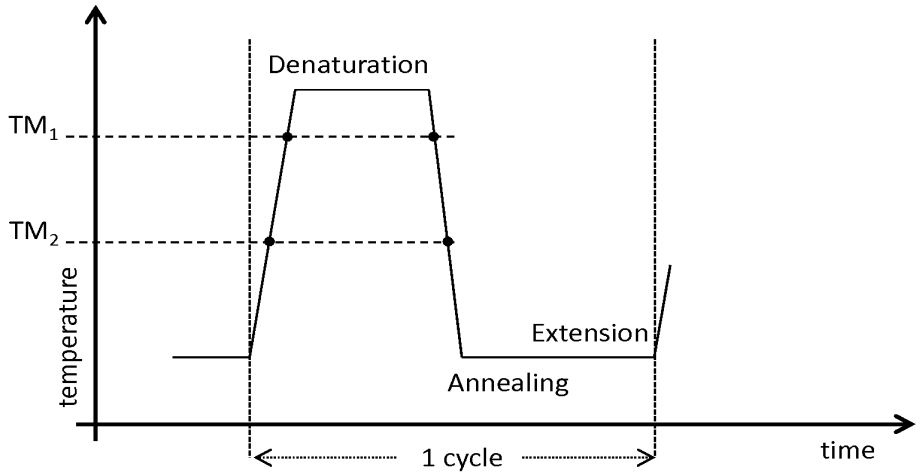

Denaturation
TM1
TM2
temperature
Extension
Annealing
1 cycle
time

FIG. 7A

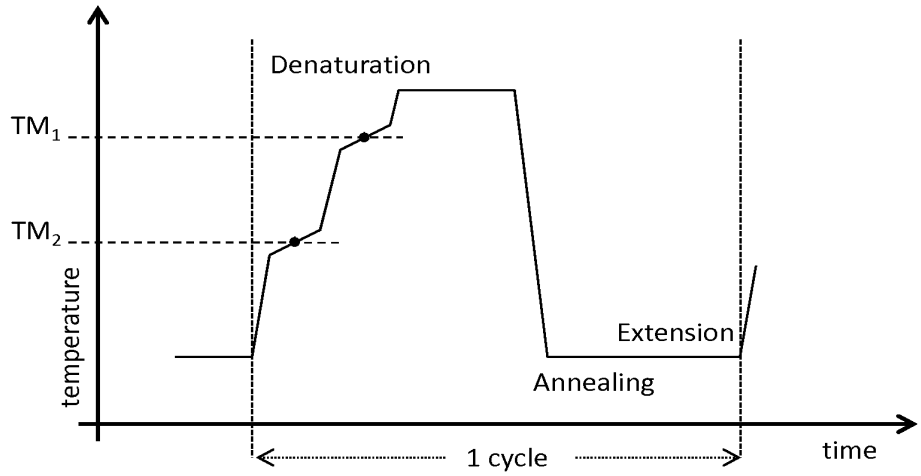

Denaturation
TM1
TM2
temperature
Extension
Annealing
1 cycle
time

FIG. 7B

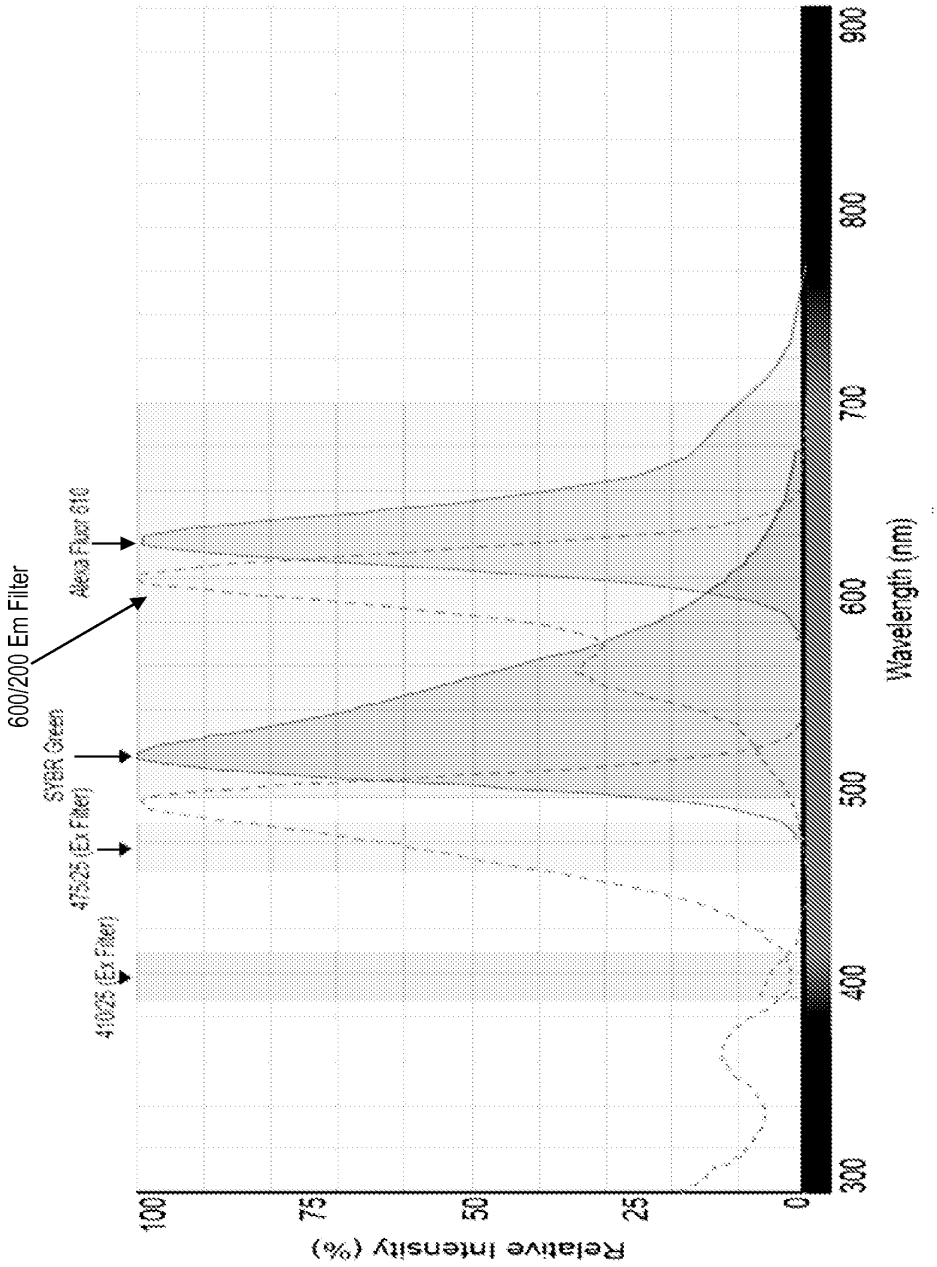

600/200 Em Filter
Alexa Fluor 616
SYBR Green
475/25 (Ex Filter)
410/25 (Ex Filter)
Relative Intensity (%)
100
75
50
25
0
300
400
500
600
700
800
900
Wavelength (nm)

FIG. 8

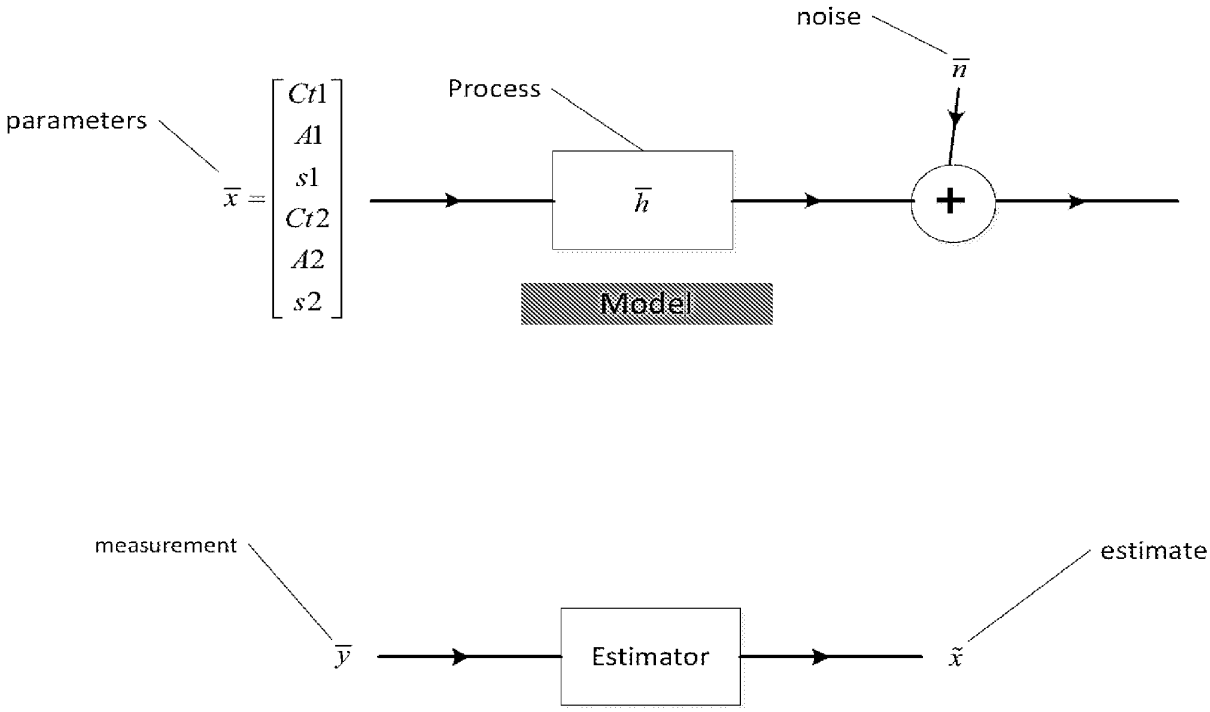
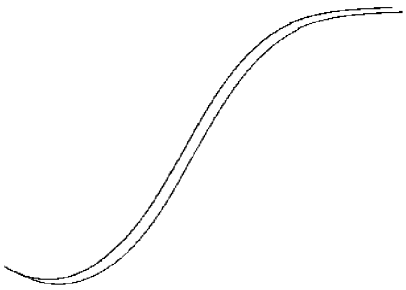
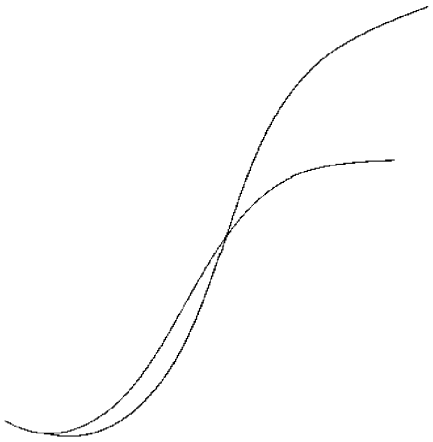

noise
$\overline{n}$
parameters
Process
$\overline{x} = \begin{bmatrix} Ct1 \\ A1 \\ s1 \\ Ct2 \\ A2 \\ s2 \end{bmatrix}$
$\overline{h}$
+
Model
measurement
estimate
$\overline{y}$
Estimator
$\tilde{x}$

FIG. 9



Amplitude normalized:
These two amplification curves with same Ct should overlap for minimum cumulative point wise distance.



Amplitude not normalized:
These two amplification curves with same Ct are not at correct location for minimum cumulative point wise distance.

FIG. 10

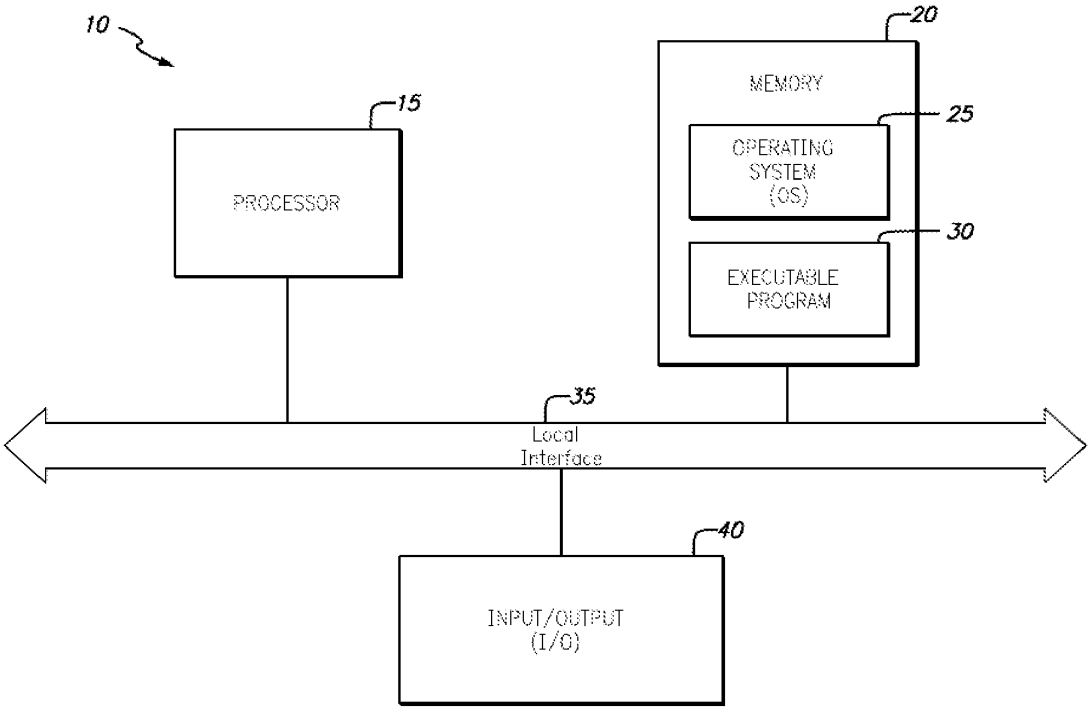

10
15
PROCESSOR
20
MEMORY
25
OPERATING SYSTEM (OS)
30
EXECUTABLE PROGRAM
35
Local Interface
40
INPUT/OUTPUT (I/O)

FIG. 11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/053512

A. CLASSIFICATION OF SUBJECT MATTER

C12Q 1/68(2006.01)i, C12M 1/38(2006.01)i, C12N 15/11(2006.01)i

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)
C12Q 1/68; C12M 1/00; G06F 19/00; G01N 21/64; C12P 19/34; G06F 19/10; C12M 1/34; C12M 1/38; C12N 15/11

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords: amplicons, multiplexed amplification, single channel detector, sum amplitude signal, processor-based hardware analyzer

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	FU et al., 'Multiplex detection and SNP genotyping in a single fluorescence channel' PLoS One, Vol.7, Issue 1, Article No.e30340, pp.1-8 (January 2012) See the whole document.	1-12,18-19,33-39
A	US 2011-0223602 A1 (WHITMAN et al.) 15 September 2011 See the whole document.	1-12,18-19,33-39
A	US 2010-0324834 A1 (TREPTOW et al.) 23 December 2010 See the whole document.	1-12,18-19,33-39
A	US 2012-0040352 A1 (WANGH et al.) 16 February 2012 See the whole document.	1-12,18-19,33-39
A	US 7507575 B2 (BEDINGHAM et al.) 24 March 2009 See the whole document.	1-12,18-19,33-39
A	US 2012-0101740 A1 (ORPANA et al.) 26 April 2012 See the whole document.	1-12,18-19,33-39

☐ 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 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
16 October 2013 (16.10.2013)	**16 October 2013 (16.10.2013)**

Name and mailing address of the ISA/KR
Korean Intellectual Property Office
189 Cheongsa-ro, Seo-gu, Daejeon Metropolitan City, 302-701, Republic of Korea
Facsimile No. +82-42-472-7140

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KIM Seung Beom
Telephone No. +82-42-481-3371

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/053512

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.: 14-17,23-26
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:
Claims 14-17 and 23-26 are unclear since they are referring to the multiple dependent claims which do not comply with PCT Rule 6.4(a).

3. ☒ Claims Nos.: 13,20-22,27-32
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 addtional 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 an additional fee, this Authority did not invite payment of any additional fee.

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

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

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
☐ No protest accompanied the payment of additional search fees.

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/US2013/053512

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/US2013/053512

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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(74) Agents: **STOPAK, Kimberly** 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, IR, IS, JP, KE, KG, 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, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, 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).

Published:

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

WO 2014/116884 A1

(54) Title: CHROMOPHORE-BASED CHARACTERIZATION AND DETECTION METHODS

(57) Abstract: This disclosure provides methods, compositions and kits for the detection of a plurality of analytes in a sample. In some examples, this disclosure provides methods, compositions, and kits for detecting analytes, genetic variations, monitoring reaction process, and monitoring analyte-analyte interactions by measuring signals. In some examples, the presence of signals or changes in signals may be used to construct signal profiles which can be used to determine the presence or absence of analytes.

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CHROMOPHORE-BASED CHARACTERIZATION AND DETECTION METHODS

CROSS-REFERENCE

[0001] This application claims the benefit of U.S. provisional application 61/756,343, filed January 24, 2013, which is incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

[0002] Nucleic acid analyte identification is a critical procedure in a variety of biomedical applications, such as in research and clinical diagnostic environments. Identification of an analyte is primarily done by sequencing or by amplification-based detection. For example, in the latter scheme, the polymerase chain reaction is often used to increase the quantity of the nucleic acid analyte present. Then, the nucleic acid analytes are discriminated using one of several additional techniques including fluorescence intensity measurement (e.g., fluorescent probes or intercalating dyes), length discrimination (e.g., using gel electrophoresis or melt curve analysis), or chromatography (e.g., haptin-based nucleic acid capture). Thus, current amplification-based detection technology indirectly detects analytes and requires a secondary technique (such as gel electrophoresis or mass spectroscopy) for analyte detection. Amplification or polymerization-based techniques that directly detect analytes would improve efficiency, time and cost.

SUMMARY OF THE INVENTION

[0003] Disclosed herein are methods, compositions, and kits for detecting analytes, particularly polynucleotides and/or polypeptides. The methods generally involve using oligonucleotides (e.g., primers, probes) attached to chromophores (e.g., fluorophores, quenchers, etc.) in amplification or polymerization reactions in order to detect a polynucleotide analyte. In some embodiments, provided herein are methods of detecting at least one polynucleotide analyte in a sample, comprising: (a) combining the sample with a first primer and a first oligonucleotide, wherein a first chromophore is attached to the first primer, a second chromophore is attached to the first oligonucleotide, the first primer and the first oligonucleotide are specific for a first polynucleotide analyte and the first chromophore is different from the second chromophore; (b) measuring a first signal generated by the first and second chromophores; (c) performing at least one polymerization reaction with the first primer using the first polynucleotide analyte as a template; and (d) measuring a second signal

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generated by the first and second chromophores; wherein the first and second signals are used to detect the first polynucleotide analyte.

[0004] In some cases, the first oligonucleotide is a second primer. In some cases, the first chromophore is attached to the 5' end of the first primer. In some cases, the first chromophore is an inorganic or organic dye, a fluorophore or a quencher. In some cases, the first chromophore is a fluorophore. In some cases, the fluorophore is 6-FAM (Fluorescein), 6-FAM (NHS Ester), Fluorescein dT, HEX, JOE (NHS Ester), MAX, TET, ROX, TAMRA, TARMA (NHS Ester), TEX 615, ATTO 488, ATTO 532, ATTO 550, ATTO 565, ATTO Rho101, ATTO 590, ATTO 633, ATTO 647N, TYE 563, TYE 665 or TYE 705. In some cases, the second chromophore is attached to the 5' end of the second primer. In some cases, the second chromophore is an inorganic or organic dye, a fluorophore or a quencher. In some cases, the second chromophore is a quencher. In some cases, the quencher is Iowa Black FG, Iowa Black RG, BHQ1, BHQ2 or BHQ3. In some cases, the first chromophore is a fluorophore and the second chromophore is a quencher.

[0005] In some cases, the methods described herein further comprise comparing the first and second signals, wherein a change in the first and second signals indicates the presence of the first polynucleotide analyte. In some cases, the change in the first and second signals is a decrease in fluorescent intensity. In some cases, the change is an increase in intensity. In some cases, the decrease in fluorescent intensity is at least about a 30% decrease in signal (or at least about 10%, 20%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, or 75%). In some cases, the second signal is measured after a second polymerization reaction. In some cases, the polymerization reaction is a polymerase chain reaction process or an isothermal process. In some cases, the polymerase chain reaction process is an end-point polymerase chain reaction process, a real-time polymerase chain reaction process, a digital polymerase chain reaction process, a droplet digital polymerase chain reaction process, or a quantitative polymerase chain reaction process. In some cases, the first primer is a forward primer and the second primer is a reverse primer. In some cases, the first primer is a reverse primer and the second primer is a forward primer. In some cases, the first and second chromophores interact through an electron-transfer process. In some cases, the first polynucleotide analyte is from about 10 to about 500 nucleotides in length. In some cases, the concentration of the first polynucleotide analyte is from about 10μM to about 10aM. In some cases, the first polynucleotide analyte is a DNA polynucleotide analyte. In some cases, the first polynucleotide analyte is an RNA polynucleotide analyte.

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[0006] In some cases, the first polynucleotide analyte comprises a genetic variation. In some cases, the genetic variation comprises a substitution, an addition, a deletion or a translocation. In some cases, the genetic variation comprises a single-nucleotide polymorphism (SNP). In some cases, the at least one polynucleotide analyte is from a source selected from a human, a non-human mammal, a plant, a bacteria, a fungus, an archaea, a parasite, or a virus. In some cases, the virus is a human immunodeficiency virus, an influenza type A virus, an influenza type B virus, a respiratory syncytial virus type A (RsvA), a respiratory syncytial virus type B virus (RsvB), a human rhinovirus (Hrv), a human metapneumovirus (Hmpv) or a human parainfluenza virus type 3 (PIV-3). In some cases, the sample is a forensic sample, a clinical sample, a food sample, an environmental sample, a pharmaceutical sample, or a sample from a consumer product. In some cases, the methods disclosed herein further comprising detecting at least one additional polynucleotide analyte within the sample with an additional fluorophore attached to a primer, wherein the first chromophore is a fluorophore with the same color as the additional fluorophore. In some cases, the first primer is not attached to two or more chromophores. In some cases, the first oligonucleotide is not attached to two or more chromophores. In some cases, step b) of the method further comprises measuring the first signal at a first denaturing step and a third signal at a first annealing step; step d) further comprises measuring the second signal at a second denaturing step and a fourth signal at a second annealing step; and the method further comprises comparing the first signal with the second signal to obtain a first ratio and comparing the third signal with the fourth signal to obtain a second ratio; wherein when the first ratio is about 1 and the second ratio is greater than or less than 1 indicate the presence of the first polynucleotide analyte.

[0007] In some cases, step a) of the method described herein further comprises combining the sample with a third primer and a second oligonucleotide, wherein a third chromophore is attached to the third primer and a fourth chromophore is attached to the second oligonucleotide , and the third primer and the second oligonucleotide are specific for a second polynucleotide analyte; step b) further comprises measuring a fifth signal generated by the third and fourth chromophores; step d) further comprises measuring a sixth signal generated by the third and fourth chromophores; wherein the fifth and sixth signals are used to detect the second polynucleotide analyte. In some cases, the methods disclosed herein further comprising comparing the fifth and sixth signals, wherein a change in the fifth and

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sixth signals indicates the presence of the second polynucleotide analyte. In some cases, the second oligonucleotide is a fourth primer.

[0008] Also disclosed herein are methods of detecting at least one genetic variation in an analyte comprising: (a) combining a first analyte with a first primer and a second primer, wherein a first chromophore is attached to the first primer, a second chromophore is attached to the second primer, at least one of the first and the second primers are specific for a first genetic variation in the first analyte and the first chromophore is different from the second chromophore; (b) measuring a first signal generated by the first and second chromophores; (c) performing at least one polymerization reaction with the first primer and the second primer using the first analyte as a template; and (d) measuring a second signal generated by the first and second chromophores; wherein the first and second signals are used to detect the first genetic variation in the first analyte. In some cases, the methods described herein further comprise comparing the first and second signals, wherein a change in the first and second signals indicates the presence of the genetic variation in the first analyte.

[0009] In some cases, the analyte is a polynucleotide analyte. In some cases, the genetic variation comprises a substitution, an addition, a deletion or a translocation. In some cases, the genetic variation comprises a single-nucleotide polymorphism (SNP). In some cases, the first primer comprises a sequence encoding the SNP or the first primer binds to a region of the analyte encoding the SNP. In some cases, the second primer comprises a sequence not encoding the SNP or the second primer comprises a sequence complementary to a region of the analyte not encoding the SNP. In some cases, the first primer encodes a region of the analyte less than 500 base pairs apart from a region of the analyte encoded by the second primer. In some cases, the change in signal is distinct for at least two of the mismatched base pairs selected from the group consisting of UU, UT, UG, UC, UA, AA, TT, GG, CC, AG, AC, TG and TC. In some cases, the change in signal from a mismatched base pair is distinct from a change in signal from a complementary base pair.

[0010] In some cases, step a) of the methods described herein further comprises combining the first analyte with a third primer and a fourth primer, wherein a third chromophore is attached to the third primer, a fourth chromophore is attached to the fourth primer, the third and the fourth primers are specific for a second genetic variation in the first analyte and the third chromophore is different from the fourth chromophore; step b) further comprises measuring a third signal; step d) further comprises measuring a fourth signal; and the method further comprises comparing the third and fourth signals; wherein a change in the

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third and fourth signals indicates the presence of the second single genetic variation in the first analyte. In some cases, step a) of the method further comprises combining a second analyte with a third primer and a fourth primer, wherein a third chromophore is attached to the third primer, a fourth chromophore is attached to the fourth primer comprises, the third and the fourth primers are specific for a second genetic variation in the second analyte and the third chromophore is different from the fourth chromophore; step b) further comprises measuring a third signal; step d) further comprises measuring a fourth signal; and the method further comprises comparing the third and fourth signals; wherein a change in the third and fourth signals indicates the presence of the second single genetic variation in the second analyte. In some cases, step a) further comprises combining a second analyte with a third primer and a fourth primer, wherein the first chromophore is attached to the third primer, the second chromophore is attached to the fourth primer, and the third and the fourth primers are specific for a second genetic variation in the second analyte. In some cases, the polymerization reaction is a PCR process or an isothermal reaction. In some cases, the PCR process is an end-point PCR process, a digital PCR process, a real-time PCR process, a droplet digital PCR process, or a quantitative PCR process. In some cases, the polymerization reaction is a quantitative PCR process. In some cases, the detecting comprises a quantitative PCR method. In some cases, the detecting comprises a quantitative PCR method and a second method. In some cases, the second method is a digital PCR process. In some cases, at least one SNP is detected in a gene. In some cases, at least one SNP is associated with a disease. In some cases, the disease is a genetic disorder, an autoimmune disease, a neurological disease, a cardiovascular disease, or a cancer.

[0011] Also disclosed herein are methods of detecting a plurality of analytes, comprising: a) combining the plurality of analytes with a plurality of primer pairs, wherein each primer pair is specific to a single analyte and each primer of the primer pair is attached to at least one chromophore; b) measuring a first set of signals generated by the chromophores; c) performing at least one polymerization reaction with the plurality of primer pairs using the plurality of analytes as templates; and d) measuring a second set of signals generated by the chromophores; wherein the first and second set of signals are used to detect each analyte of the plurality of analytes. In some cases, the method described herein further comprises: e) repeating step c and d at least once; and f) generating a set of signature profiles; wherein the presence of each analyte of the plurality of analytes is detected by comparing the set of signature profiles to a control set of signature profiles.

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[0012] In some cases, the signature profile is an end-point signature profile or a signature curve. In some cases, the plurality of analytes has different lengths. In some cases, each of the plurality of primer pairs comprises a forward primer and a reverse primer. In some cases, one, two or more chromophores are attached to the forward primer. In some cases, one chromophore is attached to the 5' end of the forward primer. In some cases, at least one chromophore is attached to the reverse primer. In some cases, one chromophore is attached to the 5' end of the reverse primer. In some cases, the chromophore is an inorganic or organic dye, a fluorophore or a quencher. In some cases, the chromophore is a fluorophore. In some cases, the chromophore is a quencher.

[0013] Also disclosed herein are methods of generating a signature curve profile for a polynucleotide analyte, comprising: (a) contacting the polynucleotide analyte with a first primer and a second primer, wherein a first chromophore is attached to the first primer and a second chromophore is attached to the second primer, the first primer and the second primer are specific for the polynucleotide analyte and the first chromophore is different from the second chromophore; (b) measuring a first signal at a first temperature; (c) performing at least one polymerization reaction with the first primer and the second primer using the polynucleotide analyte as a template; (d) measuring a second signal at the first temperature; and (e) repeating step c and d at least once; wherein the signals create the signature curve profile of the polynucleotide analyte. In some cases, the method disclosed herein further comprises: f) changing the temperature; g) measuring a third signal at a second temperature; and h) repeating steps f and g at least once; wherein the signals create the signature curve profile of the polynucleotide analyte.

[0014] In some cases, the signature curve is a length curve, a morphology curve, a melt curve, or a SNP curve. In some cases, the polymerization reaction is a polymerase chain reaction process. In some cases, the polymerase chain reaction process is an end-point polymerase chain reaction process, a real-time polymerase chain reaction process, a digital polymerase chain reaction process or a quantitative polymerase chain reaction process. In some cases, the first chromophore is an inorganic or organic dye, a fluorophore or a quencher. In some cases, the first chromophore is a fluorophore. In some cases, the fluorophore is 6-FAM (Fluorescein), 6-FAM (NHS Ester), Fluorescein dT, HEX, JOE (NHS Ester), MAX, TET, ROX, TAMRA, TARMA (NHS Ester), TEX 615, ATTO 488, ATTO 532, ATTO 550, ATTO 565, ATTO Rho101, ATTO 590, ATTO 633, ATTO 647N, TYE 563, TYE 665 or TYE 705. In some cases, the second chromophore is an inorganic or organic

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dye, a fluorophore or a quencher. In some cases, the second chromophore is a quencher. In some cases, the quencher is Iowa Black FG, Iowa Black RG, BHQ1, BHQ2 or BHQ3. In some cases, the polynucleotide analyte is a DNA polynucleotide analyte. In some cases, the polynucleotide analyte is an RNA polynucleotide analyte. In some cases, the polynucleotide analyte is from a source selected from the group consisting of a human, a non-human mammal, a plant, a bacteria, an archaea, a fungus, a parasite, and a virus. In some cases, the virus is a human immunodeficiency virus, an influenza type A virus, an influenza type B virus, a respiratory syncytial virus type A (RsvA), a respiratory syncytial virus type B virus (RsvB), a human rhinovirus (Hrv), a human metapneumovirus (Hmpv) or a human parainfluenza virus type 3 (PIV-3).

[0015] Also disclosed herein is a method of monitoring an amplification reaction comprising: (a) contacting a first polynucleotide analyte with: i) a first primer and a second primer, wherein the first primer and the second primer are specific for the first polynucleotide analyte; and ii) at least two chromophores capable of specific incorporation into a product amplified from the first polynucleotide analyte; (b) subjecting the combination in step (a) to a temperature capable of denaturing double-stranded DNA; (c) measuring a first signal in step (b) generated by the at least two chromophores; (d) subjecting the combination in step b) to a temperature capable of annealing polynucleotides; (e) measuring a second signal in step (d) generated by the at least two chromophores; and (f) repeating steps b) – e) to obtain a third signal and a fourth signal; and (g) comparing the first and the third signals to obtain a first ratio and the second and the fourth signals to obtain a second ratio; wherein an amplification reaction occurs when the first ratio is about 1 and the second ratio is greater than or less than 1. In some cases, at least one chromophore is attached to the first primer, for example exactly one chromophore may be attached to the first primer. In some cases, one chromophore (or exactly one chromophore) is attached to the 5' end of the first primer. In some cases, one, two or more chromophores are attached to the second primer. In some cases, one chromophore is attached to the 5' end of the second primer. In some cases, the chromophore is an inorganic or organic dye, a fluorophore or a quencher.

[0016] Also disclosed herein are methods of detecting a morphology of an analyte, comprising: (a) providing a sample comprising the analyte, wherein a first chromophore, a second chromophore and a third chromophore are attached to the analyte, wherein the first, second and third chromophores are different; (b) measuring a signal from the first chromophore, second chromophore and the third chromophore at a first and second

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temperature, wherein the first and second temperatures are different; and (c) using the measured signals to detect the morphology of the analyte. In some cases, the analyte is a protein, a polypeptide, a lipid or a polynucleotide. In some cases, the analyte is a polynucleotide. In some cases, the polynucleotide is a DNA polynucleotide. In some cases, the polynucleotide is an RNA polynucleotide. In some cases, the first chromophore is an inorganic or organic dye, a fluorophore or a quencher. In some cases, the first chromophore is a first fluorophore. In some cases, the first fluorophore is 6-FAM (Fluorescein), 6-FAM (NHS Ester), Fluorescein dT, HEX, JOE (NHS Ester), MAX, TET, ROX, TAMRA, TARMA (NHS Ester), TEX 615, ATTO 488, ATTO 532, ATTO 550, ATTO 565, ATTO Rho101, ATTO 590, ATTO 633, ATTO 647N, TYE 563, TYE 665 or TYE 705. In some cases, the second chromophore is an inorganic or organic dye, a fluorophore or a quencher. In some cases, the second chromophore is a second fluorophore. In some cases, the second fluorophore is 6-FAM (Fluorescein), 6-FAM (NHS Ester), Fluorescein dT, HEX, JOE (NHS Ester), MAX, TET, ROX, TAMRA, TARMA (NHS Ester), TEX 615, ATTO 488, ATTO 532, ATTO 550, ATTO 565, ATTO Rho101, ATTO 590, ATTO 633, ATTO 647N, TYE 563, TYE 665 or TYE 705. In some cases, the third chromophore is an inorganic or organic dye, a fluorophore or a quencher. In some cases, the third chromophore is a quencher. In some cases, the quencher is Iowa Black FG, Iowa Black RG, BHQ1, BHQ2 or BHQ3. In some cases, the analyte comprises more than one fluorophore. In some cases, the analyte comprises more than one quencher. In some cases, the polynucleotide comprises a quencher at its 3' end.

[0017] Also disclosed herein are methods of detecting a polynucleotide analyte comprising: (a) combining the polynucleotide analyte with at least two chromophores, wherein the at least two chromophores are each attached to a separate polynucleotide that is complementary to a region within the polynucleotide analyte; (b) performing at least one polymerization reaction to incorporate the at least two chromophores into products of the polymerization reaction; and (c) detecting a fluorescent intensity from the at least two chromophores at a first timepoint and a second timepoint, wherein the second timepoint is later than the first timepoint and wherein a change (particularly a decrease) in fluorescent intensity at the second timepoint relative to the first timepoint is indicative of the presence of the polynucleotide analyte. In some cases, the polymerization reaction is a polymerase chain reaction. In some cases, the first timepoint is after step a) and the second timepoint is after step b). In some cases, the products of the at least one polymerization reaction each comprise

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a first polynucleotide strand and a second polynucleotide strand, wherein the first polynucleotide strand and the second polynucleotide strand are complementary. In some cases, the at least two chromophores are different. In some cases, the at least two chromophores comprise a fluorophore. In some cases, the at least two chromophores comprise a fluorophore and a quencher. In some cases, the fluorophore is incorporated into the first polynucleotide strand and the quencher is incorporated into the second polynucleotide strand. In some cases, the fluorophore is incorporated at the 5' end of first polynucleotide strand and the quencher is incorporated at the 5' end of the second polynucleotide strand.

[0018] Also disclosed herein are compositions and kits, particularly for detecting polynucleotide or polypeptide analytes. In some cases, the kits comprise oligonucleotides (e.g., primers, probes, etc.) attached to a chromophore (e.g., fluorophore, quencher). In some cases, the kit comprises: (a) a first primer or probe attached to a fluorophore; and (b) a second primer or probe attached to a quencher. In some cases, the kit comprises: (a) a first primer or probe attached to exactly one first chromophore, wherein the first chromophore is a fluorophore; and (b) a second primer or probe attached to exactly one second chromophore, wherein the first chromophore is different from the second chromophore. In some cases, the second chromophore is a quencher. In some cases, the second chromophore is a fluorophore. In some cases, the first primer or probe is a primer. In some cases, the second primer or probe is a primer. In some cases, the first primer or probe comprises an oligonucleotide sequence that is complementary to the sequence of a target analyte. In some cases, the second primer or probe comprises an oligonucleotide sequence complementary to the sequence of a target analyte. In some cases, the fluorophore is 6-FAM (Fluorescein), 6-FAM (NHS Ester), Fluorescein dT, HEX, JOE (NHS Ester), MAX, TET, ROX, TAMRA, TARMA (NHS Ester), TEX 615, ATTO 488, ATTO 532, ATTO 550, ATTO 565, ATTO Rho101, ATTO 590, ATTO 633, ATTO 647N, TYE 563, TYE 665 or TYE 705. In some cases, the quencher is Iowa Black FG, Iowa Black RG, BHQ1, BHQ2 or BHQ3. In some cases, the kit comprises at least three primers, wherein each primer is attached to a different chromophore.

[0019] In some cases, the kit comprises: (a) a first primer or probe attached to a first chromophore, wherein the first chromophore is a fluorophore; and (b) a second primer or probe attached to a second chromophore, wherein the first chromophore is different from the second chromophore. In some cases, the kit comprises: (a) a first primer or probe attached to exactly one first chromophore; and (b) a second primer or probe attached to exactly one

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second chromophore, wherein the first chromophore is different from the second chromophore. In some cases, the kit comprises: (a) a first primer or probe attached to exactly one first chromophore, wherein the first chromophore is a fluorophore; and (b) a second primer or probe attached to exactly one second chromophore, wherein the first chromophore is different from the second chromophore. In some cases, the second chromophore is a quencher. In some cases, the second chromophore is a fluorophore. In some cases, the first primer or probe is a primer. In some cases, the second primer or probe is a primer. In some cases, the first primer or probe comprises an oligonucleotide sequence that is complementary to the sequence of a target analyte. In some cases, the second primer or probe comprises an oligonucleotide sequence complementary to the sequence of a target analyte. In some cases, the fluorophore is 6-FAM (Fluorescein), 6-FAM (NHS Ester), Fluorescein dT, HEX, JOE (NHS Ester), MAX, TET, ROX, TAMRA, TARMA (NHS Ester), TEX 615, ATTO 488, ATTO 532, ATTO 550, ATTO 565, ATTO Rho101, ATTO 590, ATTO 633, ATTO 647N, TYE 563, TYE 665 or TYE 705. In some cases, the quencher is Iowa Black FG, Iowa Black RG, BHQ1, BHQ2 or BHQ3. In some cases, the kit comprises at least three primers, wherein each primer is attached to a different chromophore.

INCORPORATION BY REFERENCE

[0020] All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

[0021] Various aspects 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:

[0022] **Fig. 1** exemplifies a mechanism for detection of a nucleic acid analyte using chromophore-attached primers (a fluorophore-attached forward (FWD) primer and a quencher-attached reverse (or rewind (RWD)) primer) during a polymerase chain reaction (PCR). Figs. 1A-1B illustrate the denaturation of a double-stranded polynucleotide analyte. Figs. 1C-1D illustrate the annealing of the chromophore-attached primers to opposite strands

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of the analyte and for a polymerase to extend the primers during a PCR reaction. Figs. 1E-1F illustrate the formation of a double-stranded PCR products containing both chromophores (fluorophore and a quencher) which leads to the generation of a signal (e.g., quenching of fluorescence).

[0023] **Fig. 2** exemplifies an electron transfer state. Fig. 2A illustrates an energy transfer formula. Fig. 2B illustrates a Jablonski diagram showing Förster resonance energy transfer with typical timescales indicated.

[0024] **Fig. 3** illustrates a diagnostic protocol and treatment method for use with a detection method described herein.

[0025] **Fig. 4** illustrates a conceptual schematic of an exemplary computer server to be used for processing a method described herein.

[0026] **Fig. 5** exemplifies the detection of HIV TPP analytes at varying concentrations using quantitative PCR (qPCR). Fig. 5A illustrates the qPCR detection of 80bp HIV TPP analytes at 10nM concentration. Fig. 5B illustrates the qPCR detection of 80bp HIV TPP analytes at 1nM concentration. Fig. 5C illustrates the qPCR detection of 80bp HIV TPP analytes at 100pM concentration. Fig. 5D illustrates the qPCR detection of 80bp HIV TPP analytes at 10pM concentration. All successful detection curves exhibit the inverse-sigmoidal characteristic.

[0027] **Fig. 6** illustrates the sensitivity of detection of HIV TPP using an exemplary detection method. Fig. 6A illustrates the qPCR detection of 80bp HIV TPP analytes at 1pM concentration. Fig. 6B illustrates the qPCR detection of 80bp HIV TPP analytes at 100fM concentration. Fig. 6C illustrates the qPCR detection of 80bp HIV TPP analytes at 10fM concentration. Fig. 6D illustrates the qPCR detection of 80bp HIV TPP analytes at 1fM concentration.

[0028] **Fig. 7** shows that the extent of signal generation (quenching) is strongly correlated with the distance between a quencher and a fluorophore. Analytes were varied in length from 40bp to 120bp for a single set of primers. Fig. 7A illustrates the extent of quenching using a 40mer analyte. Fig. 7B illustrates the extent of quenching using a 60mer analyte. Fig. 7C illustrates the extent of quenching using a 80mer analyte.

[0029] **Fig. 8** illustrates the effect of a single nucleotide polymorphism on signal generation. Signal level changes significantly when a mis-priming event occurs (Fig. 8A and Fig. 8B).

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

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

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

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

[0034] **Fig. 13** exemplifies signal generation as a function of temperature for an analyte containing multiple chromophores. Fig. 13A illustrates the morphology of the oligonucleotide at a first temperature (T_1). Three fluorophores (F_1, F_2 and F_3) are attached to the oligonucleotide and one quencher (Q_1) is attached at the 3' end of the oligonucleotide. Fig. 13B illustrates the fluorescence signature profile of the oligonucleotide at T_1. At T_1, Q1 is closest to F_3, and a quenching effect is observed (Fig. 13B). Fig. 13C illustrates the morphology of the oligonucleotide at a second temperature (T_2). Fig. 13D illustrates the fluorescence signature profile of the oligonucleotide at T_2. At T_2, the oligonucleotide is fully denatured and Q_1 is further away from F_3. As a result, the fluorescence signal of F_3 increased (Fig. 13D).

DETAILED DESCRIPTION OF THE INVENTION

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

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

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

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

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

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

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

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

I. Certain Terminology

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

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

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

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

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

II. Methods of Detection

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

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

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

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

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

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

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

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

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

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

A. Detection of Analytes from Intensity-Length Relationship

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

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

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

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

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

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

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

B. Detection of Genetic Variations

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

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

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

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

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

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

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

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

C. Monitoring an Amplification or Polymerization Reaction

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

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

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

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

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

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

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

D. Detection of Morphological Change

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

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

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

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

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

III. Multiplex Detection

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

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

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

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

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

A. Multiplex Detection for Genetic Variation

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

B. Multiplex Detection for SNP

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

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

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

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

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

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

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

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

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

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

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

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

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

IV. Analytes

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

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

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

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

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

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

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

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

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[0088] Exemplary 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.*

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

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

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

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

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

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

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

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

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

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

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

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

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

A. Distance

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

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

B. Sensitivity

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

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

C. Specificity

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

V. Probes and Primers

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

VI. Chromophores

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

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

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

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

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

A. Chromophore Combinations

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

B. Signals

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

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

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

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

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

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

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

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

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

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

C. Signature Profiles

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

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

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

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

VII. Analytical Techniques and Instrumentation

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

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

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

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

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

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

VIII. Diseases

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

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

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

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

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

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

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

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

IX. Compositions and Kits

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

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

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

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X. Services

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

A. Server

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

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

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

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

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

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

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

EXAMPLES

Example 1: DNA Sequences, Primers and Probes

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

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

[00165] All oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, Iowa). Diagnostic sequences were input into IDT's OligoAnalyzer 3.1 tool. Probes and primer pairs for each analyte were chosen, from the OligoAnalyzer's set of generated sequences, to minimize homology with un-intended analytes, probes and primers. Forward primers for all analytes were synthesized with a fluorophore at the 5' end, while the reverse primes for all analytes were synthesized with a quencher at the 5' end. Sequence information is tabulated in Tables 1 to 8. Nucleic acid products were synthesized and lyophilized by IDT. All nucleic acid analytes were reconstituted in TE buffer, ph7 (Life Technologies, Carlsbad, CA). Dilutions were done using UltraPure RNAse-free Water (Life Technologies, Carlsbad, CA).

Example 2: Primer Design

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

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

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

Table 1. HIV-1 Poly Protease Sequence Information

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

Table 2. Influenza type A sequence information

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

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

Table 3. Influenza type B sequence information

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

Table 4. Respiratory syncytial virus type A sequence information

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

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

Table 5. Respiratory syncytial virus type B sequence information

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

Table 6. Human rhinovirus sequence information

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

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

Table 7. Human metapneumovirus sequence information

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

Table 8. Parainfluenza virus type 3 sequence information

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

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

Example 3: Polymerase Chain Reactions

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

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

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

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Example 4: Detection of HIV TPP analytes

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

Example 5: Detection of single nucleotide polymorphisms

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

Example 6: Multiplex detection using binary coding of analytes

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

Example 7: Tagged Primer PCR

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

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

Example 8: Multiplex detection using various chromophore combinations

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

Example 9: Multiplex detection using a single chromophore combination

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

Example10: Detection of morphology

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

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

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

Table 9. Experiment 01 Cocktail

Reagents	Concentration	Volume Added
UltraPure H_2O	-	70µL
Taq 5x Master Mix	-	25µL
Templates	**Concentration**	**Volume Added**
Poly Protease 40mer	12nM	5µL
Primers	**Concentration**	**Volume Added**
Poly Protease FWD qPrimer	1µM	5µL
Poly Protease RWD qPrimer	1µM	5µL

Table 10. Experiment 02 Cocktail

Reagents	Concentration	Volume Added
UltraPure H_2O	-	70µL
Taq 5x Master Mix	-	25µL
Templates	**Concentration**	**Volume Added**
Poly Protease 60mer	10nM	5µL
Primers	**Concentration**	**Volume Added**

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Poly Protease FWD qPrimer	1μM	5μL
Poly Protease RWD qPrimer	1μM	5μL

Table 11. Experiment 03 Cocktail

Reagents	Concentration	Volume Added
UltraPure H_2O	-	70μL
Taq 5x Master Mix	-	25μL
Templates	**Concentration**	**Volume Added**
Poly Protease 80mer	10nM	5μL
Primers	**Concentration**	**Volume Added**
Poly Protease FWD qPrimer	1μM	5μL
Poly Protease RWD qPrimer	1μM	5μL

Table 12. Experiment 04 Cocktail

Reagents	Concentration	Volume Added
UltraPure H_2O	-	70μL
Taq 5x Master Mix	-	25μL
Templates	**Concentration**	**Volume Added**
Poly Protease 45mer	10nM	5μL
Primers	**Concentration**	**Volume Added**
Poly Protease FWD qPrimer	1μM	5μL
Poly Protease RWD qPrimer	1μM	5μL

Table 13. Experiment 05 Cocktail

Reagents	Concentration	Volume Added
UltraPure H_2O	-	70μL
Taq 5x Master Mix	-	25μL
Templates	**Concentration**	**Volume Added**
Poly Protease 50mer	10nM	5μL

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Primers	Concentration	Volume Added
Poly Protease FWD qPrimer	1μM	5μL
Poly Protease RWD qPrimer	1μM	5μL

Table 14. Experiment 06 Cocktail

Reagents	Concentration	Volume Added
UltraPure H_2O	-	70μL
Taq 5x Master Mix	-	25μL
Templates	**Concentration**	**Volume Added**
Poly Protease 55mer	10nM	5μL
Primers	**Concentration**	**Volume Added**
Poly Protease FWD qPrimer	1μM	5μL
Poly Protease RWD qPrimer	1μM	5μL

Table 15. Experiment 07 Cocktail

Reagents	Concentration	Volume Added
UltraPure H_2O	-	70μL
Taq 5x Master Mix	-	25μL
Templates	**Concentration**	**Volume Added**
Poly Protease 100mer	10nM	5μL
Primers	**Concentration**	**Volume Added**
Poly Protease FWD qPrimer	1μM	5μL
Poly Protease RWD qPrimer	1μM	5μL

Table 16. Experiment 08 Cocktail

Reagents	Concentration	Volume Added
UltraPure H_2O	-	70μL
Taq 5x Master Mix	-	25μL
Templates	**Concentration**	**Volume Added**

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

Table 17. Experiment 09 Cocktail

Reagents	Concentration	Volume Added
UltraPure H_2O	-	105µL
Taq 5x Master Mix	-	75µL
Templates	**Concentration**	**Volume Added**
Influenza A 85mer	10nM	5µL
Primers	**Concentration**	**Volume Added**
Influenza A FWD qPrimer	300nM	5µL
Influenza A RWD qPrimer	300nM	5µL

Table 18. Experiment 10 Cocktail

Reagents	Concentration	Volume Added
UltraPure H_2O	-	105µL
Taq 5x Master Mix	-	75µL
Templates	**Concentration**	**Volume Added**
Influenza B 81mer	10nM	5µL
Primers	**Concentration**	**Volume Added**
Influenza B FWD qPrimer	300nM	5µL
Influenza B RWD qPrimer	300nM	5µL

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CLAIMS

WHAT IS CLAIMED IS:

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

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

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

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fourth chromophore is attached to the second oligonucleotide , and the third primer and the second oligonucleotide are specific for a second polynucleotide analyte;

step b) further comprises measuring a fifth signal generated by the third and fourth chromophores;

step d) further comprises measuring a sixth signal generated by the third and fourth chromophores;

wherein the fifth and sixth signals are used to detect the second polynucleotide analyte.

36. The method of claim 35, further comprising comparing the fifth and sixth signals, wherein a change in the fifth and sixth signals indicates the presence of the second polynucleotide analyte.

