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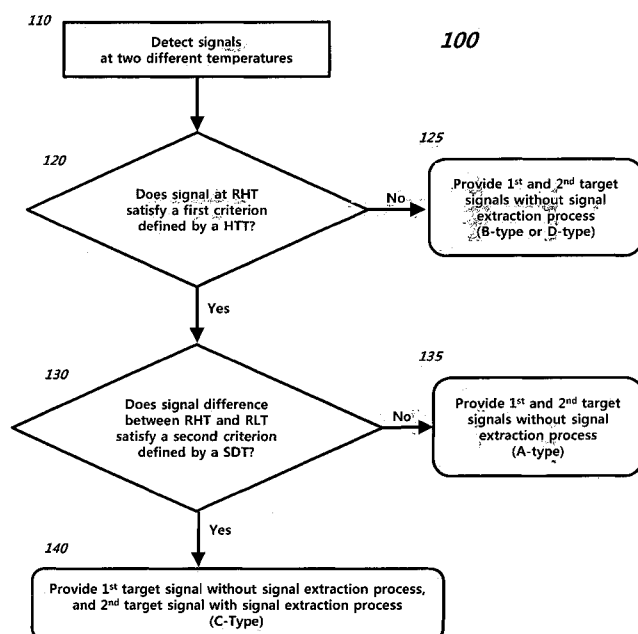
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(54) Title: METHODS FOR PROVIDING SIGNAL FOR TARGET NUCLEIC ACID SEQUENCE

Fig. 2A

(57) Abstract: The present invention is directed to a method for providing a signal for at least one target nucleic acid sequence in a sample from signals detected at different temperatures. The method of the present invention provides a signal for a target nucleic acid sequence with a signal extraction process only when particular criteria are satisfied, and provides a signal for a target nucleic acid sequence without a signal extraction process when a particular criterion is not satisfied. The method of the present invention can minimize the signal extraction process, thereby significantly reducing false positive results which may occur due to the signal extraction process. Accordingly, the method of the present invention can provide a signal for a target nucleic acid sequence using two detection temperatures in a more accurate and efficient manner, from which the presence or absence of a target nucleic acid sequence can be determined.



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METHODS FOR PROVIDING SIGNAL FOR TARGET NUCLEIC ACID SEQUENCE

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority from Korean Patent Application No. 2016-
5 0009509, filed on January 26, 2016, in the Korean Intellectual Property Office, the disclosure of which is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

The present invention relates to a method for providing a signal for at least one
10 target nucleic acid sequence in a sample from signals detected at different temperatures.

BACKGROUND OF THE INVENTION

For detection of target nucleic acid sequences, real-time detection methods are
15 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
20 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
25 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 U.S. Pat. No. 6,117,635), Scorpion primer method (Whitcombe et al, 804-807, Nature

Biotechnology v.17 AUGUST 1999 and U.S. Pat. No. 6,326,145) and TSG primer method (WO 2011/078441).

As alternative approaches, real-time detection methods using duplexes formed depending on the presence of target nucleic acid sequences have been proposed:

5 Invader assay (U.S. Pat. Nos. 5,691,142, 6,358,691 and 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 (WO 2014/104818).

10 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,
15 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 using a single type of label. However, the melting analysis has
20 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 are developed for detecting a plurality of target nucleic acid
25 sequences using a single type of label in a single reaction vessel and a single type of detector, 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.

For this purpose, the present inventors have developed a novel method for detecting a plurality of target nucleic acid sequences using a single type of detector in a single reaction vessel (also referred to as 'MuDT1' technology; see WO 2015/147412). According to the method, the presence of the target nucleic acid sequence having a relatively low detection temperature can be determined by signals detected at a relatively high detection temperature and a relatively low detection temperature, and a reference value representing a relationship of change in signals at different detection temperatures. As a representative example, where the first target nucleic acid sequence is a target nucleic acid sequence having a relatively high detection temperature (*i.e.*, capable of generating signals at both a relatively high detection temperature and a relatively low detection temperature) and the second target nucleic acid sequence is a target nucleic acid sequence having a relatively low detection temperature (*i.e.*, capable of generating a signal at only a relatively low detection temperature), the signal for the second target nucleic acid sequence can be extracted by eliminating the signal for the first target nucleic acid sequence from the signal detected at the relatively low detection temperature by using a reference value for the first target nucleic acid sequence. The extracted signal may be then used to determine the presence or absence of the second target nucleic acid sequence.

The reference value for the first target nucleic acid sequence used in the method may be obtained in a various manners. For instance, the reference value is determined by detecting two signals at a relatively low detection temperature and a relatively high detection temperature from a control sample containing only a first target nucleic acid sequence, followed by calculation of the relationship of change in the two signals, *e.g.*, the ratio of the two signals.

The method as described above is surprisingly remarkable compared with the conventional methods in that the signal for the first target nucleic acid sequence and the signal for the second target nucleic acid sequence are easily distinguishable even by using a conventional single type of detector.

In particular, the method as described above exhibits considerable accuracy in extracting a signal for a target nucleic acid sequence from a sample containing both a target nucleic acid sequence having a relatively high detection temperature and a target nucleic acid sequence having a relatively low detection temperature (a sample co-infected with two targets).

However, for a sample containing only one of two target nucleic acid sequences (*e.g.*, a sample infected with a single target) or a sample containing no target nucleic acid sequence (*e.g.*, non-infected sample), the method may lead to false positive results in extracting the signal for the target nucleic acid sequence having a relatively low detection temperature.

Accordingly, there remains a need in the art to develop a novel method for providing a signal for a target nucleic acid sequence in a more effective and accurate manner.

Throughout this application, various patents and publications are referenced and citations are provided in parentheses. The disclosure of these patents and publications in their entirety 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 improve the MuDT1 technology for detecting a target nucleic acid sequence from signals detected at different temperatures (WO 2015/147412). As a result, the present inventors have developed a novel method for providing a signal for a target nucleic acid sequence in a more accurate and effective manner while significantly reducing false positive results by applying the signal extraction process only when the signals detected at two detection temperatures satisfy particular criteria.

Accordingly, it is an object of this invention to provide a method for providing a signal for at least one target nucleic acid sequence in a sample from signals detected

at different temperatures.

It is another object of this invention to provide a computer readable storage medium containing instructions to configure a processor to perform a method for providing a signal for at least one target nucleic acid sequence in a sample from signals detected at different temperatures.

It is still another object of this invention to provide a device for providing a signal for at least one target nucleic acid sequence in a sample from signals detected at different temperatures.

It is further object of this invention to provide a computer program to be stored on a computer readable storage medium to configure a processor to perform a method for providing a signal for at least one target nucleic acid sequence in a sample from signals detected at different temperatures.

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

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 represents signals detected at two different detection temperatures (72°C and 60°C) in accordance with a PTOCE real-time PCR method (MuDT1 Technology), for four (4) types of samples: (i) A-type: a sample containing only a first target nucleic acid sequence (*Chlamydia trachomatis*, CT); (ii) B-type: a sample containing only a second target nucleic acid sequence (*Neisseria gonorrhoeae*, NG); (iii) C-type: a sample containing both the first target nucleic acid sequence (CT) and the second target nucleic acid sequence (NG); and (iv) D-type: a sample containing no target nucleic acid sequence.

Fig. 2A is a flow chart 100 representing an embodiment of this invention for providing signals for target nucleic acid sequences from signals detected at two different detection temperatures by applying two criteria. For a sample (B-type or D-type of sample) in which the signal detected at the relatively high detection

temperature (RHT) does not satisfy a first criterion defined by a high-temperature threshold (HTT), a signal for a first target nucleic acid sequence and a signal for a second target nucleic acid sequence are provided without signal extraction process **125**; whereas for a sample in which the signal detected at the RHT satisfies the first criterion, the next step **130** proceeds. Afterwards, for a sample (A-type of sample) in which a signal difference between the signal detected at the RHT and the signal detected at the relatively low detection temperature (RLT) does not satisfy a second criterion defined by a signal-difference threshold (SDT), a signal for a first target nucleic acid sequence and a signal for a second target nucleic acid sequence are provided without signal extraction process **135**; whereas for a sample (C-type of sample) in which the signal difference between the signal detected at the RHT and the signal detected at the RLT satisfies the second criterion, a signal for a first target nucleic acid sequence is provided without signal extraction process and a signal for a second target nucleic acid sequence is provided with signal extraction process **140**.

Fig. 2B represents signals for a first target nucleic acid sequence (CT) and a second target nucleic acid sequence (NG) provided according to an embodiment of this invention depicted in Fig. 2A, for four types of samples: A-type; B-type; C-type; and D-type.

Fig. 3 is a flow chart **200** representing another embodiment of this invention for providing a signal for a target nucleic acid sequence from signals detected at two different detection temperatures by applying three criteria. For a sample (D-type of sample) which does not satisfy a first criterion defined by a HTT and does not satisfy a third criterion defined by a low-temperature threshold (LTT), a signal for a first target nucleic acid sequence and a signal for a second target nucleic acid sequence are provided without signal extraction process **224**; and for a sample (B-type of sample) which does not satisfy the first criterion but satisfies the third criterion, a signal for a first target nucleic acid sequence and a signal for a second target nucleic acid sequence are provided without signal extraction process **226**. For a sample (A-type of sample) which satisfies the first criterion but does not satisfy a second

criterion defined by a signal-difference threshold (SDT), a signal for a first target nucleic acid sequence and a signal for a second target nucleic acid sequence are provided without signal extraction process **232**; whereas for a sample (C-type of sample) which satisfies both the first criterion and the second criterion, a signal for a first target nucleic acid sequence is provided without signal extraction process and a signal for a second target nucleic acid sequence is provided with signal extraction process **240**.

Fig. 4 is a flow chart **300** representing another embodiment of this invention for providing a signal for a target nucleic acid sequence from signals detected at two different detection temperatures by applying three criteria. For a sample (D-type of sample) which does not satisfy a third criterion defined by a low-temperature threshold (LTT), a signal for a first target nucleic acid sequence and a signal for a second target nucleic acid sequence are provided without signal extraction process **322**. For a sample (B-type of sample) which satisfies the third criterion but does not satisfies a first criterion defined by a high-temperature threshold (HTT), a signal for a first target nucleic acid sequence and a signal for a second target nucleic acid sequence are provided without signal extraction process **332**. For a sample (A-type of sample) which satisfies the third criterion and the first criterion but does not satisfies a second criterion defined by a signal-difference threshold (SDT), a signal for a first target nucleic acid sequence and a signal for a second target nucleic acid sequence are provided without signal extraction process **342**. For a sample (B-type of sample) which satisfies all of the criteria described above, a signal for a first target nucleic acid sequence is provided without signal extraction process and a signal for a second target nucleic acid sequence is provided with signal extraction process **350**.

25^A Figs. 5A, 5B and 5C represent signals detected at three different detection temperatures (95°C, 72°C and 60°C) for eight (8) types of samples: (i) A-type: a sample containing only a first target nucleic acid sequence; (ii) B-type: a sample containing only a second target nucleic acid sequence; (iii) C-type: a sample containing only a third target nucleic acid sequence; (iv) D-type: a sample containing

both a first target nucleic acid sequence and a second target nucleic acid sequence;
 (v) E-type: a sample containing both a first target nucleic acid sequence and a third
 target nucleic acid sequence; (vi) F-type: a sample containing both a second target
 nucleic acid sequence and a third target nucleic acid sequence; (vii) G-type: a sample
 5 containing all of a first target nucleic acid sequence, a second target nucleic acid
 sequence and a third target nucleic acid sequence; and (viii) H-type: a sample
 containing no target nucleic acid sequence.

Figs. 5D and 5E are a flow chart **400** representing an embodiment of this
 invention for providing signals for target nucleic acid sequences from signals detected
 10 at three different detection temperatures by applying various criteria, for eight (8)
 types of samples containing up to three target nucleic acid sequences. In Figs. 5D
 and 5E, RHT represents a relatively high detection temperature; RMT represents a
 relatively middle detection temperature; RLT represents a relatively low detection
 temperature; HTT represents a threshold for determining significance of the signal at
 15 RHT; MTT represents a threshold for determining significance of the signal at RMT;
 LTT represents a threshold for determining significance of the signal at RLT; End-
 Ratio_{M/H} represents the ratio of End-RFU (RFU value at the end cycle) at RMT to End-
 RFU at RHT; End-Ratio_{L/M} represents the ratio of End-RFU at RLT to End-RFU at RMT;
 RT_{M/H} represents a threshold for distinguishing between a sample containing 2nd
 20 target and a sample containing no 2nd target; and RT_{L/M} represents a threshold for
 distinguishing between a sample containing 3rd target and a sample containing no 3rd
 target.