37. The method of claim 35 or 36, wherein the second oligonucleotide is a fourth primer.

38. A method of detecting at least one genetic variation in an analyte comprising:

a) combining a first analyte with a first primer and a second primer, wherein a first chromophore is attached to the first primer, a second chromophore is attached to the second primer, at least one of the first and the second primers are specific for a first genetic variation in the first analyte and the first chromophore is different from the second chromophore;

b) measuring a first signal generated by the first and second chromophores;

c) performing at least one polymerization reaction with the first primer and the second primer using the first analyte as a template; and

d) measuring a second signal generated by the first and second chromophores;

wherein the first and second signals are used to detect the first genetic variation in the first analyte.

39. The method of claim 38, further comprising comparing the first and second signals, wherein a change in the first and second signals indicates the presence of the genetic variation in the first analyte.

40. The method of claim 38 or 39, wherein the analyte is a polynucleotide analyte.

41. The method of any of claims 38-40, wherein the genetic variation comprises a substitution, an addition, a deletion or a translocation.

42. The method of any of claims 38-40, wherein the genetic variation comprises a single-nucleotide polymorphism (SNP).

43. The method of claim 42, wherein the first primer comprises a sequence encoding the SNP or the first primer binds to a region of the analyte encoding the SNP.

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44. The method of claim 42 or 43, wherein the second primer comprises a sequence not encoding the SNP or the second primer comprises a sequence complementary to a region of the analyte not encoding the SNP.

45. The method of any of claims 38-44, wherein the first primer encodes a region of the analyte less than 500 base pairs apart from a region of the analyte encoded by the second primer.

46. The method of any of claims 38-45, wherein the change in signal is distinct for at least two of the mismatched base pairs selected from the group consisting of UU, UT, UG, UC, UA, AA, TT, GG, CC, AG, AC, TG and TC.

47. The method of any of claims 38-46, wherein the change in signal from a mismatched base pair is distinct from a change in signal from a complementary base pair.

48. The method of any of claims 38-47, wherein:
step a) further comprises combining the first analyte with a third primer and a fourth primer, wherein a third chromophore is attached to the third primer, a fourth chromophore is attached to the fourth primer, the third and the fourth primers are specific for a second genetic variation in the first analyte and the third chromophore is different from the fourth chromophore;
step b) further comprises measuring a third signal;
step d) further comprises measuring a fourth signal; and
further comprising comparing the third and fourth signals;
wherein a change in the third and fourth signals indicates the presence of the second single genetic variation in the first analyte.

49. The method of any of claims 38-48, wherein:
step a) further comprises combining a second analyte with a third primer and a fourth primer, wherein a third chromophore is attached to the third primer, a fourth chromophore is attached to the fourth primer comprises, the third and the fourth primers are specific for a second genetic variation in the second analyte and the third chromophore is different from the fourth chromophore;
step b) further comprises measuring a third signal;
step d) further comprises measuring a fourth signal; and
further comprising comparing the third and fourth signals;
wherein a change in the third and fourth signals indicates the presence of the second single genetic variation in the second analyte.

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

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61. The method of claim 60, further comprising:
e) repeating step c and d at least once; and
f) generating a set of signature profiles;
wherein the presence of each analyte of the plurality of analytes is detected by comparing the set of signature profiles to a control set of signature profiles.

62. The method of claim 61, wherein the signature profile is an end-point signature profile or a signature curve.

63. The method of any of claims 60-62, wherein the plurality of analytes has different lengths.

64. The method of any of claims 60-63, wherein each of the plurality of primer pairs comprises a forward primer and a reverse primer.

65. The method of claim 64 wherein one, two or more chromophores are attached to the forward primer.

66. The method of claim 65, wherein one chromophore is attached to the 5' end of the forward primer.

67. The method of claim 64, wherein at least one chromophore is attached to the reverse primer.

68. The method of claim 67, wherein one chromophore is attached to the 5' end of the reverse primer.

69. The method of any of claims 60-68, wherein the chromophore is an inorganic or organic dye, a fluorophore or a quencher.

70. The method of claim 69, wherein the chromophore is a fluorophore.

71. The method of claim 69, wherein the chromophore is a quencher.

72. A method of generating a signature curve profile for a polynucleotide analyte, comprising:
a) contacting the polynucleotide analyte with a first primer and a second primer, wherein a first chromophore is attached to the first primer and a second chromophore is attached to the second primer, the first primer and the second primer are specific for the polynucleotide analyte and the first chromophore is different from the second chromophore;
b) measuring a first signal at a first temperature;
c) performing at least one polymerization reaction with the first primer and the second primer using the polynucleotide analyte as a template;

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d) measuring a second signal at the first temperature; and

e) repeating step c and d at least once;

wherein the signals create the signature curve profile of the polynucleotide analyte.

73. The method of claim 72, further comprising: f) changing the temperature; g) measuring a third signal at a second temperature; and h) repeating steps f and g at least once; wherein the signals create the signature curve profile of the polynucleotide analyte.

74. The method of claim 72 or 73, wherein the signature curve is a length curve, a morphology curve, a melt curve, or a SNP curve.

75. The method of any of claims 72-74, wherein the polymerization reaction is a polymerase chain reaction process.

76. The method of claim 75, wherein the polymerase chain reaction process is an end-point polymerase chain reaction process, a real-time polymerase chain reaction process, a digital polymerase chain reaction process or a quantitative polymerase chain reaction process.

77. The method of any of claims 72-76, wherein the first chromophore is an inorganic or organic dye, a fluorophore or a quencher.

78. The method of claim 77, wherein the first chromophore is a fluorophore.

79. The method of claim 78, wherein the fluorophore is 6-FAM (Fluorescein), 6-FAM (NHS Ester), Fluorescein dT, HEX, JOE (NHS Ester), MAX, TET, ROX, TAMRA, TARMA (NHS Ester), TEX 615, ATTO 488, ATTO 532, ATTO 550, ATTO 565, ATTO Rho101, ATTO 590, ATTO 633, ATTO 647N, TYE 563, TYE 665 or TYE 705.

80. The method of any of claims 72-79, wherein the second chromophore is an inorganic or organic dye, a fluorophore or a quencher.

81. The method of claim 80, wherein the second chromophore is a quencher.

82. The method of claim 81, wherein the quencher is Iowa Black FG, Iowa Black RG, BHQ1, BHQ2 or BHQ3.

83. The method of any of claims 72-82, wherein the polynucleotide analyte is a DNA polynucleotide analyte.

84. The method of any of claims 72-82, wherein the polynucleotide analyte is an RNA polynucleotide analyte.

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85. The method of claim 72, wherein the polynucleotide analyte is from a source selected from the group consisting of a human, a non-human mammal, a plant, a bacteria, an archaea, a fungus, a parasite, and a virus.

86. The method of claim 85, wherein the virus is a human immunodeficiency virus, an influenza type A virus, an influenza type B virus, a respiratory syncytial virus type A (RsvA), a respiratory syncytial virus type B virus (RsvB), a human rhinovirus (Hrv), a human metapneumovirus (Hmpv) or a human parainfluenza virus type 3 (PIV-3).

87. A method of monitoring an amplification reaction comprising:

a) contacting a first polynucleotide analyte with: i) a first primer and a second primer, wherein the first primer and the second primer are specific for the first polynucleotide analyte; and ii) at least two chromophores capable of specific incorporation into a product amplified from the first polynucleotide analyte;

b) subjecting the combination in step (a) to a temperature capable of denaturing double-stranded DNA;

c) measuring a first signal in step (b) generated by the at least two chromophores;

d) subjecting the combination in step b) to a temperature capable of annealing polynucleotides;

e) measuring a second signal in step (d) generated by the at least two chromophores; and

f) repeating steps b) – e) to obtain a third signal and a fourth signal; and

g) comparing the first and the third signals to obtain a first ratio and the second and the fourth signals to obtain a second ratio;

wherein an amplification reaction occurs when the first ratio is about 1 and the second ratio is greater than or less than 1.

88. The method of claim 87, wherein one, two or more chromophores are attached to the first primer.

89. The method of claim 88, wherein one chromophore is attached to the 5' end of the first primer.

90. The method of claim 87, wherein one, two or more chromophores are attached to the second primer.

91. The method of claim 90, wherein one chromophore is attached to the 5' end of the second primer.

92. The method of any of claims 87-91, wherein the chromophore is an inorganic or organic dye, a fluorophore or a quencher.

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

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106. The method of claim 105, wherein the quencher is Iowa Black FG, Iowa Black RG, BHQ1, BHQ2 or BHQ3.

107. The method of any of claims 93-106, wherein the analyte comprises more than one fluorophore.

108. The method of any of claims 93-106, wherein the analyte comprises more than one quencher.

109. The method of any of claims 93-108, wherein the polynucleotide comprises a quencher at its 3' end.

110. A method of detecting a polynucleotide analyte comprising:

a. combining the polynucleotide analyte with at least two chromophores, wherein the at least two chromophores are each attached to a separate polynucleotide that is complementary to a region within the polynucleotide analyte;

b. performing at least one polymerization reaction to incorporate the at least two chromophores into products of the polymerization reaction; and

c. detecting a fluorescent intensity from the at least two chromophores at a first timepoint and a second timepoint, wherein the second timepoint is later than the first timepoint and wherein a decrease in fluorescent intensity at the second timepoint relative to the first timepoint is indicative of the presence of the polynucleotide analyte.

111. The method of claim 110, wherein the polymerization reaction is a polymerase chain reaction.

112. The method of claim 110, wherein the first timepoint is after step a) and the second timepoint is after step b).

113. The method of claim 110, wherein the products of the at least one polymerization reaction each comprise a first polynucleotide strand and a second polynucleotide strand, wherein the first polynucleotide strand and the second polynucleotide strand are complementary.

114. The method of claim 110, wherein the at least two chromophores are different.

115. The method of claim 110, wherein the at least two chromophores comprise a fluorophore.

116. The method of claim 110, wherein the at least two chromophores comprise a fluorophore and a quencher.

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117. The method of claim 116, wherein the fluorophore is incorporated into the first polynucleotide strand and the quencher is incorporated into the second polynucleotide strand.

118. The method of claim 116, wherein the fluorophore is incorporated at the 5' end of first polynucleotide strand and the quencher is incorporated at the 5' end of the second polynucleotide strand.

119. A kit comprising: (a) a first primer attached to a first chromophore; and (b) a second primer attached to a second chromophore, wherein the first and second chromophores are different chromophores.

120. The kit of claim 119, wherein the first chromophore is a first fluorophore.

121. The kit of claim 119 or 120, wherein the second chromophore is a second fluorophore.

122. The kit of claim 119 or 120, wherein the second chromophore is a quencher.

123. The kit of claim 120 or 121, wherein the first or second fluorophore is 6-FAM (Fluorescein), 6-FAM (NHS Ester), Fluorescein dT, HEX, JOE (NHS Ester), MAX, TET, ROX, TAMRA, TARMA (NHS Ester), TEX 615, ATTO 488, ATTO 532, ATTO 550, ATTO 565, ATTO Rho101, ATTO 590, ATTO 633, ATTO 647N, TYE 563, TYE 665 or TYE 705.

124. The kit of claim 122, wherein the quencher is Iowa Black FG, Iowa Black RG, BHQ1, BHQ2 or BHQ3.

125. The kit of any one of claims 119-124, wherein the kit comprises at least three primers, wherein each primer is attached to a different chromophore.

126. The kit of any one of claims 119-125, wherein the first primer comprises a sequence complementary to a target analyte.

127. The kit of any one of claims 119-125, wherein the second primer comprises a sequence complementary to a target analyte.

128. A kit comprising a first oligonucleotide probe attached to exactly one first chromophore and a second oligonucleotide probe attached to exactly one second chromophore, wherein the first and second chromophores are different.

129. The kit of claim 128, wherein the first chromophore is a fluorophore.

130. The kit of claims 128 or 129, wherein the second chromophore is a fluorophore.

131. The kit of claim 128, wherein the second chromophore is a quencher.

132. The kit of claims 129, or 130, wherein the first or second chromophore is a fluorophore selected from the group consisting of: the first or second fluorophore is 6-FAM

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

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

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Fig. 1

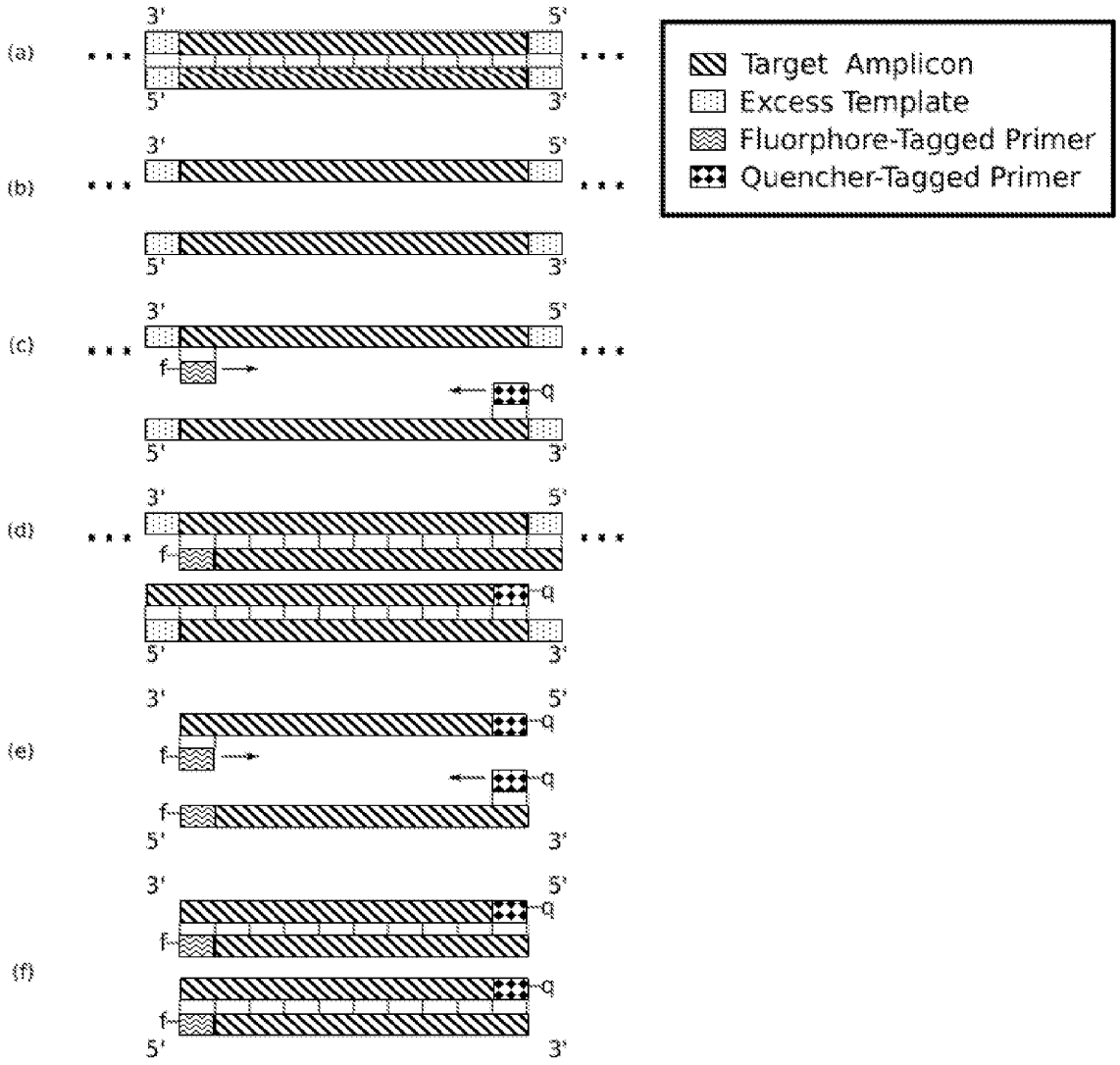

(a)
(b)
(c)
(d)
(e)
(f)
3'
5'
f
q
Target Amplicon
Excess Template
Fluorphore-Tagged Primer
Quencher-Tagged Primer

Fig. 2

A

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

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

B

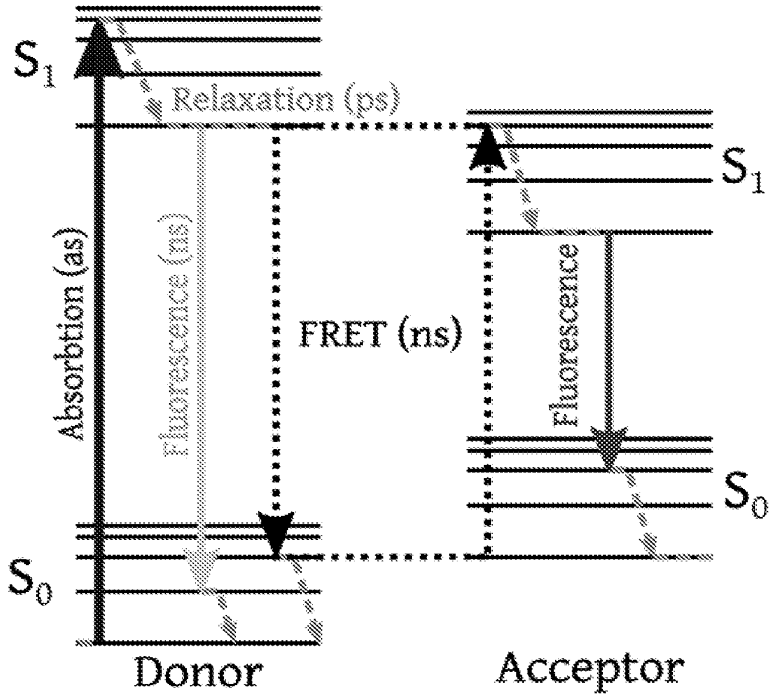


Fig. 3

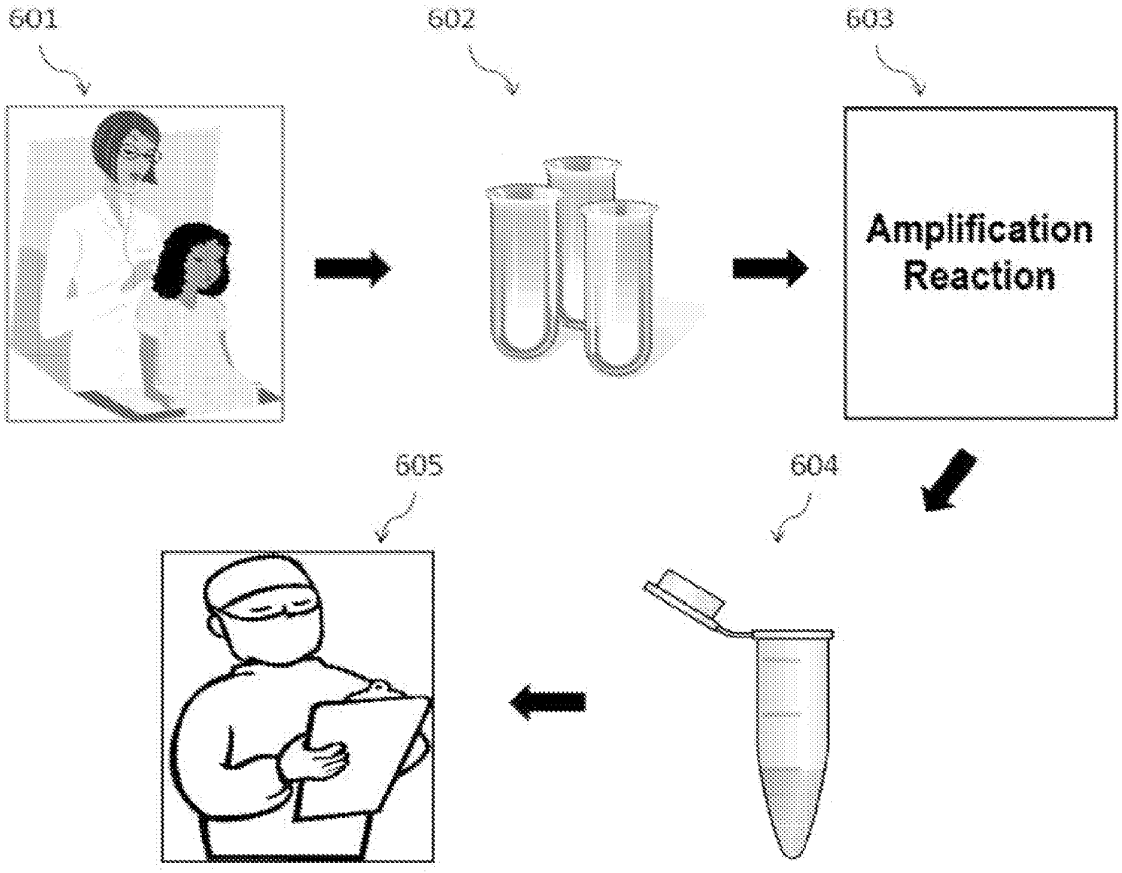


Fig. 4

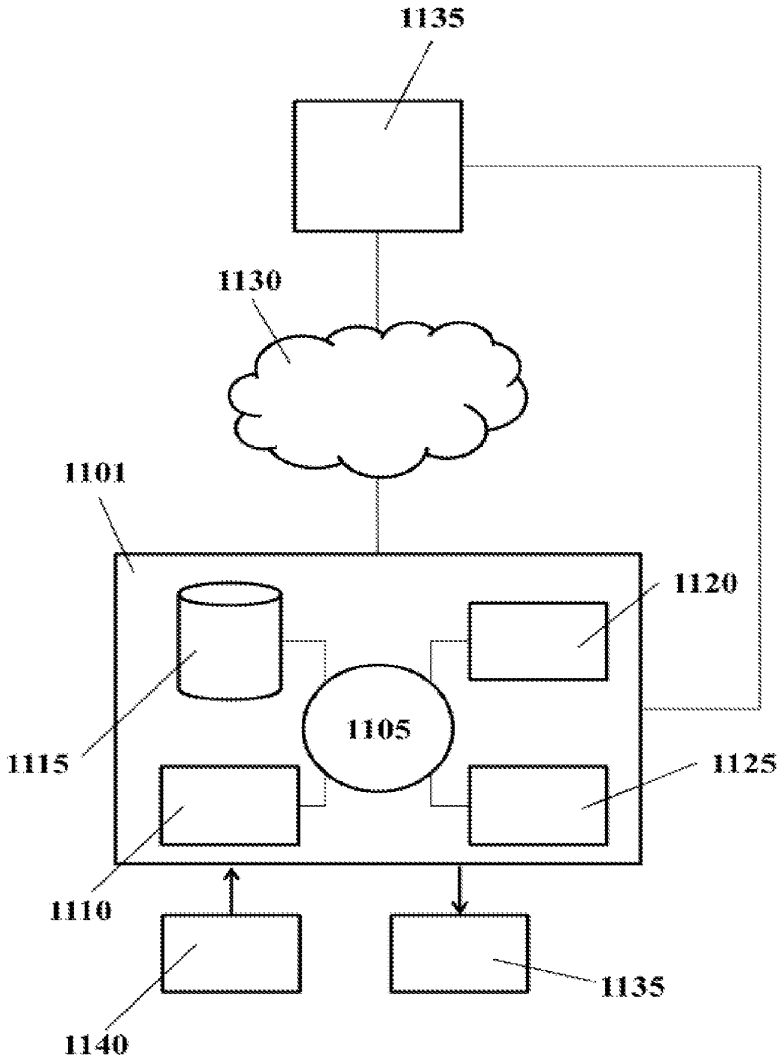


Fig. 5A

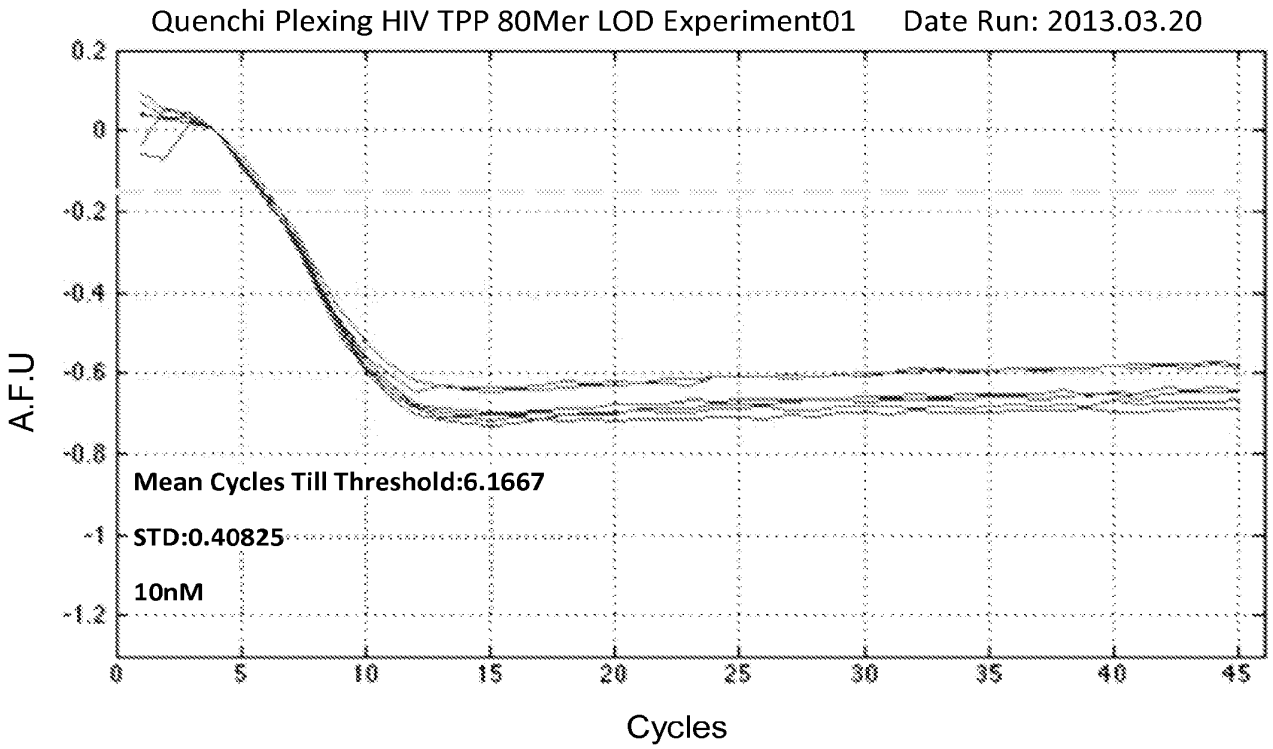

Quenchi Plexing HIV TPP 80Mer LOD Experiment01 Date Run: 2013.03.20
A.F.U
Cycles
Mean Cycles Till Threshold:6.1667
STD:0.40825
10nM
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
-1.2
0
5
10
15
20
25
30
35
40
45

Fig. 5B

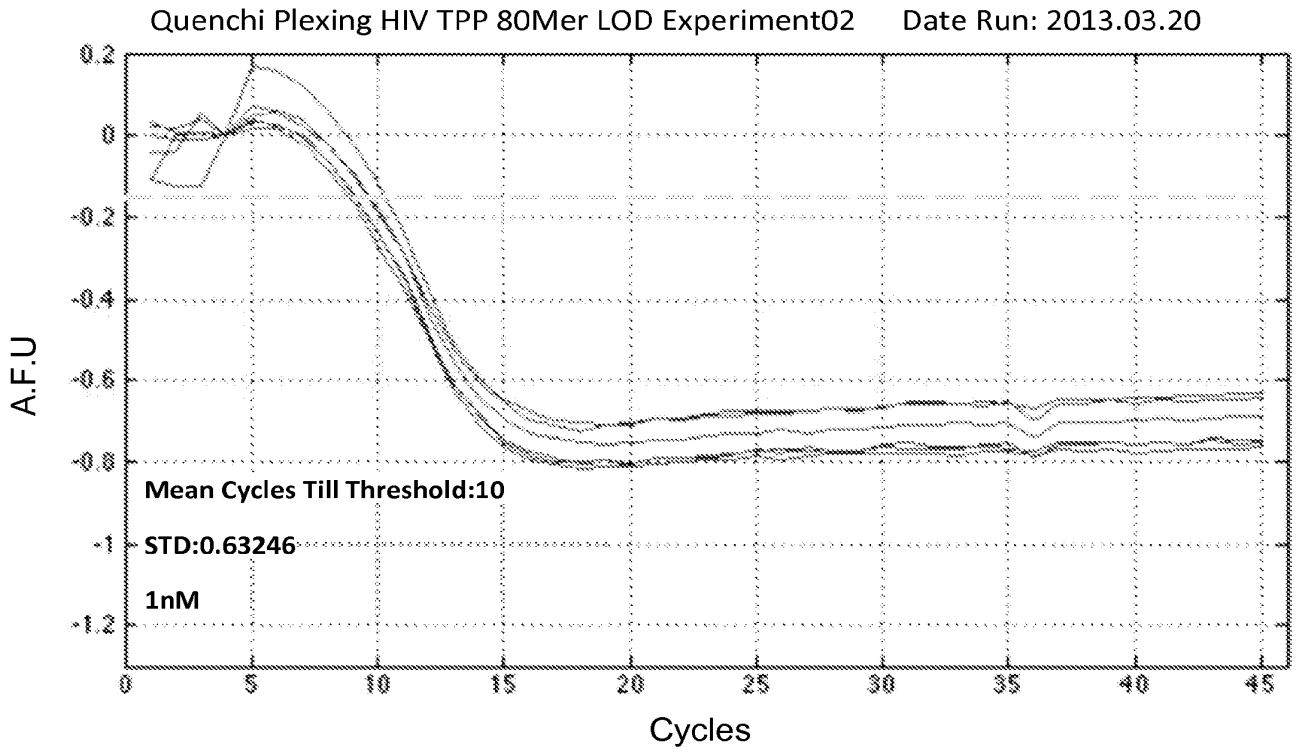

Quenchi Plexing HIV TPP 80Mer LOD Experiment02 Date Run: 2013.03.20
A.F.U
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
-1.2
Mean Cycles Till Threshold:10
STD:0.63246
1nM
0
5
10
15
20
25
30
35
40
45
Cycles

Fig. 5C

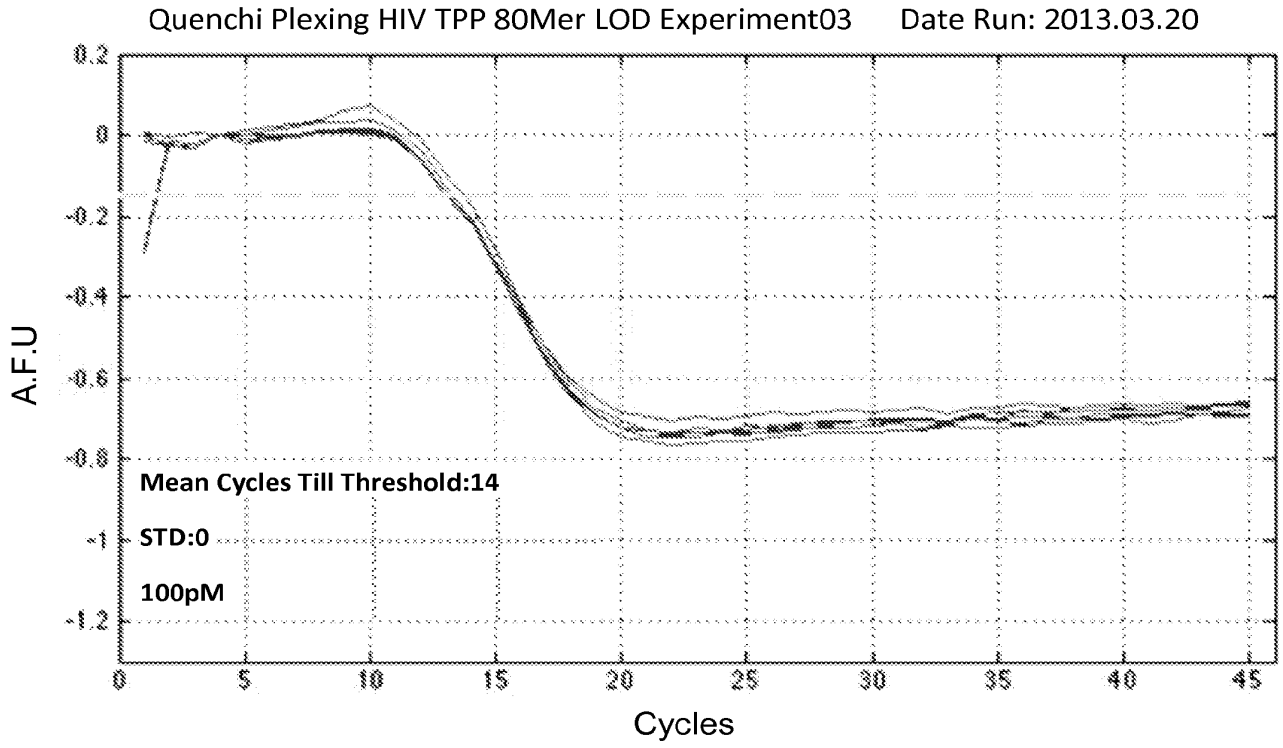

Quenchi Plexing HIV TPP 80Mer LOD Experiment03 Date Run: 2013.03.20
A.F.U
Cycles
Mean Cycles Till Threshold:14
STD:0
100pM
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
-1.2
0
5
10
15
20
25
30
35
40
45

Fig. 5D

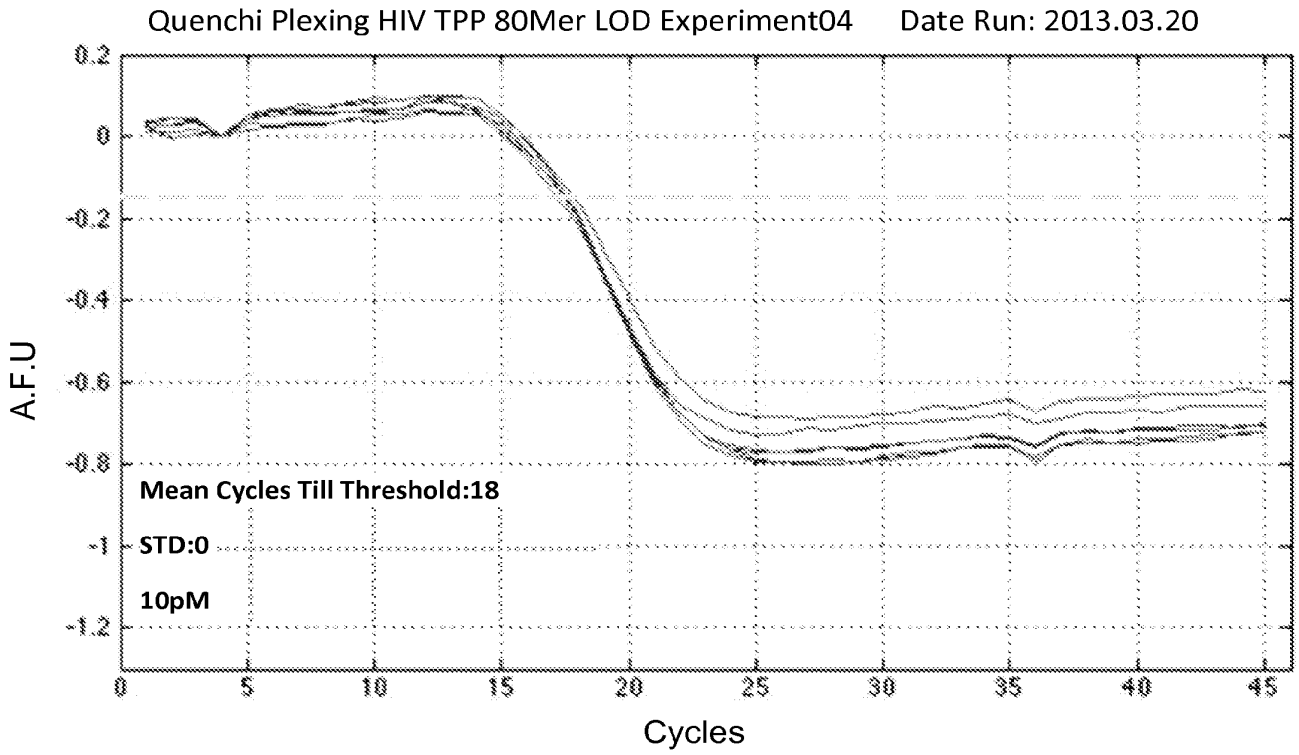

Quenchi Plexing HIV TPP 80Mer LOD Experiment04 Date Run: 2013.03.20
A.F.U
Cycles
Mean Cycles Till Threshold:18
STD:0
10pM
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
-1.2
0
5
10
15
20
25
30
35
40
45

Fig. 6A

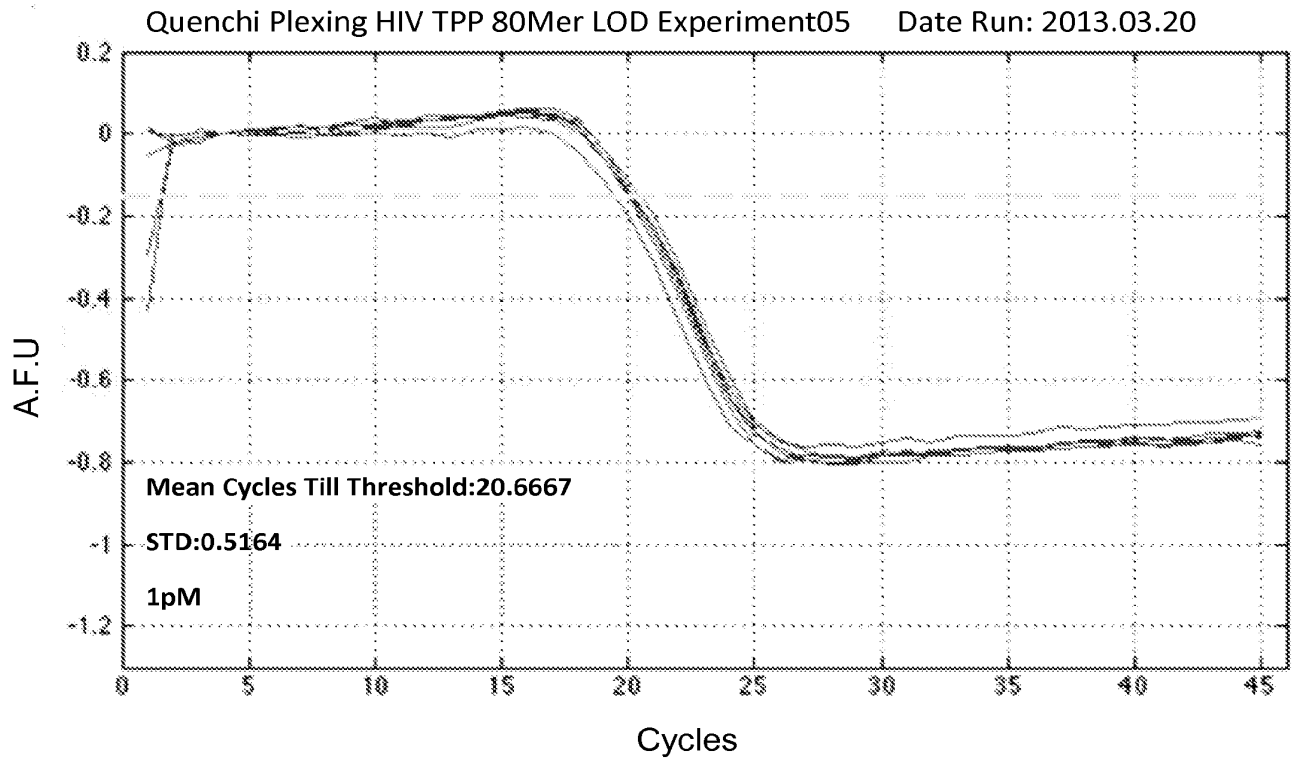

Quenchi Plexing HIV TPP 80Mer LOD Experiment05 Date Run: 2013.03.20
A.F.U
Cycles
Mean Cycles Till Threshold:20.6667
STD:0.5164
1pM
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
-1.2
0
5
10
15
20
25
30
35
40
45

Fig. 6B

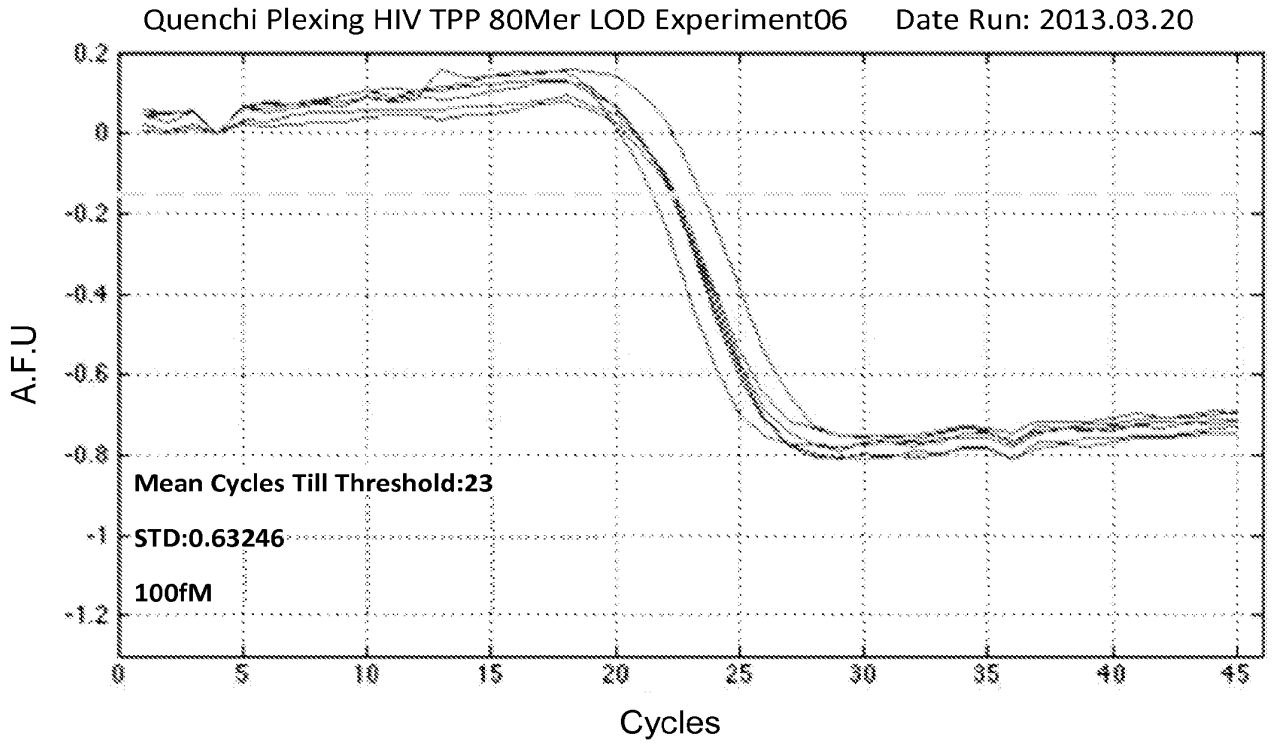

Quenchi Plexing HIV TPP 80Mer LOD Experiment06 Date Run: 2013.03.20
A.F.U
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
-1.2
Mean Cycles Till Threshold:23
STD:0.63246
100fM
0
5
10
15
20
25
30
35
40
45
Cycles

Fig. 6C

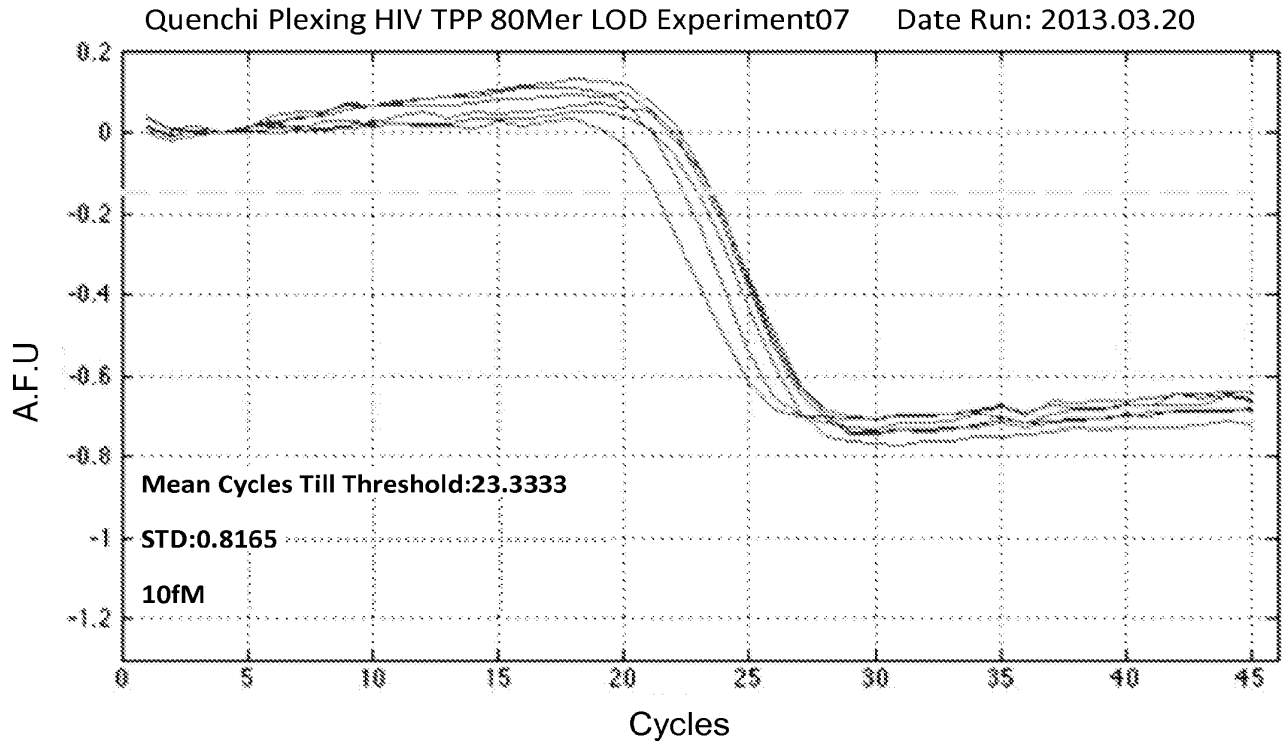

Quenchi Plexing HIV TPP 80Mer LOD Experiment07 Date Run: 2013.03.20
A.F.U
Cycles
Mean Cycles Till Threshold:23.3333
STD:0.8165
10fM
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
-1.2
0
5
10
15
20
25
30
35
40
45

Fig. 6D

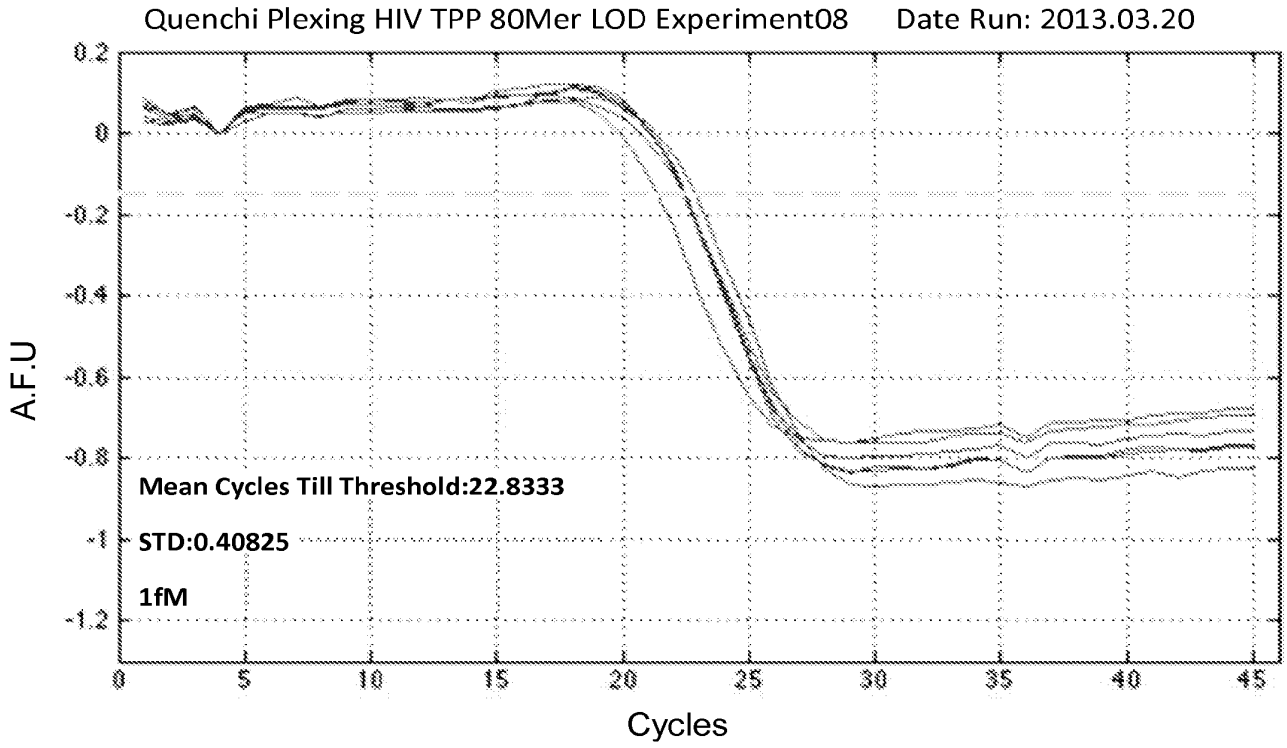

Quenchi Plexing HIV TPP 80Mer LOD Experiment08 Date Run: 2013.03.20
A.F.U
Cycles
Mean Cycles Till Threshold:22.8333
STD:0.40825
1fM
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
-1.2
0
5
10
15
20
25
30
35
40
45