DETAILED DESCRIPTION OF THIS INVENTION

25

I. Providing a Signal for a Target Nucleic Acid Sequence in a Sample

In one aspect of this invention, there is provided a method for providing a
 signal for at least one target nucleic acid sequence in a sample from signals detected
 at different temperatures, which comprises the steps of:

(a) incubating the sample with a first signal-generating means capable of generating a signal for a first target nucleic acid sequence and a second signal-generating means capable of generating a signal for a second target nucleic acid sequence in a single reaction vessel, and detecting signals at a relatively high detection temperature and a relatively low detection temperature by a single type of detector; wherein the first signal-generating means generates signals at both the relatively high detection temperature and the relatively low detection temperature in the presence of the first target nucleic acid sequence in the sample and the second signal-generating means generates a signal at the relatively low detection temperature in the presence of the second target nucleic acid sequence in the sample; wherein the two signals to be generated by the two signal-generating means are not differentiated by the single type of detector;

(b) identifying whether the signal detected at the relatively high detection temperature satisfies a first criterion defined by a high-temperature threshold (HTT); when the signal detected at the relatively high detection temperature satisfies the first criterion, the next step (c) proceeds;

(c) identifying whether a signal difference between the signal detected at the relatively low detection temperature and the signal detected at the relatively high detection temperature satisfies a second criterion defined by a signal-difference threshold (SDT); when the signal difference satisfies the second criterion, the next step (d) proceeds; and

(d) extracting the signal for the second target nucleic acid sequence by using the signal detected at the relatively high detection temperature in the step (a), the signal detected at the relatively low detection temperature in the step (a) and a reference value for the first target nucleic acid sequence, thereby providing the signal for the second target nucleic acid sequence; wherein the reference value for the first target nucleic acid sequence is a value representing a relationship of change in signals provided by the first signal-generating means at the relatively high detection temperature and the relatively low detection temperature; wherein the reference

value for the first target nucleic acid sequence is determined from a control reaction using the first target nucleic acid sequence and the first signal-generating means; wherein the steps (b) and (c) may be performed in reverse order.

The present inventors have made intensive researches to improve the MuDT1 technology for detecting a target nucleic acid sequence from signals detected at different temperatures (WO 2015/147412). As a result, the present inventors have developed a novel method for providing a signal for a target nucleic acid sequence in a more accurate and effective manner while significantly reducing false positive results by applying the signal extraction process only when the signals detected at two detection temperatures satisfy particular criteria.

Although the inventive method is expressed as a method for providing a signal for a target nucleic acid sequence throughout the specification, it can be also referred to as "a method for determining the presence of a target nucleic acid sequence using a signal for the target nucleic acid sequence", since the signal provided is directly used to determine the presence of the target nucleic acid sequence.

One embodiment of the present invention **100** is illustrated in Fig. 2A. The present invention will be described in more detail with reference to Fig. 2A as follows:

Step (a): Detecting signals at two different temperatures 110

In step (a), the sample is incubated with a first signal-generating means capable of generating a signal for a first target nucleic acid sequence and a second signal-generating means capable of generating a signal for a second target nucleic acid sequence in a single reaction vessel. Afterwards, signals are detected at a relatively high detection temperature and a relatively low detection temperature by a single type of detector. The first signal-generating means generates signals at both the relatively high detection temperature and the relatively low detection temperature in the presence of the first target nucleic acid sequence in the sample and the second signal-generating means generates a signal at the relatively low detection temperature in the presence of the second target nucleic acid sequence in the sample.

The two signals to be generated by the two signal-generating means are not differentiated by the single type of detector.

The term "sample" as used herein refers to any material undergoing the method of the present invention. Particularly, the term "sample" refers to any material containing or presumed to contain a nucleic acid of interest (one or both of a first target nucleic acid sequence and a second target nucleic acid sequence) or which is itself a nucleic acid containing or presumed to contain a target nucleic acid sequence of interest. More particularly, the term "sample" as used herein includes biological samples (*e.g.*, cells, tissues, and fluid from a biological source) and non-biological samples (*e.g.*, food, water and soil). The biological samples includes, but not limited to, virus, bacteria, tissue, cell, blood, serum, plasma, lymph, sputum, swab, aspirate, bronchoalveolar lavage fluid, milk, urine, feces, ocular fluid, saliva, semen, brain extracts, spinal cord fluid (SCF), appendix, spleen and tonsillar tissue extracts, amniotic fluid and ascitic fluid. The sample can be subjected to nucleic acid extraction process known in the art for efficient amplification reactions (see Sambrook, J. et al., Molecular Cloning, A Laboratory Manual, 3rd ed. Cold Spring Harbor (2001)). The nucleic acid extraction process may vary depending on the type of the sample. Moreover, if the extracted nucleic acid is RNA, a reverse transcription is further performed to synthesize cDNA therefrom (see *supra*).

The term used herein "target nucleic acid", "target nucleic acid sequence" or "target sequence" refers to a nucleic acid sequence of interest for analysis, detection or 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 newly generated in reactions as well as a sequence initially present in a sample.

The target nucleic acid sequence may include any DNA (gDNA and cDNA), RNA molecules and 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 glyoxal 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 methods for accomplishing this treatment are provided by Joseph Sambrook, et al.,
5 *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.(2001).

The target nucleic acid sequence includes any naturally occurring prokaryotic, eukaryotic (for example, protozoans and parasites, fungi, yeast, higher plants, lower
10 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 target nucleic acid sequence can also be any nucleic acid molecule which has been or can be recombinantly produced or chemically synthesized. Thus, the target nucleic acid sequence may or may not be found in
15 nature. The target nucleic acid sequence may comprise a known or unknown sequence.

The target nucleic acid sequence should not be construed as limiting the sequence known at a given time or the sequence available as of a given time, but instead should be read to encompass the sequence that may be available or known
20 now or at any time in the future. In other words, the target nucleic acid sequence may or may not be known at the time of practicing the present method. In case of unknown target nucleic acid, its sequence may be determined by one of conventional sequencing methods prior to performing the present method.

The target nucleic acid sequence used in the present invention comprises the
25 first target nucleic acid sequence and the second target nucleic acid sequence. The terms "first target nucleic acid sequence" and "second target nucleic acid sequence" are used herein to distinguish two different target nucleic acid sequences. For instance, the first target nucleic acid sequence and the second target nucleic acid sequence may be two different genes, two different gene regions or two different

DNA sequences of interest. Particularly, the first target nucleic acid sequence may be derived from one organism, whereas the second target nucleic acid sequence from another organism.

According to an embodiment of this invention, one of the two target nucleic acid sequences comprise a nucleotide variation, or 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 the present invention, the sample (or target nucleic acid sequences in the sample) is incubated with two signal-generating means in order to obtain signals for the target nucleic acid sequences.

The term "incubating," "incubate," or "incubation" as used herein refers to bring components together for their interaction or reaction. Particularly, the term refers to subjecting the components herein to a signal-generating process.

The term "signal" as used herein refers to a measurable output.

The signal change may serve as an indicator indicating qualitatively or

quantitatively the presence or absence of an analyte (a target nucleic acid sequence).

Examples of useful indicators include, but not limited to, fluorescence intensity, luminescence intensity, chemiluminescence intensity, bioluminescence intensity, phosphorescence intensity, charge transfer, voltage, current, power, energy, temperature, viscosity, light scatter, radioactive intensity, reflectivity, transmittance and absorbance. The most widely used indicator is fluorescence intensity.

Signals include 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 shape (or pattern) or C_t value, or values obtained by mathematically processing the characteristics.

According to an embodiment, the term "signal" with conjunction with reference value or sample analysis includes not only signals *per se* obtained at detection temperatures but also a modified signal provided by mathematically processing the signals.

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 and used for determination of target presence (intensity, C_t value or amplification curve data).

The terms "signal for a first target nucleic acid sequence" and "signal for a second target nucleic acid sequence" as used herein refer to signals representing the first target nucleic acid sequence and the second target nucleic acid sequence, respectively. In other words, the terms refer to signals which are generated and detected dependently on the presence of the first target nucleic acid sequence or the second target nucleic acid sequence, and thus a significant level of the signal for a first target nucleic acid sequence or second target nucleic acid sequence indicates the presence of the first target nucleic acid sequence or the second target nucleic acid sequence.

The signal (particularly, the signal intensity) may vary depending upon its

detection temperature as well as a signal-generating means employed.

The incubation herein is performed by a signal-generating process using signal-generating means.

The term "signal-generating process" as used herein refers to any process capable of generating signals in a dependent manner on the presence of a target nucleic acid sequence in a sample.

The term "signal-generating means" as used herein 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.

In particular, the terms "first signal-generating means" and "second signal-generating means" as used herein refer to means for generating signals for a first target nucleic acid sequence and a second target nucleic acid sequence, respectively. The first signal-generating means and the second signal-generating means may include common components (*e.g.*, a single type of label and a single type of enzyme) or different components (*e.g.*, different types of oligonucleotides) used in signal generation. In particular, the first signal-generating means and the second signal-generating means are characterized by having a single type of label (the same label). Thus, the signal derived from the label of the first signal-generating means is not differentiated from the signal derived from the label of the second signal-generating means by conventional methods.

A wide variety of the signal-generating means have been known to one of skill in the art. The signal-generating means include both labels *per se* and oligonucleotides with labels. The labels may include a fluorescent label, a luminescent label, a chemiluminescent label, an electrochemical label and a metal label. The label *per se* may serve as signal-generating means, for example, an intercalating dye. Alternatively, a single label or an interactive dual label containing a donor molecule and an acceptor molecule may be used as signal-generating means in the form of

linkage to at least one oligonucleotide.

The signal-generating means may comprise additional components for generating signals such as nucleolytic enzymes (*e.g.*, 5'-nucleases and 3'-nucleases).

The signal-generating process is accompanied with signal change. The signal
5 change may serve as an indicator indicating qualitatively or quantitatively the presence or absence of a target nucleic acid sequence.

The details of "signal-generating process" are disclosed in WO 2015/147412 filed by the present inventors, the teachings of which are incorporated herein by reference in its entirety.

10 According to an embodiment, the signal-generating process is a signal amplification process.

According to an embodiment of this invention, the signal-generating process is a process with amplification or with no amplification of a target nucleic acid sequence.

Particularly, the signal-generating process is a process with amplification of a
15 target nucleic acid molecule. More particularly, the signal-generating process is a process with amplification of a target nucleic acid molecule and capable of increasing or decreasing signals (particularly, increasing signals) upon amplifying the target nucleic acid molecule.

The term used herein "signal generation" include appearance or disappearance
20 of signals and increase or decrease in signals. Particularly, the term "signal generation" means increase in signals.

The signal-generating process may be performed in accordance with a multitude of methods known to one of skill in the art. The methods include TaqMan™ probe method (U.S. Pat. No. 5,210,015), Molecular Beacon method (Tyagi et al.,
25 Nature Biotechnology, 14(3):303(1996)), 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), CPT (Duck P, et al., Biotechniques, 9:142-148(1990)), LNA method (U.S. Pat. No. 6,977,295), Plexor method (Sherrill CB, et al.,

Journal of the American Chemical Society, 126:4550-4556(2004)), Hybeacons™ (D. J. French, et al., Molecular and Cellular Probes, 13:363-374(2001)) and U.S. Pat. No. 7,348,141), Dual-labeled, self-quenched probe (U.S. Pat. No. 5,876,930), Hybridization probe (Bernard PS, et al., Clin. Chem., 46:147-148(2000)), PTOCE (PTO
5 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 (WO 2014/104818) and CER method (WO 2011/037306).

When the signal-generating process is performed in accordance with TaqMan™
10 probe method, the signal-generation means may comprise a primer pair, a probe with an interactive dual label and DNA polymerase having 5' to 3' nuclease activity. When the signal-generating process is performed in accordance with PTOCE method, the signal-generation means may comprise a primer pair, PTO (Probing and Tagging Oligonucleotide), CTO (Capturing and Templating Oligonucleotide) and DNA
15 polymerase having 5' to 3' nuclease activity. Either PTO or CTO may be labeled with suitable labels.

According to an embodiment, the signal-generating process is performed in a process involving signal amplification together with target amplification.

According to an embodiment, the amplification reaction as the signal-
20 generating process is performed in such a manner that signals are amplified simultaneously with amplification of the target nucleic acid sequence (*e.g.*, real-time PCR). Alternatively, the amplification reaction is performed in such a manner that signals are amplified with no amplification of the target nucleic acid molecule [*e.g.*, CPT method (Duck P, et al., Biotechniques, 9:142-148 (1990)), Invader assay (U.S.
25 Pat. Nos. 6,358,691 and 6,194,149)].