Fig. 7A

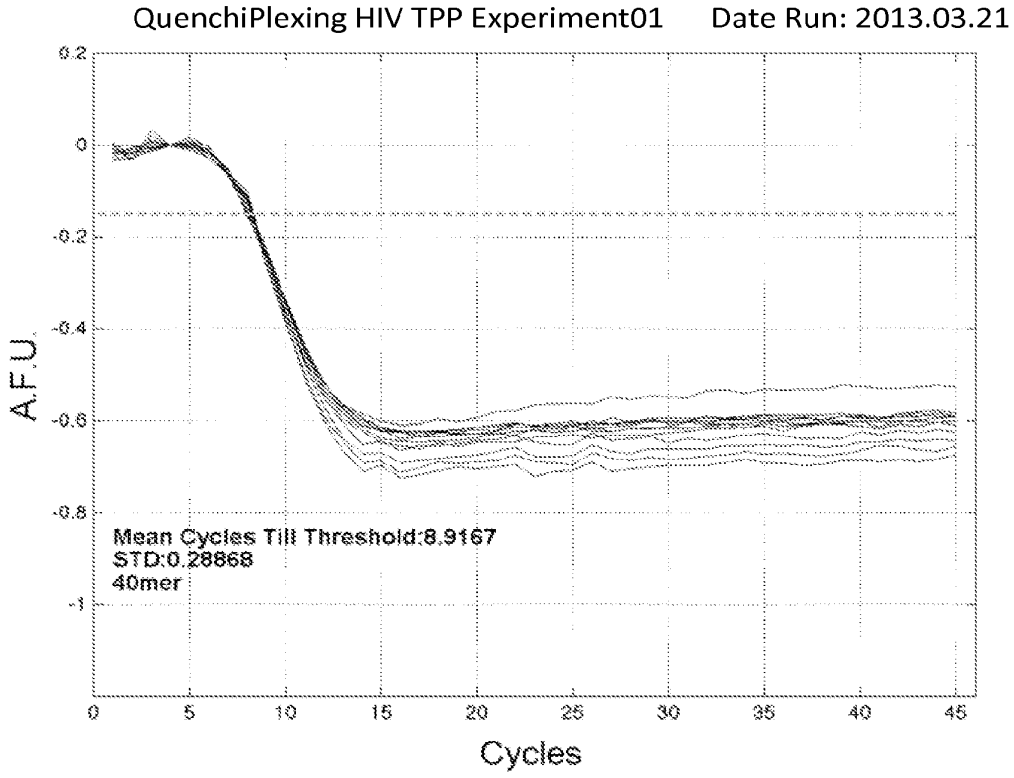

QuenchiPlexing HIV TPP Experiment01 Date Run: 2013.03.21
A.F.U.
Cycles
Mean Cycles Till Threshold:8.9167
STD:0.28868
40mer
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
0
5
10
15
20
25
30
35
40
45

Fig. 7B

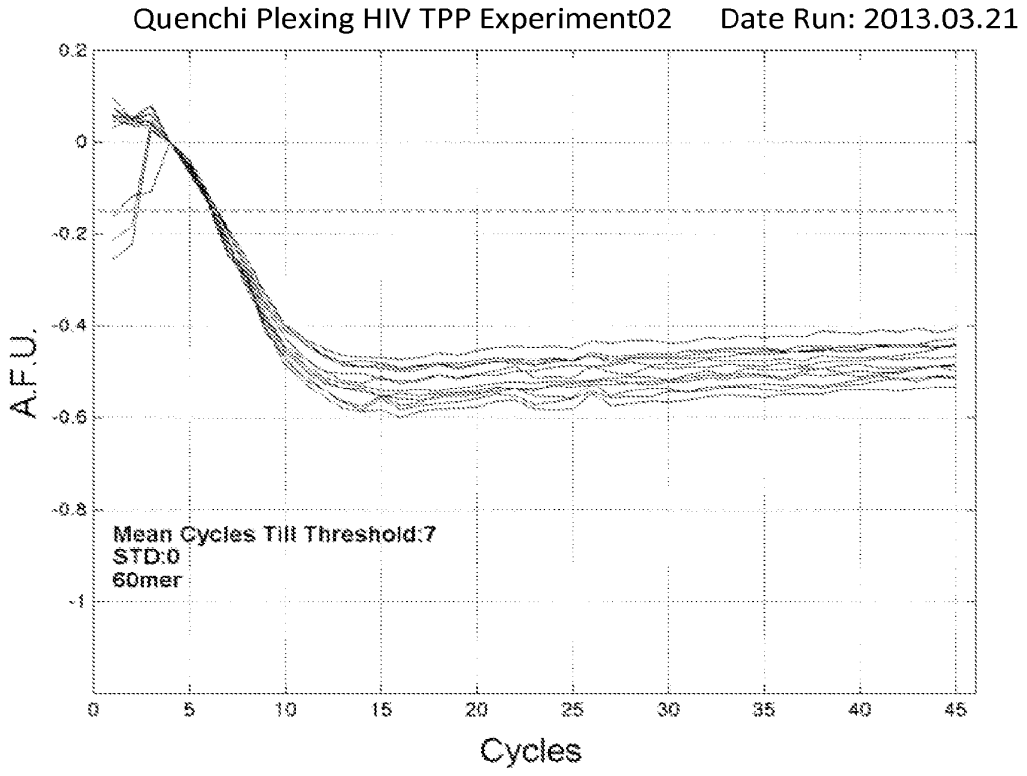

Quenchi Plexing HIV TPP Experiment02 Date Run: 2013.03.21
A.F.U.
Cycles
Mean Cycles Till Threshold:7
STD:0
60mer
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
0
5
10
15
20
25
30
35
40
45

Fig. 7C

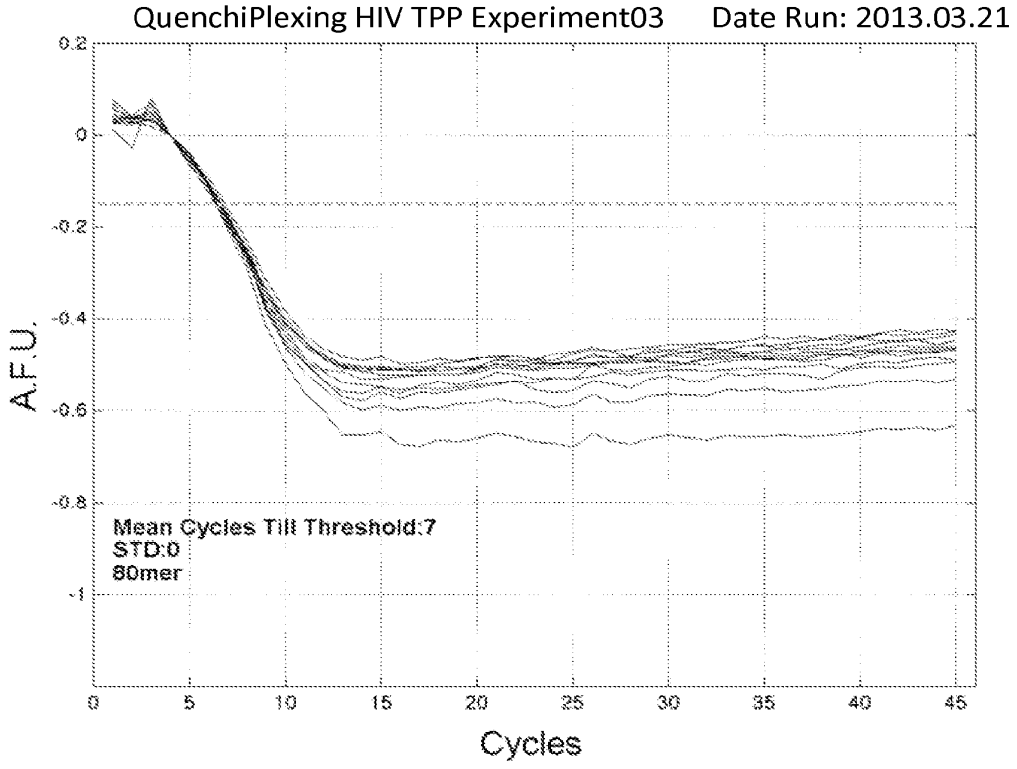

QuenchiPlexing HIV TPP Experiment03 Date Run: 2013.03.21
A.F.U.
Cycles
Mean Cycles Till Threshold:7
STD:0
60mer
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
0
5
10
15
20
25
30
35
40
45

Fig. 8A

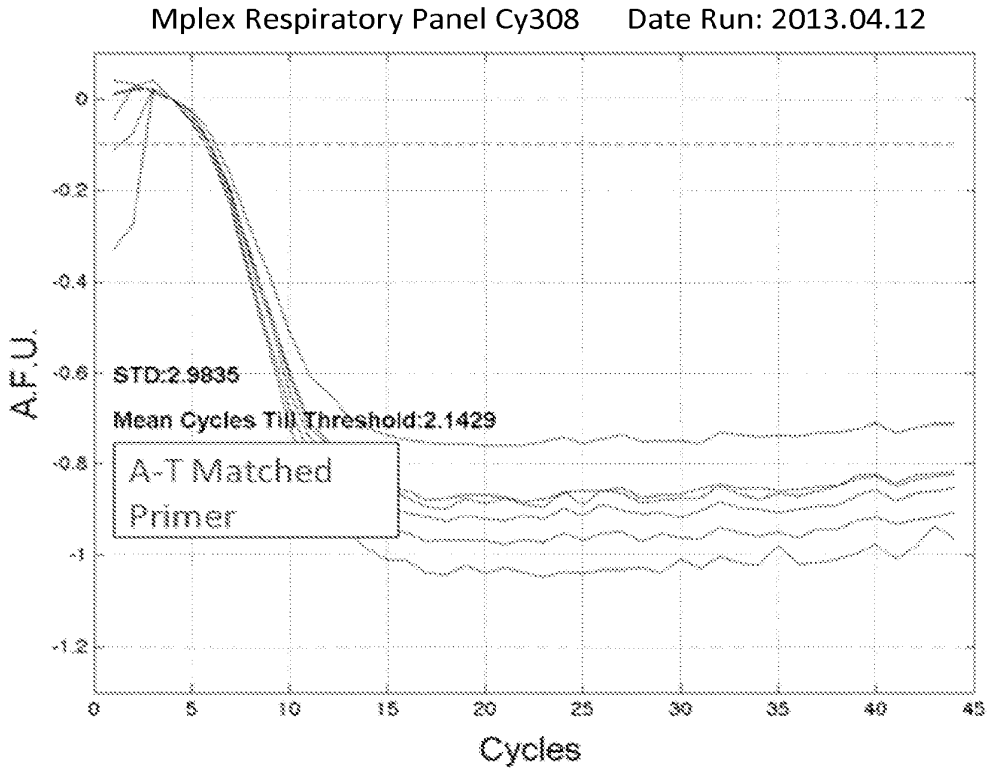

Mplex Respiratory Panel Cy308 Date Run: 2013.04.12
A.F.U.
Cycles
STD:2.9835
Mean Cycles Till Threshold:2.1429
A-T Matched Primer
0
-0.2
-0.4
-0.6
-0.8
-1
-1.2
0
5
10
15
20
25
30
35
40
45

Fig. 8B

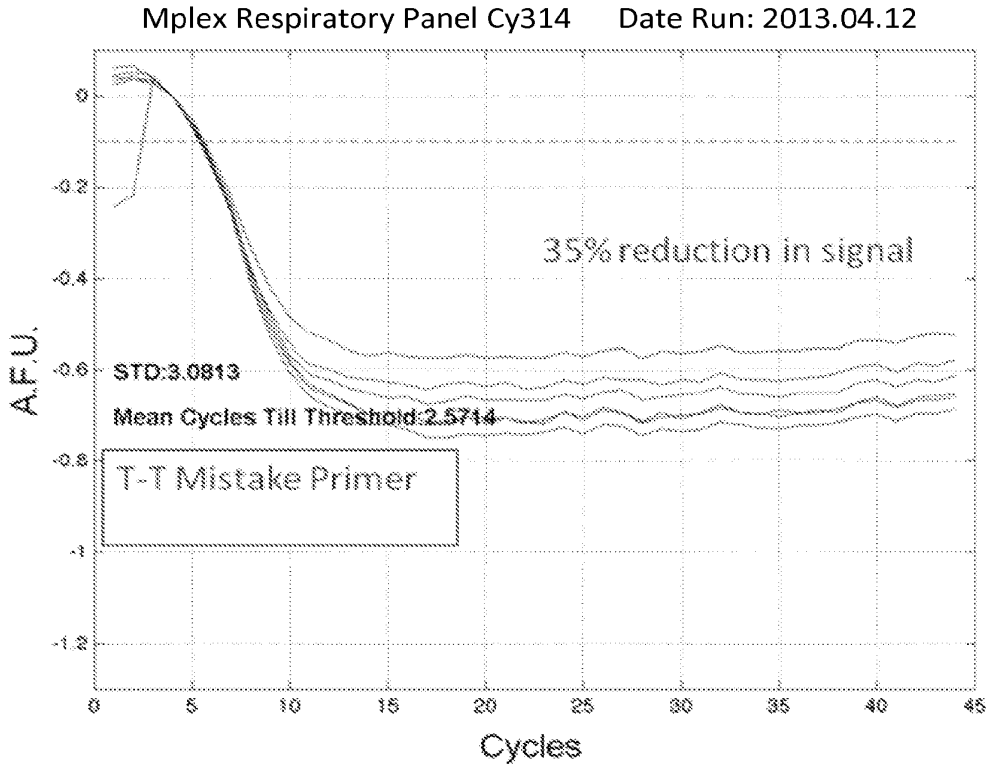

Mplex Respiratory Panel Cy314 Date Run: 2013.04.12
35% reduction in signal
STD:3.0813
Mean Cycles Till Threshold:2.5714
T-T Mistake Primer
A.F.U.
Cycles
0
-0.2
-0.4
-0.6
-0.8
-1
-1.2
0
5
10
15
20
25
30
35
40
45

Fig. 9

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

Fig. 10A

```
5'ATGGGGGGGGGGGGGGGGATGGGGGGGGGAAGGGGGGATGGGGGGGGGATGGGGATGGATG3'
3'TACCCCCCCCCCCCCCCCCTACCCCCCCCCTTCCCCCCCTACCCCCCCCCTACCCCTACCTAC5'
```

Fig. 10B

```
FWD Primer /Quencher/5'ATGGGGGGGGGGGGGGGGAT3'
RWD Primer 3'CCCCCCCTACCCCTACCTAC5'/Fluorphore/
```

Fig. 10C

```
5'ATGGGGGGGGGGGGGGGGATGGGGGGGGGAAGGGGGGATGGGGGGGGGATGGGGATGGATG3'
                          Extension <-   3'CCCCCCCTACCCCTACCTAC5'/F/

   3'TACCCCCCCCCCCCCCCCCTACCCCCCCCCTTCCCCCCCTACCCCCCCCCTACCCCTACCTAC5'
/Q/5'ATGGGGGGGGGGGGGGGGAT3'  -> Extension
```

Fig. 10D

```
/Q/5'ATGGGGGGGGGGGGGGGGATGGGGGGGGGAAGGGGGGATGGGGGGGGGATGGGGATGGATG3'
                           Extension <-   3'CCCCCCCTACCCCTACCTAC5'/F/

  3'TACCCCCCCCCCCCCCCCCTACCCCCCCCCTTCCCCCCCTACCCCCCCCCTACCCCTACCTAC5'/F/
/Q/5'ATGGGGGGGGGGGGGGGGAT3'  -> Extension
```

Fig. 10E

```
/Q/5'ATGGGGGGGGGGGGGGGGATGGGGGGGGGAAGGGGGGATGGGGGGGGGATGGGGATGGATG3'
   3'TACCCCCCCCCCCCCCCCCTACCCCCCCCCTTCCCCCCCTACCCCCCCCCTACCCCTACCTAC5'/F/

/Q/5'ATGGGGGGGGGGGGGGGGATGGGGGGGGGAAGGGGGGATGGGGGGGGGATGGGGATGGATG3'
   3'TACCCCCCCCCCCCCCCCCTACCCCCCCCCTTCCCCCCCTACCCCCCCCCTACCCCTACCTAC5'/F/
```

Fig. 11

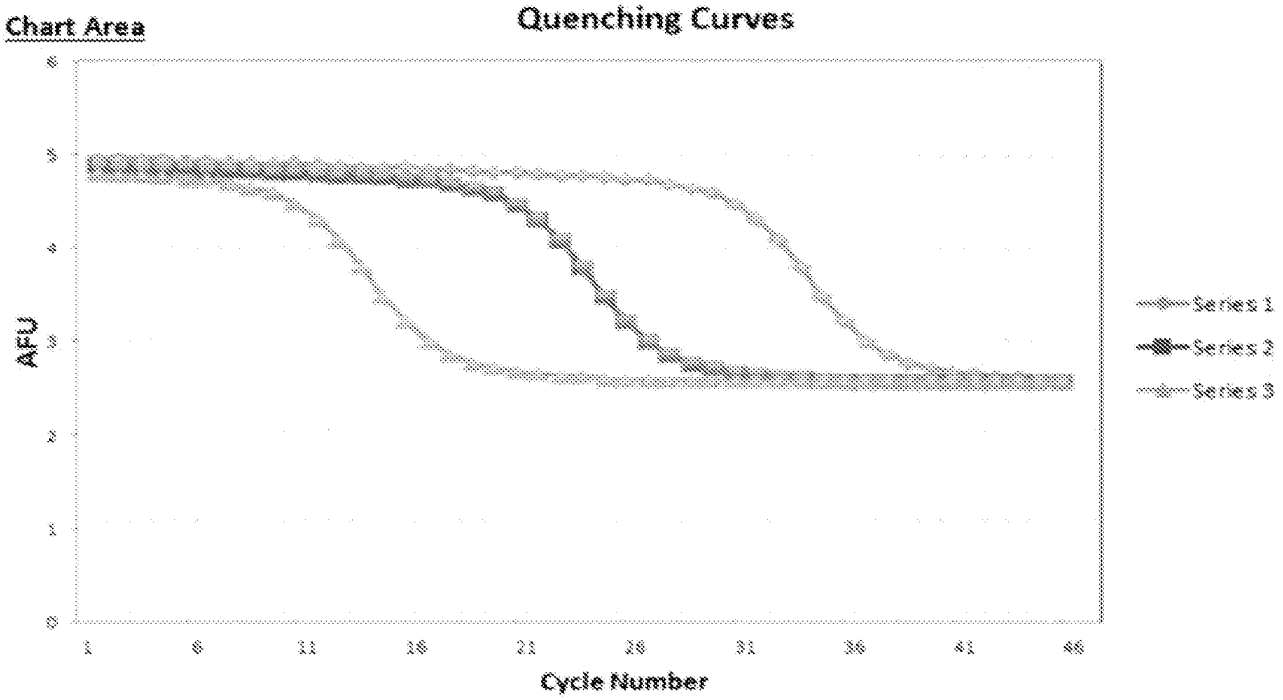

Chart Area
Quenching Curves
RFU
Cycle Number
Series 1
Series 2
Series 3

Fig. 12

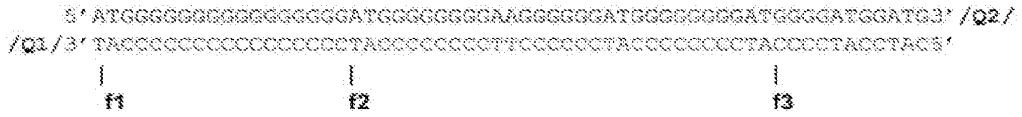
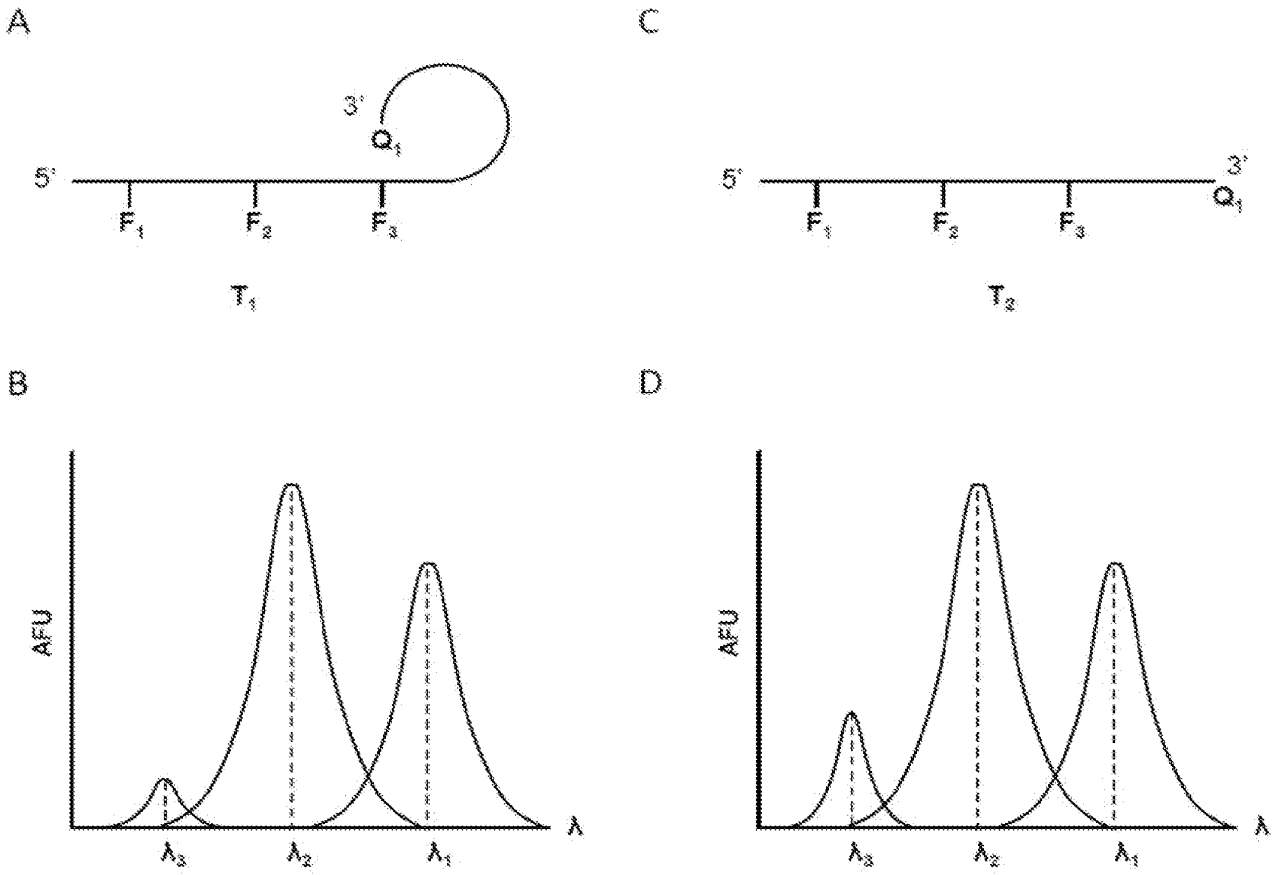

5'ATGGGGGGGGGGGGGGGGATGGGGGGGGAAGGGGGGATGGGGGGGGATGGGGATGGATG3'/Q2/
/Q1/3'TACCCCCCCCCCCCCCCCCTACCCCCCCCTTCCCCCCTACCCCCCCCTACCCCTACCTAC5'
f1
f2
f3

Fig. 13



International application No.
PCT/US 14/12836

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

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

   a. (means)

   ☐ on paper

   ☒ in electronic form

   b. (time)

   ☐ in the international application as filed

   ☐ together with the international application in electronic form

   ☒ subsequently to this Authority for the purposes of search

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

3. Additional comments:

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INTERNATIONAL SEARCH REPORT PCT/US2014/012836 13.05.2014

International application No.
PCT/US 14/12836

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

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

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

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

3. ☒ Claims Nos.: 11-27, 30-33, 35-37, 41-59, 64-71, 75-86, 101-109, 123-127, 132
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

This International Searching Authority found multiple inventions in this international application, as follows:

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

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

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

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

Remark on Protest

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

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

☐ No protest accompanied the payment of additional search fees.

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INTERNATIONAL SEARCH REPORT PCT/US2014/012836 13.05.2014

International application No.
PCT/US 14/12836

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12Q 1/68 (2014.01)
USPC - 435/6.11
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
USPC: 435/6.11

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 435/6.11, 6.12, 91.2 (text search)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Electronic data bases: PatBase; Google Scholar; Google Patents Search terms:fluorophore, two fluorescent primers, three fluorescent probes, fluorescent primer extension, 6-FAM (fluorescein), TAMRA (tetramethyl rhodamine) [fluorophore and quencher], BHQ1 [quencher] genetic variant or polymorphism, background fluorescence before PCR, PCR cycling

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	6,022,686 A (GARMAN et al.) 08 February 2000 (08.02.2000). Especially col 7 ln 36-52, col 8 23-41 and 47-50, col 9 ln 24-31 and 46-47, col 13 ln 3-5, 29-31 and 43-45.	1-9, 28, 34, 38-40, 60-63, 72-74, 87-100, 110-122, 128-131 ---------- 10, 29, 133
X	CHEHAB et al.Detection of specific DNA sequences by fluorescence amplification: a color complementation assay. Proc Nat Acad Sci December 1989 Vol 86 No 23 Pages 9178-9182. Especially pg 9179 col 2 para 2, pg 9180 fig 2.	38-49
Y	YEUNG et al. Evaluation of dual-labeled fluorescent DNA probe purity versus performance in real-time PCR. Biotechniques February 2004 Vol 36 No 2 Pages 266-270. Especially pg 237 col 3 para 2.	10, 133
Y	MERZOUKI et al. Accurate and differential quantitation of HIV-1 tat, rev and nef mRNAs by competitive PCR. J Virol Methods December 1994 Vol 50 No 1-3 Pages 115-128.	29
A	PERDOK et al. PROTOZOA INHIBITORS TO REDUCE METHANE EXCRETION FROM DAIRY COWS [online] 29 May 2007 [retrieved 10 April 2014]. Available on the internet: < http://scholar.google.co.th/scholar?q=PROTOZOA+INHIBITORS+TO+REDUCE+METHANE+EXCRETION+FROM+DAIRY+COWS&btnG=&hl=en&as_sdt=0%2C5>. Especially pg 9 para 7, pg 10 fig 4.4	1, 38, 60, 72, 87, 93,110

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

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

Date of the actual completion of the international search	Date of mailing of the international search report
10 April 2014 (10.04.2014)	13 MAY 2014
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

International application No.
PCT/US 14/12836

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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

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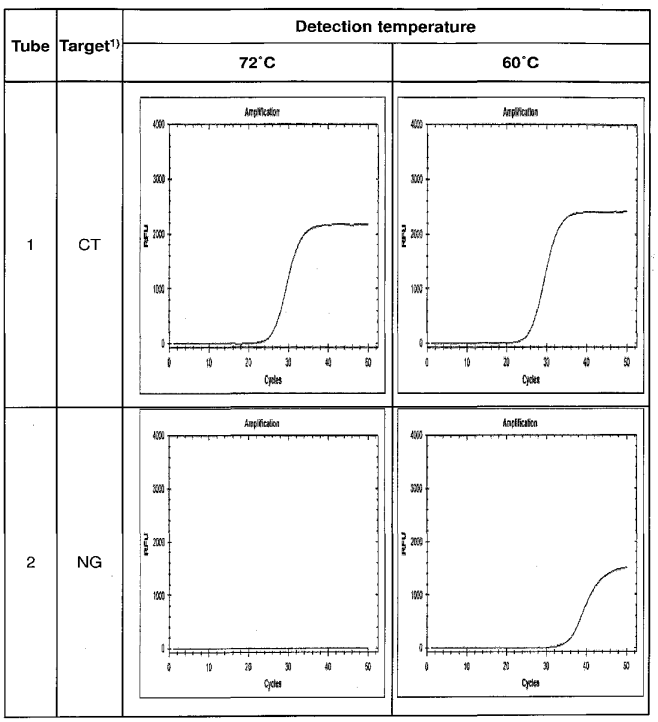
(72) Inventors: **CHUN, Jong Yoon**; Rm 1901, Cheongdam Mark-Hills 1-cha, 142gil 21, Yeongdong-daero, Gangnam-gu, Seoul 135-957 (KR). **LEE, Young Jo**; 115-1301 Park Rio Apt., 435, Olympic-ro, Seoul, Songpa-gu 138-930 (KR).

(74) Agent: **YANG, Boo-Hyun**; Suite 301, Chundong Building, 1922, Namboosunhwan-ro, Gwanak-gu, Seoul 151-832 (KR).

(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, IR, IS, JP, KE, KG, 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, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

[Continued on next page]

(54) Title: DETECTION OF TARGET NUCLEIC ACID SEQUENCES USING DIFFERENT DETECTION TEMPERATURES



(57) Abstract: The present invention relates to detection of target nucleic acid sequences using different detection temperatures. The present invention employing different detection temperatures enables to detect a plurality of target nucleic acid sequences in conventional real-time manners even with a single type of label in a single reaction vessel. The conventional technologies detect a plurality of target nucleic acid sequences by a melting analysis after target amplification. Unlikely, the present invention does not require a melting analysis after target amplification, such that the time for analysis is greatly reduced.

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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, 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).

Published:

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

DETECTION OF TARGET NUCLEIC ACID SEQUENCES USING DIFFERENT DETECTION TEMPERATURES

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

The present invention relates to detection of target nucleic acid sequences using different detection temperatures.

DESCRIPTION OF THE RELATED ART

For detection of target nucleic acid sequences, real-time detection methods are widely used to detect target nucleic acid sequences with monitoring target amplification in a real-time manner. The real-time detection methods generally use labeled probes or primers specifically hybridized with target nucleic acid sequences. The exemplified methods by use of hybridization between labeled probes and target nucleic acid sequences include Molecular beacon method using dual-labeled probes with hairpin structure (Tyagi et al, Nature Biotechnology v.14 MARCH 1996), HyBeacon method (French DJ et al., Mol. Cell Probes, 15(6):363-374(2001)), Hybridization probe method using two probes labeled each of donor and acceptor (Bernad et al, 147-148 Clin Chem 2000; 46) and Lux method using single-labeled oligonucleotides (U.S. Pat No 7,537,886). TaqMan method (U.S. Pat Nos 5,210,015 and 5,538,848) using dual-labeled probes and its cleavage by 5'-nuclease activity of DNA polymerase is also widely employed in the art.

The exemplified methods using labeled primers include Sunrise primer method (Nazarenko et al, 2516-2521 Nucleic Acids Research, 1997, v.25 no.12, and US Pat. No. 6,117,635), Scorpion primer method (Whitcombe et al, 804-807, Nature Biotechnology v.17 AUGUST 1999 and US Pat. No. 6,326,145) and TSG primer method (WO 2011/078441).

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As alternative approaches, real-time detection methods using duplexes formed depending on the presence of target nucleic acid sequences have been proposed: Invader assay (US 5,691,142, US 6,358,691 and US 6,194,149), PTOCE (PTO cleavage AND extension) method (WO 2012/096523), PCE-SH (PTO Cleavage and Extension-Dependent Signaling Oligonucleotide Hybridization) method (WO 2013/115442), PCE-NH (PTO Cleavage and Extension-Dependent Non-Hybridization) method (PCT/KR2013/012312).

The conventional real-time detection technologies described above detect signals generated from fluorescent labels at a selected detection temperature in signal amplification process associated with or with no target amplification. When a plurality of target nucleic acid sequences using a single type of label in a single reaction tube are detected in accordance with the conventional real-time detection technologies, generated signals for target nucleic acid sequences are not differentiated from each other. Therefore, the conventional real-time detection technologies generally employ different types of labels for detecting a plurality of target nucleic acid sequences. The melting analysis using T_m difference permits to detect a plurality of target nucleic acid sequences even a single type of label. However, the melting analysis has serious shortcomings in that its performance time is longer than real-time technologies and design of probes with different T_m values becomes more difficult upon increasing target sequences.

Accordingly, where novel methods or approaches being not dependent on melting analysis for detecting a plurality of target nucleic acid sequences using a single type of label in a single reaction vessel and a single type of detector are developed, they enable to detect a plurality of target nucleic acid sequences with dramatically enhanced convenience, cost-effectiveness and efficiency. In addition, the combination of the novel methods with other detection methods (*e.g.*, melting analysis) would result in detection of a plurality of target nucleic acid sequences using a single type of label in a single reaction vessel with dramatically enhanced efficiency.

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Throughout this application, various patents and publications are referenced and citations are provided in parentheses. The disclosure of these patents and publications in their entities are hereby incorporated by references into this application in order to more fully describe this invention and the state of the art to which this invention pertains.

SUMMARY OF THE INVENTION

The present inventors have made intensive researches to develop novel methods for detecting a plurality of target nucleic acid sequences using a single type of label in a single reaction vessel and a single type of detector. As a result, we have found that signals for target nucleic acid sequences are obtained at adjusted detection temperatures and then detection results are suitably interpreted, thereby enabling to detect a plurality of target nucleic acid sequences using a single type of label in a single reaction vessel and a single type of detector with dramatically enhanced convenience, cost-effectiveness and efficiency.

Accordingly, it is an object of this invention to provide a method for detecting two target nucleic acid sequences in a sample using different detection temperatures.

It is another object of this invention to provide a method for SNP genotyping of a nucleic acid sequence in a sample using different detection temperatures.

It is still another object of this invention to provide a method for detecting at least three target nucleic acid sequences in a sample using different detection temperatures.

It is further object of this invention to provide a method for detecting two target nucleic acid sequences in a sample using different detection temperatures and melting analysis.

It is still further object of this invention to provide a method for detecting at least three target nucleic acid sequences in a sample using detection temperature analysis and melting analysis.

It is another object of this invention to provide a method for detecting a target

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nucleic acid sequence selected from two target nucleic acid sequences providing non-differentiated signals by a single type of detector in a single reaction vessel.

Other objects and advantages of the present invention will become apparent from the detailed description to follow taken in conjugation with the appended claims and drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1a represents the detection results of the present invention using different detection temperatures to detect a target nucleic acid sequence (genome DNA of *Chlamydia trachomatis*, CT) having a relatively high detection temperature (72°C), a target nucleic acid sequence (genome DNA of *Neisseria gonorrhoeae*, NG) having a relatively low detection temperature (60°C) and their combination. The signal for CT was generated by TaqMan real-time PCR method, and the signal for NG was generated by PTOCE real-time PCR method.

Fig. 1b represents determination of the presence of the target nucleic acid sequences having a relatively low detection temperature by using a ratio between the signal at the relatively high detection temperature and the signal at the relatively low detection temperature.

Fig. 1c represents determination of the presence of the target nucleic acid sequences having a relatively low detection temperature by plotting the ratios between the signal at the relatively high detection temperature and the signal at the relatively low detection temperature.

Fig. 1d and 1e represents determination of the presence of the target nucleic acid sequences having a relatively low detection temperature by using difference between the signal at the relatively high detection temperature and the signal at the relatively low detection temperature, wherein the signal at the relatively high detection temperature is modified to a threshold value and used to obtain the difference.

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Fig. 2a represents the detection results of the present invention using different detection temperatures to detect a target nucleic acid sequence (genome DNA of *Chlamydia trachomatis*, CT) having a relatively high detection temperature (72°C), a target nucleic acid sequence (genome DNA of *Neisseria gonorrhoeae*, NG) having a relatively low detection temperature (60°C) and their combination. The signals for CT and NG were generated by PTOCE real-time PCR method.

Fig. 2b represents determination of the presence of the target nucleic acid sequences having a relatively low detection temperature by using a ratio between the signal at the relatively high detection temperature and the signal at the relatively low detection temperature.

Fig. 2c represents determination of the presence of the target nucleic acid sequences having a relatively low detection temperature by plotting the ratios between the signal at the relatively high detection temperature and the signal at the relatively low detection temperature.

Fig. 2d and 2e represents determination of the presence of the target nucleic acid sequences having a relatively low detection temperature by using difference between the signal at the relatively high detection temperature and the signal at the relatively low detection temperature, wherein the signal at the relatively high detection temperature is modified to a threshold value and used to obtain the difference.

Fig. 3 represents the detection results of a target nucleic acid sequence (genome DNA of *Chlamydia trachomatis*, CT) having a relatively high detection temperature (72°C), a target nucleic acid sequence (genome DNA of *Neisseria gonorrhoeae*, NG) having a relatively low detection temperature (60°C) and their combination in accordance with both a real-time PCR using different detection temperatures and a melting analysis. The signal for CT was generated by TaqMan real-time PCR method, and the signal for NG was generated by PTOCE-melting method.

Fig. 4a represents the SNP genotyping results of the present invention using

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different detection temperatures in a real-time PCR manner. MTHFR(C677T) human genomic DNA was used as templates (target sequences). The wild homozygote (CC) , the mutant homozygote (TT) and the heterozygote (CT) were detected. All signals were generated by PTOCE real-time PCR method.

Fig. 4b represents SNP genotyping by using a ratio between the signal at the relatively high detection temperature and the signal at the relatively low detection temperature.

DETAILED DESCRIPTION OF THIS INVENTION

The most prominent feature of the present invention is to detect a plurality of target nucleic acid sequences by using a single type of label and a single type of detector in a signal reaction vessel. The present invention employing different detection temperatures enables to detect a plurality of target nucleic acid sequences even with a single type of label in a single reaction vessel. The elements of the present invention are selected in compliance with the feature of the present invention and fabricated into a surprising process for detect target nucleic acid sequences.

Conventional real-time PCR methods require two types of fluorescent labels or melting analysis for detection of two target nucleic acid sequences in a single reaction vessel.

The present invention permits real-time PCR protocols to detect two target nucleic acid sequences by using a single type of fluorescent label in a single reaction vessel. Alternatively, the present invention enables to detect two target nucleic acid sequences by detecting one of two target nucleic acid sequences by real-time PCR and the other by melting analysis.

The present invention employs our findings that detection of signals is adjustable by temperatures in accordance with signal-generating means for target nucleic acid sequences.

For example, where signal-generating means by hybridization of a probe with a target nucleic acid sequence is used for detection of a first target nucleic acid

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sequence, a temperature at which the probe is hybridized with the first target nucleic acid sequence permits to generate and detect signals indicative of the presence of the first target nucleic acid sequence. In contrast, a temperature at which the probe is not hybridized with the first target nucleic acid sequence permits to generate and detect no signal. In this regard, it would be recognized that there are temperatures at which the signal is generated and temperatures at which the signal is not generated, depending on the signal-generating means.

In such signal-generating means, the temperatures at which the probe is hybridized with the first target nucleic acid sequence can be served as a detection temperature for the first target nucleic acid sequence. The temperatures at which the probe is not hybridized with the first target nucleic acid sequence cannot be served as a detection temperature.

Where probe hybridization is also used for detection of a second target nucleic acid sequence, its detection temperature may be determined in considering the fact that there is a temperature range to generate or not generate signals.

Where the T_m value of the probe used for detection of the second target nucleic acid sequence is lower than that of the probe for detection of the first target nucleic acid sequence, the signal for the first target nucleic acid sequence can be detected at a relatively higher temperature. In other words, there is difference in temperatures to generate and detect signals between the two signal-generating means for the two target nucleic acid sequences.

Where the two target nucleic acid sequences co-exist in a sample, there is a temperature range enabling to generate signal for the first target nucleic acid sequence and not to generate signal for the second target nucleic acid sequence. Meanwhile, at a temperature range lower than the temperature range, signals for the two target nucleic acid sequences are generated.

Considering the two temperature ranges, a detection temperature may be determined for each of the target nucleic acid sequences. A relatively high detection temperature can be selected from the former temperature range, and the relatively

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high detection temperature is assigned to the first target nucleic acid sequence. A relatively low detection temperature can be selected from the latter temperature range, and the relatively low detection temperature is assigned to the second target nucleic acid.

According to the present invention, the signal at the relatively high detection temperature is measured to determine the presence of the first target nucleic acid sequence. According to the present invention, the detection at the relatively high detection temperature allows a method determining the presence of the first target nucleic acid sequence.

The important technical feature of the present invention is to unveil the signal detected at the relatively low detection temperature for determining the presence of the second target nucleic acid sequence having the relatively low detection temperature, by using both the signal at the relatively high detection temperature and the signal at the relatively low detection temperature.

Interestingly, the present inventors have found that signals detected at two different detection temperatures are changed in accordance with a certain pattern, and adopted the findings to detection of target nucleic acid sequences, thereby accomplishing the present invention.

Alternatively, the present inventors have contemplated that the first target nucleic acid sequence may be detected at the relatively high detection temperature by using different detection temperatures and the second target nucleic acid sequence may be detected by melting analysis as another signal generation approach. A signal-generating means used for melting analysis may provide a signal at a certain temperature during the real-time detection process. Therefore, even when one of two target nucleic acid sequences is detected by melting analysis, the two target nucleic acid sequences are required to have different detection temperatures from each other.

The present invention can be embodied to various aspects as follows:

(a) Detection of two target nucleic acid sequences in a sample using different

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detection temperatures;

(b) SNP genotyping of a nucleic acid sequence in a sample using different detection temperatures;

(c) Detection of at least three target nucleic acid sequences in a sample using different detection temperatures;

(d) Detection of two target nucleic acid sequences in a sample using different detection temperatures and melting analysis; and

(e) Detection of at least three target nucleic acid sequences in a sample using detection temperature analysis and melting analysis.

I. Detection of Two Target Nucleic Acid Sequences in a Sample Using Different Detection Temperatures

In one aspect of this invention, there is provided a method for detecting two target nucleic acid sequences in a sample using different detection temperatures, comprising:

(a) incubating the sample with two signal-generating means for detection of the two target nucleic acid sequences in a single reaction vessel and detecting a generated signal by using a single type of detector; wherein each of the target nucleic acid sequences is detected by a corresponding signal-generating means; wherein one of the two target nucleic acid sequences has a relatively high detection temperature and the other has a relatively low detection temperature determined by the corresponding signal-generating means; wherein the relatively high detection temperature is a temperature capable of generating a signal for the target nucleic acid sequence having the relatively high detection temperature and the relatively low detection temperature is a temperature capable of generating both of a signal for the target nucleic acid sequence having the relatively low detection temperature and a signal for the target nucleic acid sequence having the relatively high detection temperature; wherein signals to be generated by the two signal-generating means are not differentiated by the single type of detector; wherein the detection is performed

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at both the relatively high detection temperature and the relatively low detection temperature; and

(b) determining the presence of the two target nucleic acid sequences by using the signal detected in the step (a); wherein (i) the presence of the target nucleic acid sequence having the relatively high detection temperature is determined by using the signal detected at the relatively high detection temperature and (ii) the presence of the target nucleic acid sequence having the relatively low detection temperature is determined by using a difference between the signal detected at the relatively high detection temperature and the signal detected at the relatively low detection temperature.

According to conventional real-time PCR methods using amplification curves, it is common knowledge in the art that a plurality of target nucleic acid sequences cannot be differentially detected by use of signal-generating means providing undistinguishable identical signals.

The present invention overcomes limitations associated with the common knowledge in the art and leads to unexpected results to detect target nucleic acid sequences in greatly improved manner.

The present invention will be described in more detail as follows:

Step (a): <u>Incubation with signal-generating means and signal detection</u>

Firstly, the sample to be analyzed is incubated with two signal-generating means for detection of the two target nucleic acid sequences in a single reaction vessel and then a generated signal is detected by using a single type of detector. Signals to be generated by the two signal-generating means are not differentiated by the single type of detector.

The present invention utilizes signal-generating means for generating signals for target nucleic acid sequences. Each of the target nucleic acid sequences is detected by a corresponding signal-generating means. The term used herein "signal-

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generating means" refers to any material used in generation of signals indicating the presence of target nucleic acid sequences, for example including oligonucleotides, labels and enzymes. Alternatively, the term used herein "signal-generating means" can be used to refer to any methods using the materials for signal generation.

According to an embodiment of this invention, incubation is preformed in the conditions allowing a signal generation by the signal-generation means.

Examples of the oligonucleotides serving as signal-generating means include: oligonucleotides to be specifically hybridized with target nucleic acid sequences (*e.g.*, probes and primers); where oligonucleotides hybridized with target nucleic acid sequences are cleaved to release a fragment, capture oligonucleotides to be specifically hybridized with the fragment; where the fragment hybridized with the capture oligonucleotide is extended to form an extended strand, oligonucleotides to be specifically hybridized with the extended strand; oligonucleotides to be specifically hybridized with the capture oligonucleotide; and combinations thereof.

While a signal generation principle is the same, the signal generating means comprising different sequences of oligonucleotides used may be considered different from each other.

The label may be linked to oligonucleotides or may be in the free form. The label may be incorporated into extended products during an extension reaction.

Where the cleavage of oligonucleotides is used in signal generation, examples of the enzyme include 5'-nuclease and 3'-nuclease, particularly nucleic acid polymerase having 5'-nuclease activity, nucleic acid polymerase having 3'-nuclease activity or FEN nuclease.

In the present invention, signals may be generated by using various materials described above in various fashions.

According to an embodiment, at least one of the two signal-generating means is a signal-generating means to generate a signal in a dependent manner on the formation of a duplex.

According to an embodiment, the duplx includes a double stranded target

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nucleic acid sequence.

The expression used herein "generate a signal in a dependent manner on the formation of a duplex" in conjunction with signal-generating means refers to that signal to be detected is provided being dependent on association or dissociation of two nucleic acid molecules. The expression includes that signal is provided by a duplex formed being dependent on the presence of a target nucleic acid sequence. In addition, the expression includes that signal is provided by inhibition of hybridization of a duplex. The expression is different from providing a signal by cleavage.

Particularly, the signal is generated by the formation of a duplex between a target nucleic acid sequence and a detection oligonucleotide specifically hybridized with the target nucleic acid sequence.

The term used herein "detection oligonucleotide" is an oligonucleotide which is involved in generation of signal to be detected. According to an embodiment of the present invention, the detection oligonucleotide includes an oligonucleotide which is specifically hybridized with a target nucleic acid sequence and is involved in an actual signal generation. For example, the hybridization or non-hybridization of a detection oligonucleotide to the target nucleic acid determines the signal generation.

The signal by the formation of a duplex between a target nucleic acid sequence and the detection oligonucleotide may be generated by various methods, including Scorpion method (Whitcombe et al, Nature Biotechnology 17:804-807 (1999)), Sunrise (or Amplifluor) method (Nazarenko et al, Nucleic Acids Research, 25(12):2516-2521 (1997), and U.S. Pat. No. 6,117,635), Lux method (U.S. Pat. No. 7,537,886), Plexor method (Sherrill C B, et al., Journal of the American Chemical Society, 126:4550-45569 (2004)), Molecular Beacon method (Tyagi et al, Nature Biotechnology v.14 MARCH 1996), HyBeacon method (French DJ et al., Mol. Cell Probes, 15(6):363-374(2001)), adjacent hybridization probe method (Bernard, P.S. et al., Anal. Biochem., 273:221(1999)) and LNA method (U.S. Pat. No. 6,977,295).

Particularly, the signal is generated by a duplex formed in a dependent manner on cleavage of a mediation oligonucleotide specifically hybridized with the target

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nucleic acid sequence.

The term used herein "mediation oligonucleotide" is an oligonucleotide which mediates production of a duplex not containing a target nucleic acid sequence.

According to an embodiment of the present invention, the cleavage of the mediation oligonucleotide *per se* does not generate signal and a fragment formed by the cleavage is involved in successive reactions for signal generation following hybridization and cleavage of the mediation oligonucleotide.

According to an embodiment, the hybridization or cleavage of the mediation oligonucleotide *per se* does not generate signal.

According to an embodiment of the present invention, the mediation oligonucleotide includes an oligonucleotide which is hybridized with a target nucleic acid sequence and cleaved to release a fragment, leading to mediate the production of a duplex.

The signal by the duplex formed in a dependent manner on cleavage of the mediation oligonucleotide may be generated by various methods, including PTOCE (PTO cleavage and extension) method (WO 2012/096523), PCE-SH (PTO Cleavage and Extension-Dependent Signaling Oligonucleotide Hybridization) method (WO 2013/115442) and PCE-NH (PTO Cleavage and Extension-Dependent Non-Hybridization) method (PCT/KR2013/012312).

The signal by the duplex formed in a dependent manner on cleavage of the mediation oligonucleotide includes the signal provided by inhibition of the formation of other duplex by the duplex formed in a dependent manner on cleavage of the mediation oligonucleotide (*e.g.* PCE-NH).

For example, where the signal by the duplex formed in a dependent manner on cleavage of the mediation oligonucleotide is generated by PTOCE method, the signal-generating means comprises an upstream oligonucleotide and a PTO (Probing and Tagging Oligonucleotide) having a hybridizing nucleotide sequence complementary to the target nucleic acid sequence, a CTO (Capturing and Templating Oligonucleotide), suitable label and a template-dependent nucleic acid polymerase having 5' nuclease

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activity. The PTO comprises (i) a 3'-targeting portion comprising a hybridizing nucleotide sequence complementary to the target nucleic acid sequence and (ii) a 5'-tagging portion comprising a nucleotide sequence non-complementary to the target nucleic acid sequence. The CTO comprises in a 3' to 5' direction (i) a capturing portion comprising a nucleotide sequence complementary to the 5'-tagging portion or a part of the 5'-tagging portion of the PTO and (ii) a templating portion comprising a nucleotide sequence non-complementary to the 5'-tagging portion and the 3'-targeting portion of the PTO.

The particular example of the signal generation by PTOCE method comprises the steps of:

(a) hybridizing the target nucleic acid sequence with the upstream oligonucleotide and the PTO; (b) contacting the resultant of the step (a) to an enzyme having a 5' nuclease activity under conditions for cleavage of the PTO; wherein the upstream oligonucleotide or its extended strand induces cleavage of the PTO by the enzyme having the 5' nuclease activity such that the cleavage releases a fragment comprising the 5'-tagging portion or a part of the 5'-tagging portion of the PTO; (c) hybridizing the fragment released from the PTO with the CTO; wherein the fragment released from the PTO is hybridized with the capturing portion of the CTO; and (d) performing an extension reaction using the resultant of the step (c) and a template-dependent nucleic acid polymerase; wherein the fragment hybridized with the capturing portion of the CTO is extended and an extended duplex is formed; wherein the extended duplex has a T_m value adjustable by (i) a sequence and/or length of the fragment, (ii) a sequence and/or length of the CTO or (iii) the sequence and/or length of the fragment and the sequence and/or length of the CTO; wherein the extended duplex provides a target signal by (i) at least one label linked to the fragment and/or the CTO, (ii) a label incorporated into the extended duplex during the extension reaction, (iii) a label incorporated into the extended duplex during the extension reaction and a label linked to the fragment and/or the CTO, or (iv) an intercalating label; and (e) detecting the extended duplex by measuring the target signal at a

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predetermined temperature that the extended duplex maintains its double-stranded form, whereby the presence of the extended duplex indicates the presence of the target nucleic acid sequence. In this case, the method further comprises repeating the steps (a)-(b), (a)-(d) or (a)-(e) with denaturation between repeating cycles.