A multitude of methods have been known for amplification of a target nucleic acid molecule, including, but not limited to, PCR (polymerase chain reaction), LCR (ligase chain reaction, see Wiedmann M, et al., "Ligase chain reaction (LCR)- overview and applications." PCR Methods and Applications, 3(4):S51-64(1994)), GLCR (gap

filling LCR, see WO 90/01069, EP 0439182 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. 5,556,751), SDA (strand displacement amplification, see G T Walker et al., Nucleic Acids Res. 20(7):16911696(1992), EP 0497272), 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. 5,888,779) or RCA (Rolling Circle Amplification, see Hutchison C.A. et al., Proc. Natl Acad. Sci. USA. 102:1733217336(2005)).

The detection of signals from the two signal-generating means at a relatively high detection temperature and a relatively low detection temperature is performed at one or more cycles of the signal-generating process to obtain a signal value at each of the one or more cycles.

The term used herein "cycle" refers to a unit of changes of conditions in a plurality of measurements accompanied with changes of conditions. For example, the changes of conditions include changes in temperature, reaction time, reaction number, concentration, pH and/or replication number of a target nucleic acid molecule sequence. Therefore, the cycle may include time or process cycle, unit operation cycle and reproductive cycle. For example, an isothermal amplification allows for a plurality of measurements for a sample in the course of reaction time under isothermal conditions and the reaction time may correspond to the changes of conditions and a unit of the reaction time may correspond to a cycle.

Particularly, when repeating a series of reactions or repeating a reaction with a time interval, the term "cycle" refers to a unit of the repetition.

For example, in a polymerase chain reaction (PCR), a cycle refers to a reaction unit comprising denaturation of a target molecule, annealing (hybridization) between the target molecule and primers and primer extension. The increases in the repetition of reactions may correspond to the changes of conditions and a unit of the repetition may correspond to a cycle.

The signals detected at the relatively high detection temperature and the

relatively low detection temperature may be a signal value at a particular cycle, a set of signal values at particular cycles, a set of signal values within a particular cycle interval, or a set of signal values at all cycles. The detection of signals provides a signal value at each cycle of the signal-generating process. The term used herein

5 "signal value" means either signal value actually measured at each cycle of the signal-generating process (*e.g.*, actual value of fluorescence intensity processed by amplification reaction) or its modification. The modification may include mathematically processed value of measured signal value (*e.g.*, intensities). Examples of mathematically processed value of measured signal value may include logarithmic

10 value and derivative of measured signal values. The term used herein "signal" is intended to encompass the term "signal value" and therefore these terms will be used interchangeably.

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

15 single type of detector" means that signals are not differentiated from each other at a certain detection temperature by a single type of detector due to their identical or substantially identical signal properties (*e.g.*, optical properties, emission wavelength and electrical signal).

The term used herein "a single type of signal" means signals providing identical

20 or substantially identical signal properties (*e.g.*, optical properties, emission wavelength and electrical signal). For example, FAM and CAL Fluor 610 provide different types of signals from one another.

According to an embodiment of the present invention, the two signal-generating means comprise an identical label and the signals from the label is not

25 differentiated from each other by a single type of detector.

The first signal-generating means generates signals at both the relatively high detection temperature and the relatively low detection temperature in a dependent manner on the presence of the first target nucleic acid sequence; whereas the second signal-generating means generates a signal at the relatively low detection

temperature in a dependent manner on the presence of the second target nucleic acid sequence. According to an embodiment of the present invention, the detection temperatures for target nucleic acid sequences are determined in considering a temperature range to allow signal generation by the signal-generating means.

5 The term "target nucleic acid sequence having a relatively high detection temperature" as used herein refers to a target nucleic acid sequence which is capable of generating a signal at the relatively high detection temperature of the two detection temperatures, and thus generating a signal at the relatively low detection temperature as well. A target nucleic acid sequence having a relatively high detection
10 temperature herein may be used interchangeably with a first target nucleic acid sequence. In contrast, the term "target nucleic acid sequence having a relatively low detection temperature" refers to a target nucleic acid sequence which is capable of generating a signal at the relatively low detection temperature of the two detection temperature, but not generating a signal at the relatively high detection temperature.
15 A target nucleic acid sequence having a relatively low detection temperature herein may be used interchangeably with the second target nucleic acid sequence.

According to an embodiment of the present invention, a detection temperature determined by a corresponding signal-generation is assigned to one target nucleic acid sequence.

20 The relatively high detection temperature is a temperature capable of generating only a signal for the target nucleic acid sequence having the relatively high detection temperature (first target nucleic acid sequence), and the relatively low detection temperature is a temperature capable of generating both a signal for the target nucleic acid sequence having the relatively low detection temperature (second
25 target nucleic acid sequence) and a signal for the target nucleic acid sequence having the relatively high detection temperature (first target nucleic acid sequence). The relatively high detection temperature may be referred to a first detection temperature, and the relatively low detection temperature may be referred to a second detection temperature.

According to MuDT1 technology, the presence of the two target nucleic acid sequences may be differentially determined by detecting the signals indicative of the presence of the two target nucleic acid sequences at different temperatures. The technology employs that there is a certain temperature range to allow signal
5 generation by signal-generating means.

For example, when a signal-generating means generates a signal upon hybridization (or association) between two nucleic acid molecules and does not generate a signal upon non-hybridization (or dissociation) between them, a signal is generated at temperatures allowing hybridization between two nucleic acid molecules,
10 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. 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.

15 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.*, 30-99°C).

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

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

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

signal at the relatively high detection temperature is determined as the target nucleic acid sequence having the relatively low detection temperature.

According to an embodiment, the detection temperature may be determined in considering the overlapped detection temperature range and the non-overlapped
5 detection temperature range.

According to an embodiment, the detection temperatures allocated for the target nucleic acid sequences are different from each other by at least 2°C, 3°C, 4°C, 5°C, 6°C, 7°C, 8°C, 9°C, 10°C, 11°C, 12°C, 15°C or 20°C.

According to the present invention, a temperature for detecting the presence
10 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
15 means suitable for the detection temperatures are constructed.

According to an embodiment, the relatively high detection temperature and the relatively low detection temperature at which the detection is carried out may be determined. For example, the relatively high detection temperature and the relatively low detection temperature are determined as 72°C and 60°C, respectively, and then
20 signal-generating means suitable for the detection temperatures are constructed.

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.

According to an embodiment of this invention, when the signal-generating
25 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, the detection of the signal is successfully done at the determined 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 determined 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 determined 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 determined 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 (*e.g.*, 30°C to 130°C).

The signals are generated by the signal-generating process, thereby providing a data set. The term used herein "data set" refers to a set of data points. The term used herein "data point" means a coordinate value comprising a cycle and a signal value at the cycle. Data points obtained by the signal-generating process may be plotted with coordinate values in a rectangular coordinate system, giving a curve (*e.g.*, amplification curve). The curve may be a fitted or normalized (*e.g.*, baseline-subtracted) curve. In particular, the signals detected are plotted against cycles.

In a particular embodiment, the signals, and the data sets, the data points and the curve thereof are baseline-subtracted.

According to the present invention, the signals detected at the relatively high detection temperature and at the relatively low detection temperature exhibit distinct

pattern depending upon the types of samples.

For instance, the samples containing up to two target nucleic acid sequences may be divided into four (4) types:

- (i) a sample containing only a first target nucleic acid sequence (A-type);
- 5 (ii) a sample containing only a second target nucleic acid sequence (B-type);
- (iii) a sample containing both a first target nucleic acid sequence and a second target nucleic acid sequence (C-type); and
- (iv) a sample containing no target nucleic acid sequence (D-type).

Typical signal patterns of the four types of samples are illustrated in Fig. 1.

10 Referring to Fig. 1, (i) for an A-type sample, significant signals are detected both at the relatively high detection temperature and the relatively low detection temperature, but the difference between the signal at the relatively low detection temperature and the signal at the relatively high detection temperature is lower than that for C-type; (ii)

15 for a B-type sample, a significant signal is not detected at the relatively high detection temperature, but is detected at the relatively low detection temperature; (iii) for a C-type sample, significant signals are detected both at the relatively high detection temperature and the relatively low detection temperature, and the difference between the signal at the relatively low detection temperature and the signal at the relatively high detection temperature is higher than that for A-type; and (iv) for a D-type

20 sample, no significant signals are detected both at the relatively high detection temperature and the relatively low detection temperature.

Such differences in signal patterns allow the samples to be distinguished into the four types using appropriate criteria as described below.

25 **Step (b): Evaluating the signal at the relatively high detection temperature by a high-temperature threshold 120**

In step (b), it is identified whether the signal detected at the relatively high detection temperature satisfies a first criterion defined by a high-temperature threshold (HTT) **120**. When the signal detected at the relatively high detection

temperature satisfies the first criterion, the next step (c) **130** proceeds.

The term "high-temperature threshold (HTT)" as used herein refers to a threshold used for determining the significance of the signal detected at the relatively high detection temperature. Determining the significance of the signal means
5 determining whether the signal is generated at a meaningful level at a certain temperature relative to a specific threshold. The significance of the signal can be determined by a suitable threshold (*e.g.*, HTT) for distinguishing a signal generated by a specific signal-generating means from other signals. Specifically, the threshold (*e.g.*, HTT) used to determine the significance of the signal is set to any value that
10 can exclude a signal not derived from a nucleic acid sequence of interest, for example, a background signal or a noise signal. As an example, if the signal is amplified as the amplification reaction proceeds, the signal above the threshold may be determined to be significant.

The significance of the signal can be applied to the "low-temperature threshold (LTT)" or the "verification threshold (VT)" in a similar manner as will be described
15 below. The HTT used herein may also be expressed as the "first signal threshold" in order to distinguish it from other thresholds. The value of the HTT used to determine the significance of the signal may differ from that of the threshold used to finally determine the presence of the target nucleic acid sequence.

20 The HTT may be determined by a conventional method for setting thresholds. For example, the HTT may be determined by considering the background signal, sensitivity, label characteristics, signal variation of the detector, or error range. The HTT may be set to any value within the exponential region of the signal-generating reaction. The HTT may be set to any value which is greater than the signal values in
25 the baseline region and which is lower than the signal values in the plateau region of the signal-generating reaction. Alternatively, the HTT may be set to a value obtained by multiplying by a certain value (*e.g.*, 10) the standard deviation of the signal values in the baseline region in a baseline-subtracted amplification curve.

The HTT may be set automatically by the detector or directly by the user.

As used herein, the expression "first criterion defined by a high-temperature threshold" refers to a criterion which is defined in consideration of the HTT in order to determine the significance of the signal detected at the relatively high detection temperature. In other words, the signal satisfying the first criterion indicates that a significant signal is generated at the relatively high detection temperature, whereas the signal not satisfying the first criterion indicates that no significant signal is generated at the relatively high detection temperature.

According to an embodiment of the present invention, the first criterion for the signal detected at the relatively high detection temperature is the magnitude relation between the signal detected at the relatively high detection temperature and the HTT. For example, the first criterion for the signal detected at the relatively high detection temperature is that the signal is not less than, above, not more than, or below the HTT. Specifically, when the signal is amplified as amplification products accumulate, the first criterion is that the signal is not less than or above the HTT. On the other hand, when the signal is decreased as amplification products accumulate, the first criterion is that the signal is not more than or below the HTT.

More specifically, the first criterion for the signal detected at the relatively high detection temperature is that the signal is not less than the HTT.

For example, when the signal amplification reaction is performed according to real-time PCR and the HTT is RFU (relative fluorescence unit) 100, "the signal value not less than RFU 100" may be the first criterion.

According to the method of the present invention, the signal detected at the relatively high detection temperature will have only the signal derived from the first signal-generating means, *i.e.*, the signal for the first target nucleic acid sequence, because the second signal-generating means is designed not to generate a signal at the relatively high detection temperature. Thus, if the signal detected at the relatively high detection temperature satisfies the first criterion, *i.e.*, if the signal detected at the relatively high detection temperature is identified as being significant, it indicates directly that the first target nucleic acid sequence is present in a sample. Such

significant signal for the first target nucleic acid sequence may be provided as the signal for the first target nucleic acid sequence. In this case, the presence or absence of the first target nucleic acid sequence may be identified by use of an additional threshold, or the presence of the first target nucleic acid sequence may be determined directly based on the result of satisfying the first criterion without using the additional threshold. Further, such significant signal for the first target nucleic acid sequence is then used to calculate the signal difference between the signal detected at the relatively low detection temperature and the signal detected at the relatively high detection temperature in the next step (c).