In the step (a) of PTOCE method, a primer set for amplification of the target nucleic acid sequence may be used instead of the upstream oligonucleotide. In this case, the method further comprises repeating the steps (a)-(b), (a)-(d) or (a)-(e) with denaturation between repeating cycles.

The PTOCE method can be classified as a process in which the PTO fragment hybridized with the CTO is extended to form an extended strand and the extended strand is then detected. The PTOCE method is characterized that the formation of the extended strand is detected by using the duplex between the extended strand and the CTO.

There is another approach to detect the formation of the extended strand. For example, the formation of the extended strand may be detected by using an oligonucleotide specifically hybridized with the extended strand (*e.g.*, PCE-SH method). In this method, the signal may be provided from (i) a label linked to the oligonucleotide specifically hybridized with the extended strand, (ii) a label linked to the oligonucleotide specifically hybridized with the extended strand and a label linked to the PTO fragment, (iii) a label linked to the oligonucleotide specifically hybridized with the extended strand and a label incorporated into the extended strand during the extension reaction, or (iv) a label linked to the oligonucleotide specifically hybridized with the extended strand and an intercalating dye. Alternatively, the signal may be provided from (i) a label linked to the extended strand or (ii) an intercalating dye.

Alternatively, the detection of the formation of the extended strand is performed by another method in which inhibition of the hybridization between the CTO and an oligonucleotide being specifically hybridizable with the CTO is detected(*e.g.* PCE-NH method). Such inhibition is considered to be indicative of the presence of a target nucleic acid sequence. The signal may be provided from (i) a

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label linked to the oligonucleotide being hybridizable with the CTO, (ii) a label linked to the CTO, (iii) a label linked to the oligonucleotide being hybridizable with the CTO and a label linked to the CTO, or (iv) an intercalating label.

According to an embodiment, the oligonucleotide being specifically hybridizable with the CTO has an overlapping sequence with the PTO fragment.

According to an embodiment, the detection oligonucleotide includes the oligonucleotide being specifically hybridizable with the extended strand (*e.g.*, PCE-SH method) and oligonucleotide being specifically hybridizable with the CTO (*e.g.* PCE-NH method). According to an embodiment, the detection oligonucleotide includes the extended strand produced during a reaction or CTO.

The PTOCE-based methods commonly involve the formation of the extended strand depending on the presence of a target nucleic acid sequence. The term "PTOCE-based method" is used herein to intend to encompass various methods for providing signals comprising the formation of an extended strand through cleavage and extension of PTO.

The example of signal generation by the PTOCE-based methods comprises the steps of: (a) hybridizing the target nucleic acid sequence with the upstream oligonucleotide and the PTO; (b) contacting the resultant of the step (a) to an enzyme having a 5′ nuclease activity under conditions for cleavage of the PTO; wherein the upstream oligonucleotide or its extended strand induces cleavage of the PTO by the enzyme having the 5′ nuclease activity such that the cleavage releases a fragment comprising the 5′-tagging portion or a part of the 5′-tagging portion of the PTO; (c) hybridizing the fragment released from the PTO with the CTO; wherein the fragment released from the PTO is hybridized with the capturing portion of the CTO; (d) performing an extension reaction using the resultant of the step (c) and a template-dependent nucleic acid polymerase; wherein the fragment hybridized with the capturing portion of the CTO is extended to form an extended strand; and (e) detecting the formation of the extended strand by detecting signal generated dependent on the presence of the extended strand. In the step (a), a primer set for

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amplification of the target nucleic acid sequence may be used instead of the upstream oligonucleotide. In this case, the method further comprises repeating the steps (a)-(b), (a)-(d) or (a)-(e) with denaturation between repeating cycles.

According to an embodiment, the signal generated by the formation of a duplex includes signals induced by hybridization of the duplex (*e.g.*, hybridization of the duplex *per se*, or hybridization of a third oligonucleotide) or by inhibition of hybridization of a third oligonucleotide due to the formation of a duplex.

According to an embodiment, signal-generating means to generate a signal in a dependent manner on the formation of a duplex may be used for either the target nucleic acid sequence having the relatively high detection temperature or the target nucleic acid sequence having the relatively low detection temperature.

According to an embodiment, at least one of the two signal-generating means is a signal-generating means to generate a signal in a dependent manner on cleavage of a detection oligonucleotide.

Particularly, the signal is generated by hybridization of the detection oligonucleotide with a target nucleic acid sequence and then cleavage of the detection oligonucleotide.

The signal by hybridization of the detection oligonucleotide with a target nucleic acid sequence and then cleavage of the detection oligonucleotide may be generated by various methods, including TaqMan probe method (U.S. Pat. No. 5,210,015).

Where the signal is generated by TaqMan probe method, the signal-generating means includes a primer set for amplification of a target nucleic acid sequence, TaqMan probe having a suitable label (*e.g.*, interactive dual label) and nucleic acid polymerase having 5'-nuclease activity. The TaqMan probe hybridized with a target nucleic acid sequence is cleaved during target amplification and generates signal indicating the presence of the target nucleic acid sequence.

The particular example generating signal by TaqMan probe method comprises

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the step of: (a) hybridizing the primer set and TaqMan probe having a suitable label (*e.g.*, interactive dual label) with the target nucleic acid sequence; (b) amplifying the target nucleic acid sequence by using the resultant of the step (a) and nucleic acid polymerase having 5'-nuclease activity, wherein the TaqMan probe is cleaved to release the label; and (c) detecting a signal generation from the released label.

Particularly, the signal is generated by cleavage of the detection oligonucleotide in a dependent manner on cleavage of a mediation oligonucleotide specifically hybridized with the target nucleic acid sequence.

The signal by cleavage of the detection oligonucleotide in a dependent manner on cleavage of the mediation oligonucleotide may be generated by various methods, including Invader assay (U.S. Pat. No. US 5,691,142), PCEC (PTO Cleavage and Extension-Dependent Cleavage) method (WO 2012/134195) and a method described in US Pat. No. 7,309,573. In particular, the method described in US Pat No. 7,309,573 may be considered as one of PTOCE-based methods using signal generation by cleavage, and in the method, the formation of the extended strand may be detected by detecting cleavage of an oligonucleotide specifically hybridized with the CTO by the formation of the extended strand. Invader assay forms a fragment by cleavage of a mediation oligonucleotide and induces successive cleavage reactions with no extension of the fragment.

According to an embodiment, the signal generation being dependent manner on cleavage of the detection oligonucleotide is used for the target nucleic acid sequence having the relatively high detection temperature. Where the signal is generated by cleavage of the detection oligonucleotide, a released label by the cleavage may be detected at any temperatures. Therefore, the signal generated by cleavage of the detection oligonucleotide may not be employed for the target nucleic acid sequence having the relatively low detection temperature requiring restricted detection temperatures.

According to an embodiment, the signal generation being dependent on cleavage of the detection oligonucleotide is used for solely one target nucleic acid

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sequence. Where the signal generation being dependent on cleavage of the detection oligonucleotide is used for both of the two target nucleic acid sequences, the two target nucleic acid sequences may not be differentially detected depending on detection temperatures.

According to the embodiment of this invention, the signal-generating means for the target nucleic acid sequence having the relatively high detection temperature is a signal-generating means by cleavage of a detection oligonucleotide, and the signal-generating means for the target nucleic acid sequence having the relatively low detection temperature is a signal-generating means by the formation of a duplex.

According to an embodiment of this invention, the step (a) is performed in a signal amplification process concomitantly with a nucleic acid amplification.

In the present invention, the signal generated by signal-generating means may be amplified simultaneously with target amplification. Alternatively, the signal may be amplified with no target amplification.

According to an embodiment of this invention, the signal generation is performed in a process involving signal amplification together with target amplification.

According to an embodiment of this invention, the target amplification is performed in accordance with PCR (polymerase chain reaction). PCR is widely employed for target amplification in the art, including cycles of denaturation of a target sequence, annealing (hybridization) between the target sequence and primers and primer extension (Mullis et al. U.S. Pat. Nos. 4,683,195, 4,683,202 and 4,800,159; Saiki et al., (1985) Science 230, 1350-1354). The signal may be amplified by applying the signal generation methods described above (*e.g.*, TaqMan method and PTOCE-based methods) to the PCR process. According to an embodiment, the present invention provides signals by real-time PCR method. According to an embodiment, the amplification of the target nucleic acid sequence is performed by PCR (polymerase chain reaction), LCR (ligase chain reaction, see Wiedmann M, et al., "Ligase chain reaction (LCR)- overview and applications." PCR Methods and Applications 1994

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Feb;3(4):S51-64), GLCR (gap filling LCR, see WO 90/01069, EP 439182 and WO 93/00447), Q-beta (Q-beta replicase amplification, see Cahill P, et al., Clin Chem., 37(9):1482-5(1991), U.S. Pat. No. 5556751), SDA (strand displacement amplification, see G T Walker et al., Nucleic Acids Res. 20(7):16911696(1992), EP 497272), NASBA (nucleic acid sequence-based amplification, see Compton, J. Nature 350(6313):912(1991)), TMA (Transcription-Mediated Amplification, see Hofmann WP et al., J Clin Virol. 32(4):289-93(2005); U.S. Pat. No. 5888779).) or RCA (Rolling Circle Amplification, see Hutchison C.A. et al., Proc. Natl Acad. Sci. USA. 102:1733217336(2005)).

The amplification methods described above may amplify target sequences through repeating a series of reactions with or without changing temperatures. The unit of amplification comprising the repetition of a series of reactions is expressed as a "cycle". The unit of cycles may be expressed as the number of the repetition or time being dependent on amplification methods.

For example, the detection of signals may be performed at each cycle of amplification, selected several cycles or end-point of reactions. According to an embodiment, where signals are detected at at least two cycles, the detection of signal in an individual cycle may be performed at all detection temperatures or some selected detection temperatures. According to an embodiment of this invention, the detection is performed at the relatively high detection temperature in odd numbered cycles and at the relatively high detection temperature in even numbered cycles.

According to an embodiment of this invention, incubation is preformed in the conditions allowing target amplification well as signal generation by the signal-generation means.

According to an embodiment of this invention, the step (a) is performed in a signal amplification process without a nucleic acid amplification.

Where the signal is generated by methods including cleavage of an oligonucleotide, the signal may be amplified with no target amplification. For example, the step (a) may be performed with amplification of signals but with no amplification

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of target sequences in accordance with CPT method (Duck P, et al., Biotechniques, 9:142-148 (1990)), Invader assay (U.S. Pat. Nos. 6,358,691 and 6,194,149), PTOCE-based methods (*e.g.*, PCE-SH method, PCE-NH method and PCEC method) or CER method (WO 2011/037306).

The signal amplification methods described above may amplify signals through repeating a series of reactions with or without changing temperatures. The unit of signal amplification comprising the repetition of a series of reactions is expressed as a "cycle". The unit of cycles may be expressed as the number of the repetition or time being dependent on amplification methods.

For example, the generation and detection of signals may be performed at each cycle of amplification, selected several cycles or end-point of reactions.

The signals generated by the two signal-generating means are not differentiated by a single type of detector. The term "signals not differentiated by a single type of detector" means that signals are not differentiated from each other by a single type of detector due to their identical or similar signal properties (*e.g.*, optical properties, emission wavelength and electrical signal).

For example, where the same label (*e.g.*, FAM) is used for two target nucleic acid sequences and a single type of detector for detection of emission wavelength from FAM is used, signals are not differentially detected.

The term used herein "a single type of detector" means a detection means for a singly type of signal. In a detector comprising several channels (e.g., photodiodes) for several signals, each channel (e.g., a photodiode) corresponds to "a single type of detector".

According to an embodiment of this invention, the two signal-generating means comprise an identical label and signals from the label are not differentiated by the single type of detector.

The label useful in the present invention includes various labels known in the art. For example, the label useful in the present invention includes a single label, an interactive dual label, an intercalating dye and an incorporating label.

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The single label includes, for example, a fluorescent label, a luminescent label, a chemiluminescent label, an electrochemical label and a metal label. According to an embodiment, the single label provides a different signal (*e.g.*, different signal intensities) depending on its presence on a double strand or single strand. According to an embodiment, the single label is a fluorescent label. The preferable types and binding sites of single fluorescent labels used in this invention are disclosed U.S. Pat. Nos. 7,537,886 and 7,348,141, the teachings of which are incorporated herein by reference in their entity. For example, the single fluorescent label includes JOE, FAM, TAMRA, ROX and fluorescein-based label. The single label may be linked to oligonucleotides by various methods. For instance, the label is linked to probes through a spacer containing carbon atoms (*e.g.*, 3-carbon spacer, 6-carbon spacer or 12-carbon spacer).

As a representative of the interactive label system, the FRET (fluorescence resonance energy transfer) label system includes a fluorescent reporter molecule (donor molecule) and a quencher molecule (acceptor molecule). In FRET, the energy donor is fluorescent, but the energy acceptor may be fluorescent or non-fluorescent. In another form of interactive label systems, the energy donor is non-fluorescent, *e.g.*, a chromophore, and the energy acceptor is fluorescent. In yet another form of interactive label systems, the energy donor is luminescent, *e.g.* bioluminescent, chemiluminescent, electrochemiluminescent, and the acceptor is fluorescent. The interactive label system includes a dual label based on "on contact-mediated quenching" (Salvatore et al., Nucleic Acids Research, 2002 (30) no.21 e122 and Johansson et al., J. AM. CHEM. SOC 2002 (124) pp 6950-6956). The interactive label system includes any label system in which signal change is induced by interaction between at least two molecules (*e.g.* dye).

The reporter molecule and the quencher molecule useful in the present invention may include any molecules known in the art. Examples of those are: Cy2™ (506), YO-PRO™-1 (509), YOYO™-1 (509), Calcein (517), FITC (518), FluorX™ (519), Alexa™ (520), Rhodamine 110 (520), Oregon Green™ 500 (522), Oregon Green™

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488 (524), RiboGreen™ (525), Rhodamine Green™ (527), Rhodamine 123 (529), Magnesium Green™(531), Calcium Green™ (533), TO-PRO™-1 (533), TOTO1 (533), JOE (548), BODIPY530/550 (550), DiI (565), BODIPY TMR (568), BODIPY558/568 (568), BODIPY564/570 (570), Cy3™ (570), Alexa™ 546 (570), TRITC (572), Magnesium Orange™ (575), Phycoerythrin R&B (575), Rhodamine Phalloidin (575), Calcium Orange™(576), Pyronin Y (580), Rhodamine B (580), TAMRA (582), Rhodamine Red™ (590), Cy3.5™ (596), ROX (608), Calcium Crimson™ (615), Alexa™ 594 (615), Texas Red(615), Nile Red (628), YO-PRO™-3 (631), YOYO™-3 (631), R-phycocyanin (642), C-Phycocyanin (648), TO-PRO™-3 (660), TOTO3 (660), DiD DiIC(5) (665), Cy5™ (670), Thiadicarbocyanine (671), Cy5.5 (694), HEX (556), TET (536), Biosearch Blue (447), CAL Fluor Gold 540 (544), CAL Fluor Orange 560 (559), CAL Fluor Red 590 (591), CAL Fluor Red 610 (610), CAL Fluor Red 635 (637), FAM (520), Fluorescein (520), Fluorescein-C3 (520), Pulsar 650 (566), Quasar 570 (667), Quasar 670 (705) and Quasar 705 (610). The numeric in parenthesis is a maximum emission wavelength in nanometer. Preferably, the reporter molecule and the quencher molecule include JOE, FAM, TAMRA, ROX and fluorescein-based label.

Suitable fluorescence molecule and suitable pairs of reporter-quencher are disclosed in a variety of publications as follows: Pesce et al., editors, Fluorescence Spectroscopy (Marcel Dekker, New York, 1971); White et al., Fluorescence Analysis: A Practical Approach (Marcel Dekker, New York, 1970); Berlman, Handbook of Fluorescence Spectra of Aromatic Molecules, 2nd Edition (Academic Press, New York, 1971); Griffiths, Color AND Constitution of Organic Molecules (Academic Press, New York, 1976); Bishop, editor, Indicators (Pergamon Press, Oxford, 1972); Haugland, Handbook of Fluorescent Probes and Research Chemicals (Molecular Probes, Eugene, 1992); Pringsheim, Fluorescence and Phosphorescence (Interscience Publishers, New York, 1949); Haugland, R. P., Handbook of Fluorescent Probes and Research Chemicals, 6th Edition (Molecular Probes, Eugene, Oreg., 1996) U.S. Pat. Nos. 3,996,345 and 4,351,760.

It is noteworthy that a non-fluorescent quencher molecule (e.g. black quencher

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or dark quencher) capable of quenching a fluorescence of a wide range of wavelengths or a specific wavelength may be used in the present invention.

In the signaling system comprising the reporter and quencher molecules, the reporter encompasses a donor of FRET and the quencher encompasses the other partner (acceptor) of FRET. For example, a fluorescein dye is used as the reporter and a rhodamine dye as the quencher.

The interactive dual label may be linked to one strand of a duplex. Where the strand containing the interactive dual label leaves in a single stranded state, it forms a hairpin or random coil structure to induce quenching between the interactive dual label. Where the strand forms a duplex, the quenching is relieved. Alternatively, where the interactive dual label is linked to nucleotides adjacently positioned on the strand, the quenching between the interactive dual label occurs. Where the strand forms a duplex and then is cleaved, the quenching becomes relieved.

Each of the interactive dual label may be linked to each of two strands of the duplex. The formation of the duplex induces quenching and denaturation of the duplex induces unquenching. Alternatively, where one of the two stands is cleaved, the unquenching may be induced.

Exemplified intercalating dyes useful in this invention include SYBR™ Green I, PO-PRO™-1, BO-PRO™-1, SYTO™43, SYTO™44, SYTO™45, SYTOX™Blue, POPO™-1, POPO™-3, BOBO™-1, BOBO™-3, LO-PRO™-1, JO-PRO™-1, YO-PRO™1, TO-PRO™1, SYTO™11, SYTO™13, SYTO™15, SYTO™16, SYTO™20, SYTO™23, TOTO™-3, YOYO™3, GelStar™ and thiazole orange. The intercalating dyes intercalate specifically into double-stranded nucleic acid molecules to generate signals.

The incorporating label may be used in a process to generate signals by incorporating a label during primer extension (*e.g.*, Plexor method, Sherrill C B, et al., Journal of the American Chemical Society, 126:4550-45569(2004)). The incorporating label may be also used in a signal generation by a duplex formed in a dependent manner on cleavage of a mediation oligonucleotide hybridized with the target nucleic acid sequence.

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The incorporating label may be generally linked to nucleotides. The nucleotide having a non-natural base may be also used.

The term used herein "non-natural base" refers to derivatives of natural bases such as adenine (A), guanine (G), thymine (T), cytosine (C) and uracil (U), which are capable of forming hydrogen-bonding base pairs. The term used herein "non-natural base" includes bases having different base pairing patterns from natural bases as mother compounds, as described, for example, in U.S. Pat. Nos. 5,432,272, 5,965,364, 6,001,983, and 6,037,120. The base pairing between non-natural bases involves two or three hydrogen bonds as natural bases. The base pairing between non-natural bases is also formed in a specific manner. Specific examples of non-natural bases include the following bases in base pair combinations: iso-C/iso-G, iso-dC/iso-dG, K/X, H/J, and M/N (see U.S. Pat. No. 7,422,850).

Where the signal is generated by the PTOCE method, a nucleotide incorporated during the extension reaction may have a first non-natural base and the CTO may have a nucleotide having a second non-natural base with a specific binding affinity to the first non-natural base.

The term used herein "target nucleic acid", "target nucleic acid sequence" or "target sequence" refers to a nucleic acid sequence of interest for quantification. The target nucleic acid sequence comprises a sequence in a single strand as well as in a double strand. The target nucleic acid sequence comprises a sequence initially present in a nucleic acid sample as well as a sequence newly generated in reactions.

The target nucleic acid sequence may include any DNA (gDNA and cDNA), RNA molecules their hybrids (chimera nucleic acid). The sequence may be in either a double-stranded or single-stranded form. Where the nucleic acid as starting material is double-stranded, it is preferred to render the two strands into a single-stranded or partially single-stranded form. Methods known to separate strands includes, but not limited to, heating, alkali, formamide, urea and glycoxal treatment, enzymatic methods (e.g., helicase action), and binding proteins. For instance, strand separation can be achieved by heating at temperature ranging from 80°C to 105°C. General

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methods for accomplishing this treatment are provided by Joseph Sambrook, et al., *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.(2001).

Where a mRNA is employed as starting material, a reverse transcription step is necessary prior to performing annealing step, details of which are found in Joseph Sambrook, et al., *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.(2001); and Noonan, K. F. et al., *Nucleic Acids Res.* 16:10366 (1988)). For reverse transcription, an oligonucleotide dT primer hybridizable to poly A tail of mRNA, random primers or target-specific primers may be used.

The target nucleic acid sequence includes any naturally occurring prokaryotic, eukaryotic (for example, protozoans and parasites, fungi, yeast, higher plants, lower and higher animals, including mammals and humans), viral (for example, Herpes viruses, HIV, influenza virus, Epstein-Barr virus, hepatitis virus, polio virus, etc.), or viroid nucleic acid. The nucleic acid molecule can also be any nucleic acid molecule which has been or can be recombinantly produced or chemically synthesized. Thus, the nucleic acid sequence may or may not be found in nature. The target nucleic acid sequence may include known or unknown sequences.

The term used herein "sample" refers to any cell, tissue, or fluid from a biological source, or any other medium that can advantageously be evaluated according to this invention, including virus, bacteria, tissue, cell, blood, serum, plasma, lymph, milk, urine, faeces, ocular fluid, saliva, semen, brain extracts, spinal cord fluid (SCF), appendix, spleen and tonsillar tissue extracts, amniotic fluid, ascitic fluid and non-biological samples (*e.g.*, food and water). In addition, the sample includes natural-occurring nucleic acid molecules isolated from biological sources and synthetic nucleic acid molecules.

The amplification of the target nucleic acid sequence is accomplished by target amplification means including a primer set for amplification and nucleic acid polymerase.

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The nucleic acid polymerase useful in the present invention is a thermostable DNA polymerase obtained from a variety of bacterial species, including *Thermus aquaticus* (Taq), *Thermus thermophilus* (Tth), *Thermus filiformis*, *Thermis flavus*, *Thermococcus literalis*, *Thermus antranikianii*, *Thermus caldophilus*, *Thermus chliarophilus*, *Thermus flavus*, *Thermus igniterrae*, *Thermus lacteus*, *Thermus oshimai*, *Thermus ruber*, *Thermus rubens*, *Thermus scotoductus*, *Thermus silvanus*, *Thermus species Z05*, *Thermus species sps 17*, *Thermus thermophilus*, *Thermotoga maritima*, *Thermotoga neapolitana*, *Thermosipho africanus*, *Thermococcus litoralis*, *Thermococcus barossi*, *Thermococcus gorgonarius*, *Thermotoga maritima*, *Thermotoga neapolitana*, *Thermosipho africanus*, *Pyrococcus woesei*, *Pyrococcus horikoshii*, *Pyrococcus abyssi*, *Pyrodictium occultum*, *Aquifex pyrophilus* and *Aquifex aeolieus*. Particularly, the thermostable DNA polymerase is Taq polymerase.

According to an embodiment of the present invention, the amplification of the target nucleic acid sequence is accomplished by an asymmetric PCR. The ratio of primers may be selected in consideration of cleavage or hybridization of downstream oligonucleotides.

After the incubation (reaction) of the sample with two signal-generating means to generate signal, the generated signal is detected by using a single type of detector.

One of the two target nucleic acid sequences has a relatively high detection temperature and the other has a relatively low detection temperature determined by the corresponding signal-generating means.

The expression used herein "a target nucleic acid sequence has a detection temperature determined by the corresponding signal-generating means" refers to that a target nucleic acid sequence is detectable at a detection temperature pre-assigned to the target nucleic acid sequence allowing to detect a generated signal from a signal-generating means designed to generate the signal at the detection temperature.

The relatively high detection temperature is a temperature capable of generating a signal for the target nucleic acid sequence having the relatively high detection temperature and the relatively low detection temperature is a temperature

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capable of generating both of a signal for the target nucleic acid sequence having the relatively low detection temperature and a signal for the target nucleic acid sequence having the relatively high detection temperature.

One of features of the present invention is to determine differentially the presence of the two target nucleic acid sequences by detecting at different detection temperatures signals indicative of the presence of the two target nucleic acid sequences.

According to an embodiment, the detection temperatures for target nucleic acid sequences are predetermined in considering a temperature range to allow signal generation by the signal-generating means.

The present invention uses that there is a certain temperature range to allow signal generation in a dependent manner on signal-generating means.

For example, when a signal-generating means generates a signal upon hybridization (or association) between two nucleic acid molecules and do not generate a signal upon non-hybridization (or dissociation) between them, a signal is generated at temperatures allowing hybridization between two nucleic acid molecules, however, no signal is generated at temperatures failing to hybridize between two nucleic acid molecules. As such, there is a certain temperature range to allow signal generation (*i.e.*, signal detection) and other temperature range not to allow signal generation (*i.e.*, signal detection). The temperature ranges are affected by the T_m value of the hybrid of the two nucleic acid molecules employed in the signal-generation means.

Where the signal generation method using a released fragment with a label after cleavage is employed, the signal may be theoretically detected at any temperature (*e.g.*, 30-95°C).

A detection temperature is selected from the temperature range to allow signal generation by the signal generation mean.

The term "the detection temperature range" is used herein to particularly describe the temperature range to allow signal generation (*i.e.*, signal detection).

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Where there are different detection temperature ranges depending on signal-generating means for the two target nucleic acid sequences, a non-overlapped detection temperature range may be selected as the relatively high detection temperature. A target nucleic acid sequence detected by signal-generating means providing the relatively high detection temperature is determined as the target nucleic acid sequence having the relatively high detection temperature. An overlapped detection temperature range may be selected as the relatively low detection temperature. A target nucleic acid sequence detected by signal-generating means providing the relatively low detection temperature and not providing the relatively high detection temperature is determined as the target nucleic acid sequence having the relatively low detection temperature.

According to the present invention, a temperature for detecting the presence of each of target nucleic acid sequences may be allocated in considering signal-generating means.

According to an embodiment of this invention, one of the two target nucleic acid sequences is assigned with a relatively high detection temperature and the other is assigned with a relatively low detection temperature, and then signal-generating means suitable for the detection temperatures are constructed, followed by performing the step (a).

According to an embodiment, the relatively high detection temperature and the relatively low detection temperature at which the detection is carried out may be predetermined. For example, the relatively high detection temperature and the relatively low detection temperature are predetermined as 72°C and 60°C, respectively, and then signal-generating means suitable for the detection temperatures are constructed, followed by performing the step (a).

According to an embodiment, signal-generating means for the two target nucleic acid sequences are firstly constructed and then detection temperatures for the two target nucleic acid sequences are allocated, followed by performing the step (a).

According to an embodiment of this invention, when the signal-generating

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means generates a signal in a dependent manner on the formation of a duplex, the detection temperature is selected based on a T_m value of the duplex.

According to an embodiment of this invention, when the signal-generating means generates a signal in a dependent manner on the formation of a duplex, the detection temperature is controllable by adjusting a T_m value of the duplex.

For example, where the signal is generated by a detection oligonucleotide specifically hybridized with the target nucleic acid sequence (*e.g.*, Lux probe, Molecular Beacon probe, HyBeacon probe and adjacent hybridization probe), the detection of the signal is successfully done at the predetermined temperature by adjusting the T_m value of the oligonucleotide. Where Scorpion primer is used, the detection of the signal is successfully done at the predetermined temperature by adjusting the T_m value of a portion to be hybridized with extended strand.

Where the signal is generated by the duplex formed dependent on the presence of the target nucleic acid sequence, the detection of the signal is successfully done at the predetermined temperature by adjusting the T_m value of the duplex. For example, where the signal is generated by the PTOCE method, the detection of the signal is successfully done at the predetermined temperature by adjusting the T_m value of the extended duplex formed by the extension of the PTO fragment on the CTO.

The PTOCE-based methods have advantages to readily adjust T_m values of the duplex or a third hybrid whose hybridization is affected by the duplex.

According to an embodiment of this invention, when the signal-generating means generates a signal in a dependent manner on cleavage of a detection oligonucleotide, the detection temperature is arbitrarily selected. As described above, where the signal is generated being dependent manner on cleavage of the detection oligonucleotide, the label released by the cleavage may be detected at various temperatures.

According to an embodiment, where the signal is generated being dependent manner on cleavage of the detection oligonucleotide, the detection temperature is

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selected to be a relatively highest detection temperature.

As discussed above, the detection temperature is determined in considering a detection temperature range depending on signal-generating means. Therefore, the signal detection at a certain detection temperature may be described as follows: the detection at the relatively high detection temperature is to detect the target nucleic acid sequence having the relatively high detection temperature, and the detection at the relatively low detection temperature is to detect both the target nucleic acid sequence having the relatively low detection temperature and the target nucleic acid sequence having the relatively high detection temperature.

For instance, where both signals for the target nucleic acid sequence having the relatively low detection temperature and the target nucleic acid sequence having the relatively high detection temperature are generated by the PTOCE method, the signal for the target nucleic acid sequence having the relatively high detection temperature is generated by an extended duplex having a T_m value suitable for the relatively high detection temperature, and the signal for the target nucleic acid sequence having the relatively low detection temperature is generated by an extended duplex having a T_m value suitable for the relatively low detection temperature. When the signal is detected at the relatively high detection temperature, the extended duplex having a T_m value suitable for the relatively low detection temperature is dissociated to be in a single strand and therefore no signal is generated, thereby detecting only the signal for the target nucleic acid sequence having the relatively high detection temperature. When the signal is detected at the relatively low detection temperature, all of the extended duplex having a T_m value suitable for the relatively high detection temperature and the extended duplex having a T_m value suitable for the relatively low detection temperature have their duplex form, thereby detecting both of the signal for the target nucleic acid sequence having the relatively low detection temperature and the signal for the target nucleic acid sequence having the relatively high detection temperature.

In another example, where the signal for the target nucleic acid sequence

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having the relatively high detection temperature is generated by TaqMan method and the signal for the target nucleic acid sequence having the relatively low detection temperature is generated by the PTOCE method, the signal for the target nucleic acid sequence having the relatively high detection temperature is provided by a released fluorescent label and the signal for the target nucleic acid sequence having the relatively low detection temperature is provided by an extended duplex having a T_m value suitable for the relatively low detection temperature. When the signal is detected at the relatively high detection temperature, the extended duplex having a T_m value suitable for the relatively low detection temperature is dissociated to be in a single strand and therefore no signal is generated, thereby detecting only the signal from the released fluorescent label for the target nucleic acid sequence having the relatively high detection temperature. When the signal is detected at the relatively low detection temperature, not only the signal provided from the extended duplex having a T_m value suitable for the relatively low detection temperature but also the signal from the released fluorescent label are detected, thereby detecting both of the signal for the target nucleic acid sequence having the relatively low detection temperature and the signal for the target nucleic acid sequence having the relatively high detection temperature.

The detector used in the present invention includes any means capable of detecting signals. For example, where fluorescent signals are used, photodiodes suitable in detection of the fluorescent signals may be employed as detectors. The detection using a single type of detector means that the detection is performed by using a detect capable of single type of signal or using each channel (*i.e.*, photodiode) of a detector carrying several channels (*i.e.*, photodiodes).

According to an embodiment, the generation of signals includes "signal generation or extinguishment" and "signal increase or decrease" from labels.

Step (b): <u>Determining the presence of target nucleic acid sequences</u>

Following the detection of the signal, the presence of the two target nucleic

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acid sequences is determined by using the signal detected in the step (a).

The presence of the target nucleic acid sequence having the relatively high detection temperature is determined by using the signal detected at the relatively high detection temperature. The presence of the target nucleic acid sequence having the relatively low detection temperature is determined by using a difference between the signal detected at the relatively high detection temperature and the signal detected at the relatively low detection temperature.

The signals used for determination of target presence includes that various signal characteristics from the signal detection, *e.g.*, signal intensity [*e.g.*, RFU (relative fluorescence unit) value or in the case of performing amplification, RFU values at a certain cycle, at selected cycles or at end-point], signal change pattern or C_t value, or values obtained by mathematically processing the characteristics.

According to an embodiment of this invention, when an amplification curve is obtained by real-time PCR, various signal values (or characteristics) from the amplification curve may be selected used for determination of target presence.

The characteristics of the signal obtained at the relatively high detection temperature *per se* may be used to determine the presence of the target nucleic acid sequence having the relatively high detection temperature.

Alternatively, a modified signal provided by mathematically processing the characteristics of the signal may be used to determine the presence of the target nucleic acid sequence having the relatively high detection temperature.

The characteristics of the signals at the relatively high detection temperature *per se* and the relatively high detection temperature *per se* may be used to obtain the difference between the signals at relatively high detection temperature and a relatively low detection temperature.

Alternatively, one or both of the signals at the relatively high detection temperature and the relatively high detection temperature may be modified by mathematically processing the characteristics of the signal and used to obtain the difference between the signals at relatively high detection temperature and a

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relatively low detection temperature.

According to an embodiment, where the mathematical processing is done, the characteristics of the signal should be characteristics vulnerable to the mathematical processing. In certain embodiment, the mathematical processing includes calculation (*e.g.*, addition, multiplication, subtraction and division) using signals.

The signals used for determination of the presence of target nucleic acid sequences in the present invention generally are a significant signal. In other words, the signals are signal to be generated being dependent on the presence of target nucleic acid sequences. In the meantime, where the difference between the signal detected at the relatively high detection temperature and the signal detected at the relatively low detection temperature is calculated, signal without significance such as background signals may be used to calculate the difference. In this regard, it would be understood that the signals used for determination of the presence of target nucleic acid sequences encompass not only signals with significance but also signals without significance so long as they can be used to calculate the difference or involved in a determination process.

According to an embodiment, significance of signals detected may be determined using a threshold value. For example, a threshold value is predetermined from a negative control in considering background signals of detector, sensitivity or label used, and then the significance of signals from samples may be determined.

Where a signal (*i.e.,* a significant signal) is detected at the relatively high detection temperature, it is determined that the target nucleic acid sequence having the relatively high detection temperature is present.

The term used herein "using a signal" includes using numerical values of signals or their modifications and using the presence/absence of signals.

According to an embodiment of this invention, the presence of the target nucleic acid sequence having the relatively low detection temperature is determined by using the signal detected at the relatively high detection temperature and the

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signal detected at the relatively low detection temperature.

Interestingly, the present inventors have found that when signals indicating the presence of a single target nucleic acid sequence are detected in a single reaction vessel at predetermined two detection temperatures, there is a signal change in a certain pattern (rule). The feature of the present invention is to adopt the findings to detection of target nucleic acid sequences. Because signals for a target nucleic acid sequence in a single reaction vessel are detected with differing only detection temperatures (*e.g.* no change of amount of the target or no variation of buffer conditions), there is a certain pattern (rule) in a signal change between the two detection temperatures.

According to an embodiment, the present method is performed in a condition that permits a certain pattern (rule) in a signal change between the two detection temperatures.

Under the principle, the presence of the target nucleic acid sequence having the relatively low detection temperature may be determined by using the signal detected at the relatively high detection temperature and the signal detected at the relatively low detection temperature. According to an embodiment of this invention, the extent (or portion) of signal of the target nucleic acid sequence having the relatively low detection temperature among the signal detected at the relatively low detection temperature may be obtained under the principle by using the signal at the relatively high detection temperature.

According to an embodiment, the presence of the target nucleic acid sequence having the relatively low detection temperature is determined by using a difference between the signal detected at the relatively high detection temperature and the signal detected at the relatively low detection temperature.

Where a signal is detected at the relatively low detection temperature, said signal *per se* does not permit to determine the presence of the target nucleic acid sequence having the relatively low detection temperature. The reason for those is that the signal for the target nucleic acid sequence having the relatively high detection

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temperature may be detected at the relatively low detection temperature. One of the features of the present invention is to employ the signal detected at the relatively high detection temperature for analyzing the signal detected at the relatively low detection temperature, whereby the presence of the target nucleic acid sequence having the relatively low detection temperature can be determined.

For example, (i) where only the target nucleic acid sequence having the relatively high detection temperature is present in the sample, signal is detected at both the relatively high detection temperature and the relatively low detection temperature. The signal detected at the relatively high detection temperature is likely to be different from that detected at the relatively low detection temperature. Such difference is very likely to be within a certain range because all conditions except for detection temperatures are common. Where the difference calculated for a sample falls within the certain range, the signal detected at the relatively low detection temperature is due to only the target nucleic acid sequence having the relatively high detection temperature. In other words, the target nucleic acid sequence having the relatively low detection temperature can be determined to be absent in the sample.

(ii) Where both the target nucleic acid sequence having the relatively high detection temperature and the target nucleic acid sequence having the relatively low detection temperature are present in a sample, signals are detected at both the relatively high detection temperature and the relatively low detection temperature. The difference between the signals is distinctly greater than the difference in the case (i) because the target nucleic acid sequence having the relatively low detection temperature is present. The presence of the target nucleic acid sequence having the relatively low detection temperature can be determined by using the difference.

(iii) Where only the target nucleic acid sequence having the relatively low detection temperature is present in a sample, signal is detected at the relatively low detection temperature and not at the relatively high detection temperature. No signal detection at the relatively high detection temperature indicates the absence of the target nucleic acid sequence having the relatively high detection temperature, such

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that the signal detected at the relatively low detection temperature can be recognized to be due to the target nucleic acid sequence having the relatively low detection temperature, whereby the presence of the target nucleic acid sequence having the relatively low detection temperature can be determined.

Alternatively, in the case (iii), the difference may be obtained by using signal without significance (*e.g.*, background signal) detected at the relatively high detection temperature. In this alternative, the difference is very likely to be distinctly different from the difference in the case (i), whereby the presence of the target nucleic acid sequence having the relatively low detection temperature can be determined.

The term used herein "difference" with conjunction to "using the difference between the signals" includes not only a difference to be obtained by mathematically processing signals *per se* or modified signals but also a difference due to the presence and absence of signals.

According to an embodiment of this invention, using the difference between the signal detected at the relatively high detection temperature and the signal detected at the relatively low detection temperature comprises using a difference to be obtained by mathematically processing the signal detected at the relatively high detection temperature and the signal detected at the relatively low detection temperature.

According to an embodiment of this invention, when the signal is not detected at the relatively high detection temperature, using the difference between the signal detected at the relatively high detection temperature and the signal detected at the relatively low detection temperature comprises using the signal detected at the relatively low detection temperature with considering no detection of the signal at the relatively high detection temperature.

According to an embodiment, a background signal detected at the relatively high detection temperature may be treated as "0" or "1" for calculating the difference.

According to an embodiment, where a minus value is obtained during calculation, it is converted to absolute value and used to obtain the difference

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According to an embodiment of this invention, the signal for the target nucleic acid sequence having the relatively high detection temperature is a calculation parameter to analyze the signal for the target nucleic acid sequence having the relatively low detection temperature.

The signals for determining the presence of the target nucleic acid sequence having the relatively high detection temperature and the difference between the signal detected at the relatively high detection temperature and the signal detected at the relatively low detection temperature may have different dimensions or units from each other or have same dimensions or units from each other.

The term used herein "using a signal" includes using numerical values of signals or their modifications and using the presence/absence of signals.

The term used herein "using a difference" includes using occurrence/non-occurrence of a difference, using value or range of a difference with a numerical value and using a plotting result of the difference. Furthermore, "using a difference" includes obtaining a value (e.g. C_T) for the target nucleic acid having a relative low detection temperature on the basis of the difference.

The mathematical processing of the signals may be carried out by various calculation methods and their modifications.

According to an embodiment of this invention, the mathematical processing of the signals to obtain the difference between the signals is a calculation of a ratio of the signal detected at the relatively low detection temperature to the signal detected at the relatively high detection temperature.

According to an embodiment of this invention, the mathematical processing of the signals to obtain the difference between the signals is a calculation of a ratio of the signal detected at the relatively high detection temperature to the signal detected at the relatively low detection temperature.

The term used herein "ratio" means a relationship between two numbers. By using the ratio, the presence of the target nucleic acid sequence having the relatively low detection temperature may be determined. Where the ratio of the signal detected

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at the relatively low detection temperature to the signal detected at the relatively high detection temperature is significant, it becomes entitled as an indicator for the presence of the target nucleic acid sequence having the relatively low detection temperature. For instance, where the ratio of the end-point intensity of the signal detected at the relatively low detection temperature to the end-point intensity of the signal detected at the relatively high detection temperature is significant (e.g., increase in the end-point intensity), it indicates the presence of the target nucleic acid sequence having the relatively low detection temperature.

The mathematical processing may be carried out in various fashions.

The mathematical processing may be carried out by use of a machine. For example, the signals may be undergone a mathematical processing by a processor in a detector or real-time PCR device. Alternatively, the signals may be manually undergone a mathematical processing.

According to an embodiment of this invention, the method uses a reference value, for determining the presence of the target nucleic acid sequence having the relatively low detection temperature, obtained by (i) incubating the target nucleic acid sequence having the relatively high detection temperature with a signal-generating means for detection of the target nucleic acid sequence having the relatively high detection temperature in a reaction vessel other than the reaction vessel in the step (a), (ii) detecting signals at both the relatively high detection temperature and the relatively low detection temperature, and (iii) then obtaining a difference between the signal detected at the relatively high detection temperature and the signal detected at the relatively low detection temperature.

According to an embodiment of this invention, signal-generating means for the reference value may be the same as that for the detection of the target nucleic acid sequence.

For a target nucleic acid sequence, the reference values may be obtained in various concentrations of the target nucleic acid sequence. Particularly, the reference value may be modified in considering standard variation, specificity or sensitivity.

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According to an embodiment of this invention, the reference value is a value reflecting a pattern (rule) of a signal change in the difference temperatures.

According to an embodiment of this invention, where there is a significant difference between the reference value and the difference obtained for determining the presence of the target nucleic acid sequence having the relatively low detection temperature, the target nucleic acid sequence having the relatively low detection temperature is then determined to be present. The reference value may be expressed with the same value type as the difference obtained for determining the presence of the target nucleic acid sequence having the relatively low detection temperature (*e.g.*, ratio of end-point values of signal intensities).

In a particular example, where the ratio of the end-point value of the signal intensity detected at the relatively high detection temperature to the end-point value of the signal intensity detected at the relatively low detection temperature is 1.8 and the reference value is 1.1, it can be determined that there is a significant difference between the reference value and the difference obtained for determining the presence of the target nucleic acid sequence having the relatively low detection temperature. It indicates the presence of the target nucleic acid sequence having the relatively low detection temperature.

According to an embodiment, where the difference for determining the presence of the target nucleic acid sequence having the relatively low detection temperature is same or higher than the reference value, the target nucleic acid sequence having the relatively low detection temperature is then determined to be present.

According to an embodiment, where the difference for determining the presence of the target nucleic acid sequence having the relatively low detection temperature is same or lower than the reference value, the target nucleic acid sequence having the relatively low detection temperature is then determined to be present.

Alternatively, the reference value may be used to calculate the difference

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between the signal detected at the relatively high detection temperature and the signal detected at the relatively low detection temperature. For example, the difference for determining the presence of the target nucleic acid sequence having the relatively low detection temperature is calculated such a manner that the signal (*e.g.* RFU) detected at the relatively high detection temperature is multiplied by the reference value and then the multiplication result is subtracted with the signal (*e.g.* RFU) detected at the relatively low detection temperature. Where a difference is obtained, the target nucleic acid sequence having the relatively low detection temperature can be determined to be present.

According to an embodiment, the reference value is used to determine the presence of the target nucleic acid sequence having the relatively low detection temperature, when a signal for the target nucleic acid sequence having the relatively high detection temperature is detected or when a difference between the signals at . the relatively high detection temperature and the relatively low detection temperature is obtained by mathematical process.

According to an embodiment, where signals are generated in a real-time manner associated with target amplification by PCR, the mathematical processing of the signals comprises calculations of the ratio of a signal intensity detected at the relatively high detection temperature to a signal intensity detected at the relatively low detection temperature at each amplification cycle. The calculation results are plotted against cycles and used for determination of the presence of the target nucleic acid sequence having the relatively low detection temperature.

According to an embodiment, where signals are generated in a real-time manner associated with target amplification by PCR, C_t value is a signal for detection target.

The C_t value of the target nucleic acid sequence having the relatively low detection temperature may be determined using the signals detected at the relatively high detection temperature and at the relatively low detection temperature, which is exemplified as follows: Firstly, a real-time PCR is performed for a sample to be

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analyzed and the signals detected at the relatively high detection temperature and at the relatively low detection temperature are obtained, followed by obtaining amplification curves of the two detection temperatures.

(a) In the detection at the relatively high detection temperature, where there is no C_t value of the target nucleic acid sequence having the relatively high detection temperature, it can be determined that the target nucleic acid sequence having the relatively high detection temperature is not present. Then, the C_t value of the target nucleic acid sequence having the relatively low detection temperature is calculated from the amplification curve obtained in relatively low detection temperature. Where the target nucleic acid sequence having the relatively low detection temperature is also absent, there is no C_t value of the target nucleic acid sequence having the relatively low detection temperature.

(b) In the detection at the relatively high detection temperature, where there is C_t value of the target nucleic acid sequence having the relatively high detection temperature, a ratio of the RFU value obtained at the relatively low detection temperature to the RFU value obtained at the relatively high detection temperature at the cycle showing the C_t value is then calculated. Ratios of RFU values obtained at cycles following the cycle showing the C_t value are also calculated. (i) Where all ratios of the RFU values are lower than a reference value (*e.g.* a value obtained using only the target nucleic acid sequence having the relatively high detection temperature as described above), the target nucleic acid sequence having the relatively low detection temperature is determined to be absent. Therefore, there is no C_t value of the target nucleic acid sequence having the relatively low detection temperature. (ii) Where all ratios of the RFU values are higher than the reference value, C_t value calculated from amplification curve obtained at the relatively low detection temperature is determined as C_t value of the target nucleic acid sequence having the relatively low detection temperature. (iii) Where the ratio of the RFU values at the cycle showing the C_t value is lower than the reference value and the ratio of the RFU values after a certain cycle are higher than the reference value, the certain cycle is determined as C_t value of the

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target nucleic acid sequence having the relatively low detection temperature.

Where ratios calculated are the same as the reference value, the determination may be arbitrarily made. For example, the examples described above describe that the determination is made with considering whether the ratios are less than or no less than reference values. In addition, the determination may be made with considering whether the ratios are no more than or more than reference values.

The C_t value of the target nucleic acid sequence having the relatively low detection temperature may be alternatively calculated as follows: the ratio of the RFU value obtained at the relatively low detection temperature to the RFU value obtained at the relatively high detection temperature is calculated for each cycle; and C_t value is then calculated with consideration of a threshold value.

The C_t value of the target nucleic acid sequence having the relatively low detection temperature may be alternatively calculated as follows: The RFU value obtained at the relatively high detection temperature at each cycle is modified with a reference value of each cycle; the ratio of the RFU value obtained at the relatively low detection temperature to the modified RFU value is calculated for each cycle; and C_t value is then calculated.

According to an embodiment of this invention, using the signal detected at the relatively high detection temperature comprises obtaining a qualifying value for determining the presence of the target nucleic acid sequence having the relatively high detection temperature and said using the difference comprises obtaining a qualifying value for determining the presence of the target nucleic acid sequence having the relatively low detection temperature.

According to an embodiment of this invention, using the difference comprises obtaining a qualifying value for determining the presence of the target nucleic acid sequence having the relatively low detection temperature, and the qualifying value is obtained by (i) mathematically processing the signal detected at the relatively high detection temperature and the signal detected at the relatively low detection temperature or (ii) using the signal detected at the relatively low detection

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temperature with considering no detection of the signal at the relatively high detection temperature when the signal is not detected at the relatively high detection temperature.

The qualifying values may be further mathematically processed to obtain modified values. The qualifying values are used to determine the presence of the two target nucleic acid sequences in the sample.

According to an embodiment of this invention, the two target nucleic acid sequences comprises a nucleotide variation and one of the two target nucleic acid sequences comprises one type of the nucleotide variation and the other comprises the other type of the nucleotide variation.

The term "nucleotide variation" used herein refers to any single or multiple nucleotide substitutions, deletions or insertions in a DNA sequence at a particular location among contiguous DNA segments that are otherwise similar in sequence. Such contiguous DNA segments include a gene or any other portion of a chromosome. These nucleotide variations may be mutant or polymorphic allele variations. For example, the nucleotide variation detected in the present invention includes SNP (single nucleotide polymorphism), mutation, deletion, insertion, substitution and translocation. Exemplified nucleotide variation includes numerous variations in a human genome (*e.g.*, variations in the MTHFR (methylenetetrahydrofolate reductase) gene), variations involved in drug resistance of pathogens and tumorigenesis-causing variations. The term nucleotide variation used herein includes any variation at a particular location in a nucleic acid sequence. In other words, the term nucleotide variation includes a wild type and its any mutant type at a particular location in a nucleic acid sequence.

According to an embodiment of this invention, the nucleotide variation detected by the present invention is a SNP (single nucleotide polymorphism).

According to an embodiment of this invention, one of the SNP alleles has a relatively high detection temperature and the other has a relatively low detection

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temperature determined by the corresponding signal-generating means.

The advantages of the present invention become more prominent for detection of SNP.

Where a detection temperature for the wild type allele is the relatively high detection temperature, a detection temperature for the mutant allele is the relatively low detection temperature and the sample is mutant homozygous, a signal will be not detected at the relatively high detection temperature and a signal will be detected at the relatively high detection temperature. The sample will be determined to contain no wild type allele and contain mutant type allele. Meanwhile, even when a false signal at the relatively high detection temperature is generated for the mutant homozygote sample, calculating result of the difference between the signal detected at the relatively high detection temperature and the signal detected at the relatively low detection temperature for determining the presence of the SNP allele having the relatively low detection temperature permits to verify whether the signal detected at the relatively high detection temperature is a false positive signal or not. The reason is that a heterozygote for SNP comprises the wild type allele and the mutant allele in 1:1 ratio.

II. SNP Genotyping Using Different Detection Temperatures

In another aspect of this invention, there is provided a method for SNP genotyping of a nucleic acid sequence in a sample using different detection temperatures, comprising:

(a) incubating the sample comprising the nucleic acid sequence containing a SNP (single nucleotide polymorphism) site and a signal-generating means for detection of SNP alleles in a single reaction vessel and detecting a generated signal by using a single type of detector; wherein each of the SNP alleles is detected by a corresponding signal-generating means; wherein one of the SNP alleles has a relatively high detection temperature and the other has a relatively low detection temperature determined by the corresponding signal-generating means; wherein the

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relatively high detection temperature is a temperature capable of generating a signal for the SNP allele having the relatively high detection temperature and the relatively low detection temperature is a temperature capable of generating both of a signal for the SNP allele having the relatively low detection temperature and a signal for the SNP allele having the relatively high detection temperature; wherein signals to be generated by the signal-generating means are not differentiated by the single type of detector; wherein the detection is performed at both the relatively high detection temperature and the relatively low detection temperature; and

(b) determining a SNP genotype by using a difference between the signal detected at the relatively high detection temperature and the signal detected at the relatively low detection temperature in the step (a).

Since the present invention follows in principle the first aspect of this invention described above, the common descriptions between them are omitted in order to avoid undue redundancy leading to the complexity of this specification.

Step (a): <u>Incubation with signal generating means and signal detection</u>

Firstly, the sample comprising the nucleic acid sequence containing a SNP (single nucleotide polymorphism) site is incubated with a signal-generating means for detection of SNP alleles in a single reaction vessel and then a generated signal is detected by using a single type of detector. Signals to be generated by the signal-generating means are not differentiated by the single type of detector.

The nucleic acid sequence containing a SNP site may include a chromosome pair of human.

One of the SNP alleles has a relatively high detection temperature and the other has a relatively low detection temperature determined by the corresponding signal-generating means; wherein the relatively high detection temperature is a temperature capable of generating a signal for the SNP allele having the relatively high detection temperature and the relatively low detection temperature is a

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temperature capable of generating both of a signal for the SNP allele having the relatively low detection temperature and a signal for the SNP allele having the relatively high detection temperature.

Step (b): Determining a SNP genotype

Following the detection of the signal, SNP genotyping is determined by using difference between the signal detected at the relatively high detection temperature and the signal detected at the relatively low detection temperature in the step (a).

The present invention allows for SNP genotyping only by using difference between the signal detected at the relatively high detection temperature and the signal detected at the relatively low detection temperature with no determining for the presence of the SNP allele having the relatively high detection temperature.

According to an embodiment, the difference is obtained by mathematically processing the signal detected at the relatively high detection temperature and the signal detected at the relatively low detection temperature.

According to an embodiment, a background signal detected at the relatively high detection temperature is used to calculate the difference.

According to an embodiment, a background signal detected at the relatively high detection temperature may be treated as "0" or "1" for calculating the difference.

According to an embodiment, where a minus value is obtained during calculation, it is converted to absolute value and used to obtain the difference.

According to an embodiment of this invention, the step (b) for determining SNP genotype is performed with no determining the presence of the SNP allele having the relatively high detection temperature. SNP genotyping is performed by using the difference between the signal detected at the relatively high detection temperature and the signal detected at the relatively low detection temperature.

The reason for that no requirement for determining the presence of the SNP allele having the relatively high detection temperature is that a heterozygote for SNP comprises the wild type allele and the mutant allele in 1:1 ratio. By combining the

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reason with the principle of the present invention, SNP genotyping can be made with no determining the presence of the SNP allele having the relatively high detection temperature.

Where a detection temperature for the wild type allele is the relatively high detection temperature, a detection temperature for the mutant allele is the relatively low detection temperature, and the difference between signals detected at the relatively high detection temperature and the relatively low detection temperature is processed to obtain a ratio, the wild homozygote sample show a ratio within a certain range and particularly, the heterozygote sample shows a ratio within a certain range. For example, the wild homozygote sample shows a ratio of around for 1.0 and the heterozygote sample for SNP shows a ratio of around 2.0. Where the sample is mutant homozygous, a background signal detected at the relatively high detection temperature may be used for the calculation of the ratio between the signal detected at the relatively high detection temperature and the signal detected at the relatively low detection temperature. In such case, ratio calculated may show a value much higher than 2.0, which indicates that the SNP genotype of the sample is mutant homozygous.

Furthermore, even when the false signal at the relatively high detection temperature is generated for the mutant homozygote sample, the ratio will show a value much higher than 2.0, which indicates that the SNP genotype of the sample is mutant homozygous.

Therefore, the present invention for SNP genotype enables to determine the SNP genotype by using only the difference between the signal detected at the relatively high detection temperature and the signal detected at the relatively low detection temperature.

III. Detection of at least Three Target Nucleic Acid Sequences in a Sample Using Different Detection Temperatures

In still another aspect of this invention, there is provided a method for

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detecting at least three target nucleic acid sequences in a sample using different detection temperatures, comprising:

(a) incubating the sample with at least three signal-generating means for detection of the at least three target nucleic acid sequences in a single reaction vessel and detecting a generated signal by using a single type of detector; wherein each of the at least three target nucleic acid sequences is detected by a corresponding signal-generating means; wherein each of the at least three target nucleic acid sequences has a different detection temperature determined by the corresponding signal-generating means; wherein a detection temperature is a temperature capable of generating not only a signal for a target nucleic acid sequence having the detection temperature but also a signal for a target nucleic acid sequence having a higher detection temperature than the detection temperature; wherein signals to be generated by the signal-generating means are not differentiated by the single type of detector; wherein the detection is performed at each of the different detection temperatures; and

(b) determining the presence of the at least three target nucleic acid sequences by using the signal detected in the step (a); wherein when the presence of a target nucleic acid sequence having a certain detection temperature among the at least three target nucleic acid sequences is determined, the presence of the target nucleic acid sequence having the certain detection temperature is determined by using a difference between the signal detected at one or more detection temperatures higher than the certain detection temperature and a signal detected at the certain detection temperature; wherein when the certain detection temperature is a relatively highest detection temperature among the detection temperatures, the presence of a target nucleic acid sequence is determined by using the signal detected at the certain detection temperature.

Since the present invention follows in principle the first aspect of this invention described above, the common descriptions between them are omitted in order to

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avoid undue redundancy leading to the complexity of this specification.

Step (a): Incubation with signal-generating means and signal detection

Firstly, the sample to be analyzed is incubated with at least three signal-generating means for detection of the at least three target nucleic acid sequences in a single reaction vessel and then a generated signal is detected by using a single type of detector. Signals to be generated by the at least three signal-generating means are not differentiated by the single type of detector.

The number of the target nucleic acid sequences to be detected by the present invention is not limited, including more than 3, 4, 5, 6, 7, 8, 9 and 10 target nucleic acid sequences in the single reaction vessel.

Each of the at least three target nucleic acid sequences is detected by a corresponding signal-generating means. Each of the at least three target nucleic acid sequences has a different detection temperature determined by the corresponding signal-generating means.

One of the target nucleic acid sequences has a relatively highest detection temperature. A signal-generating means capable of providing a signal at a relatively highest detection temperature is used to detect the target nucleic acid sequence.

A detection temperature is a temperature capable of generating not only a signal for a target nucleic acid sequence having the detection temperature but also a signal for a target nucleic acid sequence having a higher detection temperature than the detection temperature. The detection is performed at each of the different detection temperatures.

According to an embodiment, the step (a) is performed in a signal amplification process concomitantly with a nucleic acid amplification.

According to an embodiment, the step (a) is performed in a signal amplification process without a nucleic acid amplification.

According to an embodiment, at least one of the signal-generating means is a signal-generating means to generate a signal in a dependent manner on the

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formation of a duplex. According to an embodiment, the signal is generated by the formation of a duplex between a target nucleic acid sequence and a detection oligonucleotide specifically hybridized with the target nucleic acid sequence. According to an embodiment, the signal is generated by a duplex formed in a dependent manner on cleavage of a mediation oligonucleotide specifically hybridized with the target nucleic acid sequence.

According to an embodiment, at least one of the signal-generating means is a signal-generating means to generate a signal in a dependent manner on cleavage of a detection oligonucleotide. According to an embodiment, the signal is generated by hybridization of the detection oligonucleotide with a target nucleic acid sequence and then cleavage of the detection oligonucleotide. According to an embodiment, the signal is generated by cleavage of the detection oligonucleotide in a dependent manner on cleavage of a mediation oligonucleotide specifically hybridized with the target nucleic acid sequence.

According to an embodiment, the signal generation being dependent manner on cleavage of the detection oligonucleotide is used for the target nucleic acid sequence having the relatively highest detection temperature among the at least three target nucleic acid sequences.

According to an embodiment of this invention, the signal-generating means for the target nucleic acid sequence having the relatively highest detection temperature is a signal-generating means by cleavage of a detection oligonucleotide, and the signal-generating means for the other target nucleic acid sequences are a signal-generating means by the formation of a duplex.

According to an embodiment of this invention, the at least three signal-generating means comprise an identical label and signals from the label are not differentiated by the single type of detector.

According to an embodiment of this invention, when the signal-generating means generates a signal in a dependent manner on the formation of a duplex, the detection temperature is selected based on a T_m value of the duplex.

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According to an embodiment of this invention, when the signal-generating means generates a signal in a dependent manner on cleavage of a detection oligonucleotide, the detection temperature is arbitrarily selected. According to an embodiment of this invention, the signal-generating means by cleavage of a detection oligonucleotide can provide a relatively highest detection temperature.

According to an embodiment, the detection temperatures for target nucleic acid sequences are predetermined in considering a temperature range to allow signal generation by the signal-generating means.

According to an embodiment, the detection temperature for each target nucleic acid sequence is predetermined in considering a temperature range to allow signal generation by the signal-generating means for detection of each target nucleic acid sequence. The detection temperatures may be predetermined in considering non-overlapped detection temperature range and overlapped detection temperature range among the detection temperatures.

The detection at a certain detection temperature is to detect a signal for a target nucleic acid sequence having the detection temperature but also a signal for a target nucleic acid sequence having a higher detection temperature than the detection temperature.

For example, where the target nucleic acid sequences comprise three target sequences and detection temperatures 72°C, 60°C and 50°C are assigned to the three target sequences, respectively, the detection at 50°C includes not only detection of the signal for a target nucleic acid sequence having 50°C detection temperature but also detection of signals for target nucleic acid sequences having 70°C and 60°C detection temperature, respectively.

One of features of the present invention is to determine differentially the presence of the at least three target nucleic acid sequences by detecting at different detection temperatures signals indicative of the presence of the at least three target nucleic acid sequences.

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Step (b): Determining the presence of the at least three target nucleic acid sequences

Following the detection of the signal, the presence of the at least three target nucleic acid sequences are determined by using the signal detected in the step (a).

When the presence of a target nucleic acid sequence having a certain detection temperature among the at least three target nucleic acid sequences is to be determined, the presence of the target nucleic acid sequence having the certain detection temperature is determined by using a difference between the signal detected at one or more detection temperatures higher than the certain detection temperature and the signal detected at the certain detection temperature. Where the certain detection temperature is a relatively highest detection temperature among the detection temperatures, the presence of a target nucleic acid sequence is determined by using the signal detected at the certain detection temperature.

According to an embodiment, using the difference comprises using a difference to be obtained by mathematically processing the signal detected at one or more detection temperatures higher than the certain detection temperature and a signal detected at the certain detection temperature.

According to an embodiment, when the signal is not detected at the detection temperatures higher than the certain detection temperature, using the difference comprises using the signal detected at the certain detection temperature with considering no detection of the signal at the detection temperatures higher than the certain detection temperature.

For instance, assuming that target nucleic acid sequences comprise four target sequences, detection temperatures 72°C, 60°C, 50°C and 40°C are assigned to the four target sequences, respectively, and signals at detection temperatures 60°C, 50°C and 40°C are detected in the step (a). When the presence of a target nucleic acid sequence having 40°C detection temperature is to be determined, the presence of the target nucleic acid sequence having the certain detection temperature (40°C) is determined by using a difference between a signal detected at one or more detection

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temperatures (60°C and 50°C) higher than the certain detection temperature (40°C) and the signal detected at the certain detection temperature. The presence of target nucleic acid sequences having the other detection temperatures (*i.e.*, 60°C and 50°C) is determined.

Alternatively, according to an embodiment, the presence of the target nucleic acid sequence having the certain detection temperature (60°C) is determined by using a difference to be obtained by mathematically processing the signal detected at one or more detection temperatures (72°C) higher than the certain detection temperature (60°C) and the signal detected at the certain detection temperature. As the signal is not detected at the higher detection temperature (72°C), the background signal may be used for calculation the difference.

According to an embodiment, as the signal is not detected at the detection temperature (72°C) higher than the certain detection temperature (60°C), the presence of the target nucleic acid sequence having the certain detection temperature (60°C) is determined by using the signal detected at the certain detection temperature (60°C) with considering no detection of the signal at the detection temperature (72°C) higher than the certain detection temperature (60°C).

If a signal is detected at the highest detection temperature (72°C), it can be determined that the target nucleic acid sequence having the relatively highest detection temperature is present.

According to an embodiment, when the presence of a target nucleic acid sequence having a certain detection temperature among the at least three target nucleic acid sequences is to be determined, the presence of the target nucleic acid sequence having the certain detection temperature is determined by using a difference between the signal detected at a detection temperature immediately higher than the certain detection temperature and the signal detected at the certain detection temperature.

According to an embodiment, using the difference comprises using a difference to be obtained by mathematically processing the signal detected at a detection

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temperature immediately higher than the certain detection temperature and a signal detected at the certain detection temperature.

According to an embodiment, when the signal is not detected at the detection temperature immediately higher than the certain detection temperature, using the difference comprises using the signal detected at the certain detection temperature with considering no detection of the signal at the detection temperature immediately higher than the certain detection temperature.

For example, when the presence of a target nucleic acid sequence having 40°C detection temperature is to be determined, the presence of the target nucleic acid sequence having the certain detection temperature is determined by using a difference between the signal detected at the detection temperature (50°C) immediately higher than the certain detection temperature (40°C) and the signal detected at the certain detection temperature.

According to an embodiment, a reference value is required to determine whether signals detected at the 50°C and 40°C detection temperatures are indicative of the presence of target nucleic acid sequences having the 50°C and 40°C detection temperatures.

According to an embodiment of this invention, the method uses a reference value for determining the presence of the target nucleic acid sequence having the certain detection temperature, obtained by (i) incubating all combinations of target nucleic acid sequences having detection temperatures higher than the certain detection temperature with signal-generating means in a reaction vessel other than the reaction vessel in the step (a), (ii) detecting signals at not only one or more detection temperatures higher than the certain detection temperature but also the certain detection temperature, and (iii) then obtain a difference between the signal detected at the one or more detection temperatures higher than the certain detection temperature and the signal detected at the certain detection temperature.

In the above example, for obtaining a reference value for determining the presence of the target nucleic acid sequence having the certain detection temperature

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(40°C), all combinations (*i.e.*, target nucleic acid sequence having the 60°C detection temperature, target nucleic acid sequence having the 50°C detection temperature, and a combination of target nucleic acid sequences having the 60°C and 50°C detection temperatures) of target nucleic acid sequences having detection temperatures (60°C and 50°C) higher than the certain detection temperature (40°C) are incubated with signal-generating means to generate signals; signals are detected at not only one or more detection temperatures (60°C and 50°C) higher than the certain detection temperature (40°C) but also the certain detection temperature (40°C); and then the signal detected at the one or more detection temperatures higher than the certain detection temperature and the signal detected at the certain detection temperature are used to obtain the difference between the signals. In accordance with the same method, reference values for the qualifying values for determining the presence of target nucleic acid sequence having the other detection temperatures (*i.e.*, 60°C and 50°C) are obtained.

The references values may be pre-prepared for all combinations. The reference values may be used to determine a significance of the difference obtained in the sample, in considering method for obtaining qualifying values and practical detection results. The reference values may be used to obtain the difference in the sample, whereby involved in the determination of the presence of target nucleic acid.

Alternatively, the method further uses a reference value for determining the presence of the target nucleic acid sequence having the certain detection temperature, obtained by (i) incubating all combinations of target nucleic acid sequences having detection temperatures higher than the certain detection temperature with signal-generating means in a reaction vessel other than the reaction vessel in the step (a), (ii) detecting signals at both a detection temperature immediately higher than the certain detection temperature and the certain detection temperature, and (iii) then obtaining a difference between the signal detected at the detection temperature immediately higher than the certain detection temperature and the signal detected at the certain detection temperature to obtain the difference between the signals.

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In the above example, for obtaining a reference value for determining the presence of the target nucleic acid sequence having the certain detection temperature (40°C), all combinations (*i.e.*, target nucleic acid sequence having the 60°C detection temperature, target nucleic acid sequence having the 50°C detection temperature, and a combination of target nucleic acid sequences having the 60°C and 50°C detection temperatures) of target nucleic acid sequences having detection temperatures (60°C and 50°C) higher than the certain detection temperature (40°C) are incubated with signal-generating means to generate signals; signals are detected at both a detection temperature (50°C) immediately higher than the certain detection temperature (40°C) and the certain detection temperature (40°C); then the signal detected at a detection temperature (50°C) immediately higher than the certain detection temperature and the signal detected at the certain detection temperature (40°C) are used to obtain the difference between the signals. In accordance with the same method, reference values for determining the presence of target nucleic acid sequence having the other detection temperatures (*i.e.*, 60°C and 50°C) are obtained.

According to an embodiment, using the signal detected at the certain detection temperature comprises obtaining a qualifying value for determining the presence of the target nucleic acid sequence having the relatively highest detection temperature and using the difference comprises obtaining a qualifying value for determining the presence of the target nucleic acid sequence having the certain detection temperature.

According to an embodiment of this invention, using the difference comprises obtaining a qualifying value for determining the presence of the target nucleic acid sequence having the certain detection temperature, and the qualifying value is obtained by (i) mathematically processing the signal detected at one or more detection temperatures higher than the certain detection temperature and the signal detected at the certain detection temperature or (ii) using the signal detected at the certain detection temperature with considering no detection of the signal at the one or more detection temperature higher than the certain detection temperature when the signal is not detected at the one or more detection temperature higher than the

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certain detection temperature.

According to an embodiment of this invention, the step (b) is performed by determining firstly the presence of a target nucleic acid sequence having a relatively highest detection temperature and then sequentially determining the presence of target nucleic acid sequences having relatively lower detection temperatures in a descending order.

According to an embodiment of this invention, the target nucleic acid sequences comprise a nucleotide variation (particularly SNP).

IV. Detection of Two Target Nucleic Acid Sequences Using Different Detection Temperatures and Melting Analysis

In a further aspect of this invention, there is provided a method for detecting two target nucleic acid sequences in a sample using different detection temperatures and melting analysis, comprising:

(a) incubating the sample with two signal-generating means for detection of the two target nucleic acid sequences in a single reaction vessel and detecting a generated signal by using a single type of detector; wherein each of the target nucleic acid sequences is detected by a corresponding signal-generating means; wherein one of the two target nucleic acid sequences has a relatively high detection temperature and the other has a relatively low detection temperature determined by the corresponding signal-generating means; wherein the relatively high detection temperature is a temperature capable of generating a signal for the target nucleic acid sequence having the relatively high detection temperature and the relatively low detection temperature is a temperature capable of generating both of a signal for the target nucleic acid sequence having the relatively low detection temperature and a signal for the target nucleic acid sequence having the relatively high detection temperature; wherein signals to be generated by the two signal-generating means are not differentiated by a single type of detector; wherein the detection is performed at the relatively high detection temperature;

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(b) performing a melting analysis of the incubation resultant of the step (a) over a range of temperatures for determining the presence of the target nucleic acid sequence having the relatively low detection temperature; and

(c) determining the presence of the target nucleic acid sequence having the relatively high detection temperature by using the signal detected in the step (a) and the presence of the target nucleic acid sequence having the relatively low detection temperature by using the result of the melting analysis in the step (b).

Step (a): <u>Incubation with signal generating means and signal detection</u>

Firstly, the sample to be analyzed is incubated with two signal-generating means for detection of the two target nucleic acid sequences in a single reaction vessel and then a generated signal is detected by using a single type of detector. Signals to be generated by the two signal-generating means are not differentiated by the single type of detector.

Each of the target nucleic acid sequences is detected by a corresponding signal generating means. One of the two target nucleic acid sequences has a relatively high detection temperature and the other has a relatively low detection temperature determined by the corresponding signal-generating means.

According to an embodiment of the present invention, the target nucleic acid sequence having a relatively high detection temperature is detected by a real-time detection method using a different detection temperature, and the target nucleic acid sequence having a relatively low detection temperature is detected by melting analysis.

The detection in the step (a) is performed at the relatively high detection temperature and not at the relatively low detection temperature.

The signal generating means capable of generating a signal during melting analysis is chosen for the target nucleic acid sequence having the relatively low detection temperature.

According to an embodiment, because the signal-generating means for the

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melting analysis employs hybridization and denaturation of a duplex, it may generate a signal depending on reaction conditions in the step (a).

According to an embodiment, the signal-generating means for generating a signal for the target nucleic acid sequence having the relatively low detection temperature has to be constructed to generate no signal at the relatively high detection temperature.

In the fourth aspect of this invention, the signal generating means for generating a signal for the target nucleic acid sequence having the relatively low detection temperature may generate or not generate signal depending on reaction conditions in the step (a). Even if a signal for the target nucleic acid sequence having the relatively low detection temperature may generate in a certain reaction condition, the generated signal is not detected as a signal detection is performed at the relatively high detection temperature in the step (a).

Since the signal for the target nucleic acid sequence having the relatively low detection temperature may be generated and detected in the step (b), the signal is not compelled to be generated in the step (a).

According to an embodiment, the signal-generating means for the melting analysis does not include a means generating signal by cleavage of labeled probes or labeled duplex. As the melting analysis employs T_m value of hybrids, the cleavage of hybrids to be detected has to be excluded. Upon cleavage, melting peaks may be not produced or detection sensitivity may be greatly reduced. In addition, signal by cleavage is likely to be a false positive signal in the real-time detection method.

The step (a) is performed under conditions capable of generating at least the signal for the target nucleic acid sequence having the relatively high detection temperature.

According to an embodiment, the step (a) is performed in a signal amplification process concomitantly with a nucleic acid amplification.

According to an embodiment, the step (a) is performed in a signal amplification process without a nucleic acid amplification.

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According to an embodiment, at least one of the signal-generating means is a signal-generating means to generate a signal in a dependent manner on the formation of a duplex. According to an embodiment, the signal is generated by the formation of a duplex between a target nucleic acid sequence and a detection oligonucleotide specifically hybridized with the target nucleic acid sequence. According to an embodiment, the signal is generated by a duplex formed in a dependent manner on cleavage of a mediation oligonucleotide specifically hybridized with the target nucleic acid sequence.

According to an embodiment, at least one of the signal-generating means is a signal-generating means to generate a signal in a dependent manner on cleavage of a detection oligonucleotide. According to an embodiment, the signal is generated by hybridization of the detection oligonucleotide with a target nucleic acid sequence and then cleavage of the detection oligonucleotide. According to an embodiment, the signal is generated by cleavage of the detection oligonucleotide in a dependent manner on cleavage of a mediation oligonucleotide specifically hybridized with the target nucleic acid sequence.

According to an embodiment, the signal generation being dependent manner on cleavage of the detection oligonucleotide is used for the target nucleic acid sequence having the relatively high detection temperature.

Particularly, it is very advantageous to use the signal generation being dependent manner on cleavage of the detection oligonucleotide for the target nucleic acid sequence having the relatively high detection temperature for the following reasons: (i) a detection temperature may be selected with a relatively wide temperature range; (ii) a much higher temperature compared with hybridization approaches may be selected; and (iii) it is possible to provide no signal in melting analysis by selecting suitable signal-generating means and reaction conditions (*e.g.*, selection of signal-generating means incapable of providing signals by only hybridization without cleavage or selection of conditions allowing for cleavage of most of detection oligonucleotides).

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The signal-generating means to generate a signal in a dependent manner on the formation of a duplex (*e.g.*, Molecular beacon) may also provide signal through cleavage by 5'-nuclease being dependent on reaction conditions. The signal-generating means may be considered as that capable of providing signal by cleavage, so long as it is used to generate signal by cleavage.

According to an embodiment, the signal-generating means for the target nucleic acid sequence having the relatively high detection temperature is a signal-generating means by cleavage of a detection oligonucleotide, and the signal-generating means for the target nucleic acid sequence having the relatively low detection temperature is a signal-generating means by the formation of a duplex.

According to an embodiment, the signal-generating means for the target nucleic acid sequence having the relatively high detection temperature is a signal-generating means by cleavage of a detection oligonucleotide, and the signal-generating means for the target nucleic acid sequence having the relatively low detection temperature generates a signal by a duplex formed in a dependent manner on cleavage of a mediation oligonucleotide specifically hybridized with the target nucleic acid sequence.

According to an embodiment, the two signal-generating means comprises an identical label and signals from the label are not differentiated by the single type of detector.

After the incubation (reaction) of the sample with two signal-generating means to generate signal, the generated signal is detected by using a single type of detector. The detection is performed at the relatively high detection temperature capable of generating a signal for the target nucleic acid sequence having the relatively high detection temperature.

Step (b): <u>Melting analysis</u>

Afterwards, the melting analysis of the incubation resultant of the step (a) is performed over a range of temperatures for determining the presence of the target

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nucleic acid sequence having the relatively low detection temperature.

The present specification describes performing the step (b) after the detection in the step (a) only for convenience in description. Given the principle underlying the present invention, it would be understood that the step (b) may be performed before the detection in the step (a). Therefore, the process comprising the step (b) before the detection in the step (a) is also encompassed by the scope of the present invention.

The step (b) may be carried out by various melting analysis processes known to one of skill in the art. The term "melting analysis" used herein is intended to encompass not only a melting analysis in a narrow sense but also a hybridization analysis, unless otherwise indicated. The melting analysis in a narrow sense refers to a method in which the dissociation of duplexes is measured under increasing stringency conditions by adjusting temperatures. The hybridization analysis in a narrow sense refers to a method in which the association of duplexes is measured under decreasing stringency conditions by adjusting temperatures. The term "melting curve" or "melting peak curve" used herein is intended to encompass not only a melting curve or melting peak curve from a melting analysis in a narrow sense but also a hybridization curve or hybridization peak curve from a hybridization analysis, unless otherwise indicated. The melting curve or hybridization curve may be obtained by conventional technologies, for example, as described in U.S. Pat Nos. 6,174,670 and 5,789,167, Drobyshev et al, *Gene* 188: 45(1997); Kochinsky and Mirzabekov *Human Mutation* 19:343(2002); Livehits et al *J. Biomol. Structure Dynam.* 11:783(1994); and Howell et al *Nature Biotechnology* 17:87(1999). For example, a melting curve or hybridization curve may consist of a graphic plot or display of the variation of the output signal with the parameter of hybridization stringency. Output signal may be plotted directly against the hybridization parameter. Typically, a melting curve or hybridization curve will have the output signal, for example fluorescence, which indicates the degree of duplex structure (*i.e.* the extent of hybridization), plotted on the Y-axis and the hybridization parameter on the X axis.

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The melting (hybridization) curve analysis and the melting (hybridization) peak analysis will be described with reference to disclosures of US Pat No 8,039,215.

The melting analysis uses "T_m" values. The term used herein "T_m" refers to a melting temperature at which half a population of double stranded nucleic acid molecules are dissociated to single-stranded molecules. The T_m value is determined by length and G/C content of nucleotides hybridized. The T_m value may be calculated by conventional methods such as Wallace rule (R.B. Wallace, et al., *Nucleic Acids Research*, 6:3543-3547(1979)) and nearest-neighbor method (SantaLucia J. Jr., et al., *Biochemistry*, 35:3555–3562(1996)); Sugimoto N., et al., *Nucleic Acids Res.*, 24:4501-4505(1996)).

According to an embodiment, the step (b) is performed by detecting signals generated with increasing temperatures (melting analysis in a narrow sense). Alternatively, the step (b) is performed by detecting signals generated with decreasing temperatures (hybridization analysis in a narrow sense).

According to an embodiment, the signal-generating means used for the melting analysis includes any means generating signal from a duplex formation over a range of temperatures. Among signal-generating means for real-time detection methods, means generating signal by hybridization of a detection oligonucleotide rather than cleavage reaction is also used for the melting analysis.

According to an embodiment, the step (b) is performed by using a signal-generating means to generate a signal in a dependent manner on the formation of a duplex. Particularly, the step (b) is performed by using a signal generating means generated signal by a duplex formed in a dependent manner on cleavage of a mediation oligonucleotide specifically hybridized with the target nucleic acid sequence.

The signal by the duplex formed in a dependent manner on cleavage of the mediation oligonucleotide may be generated by various methods, including PTOCE-melting method (WO 2012/096523), PCE-SH-melting method (WO 2013/115442) and PCE-NH-melting method (PCT/KR2013/012312). The PTOCE-melting method, PCE-SH-melting method and PCE-NH-melting method correspond to a PTOCE-based method

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with melting analysis, a PCE-SH-melting method with melting analysis and a PCE-NH-melting method with melting analysis, respectively. Among the PTOCE-based methods, methods to generate no signal by cleavage are employed for the melting analysis in the step (b).

The PTOCE-melting method, PCE-SH-melting method and PCE-NH-melting method are also described in the prior patent documents.

The steps (a)-(b) performed by the PTOCE-melting method comprise the following steps:

(a) hybridizing the target nucleic acid sequence with the upstream oligonucleotide and the PTO; (b) contacting the resultant of the step (a) to an enzyme having a 5′ nuclease activity under conditions for cleavage of the PTO; wherein the upstream oligonucleotide or its extended strand induces cleavage of the PTO by the enzyme having the 5′ nuclease activity such that the cleavage releases a fragment comprising the 5′-tagging portion or a part of the 5′-tagging portion of the PTO; (c) hybridizing the fragment released from the PTO with the CTO; wherein the fragment released from the PTO is hybridized with the capturing portion of the CTO; (d) performing an extension reaction using the resultant of the step (c) and a template-dependent nucleic acid polymerase; wherein the fragment hybridized with the capturing portion of the CTO is extended and an extended duplex is formed; wherein the extended duplex has a T_m value adjustable by (i) a sequence and/or length of the fragment, (ii) a sequence and/or length of the CTO or (iii) the sequence and/or length of the fragment and the sequence and/or length of the CTO; (e) melting the extended duplex over a range of temperatures to give a target signal indicative of the presence of the extended duplex; wherein the target signal is provided by (i) at least one label linked to the fragment and/or the CTO, (ii) a label incorporated into the extended duplex during the extension reaction, (iii) a label incorporated into the extended duplex during the extension reaction and a label linked to the fragment and/or the CTO, or (iv) an intercalating label; and (f) detecting the extended duplex by measuring the target signal; whereby the presence of the extended duplex indicates

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the presence of the target nucleic acid sequence.

In this case, the PTOCE melting method further comprises repeating the steps (a)-(b), (a)-(d) or (a)-(f) with denaturation between repeating cycles. In the step (a) of PTOCE-melting method, a primer set for amplification of the target nucleic acid sequence may be used instead of the upstream oligonucleotide. In this case, the method further comprises repeating the steps (a)-(b), (a)-(d) or (a)-(f) with denaturation between repeating cycles.

The steps (a)-(b) performed by the PTOCE-based melting methods comprise the following steps:

(a) hybridizing the target nucleic acid sequence with the upstream oligonucleotide and the PTO; (b) contacting the resultant of the step (a) to an enzyme having a 5′ nuclease activity under conditions for cleavage of the PTO; wherein the upstream oligonucleotide or its extended strand induces cleavage of the PTO by the enzyme having the 5′ nuclease activity such that the cleavage releases a fragment comprising the 5′-tagging portion or a part of the 5′-tagging portion of the PTO; (c) hybridizing the fragment released from the PTO with the CTO; wherein the fragment released from the PTO is hybridized with the capturing portion of the CTO; (d) performing an extension reaction using the resultant of the step (c) and a template-dependent nucleic acid polymerase; wherein the fragment hybridized with the capturing portion of the CTO is extended to form an extended strand; and (e) performing a melting analysis over a range of temperatures by detecting signal generated dependent on the presence of the extended strand.

Step (c): <u>Determining the presence of target nucleic acid sequences</u>

Finally, the presence of the target nucleic acid sequence having the relatively high detection temperature is determined by using the signal detected in the step (a) and the presence of the target nucleic acid sequence having the relatively low detection temperature is determined by using the result of the melting analysis in the step (b).

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According to an embodiment of this invention, the target nucleic acid sequences comprise a nucleotide variation (particularly SNP).

The unexpected results of the present invention may be yielded when a signal-generating means to generate signal by cleavage for a real-time detection is combined with a signal-generating means to generate signal by a duplex formed in a dependent manner on cleavage of a mediation oligonucleotide hybridized with the target nucleic acid sequence for the melting analysis. In such case, a signal-generating means involving the formation of duplex and generating signal directly by cleavage should be excluded for the melting analysis.

It is noteworthy that the present invention is performed in a combination of the signal-generating means to generate signal by cleavage (e.g. the TaqMan method) as a real-time process and the PTOCE-based melting method as a melting analysis, providing the most striking results.

According to an embodiment, using the signal detected in the step (a) comprises obtaining a qualifying value for determining the presence of the target nucleic acid sequence having the relatively high detection temperature.

According to an embodiment of this invention, the target nucleic acid sequences comprise a nucleotide variation (particularly SNP).

V. Detection of at least Three Target Nucleic Acid Sequences Using Different Detection Temperatures and Melting Analysis

In still further aspect of this invention, there is provided a method for detecting at least three target nucleic acid sequences in a sample using detection temperature analysis and melting analysis, comprising:

(a) incubating the sample with at least three signal-generating means for detection of the at least three target nucleic acid sequences in a single reaction vessel and detecting a generated signal by using a single type of detector; wherein each of the at least three target nucleic acid sequences is detected by a corresponding signal-generating means; wherein each of the at least three target nucleic acid sequences

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has a different detection temperature determined by the corresponding signal-generating means; wherein a detection temperature is a temperature capable of generating not only a signal for a target nucleic acid sequence having the detection temperature but also a signal for a target nucleic acid sequence having a higher detection temperature than the detection temperature; wherein signals to be generated by the signal-generating means are not differentiated by the single type of detector; wherein some of the at least three target nucleic acid sequences are detected by detection temperature analysis and the detection is performed at both the detection temperature of said some of the at least three target nucleic acid sequences and one or more detection temperatures higher than the detection temperatures;

(b) performing a melting analysis of the incubation resultant of the step (a) over a range of temperatures for determining the presence of the other target nucleic acid sequences than said some of the at least three the target nucleic acid sequences; and

(c) determining (i) the presence of said some of the target nucleic acid sequences by using the signal detected in the step (a); wherein when the presence of a target nucleic acid sequence having a certain detection temperature among said some of the at least three target nucleic acid sequences is determined, the presence of the target nucleic acid sequence having the certain detection temperature is determined by using a difference between the signal detected at one or more detection temperatures higher than the certain detection temperature and the signal detected at the certain detection temperature; wherein when the certain detection temperature is a relatively highest detection temperature among the detection temperatures, the presence of the target nucleic acid sequence is determined by using the signal detected at the certain detection temperature; and (ii) the presence of the other target nucleic acid sequences than said some of the at least three target nucleic acid sequences is determined by the result of the melting analysis in the step (b).

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Since the present invention follows in principle, the first aspect to the fourth aspect of this invention described above, the common descriptions between them are omitted in order to avoid undue redundancy leading to the complexity of this specification.

Step (a): Incubation with signal-generating means and signal detection

Firstly, the sample to be analyzed is incubated with at least three signal-generating means for detection of the at least three target nucleic acid sequences in a single reaction vessel and then a generated signal is detected by using a single type of detector. Signals to be generated by the at least three signal generating means are not differentiated by the single type of detector.

One of the target nucleic acid sequences has a relatively highest detection temperature. A signal-generating means capable of providing a signal at a relatively highest detection temperature is used to detect the target nucleic acid sequence.

In the present invention, some of the at least three target nucleic acid sequences are detected by the detection temperature analysis and the other are detected by the melting analysis.

The tem "some" in the expression "some of the at least three target nucleic acid sequences" includes one or more. The tem used herein "detection temperature analysis" refers to a real-time detection method comprising detection at different temperatures to detect a target nucleic acid sequence, unless otherwise indicated.

The signal-generating means for generating a signal for the target nucleic acid sequence to be analyzed the melting analysis may generate or not generate signal depending on reaction conditions in the step (a). Since the signal for the target nucleic acid sequence to be analyzed the melting analysis may be generated and detected in the step (b), the signal is not compelled to be generated in the step (a).

Each of the at least three target nucleic acid sequences has to use a signal-generating means having a different detection temperature from one another.

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According to an embodiment, because the signal-generating means for the melting analysis employs hybridization and denaturation of a duplex, it may generate a signal depending on reaction conditions in the step (a).

According to an embodiment, the step (a) is carried out under conditions suitable in generation of at least a signal for a target nucleic acid sequence to be analyzed by the detection temperature analysis.

According to an embodiment, the selection of target nucleic acid sequences to be analyzed by the detection temperature analysis is done in considering detection temperatures. Particularly, a target nucleic acid sequence having the highest detection temperature is firstly selected and then some sequences are selected in a detection temperature order.

According to an embodiment, the step (a) is performed in a signal amplification process concomitantly with a nucleic acid amplification.

According to an embodiment, the step (a) is performed in a signal amplification process without a nucleic acid amplification.

According to an embodiment, at least one of the signal-generating means is a signal-generating means to generate a signal in a dependent manner on the formation of a duplex. According to an embodiment, the signal is generated by the formation of a duplex between a target nucleic acid sequence and a detection oligonucleotide specifically hybridized with the target nucleic acid sequence. According to an embodiment, the signal is generated by a duplex formed in a dependent manner on cleavage of a mediation oligonucleotide specifically hybridized with the target nucleic acid sequence.

According to an embodiment, at least one of the signal-generating means is a signal-generating means to generate a signal in a dependent manner on cleavage of a detection oligonucleotide. According to an embodiment, the signal is generated by hybridization of the detection oligonucleotide with a target nucleic acid sequence and then cleavage of the detection oligonucleotide. According to an embodiment, the signal is generated by cleavage of the detection oligonucleotide in a dependent

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manner on cleavage of a mediation oligonucleotide specifically hybridized with the target nucleic acid sequence.

According to an embodiment, the signal generation being dependent manner on cleavage of the detection oligonucleotide is used only for the target nucleic acid sequence having the relatively highest detection temperature among the at least three target nucleic acid sequences.

According to an embodiment of this invention, the signal-generating means for the target nucleic acid sequence having the relatively highest detection temperature is a signal-generating means by cleavage of a detection oligonucleotide, and the signal-generating means for the other target nucleic acid sequences are a signal-generating means by the formation of a duplex.

According to an embodiment of this invention, the signal-generating means for the target nucleic acid sequence having the relatively highest detection temperature is a signal-generating means by cleavage of a detection oligonucleotide, and the signal-generating means for the other target nucleic acid sequences generate signals by the formation of a duplex in a dependent manner on cleavage of a mediation oligonucleotide specifically hybridized with the target nucleic acid sequence.

For example, the signal for the target nucleic acid sequence having the relatively highest detection temperature is generated by TaqMan method and signals for the other target nucleic acid sequences are generated by the PTOCE method, PCE-SH method or PCE-NH method.

According to an embodiment of this invention, the at least three signal-generating means comprise an identical label and signals from the label are not differentiated by the single type of detector.

The number of the target nucleic acid sequences to be detected by the present invention is not limited, including more than 3, 4, 5, 6, 7, 8, 9 and 10 target nucleic acid sequences in the single reaction vessel.

After the incubation (reaction) of the sample with the at least three signal-

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generating means to generate signal, the generated signal is detected by using a single type of detector. some of the at least three target nucleic acid sequences are detected by the detection temperature analysis and the detection is performed at both the detection temperatures of said some of the at least three target nucleic acid sequences and one or more detection temperatures higher than the detection temperatures.

According to an embodiment, signals are detected at all detection temperatures required for performing the detection temperature analysis.

The generated signal is detected by using a single type of detector. Each of the at least three target nucleic acid sequences has a different detection temperature determined by the corresponding signal-generating means. A detection temperature is a temperature capable of generating not only a signal for a target nucleic acid sequence having the detection temperature but also a signal for a target nucleic acid sequence having a higher detection temperature than the detection temperature.

According to an embodiment, the target nucleic acid sequences to be detected by the detection temperature analysis have detection temperatures higher than those to be detected by the melting analysis.

Step (b): <u>Melting analysis</u>

The melting analysis of the incubation resultant of the step (a) is performed over a range of temperatures for determining the presence of the other target nucleic acid sequences than said some of the at least three target nucleic acid sequences.

The present specification describes performing the step (b) after the detection in the step (a) only for convenience in description. Given the principle underlying the present invention, it would be understood that the step (b) may be performed before the detection in the step (a). Therefore, the process comprising the step (b) before the detection in the step (a) is also encompassed by the scope of the present invention.

According to an embodiment, the step (b) is performed by using a signal-

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generating means to generate a signal in a dependent manner on the formation of a duplex. Particularly, the step (b) is performed in accordance with the PTOCE based-melting method.

Step (c): <u>Determining the presence of target nucleic acid sequences</u>

Finally, the presence of the at least three target nucleic acid sequences in the sample is determined by using the signal in the step (a) and the result of the melting analysis in the step (b).

The presence of some of the target nucleic acid sequences is determined by using the signal detected in the step (a). When the presence of a target nucleic acid sequence having a certain detection temperature among said some of the at least three target nucleic acid sequences is determined, the presence of the target nucleic acid sequence having the certain detection temperature is determined by using a difference between the signal detected at one or more detection temperatures higher than the certain detection temperature and the signal detected at the certain detection temperature. When the certain detection temperature is a relatively highest detection temperature among the detection temperatures, the presence of the target nucleic acid sequence is determined by using the signal detected at the certain detection temperature;

According to an embodiment, the method further uses a reference value for determining the presence of the target nucleic acid sequence having the certain detection temperature, obtained by (i) incubating all combinations of target nucleic acid sequences having detection temperatures higher than the certain detection temperature with signal-generating means in a reaction vessel other than the reaction vessel in the step (a), (ii) detecting signals at not only one or more detection temperatures higher than the certain detection temperature but also the certain detection temperature, and (iii) then obtaining a difference between the signal detected at the one or more detection temperatures higher than the certain detection temperature and the signal detected at the certain detection temperature.

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Alternatively, the method further uses a reference value for determining the presence of the target nucleic acid sequence having the certain detection temperature, obtained by (i) incubating all combinations of target nucleic acid sequences having detection temperatures higher than the certain detection temperature with signal-generating means in a reaction vessel other than the reaction vessel in the step (a), (ii) detecting signals at both the detection temperature immediately higher than the certain detection temperature and the certain detection temperature, and (iii) then obtaining a difference between the signal detected at the detection temperature immediately higher than the certain detection temperature and the signal detected at the certain detection temperature.

According to an embodiment, where signal detection at detection temperatures for target nucleic acid sequences to be detected by the melting analysis is required for obtaining the difference between signals or reference values for target nucleic acid sequences to be detected by the detection temperature analysis, signals may be collected at the detection temperatures for target nucleic acid sequences to be detected by the melting analysis and be used.

The presence of the other target nucleic acid sequences than some of the at least three target nucleic acid sequences to be determined by the detection temperature analysis is determined by the result of the melting analysis in the step (b).

According to an embodiment, the step (d) is performed by determining firstly the presence of a target nucleic acid sequence having a relatively highest detection temperature and then sequentially determining the presence of target nucleic acid sequences having relatively lower detection temperatures in a descending order.

According to an embodiment, the determination of the presence of a target nucleic acid sequence by the melting analysis may be used in determination of the presence of a target nucleic acid sequence by the detection temperature analysis (*e.g.*, for selection of reference values).

Where the at least three target nucleic acid sequences are detected in a real-

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time manner, a signal generation by cleavage is used only for one target nucleic acid sequence. For the other target nucleic acid sequences, the PTOCE-based methods may be employed to improve efficiency and readiness of analysis.

According to an embodiment of this invention, the target nucleic acid sequences comprise a nucleotide variation (particularly SNP).

VI. Detection of a Target Nucleic Acid Sequence from Two Target Nucleic Acid Sequences

In another aspect of this invention, there is provided a method for detecting a target nucleic acid sequence selected from two target nucleic acid sequences providing non-differentiated signals by a single type of detector in a single reaction vessel, comprising:

(a) assigning different detection temperatures to the two target nucleic acid sequences from each other; wherein the target nucleic acid sequence selected among the two target nucleic acid sequences has a relatively high detection temperature and the other has a relatively low detection temperature;

(b) incubating the two target nucleic acid sequences with two signal-generating means for detection of the two target nucleic acid sequences in a single reaction vessel; wherein each of the target nucleic acid sequences is detected by a corresponding signal-generating means; wherein the relatively high detection temperature is a temperature capable of generating a signal for the target nucleic acid sequence having the relatively high detection temperature and the relatively low detection temperature is a temperature capable of generating both of a signal for the target nucleic acid sequence having the relatively low detection temperature and a signal for the target nucleic acid sequence having the relatively high detection temperature; wherein signals to be generated by the two signal-generating means are not differentiated by the single type of detector; and

(c) detecting a generated signal at the relatively high detection temperature by the single type of detector to obtain only the signal for the target nucleic acid

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sequence having the relatively high detection temperature, whereby the detected signal is indicative of the presence of the target nucleic acid sequence having the relatively high detection temperature.

Since the present invention has the common descriptions with the first aspect of this invention, the common descriptions between them are omitted in order to avoid undue redundancy leading to the complexity of this specification.

Step (a): <u>Assigning different detection temperatures</u>

The different detection temperatures are assigned to the two target nucleic acid sequences from each other. The target nucleic acid sequence selected among the two target nucleic acid sequences has a relatively high detection temperature and the other has a relatively low detection temperature.

The relatively high detection temperature is assigned to the target nucleic acid sequences to be selectively detected.

Step (b): <u>Incubation with signal-generating means</u>

The two target nucleic acid sequences are incubated with the two signal-generating means for detection of the two target nucleic acid sequences in a single reaction vessel. The signals to be generated by the two signal-generating means are not differentiated by the single type of detector.

Each of the target nucleic acid sequences is detected by a corresponding signal-generating means. According to an embodiment, the detection temperature is determined by the corresponding signal-generating means.

The relatively high detection temperature is a temperature capable of generating a signal for the target nucleic acid sequence having the relatively high detection temperature and the relatively low detection temperature is a temperature capable of generating both of a signal for the target nucleic acid sequence having the relatively low detection temperature and a signal for the target nucleic acid sequence having the relatively high detection temperature.

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Step (c): Detection of the presence of a selected target nucleic acid sequence

Afterwards, a generated signal at the relatively high detection temperature is detecting by the single type of detector to obtain only the signal for the target nucleic acid sequence having the relatively high detection temperature. The detected signal is indicative of the presence of the target nucleic acid sequence having the relatively high detection temperature.

According to an embodiment, the signal for the target nucleic acid sequence having the relatively high detection temperature is a calculation parameter to analyze the signal for the target nucleic acid sequence having the relatively low detection temperature. The signal for the target nucleic acid sequence having the relatively high detection temperature plays a role as not only an indicator for the presence of the target nucleic acid sequence having the relatively high detection temperature but also a calculation (*e.g.*, ratio calculation of signal intensities) parameter to analyze the signal for the target nucleic acid sequence having the relatively low detection temperature when signal is also detected at the relatively low detection temperature.

According to an embodiment, the signal for the target nucleic acid sequence having the relatively high detection temperature is generated by a signal-generating means to generate a signal in a dependent manner on the formation of a duplex.

According to an embodiment, the signal for the target nucleic acid sequence having the relatively high detection temperature is generated by a signal-generating means to generate a signal in a dependent manner on cleavage of a detection oligonucleotide.

VII. Processor and Device for Detection of a Target Nucleic Acid Sequence by Using Two Detection Temperatures

In another aspect of this invention, there is provided a computer readable storage medium containing instructions to configure a processor to perform a method

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for determining the presence of two target nucleic acid sequences in a sample using different detection temperatures, the method comprising:

(a) receiving both a signal detected at a relatively high detection temperature and a signal detected at a relatively low detection temperature, wherein each of the target nucleic acid sequences is detected by a corresponding signal-generating means; wherein a generated signal is detected by using a single type of detector; wherein one of the two target nucleic acid sequences has the relatively high detection temperature and the other has the relatively low detection temperature determined by corresponding signal-generating means; wherein the relatively high detection temperature is a temperature capable of generating a signal for the target nucleic acid sequence having the relatively high detection temperature and the relatively low detection temperature is a temperature capable of generating both of a signal for the target nucleic acid sequence having the relatively low detection temperature and a signal for the target nucleic acid sequence having the relatively high detection temperature; and

(b) determining the presence of the two target nucleic acid sequences by using the signal detected; wherein (i) the presence of the target nucleic acid sequence having the relatively high detection temperature is determined by using the signal detected at the relatively high detection temperature and (ii) the presence of the target nucleic acid sequence having the relatively low detection temperature is determined by using a difference between the signal detected at the relatively high detection temperature and the signal detected at the relatively low detection temperature.

The types of the computer readable storage medium include various storage medium such as CD-R, CD-ROM, DVD, flash memory, floppy disk, hard drive, magnetic tape, MINIDISC, nonvolatile memory card, EEPROM, optical disk, optical storage medium, RAM, ROM, system memory and web server.

In still another aspect of this invention, there is provided a device for detecting

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a target nucleic acid sequence in a sample using different detection temperatures, comprising (a) a computer processor and (b) the computer readable storage medium described above coupled to the computer processor.

According to an embodiment, the device further comprises a reaction vessel to accommodate the sample and signal-generating means, a temperature controlling means to control temperatures of the reaction vessel and/or a single type detector to detect signals to be generated by the signal-generating means.

According to an embodiment, the computer processor permits not only the single type of detector to detect signals generated by the signal-generating means at a relatively high detection temperature and a relatively low detection temperature but also to calculate a difference between the signal detected at the relatively high detection temperature and the signal detected at the relatively low detection temperature. The processor may be prepared in such a manner that a single processor can do two performances: direction of detection at two detection temperatures and calculation of the difference. Alternatively, the processor unit may be prepared in such a manner that two processors do two performances, respectively.