Conversely, if the signal detected at the relatively high detection temperature does not satisfy the first criterion, *i.e.*, if the signal detected at the relatively high detection temperature is identified as being insignificant, it indicates directly that the first target nucleic acid sequence is absent in a sample. Such insignificant signal for the first target nucleic acid sequence may be provided as the signal for the first target nucleic acid sequence. In this case, the presence or absence of the first target nucleic acid sequence may be identified by use of an additional threshold, or the absence of the first target nucleic acid sequence may be determined directly based on the result of not satisfying the first criterion without using the additional threshold. Further, it may also indicate that the signal detected at the relatively low detection temperature includes only the signal provided by the second target nucleic acid sequence.

The first criterion defined by the HTT as used in this step allows distinguishing A-type or C-type from B-type or D-type among the typical four (4) types of samples (see Fig. 1). Typically, an A-type or C-type of samples generates significant signals at the relatively high detection temperatures, whereas a B-type or D-type of samples generates no significant signals at the relatively high detection temperatures. Therefore, if the signal detected at the relatively high detection temperature satisfies the first criterion defined by the HTT, the sample is determined to be either an A-type or C-type sample, whereas if the signal detected at the relatively high detection temperature does not satisfy the first criterion defined by the HTT, the sample is

determined to be either a B-type or D-type sample.

According to an embodiment of the present invention, when the signal detected at the relatively high detection temperature does not satisfy the first criterion defined by the HTT, the signal detected at the relatively low detection temperature in the step (a) is provided as the signal for the second target nucleic acid sequence, without the signal extraction process of the step (d) **125**. This embodiment may provide the signal detected at the relatively low detection temperature in the step (a) as the signal for the second target nucleic acid sequence, without using the third criterion defined by the low-temperature threshold as will be described below.

For example, when a signal is detected at the relatively high detection temperature in a real-time PCR reaction but the signal values of the signal are all below the HTT, the signal detected at the relatively low detection temperature in the step (a) may be provided as the signal for the second target nucleic acid sequence, and the signal provided may be used to determine the presence of the second target nucleic acid sequence. The provided signal for the second target nucleic acid sequence is analyzed using an additional threshold for determining the presence of the second target nucleic acid sequence.

According to one embodiment of the present invention, the signal detected at the relatively high detection temperature in the step (a) is provided as the signal for the first target nucleic acid sequence.

According to an alternative embodiment of the present invention, when the signal detected at the relatively high detection temperature does not satisfy the first criterion defined by the HTT, any signal indicative of the absence of a target nucleic acid sequence is provided as the signal for the absence of the first target nucleic acid sequence.

As used herein, the expression "signal indicative of the absence of a target nucleic acid sequence" is used to encompass a signal having signal values by which the absence of the target nucleic acid sequence is determined. For example, the expression is used to encompass a signal indicating that there is no or substantially

no target nucleic acid present in a sample (*e.g.*, a signal having signal values of RFU zero or signal values close to RFU zero), or a signal having such low signal values that the target nucleic acid sequence is determined not to be significantly present by a predetermined threshold (*e.g.*, a signal having signal values lower than the threshold).

5 According to one embodiment of the present invention, when the signal detected at the relatively high detection temperature does not satisfy the first criterion defined by the HTT, any of the following signals is provided as the signal for the first target nucleic acid sequence: (i) the signal detected at the relatively high detection temperature in the step (a); (ii) the signal obtained from a negative control
10 that does not contain the target nucleic acid sequence; or (iii) any signal indicative of the absence of a target nucleic acid sequence. These signals indicate that the sample does not contain the first target nucleic acid sequence.

According to one embodiment of the present invention, when the signal detected at the relatively high detection temperature does not satisfy the first
15 criterion defined by the HTT **120**, any of the signals (i)-(iii) is provided as the signal for the first target nucleic acid sequence, and the signal detected at the relatively low detection temperature in the step (a) is provided as the signal for the second target nucleic acid sequence **125**.

Therefore, according to the embodiment of the present invention, in the case
20 of a sample containing only the second target nucleic acid sequence (B-type), any of the signals (i)-(iii) is provided as the signal for the first target nucleic acid sequence, and the significant signal detected at the relatively low detection temperature in the step (a) is provided as the signal for the second target nucleic acid sequence. Also, in the case of a sample not containing the first target nucleic acid sequence and the
25 second target nucleic acid sequence (D-type), any of the signals (i)-(iii) is provided as the signal for the first target nucleic acid sequence, and the insignificant signal detected at the relatively low detection temperature in the step (a) is provided as the signal for the second target nucleic acid sequence.

The provided signals may be used to determine the presence or absence of

each target nucleic acid sequence by applying new additional thresholds.

When the signal detected at the relatively high detection temperature satisfies the first criterion, the next step (c) **130** proceeds.

5 **Step (c): Evaluating the signals detected at the relatively low detection temperature and the relatively high detection temperature by a signal-difference threshold 130**

When the first criterion is satisfied in the step (b), it is then performed to identify whether the signal difference between the signal detected at the relatively
10 low detection temperature and the signal detected at the relatively high detection temperature satisfies a second criterion defined by a signal-difference threshold (SDT) **130**. When the signal difference satisfies the second criterion, the next step **140** proceeds.

The signal difference and SDT may be used to determine the presence or
15 absence of a second target nucleic acid sequence.

The "signal difference" between the signal detected at the relatively low detection temperature and the signal detected at the relatively high detection temperature as used in this step is a value representing a change, a signal change or a signal difference between the signals detected at the relatively low detection
20 temperature and the relatively high detection temperature from the sample (both signals obtained in the step (a)). That is, the "signal difference" includes any value reflecting the change of signals at the different detection temperatures from the sample. The signal difference may be expressed in various manners. For example, a signal difference may be expressed by a numerical value, the presence/absence of a
25 signal, or a plot of signal characteristics.

The signal difference is a value representing a change of the signals detected at the relatively high detection temperature and the relatively low detection temperature from the sample of interest.

According to one embodiment of the present invention, the signal difference

may be obtained by mathematically processing the signals detected at the relatively high detection temperature and the relatively low detection temperature from the sample.

According to one embodiment of the present invention, the mathematical processing includes calculation (*e.g.*, addition, subtraction, multiplication and division) using signals or other values derived from the signals.

Particularly, the mathematical processing is a subtraction or ratio of the signal detected at the relatively low detection temperature and the signal detected at the relatively high detection temperature, more particularly a ratio, and most particularly a ratio of the signal detected at the relatively low detection temperature to the signal detected at the relatively high detection temperature.

In a particular embodiment of the invention, the signal difference is a ratio of signal values at one cycle, *i.e.*, a ratio of a signal value at one cycle in the signal detected at the relatively high detection temperature and a signal value at the same cycle in the signal detected at the relatively low detection temperature. The cycle selected for the calculation of the ratio is any cycle following the baseline region, particularly any cycle within the plateau region, and more particularly the end cycle in the amplification curve. According to one embodiment of the present invention, the ratio may be an average value of the ratios in several cycles, for example, two, three, four or five consecutive cycles. According to an embodiment of the present invention, the signal difference is a ratio of the signal value at the end cycle in the signal detected at the relatively low detection temperature versus the signal value at the end cycle in the signal detected at the relatively high detection temperature.

The mathematical processing for obtaining the signal difference may be performed in various ways. Mathematical processing may be performed using a machine. For example, the signals may be mathematically processed by a processor in a detector or real-time PCR instrument. Alternatively, the signals may be mathematically processed manually, in particular according to a certain algorithm.

The term "signal-difference threshold (SDT)" as used herein refers to a

determined value for distinguishing a sample containing only the first target nucleic acid sequence (A-type) from a sample containing both the first target nucleic acid sequence and the second target nucleic acid sequence (C-type). The SDT is compared with the signal difference as described above and the comparison result allows for distinguishing between the sample containing only the first target nucleic acid sequence and the sample containing both the first target nucleic acid sequence and the second target nucleic acid sequence. The SDT may be referred to as a second criterion.

The SDT may be set in various manners and applied to the present invention. According to one embodiment, the SDT is determined by considering: (i) the signal difference between the signals at the relatively low detection temperature and the relatively high detection temperature for a control sample containing only the first target nucleic acid sequence; and (ii) the signal difference between the signals at the relatively low detection temperature and the relatively high detection temperature for the control sample containing both the first target nucleic acid sequence and the second target nucleic acid sequence. The signal differences which are obtained from the control samples and used to set the SDT and which are obtained from the control samples may be calculated in the same manner as the signal difference which is obtained from the test sample as described above. However, it is noted that the signal difference is obtained by analyzing the signals from a test sample of interest (*i.e.*, unknown sample), while the SDT is obtained by analyzing the signals from two control samples to acquire two signal differences and then selecting a value in considering the two signal differences.

Specifically, the SDT may be determined as follows:

First, a control sample (a first control sample) containing only the first target nucleic acid sequence is incubated with the first signal generating means and two signals are detected at the relatively high detection temperature and the relatively low detection temperature. Afterwards, a signal difference is calculated from the signals at the relatively high detection temperature and the relatively low detection temperature

for the first control sample.

Second, a control sample (a second control sample) containing both the first target nucleic acid sequence and the second target nucleic acid sequence is also incubated with the first signal generating means and the second signal generating means, and two signals are detected at the relatively high detection temperature and the relatively low detection temperature. Afterwards, the signal difference is calculated from the signals at the relatively high detection temperature and the relatively low detection temperature for the second control sample.

Finally, a SDT is set in consideration of both the signal difference for the first control sample and the signal difference for the second control sample.

For example, when the signal difference (*e.g.*, a ratio) for the first control sample is 1.15 and the signal difference (*e.g.*, a ratio) for the second control sample is 2.09, the SDT may be set to any appropriate value between 1.15 and 2.09 (*e.g.*, 1.50).

According to one embodiment, the SDT is any value between (i) the signal difference between the signals at the relatively high detection temperature and the relatively low detection temperature for the control sample containing only the first target nucleic acid sequence and (ii) the signal difference between the signals at the relatively high detection temperature and the relatively low detection temperature for the control sample containing both the first target nucleic acid sequence and the second target nucleic acid sequence.

The signal difference for the control samples may be both acquired in a specific range via repeated experiments. For example, when the signal difference for the control sample containing only the first target nucleic acid sequence is acquired in the range of 1.05 to 1.30 via repeated experiments, and the signal difference for the control sample containing both the first target nucleic acid sequence and the second target nucleic acid sequence is acquired in the range of 1.80 to 2.30 via repeated experiments, the SDT may be set to a value which is between the two ranges and does not overlap the two range, for example a value higher than 1.30 and lower than

1.80.

According to one embodiment, the SDT is set to any value which is higher than the ratio of the signal values at end cycles in the signals detected at the relatively low detection temperature and the relatively high detection temperature from a control containing only the first target nucleic acid sequence and which is lower than the ratio of the signal values at end cycles in the signals detected at the relatively low detection temperature and the relatively high detection temperature from a control containing both the first target nucleic acid sequence and the second target nucleic acid sequence.

When the SDT is expressed by a ratio, the SDT value may or may not be identical to the reference value as will be described below.

Although the SDT is explained mainly by the ratio of the signal values in the specification, it will be appreciated by those skilled in the art that various other modified signal-difference thresholds can be devised by considering the calculating method for a signal difference or considering the signal pattern difference between a control sample containing only the first target nucleic acid sequence and a control sample containing both the first target nucleic acid sequence and the second target nucleic acid sequence.

The expression "second criterion defined by a signal-difference threshold (SDT)" as used herein refers to a criterion which is defined in consideration of the SDT. For example, the second criterion defined by the signal-difference threshold is that the signal difference is more than, not less than, not more than, or less than the signal-difference threshold. For example, if the SDT is set to 1.5 based on the ratio of the signal values at the end cycle, the second criterion defined by the SDT may be that the signal difference is 1.5 or more.

In the method of the present invention, the SDT and the signal difference should be calculated by the same mathematical processing. For example, if the SDT is calculated by subtraction of the signals, the signal difference should be also calculated by subtraction of the signals. Alternatively, if the SDT is calculated by the ratio of the

signals, the signal difference should also be calculated by the ratio of the signals.

According to an embodiment, when the signal difference calculated in the step (c) does not satisfy the second criterion defined by the SDT, the method of the present invention provides any signal indicative of the absence of a target nucleic acid sequence as the signal for the absence of the second target nucleic acid sequence, without signal extraction process **135**. Such a signal indicates that the sample contains the first target nucleic acid sequence but does not contain the second target nucleic acid sequence. In this case, the signal detected at the relatively high detection temperature or at the relatively low detection temperature in the step (a) may be provided as the signal for the first target nucleic acid sequence without signal extraction.

For example, if the signal difference is calculated as the ratio of the signal value at the end cycle in the signal detected at the relatively low detection temperature to the signal value at the corresponding cycle in the signal detected at the relatively high detection temperature, the signal difference of less than the SDT indicates that the sample contains only the first target nucleic acid sequence (A-type of sample).

According to one embodiment of the present invention, the signal detected at the relatively high detection temperature in the step (a) is provided as the signal for the first target nucleic acid sequence.

The signals provided may be used to determine the presence or absence of each target nucleic acid sequence by applying a new additional threshold.

When the first criterion defined by the HTT is satisfied in the step (b), the sample is determined to be either A-type or C-type. For the A-type or C-type sample, signals are generated at both the relatively high detection temperature and the relatively low detection temperature. However, the signal difference between the signal at the relatively high detection temperature and the signal at the relatively low detection temperature is much higher in C-type compared to A-type. Therefore, in this step, it is possible to distinguish A-type and C-type samples by analyzing the

signal differences by the second criterion.

When the signal difference satisfies the second criterion, the next step (c) **140** proceeds.

5 It will be appreciated by those skilled in the art that the steps (b) and (c) may be performed in reverse order.

According to an embodiment, the method may be performed as follows:

(a) incubating the sample with a first signal-generating means capable of generating a signal for a first target nucleic acid sequence and a second signal-
10 generating means capable of generating a signal for a second target nucleic acid sequence in a single reaction vessel, and detecting signals at a relatively high detection temperature and a relatively low detection temperature by a single type of detector; wherein the first signal-generating means generates signals at both the relatively high detection temperature and the relatively low detection temperature in
15 the presence of the first target nucleic acid sequence in the sample and the second signal-generating means generates a signal at the relatively low detection temperature in the presence of the second target nucleic acid sequence in the sample; wherein the two signals to be generated by the two signal-generating means are not differentiated by the single type of detector;

20 (c) identifying whether a signal difference between the signal detected at the relatively low detection temperature and the signal detected at the relatively high detection temperature satisfies a second criterion defined by a signal-difference threshold (SDT); when the signal difference satisfies the second criterion, the next step (b) proceeds;

25 (b) identifying whether the signal detected at the relatively high detection temperature satisfies a first criterion defined by a high-temperature threshold (HTT); when the signal detected at the relatively high detection temperature satisfies the first criterion, the next step (d) proceeds; and

(d) extracting the signal for the second target nucleic acid sequence by using

the signal detected at the relatively high detection temperature in the step (a), the signal detected at the relatively low detection temperature in the step (a) and a reference value for the first target nucleic acid sequence, thereby providing the signal for the second target nucleic acid sequence; wherein the reference value for the first target nucleic acid sequence is a value representing a relationship of change in signals provided by the first signal-generating means at the relatively high detection temperature and the relatively low detection temperature; wherein the reference value for the first target nucleic acid sequence is determined from a control reaction using the first target nucleic acid sequence and the first signal-generating means.

10 According to the embodiment, when the signal difference does not satisfy the second criterion, any signal indicative of the absence of a target nucleic acid sequence is provided as the signal for the absence of the second target nucleic acid sequence without signal extraction process, and the signal detected at the relatively high detection temperature in the step (a) is provided as the signal for the first target
15 nucleic acid sequence without signal extraction.

According to the embodiment, when the signal detected at the relatively high detection temperature does not satisfy the first criterion, the signal detected at the relatively low detection temperature in the step (a) is provided as the signal for the second target nucleic acid sequence, and any signal indicative of the absence of a
20 target nucleic acid sequence or the signal detected at the relatively high detection temperature in the step (a) is provided as the signal for the first target nucleic acid sequence.

According to the embodiment, when the second criterion and the first criterion are all satisfied, the step (d) is performed to extract the signal for the second target
25 nucleic acid sequence.

Step (d): Providing a signal for a second target nucleic acid sequence using a reference value (140) – signal extraction process

When the first criterion in the step (b) and the second criterion in the step (c)

are all satisfied, a signal for the second target nucleic acid sequence is extracted using the signal detected at the relatively high detection temperature in the step (a), the signal detected at the relatively low detection temperature in the step (b) and a reference value for the first target nucleic acid sequence, thereby providing the signal
5 for the second target nucleic acid sequence. In the step (d), the reference value for the first target nucleic acid sequence is a value representing a relationship of change in signals provided by the first signal-generating means at the relatively high detection temperature and the relatively low detection temperature. The reference value for the first target nucleic acid sequence is determined from a control reaction
10 using the first target nucleic acid sequence and the first signal-generating means.

As described above, a process of extracting a signal for a specific target nucleic acid sequence by using signals detected at two different detection temperatures and a reference value is referred herein to as a "signal extraction process". Specifically, the signal extracting process refers to a process of extracting only signal derived from
15 the target nucleic acid sequence of interest by eliminating other signals from the signal detected at a particular detection temperature.

The term "reference value" as used herein describes a relationship or degree of change in signals, a signal change or a signal difference, in particular numerically, when two signals are generated at different detection temperatures (*i.e.*, relatively
20 high detection temperature and relatively low detection temperature). Stated otherwise, the "reference value" includes any value reflecting a pattern (rule) of a signal change at different detection temperatures. Also, the term "reference value" indicates a value representing the degree of change between a signal detected at relatively high detection temperature and a signal detected at relatively low detection
25 temperature for a particular target nucleic acid sequence. The reference value may indicate a value used herein to transform, convert, adjust or modify a signal detected at one temperature into a signal at another temperature. The reference value may vary depending upon the types of the target nucleic acid sequences, the types of the signal-generating means and the conditions of incubation and detection. Thus, a

variety of reference values may be determined for different or same target nucleic acid sequences.

The reference value may be expressed in various aspects. For example, the reference value may be expressed as numerical values, the presence/absence of signal or plot with signal characteristics.

Particularly, the term "reference value" as used herein refers to a reference value which is determined for the first target nucleic acid sequence and which is used in the signal extraction of the second target nucleic acid sequence.

In an embodiment, the reference value may be determined considering a signal value at a selected cycle. In other words, the reference value may be determined considering a signal value at a selected cycle among signals detected at a relatively high detection temperature and at a relatively low detection temperature. In such case, the selected cycle may be one of cycles following a baseline region of an amplification curve, particularly one of cycles in a plateau region, more particularly an end cycle.

In an alternative embodiment, the reference value may be determined considering a plurality of signal values at different selected cycles. For instance, the reference value may be determined considering a mean of a plurality of signal values at different selected cycles.

The reference values will be described in detail below.

The reference value for the first target nucleic acid sequence is a value representing a relationship of change in signals provided by the first signal-generating means at the relatively high detection temperature and the relatively low detection temperature.

According to an embodiment, the reference value for the first target nucleic acid sequence may be determined by incubating a first target nucleic acid sequence with a signal-generating means, detecting signals at a relatively high detection temperature and a relatively low detection temperature, and then obtaining a relationship of change in signals detected at the relatively high detection temperature

and the relatively low detection temperature.

According to an embodiment, the reference value for the first target nucleic acid sequence is determined by using a standard material corresponding to a first target nucleic acid sequence.

5 According to an embodiment, the reference value for the first target nucleic acid sequence is determined from a control reaction. For example, the reference value for the first target nucleic acid sequence is determined from a control reaction using the first target nucleic acid sequence and the first signal-generating means.

10 According to an embodiment, the reference value for the first target nucleic acid sequence may be determined by using a control sample containing the first target nucleic acid sequence.

The reference value for the first target nucleic acid sequence may be determined by acquiring a certain range of values via iterative reactions for the control reaction (or control sample) under various conditions (*e.g.*, concentrations of
15 a target nucleic acid sequence and types of primers) and selecting a suitable one among the acquired values.

In an embodiment, the reference value for the first target nucleic acid sequence may be selected such that the signal for the first target nucleic acid sequence not to be extracted is eliminated. In other embodiment, the reference value
20 for the first target nucleic acid sequence may be selected such that the signal for the second target nucleic acid sequence to be extracted is not eliminated or eliminated as little as possible. In another embodiment, the reference value for the first target nucleic acid sequence may be selected such that the signal for the first target nucleic acid sequence not to be extracted is eliminated and the signal for the second target
25 nucleic acid sequence to be extracted is not eliminated or eliminated as little as possible.

For example, when the target nucleic acid sequences comprise "*Chlamydia trachomatis* (CT)" and "*Neisseria gonorrhoeae* (NG)" and a signal for "NG" is intended to be extracted, the reference value for CT may be selected such that the signal for

CT is completely eliminated and the signal for NG is not eliminated or eliminated as little as possible.

The reference value for the first target nucleic acid sequence may be empirically obtained by repetitive experiments.

5 The reference value for the first target nucleic acid sequence may be selected among a certain range empirically obtained by repetitive experiments. In this regard, it is advantageous that a relatively high value within the range is selected as a reference value, because the relatively high reference value has a higher propensity to eliminate a signal intended not to be extracted, compared to relatively low
10 reference values. For example, when a range from 1.1 to 1.50 is obtained, a value around 1.50 may be more suitable as a reference value. Alternatively, a value exceeding the range may be selected as a reference value. For example, a value of 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100% greater than the upper limit of the range may be selected a reference value. However, it is noted that
15 too high reference value is not desirable, since it may rather eliminate a signal intended to be extracted.

According to an embodiment, the reference value for the first target nucleic acid sequence can be calculated by the difference between signals provided by the first signal-generating means at the relatively high detection temperature and the
20 relatively low detection temperature.

According to an embodiment, the reference value for the first target nucleic acid sequence can be calculated by mathematically processing the signals provided by the first signal-generating means at the relatively high detection temperature and the relatively low detection temperature.

25 In certain embodiment, the mathematical processing includes calculation (*e.g.*, addition, multiplication, subtraction and division) using signals or other values derived from signals.

According to an embodiment of this invention, the mathematical processing of the signals to obtain the reference value for the first target nucleic acid sequence is a

calculation of a ratio of the signal provided by the first signal-generating means at the relatively low detection temperature to the signal provided by the first signal-generating means at the relatively high detection temperature. According to an embodiment of this invention, the mathematical processing of the signals to obtain
5 the reference value for the first target nucleic acid sequence is a calculation of a ratio of the signal provided by the first signal-generating means at the relatively high detection temperature to the signal provided by the first signal-generating means at the relatively low detection temperature.

The ratio may be a ratio of a signal value at a cycle of signals detected at the
10 relatively low detection temperature to a signal value at the cycle of signals detected at the relatively high detection temperature. Alternatively, the ratio may be a ratio of a signal value at a cycle of signals detected at the relatively high detection temperature to a signal value at the cycle of signals detected at the relatively low detection temperature. The cycle selected for ratio calculation may be one of cycles
15 following a baseline region of an amplification curve. Particularly, the cycle for ratio calculation may be one of cycles in a plateau region. More particularly, the cycle for ratio calculation may be the end cycle.

In an embodiment, the reference value for the first target nucleic acid sequence can be obtained by a mean of ratios at several cycles, *e.g.*, consecutive two
20 cycles, three cycles, four cycles, five cycles, etc. Further, the reference value for the first target nucleic acid sequence can be suitably selected in consideration of ratios at several cycles. For example, the reference value for the first target nucleic acid sequence can be selected as being a slightly higher than a ratio of a signal value at a cycle or ratios of signal values at several cycles.

25 In a particular embodiment, the reference value for the first target nucleic acid sequence may be calculated in accordance with the mathematical equation III:

<Equation III>

Reference value for the first target nucleic acid sequence = [signal at the relatively low temperature for a sample containing only the first target nucleic acid

sequence] ÷ [signal at the relatively high temperature for a sample containing only the first target nucleic acid sequence]

The mathematical processing for obtaining the reference value may be carried out in various fashions. The mathematical processing may be carried out by use of a machine. For example, the signals may undergo a mathematical processing by a processor in a detector or real-time PCR device. Alternatively, the signals may manually undergo a mathematical processing particularly according to a determined algorithm.

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

According to an embodiment, the signal for the second target nucleic acid sequence is provided either (i) by using the signal detected at the relatively high detection temperature in the step (a) and the reference value for the first target nucleic acid sequence to eliminate the signal for the first target nucleic acid sequence (*i.e.*, the signal generated by the first signal generating means) at the relatively low detection temperature from the signal detected at the relatively low detection temperature in the step (a), or (ii) by using the signal detected at the relatively low detection temperature in the step (a) and the reference value for the first target nucleic acid sequence to eliminate the signal for the first target nucleic acid sequence (*i.e.*, the signal generated by the first signal generating means) at the relatively high detection temperature from the signal detected at the relatively high detection temperature in the step (a).