The first essential feature of the device carries the processor to permit the device to detect signals to be generated at the two detection temperatures. According to an embodiment, where the signal is generated along with amplification of the target nucleic acid sequence, the device comprises a processor to permit the device to detect signals to be generated at the two detection temperatures at each amplification cycle.

The second essential feature of the device is to carry the processor to process the signal detected at the two detection temperatures to obtain the difference between the signals. According to an embodiment, the difference between the signals is expressed as numeric values by a mathematical processing.

According to an embodiment, the processor may be embodied by installing software into conventional devices for detection of target nucleic acid sequences (e.g. real-time PCR device). According to an embodiment, the device comprises a

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processor to permit the device to detect signals at at least two detection temperatures and to mathematically process at least two detection results.

VIII. Detection of a Target Nucleic Acid Sequence by using two detection temperatures.

In another aspect of this invention, there is provided a method for detecting a target nucleic acid sequence in a sample using two different detection temperatures, comprising:

(a) incubating the sample with a signal-generating means for detection of the target nucleic acid sequence in a single reaction vessel and detecting signals generated at a relatively high detection temperature and at a relatively low detection temperature; and

(b) determining the presence of the target nucleic acid sequence by using a difference between the signal detected at the relatively high detection temperature and the signal detected at the relatively low detection temperature.

Where several samples whose each contains an identical target nucleic acid sequence are prepared and used for detection, there may be difference of detection results from each other in reaction vessels. Such difference may be owing to differences of reaction conditions between reaction vessels. Furthermore, where different detection devices are used, such difference of detection results may be more severe due to differences of components or setting in the devices.

The difference becomes more considerable factor when the amount of target nucleic acid sequences becomes smaller or results obtained from repetitive experiments are compared.

To decrease deviations in samples between detection apparatuses, conventional technologies are seeking solutions in view of software or hardware.

The present invention detects signals at two detection temperatures for a single target nucleic acid sequence in a reaction vessel. The present invention permits

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to yield detection results under substantially identical reaction conditions except for detection temperatures. The present invention permits to provide results being free from influences of reaction conditions by suitably processing detection results obtained at two different detection temperatures, which is employed to determine the presence of target nucleic acid sequences.

In another aspect of this invention, there is provided a computer readable storage medium containing instructions to configure a processor to perform a method for determining the presence of a target nucleic acid sequence in a sample using two different detection temperatures, the method comprising:

(a) receiving both a signal detected at a relatively high detection temperature and a signal detected at a relatively low detection temperature; and

(b) determining the presence of the target nucleic acid sequence by using a difference between the signal detected at the relatively high detection temperature and the signal detected at the relatively low detection temperature.

In still another aspect of this invention, there is provided a device for detecting a target nucleic acid sequence in a sample using two different detection temperatures, comprising (a) a computer processor and (b) the computer readable storage medium described above coupled to the computer processor.

The features and advantages of this invention will be summarized as follows:

(a) The present invention employing different detection temperatures enables to detect a plurality of target nucleic acid sequences in conventional real-time manners even with a single type of label in a single reaction vessel. The conventional technologies detect a plurality of target nucleic acid sequences by a melting analysis after target amplification. Unlikely, the present invention does not require a melting analysis after target amplification, such that the time for analysis is greatly reduced.

(b) Interestingly, the present inventors have found that when a signal for a target nucleic acid sequence is generated using a signal-generating means, (i) a signal detection at a certain temperature becomes adjustable depending on the type

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of signal-generating means, and (ii) in the case of detecting signal at selected two detection temperatures, signals detected at the two different detection temperatures are changed in accordance with a certain pattern. The present inventors adopted the findings to detection of target nucleic acid sequences, thereby accomplishing the present invention.

(c) In an embodiment of this invention to detect a plurality of target nucleic acid sequences by use of different detection temperatures in a real-time manner, the combination of (i) for a target nucleic acid sequence, a signal-generating means to generate a signal in a dependent manner on cleavage of a detection oligonucleotide (*e.g.*, TaqMan method) and (ii) for another target nucleic acid sequence, a signal-generating means to provide a signal by a duplex formed in a dependent manner on cleavage of a mediation oligonucleotide specifically hybridized with the target nucleic acid sequence (*e.g.*, PTOCE-based methods) can induce the unexpected results.

The method using cleavage of the detection oligonucleotide generally uses enzyme having 5′ nuclease activity (particularly, Taq polymerase) for cleavage of the detection oligonucleotide. In the conventional methods to generate signal by direct hybridization of detection probes (*e.g.*, Molecular beacon method, hybridization probe method or Hybeacon method), the detection probes are very likely to be cleaved by the enzyme having 5′ nuclease activity (particularly, Taq polymerase). The cleavage of the detection probes may cause decease in sensitivity due to consumption of the detection probes (*e.g.*, hybridization probe method) or false positive signal in methods with cleavage-dependent signaling (*e.g.*, Molecular beacon method). Although the labeled primer methods (*e.g.*, Sunrise method or Scorpion method) do not suffer from the cleavage as the probe methods, they have shortcomings in which T_m value of amplicon *per se* has to be controlled to adjust detection temperatures. In contrast, because the PTOCE-based methods employ cleavage of the mediation oligonucleotide specifically hybridized with the target nucleic acid sequence, they are not affected by enzyme having 5′ nuclease activity (particularly, Taq polymerase). In addition, the PTOCE-based methods can readily adjust T_m value of duplex formed to

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ensure convenient selection of detection temperatures.

(d) In an embodiment of this invention to detect a plurality of target nucleic acid sequences in a single reaction vessel by using a real-time detection for some targets and a melting analysis for other targets, signal-generating means suitable to characteristics of a target nucleic acid sequence to be analyzed may be selected and applied, which enables to detect a plurality of target nucleic acid sequences in a much more efficient manner.

(e) In an embodiment of this invention to detect a plurality of target nucleic acid sequences by using a real-time detection and a melting analysis, a signal-generating means to generate a signal in a dependent manner on cleavage of a detection oligonucleotide (*e.g.*, TaqMan method) for a target nucleic acid sequence detected by a real-time manner permits to detect a plurality of target nucleic acid sequences in greatly enhanced convenience and efficiency. The method using cleavage of a detection oligonucleotide as TaqMan method is undergone cleavage of a detection probe. In a certain reaction condition, it may be possible to cleave most of the detection probes. In that case, there is no duplex capable of generating signal in the melting analysis after the real-time reaction, thereby T_m value for other target nucleic acid sequences to be detected by the melting analysis may be easily selected.

(f) In an embodiment of this invention to detect a plurality of target nucleic acid sequences by using a real-time detection and a melting analysis, the combination of (i) for a target nucleic acid sequence, a signal-generating means to generate a signal in a dependent manner on cleavage of a detection oligonucleotide (*e.g.*, TaqMan method) and (ii) for another target nucleic acid sequence, a signal-generating means to provide a signal by a duplex formed in a dependent manner on cleavage of a mediation oligonucleotide specifically hybridized with the target nucleic acid sequence (*e.g.*, PTOCE-based methods) can induce the unexpected results.

According to conventional technologies by use of hybridization between target nucleic acid sequences and detection oligonucleotides, there are serious problems such as sensitivity decrease (including sensitivity decrease in melting analysis) due to

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probe cleavage and false signal generation due to cleavage. The present invention is completely free from such problems. The PTOCE-based methods can readily adjust T_m value of duplexes used for detection, such that detection temperatures used for real-time detection and peak T_m value used for melting analysis may be readily selected.

The present invention will now be described in further detail by examples. It would be obvious to those skilled in the art that these examples are intended to be more concretely illustrative and the scope of the present invention as set forth in the appended claims is not limited to or by the examples.

Examples

Example 1: Multiple target detection by TaqMan/PTOCE real-time PCR comprising signal detection at different temperatures.

We examined whether a plural of target nucleic acids can be detected in a single reaction vessel by using a single detection channel and TaqMan/PTOCE real-time PCR comprising signal detection at different temperatures.

Taq DNA polymerase having a 5′ nuclease activity was used for the extension of upstream primers and downstream primers, the cleavage of TaqMan probe, the cleavage of PTO, and the extension of PTO fragment. Genomic DNA of *Neisseria gonorrhoeae* (NG) and genomic DNA of *Chlamydia trachomatis* (CT) were used as target nucleic acid sequences.

TaqMan real-time PCR was employed to detect CT. If CT is present, a TaqMan probe specifically hybridized to CT is cleaved and a labeled fragment is released. An amplification curve can be obtained by measuring a signal from the labeled fragment. PTOCE real-time PCR was used to detect NG. If NG is present, a PTO specifically hybridized to NG is cleaved and a PTO fragment is produced. The PTO fragment is annealed to the capturing portion of the CTO, extended on the

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templating portion of the CTO and forms an extended duplex with CTO (Duplexed CTO). The formation of the extended duplex provides a signal and an amplification curve can be obtained by measuring the signal at the extended duplex-forming temperature.

In this Example, "72°C" was selected as a signal detection temperature for CT, and "60°C" was selected as a signal detection temperature for NG with consideration of the signal generating means. The extended duplex produced depending on the presence of the NG has a controllable Tm value adjusted by its sequence and length. In this Example, the sequence and length of the extended duplex is designed to provide a signal as it forms the duplex at 60°C, but not to provide a signal as it is dissociated not to forms the duplex at 72°C. In the detection temperature of 72°C, the signal for CT will be generated and detected. In the detection temperature of 60°C, the signal for NG will be generated and detected as well as the signal for CT.

TaqMan probes are labeled with a fluorescent reporter molecule (CAL Fluor Red 610) at their 5'-ends and a quencher molecule at their 3'-ends (BHQ-2) (SEQ ID NO: 7). The PTO and CTO are blocked with a carbon spacer at their 3'-ends to prohibit their extension. CTO is labeled with a quencher molecule (BHQ-2) and a fluorescent reporter molecule (CAL Fluor Red 610) in its templating portion (SEQ ID NO: 4).

Four reaction tubes were prepared containing CT, NG, a mixture of CT and NG and no target control respectively.

The sequences of upstream primer, downstream primer, PTO, CTO, and TaqMan probe used in this Example are:

NG-F 5'-TACGCCTGCTACTTTCACGCTIIIIIGTAATCAGATG-3' (SEQ ID NO: 1)

NG-R 5'-CAATGGATCGGTATCACTCGCIIIIICGAGCAAGAAC-3' (SEQ ID NO: 2)

NG-PTO 5'-GTACGCGATACGGGCCCCTCATTGGCGTGTTTCG[C3 spacer]-3' (SEQ ID NO: 3)

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NG-CTO 5'-[BHQ-2]TTTTTTTTTTTTTTTTTTTTG[T(CAL Fluor Red 610)]ACTGCCCGTATCGCGTAC[C3 spacer]-3' (SEQ ID NO: 4)

CT-F 5'-GAGTTTTAAAATGGGAAATTCTGGTIIIIIITTTGTATAAC-3' (SEQ ID NO: 5)

CT-R 5'-CCAATTGTAATAGAAGCATTGGTTGIIIIIITTATTGGAGA-3' (SEQ ID NO: 6)

CT-P 5'-[CAL Fluor Red 610]CATCAGAAGCTGTCATTTTGGCTGCG[BHQ-2]-3' (SEQ ID NO: 7)

(I: Deoxyinosine)

(Underlined letters indicate the 5'-tagging portion of PTO)

The real-time PCR was conducted in the final volume of 20 μl containing a target nucleic acid (10 pg of NG genomic DNA, 10 pg of CT genomic DNA or a mixture of 10 pg of NG genomic DNA and 10 pg of CT genomic DNA), 10 pmole of upstream primer (SEQ ID NO: 1) and 10 pmole of downstream primer (SEQ ID NO: 2) for NG target amplification, 5 pmole of PTO (SEQ ID NO: 3), 1 pmole of CTO (SEQ ID NO: 4), 10 pmole of upstream primer (SEQ ID NO: 5) and 12 pmole of downstream primer (SEQ ID NO: 6) for CT target amplification, 1 pmole of TaqMan probe (SEQ ID NO: 7), and 10 μl of 2X Master Mix [final, 200 uM dNTPs, 2 mM $MgCl_2$, 2 U of *Taq* DNA polymerase (Enzynomics, Korea)]. The tubes containing the reaction mixture were placed in the real-time thermocycler (CFX96, Bio-Rad) for 5 min at 50°C, denatured for 15 min at 95°C and subjected to 50 cycles of 30 sec at 95°C, 60 sec at 60°C, 30 sec at 72°C. Detection of a signal was performed at 60°C and 72°C of each cycle.

As shown in Fig. 1A, signals were detected at 72°C and 60°C in the presence of the CT (Tube 1) or in the presence of both the NG and CT (Tube 3). In the presence of the NG, a signal was detected at 60°C but not at 72°C (Tube 2). No signal was detected in the absence of the target nucleic acids (Tube 4). The result of Fig. 1A shows that the signal for the CT having the relatively high detection temperature is generated, but the signal for the NG having the relatively low detection temperature not generated at the relatively high detection temperature, 72°C. Therefore, it would be appreciated that the signal detection at the relatively high detection temperature allows detecting at least the target nucleic acid sequence

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having the relatively high detection temperature. Also, the signal detection at the relatively low detection temperature enables detecting the target nucleic acid sequence having the relatively low detection temperature in the case that a signal is not detected at the relatively high detection temperature.

We employed several analytical methods to examine whether the presence of the NG having the relatively low detection temperature can be detected even in the presence or absence of the signal from the CT having the relative high detection temperature (Fig. 1B ~ 1E).

The Fig. 1B shows the ratio of RFU calculated with the RFU values obtained at 72°C and 60°C in the end point (All RFU values shown in Fig. 1B and processed for the ratio were derived and exported from "Baseline subtracted curve fit" analysis data in instrumental software). The ratio in the sole presence of CT (Tube 1) was 1.1 but, that in the presence of both CT and NG (Tube 3) was 1.8. Also, the ratio in the sole presence of NG (Tube 2) and in absence of any target nucleic acid (Tube 4) was 1278.0 and 1.0, respectively. Cut-off ratio, 1.2, was applied to determine the presence or absence of NG. In accordance with the cut-off ratio, the presence of NG was confirmed in Tube 2 and Tube 3, and there is no NG in Tube 1 and Tube 4. Thus, it would be appreciated that use of the ratio and its cut-off value allows detecting the signal of the target nucleic acid sequence having the relatively low detection temperature even in the presence or absence of the signal from the target nucleic acid having the relative high detection temperature.

In other aspect to using the signals at 72°C and 60°C, there is one to calculate the ratio of the fluorescence signals at 72°C and 60°C in each of cycles and plot the ratio against the cycle (All RFU values processed for the plotting were derived and exported from "No baseline subtraction" analysis data in instrumental software). Threshold, 0.1, was applied to determine the presence or absence of NG. As shown in Fig. 1C, the presence of NG was ascertained in Tube 2 and Tube 3 and its C_T value were 37.88 and 37.20, respectively. There is no C_T value obtained in Tube 1 and Tube 4. Hence, it would be appreciated that the plotting method enables detecting the

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signal of the target nucleic acid sequence having the relatively low detection temperature even in the presence or absence of the signal from the target nucleic acid having the relative high detection temperature.

Application of individual thresholds for the signal of each tube at 60°C is the other method to detect the presence of the NG having the relatively low detection temperature by using the signal at the relatively high detection temperature. In the case that the signal is detected at 72°C, the individual threshold for the signal of tubes at 60°C were calculated by multiplying each End-RFU value at 72°C by the cut-off ratio, 1.2, (refer to Fig. 1B). In the case that the signal is not detected at 72°C, a single threshold (i.e. 200) for the signal of tubes at 60°C is chosen and used with consideration of the background signal, sensitivity, signal variation or error range of device. The threshold for the signal of tubes at 72°C is chosen and used as above.

As shown in Fig. 1D and Fig. 1E, according to the individual threshold at 60°C, the presence of NG was confirmed in Tube 2 and Tube 3 and its C_T value were 36.21 and 37.07, respectively. No C_T value for the NG was available in Tube 1 and Tube 4. Therefore, it would be appreciated that application of the individual threshold derived from the cut-off ratio and the RFU values at 72°C allows detecting the signal of the target nucleic acid sequence having the relatively low detection temperature even in the presence or absence of the signal from the target nucleic acid having the relative high detection temperature.

These results indicates that in the TaqMan/PTOCE real-time method comprising signal detection at two temperatures, (i) the signal detection at the relatively high detection temperature allows detecting the target nucleic acid sequence having the relatively high detection temperature and (ii) the signals obtained at the relatively high detection temperature and the relatively low detection temperature can be used to detect the target nucleic acid sequence having the relatively low detection temperature.

Therefore, a plural of target nucleic acids can be detected in a single reaction vessel by using a single detection channel and TaqMan/PTOCE real-time PCR

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comprising signal detection at different temperatures.

Example 2: Multiple target detection by PTOCE real-time PCR comprising signal detection at different temperatures.

We further examined whether a plural of target nucleic acids can be detected in a single reaction vessel by using a single detection channel and PTOCE real-time PCR comprising signal detection at different temperatures.

Taq DNA polymerase having a 5' nuclease activity was used for the extension of upstream primers and downstream primers, the cleavage of PTO, and the extension of PTO fragment. Genomic DNA of *Neisseria gonorrhoeae* (NG) and genomic DNA of *Chlamydia trachomatis* (CT) were used as target nucleic acid sequences.

PTOCE real-time PCR was used to detect CT and NG. If a target is present, a PTO specifically hybridized to the target is cleaved and a PTO fragment is produced. The PTO fragment is annealed to the capturing portion of the CTO, extended on the templating portion of the CTO and forms an extended duplex with CTO (Duplexed CTO). The formation of the extended duplex provides a signal and an amplification curve can be obtained by measuring the signal at the extended duplex-forming temperature.

In this Example, "72°C" was selected as a signal detection temperature for CT, and "60°C" was selected as a signal detection temperature for NG with consideration of the signal generating means. The extended duplex produced depending on the presence of the CT or NG has a controllable Tm value adjusted by its sequence and length. In this Example, the sequence and length of the extended duplex for CT is designed to provide a signal as it forms the duplex at 72°C. Meanwhile, the sequence and length of the extended duplex for NG is designed to provide a signal as it forms the duplex at 60°C, but not to provide a signal as it is dissociated not to forms the duplex at 72°C. In the detection temperature of 72°C, the signal for CT will be generated and detected. In the detection temperature of

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60°C, the signal for NG will be generated and detected as well as the signal for CT.

The PTO and CTO are blocked with a carbon spacer at their 3'-ends to prohibit their extension. CTO is labeled with a quencher molecule (BHQ-2) and a fluorescent reporter molecule (CAL Fluor Red 610) in its templating portion (SEQ ID NO: 4 and 9).

Four reaction tubes were prepared containing CT, NG, a mixture of CT and NG and no target control respectively.

The sequences of upstream primer, downstream primer, PTO, and CTO used in this Example are:

NG-F 5'-TACGCCTGCTACTTTCACGCTIIIIIGTAATCAGATG-3' (SEQ ID NO: 1)

NG-R 5'-CAATGGATCGGTATCACTCGCIIIIICGAGCAAGAAC-3' (SEQ ID NO: 2)

NG-PTO 5'-GTACGCGATACGGGCCCCTCATTGGCGTGTTTCG[C3 spacer]-3' (SEQ ID NO: 3)

NG-CTO 5'-[BHQ-2]TTTTTTTTTTTTTTTTTTTG[T(CAL Fluor Red 610)]ACTGCCCGTATCGCGTAC[C3 spacer]-3' (SEQ ID NO: 4)

CT-F 5'-GAGTTTTAAAATGGGAAATTCTGGTIIIIITTTGTATAAC-3' (SEQ ID NO: 5)

CT-R 5'-CCAATTGTAATAGAAGCATTGGTTGIIIIITTATTGGAGA-3' (SEQ ID NO: 6)

CT-PTO 5'-GATTACGCGACCGCATCAGAAGCTGTCATTTTGGCTGCG[C3 spacer]-3' (SEQ ID NO: 8)

CT-CTO 5'-[BHQ-2]GCGCTGGATACCCTGGACGA[T(CAL Fluor Red 610)]ATGTGCGGTCGCGTAATC[C3 spacer]-3' (SEQ ID NO: 9)

(I: Deoxyinosine)

(Underlined letters indicate the 5'-tagging portion of PTO)

The real-time PCR was conducted in the final volume of 20 μl containing a target nucleic acid (10 pg of NG genomic DNA, 10 pg of CT genomic DNA or a mixture of 10 pg of NG genomic DNA and 10 pg of CT genomic DNA), 5 pmole of upstream primer (SEQ ID NO: 1) and 5 pmole of downstream primer (SEQ ID NO: 2) for NG target amplification, 3 pmole of PTO (SEQ ID NO: 3), 1 pmole of CTO (SEQ ID NO: 4),

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5 pmole of upstream primer (SEQ ID NO: 5) and 5 pmole of downstream primer (SEQ ID NO: 6) for CT target amplification, 3 pmole of PTO (SEQ ID NO: 8), 1 pmole of CTO (SEQ ID NO: 9), and 10 μl of 2X Master Mix [final, 200 uM dNTPs, 2 mM $MgCl_2$, 2 U of *Taq* DNA polymerase (Enzynomics, Korea)]. The tubes containing the reaction mixture were placed in the real-time thermocycler (CFX96, Bio-Rad) for 5 min at 50°C, denatured for 15 min at 95°C and subjected to 50 cycles of 30 sec at 95°C, 60 sec at 60°C, 30 sec at 72°C. Detection of a signal was performed at 60°C and 72°C of each cycle.

As shown in Fig. 2A, signals were detected at 72°C and 60°C in the presence of the CT (Tube 1) or in the presence of both the NG and CT (Tube 3). In the presence of the NG, a signal was detected at 60°C but not at 72°C (Tube 2). No signal was detected in the absence of the target nucleic acids (Tube 4). The result of Fig. 2A shows that the signal for the CT having the relatively high detection temperature is generated, but the signal for the NG having the relatively low detection temperature not generated at the relatively high detection temperature, 72°C. Therefore, it would be appreciated that the signal detection at the relatively high detection temperature allows detecting at least the target nucleic acid sequence having the relatively high detection temperature. Also, the signal detection at the relatively low detection temperature enables detecting the target nucleic acid sequence having the relatively low detection temperature in the case that a signal is not detected at the relatively high detection temperature.

We employed several analytical methods to examine whether the presence of the NG having the relatively low detection temperature can be detected even in the presence or absence of the signal from the CT having the relative high detection temperature (Fig. 2B ~ 2E).

The Fig. 2B shows the ratio of RFU calculated with the RFU values obtained at 72°C and 60°C in the end point (All RFU values shown in Fig. 2B and processed for the ratio were derived and exported from "Baseline subtracted curve fit" analysis data in instrumental software). The ratio in the sole presence of CT (Tube 1) was 1.2 but,

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that in the presence of both CT and NG (Tube 3) was 2.1. Also, the ratio in the sole presence of NG (Tube 2) and in absence of any target nucleic acid (Tube 4) was 36.7 and 0.7, respectively. Cut-off ratio, 1.5, was applied to determine the presence or absence of NG. In accordance with the cut-off ratio, the presence of NG was confirmed in Tube 2 and Tube 3, and there is no NG in Tube 1 and Tube 4. Thus, it would be appreciated that use of the ratio and its cut-off value allows detecting the signal of the target nucleic acid sequence having the relatively low detection temperature even in the presence or absence of the signal from the target nucleic acid having the relative high detection temperature.

In other aspect to using the signals at 72°C and 60°C, there is one to calculate the ratio of the fluorescence signals at 72°C and 60°C in each of cycles and plot the ratio against the cycle (All RFU values processed for the plotting were derived and exported from "No baseline subtraction" analysis data in instrumental software). Threshold, 0.1, was applied to determine the presence or absence of NG. As shown in Fig. 2C, the presence of NG was ascertained in Tube 2 and Tube 3 and its C_T value were 32.90 and 33.18, respectively. There is no C_T value obtained in Tube 1 and Tube 4. Hence, it would be appreciated that the plotting method enables detecting the signal of the target nucleic acid sequence having the relatively low detection temperature even in the presence or absence of the signal from the target nucleic acid having the relative high detection temperature.

Application of individual thresholds for the signal of each tube at 60°C is the other method to detect the presence of the NG having the relatively low detection temperature by using the signal at the relatively high detection temperature. In the case that the signal is detected at 72°C, the individual threshold for the signal of tubes at 60°C were calculated by multiplying each End-RFU value at 72°C by the cut-off ratio, 1.5, (refer to Fig. 2B). In the case that the signal is not detected at 72°C, a single threshold (i.g. 200) for the signal of tubes at 60°C is chosen and used with consideration of the background signal, sensitivity, signal variation or error range of device. The threshold for the signal of tubes at 72°C is chosen and used as above.

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As shown in Fig. 2D and Fig. 2E, according to the individual threshold at 60°C, the presence of NG was confirmed in Tube 2 and Tube 3 and its C_T value were 31.32 and 35.83, respectively. No C_T value for the NG was available in Tube 1 and Tube 4. Therefore, it would be appreciated that application of the individual threshold derived from the cut-off ratio and the RFU values at 72°C allows detecting the signal of the target nucleic acid sequence having the relatively low detection temperature even in the presence or absence of the signal from the target nucleic acid having the relative high detection temperature.

These results indicates that in the PTOCE real-time method comprising signal detection at two temperatures, (i) the signal detection at the relatively high detection temperature allows detecting the target nucleic acid sequence having the relatively high detection temperature and (ii) the signals obtained at the relatively high detection temperature and the relatively low detection temperature can be used to detect the target nucleic acid sequence having the relatively low detection temperature.

Therefore, a plural of target nucleic acids can be detected in a single reaction vessel by using a single detection channel and PTOCE real-time PCR comprising signal detection at different temperatures.

Example 3: Multiple target detection by real-time PCR and melting analysis

We examined whether a plural of target nucleic acids can be detected in a single reaction vessel by using a single detection channel and combination of real-time PCR and melting analysis.

Taq DNA polymerase having a 5′ nuclease activity was used for the extension of upstream primers and downstream primers, the cleavage of TaqMan probe, the cleavage of PTO, and the extension of PTO fragment. Genomic DNA of *Neisseria gonorrhoeae* (NG) and genomic DNA of *Chlamydia trachomatis* (CT) were used as target nucleic acid sequences.

TaqMan real-time PCR was employed to detect CT and PTOCE melting

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analysis was used to detect NG. If CT is present, a TaqMan probe specifically hybridized to CT is cleaved and a labeled fragment is released. If NG is present, a PTO specifically hybridized to NG is cleaved and a PTO fragment is produced. The PTO fragment is annealed to the capturing portion of the CTO, extended on the templating portion of the CTO and forms an extended duplex with CTO (Duplexed CTO).

In order to detect only the florescent signal generated by cleavage of TaqMan probe during real-time PCR process, the fluorescent signal detection is performed, at the temperature where the extended duplex is dissociated not to form the duplex and the signal from the extended duplex formed by PTOCE is not generated. In the melting process, a signal is measured to obtain a melting peak indicating the presence of the extended duplex formed depending on the presence of NG.

TaqMan probes are labeled with a fluorescent reporter molecule (CAL Fluor Red 610) at their 5'-ends and a quencher molecule at their 3'-ends (BHQ-2) (SEQ ID NO: 7). The PTO and CTO are blocked with a carbon spacer at their 3'-ends to prohibit their extension. CTO is labeled with a quencher molecule (BHQ-2) and a fluorescent reporter molecule (CAL Fluor Red 610) in its templating portion (SEQ ID NO: 4).

The sequences of upstream primer, downstream primer, PTO, CTO, and TaqMan probe used in this Example are:

NG-F 5'-TACGCCTGCTACTTTCACGCTIIIIIGTAATCAGATG-3' (SEQ ID NO: 1)

NG-R 5'-CAATGGATCGGTATCACTCGCIIIIICGAGCAAGAAC-3' (SEQ ID NO: 2)

NG-PTO 5'-GTACGCGATACGGGCCCCTCATTGGCGTGTTTCG[C3 spacer]-3' (SEQ ID NO: 3)

NG-CTO 5'-[BHQ-2]TTTTTTTTTTTTTTTTTTTTG[T(CAL Fluor Red 610)]ACTGCCCGTATCGCGTAC[C3 spacer]-3' (SEQ ID NO: 4)

CT-F 5'-GAGTTTTAAAATGGGAAATTCTGGTIIIIITTTGTATAAC-3' (SEQ ID NO: 5)

CT-R 5'-CCAATTGTAATAGAAGCATTGGTTGIIIIITTATTGGAGA-3' (SEQ ID NO: 6)

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CT-P 5'-[CAL Fluor Red 610]CATCAGAAGCTGTCATTTTGGCTGCG[BHQ-2]-3' (SEQ ID NO: 7)

(I: Deoxyinosine)

(Underlined letters indicate the 5'-tagging portion of PTO)

The real-time PCR was conducted in the final volume of 20 μl containing a target nucleic acid (10 pg of NG genomic DNA, 10 pg of CT genomic DNA or a mixture of 10 pg of NG genomic DNA and 10 pg of CT genomic DNA), 10 pmole of upstream primer (SEQ ID NO: 1) and 10 pmole of downstream primer (SEQ ID NO: 2) for NG target amplification, 5 pmole of PTO (SEQ ID NO: 3), 1 pmole of CTO (SEQ ID NO: 4), 10 pmole of upstream primer (SEQ ID NO: 5) and 12 pmole of downstream primer (SEQ ID NO: 6) for CT target amplification, 1 pmole of TaqMan probe (SEQ ID NO: 7), and 10 μl of 2X Master Mix [final, 200 uM dNTPs, 2 mM $MgCl_2$, 2 U of *Taq* DNA polymerase (Enzynomics, Korea)]. The tubes containing the reaction mixture were placed in the real-time thermocycler (CFX96, Bio-Rad) for 5 min at 50°C, denatured for 15 min at 95°C and subjected to 50 cycles of 30 sec at 95°C, 60 sec at 60°C, 30 sec at 72°C. Detection of a signal was performed at 72°C of each cycle. After the reaction, the tube was placed for 5 min at 55°C. Melting curve analysis was performed by measuring a florescent signal during the temperature rise from 55°C to 95°C in an interval of 0.5°C.

As shown in Fig. 3, in the presence of CT, an amplification curve was obtained during the real-time PCR, but no melting peak was observed in the melting analysis (Tube 1). In the presence of NG, an amplification curve was not obtained during the real-time PCR, but a melting peak having the expected Tm value of the extended duplex formed depending on the presence of NG was observed in the melting analysis (Tube 2). In the presence of CT and NG, signals were observed during both real-time PCR process and melting analysis process (Tube 3). No signal was detected in the absence of the target nucleic acids (Tube 4). These results indicate that a plural of target nucleic acids can be detected with a single detection channel by combination of real-time PCR and melting analysis.

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Example 4: SNP Genotyping by real-time PCR comprising signal detection at different temperatures.

We examined whether a real-time PCR comprising signal detection at a different temperature can be applied to SNP genotyping in a single reaction vessel by using a single detection channel.

Taq DNA polymerase having a 5′ nuclease activity was used for the extension of upstream primer and downstream primer, the cleavage of PTO, and the extension of PTO fragment. Wild (C) homozygote, mutant type (T) homozygote, and heterozygote of MTHFR (C677T) human genomic DNA were used as target nucleic acid sequences.

PTOCE real-time PCR was used to detect the wild (C) allele and mutant type (T) allele of the MTHFR (C677T) human genomic DNA. If a target allele is present, a PTO specifically hybridized to the target allele is cleaved and a PTO fragment is produced. The PTO fragment is annealed to the capturing portion of the CTO, extended on the templating portion of the CTO and forms an extended duplex with CTO (Duplexed CTO). The formation of the extended duplex provides a signal and an amplification curve can be obtained by measuring the signal at the extended duplex-forming temperature.

In this Example, "72°C" was selected as a signal detection temperature for the wild (C) allele and "55°C" was selected as a signal detection temperature for the mutant type (T) allele with consideration of the signal generating means. The extended duplex produced depending on the presence of the wild (C) allele or the mutant type (T) allele has a controllable Tm value adjusted by their sequence and length. In this Example, the sequence and length of the extended duplex for the wild (C) allele is designed to provide a signal as it forms the duplex at 72°C. Meanwhile, the sequence and length of the extended duplex for the mutant type (T) allele is designed to provide a signal as it forms the duplex at 55°C, but not to provide a signal as it is dissociated not to forms the duplex at 72°C. In the detection

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temperature of 72°C, the signal for the wild (C) allele will be generated and detected. In the detection temperature of 55°C, the signal for the mutant type (T) allele will be generated and detected as well as the signal for the wild (C) allele.

The PTO and CTO are blocked with a carbon spacer at their 3'-ends to prohibit their extension. CTO is labeled with a quencher molecule (BHQ-2) and a fluorescent reporter molecule (CAL Fluor Red 610) in its templating portion (SEQ ID NO: 13 and 15).

The sequences of upstream primer, downstream primer, PTO, and CTO used in this Example are:

M677-F 5'-CCACCCCGAAGCAGGGAIIIIIGAGGCTGACC-3' (SEQ ID NO: 10)

M677-R 5'-CAAGTGATGCCCATGTCGGIIIIIGCCTTCACAA-3' (SEQ ID NO: 11)

M677-W-PTO 5'-GGTCCCGACGTTAGCTCCCGCAGACACCTTCTCCTTC[C3 spacer]-3' (SEQ ID NO: 12)

M677-W-CTO 5'-[BHQ-2]CCTCGGTGCCACGCCATCGG[T(CAL Fluor Red 610)]TCTTCTAACGTCGGGACC[C3 spacer]-3' (SEQ ID NO: 13)

M677-M-PTO 5'-ACGTCGATTCGCACTCCCGCAGACACCTTCTCCTTCAA[C3 spacer]-3' (SEQ ID NO: 14)

M677-M-CTO 5'-[BHQ-2]TTTTTTTTTTTTTTTTTTTT[T(CAL Fluor Red 610)]ATTCTGCGAATCGACGT[C3 spacer] -3' (SEQ ID NO: 15)

(I: Deoxyinosine)

(Underlined letters indicate the 5'-tagging portion of PTO)

The real-time PCR was conducted in the final volume of 20 μl containing a target nucleic acid (1 ng of wild (C) homozygous MTHFR (C677T) human genomic DNA, 1 ng of mutant (T) homozygous MTHFR (C677T) human genomic DNA, or 1 ng of heterozygous MTHFR (C677T) human genomic DNA), 5 pmole of upstream primer (SEQ ID NO: 10) and 5 pmole of downstream primer (SEQ ID NO: 11) for human MTHFR gene amplification, 3 pmole of PTO (SEQ ID NOs: 12 and 14), 1 pmole of CTO (SEQ ID NOs: 13 and 15), and 10 μl of 2X Master Mix [final, 200 uM dNTPs, 2 mM

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$MgCl_2$, 2 U of *Taq* DNA polymerase (Enzynomics, Korea)]. The tubes containing the reaction mixture were placed in the real-time thermocycler (CFX96, Bio-Rad) for 5 min at 50°C, denatured for 15 min at 95°C and subjected to 50 cycles of 30 sec at 95°C, 60 sec at 55°C, 30 sec at 72°C. Detection of a signal was performed at 55°C and 72°C of each cycle.

As shown in Fig. 4A, signals were detected at 72°C and 55°C in the presence of the wild (C) homozygote (Tube 1) or in the presence of the heterozygote (Tube 3). In the presence of the mutant (T) homozygote, an intense signal was detected at 55°C but a very weak signal at 72°C (Tube 2). No signal was detected in the absence of the target nucleic acids (Tube 4).

The Fig. 4B shows the ratio of RFU calculated with the RFU values obtained at 72°C and 55°C in the end point (All RFU values shown in Fig. 4B and processed for the ratio were derived and exported from "Baseline subtracted curve fit" analysis data in instrumental software). The ratio in the presence of the wild (C) homozygote (Tube 1) was 1.2 but, that in the presence of the heterozygote (Tube 3) was 1.9. Such difference between the two ratios indicates that the mutant (T) allele exists with the wild (C) allele in the tube comprising the heterozygote. Meanwhile, the ratio obtained from the mutant (T) homozygote was relatively very large, as the RFU value in 72°C was low (Tube 2). Considering the fact that a wild allele and a mutant allele are present in the ratio of 1:1 in a heterozygote, the weak signal detected at 72°C in the presence of mutant type homozygote can be appreciated to be a false positive signal. According to the present method for SNP genotyping, the ratio value serves to determine whether the signal at the relative high detection temperature is a false signal or not.

These results indicate a real-time PCR comprising signal detection at a different temperature can be applied to SNP genotyping with a single detection channel.

Having described a preferred embodiment of the present invention, it is to

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be understood that variants and modifications thereof falling within the spirit of the invention may become apparent to those skilled in this art, and the scope of this invention is to be determined by appended claims and their equivalents.

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What is claimed is:

1. A method for detecting two target nucleic acid sequences in a sample using different detection temperatures, comprising:

(a) incubating the sample with two signal-generating means for detection of the two target nucleic acid sequences in a single reaction vessel and detecting a generated signal by using a single type of detector; wherein each of the target nucleic acid sequences is detected by a corresponding signal-generating means; wherein one of the two target nucleic acid sequences has a relatively high detection temperature and the other has a relatively low detection temperature determined by the corresponding signal-generating means; wherein the relatively high detection temperature is a temperature capable of generating a signal for the target nucleic acid sequence having the relatively high detection temperature and the relatively low detection temperature is a temperature capable of generating both of a signal for the target nucleic acid sequence having the relatively low detection temperature and a signal for the target nucleic acid sequence having the relatively high detection temperature; wherein signals to be generated by the two signal-generating means are not differentiated by the single type of detector; wherein the detection is performed at both the relatively high detection temperature and the relatively low detection temperature;

(b) determining the presence of the two target nucleic acid sequences by using the signal detected in the step (a); wherein (i) the presence of the target nucleic acid sequence having the relatively high detection temperature is determined by using the signal detected at the relatively high detection temperature and (ii) the presence of the target nucleic acid sequence having the relatively low detection temperature is determined by using a difference between the signal detected at the relatively high detection temperature and the signal detected at the relatively low detection temperature.

2. The method according to claim 1, wherein the step (a) is performed in a signal

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amplification process concomitantly with a nucleic acid amplification.

3. The method according to claim 1, wherein the step (a) is performed in a signal amplification process without a nucleic acid amplification.

4. The method according to claim 1, wherein at least one of the two signal-generating means is a signal-generating means to generate a signal in a dependent manner on the formation of a duplex.

5. The method according to claim 1, wherein at least one of the two signal-generating means is a signal-generating means to generate a signal in a dependent manner on cleavage of a detection oligonucleotide.

6. The method according to claim 1, wherein the signal-generating means for the target nucleic acid sequence having the relatively high detection temperature is a signal-generating means by cleavage of a detection oligonucleotide, and the signal-generating means for the target nucleic acid sequence having the relatively low detection temperature is a signal-generating means by the formation of a duplex.

7. The method according to claim 1, wherein the two signal-generating means comprise an identical label and signals from the label are not differentiated by the single type of detector.

8. The method according to claim 1, wherein when the signal-generating means generates a signal in a dependent manner on the formation of a duplex, the detection temperature is selected based on a T_m value of the duplex.

9. The method according to claim 1, wherein when the signal-generating means generates a signal in a dependent manner on cleavage of a detection oligonucleotide,

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the detection temperature is arbitrarily selected.

10. The method according to claim 1, wherein said using the difference comprises using a difference to be obtained by mathematically processing the signal detected at the relatively high detection temperature and the signal detected at the relatively low detection temperature.

11. The method according to claim 1, wherein when the signal is not detected at the relatively high detection temperature, said using the difference comprises using the signal detected at the relatively low detection temperature with considering no detection of the signal at the relatively high detection temperature.

12. The method according to claim 1, wherein said using the signal detected at the relatively high detection temperature comprises obtaining a qualifying value for determining the presence of the target nucleic acid sequence having the relatively high detection temperature and said using the difference comprises obtaining a qualifying value for determining the presence of the target nucleic acid sequence having the relatively low detection temperature.

13. The method according to claim 1, wherein the two target nucleic acid sequences comprise a nucleotide variation and one of the two target nucleic acid sequences comprises one type of the nucleotide variation and the other comprises the other type of the nucleotide variation.

14. The method according to claim 13, wherein the nucleotide variation is a SNP (single nucleotide polymorphism).

15. A method for SNP genotyping of a nucleic acid sequence in a sample using different detection temperatures, comprising:

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(a) incubating the sample comprising the nucleic acid sequence containing a SNP (single nucleotide polymorphism) site and a signal-generating means for detection of SNP alleles in a single reaction vessel and detecting a generated signal by using a single type of detector; wherein each of the SNP alleles is detected by a corresponding signal-generating means; wherein one of the SNP alleles has a relatively high detection temperature and the other has a relatively low detection temperature determined by the corresponding signal-generating means; wherein the relatively high detection temperature is a temperature capable of generating a signal for the SNP allele having the relatively high detection temperature and the relatively low detection temperature is a temperature capable of generating both of a signal for the SNP allele having the relatively low detection temperature and a signal for the SNP allele having the relatively high detection temperature; wherein signals to be generated by the signal-generating means are not differentiated by the single type of detector; wherein the detection is performed at both the relatively high detection temperature and the relatively low detection temperature;

(b) determining a SNP genotype by using a difference between the signal detected at the relatively high detection temperature and the signal detected at the relatively low detection temperature in the step (a).

16. A method for detecting at least three target nucleic acid sequences in a sample using different detection temperatures, comprising:

(a) incubating the sample with at least three signal-generating means for detection of the at least three target nucleic acid sequences in a single reaction vessel and detecting a generated signal by using a single type of detector; wherein each of the at least three target nucleic acid sequences is detected by a corresponding signal-generating means; wherein each of the at least three target nucleic acid sequences has a different detection temperature determined by the corresponding signal-generating means; wherein a detection temperature is a temperature capable of generating not only a signal for a target nucleic acid sequence having the detection

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temperature but also a signal for a target nucleic acid sequence having a higher detection temperature than the detection temperature; wherein signals to be generated by the signal-generating means are not differentiated by the single type of detector; wherein the detection is performed at each of the different detection temperatures;

(b) determining the presence of the at least three target nucleic acid sequences by using the signal detected in the step (a); wherein when the presence of a target nucleic acid sequence having a certain detection temperature among the at least three target nucleic acid sequences is determined, the presence of the target nucleic acid sequence having the certain detection temperature is determined by using a difference between the signal detected at one or more detection temperatures higher than the certain detection temperature and the signal detected at the certain detection temperature; wherein when the certain detection temperature is a relatively highest detection temperature among the detection temperatures, the presence of a target nucleic acid sequence is determined by using the signal detected at the certain detection temperature.

17. The method according to claim 16, wherein the step (b) is performed by determining firstly the presence of a target nucleic acid sequence having a relatively highest detection temperature and then sequentially determining the presence of target nucleic acid sequences having relatively lower detection temperatures in a descending order.

18. The method according to claim 16, wherein the step (a) is performed in a signal amplification process concomitantly with a nucleic acid amplification.

19. The method according to claim 16, wherein the step (a) is performed in a signal amplification process without a nucleic acid amplification.

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20. The method according to claim 16, wherein at least one of the signal-generating means is a signal-generating means to generate a signal in a dependent manner on the formation of a duplex.

21. The method according to claim 16, wherein at least one of the signal-generating means is a signal-generating means to generate a signal in a dependent manner on cleavage of a detection oligonucleotide.

22. The method according to claim 16, wherein the signal-generating means for the target nucleic acid sequence having the relatively highest detection temperature is a signal-generating means by cleavage of a detection oligonucleotide, and the signal-generating means for the other target nucleic acid sequences are a signal-generating means by the formation of a duplex.

23. The method according to claim 16, wherein the at least three signal-generating means comprise an identical label and signals from the label are not differentiated by the single type of detector.

24. The method according to claim 16, wherein when the signal-generating means generates a signal in a dependent manner on the formation of a duplex, the detection temperature is selected based on a T_m value of the duplex.

25. The method according to claim 16, wherein when the signal-generating means generates a signal in a dependent manner on cleavage of a detection oligonucleotide, the detection temperature is arbitrarily selected.

26. The method according to claim 16, wherein said using the difference comprises using a difference to be obtained by mathematically processing the signal detected at one or more detection temperatures higher than the certain detection temperature

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and the signal detected at the certain detection temperature.

27. The method according to claim 16, wherein when the signal is not detected at the detection temperatures higher than the certain detection temperature, said using the difference comprises using the signal detected at the certain detection temperature with considering no detection of the signal at the detection temperatures higher than the certain detection temperature.

28. The method according to claim 16, wherein said using the signal detected at the certain detection temperature comprises obtaining a qualifying value for determining the presence of the target nucleic acid sequence having the relatively highest detection temperature and said using the difference comprises obtaining a qualifying value for determining the presence of the target nucleic acid sequence having the certain detection temperature.

29. The method according to claim 16, wherein the target nucleic acid sequences comprise a nucleotide variation.

30. A method for detecting two target nucleic acid sequences in a sample using different detection temperatures and melting analysis, comprising:

(a) incubating the sample with two signal-generating means for detection of the two target nucleic acid sequences in a single reaction vessel and detecting a generated signal by using a single type of detector; wherein each of the target nucleic acid sequences is detected by a corresponding signal-generating means; wherein one of the two target nucleic acid sequences has a relatively high detection temperature and the other has a relatively low detection temperature determined by the corresponding signal-generating means; wherein the relatively high detection temperature is a temperature capable of generating a signal for the target nucleic acid sequence having the relatively high detection temperature and the relatively low

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detection temperature is a temperature capable of generating both of a signal for the target nucleic acid sequence having the relatively low detection temperature and a signal for the target nucleic acid sequence having the relatively high detection temperature; wherein signals to be generated by the two signal-generating means are not differentiated by a single type of detector; wherein the detection is performed at the relatively high detection temperature;

(b) performing a melting analysis of the incubation resultant of the step (a) over a range of temperatures for determining the presence of the target nucleic acid sequence having the relatively low detection temperature; and

(c) determining the presence of the target nucleic acid sequence having the relatively high detection temperature by using the signal detected in the step (a) and the presence of the target nucleic acid sequence having the relatively low detection temperature by using the result of the melting analysis in the step (b).

31. The method according to claim 30, wherein the step (a) is performed in a signal amplification process concomitantly with a nucleic acid amplification.

32. The method according to claim 30, wherein the step (a) is performed in a signal amplification process without a nucleic acid amplification.

33. The method according to claim 30, wherein at least one of the two signal-generating means is a signal-generating means to generate a signal in a dependent manner on the formation of a duplex.

34. The method according to claim 30, wherein at least one of the two signal-generating means is a signal-generating means to generate a signal in a dependent manner on cleavage of a detection oligonucleotide.

35. The method according to claim 30, wherein the signal-generating means for the

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target nucleic acid sequence having the relatively high detection temperature is a signal-generating means by cleavage of a detection oligonucleotide, and the signal-generating means for the target nucleic acid sequence having the relatively low detection temperature is a signal-generating means by the formation of a duplex.

36. The method according to claim 30, wherein the two signal-generating means comprise an identical label and signals from the label are not differentiated by the single type of detector.

37. The method according to claim 30, wherein when the signal-generating means generates a signal in a dependent manner on the formation of a duplex, the detection temperature is selected based on a T_m value of the duplex.

38. The method according to claim 30, wherein when the signal-generating means generates a signal in a dependent manner on cleavage of a detection oligonucleotide, the detection temperature is arbitrarily selected.

39. The method according to claim 30, wherein said using the signal detected in the step (a) comprises obtaining a qualifying value for determining the presence of the target nucleic acid sequence having the relatively high detection temperature.

40. The method according to claim 30, wherein the target nucleic acid sequences comprise a nucleotide variation.

41. A method for detecting at least three target nucleic acid sequences in a sample using detection temperature analysis and melting analysis, comprising:

(a) incubating the sample with at least three signal-generating means for detection of the at least three target nucleic acid sequences in a single reaction vessel and detecting a generated signal by using a single type of detector; wherein each of

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the at least three target nucleic acid sequences is detected by a corresponding signal-generating means; wherein each of the at least three target nucleic acid sequences has a different detection temperature determined by the corresponding signal-generating means; wherein a detection temperature is a temperature capable of generating not only a signal for a target nucleic acid sequence having the detection temperature but also a signal for a target nucleic acid sequence having a higher detection temperature than the detection temperature; wherein signals to be generated by the signal-generating means are not differentiated by the single type of detector; wherein some of the at least three target nucleic acid sequences are detected by detection temperature analysis and the detection is performed at both the detection temperature of said some of the at least three target nucleic acid sequences and one or more detection temperatures higher than the detection temperatures;

(b) performing a melting analysis of the incubation resultant of the step (a) over a range of temperatures for determining the presence of the other target nucleic acid sequences than said some of the at least three the target nucleic acid sequences; and

(c) determining (i) the presence of said some of the target nucleic acid sequences by using the signal detected in the step (a); wherein when the presence of a target nucleic acid sequence having a certain detection temperature among said some of the at least three target nucleic acid sequences is determined, the presence of the target nucleic acid sequence having the certain detection temperature is determined by using a difference between the signal detected at one or more detection temperatures higher than the certain detection temperature and the signal detected at the certain detection temperature; wherein when the certain detection temperature is a relatively highest detection temperature among the detection temperatures, the presence of the target nucleic acid sequence is determined by using the signal detected at the certain detection temperature; and (ii) the presence of the other target nucleic acid sequences than said some of the at least three target

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nucleic acid sequences is determined by the result of the melting analysis in the step (b).

42. The method according to claim 41, wherein the step (c) is performed by determining firstly the presence of a target nucleic acid sequence having a relatively highest detection temperature and then sequentially determining the presence of target nucleic acid sequences having relatively lower detection temperatures in a descending order.

43. The method according to claim 41, wherein the step (a) is performed in a signal amplification process concomitantly with a nucleic acid amplification.

44. The method according to claim 41, wherein the step (a) is performed in a signal amplification process without a nucleic acid amplification.

45. The method according to claim 41, wherein at least one of the signal-generating means is a signal-generating means to generate a signal in a dependent manner on the formation of a duplex.

46. The method according to claim 41, wherein at least one of the signal-generating means is a signal-generating means to generate a signal in a dependent manner on cleavage of a detection oligonucleotide.