More particularly, the elimination of the signal generated by the first signal generating means at the relatively low detection temperature is to mathematically eliminate the signal generated by the first signal generating means at the relatively low detection temperature from the signal detected at the relatively low detection temperature in the step (a), and the elimination of the signal generated by the first signal generating means at the relatively high detection temperature is to

mathematically eliminate the signal generated by the first signal generating means at the relatively high detection temperature from the signal detected at the relatively high detection temperature in the step (a).

Still more particularly, the signal for the second target nucleic acid sequence may be provided by the following mathematical equation I:

<Equation I>

Signal for the second target nucleic acid sequence = [signal at the relatively low detection temperature in the step (a)] - [(signal at the relatively high detection temperature in the step (a)) x (the reference value for the first target nucleic acid sequence)];

wherein the reference value for the first target nucleic acid sequence is a ratio of a signal provided by the first signal-generating means at the relatively low detection temperature to a signal provided by the first signal-generating means at the relatively high detection temperature.

In the mathematical equations described herein, the symbol "-" represents minus, particularly signal subtraction. For example, the signal subtraction may be performed at each cycle by subtracting a signal value at a cycle in one signal from a signal value at a corresponding cycle in another signal.

In the mathematical equations described herein, the symbol "x" represents multiplication.

In the mathematical equations described herein, the symbol "÷" represents division.

According to the extraction of the signal for the second target nucleic acid sequence by the mathematical equation I, the signal detected at the relatively low detection temperature reflects a combination of the signal for the first target nucleic acid sequence and the signal for the second target nucleic acid sequence, whereas the signal detected at the relatively high detection temperature only reflects the signal for the first target nucleic acid sequence. Thus, the signal for the second target nucleic acid sequence may be obtained by subtracting the signal detected at the

relatively high detection temperature from the signal detected at the relatively low detection temperature, with a proviso that the signals for the first target nucleic acid sequence at the relatively high detection temperature and the relatively low detection temperature are not changed. However, considering that the signals vary depending upon the detection temperatures, it is necessary to adjust (transform) the signal detected at the relatively high detection temperature into a signal to be expected at the relatively low detection temperature, prior to the signal subtraction. For this purpose, the reference value for the first target nucleic acid sequence is used.

Still more particularly, the signal for the second target nucleic acid sequence may be provided by the following mathematical equation II:

<Equation II>

Signal for the second target nucleic acid sequence = [signal at the relatively high detection temperature in the step (a)] - [(signal at the relatively low detection temperature in the step (a)) ÷ (the reference value for the first target nucleic acid sequence)];

wherein the reference value for the first target nucleic acid sequence is a ratio of a signal provided by the first signal-generating means at the relatively low detection temperature to a signal provided by the first signal-generating means at the relatively high detection temperature.

Unlike the signal extraction using the mathematical equation I, the signal extraction using the mathematical equation II is intended to convert the signal generated from the first signal-generating means at the relatively low detection temperature in the step (a) into a signal expected to be detected at the relatively high detection temperature and then subtract it from the signal generated from the first signal-generating means at the relatively high detection temperature in the step (a). Although the signal extracted by using the mathematical equation II is slightly lower in its intensity compared to that of the signal extracted by using the mathematical equation I, the former can be used for the qualitative determination of the target nucleic acid sequence in a similar manner to the latter.

In the mathematical equations I and II, the reference value for the first target nucleic acid sequence is represented by the ratio of the signal at the relatively low detection temperature to the signal at the relatively high detection temperature. However, it would be understood by one of skill in the art that the signal extraction
5 for the second target nucleic acid sequence may be accomplished with a modification using a reference value represented by the ratio of the signal at the relatively high detection temperature to the signal at the relatively low detection temperature. Those of skill in the art would understand that such modification falls within the spirit and scope of the invention.

10 Meanwhile, the signal for the second target nucleic acid sequence provided by using the reference value according to the present invention may be used without further analysis for determination of the presence or absence of the second target nucleic acid sequence. In other words, the presence of the second target nucleic acid sequence may be determined without using an additional threshold. Alternatively, the
15 signal for the second target nucleic acid sequence provided by using the reference value according to the present invention may be analyzed for determination of the presence or absence of the second target nucleic acid sequence. In other words, the presence of the second target nucleic acid sequence (or the significance of the second target nucleic acid sequence) may be verified by applying an additional threshold to
20 the signal.

According to one embodiment, the method further comprises the following step (e): (e) identifying whether the signal for the second target nucleic acid sequence provided in the step (d) satisfies a fourth criterion defined by a verification threshold (VT); when the signal for the second target nucleic acid sequence does not satisfy the
25 fourth criterion, any signal indicative of the absence of a target nucleic acid sequence is provided as the signal for the absence of the second target nucleic acid sequence. In this case, the signal detected at the relatively high detection temperature of the step (a) or the signal detected at the relatively low detection temperature of the step (a) may be provided as the signal for the first target nucleic acid sequence.

The "verification threshold (VT)" as used herein refers to a threshold used for verifying the significance of the signal provided by the signal extraction process. The VT is similar to the HTT and the LTT in the sense that it is used to check the significance of the signal. The details of the VT can be referred to the disclosures of the HTT and the LTT. The VT may also be expressed as a fourth threshold.

The VT used to determine the significance of the signal may or may not differ from a threshold used to finally determine the presence of a target nucleic acid sequence.

The expression "fourth criterion defined by the verification threshold (VT)" as used herein means a criterion which is defined in consideration of the VT. For example, if the VT value is RFU 300, the fourth criterion defined by the VT may be that the signal is not less than RFU 300.

If the signal for the second target nucleic acid sequence provided using the reference value satisfies the fourth criterion defined by the VT, it can be again verified that the signal provided using the reference value *per se* is a signal for the second target nucleic acid sequence. If not, the signal provided using the reference value is used to indicate that the second target nucleic acid sequence is absent in a sample, or any signal indicative of the absence of a target nucleic acid sequence is provided as the signal for the absence of the second target nucleic acid sequence instead of the signal provided using the reference value.

According to one embodiment, when the signal for the second target nucleic acid sequence is provided by the mathematical equation I, the fourth criterion defined by the VT is that the signal is not less than the VT. In other words, when the signal for the second target nucleic acid sequence is not less than the VT, the significance of the signal and thus the presence of the second target nucleic acid sequence are determined.

According to one embodiment, when the signal for the second target nucleic acid sequence is provided by the mathematical equation II, the fourth criterion defined by the VT value is that the signal is not more than the verification threshold.

The VT for verifying the signal for the second target nucleic acid sequence provided using the reference value can be set by referring to the disclosure of the above-mentioned HTT. For example, if the signal for the second target nucleic acid sequence is provided by the mathematical equation I, the VT of RFU 300 and the
5 fourth criterion of not less than RFU 300 may be determined by taking into account background signal, sensitivity, characteristics of label or error range.

The provided signals may be used to determine the presence or absence of each target nucleic acid sequence by applying an additional threshold.

10 According to the embodiments of the present invention, a signal for each target nucleic acid sequence can be provided for a sample containing one or more target nucleic acid sequences. The embodiments has technical features: (i) a signal for a target nucleic acid sequence, particularly the second target nucleic acid sequence is provided with "signal extraction process" only when the criteria of the steps (b) and (c)
15 are satisfied, and (ii) any appropriate signal selected from the signal detected in the step (a) and the signal indicative of the absence of a target nucleic acid sequence is provided as the signal for the absence of the second target nucleic acid sequence without "signal extraction process" when any one of the criteria of the steps (b) and (c) is not satisfied.

20

The method of the present invention may be performed with a little modification. Hereinafter, two different embodiments with a little modification will be described in detail. These two different embodiments are shown in Figures 3 and 4, respectively.

The method according to another embodiment **200** will now be described with
25 reference to Fig. 3.

Step (a): Detecting signals at two different temperatures 210

Since this step (a) **210** is the same as the step (a) **110** in the embodiment of the present invention **100**, a detailed description thereof is omitted.

Step (b): Evaluating the signal at the relatively high detection temperature by a high-temperature threshold 220

5 This step (b) **220** will be referred to the step (b) **120** in the embodiment of the present invention **100**.

According to the embodiment of the present invention **100**, when the signal detected at the relatively high detection temperature does not satisfy the first criterion defined by the HTT, the signal detected at the relatively low detection temperature in the step (a) is provided as the signal for the second target nucleic acid
10 sequence **125**.

In contrast, according to the another embodiment of the present invention **200**, when the signal detected at the relatively high detection temperature does not satisfy the first criterion defined by the HTT, the method further comprises the step of identifying whether the signal detected at the relatively low detection temperature
15 satisfies a third criterion defined by a low-temperature threshold (LTT) **222**.

The signal detected at the relatively low detection temperature satisfies the third criterion, the next step **230** proceeds.

Hereinafter, the step **222** will be described.

20 **Step (b'): Evaluating the signal at the relatively low detection temperature by a low-temperature threshold 222**

In the step (b'), the signal detected at the relatively low detection temperature is evaluated in order to determine whether a second target nucleic acid sequence is present in the sample which had been determined to contain no first target nucleic
25 acid sequence **222**.

The description of the LTT for evaluating the signal detected at the relatively low detection temperature can be referred to the disclosure to the HTT as described above.

The expression "low-temperature threshold (LTT)" as used in this step (b')

means a threshold for determining the significance of the signal detected at the relatively low detection temperature. The LTT may be expressed as a third threshold.

As used herein, the expression "third criterion determined by the low-temperature threshold" means a criterion which is defined by considering the LTT for
5 determining the significance of the signal detected at the relatively low detection temperature. For example, if the LTT is RFU 500, the third criterion defined by the LTT may be RFU 500 or higher.

When the signal detected at the relatively low detection temperature satisfies the third criterion defined by the LTT, it indicates that the second target nucleic acid
10 sequence is present in the sample. If it is determined in the prior step (b) that the first target nucleic acid sequence is not present, the signal detected at the relatively low detection temperature will have only the signal provided by the second target nucleic acid sequence. Thus, when the signal detected at the relatively low detection temperature satisfies the third criterion defined by the LTT, it indicates that the
15 second target nucleic acid sequence is likely to be present in the sample.

In other words, when the signal detected at the relatively high detection temperature does not satisfy the first criterion defined by the HTT, but satisfies the third criterion defined by the LTT, the method provides the signal detected at the relatively low detection temperature as the signal for the second target nucleic acid
20 sequence without signal extraction process **226**. This signal provided indicates that the sample does not contain the first target nucleic acid sequence but contains the second target nucleic acid sequence (B-type of sample). In this case, the signal detected at the relatively high detection temperature in the step (a) or any signal indicative of the absence of a target nucleic acid sequence may be provided as the
25 signal for the absence of the first target nucleic acid sequence.

When the signal detected at the relatively low detection temperature in the step (b') does not satisfy the third criterion defined by the LTT, the method provides any signal indicative of the absence of a target nucleic acid sequence as the signal for the absence of the second target nucleic acid sequence **224**.

The signal indicative of the absence of a target nucleic acid sequence may be any one selected from the group consisting of (i) the signal detected at the relatively low detection temperature in the step (a), (ii) a signal obtained from a negative control containing no target nucleic acid sequence, and (iii) any signal in which the target nucleic acid sequence is absent. This signal indicates that the sample does not contain both the first target nucleic acid sequence and the second target nucleic acid sequence. In this case, any signal indicative of the absence of a target nucleic acid sequence may be provided as the signal for the absence of the first target nucleic acid sequence.

According to an embodiment, the signal detected at the relatively high detection temperature in the step (a) is provided as the signal for the first target nucleic acid sequence.

Step (c): Evaluating the signals detected at the relatively low detection temperature and the relatively high detection temperature by a signal-difference threshold 230

Since this step (c) **230** is the same as the step (c) **130** in the embodiment of the present invention **100**, a detailed description thereof is omitted.

Step (d): Providing the signal for the second target nucleic acid sequence using the reference value 240

Since this step (d) **240** is the same as the step (d) **140** in the embodiment of the present invention **100**, a detailed description thereof is omitted.

Next, the method according to still another embodiment **300** will now be described with reference to Fig. 4.

Step (a): Detecting signals at two different temperatures 310

Since this step (a) **310** is the same as the step (a) **110** in the embodiment of

the present invention **100**, a detailed description thereof is omitted.

Step (b''): Evaluating the signal at the relatively low detection temperature by a low-temperature threshold **320**

5 In the step (b''), the signal detected at the relatively low detection temperature is evaluated to determine whether the signal at the relatively low detection temperature is significant **320**.

The another embodiment of the present invention **300** is characterized by further comprising the step of identifying whether the signal detected at the relatively
10 low detection temperature satisfies a third criterion defined by a LTT **320** prior to the step (b), compared with the embodiment of the present invention **100**.

In the step (b''), the description of the LTT for evaluating the signal detected at the relatively low detection temperature is as described above. However, satisfying the third criterion defined by the LTT as used in the step (b') means that the second
15 target nucleic acid sequence is likely to be present in the sample; whereas satisfying the third criterion defined by the LTT as used in the step (b'') means that either or both of the first target nucleic acid sequence and the second target nucleic acid sequence are likely to be present in the sample.

When the signal detected at the relatively low detection temperature does not
20 satisfy the third criterion defined by the LTT, the method provides any signal indicative of the absence of a target nucleic acid sequence as the signal for the absence of the second target nucleic acid sequence **322**. The signal indicative of the absence of a target nucleic acid sequence may be any one selected from the group consisting of (i) the signal detected at the relatively low detection temperature in the
25 step (a), (ii) a signal obtained from a negative control containing no target nucleic acid sequence, and (iii) any signal in which a target nucleic acid sequence is absent. This signal indicates that the sample does not contain both the first target nucleic acid sequence and the second target nucleic acid sequence. In this case, any signal indicative of the absence of a target nucleic acid sequence may be provided as the

signal for the absence of the first target nucleic acid sequence.

Meanwhile, when the signal detected at the relatively low detection temperature satisfy the third criterion defined by the LTT, the next step (b) **330** proceeds.

5 **Step (b): Evaluating the signal at the relatively high detection temperature by a high-temperature threshold 330**

The step (b) **330** is referred to the step (b) **110** in the embodiment of the present invention **100**.

10 According to the embodiment of the present invention **100**, when the signal detected at the relatively high detection temperature does not satisfy the first criterion defined by the HTT, it indicates that the sample contains only the second target nucleic acid sequence or the sample contains no target nucleic acid sequence; whereas according to another embodiment of the present invention **300**, it indicates that the sample contains only the second target nucleic acid sequence.

15

Step (c): Evaluating the signals detected at the relatively low detection temperature and the relatively high detection temperature by a signal-difference threshold 340

20 Since this step (c) **340** is the same as the step (c) **130** in the embodiment of the present invention **100**, a detailed description thereof is omitted.

Step (d): Providing the signal for the second target nucleic acid sequence using a reference value 350

25 Since this step (d) **350** is the same as the step (c) **140** in the embodiment of the present invention **100**, a detailed description thereof is omitted.

Although the invention has been described in terms of three embodiments providing a signal for each target nucleic acid sequence for a sample containing up to two target nucleic acid sequences, it will be appreciated that those skilled in the art

will be able to devise various modifications, which are within the scope of the present invention.

II. Storage medium, Computer program and Device for Signal Extraction

5 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 providing a signal for at least one target nucleic acid sequence in a sample from signals detected at different temperatures, comprising the steps of:

10 (a) incubating the sample with a first signal-generating means capable of generating a signal for a first target nucleic acid sequence and a second signal-generating means capable of generating a signal for a second target nucleic acid sequence in a single reaction vessel, and receiving signals detected at a relatively high detection temperature and a relatively low detection temperature by a single type of detector; wherein the first signal-generating means generates signals at both
15 the relatively high detection temperature and the relatively low detection temperature in the presence of the first target nucleic acid sequence in the sample and the second signal-generating means generates a signal at the relatively low detection temperature in the presence of the second target nucleic acid sequence in the sample; wherein the two signals to be generated by the two signal-generating means are not
20 differentiated by the single type of detector;

(b) identifying whether the signal detected at the relatively high detection temperature satisfies a first criterion defined by a high-temperature threshold (HTT); when the signal detected at the relatively high detection temperature satisfies the first criterion, the next step (c) proceeds;

25 (c) identifying whether a signal difference between the signal detected at the relatively low detection temperature and the signal detected at the relatively high detection temperature satisfies a second criterion defined by a signal-difference threshold (SDT); when the signal difference satisfies the second criterion, the next step (d) proceeds; and

(d) extracting the signal for the second target nucleic acid sequence by using the signal detected at the relatively high detection temperature in the step (a), the signal detected at the relatively low detection temperature in the step (a) and a reference value for the first target nucleic acid sequence, thereby providing the signal for the second target nucleic acid sequence; wherein the reference value for the first target nucleic acid sequence is a value representing a relationship of change in signals provided by the first signal-generating means at the relatively high detection temperature and the relatively low detection temperature; wherein the reference value for the first target nucleic acid sequence is determined from a control reaction using the first target nucleic acid sequence and the first signal-generating means; wherein the steps (b) and (c) may be performed in reverse order.

In still another aspect of this invention, there is provided a computer program to be stored on a computer readable storage medium to configure a processor to perform a method for providing a signal for at least one target nucleic acid sequence in a sample from signals detected at different temperatures, comprising the steps of:

(a) incubating the sample with a first signal-generating means capable of generating a signal for a first target nucleic acid sequence and a second signal-generating means capable of generating a signal for a second target nucleic acid sequence in a single reaction vessel, and receiving signals detected at a relatively high detection temperature and a relatively low detection temperature by a single type of detector; wherein the first signal-generating means generates signals at both the relatively high detection temperature and the relatively low detection temperature in the presence of the first target nucleic acid sequence in the sample and the second signal-generating means generates a signal at the relatively low detection temperature in the presence of the second target nucleic acid sequence in the sample; wherein the two signals to be generated by the two signal-generating means are not differentiated by the single type of detector;

(b) identifying whether the signal detected at the relatively high detection temperature satisfies a first criterion defined by a high-temperature threshold (HTT);

when the signal detected at the relatively high detection temperature satisfies the first criterion, the next step (c) proceeds;

(c) identifying whether a signal difference between the signal detected at the relatively low detection temperature and the signal detected at the relatively high detection temperature satisfies a second criterion defined by a signal-difference threshold (SDT); when the signal difference satisfies the second criterion, the next step (d) proceeds; and

(d) extracting the signal for the second target nucleic acid sequence by using the signal detected at the relatively high detection temperature in the step (a), the signal detected at the relatively low detection temperature in the step (a) and a reference value for the first target nucleic acid sequence, thereby providing the signal for the second target nucleic acid sequence; wherein the reference value for the first target nucleic acid sequence is a value representing a relationship of change in signals provided by the first signal-generating means at the relatively high detection temperature and the relatively low detection temperature; wherein the reference value for the first target nucleic acid sequence is determined from a control reaction using the first target nucleic acid sequence and the first signal-generating means; wherein the steps (b) and (c) may be performed in reverse order.

Since the storage medium, the device and the computer program of the present invention are intended to perform the present methods in a computer, the common descriptions between them are omitted in order to avoid undue redundancy leading to the complexity of this specification.

The program instructions are operative, when performed by the processor, to cause the processor to perform the present method described above. The program instructions for performing the present method may comprise: (i) an instruction to identify whether the signal detected at the relatively high detection temperature satisfies the first criterion defined by the HTT; (ii) an instruction to identify whether the signal difference between the signal detected at the relatively low detection temperature and the signal detected at the relatively high detection temperature

satisfies the second criterion defined by the SDT; and (iii) an instruction to provide a signal for the second target nucleic acid sequence by using the signal detected at the relatively high detection temperature in the step (a), the signal detected at the relatively low detection temperature in the step (a) and the reference value for the first target nucleic acid sequence.

The present method described above is implemented in a processor, such as a processor in a stand-alone computer, a network attached computer or a data acquisition device such as a real-time PCR machine.

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, portable HDD, USB, magnetic tape, MINIDISC, nonvolatile memory card, EEPROM, optical disk, optical storage medium, RAM, ROM, system memory and web server.

The signals from the signal-generating process may be received through several mechanisms. For example, the signals may be acquired by a processor resident in a PCR data acquiring device. The signals may be provided to the processor in real time as the signals are being collected, or it may be stored in a memory unit or buffer and provided to the processor after the experiment has been completed. Similarly, the signals may be provided to a separate system such as a desktop computer system via a network connection (*e.g.*, LAN, VPN, intranet and Internet) or direct connection (*e.g.*, USB or other direct wired or wireless connection) to the acquiring device, or provided on a portable medium such as a CD, DVD, floppy disk, portable HDD or the like to a stand-alone computer system. Similarly, the signals may be provided to a server system via a network connection (*e.g.*, LAN, VPN, intranet, Internet and wireless communication network) to a client such as a notebook or a desktop computer system.

The instructions to configure the processor to perform the present invention may be included in a logic system. The instructions may be downloaded and stored in a memory module (*e.g.*, hard drive or other memory such as a local or attached RAM or ROM), although the instructions can be provided on any software storage medium

such as a portable HDD, USB, floppy disk, CD and DVD. A computer code for implementing the present invention may be implemented in a variety of coding languages such as C, C++, Java, Visual Basic, VBScript, JavaScript, Perl and XML. In addition, a variety of languages and protocols may be used in external and internal
5 storage and transmission of data and commands according to the present invention.

In a further aspect of this invention, there is provided a device for providing a signal for at least one target nucleic acid sequence in a sample from signals detected at different temperatures, comprising (a) a computer processor and (b) the computer
10 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 detector to detect signals at cycles.

15 The computer processor may be prepared in such a manner that a single processor can do several performances. Alternatively, the processor unit may be prepared in such a manner that several processors do the several performances, respectively.

According to an embodiment, the processor may be embodied by installing
20 software into conventional devices for detection of target nucleic acid sequences (*e.g.* real-time PCR device).

For example, the present device may be embodied into a real-time PCR system. The system comprises a real-time PCR device for performing a real-time PCR amplification, and a computer system as a logic system connected to the real-time
25 PCR device via a cable for providing and displaying signals. The computer system may display the resultants in various forms such as graphs, tables and words according to demands of users. The computer system may comprise instructions contained in a computer readable storage medium for performing the present method. The real-time PCR device and the computer system may be integrated into a system.

The signals may be received with amplification curves in various fashions. For example, the signals may be received and collected by a processor in a data collector of the real-time PCR device. Upon collecting the signals, they may be provided to a processor in a real-time manner, or stored in a memory unit or buffer and then
5 provided to a processor after experiments.

Likely, the signals may be provided from the real-time PCR device to the computer system such as a desktop computer system via network connection (*e.g.*, LAN, VPN, intranet and internet) or direct connection (*e.g.*, USB and wired or wireless direct connections), or via portable media such as CD, DVD, floppy disk and portable
10 HDD. Alternatively, the signals may be provided to a server system via network connections (*e.g.*, LAN, VPN, intranet, internet and wireless communication network) connected to a client such as notebook and desktop computer systems.

After the signals are received or obtained, a signal processor proceeds to evaluate the signals based on the determined HTT (optionally, the LTT and/or the VT)
15 and SDT, and to provide a signal for a desired target nucleic acid sequence by using a determined reference value.

The HTT (optionally, the LTT and/or the VT), the SDT and the reference value may be determined by the product supplier or the user. Alternatively, such values may be determined by the apparatus of the present invention.

20 The process of the present invention may be undertaken by an application (*i.e.*, program) installed into the computer system. Alternatively, it may be made by an application directly installed into the computer system through application store server or application provider servers in which the application is operable in an operating system of the computer system. The operating system includes Window, Macintosh
25 and mobile operating systems such as iOS and Android that are installed into mobile terminals such as Smartphones and Tablet PC.

As described above, the present method may be embodied by an application (*i.e.*, program) supplier-installed or user-direct installed into the computer system, and recorded in a computer readable storage medium.

A computer program embodying the present method may implement all functions. The computer program may be a program comprising program instructions stored on a computer readable storage medium to configure a processor to perform the present method.

5 The computer program may be coded by using suitable computer languages such as C, C++, JAVA, Visual basic, VBScript, JavaScript, Perl, XML and machine languages. The program codes may include function codes for mathematical functions described above and control codes for implementing process in order by a processor of the computer system.

10 The codes may further comprise memory reference codes by which additional information or media required in implementing the above-described functions by the processor is referred at location (address) of internal or external memory of the computer system.

15 When the computer system requires communication with another computer or server in remote for implementing functions of the processor, the codes may further comprise communication-relating codes encoding how the processor is communicated with another computer or server in remote by using communication module (*e.g.*, wired and/or wireless communication module) or what information or media is transmitted.

20 Functional programs and codes (code segments) for embodying the present invention may be easily inferred or modified by programmers in the art in considering system environments of computers reading storage media and executing programs.

25 The storage medium network-connected to the computer system may be distributed and computer-readable codes may be stored and executed in a distribution manner. In such case, at least one computer among a plurality of distributed computers may implement a portion of the functions and transmit results of the implementation to at least one computer that may also implement a portion of the functions and transmit results of the implementation to at least one computer.