47. The method according to claim 41, wherein the signal-generating means for the target nucleic acid sequence having the relatively highest detection temperature is a signal-generating means by cleavage of a detection oligonucleotide, and the signal-generating means for the other target nucleic acid sequences are a signal-generating means by the formation of a duplex.

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48. The method according to claim 41, wherein the target nucleic acid sequences comprise a nucleotide variation.

49. A method for detecting a target nucleic acid sequence selected from two target nucleic acid sequences providing non-differentiated signals by a single type of detector in a single reaction vessel, comprising:

(a) assigning different detection temperatures to the two target nucleic acid sequences from each other; wherein the target nucleic acid sequence selected among the two target nucleic acid sequences has a relatively high detection temperature and the other has a relatively low detection temperature;

(b) incubating the two target nucleic acid sequences with two signal-generating means for detection of the two target nucleic acid sequences in a single reaction vessel; wherein each of the target nucleic acid sequences is detected by a corresponding signal-generating means; wherein the relatively high detection temperature is a temperature capable of generating a signal for the target nucleic acid sequence having the relatively high detection temperature and the relatively low detection temperature is a temperature capable of generating both of a signal for the target nucleic acid sequence having the relatively low detection temperature and a signal for the target nucleic acid sequence having the relatively high detection temperature; wherein signals to be generated by the two signal-generating means are not differentiated by the single type of detector; and

(c) detecting a generated signal at the relatively high detection temperature by the single type of detector to obtain only the signal for the target nucleic acid sequence having the relatively high detection temperature, whereby the detected signal is indicative of the presence of the target nucleic acid sequence having the relatively high detection temperature.

50. The method according to claim 49, wherein the signal for the target nucleic acid sequence having the relatively high detection temperature is a calculation parameter

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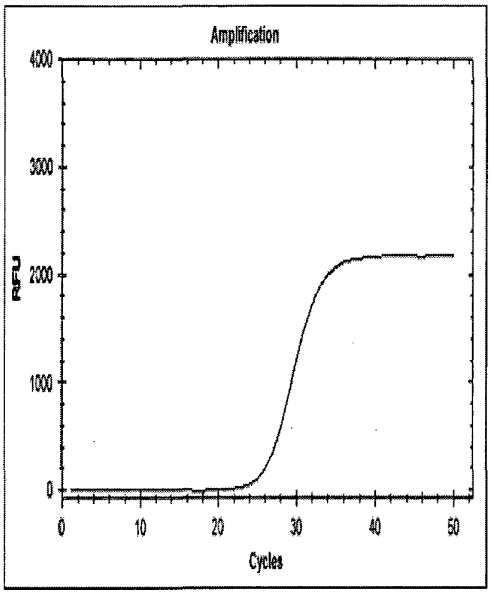
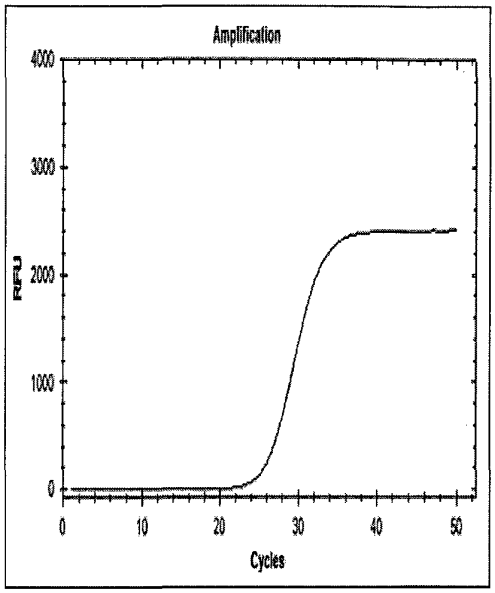
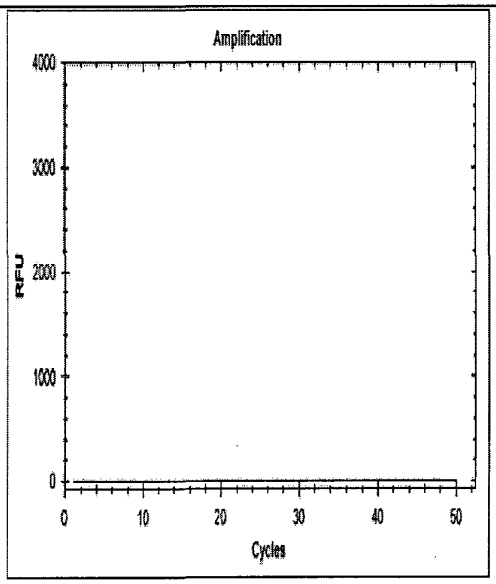
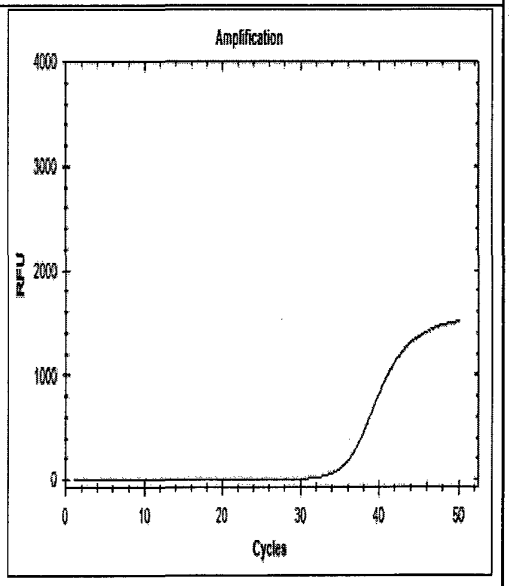
to analyze the signal for the target nucleic acid sequence having the relatively low detection temperature.

51. The method according to claim 49, wherein the signal for the target nucleic acid sequence having the relatively high detection temperature is generated by a signal-generating means to generate a signal in a dependent manner on the formation of a duplex.

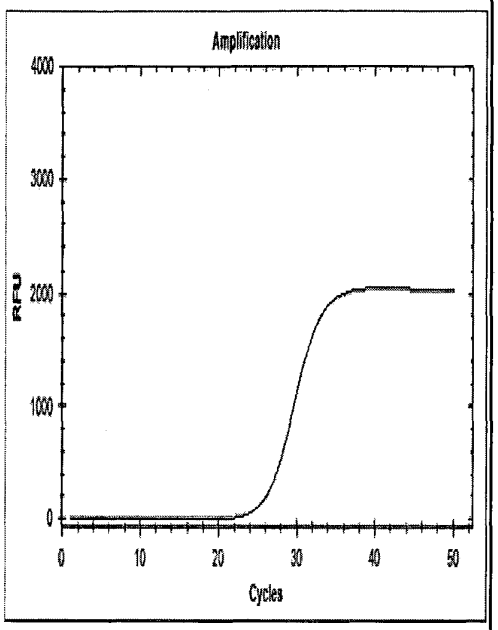
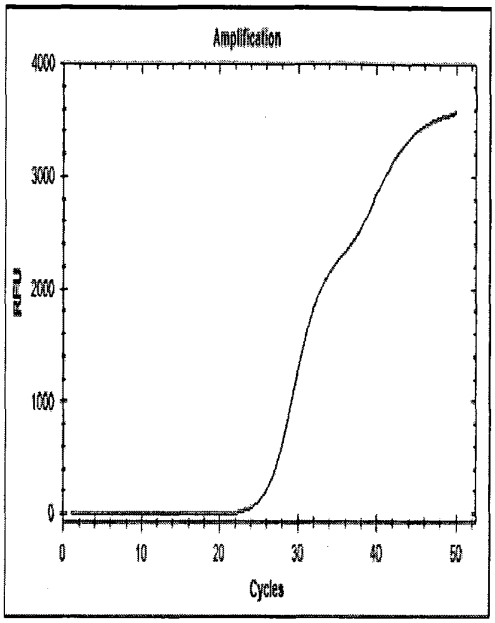
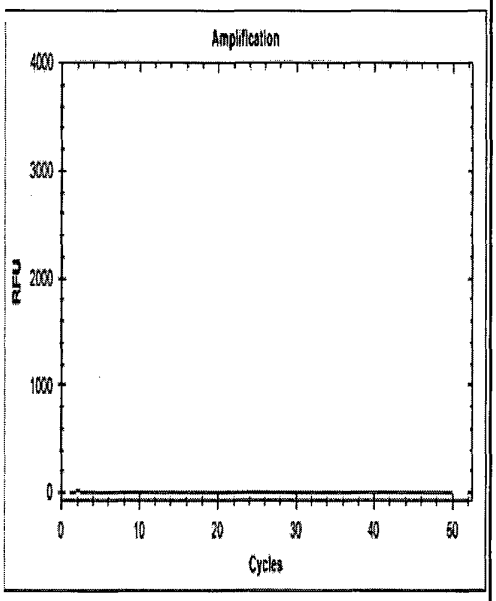
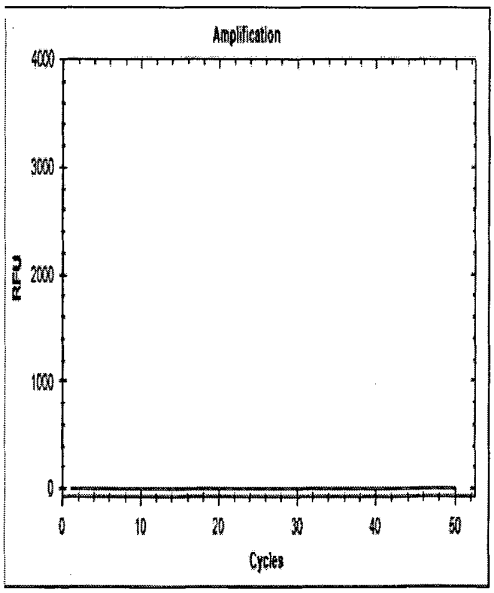
52. The method according to claim 49, wherein the signal for the target nucleic acid sequence having the relatively high detection temperature is generated by a signal-generating means to generate a signal in a dependent manner on cleavage of a detection oligonucleotide.

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Fig. 1A

Tube	Target[1)]	Detection temperature	
		72˚C	60˚C
1	CT		
2	NG		

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3	CT + NG		
4	NTC		

1) **Target is genomic DNA of *Chlamydia trachomatis* (CT) and *Neisseria gonorrhoeae* (NG). NTC represents No Target Control.**

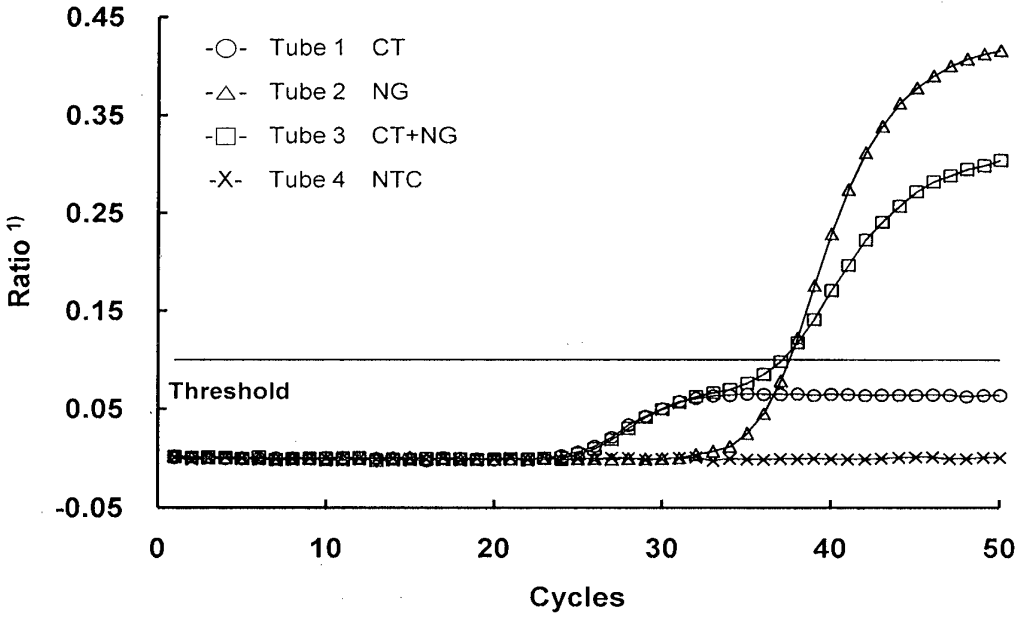
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Fig. 1B

Tube	Target[1]	End-RFU[2]		End-Ratio[3]	Cut-off ratio[4]	Result of NG
		72°C	60°C			
1	CT	2172	2413	1.1		-
2	NG	1.18	1508	1278.0	1.2	+
3	CT + NG	2030	3572	1.8		+
4	NTC	1.36	1.35	1.0		-

1) Target is genomic DNA of *Chlamydia trachomatis* (CT) and *Neisseria gonorrhoeae* (NG). NTC represents No Target Control.
2) End-RFU represents relative fluorescence units at 50th cycle.
3) End-Ratio represents the ratio of End-RFU at 60°C to End-RFU at 72°C.
4) Cut-off ratio allows determining the presence or absence of NG.

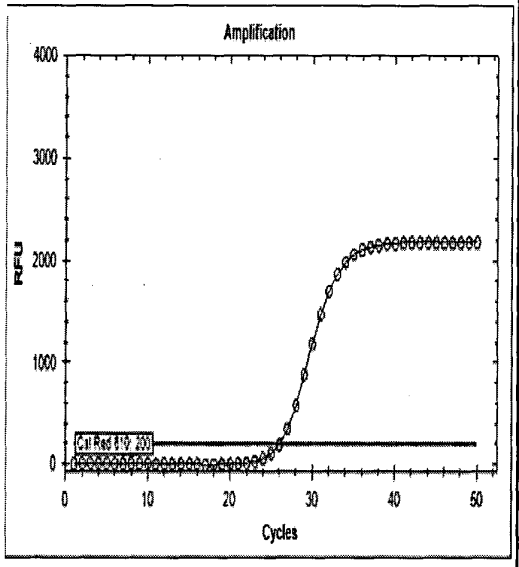
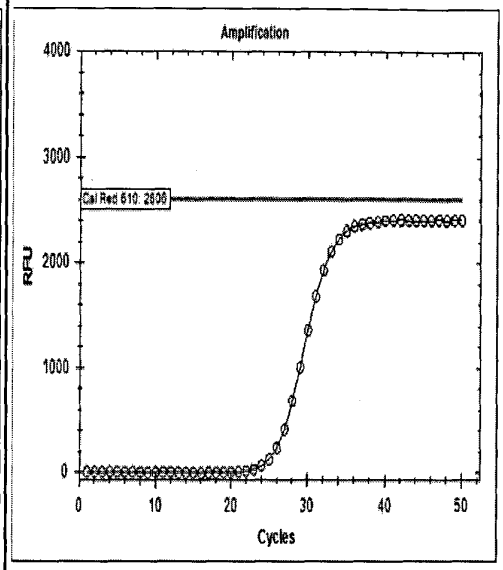
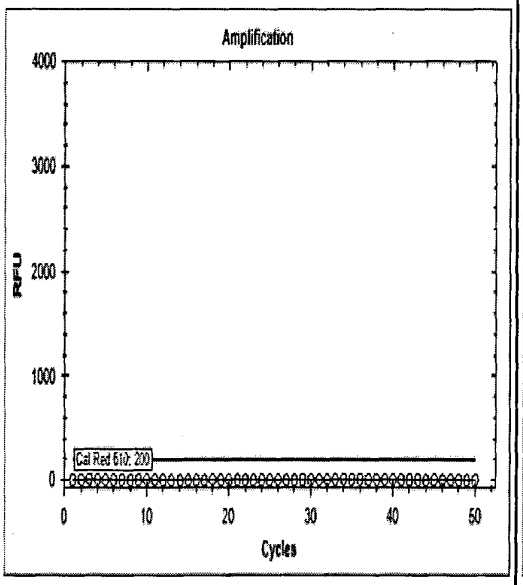
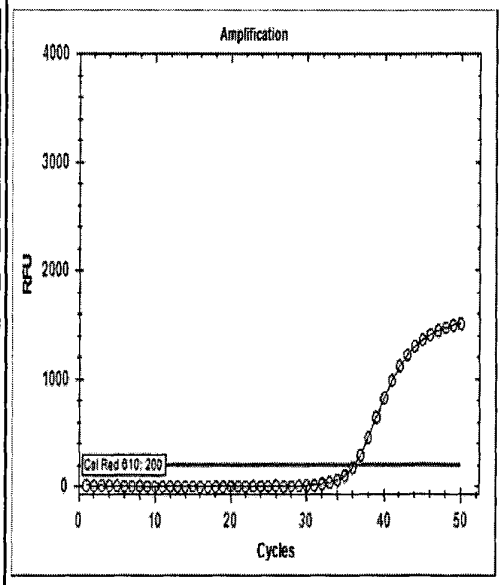
Fig. 1C



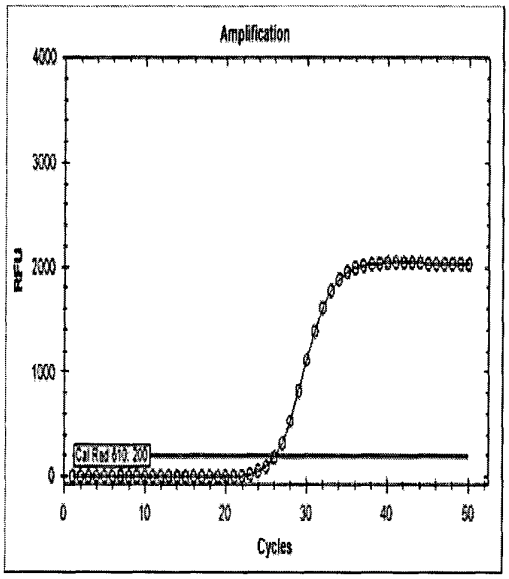
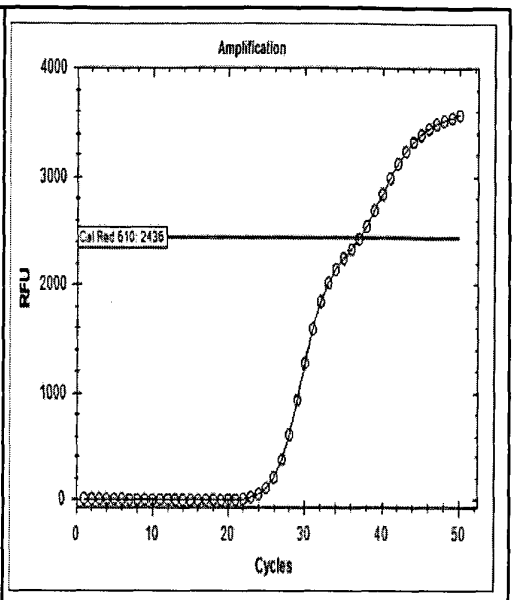
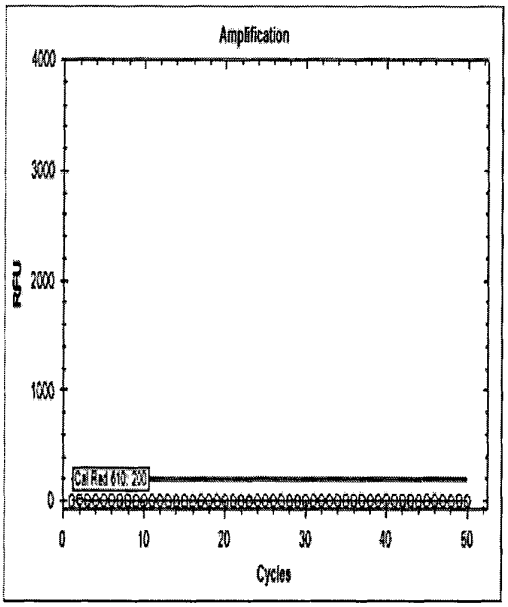
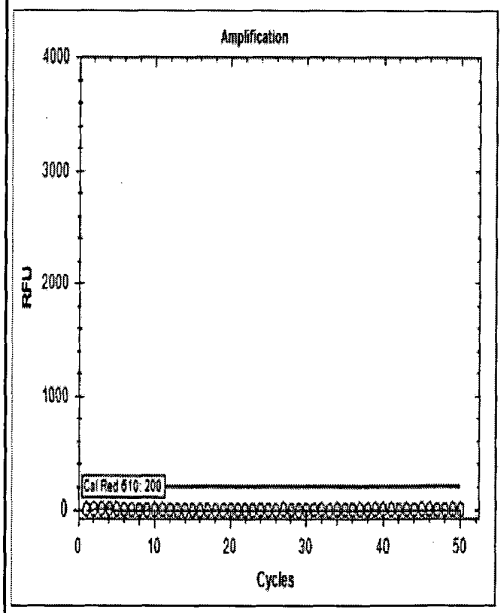
Tube	Target[2)]	Threshold[3)]	C_T value for NG
1	CT	0.10	-
2	NG		37.88
3	CT + NG		37.20
4	NTC		-

1) Ratio represents the ratio of RFU at 60°C to RFU at 72°C at each cycle.
2) Target is genomic DNA of *Chlamydia trachomatis* (CT) and *Neisseria gonorrhoeae* (NG). NTC represents No Target Control.
3) Threshold enables determining the presence or absence of NG.

Fig. 1D

Tube	Target[1)]	Detection temperature	
		72˚C	60˚C
1	CT		
2	NG		

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3	CT + NG		
4	NTC		

1) **Target is genomic DNA of *Chlamydia trachomatis* (CT) and *Neisseria gonorrhoeae* (NG). NTC represents No Target Control.**

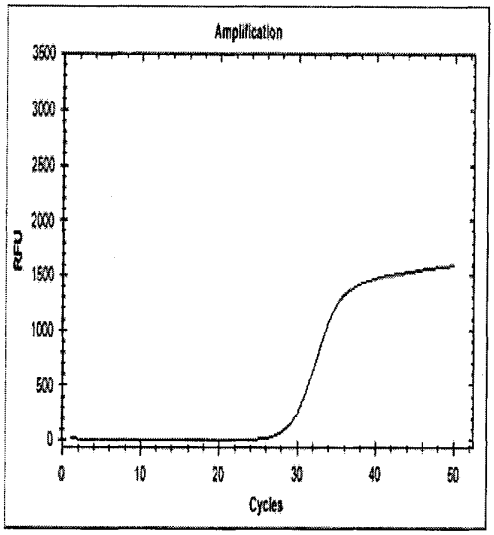
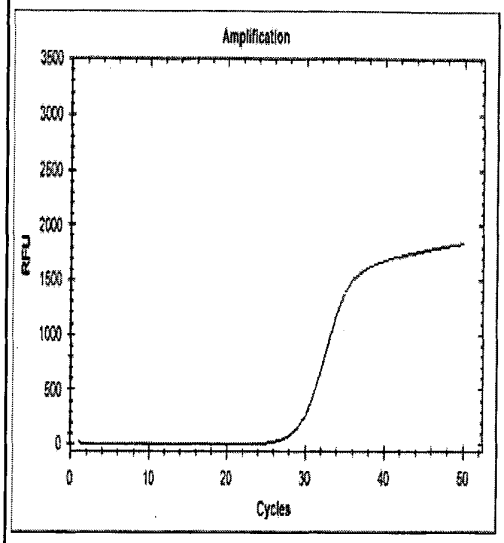
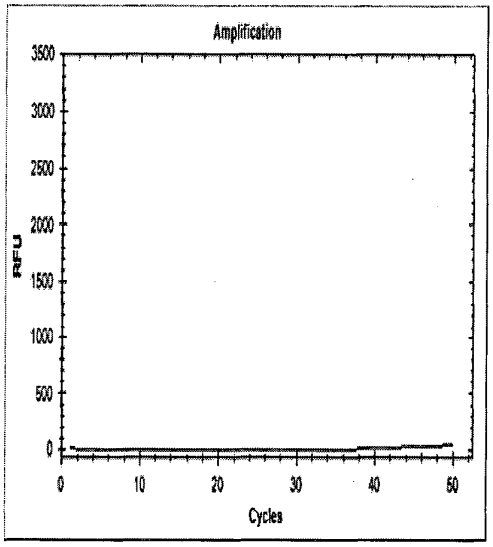
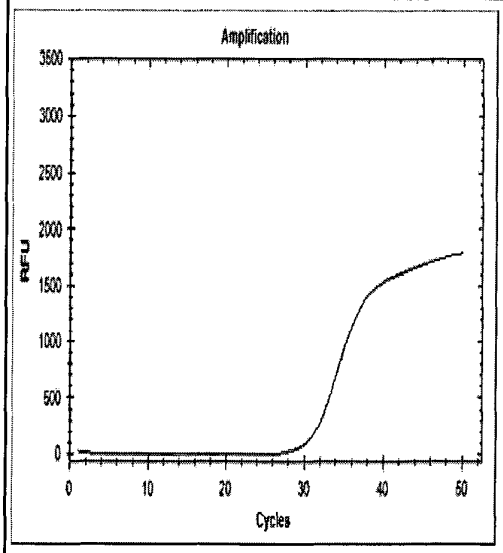
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Fig. 1E

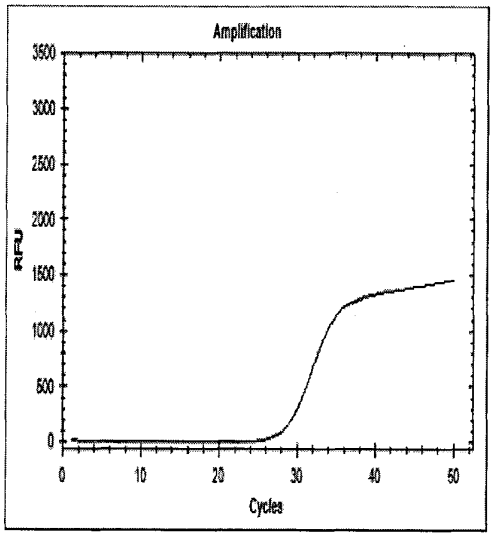
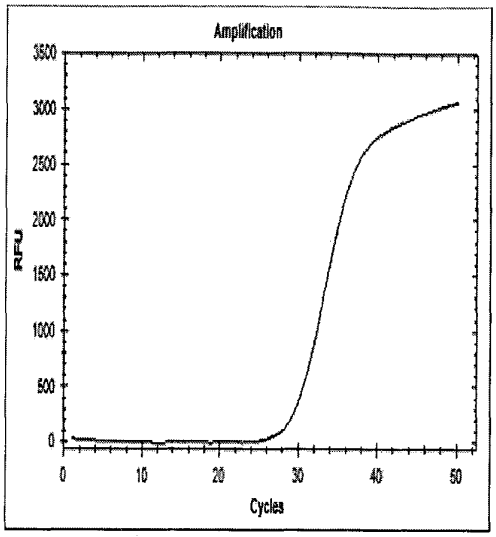
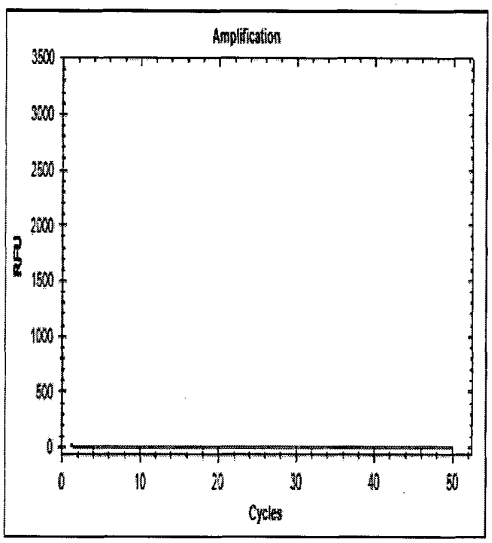
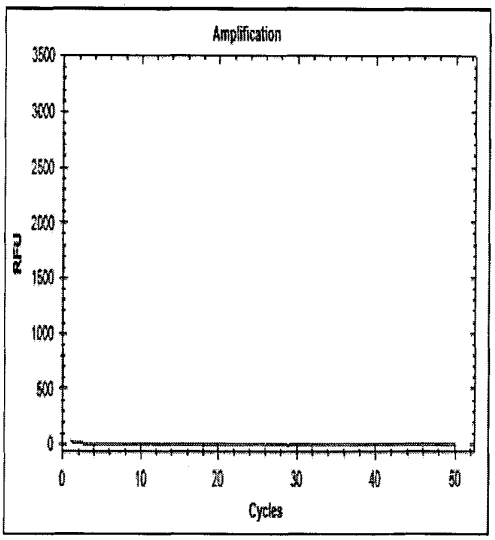
Tube	Target[1)]	CT (72°C)		NG (60°C)	
		Threshold[2)]	C_T value	Individual Threshold[3)]	C_T value
1	CT	200	26.04	2606	-
2	NG		-	200	36.21
3	CT + NG		26.13	2436	37.07
4	NTC		-	200	-

1) Target is genomic DNA of *Chlamydia trachomatis* (CT) and *Neisseria gonorrhoeae* (NG). NTC represents No Target Control.
2) Threshold at 72°C enables determining the presence or absence of CT.
3) Individual threshold for the signal of each tube at 60°C enables determining the presence or absence of NG in each tube and was obtained by multiplying End-RFU at 72°C by Cut-off ratio (Referred to Fig. 1B) on the condition that the signal at 72°C was detected (If no signal detected at 72°C, a single threshold, 200, was applied).

Fig. 2A

Tube	Target 1)	Detection temperature	
		72˚C	60˚C
1	CT		
2	NG		

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3	CT + NG		
4	NTC		

1) **Target is genomic DNA of *Chlamydia trachomatis* (CT) and *Neisseria gonorrhoeae* (NG). NTC represents No Target Control.**

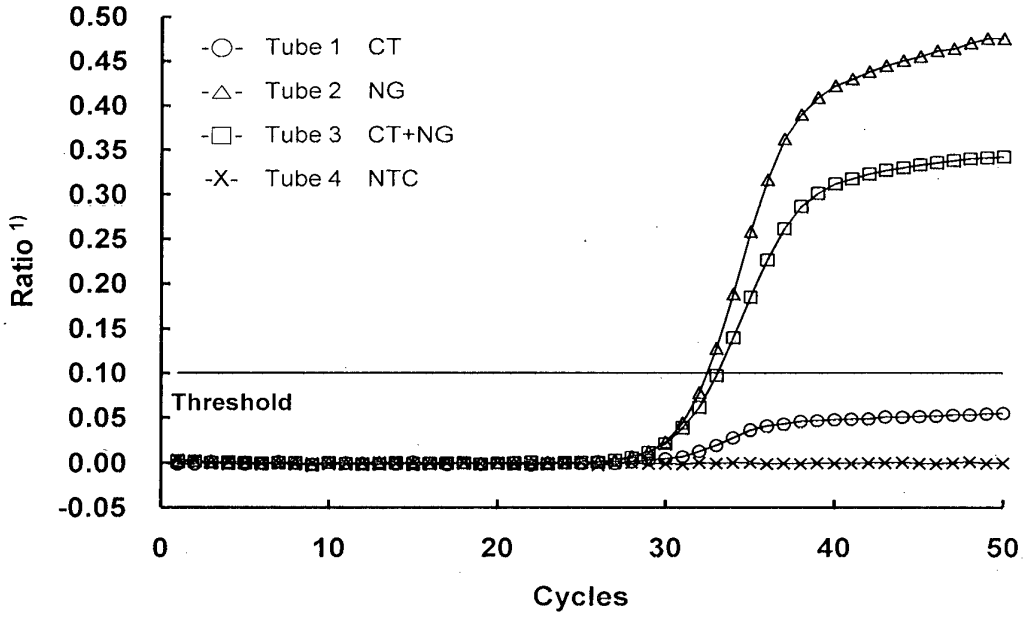
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Fig. 2B

Tube	Target[1)]	End-RFU[2)]		End-Ratio[3)]	Cut-off ratio[4)]	Result of NG
		72°C	60°C			
1	CT	1595	1839	1.2		-
2	NG	48.7	1787	36.7	1.5	+
3	CT + NG	1454	3062	2.1		+
4	NTC	3.41	2.33	0.7		-

1) Target is genomic DNA of *Chlamydia trachomatis* (CT) and *Neisseria gonorrhoeae* (NG). NTC represents No Target Control.
2) End-RFU represents relative fluorescence units at 50th cycle.
3) End-Ratio represents the ratio of End-RFU at 60°C to End-RFU at 72°C.
4) Cut-off ratio allows determining the presence or absence of NG.

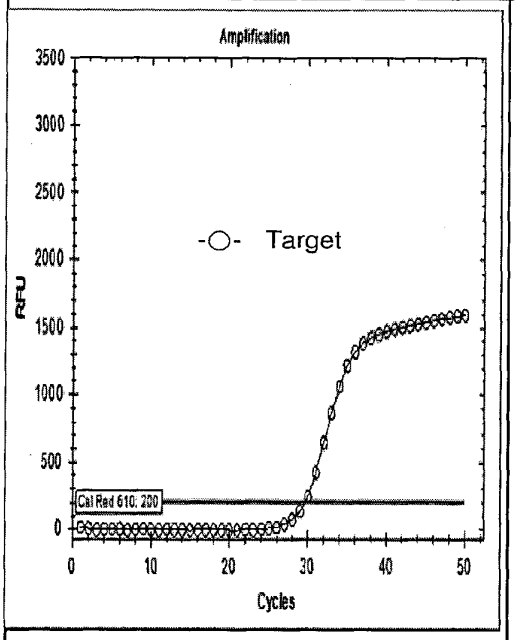
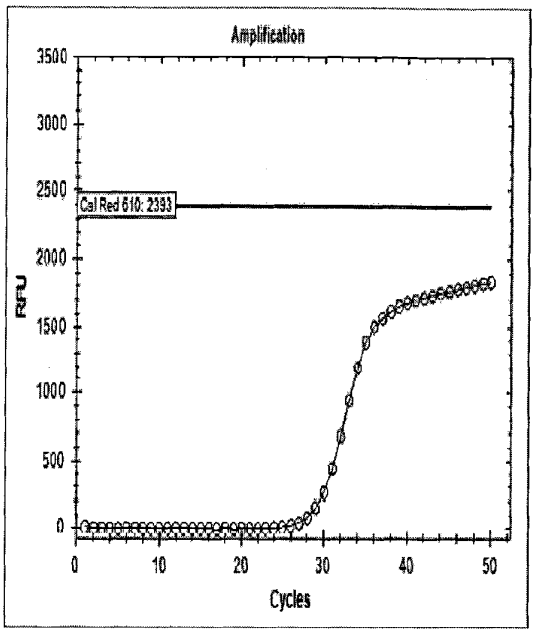
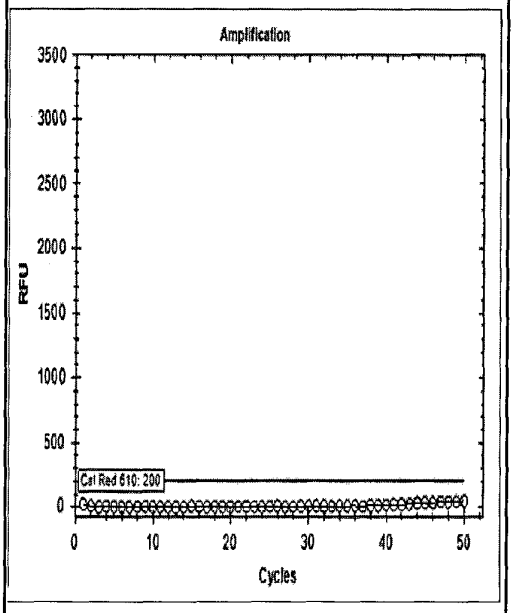
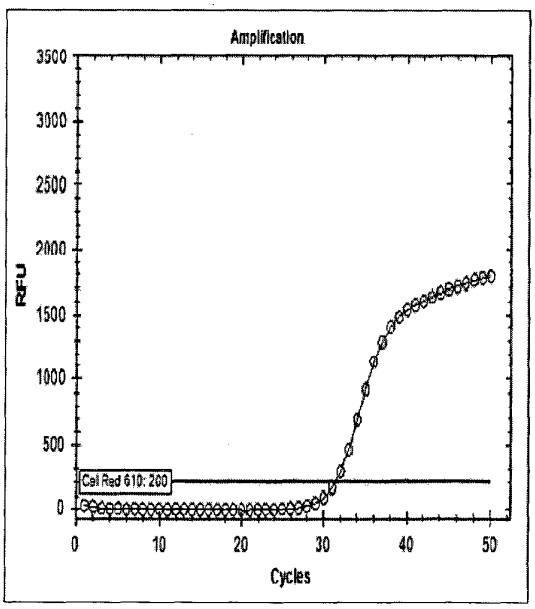
Fig. 2C



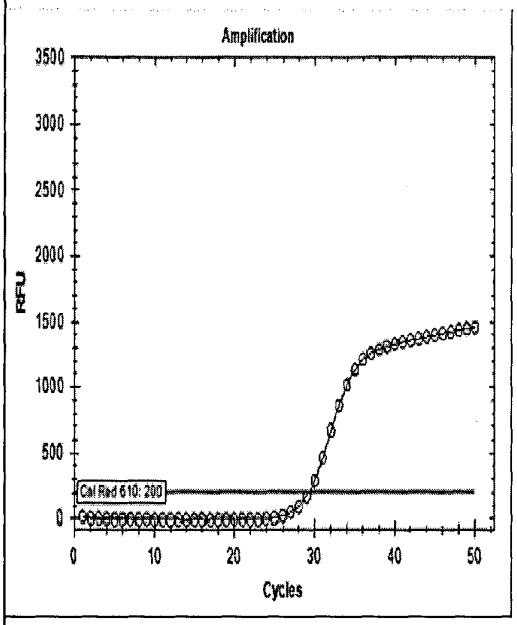
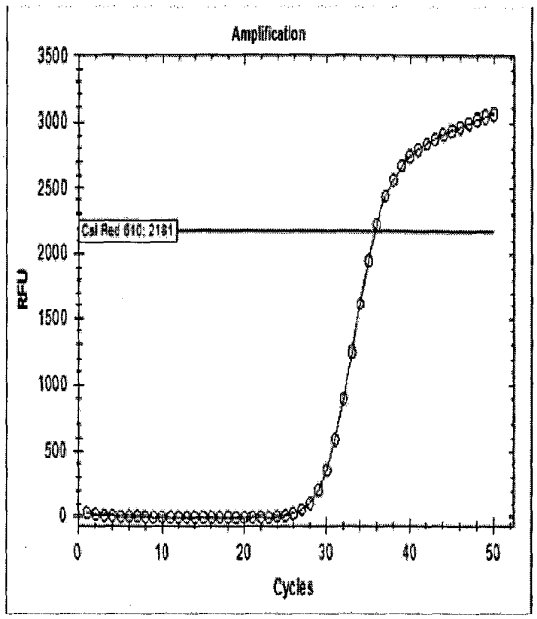
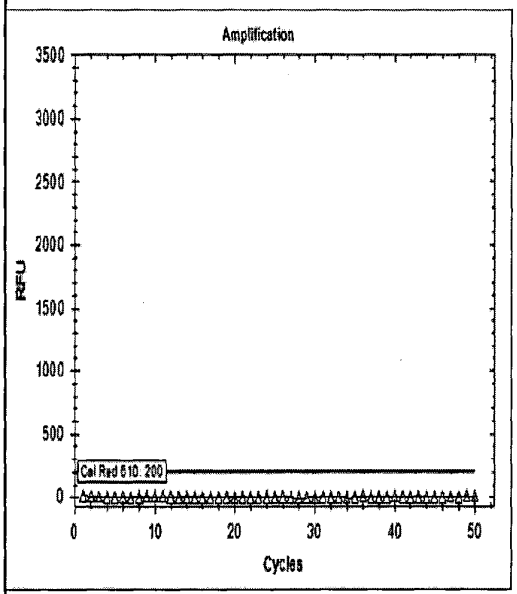
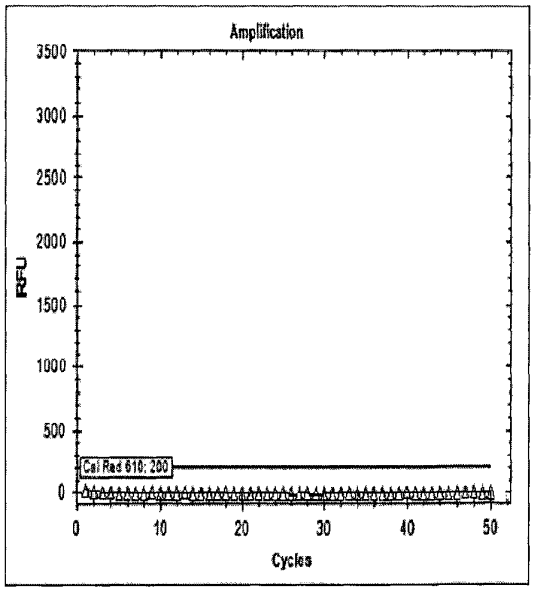
Tube	Target[2)]	Threshold[3)]	C_T value for NG
1	CT	0.10	-
2	NG		32.90
3	CT + NG		33.18
4	NTC		-

1) Ratio represents the ratio of RFU at 60°C to RFU at 72°C at each cycle.
2) Target is genomic DNA of *Chlamydia trachomatis* (CT) and *Neisseria gonorrhoeae* (NG). NTC represents No Target Control.
3) Threshold enables determining the presence or absence of NG.

Fig. 2D

Tube	Target 1)	Detection temperature	
		72°C	60°C
1	CT		
2	NG		

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3	CT + NG		
4	NTC		

1) **Target is genomic DNA of *Chlamydia trachomatis* (CT) and *Neisseria gonorrhoeae* (NG). NTC represents No Target Control.**

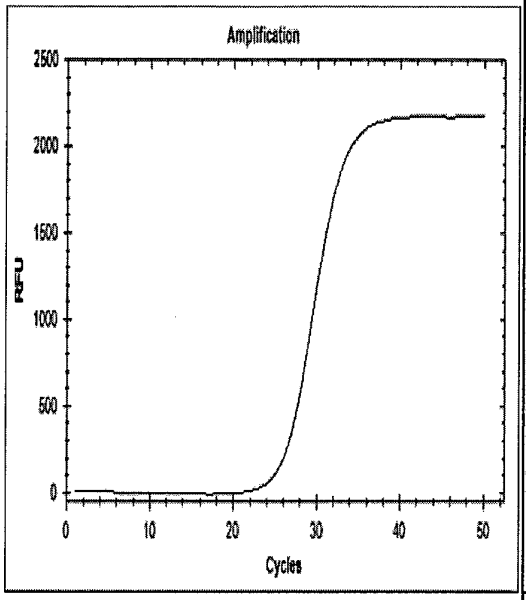
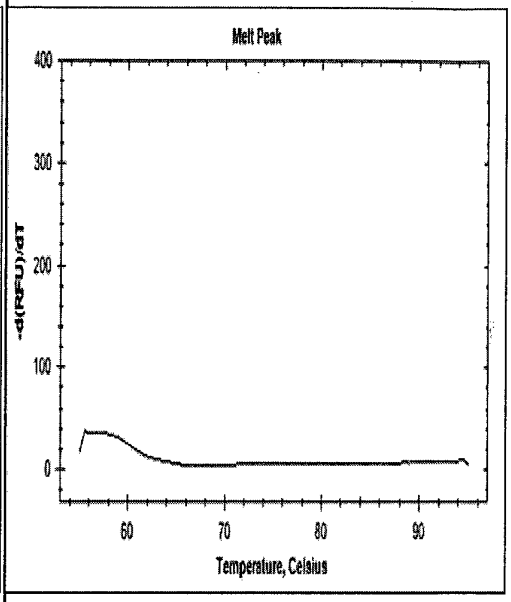
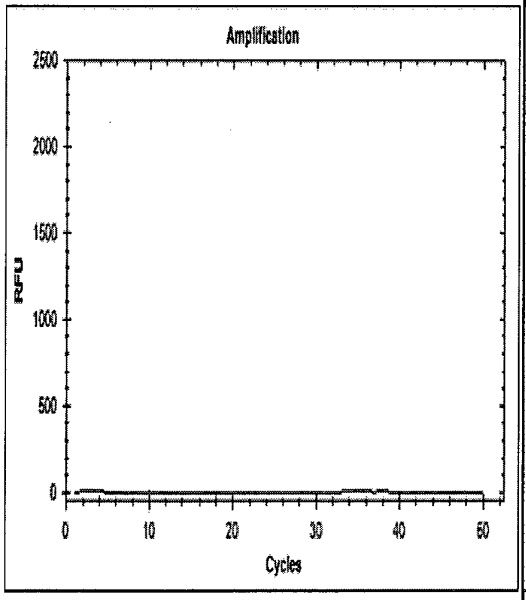
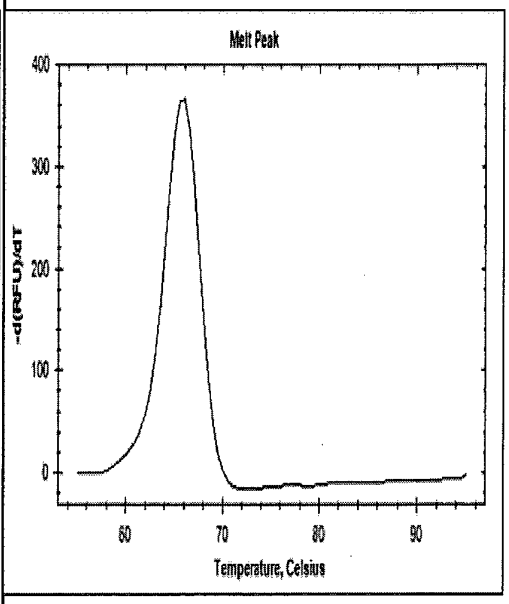
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Fig. 2E

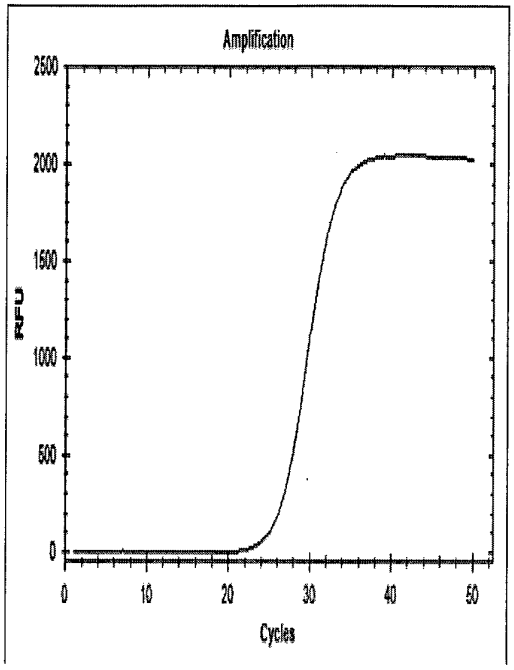
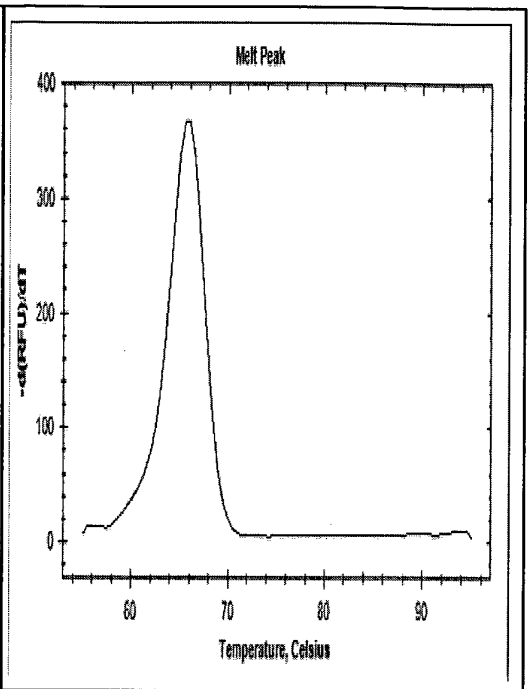
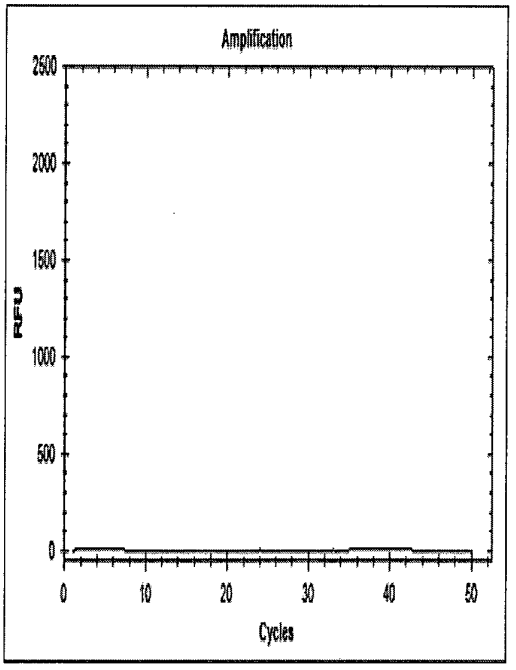
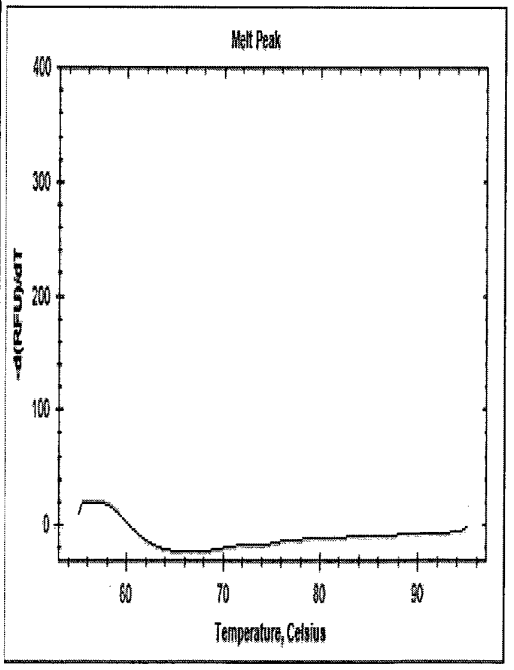
Tube	Target[1)]	CT (72°C)		NG (60°C)	
		Threshold[2)]	C_T value	Individual Threshold[3)]	C_T value
1	CT	200	29.58	2393	-
2	NG		-	200	31.32
3	CT + NG		29.26	2181	35.83
4	NTC		-	200	-

1) Target is genomic DNA of *Chlamydia trachomatis* (CT) and *Neisseria gonorrhoeae* (NG). NTC represents No Target Control.
2) Threshold at 72°C enables determining the presence or absence of CT.
3) Individual threshold for the signal of each Tube at 60°C enables determining the presence or absence of NG in each tube and was obtained by multiplying End-RFU at 72°C by Cut-off ratio (Referred to Fig. 2B) on the condition that the signal at 72°C was detected (If no signal detected at 72°C, a single threshold, 200, was applied).

Fig. 3

Tube	Target 1)	Real-time Detection	Melting
		72˚C	
1	CT		
2	NG		

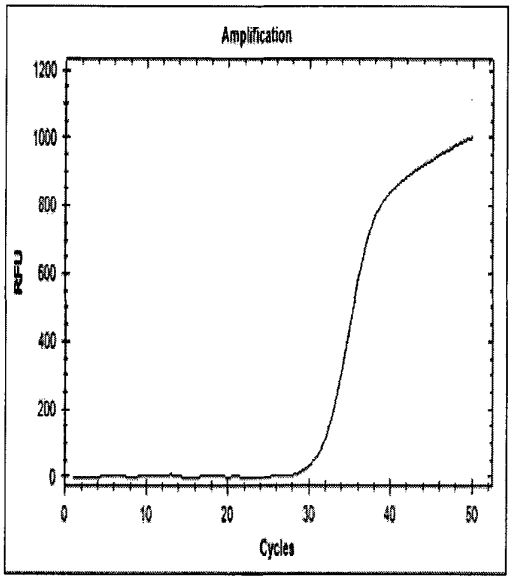
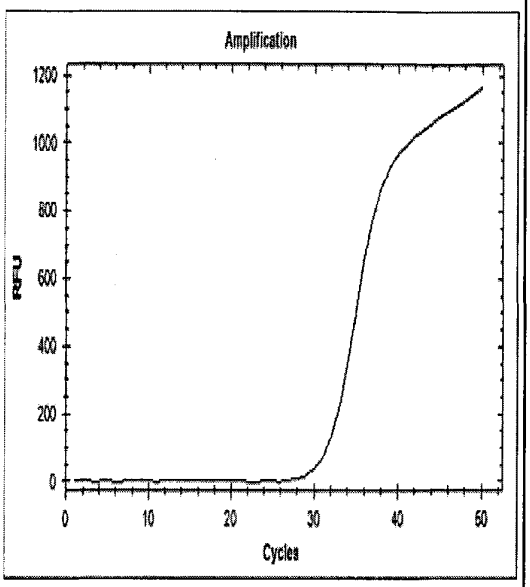
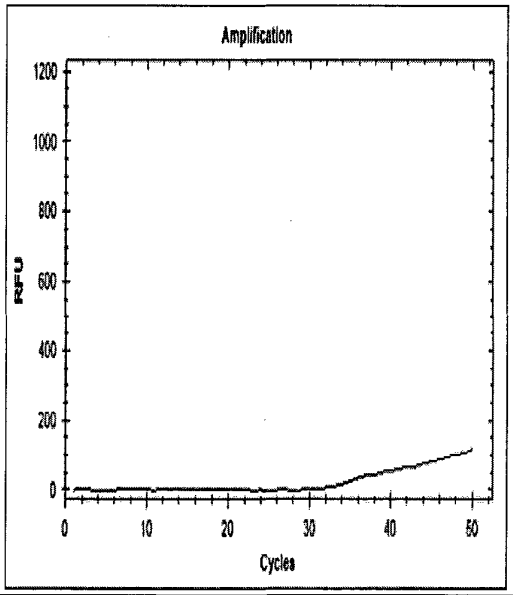
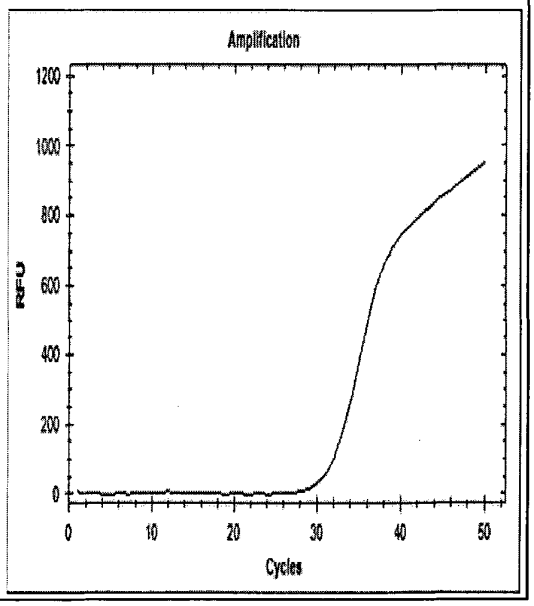
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3	CT + NG		
4	NTC		

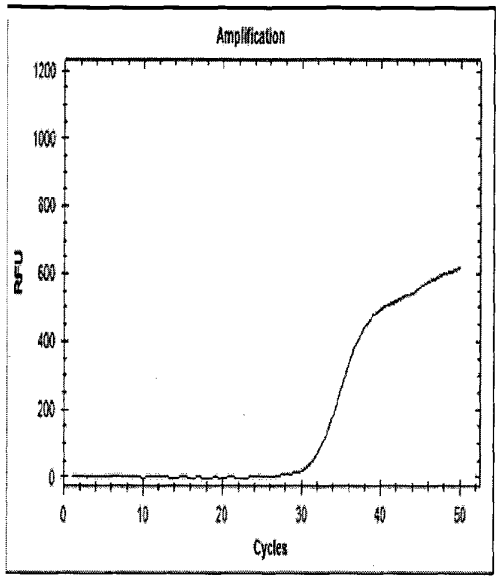
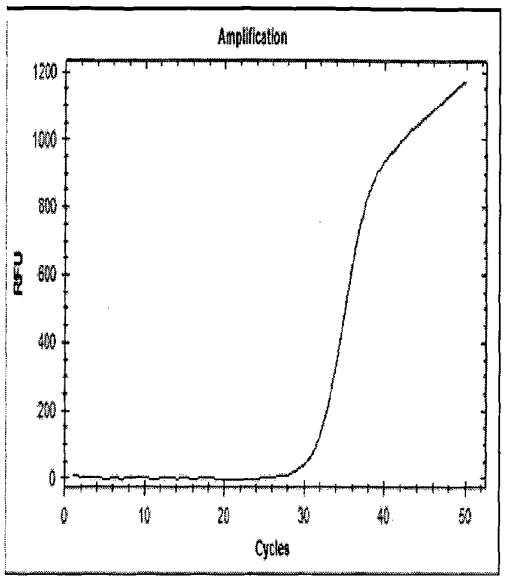
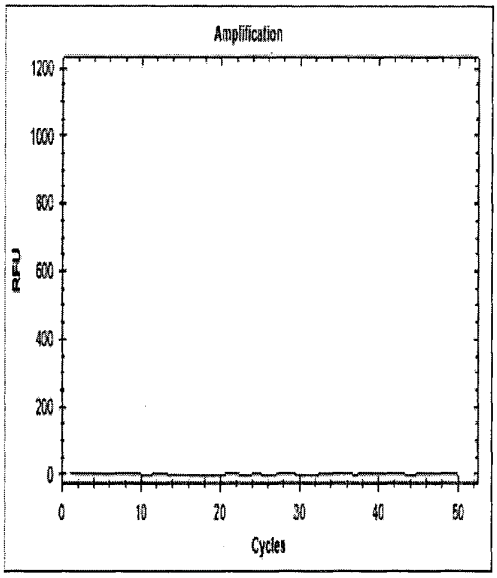
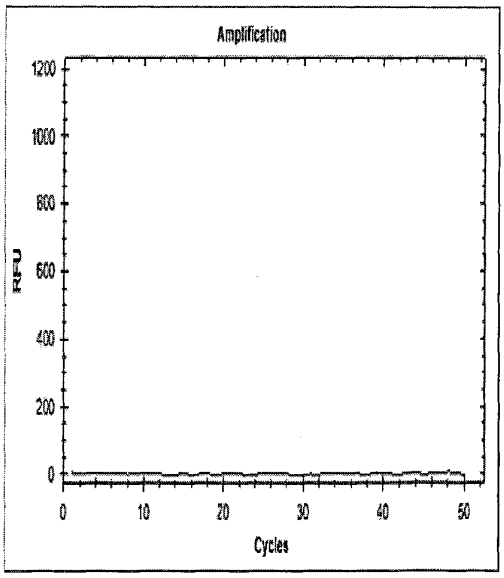
1) **Target is genomic DNA of *Chlamydia trachomatis* (CT) and *Neisseria gonorrhoeae* (NG). NTC represents No Target Control.**

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Fig. 4A

Tube	Target 1)	Detection temperature	
		72˚C	55˚C
1	Wild		
2	Mutant		

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3	Hetero		
4	NTC		

1) **Target is human genomic DNA of wild, mutant, and hetero-type MTHFR (C677T). NTC represents No Target Control.**

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Fig. 4B

Tube	Target[1)]	End-RFU[2)]		End-Ratio[3)]
		55°C	72°C	
1	Wild-type	1164	1001	1.2
2	Mutant-type	953	117	8.1
3	Hetero-type	1172	621	1.9
4	NTC	1.56	1.58	1.0

1) Target is human genomic DNA of wild, mutant, and hetero-type MTHFR (C677T). NTC represents No Target Control.
2) End-RFU represents relative fluorescence units at 50th cycle.
3) End-Ratio represents the ratio of End-RFU at 55°C to End-RFU at 72°C.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2014/004173

A. CLASSIFICATION OF SUBJECT MATTER

C12Q 1/68(2006.01)i

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)
C12Q 1/68

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords: real time PCR, melting analysis, different detection temperatures

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CHAKRAVORTY et al., 'Rapid detection of fluoroquinolone-resistant and heteroresistant Mycobacterium tuberculosis by use of sloppy molecular beacons and dual melting-temperature codes in a real-time PCR assay' Journal of Clinical Microbiology, Vol.49, No.3, pp.932-940 (2011) See abstract; materials and methods; page 935; table 1.	1-52
A	LIU et al., 'Triplex real-time PCR melting curve analysis for detecting Mycobacterium tuberculosis mutations associated with resistance to second-line drugs in a single reaction' Journal of Antimicrobial Chemotherapy, Vol.68, No.5, pp.1097-1103 (2013) See tables 1-2; pages 1099-1100.	1-52
A	PIERCE et al., 'Rapid detection and identification of hepatitis C virus (HCV) sequences using mismatch-tolerant hybridization probes: A general method for analysis of sequence variation' Biotechniques, Vol.55, No.3, pp.125-132 (2013) See the whole document.	1-52

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

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
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"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
15 December 2014 (15.12.2014)	**15 December 2014 (15.12.2014)**

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Form PCT/ISA/210 (second sheet) (July 2009)

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2014/004173

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ALVANDI et al., 'Zip nucleic acid: a new reliable method to increase the melting temperature of real-time PCR probes' Journal of Diabetes & Metabolic Disorders, Vol.13, Article No.26, (internal pages 1-4) (Epub. February 2014) See the whole document.	1-52
A	US 2012-0116686 A1 (PALAIS et al.) 10 May 2012 See abstract; claims 1-37; figure 11.	1-52

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INTERNATIONAL SEARCH REPORT

Information on patent family members

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(54) Title: DIGITAL ANALYSIS OF CIRCULATING TUMOR CELLS IN BLOOD SAMPLES

(57) Abstract: This disclosure relates to new assay methods for analysis of circulating tumor cells (CTCs) in blood samples for detection, e.g., early detection, and/or monitoring of disease, e.g., cancer. The methods provide ultra-high sensitivity and specificity, and include the use of microfluidic isolation of CTCs and digital detection of RNA derived from the CTCs.

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Digital Analysis of Circulating Tumor Cells in Blood Samples

TECHNICAL FIELD

This invention relates to blood sampling techniques, and more particularly to methods and systems for detecting and analyzing cells in blood samples.

BACKGROUND

The ability to detect the presence of rare circulating tumor cells (CTCs) using a simple blood test, or "liquid biopsy," has the potential to greatly enhance the monitoring of epithelial cancers, providing instant sampling of tumor cell numbers, genetic composition, and drug response parameters, without requiring invasive tumor biopsies. Thus, the detection of CTCs for early cancer detection has the potential to revolutionize the treatment of cancer, enabling the diagnosis of invasive cancer at a stage before it has metastasized, when curative treatment is expected.

However, CTCs are very rare, and identifying, visualizing, and scoring these tumor cells admixed with normal blood components remains a significant challenge, even after partial purification with known microfluidic devices or similar technologies. For example, per milliliter of whole blood, there are only 1-10 CTCs amongst more than 5 billion red blood cells (RBCs) and more than 5 million white blood cells (WBCs)(Plaks et al., "Cancer Circulating Tumor Cells," *Science,* 341:1186; 2013). In addition, antibody staining of tumor cells is highly variable, due to high heterogeneity among cancer cells, even within an individual patient, as well as the poor physical condition of many tumor cells that circulate in the bloodstream, many of which have begun to undergo programmed cell death or anoikis. In addition, accurate scoring of antibody-stained tumor cells requires differentiation from large numbers of contaminating white blood cells, some of which bind to antibody reagents non-specifically. As such, only a subset of candidate tumor cells can be robustly identified by antibody staining, and as many as half of patients tested have no detectable cells, despite having widely metastatic cancer.

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Thus, current protocols for imaging CTCs are seeking higher and higher levels of purity in the isolation of CTCs, especially from other nucleated blood cells, such as white blood cells (WBCs).

SUMMARY

The present disclosure relates to methods, uses, and systems to obtain the highest possible sensitivity of data relating to rare CTCs in standard blood samples, while avoiding the need for extremely high levels of purity of the CTCs. In particular, the new methods do not need the CTCs to be completely isolated from contaminating WBCs, and instead can reliably detect as few as one CTC in products containing, e.g., up to 10,000 WBCs or more. The new assay methods and systems combine (1) an isolation system that can consistently obtain CTCs as intact, whole cells (with high quality ribonucleic acid (RNA)) from blood with (2) a droplet-based digital polymerase chain reaction (PCR) assay focused on ribonucleic acid RNA markers of specific cancer lineages for each tumor type that are absent in blood of healthy patients.

When combined as described herein, these two concepts provide a CTC digital droplet PCR assay method ("CTC ddPCR") or simply stated a "digital-CTC" assay ("d-CTC"). In some embodiments, the isolation system is a microfluidic system, such as a negative depletion microfluidic system (e.g., a so-called "CTC-Chip," that uses negative depletion of hematopoietic cells, e.g., red blood cells (RBCs), WBCs, and platelets, to reveal untagged non-hematopoietic cells such as CTCs in a blood sample).

In general, the disclosure relates to methods for early detection of cancer with ultra-high sensitivity and specificity, wherein the methods include the use of microfluidic isolation of circulating tumor cells (CTCs) and digital detection of RNA derived from the CTCs. In some embodiments, the CTC-derived RNA can be converted into cDNA and encapsulated into individual droplets for amplification in the presence of reporter groups that are configured to bind specifically to cDNA from CTCs and not to cDNA from other cells. The droplets positive for reporter groups can be counted to assess the presence of disease, e.g., various types of cancer.

In another aspect, the disclosure relates to methods of analyzing circulating tumor cells (CTCs) in a blood sample. The methods include or consist of isolating

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from the blood sample a product comprising CTCs and other cells present in blood; isolating ribonucleic acid (RNA) molecules from the product; generating cDNA molecules in solution from the isolated RNA; encapsulating cDNA molecules in individual droplets; amplifying cDNA molecules within each of the droplets in the presence of reporter groups configured to bind specifically to cDNA from CTCs and not to cDNA from other cells; detecting droplets that contain the reporter groups as an indicator of the presence of cDNA molecules from CTCs in the droplets; and analyzing CTCs in the detected droplets.

The methods described herein can further include reducing a volume of the product before isolating RNA and/or removing contaminants from the cDNA-containing solution before encapsulating the cDNA molecules.

In various implementations of the new methods, generating cDNA molecules from the isolated RNA can include conducting reverse transcription (RT) polymerase chain reaction (PCR) of the isolated RNA molecules and/or amplifying cDNA molecules within each of the droplets can include conducting PCR in each droplet. In the new methods, encapsulating individual cDNA molecules and PCR reagents in individual droplets can include forming at least 1000 droplets of a non-aqueous liquid, such as one or more fluorocarbons, hydrofluorocarbons, mineral oils, silicone oils, and hydrocarbon oils and/or one or more surfactants. Each droplet can contain, on average, one target cDNA molecule obtained from a CTC. In some embodiments, the reporter groups can be or include a fluorescent label.

The new methods can include removing contaminants from the cDNA-containing solution by use of Solid Phase Reversible Immobilization (SPRI), e.g., immobilizing cDNA in the solution, e.g., with magnetic beads that are configured to specifically bind to the cDNA; removing contaminants from the solution; and eluting purified cDNA.

In various implementations, the methods described herein include using probes and primers in amplifying the cDNA molecules within each of the droplets that correspond to one or more genes selected from the list of cancer-selective genes in Table 1 herein. For example, the selected genes can include prostate cancer-selective genes, e.g., any one or more of AGR2, FOLH1, HOXDB13, KLK2, KLK3, SCHLAP1/SET4, SCHLAP1/SET5, AMACR, AR variants, UGT2B15/SET1,

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UGT2B15/SET5, and STEAP2 (as can be easily determined from Table 1). In another example, any one or more of ALDH1A3, CDH11, EGFR, FAT1, MET, PKP3, RND3, S100A2, and STEAP2 are selective for pancreatic cancer. Similar lists can be generated for the other types of cancers listed in Table 1.

In other examples, the selected genes include any one or more of the breast cancer-selective genes listed in Table 1. In other examples, the selected genes include genes selective for one or more of lung, liver, prostate, pancreatic, and melanoma cancer. For example, a multiplexed assay can include 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 or even all of the selected genes that are listed in Table 1 as being selective for a particular type of cancer, e.g., breast cancer, lung cancer, prostate cancer, pancreatic cancer, liver cancer, and melanoma. Typically a group of primers and probes for 5 to 12 cancer-selective genes from Table 1 are used for a particular type of cancer. Other specific combinations of selected genes (markers for those genes) are described in the Examples below.

The methods can also include using one or more genes selective for two or more, three or more, four or more, or five or more different types of cancer. For example, the genes can be selective for breast cancer and lung cancer; breast cancer, lung cancer, and liver cancer; breast cancer, lung cancer, and pancreatic cancer; breast cancer, lung cancer, and prostate cancer; breast cancer, liver cancer, and melanoma; breast cancer, lung cancer, and melanoma; breast cancer, lung cancer, liver cancer, and prostate cancer; breast cancer, lung cancer, liver cancer, and melanoma; breast cancer, lung cancer, liver cancer, and pancreatic cancer; breast cancer, lung cancer, prostate cancer, and pancreatic cancer; breast cancer, lung cancer, liver cancer, melanoma, and pancreatic cancer; or breast cancer, lung cancer, liver cancer, melanoma, pancreatic cancer, and prostate cancer.

In the methods described herein, the CTCs can arise from metastatic or primary/localized cancers. In the new methods, the step of analyzing the CTCs in the detected droplets cam include monitoring CTCs from a blood sample from a patient, e.g., with a known cancer, e.g., over time, and testing and/or imaging the CTCs (e.g., using standard techniques) to provide a prognosis for the patient. In other embodiments, the step of analyzing the CTCs in the detected droplets can include testing and/or imaging the CTCs (e.g., using standard techniques) from a blood

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sample from a patient to provide an indication of a response by the CTCs to a therapeutic intervention.

In other embodiments, the step of analyzing the CTCs in the detected droplets includes determining a number or level of CTCs per unit volume of a blood sample from a patient to provide a measure of tumor burden in the patient. The methods can then further include using the measure of tumor burden in the patient to select a therapy or can further include determining the measure of tumor burden in the patient at a second time point to monitor the tumor burden over time, e.g., in response to a therapeutic intervention, e.g., for dynamic monitoring of tumor burden.

The methods and assays described herein can be used to amplify and detect CTCs in a wide variety of diagnostic, prognostic, and theranostic methods.

As used herein, the phrase "circulating tumor cells" (CTCs) refers to cancer cells derived from solid tumors (non-hematogenous cancers) that are present in very rare numbers in the blood stream of patients (e.g., about 1 CTC in about 10,000,000 WBCs in whole blood). CTCs can arise from both metastatic as well as primary/localized cancers.

As used herein, a "product" means a group of isolated rare cells and other contaminating blood cells, e.g., red blood cells, white blood cells (e.g., leukocytes), e.g., in some sort of liquid, e.g., a buffer, such as a pluronic buffer, that arise from processing in the methods described herein, e.g., using the systems described herein. A typical product may contain only about one to ten CTCs admixed with 500 to 2,500 or more WBCs, e.g., one to ten CTCs in a mixture of 1000 to 2000 WBCs. However, the limit of detection of the present methods can be about 1 CTC in 10,000 WBC. Thus, while the present methods can achieve a level of purity of about 1 CTC in 500 WBCs, the present methods do not require highly purified CTCs, as is required in some known methods of CTC analysis.

As used herein a Solid Phase Reversible Immobilization (SPRI) cleanup is a technique using coated magnetic beads to perform size selection on cDNA created from Reverse Transcription (RT)-PCR of a product. In the new assay methods described herein this accomplishes the two-fold purpose of (a) selecting only the cDNA of the correct size, and (b) removing harsh lysis detergents incompatible with the stability of the droplets.

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The polymerase chain reaction (PCR) is a process of amplification of known DNA fragments by serial annealing and re-annealing of small oligonucleotide primers, resulting in a detectable molecular signal.

Reverse Transcription (RT)-PCR refers to the use of reverse transcription to generate a complementary c-DNA molecule from an RNA template, thereby enabling the DNA polymerase chain reaction to operate on RNA. An important aspect of the new methods disclosed herein is the availability of high quality RNA from whole cell CTCs that are not lysed or treated in such a way that might destroy or degrade the RNA.

As used herein, "positive droplets" are lipid-encapsulated molecules in which a PCR reaction performed with tagged primers allows visualization of the PCR amplified product. Thus, a droplet that contained a single template cDNA molecule of a particular targeted gene can become visible using fluorescence microscopy, while an "empty" or "negative" droplet is one that contains no targeted cDNA.

The new methods and systems provide numerous advantages and benefits. For example, the current methods and systems provide results that are far more accurate and robust than either of the prior known systems when used alone. By breaking down the signal from a single CTC into hundreds or thousands of brightly fluorescent droplets, each derived from a single cDNA molecule, the new digital-CTC assays enable dramatic signal amplification. Given the strict criteria in selecting and optimizing the biomarker genes described herein, the background signal from normal blood cells is negligible in d-CTC. Thus, d-CTC enables greatly amplified signal from patients with advanced cancer (nearly 100% of patients with prostate, lung, breast, and liver cancers). Not only is the fraction of patients with a positive score significantly increased, but the high level of signal enables dynamic measurements as tumor load declines following cancer therapy. In addition, the signal amplification permits detection of CTC-derived signatures even in patients with a very low tumor burden (something that is not readily accomplished with CTC cell imaging), thus enabling significantly earlier detection of cancer.

In sum, this novel microfluidics platform provides a streamlined, ultrahigh-throughput, rapid (e.g., 3 hours per run), and extremely high sensitivity method of enriching, detecting, and analyzing CTCs in patient blood samples. The platform

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provides rich, clinically actionable information, and with further optimization may enable early detection of cancer.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

DESCRIPTION OF DRAWINGS

FIG. 1A is a graph showing cDNA dilutions prepared from total RNA of LNCaP prostate cancer cells, mixed with leukocytes and analyzed by droplet PCR using two different prostate primer sets. The results represent several purities and show good response of positive droplet number across this range.

FIG. 1B is a graph of manually isolated LNCaP cells spiked into healthy donor (HD) blood samples, run through the CTC-iChip, and subjected to droplet RT-PCR (KLK3 primer set). The results show excellent sensitivity down to low numbers of target cells.

FIG. 1C is a graph that shows the analysis of blood samples from healthy controls, patients with localized (resectable) prostate cancer and metastatic prostate cancer, processed through the CTC-iChip, subjected to RT-PCR and droplet analysis using three prostate-specific and one epithelial-specific biomarkers (KLK3, AMACR, FOLH1, EpCAM). The results are shown for the total number of droplets/ml for all four markers combined.

FIG. 2 is a signal intensity plot that shows KLK3 positive droplets derived from LNCAP prostate cancer cells spiked into blood and recovered using the CTC-iChip.

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FIG. 3 is a bar graph that shows the minimal variation between experimental replicates and the retention of signal after sample processing through the CTC-iChip and shows increased detection sensitivity using the new assays described herein.

FIG. 4 is a signal intensity plot that shows the absence of four different cancer-specific marker-positive droplets in healthy donors using the new CTC digital droplet PCR assay methods described here ("CTC ddPCR" assay or simply "d-CTC" assay).

FIG. 5 is a signal intensity plot that shows a d-CTC assay multiplexed for four different lineage specific transcripts to detect prostate cancer cell lines spiked into blood.

FIGs. 6A to 7B are signal intensity plots showing d-CTC assays multiplexed for four different prostate cancer-specific transcripts per reaction. Both the theoretical model (FIGs. 6A and 7A) and cancer cell line data (FIGs. 6B and 7B) shown for two such reactions, Reactions 1 and 2, demonstrate that the theoretical model accurately predicts the experimental data.

FIGs. 8A to 13B are signal intensity plots showing d-CTC assays multiplexed for four different breast and lung cancer specific transcripts per reaction. Both the theoretical models (FIGs. 8A, 9A, 10A, 11A, 12A, and 13A) and cancer cell line data (FIGs. 8B, 9B, 10B, 11B, 12B, and 13B) shown for six such reactions, Reactions 1 through 6, each with different combinations of markers, demonstrate that the theoretic model accurately predicts the experimental data.

FIG. 14 is a bar graph showing droplet PCR signal for seven different biomarkers (PIP, PRAME, RND3, PKP3, FAT1, S100A2, and AGR2) from 1 ng of non-amplified cell-line cDNA and from 1 µl of pre-amplified product after 10, 14, and 18 cycles of Specific Target Amplification (STA) pre-amplification, demonstrating the significant enhancement of droplet PCR signal from STA pre-amplification.

FIGs. 15A to 15C are graphs that show the results of CTC detection in patients using the new d-CTC assay methods for three different sets of patients with lung cancer (FIG. 15A), breast cancer (FIG. 15B), and prostate cancer (FIG. 15C). In each, the healthy patients had no CTCs.

FIG. 16 is a horizontal bar graph that shows the results of patient prostate cancer data using a multiplexed d-CTC assay method described herein testing for the nine biomarkers recited in the figure (AGR2, Dual, FAT1, FOLH1, HOXB13, KLK2,

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KLK3, STEAP2, and TMPRSS2). 91 percent of cancer patients had detectable CTCs (10 of 11 patients), 24 of 28 samples contained detectable CTCs (86%), and 0 of 12 (0 percent) of healthy donor (HD) blood samples contained CTCs.

FIG. 17 is a series of signal intensity plots showing d-CTC assays multiplexed for for two different reactions (Reaction 1 (TMPRSS2, FAT1, KLK2, and STEAP2), left column, and Reaction 2 (KLK3, HOXB13, AGR2, and FOLH1), right column) for blood samples from a metastatic prostate cancer patient (top row), a localized prostate cancer patient (middle row), and from a healthy donor control sample (bottom row). In each case there were no CTCs in the healthy donor (HD) samples, but clear evidence of CTCs in the cancer samples.

FIG. 18 is a multiple bar graph illustrating the relative proportion of androgen receptor signaling genes in CTCs measured over time to provide a readout of drug response in a prostate cancer patient treated with Abiraterone®.

FIGs. 19A and 19B are graphs showing non-amplification versus 18 cycles of SMARTer pre-amplication. FIG. 19A is a bar graph that shows the level of amplicon amplification efficiency for different target regions that is consistent among the three replicates (WTA1, WTA2, WTA3). FIG. 19B is a graph that shows that using 18 cycles of SMARTer pre-amplification provides an increase in signal of approximately four orders of magnitude (10^8 vs 10^4) compared to a non-pre-amplified sample.

FIGs. 20A to 20C are graphs that show the results of testing of 11 markers in a multiplexed liver cancer assay. FIGs. 20A to 20C show the total droplet numbers in 21 hepatocellular carcinoma (HCC) patients (FIG. 20A), 13 chronic liver disease (CLD) patients (FIG. 20B, no significant detectable droplets) and 15 healthy donors (HDs)(FIG. 20C, no significant detectable droplets).

FIGs. 21A and 21B are graphs that show the results of a 14 marker multiplexed lung cancer assay. FIG. 21A shows the assay results for the 8 metastatic lung cancer patients and 8 healthy donors (all negative). FIG. 21B shows that all of the droplet counts per ml of blood in the cancer patients (8 of 8) were higher than in all healthy donors giving a detection rate of 100% in this assay.

FIG. 22 is a graph that shows the results of a breast cancer assay for a multiplexed eleven marker assay used in a field of 9 metastatic breast cancer patient, 5 localized breast cancer patients, and 15 healthy donors. The results show that the

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assay detects cancer in 7 of 9 metastatic breast cancer patients, 2 of 5 localized breast cancer patients, and none of the healthy donor samples.

FIGs. 23A and 23B are graphs that show the results of ARv7 detection in metastatic breast cancer patients. FIG. 23A is a bar graphs that shows the results for samples from 10 metastatic breast cancer patients and 7 healthy donors processed though the CTC-Chip as described herein. FIG. 23B shows that five of the ten cancer patient samples were above the healthy donor background level giving a detection rate of 5 in 10, or 50%.

FIG. 24A is a bar graph showing the detection rate of individual markers (PMEL, MLANA, MAGEA6, PRAME, TFAP2C, and SOX10) and a combined marker cocktail (SUM) in 34 melanoma patients.

FIG. 24B is a dot plot distribution of droplet signals detected in 34 melanoma patients for 182 draw points as compared to 15 healthy donors demonstrating an overall detection sensitivity above healthy donor background signal of 81% (based on draw points) and a specificity of 100% (by draw points).

DETAILED DESCRIPTION

The present disclosure relates to methods and systems to obtain information from rare cancer cells in blood samples. These methods and systems combine the power of isolation techniques such as ultrahigh-throughput microfluidic techniques, for example, negative depletion techniques, e.g., those using negative depletion of hematopoietic cells to isolate untagged CTCs in a blood sample, with analysis techniques, such as droplet-based digital polymerase chain reaction (PCR) assays focused on ribonucleic acid (RNA) markers of specific cancer lineages. This strategy can also be applied to other CTC isolation technologies that provide partially purification of cells (e.g., filtration, positive tumor cell selection), although the quality of the RNA and hence the sensitivity of the assay will be inferior to the microfluidic technologies. Similarly, other digital PCR technologies applied to RNA are capable of detecting lineage-specific primers, although the sensitivity of the droplet-based assay is likely to be the highest.

The new methods described herein can be used not only for early detection of cancers based on the presence of the CTCs in the blood, but also for tumor burden quantification as well as to monitor CTCs from a particular tumor over time, e.g., to

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determine any potential changes in specific tumor marker genes present in the CTCs as well changes in the tumor as the result of specific therapies, e.g., in the context of a clinical trial or a particular therapy.

General Concepts of the Assay Methods

The isolation techniques are used to enrich CTCs from a blood sample, e.g., using ultrahigh-throughput microfluidic such as the so-called "CTC-iChip" described in, for example, International PCT Application WO 2015/058206 and in Ozkumur et al., "Inertial Focusing for Tumor Antigen-Dependent and -Independent Sorting of Rare Circulating Tumor Cells," *Sci. Transl. Med.*, 5:179ra47 (2013). The CTC-iChip uses a CTC antigen-independent approach in which WBCs in the blood sample are labeled with magnetic beads, and the sample is then processed through two enrichment stages. The first stage uses deterministic lateral displacement to remove small and flexible cells/particles (RBCs, platelets, unbound magnetic beads, and plasma) while retaining larger cells (CTCs and WBCs). The second stage moves all cells into a narrow fluid stream using inertial focusing and then uses a magnetic field to pull bead-labeled WBCs out of the focused stream, leaving highly enriched CTCs. The CTC-iChip product from 10 ml of whole blood typically contains < 500,000 RBCs, < 5,000 WBCs, and a variable number of CTCs.

Some analysis techniques further enrich and analyze the isolated CTCs, e.g., as obtained from the CTC-iChip, e.g., using droplet microfluidics. Some basic information on droplet microfluidics is described generally in Jeremy et al., "Ultrahigh-Throughput Screening in Drop-Based Microfluidics for Directed Evolution," Proc. Natl. Acad. Sci. USA, 107:4004 (2010).

As used herein, the droplet microfluidic techniques can, in certain implementations, include encapsulation of single cells, RT-PCR reagents, and lysis buffer into droplets of typically non-aqueous liquids (e.g., fluorocarbons, hydrofluorocarbons, mineral oil, silicone oil, and hydrocarbon oil; surfactants can also be include in the non-aqueous liquid, e.g., Span80, Monolein/oleic acid, Tween20/80, SDS, n-butanol, ABIL EM90, and phospholipids), in the size range of, e.g., about 0.5 pL to 15 nL in volume and, e.g., 10 to 300 μm, e.g., 20 to 100 μm, e.g., 30 to 50 μm, e.g., 35 μm in diameter. As used in the new methods described in the present

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disclosure, these techniques further include amplification of cancer-specific transcripts within the droplets to produce a fluorescent signal, and sorting of amplification-positive drops. This approach results in isolation of pure CTCs that can be sequenced and analyzed for the purposes of diagnosis and individualized drug therapy. Due to the high heterogeneity of CTCs, it is useful to use multiplexed amplification to detect as many CTCs as possible. Thus, instead of using one pair of primers in the PCR mixture, one can increase the probability of detecting and sorting CTCs using a combination of tumor specific primers. For additional information on the use of PCR for sorting cancer cells, see, e.g., Eastburn et al., "Identification and genetic analysis of cancer cells with PCR-activated cell sorting," Nucleic Acids Research, 2014, Vol. 42, No. 16 e128.

In the new assay methods CTCs are lysed to release RNA molecules, which are representative of the genes expressed in a cancer cell. Most are "lineage" specific, rather than cancer specific, for example any prostate cell (whether cancerous or not) expresses these markers. However, normal blood cells do not, and the fact that the signal is derived from a cell circulating in the bloodstream defines it as an abnormal signal. By converting the RNA to a cDNA, we can now PCR amplify this lineage signal. We use droplet digital PCR, which is extraordinarily sensitive, allowing to convert the signal from a single cancer cell (i.e., one signal in an imaging assay) into thousands of positive immunofluorescent droplets. The combination of multiple, highly curated gene transcripts ensures high sensitivity and specificity for cancer, and also allows for functional insights (as in the status of hormone responsive pathways in prostate and breast cancers).

As noted, the new assay methods focus on the detection and analysis of high quality RNA rather than DNA. While there has been considerable work on DNA mutation detection in plasma and in CTCs, the present methods rely on RNA markers for the following reasons:

1. DNA mutations are not tumor specific, and the discovery that a healthy individual has some unidentified cancer cells in the blood is a very difficult clinical situation. In contrast, by selecting tumor-specific RNAs (e.g., prostate vs lung), the new methods can identify the source of cancer cells in the blood.

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2. DNA mutations are very heterogeneous and besides a few recurrent mutations shared by many cancers, most blood-based mutation detection strategies require pre-existing knowledge of the mutations present in the primary tumor (i.e. not appropriate for screening for unknown cancers). In contrast, all tumor cells derived from specific organs express common lineage markers at the RNA level. Thus, a single cocktail of markers is used in the new methods for each individual type of cancer.

3. Low levels of CTCs are shed by invasive cancers before metastases are established (i.e., it is not too late for blood-based detection), but the presence of tumor cells in the blood connotes vascular invasion (i.e., invasive rather than indolent cancer). That is not the case for plasma DNA or plasma protein markers, which are leaked from dying cells in the primary tumor, and do not necessarily indicate vascular invasion. For example, serum PSA protein in the blood is shed by both benign prostate cells as well as primary prostate cancers. On the other hand, CTCs expressing PSA are shed only by invasive prostate cancers.

4. The analysis of RNA using the novel digital scoring technologies described herein is extraordinarily sensitive. However, free RNA is degraded in the bloodstream, and the use of isolation systems as described herein, such as microfluidic negative depletion systems (e.g., the CTC- Chip system) is unique in that the untagged tumor cells have high quality RNA which is extractable.

The choice of cDNA as a target molecule over DNA was made to not only to boost the signal originating from each tumor cell, but also to specifically target only tumor cell transcripts to the exclusion of white blood cell (WBC) transcripts. The boost in signal is a significant advantage, as it avoids the need for the isolation of CTCs to very high levels of purity. That is, it enables robust and repeatable results with products that contain one or more "isolated" CTCs that are still surrounded by hundreds or thousands of contaminating WBCs, e.g., leukocytes, in the same product. Nevertheless, the strategy of targeting cDNA made from RNA as used in the new methods allows the new assay methods to be exquisitely tailored for maximum specificity with minimal levels of CTC purity compared to prior approaches.

The CTC-iChip technology is highly efficient at isolating non-hematopoietic cells by microfluidic depletion of antibody tagged leukocytes. This feature of the

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CTC-iChip provides intact tumor-derived RNA (at levels far above those obtained using other technologies), and it is independent of tumor cell surface epitopes (which are highly heterogeneous among cancers and among epithelial vs mesenchymal cell subtypes within an individual cancer). Furthermore, even pre-apoptotic cancer cells whose antibody staining and selection is suboptimal for imaging analysis can provide a source of tumor-specific RNA that can be scored using the methods described herein. For all these reasons, an isolation technology or system that provides high quality RNA from intact CTCs with at least some reduction in the WBCs found in the sample along with the rare CTCs, such as a microfluidic negative depletion system, e.g., the CTC-iChip, is an important first step isolation before the tumor-specific digital readout is applied to the product.

The droplet-based digital detection of extremely rare molecules within a heterogeneous mixture was originally developed for PCR amplification of individual DNA molecules that are below detection levels when present within a heterogeneous mixture, but which are readily identified when sequestered within a lipid droplet before being subjected to PCR. The basic technology for droplet-based digital PCR ("Droplet Digital PCR (ddPCR)") has been commercialized by RainDance and Bio-Rad, which provide equipment for lipid encapsulation of target molecules followed by PCR analysis. Important scientific advances that made this possible include work in the laboratory of David Weitz at Harvard and Bert Vogelstein at Johns Hopkins. For example, see U.S. Patent Nos. 6,767,512; 7,074,367; 8,535, 889; 8,841,071; 9,074,242; and U.S. Published Application No. 2014/0303005. See also U.S. Patent No. 9,068,181.

However, droplet digital PCR itself is not biologically significant unless coupled to a biological source of material, which is key to the new methods described herein. For instance detection of lineage-specific RNAs (the central focus of our detection strategy) does not distinguish between normal prostate epithelial cells and cancerous prostate cells. As such, detection of prostate-derived transcripts in the blood is not meaningful: they are present within debris from normal prostate cells or exosomes. It is only when coupled with the isolation of whole CTCs (i.e., intact CTCs in the blood) that the ddPCR assay achieves both extraordinary sensitivity and specificity. Hence these two technologies are ideally suited for each other, because

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the isolation systems provide high quality RNA, and the droplet-based digital PCR assays are focused on RNA markers in the new methods.

One additional aspect is important to the overall success of the new assay methods. As noted, the new assay methods described herein use cDNA made from total RNA, but key to this use is the identification of appropriate biomarkers that are tumor lineage-specific for each type cancer, yet are so unique as to be completely absent in normal blood cells (even with ddPCR sensitivity). The selection, testing, and validation of the multiple target RNA biomarkers for each type of cancer described herein enable the success of the new assay methods.

Assay Method Steps

The new assay methods start with the isolation of partially pure CTCs using an isolation system, such as a microfluidic negative depletion system, up to and including the analysis of data from a droplet digital PCR instrument. There are eight main assay steps, some of which are optional, though generally provide better results:

1. isolating from the blood sample a product including CTCs and other cells present in blood; e.g. from a patient or a subject;
2. reducing a volume of the rare cell-containing product (optional);
3. isolating ribonucleic acid (RNA) molecules from the product, e.g., by cell lysis, and generating cDNA molecules in solution from the isolated RNA; e.g., by RT-PCR of RNA released from cells contained in the product;
4. cleanup of cDNA synthesized during the RT-PCR step (optional);
5. pre-amplifying the cDNA using gene-specific targeted preamplification probes, e.g., using the Fluidigm BioMark™ Nested PCR approach, or non-specific whole-transcriptome amplification, e.g., using the Clontech SMARTer™ approach (optional);
6. encapsulating cDNA molecules in individual droplets, e.g., along with PCR reagents;
7. amplifying cDNA molecules within each of the droplets in the presence of reporter groups configured to bind specifically to cDNA from CTCs and not to cDNA from other cells, e.g., using PCR;

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8. detecting droplets that contain the reporter groups (e.g., "positive" droplets) as an indicator of the presence of cDNA molecules from CTCs in the droplets; and

9. analyzing CTCs in the detected droplets, e.g., to determine the presence of a particular disease in a patient or subject.

As described in further detail below, one of the important features of the new d-CTC assay methods is the careful selection of a number of target gene biomarkers (and corresponding primers) that deliver excellent sensitivity, while simultaneously maintaining nearly perfect specificity. A unique list of target gene biomarkers described herein (**Table 1**, below) was determined using bioinformatics analyses of publicly available datasets and proprietary RNAseq CTC data. Great care was taken to select markers that are not expressed in any subpopulations of leukocytes, but are expressed at a high enough frequency and intensity in CTCs to provide a reliable signal in a reasonably wide array of different and distinct patients. A specific set of markers was selected for each cancer type (e.g. prostate cancer, breast cancer, melanoma, lung cancer, pancreatic cancer, among others.)

The separate steps of the assay methods will now be described in more detail.

1. CTC Isolation

Patient blood is run through the CTC-iChip, e.g., version 1.3M or 1.4.5T and a sample is collected in a 15 mL conical tube on ice. CTC-iChips were designed and fabricated as previously described (Ozkumur et al., "Inertial Focusing for Tumor Antigen-Dependent and -Independent Sorting of Rare Circulating Tumor Cells," Science Translational Medicine, 5(179):179ra47 (DOI: 10.1126/scitranslmed.3005616) (2013)).

The blood samples (~ 20 mls per cancer patient) are collected in EDTA tubes using approved protocols. These samples are then incubated with biotinylated antibodies against CD45 (R&D Systems) and CD66b (AbD Serotec, biotinylated in house) and followed by incubation with Dynabeads® MyOne® Streptavidin T1 (Invitrogen) to achieve magnetic labeling of white blood cells (Ozkumur et al., 2013).

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The sample is then processed through the CTC-iChip, which separates the blood components (red and white blood cells and platelets) as well as unconjugated beads away from the CTCs. The CTCs are collected in solution while the red blood cells, platelets, unconjugated beads and the tagged white blood cells are collected in a waste chamber. The process is automated and 10 ml of blood is processed in 1 hour.

2. Volume Reduction and Storage of the Rare Cell-Containing Product

To fully lyse all cells isolated in the product, it is preferable to reduce the product volume from a typical starting point of several milliliters to a final volume of about 100 µl. This can be achieved, for example, by centrifuging the product, and resuspending in pluronic buffer in preparation for cell lysis and generation of cDNA. At this point samples can be processed for long-term storage by adding RNAlater™ (ThermoFisher), followed by flash-freezing in liquid nitrogen and storage at -80C.

3. Isolating RNA and Generation of cDNA from Cells in the Product

The RNA isolation step is important to the process to fully release all RNA molecules from cells in preparation for RT-PCR. A one-step, in-tube reaction can be used to minimize the risk of cell and RNA loss likely to be incurred during standard transfer steps. For example, one can use the lnvitrogen SuperScript III® First-Strand Synthesis Supermix® for qRT-PCR kit, by adding the RT-PCR mastermix directly to the pelleted product, pipetting to lyse fully, and performing the reaction according to the kit protocol targeting a 1:1 RNA:cDNA ratio. Once cDNA has been synthesized, RNase H is applied to the reaction to remove any remaining RNA. Alternatively, if one wants to perform whole transcriptome pre-amplification of the sample in a later step, cDNA can be synthesized using the SMARTer™ Ultra Low Input RNA Kit protocol, which uses proprietary oligonucleotides and reverse transcriptase enzyme.

4. Cleanup of cDNA Synthesized During RT-PCR

Another useful, yet optional, step in the process involves the removal of lysis reagents from the cDNA-containing solution. The presence of harsh detergents can lead to the destabilization of the droplets used in the ddPCR method, once the cDNA-containing solution is transferred to the ddPCR instrument. Detergent removal can be

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accomplished, e.g., through the use of Solid Phase Reversible Immobilization (SPRI). This technique uses coated magnetic beads to first bind cDNA of a specific size range, then allows removal of detergent-containing supernatant, and finally elution of pure cDNA for input into the ddPCR instrument. In addition to the cleanup of the RT-PCR, the SPRI process also accomplishes a size selection of cDNA, which reduces the number of non-target cDNA molecules that enter the ddPCR phase of the process, which in turn reduces background and noise.

5. Pre-Amplification

Pre-amplification of the cDNA is an optional step that increases the number of template molecules that can be detected in the droplet PCR step thus improving signal-to-noise ratio and boosting the confidence in a positive read-out. It can be a very powerful approach for the detection of markers that are expressed at low levels in CTCs, and for analyzing samples that contain very small numbers of possibly apoptotic CTCs, such as in the context of early detection of pre-metastatic disease. These two approaches have been modified to be applied in the workflow of d-CTC assay. Specific Targeted Amplification (STA), based on the Fluidigm BioMark™ Nested PCR protocol, relies on the use of primers specifically designed to amplify the region targeted by the probes used in the droplet PCR step (see Table 2). These primers were carefully designed and tested in conjuncture with their respective fluorescent probes to ensure efficient and specific amplification without increase in noise in healthy controls. Alternatively, whole transcriptome amplification, based on the SMARTer™ Ultra Low Input RNA Kit protocol, relies on the amplification of every transcript in the product, including both those found in WBCs and those found in CTCs, using random primers.

6. Encapsulation of cDNA plus PCR Reagents in Droplets

Once cDNA has been synthesized and purified of contaminating detergents, the entire aggregate of cDNA molecules in solution plus qPCR reagents is divided into many tiny compartmentalized reactions, for example, by a droplet making instrument, e.g., a droplet generator such as the Biorad Automated Droplet Generator, which generates 20,000 droplets per sample. Each reaction consists of an extremely

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small droplet of non-aqueous fluid, e.g., oil (PCR stable, e.g., proprietary formulation from vendor), which contains Taqman-type PCR reagents with gene-specific primers and an oligonucleotide probe, and a small amount of sample. Once droplet generation is complete, the sample consists of an emulsion containing a vast number of individual PCR-ready reactions.

For this step, one can use the PCR probes and related primers for any one or two or more different target genes listed in Table 1 below for overall determination of tumor load, e.g., to determine tumor progression or response to therapy, in single or multiplex reactions. Thus, although in some cases a single set of PCR primers and probes for a particular gene from Table 1 can be included in each droplet, it is also possible to multiplex PCR primers and probes for two or more different genes in each droplet using different fluorescent probes for each primer/probe set, to maximize the detection of tumor cells, given the heterogeneity of gene expression in CTCs. It is also possible to multiplex PCR primers and probes for multiple genes targeting different cancer types in each droplet, thus enabling the broad yet specific detection of multiple tumor types in a single assay.

7. PCR of Droplet Encapsulated cDNA Molecules

Standard PCR cycling is performed on the entire emulsion sample using qPCR cycling conditions. The reaction is carried to 45 cycles to ensure that the vast majority of individual droplet-PCR volumes are brought to endpoint. This is important because, although the reaction is performed with Taqman-type qPCR reagents and cycled under qPCR conditions, the fluorescent intensity of the sample will not be measured during the PCR cycling, but rather in the next step.

8. Detection of Positive Droplets

Since each individual partitioned PCR is brought fully to endpoint before any measurement of fluorescence is performed, each individual droplet will be either a fully fluorescent droplet or will contain virtually no fluorescence at all. This enables the simple enumeration of all positive (fluorescent) and negative (non-fluorescent) droplets.

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9. Analysis

Because the upstream RT-PCR targeted a 1:1 RNA:cDNA ratio, each positive droplet should represent a single originating RNA transcript. This interpretation depends on the number of individual droplets far exceeding the number of target cDNA molecules. In the new process, at one extreme we consider the possibility of a single CTC being isolated and lysed, releasing some number of RNA transcripts which are then reverse-transcribed 1:1 into cDNA, partitioned, PCR-amplified, and enumerated.

We estimate that in the case of a moderately expressed gene, such as the KLK3 gene in prostate cancer cells, each cell contains approximately 80-120 copies of KLK3 mRNA. The Biorad QX200 ddPCR System generates 20,000 droplets, which ensures that for small numbers of isolated CTCs and moderately-expressed target genes there will never be more than one target cDNA molecule per droplet. On the other hand, in cases where the numbers of CTCs reach dozens or hundreds, for moderately-expressing genes there will likely be multiple copies of target cDNA per droplet. In such cases, approximate numbers of originating transcript can be estimated using Poisson statistics.

Novel Gene Panels to Enable Lineage-Specific Identification of CTCs

As discussed above, the identification of gene transcripts that are highly specific for cancer cells within the context of surrounding normal blood cells is central to the new methods. While many genes are known to be more highly expressed in cancer cells, the vast majority of these genes also typically have at least limited expression in normal tissues, including blood. Given the extraordinary sensitivity required for this assay, complete absence of signal in normal blood cells is essential for high confidence identification of tumor cells in the bloodstream.

Candidate tumor-specific transcripts used to detect CTCs in blood are first selected by analyzing publicly available gene expression data sets derived from breast, prostate, lung, pancreas, and liver cancers and melanoma, as well as our lab-generated single cell RNASeq data from CTCs isolated from breast, prostate and pancreatic cancer patients and mouse models of these cancers. Transcripts whose expression is restricted to tumors and absent or undetectable in blood components are

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