The storage medium in which application (*i.e.*, program) is recorded for

executing the present invention includes a storage medium (*e.g.*, hard disk) contained in application store servers or application provider servers, application provider servers *per se*, another computer having the program and its storage medium.

The computer system capable of reading the storage medium may include
5 general PC such as desk top or notebook computers, mobile terminals such as Smartphone, Tablet PC, PDA (Personal Digital Assistants) and mobile communication terminals as well as all computing-executable devices.

III. Providing a Signal for a Target Nucleic Acid Sequence in a Sample 10 Containing Three or More Target Nucleic Acid Sequences

The method for providing a signal for a target nucleic acid sequence described above can be applied in the similar manner to a sample containing three or more target nucleic acid sequences.

15 A method for providing a signal for each target nucleic acid sequence from a sample containing three or more target nucleic acid sequences is characterized in that the step (a) further comprises incubating the sample with at least one additional signal-generating means capable of generating a signal for at least one additional target nucleic acid sequence in a single reaction vessel, and detecting signals at at
20 least one additional detection temperature by a single type of detector; wherein the at least one additional detection temperature is lower than the relatively high detection temperature and the relatively low detection temperature.

In addition, a method for providing a signal for each target nucleic acid sequence from a sample containing three or more target nucleic acid sequences is
25 characterized by comprising the method for providing a signal for each target nucleic acid sequence from a sample containing one or two target nucleic acid sequences described above. In other words, even if three or more detection temperatures are used, the signal provision using signals detected at at least two temperatures, *i.e.*, the highest detection temperature and the second highest detection temperature, is

performed according to the method described above.

Hereinafter, one embodiment for providing a signal for each target nucleic acid sequence from a sample containing up to three target nucleic acid sequences is illustrated.

5 The signals detected at three different temperatures for typical samples containing up to three target nucleic acid sequences are shown in Figs. 5A, 5B and 5C.

As shown in Figs. 5A, 5B and 5C, samples containing up to three target nucleic acid sequences can be divided into a total of eight (8) types: (i) A-type: a sample containing only a first target nucleic acid sequence; (ii) B-type: a sample containing only a second target nucleic acid sequence; (iii) C-type: a sample containing only a third target nucleic acid sequence; (iv) D-type: a sample containing both a first target nucleic acid sequence and a second target nucleic acid sequence; (v) E-type: a sample containing both a first target nucleic acid sequence and a third target nucleic acid sequence; (vi) F-type: a sample containing both a second target nucleic acid sequence and a third target nucleic acid sequence; (vii) G-type: a sample containing all of a first target nucleic acid sequence, a second target nucleic acid sequence and a third target nucleic acid sequence; and (viii) H-type: a sample containing no target nucleic acid sequence.

20 The procedures for providing signals for a first target nucleic acid sequence, a second target nucleic acid sequence and a third target nucleic acid sequence for the eight samples are shown in Figs. 5C and 5D.

First, an unknown sample is incubated with a first signal-generating means capable of generating a signal for a first target nucleic acid sequence, a second signal-generating means capable of generating a signal for a second target nucleic acid sequence, and a third signal-generating means capable of generating a signal for a third target nucleic acid sequence in a single reaction vessel, and signals are detected at a relatively high detection temperature (RHT), a relatively middle detection temperature (RMT) and a relatively low detection temperature (RLT) by a

single type of detector, respectively **410**. The first signal-generating means generates signals at the relatively high detection temperature, the relatively middle detection temperature and the relatively low detection temperature, the second signal-generating means generates signals at the relatively middle detection temperature and the relatively low detection temperature, and the third signal-generating means generates a signal at the relatively low detection temperature. The signals generated by the three signal-generating means are not differentiated by the single type of detector. The relatively high detection temperature used in this embodiment may be expressed as a first detection temperature, the relatively middle detection temperature as a second detection temperature, and the relatively low detection temperature as a third detection temperature. Likewise, when this embodiment is applied to the analysis of signals detected at n detection temperatures (n is an integer of 1 or more) from samples containing up to n target nucleic acid sequences (n is an integer of 1 or more), each of the detection temperatures used may be referred to a first detection temperature, a second detection temperature, a third detection temperature,... a n^{th} detection temperature, starting from the highest temperature.

Afterwards, it is identified whether the signal detected at the relatively high detection temperature is not less than a high-temperature threshold (HTT) **420**. When the signal detected at the relatively high detection temperature is not less than the HTT, the step **430** proceeds; while when the signal detected at the relatively high detection temperature is less than the HTT, the step **450** proceeds. The step **420** corresponds to the step **120** in the embodiment of the present invention **100** of identifying whether the first criterion defined by the HTT is satisfied. The HTT herein is a threshold for determining the significance of the signal detected at the relatively high detection temperature. The HTT in this step is referred to the description of the HTT in the embodiment of the present invention **100**. When the signal detected at the relatively high detection temperature is not less than the HTT, it can be determined that the first target nucleic acid sequence is present in a sample; while when the signal detected at the relatively high detection temperature is less than the

HTT, it can be determined that the first target nucleic acid sequence is absent in a sample.

When the signal detected at the relatively high detection temperature is not less than the HTT, it is then performed to identify whether $\text{End-Ratio}_{M/H}$ is not less than a $\text{RT}_{M/H}$ **430**. When the $\text{End-Ratio}_{M/H}$ is not less than the $\text{RT}_{M/H}$, the step **440** proceeds; while when the $\text{End-Ratio}_{M/H}$ is less than the $\text{RT}_{M/H}$, the step **432** proceeds. The $\text{End-Ratio}_{M/H}$ represents a difference, particularly a ratio, between the signal detected at the relatively middle detection temperature and the signal detected at the relatively high detection temperature, which corresponds to one example of the signal difference described above. Since the $\text{End-Ratio}_{M/H}$ is used in a similar manner to the signal difference as described above, its detailed description will be referred to the explanation of the signal difference described above. Although the $\text{End-Ratio}_{M/H}$ is described as an example of the signal difference in step **430** of this embodiment **400**, it will be appreciated by those skilled in the art that various other signal differences may be used as well by reference to the embodiment of the present invention **100**. For example, the signal difference may be calculated by mathematically processing the signal detected at the relatively middle detection temperature and the signal detected at the relatively high detection temperature. More specifically, the mathematical processing may be a subtraction or a ratio of the signal detected at the relatively middle detection temperature and the signal detected at the relatively high detection temperature. Still more specifically, the signal difference may be an $\text{End-Ratio}_{M/H}$, and still more specifically, the $\text{End-Ratio}_{M/H}$ is a ratio of the signal value at an end cycle in the signal detected at the middle detection temperature to the signal value at the end cycle in the signal detected at the relatively high detection temperature. The $\text{RT}_{M/H}$ is a threshold for distinguishing a sample containing a second target nucleic acid sequence from a sample containing no second target nucleic acid sequence, which corresponds to one example of the aforementioned signal-difference threshold (SDT). Since the $\text{RT}_{M/H}$ is used in a similar manner to the above-described SDT, the $\text{RT}_{M/H}$ will be referred to the above description of the SDT. With respect to

this embodiment **400**, while the $RT_{M/H}$ is mentioned as an example of the SDT in step **430**, it will be appreciated by those skilled in the art that various other signal-difference thresholds may be used as well by reference to the embodiment of the present invention **100**. The SDT may be any value between a difference in signals at the relatively middle detection temperature and the relatively high detection temperature for a control sample containing only a first target nucleic acid sequence and a difference in signals at the relatively middle detection temperature and the relatively high detection temperature for a control sample containing both a first target nucleic acid sequence and a second target nucleic acid sequence. The difference in signals at the relatively middle detection temperature and the relatively high detection temperature for a control sample containing only a first target nucleic acid sequence and a difference in signals at the relatively middle detection temperature and the relatively high detection temperature for a control sample containing both a first target nucleic acid sequence and a second target nucleic acid sequence can be obtained in the same manner as the signal difference described above. Specifically, the SDT may be $RT_{M/H}$, and the $RT_{M/H}$ is any value between the ratio of the signal at the relatively middle detection temperature to the signal at the relatively high detection temperature for a control sample containing only the first target nucleic acid sequence and the ratio of the signal at the relatively middle detection temperature to the signal at the relatively high detection temperature for a control sample containing both the target nucleic acid sequence and the second target nucleic acid sequence.

When the $End-Ratio_{M/H}$ is not less than the $RT_{M/H}$, it can be determined that the second target nucleic acid sequence is present in a sample; while when the $End-Ratio_{M/H}$ is less than the $RT_{M/H}$, it can be determined that the second target nucleic acid sequence is absent in a sample.

When the $End-Ratio_{M/H}$ is not less than the $RT_{M/H}$, it is identified whether $End-Ratio_{L/M}$ is not less than a $RT_{L/M}$ **440**. The $End-Ratio_{L/M}$ represents a difference, particularly a ratio, between the signal detected at the relatively low detection

temperature and the signal detected at the relatively middle detection temperature, which corresponds to one example of the signal difference described above. Since the $\text{End-Ratio}_{L/M}$ is used in a similar manner to the above-described signal difference, the detailed description thereof will be referred to the description of the signal difference as described above. Although the $\text{End-Ratio}_{L/M}$ is described as an example of the signal difference in the step **440** of this embodiment **400**, it will be appreciated by those skilled in the art that various other signal differences may be used as well by reference to the embodiment of the present invention **100**. Further, the $\text{RT}_{L/M}$ is a threshold for distinguishing a sample containing a third target nucleic acid sequence from a sample containing no third target nucleic acid sequence, which corresponds to one example of the aforementioned signal-difference threshold (SDT). Since the $\text{RT}_{L/M}$ is used in a similar manner to the above-described SDT, a detailed description thereof will be referred to the description of the SDT value. Although the $\text{RT}_{L/M}$ is mentioned as an example of the SDT in step **440** of this embodiment **400**, it will be appreciated by those skilled in the art that various other signal-difference thresholds may be used as well by reference to the embodiment of the present invention **100**. Specifically, the SDT may be $\text{RT}_{L/M}$, and the $\text{RT}_{L/M}$ may be any value between the ratio of the signal at the relatively low detection temperature to the signal at the relatively middle detection temperature for a control sample containing both a first target nucleic acid sequence and a second target nucleic acid sequence and the ratio of the signal at the relatively low detection temperature to the signal at the relatively middle detection temperature for a control sample containing a first target nucleic acid sequence, a second target nucleic acid sequence and a third target nucleic acid sequence. When the $\text{End-Ratio}_{L/M}$ is not less than the $\text{RT}_{L/M}$, it can be determined that the third target nucleic acid sequence is present in a sample **442** (G-type of sample); while when the $\text{End-Ratio}_{L/M}$ is less than the $\text{RT}_{L/M}$, it can be determined that the third target nucleic acid sequence is absent in a sample **444** (D-type of sample).

When the steps **420**, **430** and **440** are all satisfied, the signal detected at the relatively high detection temperature is provided without signal extraction as the

signal for the first target nucleic acid sequence, and a signal for the second target nucleic acid sequence is provided by signal extraction process of eliminating the signal for the first target nucleic acid sequence from the signal detected at the relatively middle detection temperature **442**. In this case, the signal for the second target nucleic acid sequence may be extracted by using a reference value calculated from the signal detected at the relatively high detection temperature and the signal detected at the relatively middle detection temperature for a control sample containing only the first target nucleic acid sequence. Meanwhile, a signal for the third target nucleic acid sequence may be provided by signal extraction process of eliminating the signals for the first target nucleic acid sequence and the second target nucleic acid sequence from the signal detected at the relatively low detection temperature **442**. In this case, the signal for the third target nucleic acid sequence may be extracted by using a reference value calculated from the signal detected at the relatively middle detection temperature and the signal detected at the relatively low detection temperature for a control sample containing only the first target nucleic acid sequence and the second target nucleic acid sequence.

When the steps **420** and **430** are satisfied but the step **440** is not satisfied, the signal detected at the relatively high detection temperature is provided without signal extraction as the signal for the first target nucleic acid sequence, a signal for the second target nucleic acid sequence is provided by signal extraction process of eliminating the signal for the first target nucleic acid sequence from the signal detected at the relatively middle detection temperature, and any signal indicative of the absence of a target nucleic acid sequence is provided without signal extraction as the signal for the absence of the third target nucleic acid sequence **444**.

Further, when the step **420** is satisfied but the step **430** is not satisfied, it is identified whether the $\text{End-Ratio}_{L/M}$ is not less than $\text{RT}_{L/M}$, in order to determine whether the third target nucleic acid sequence is present in a sample **432**. The $\text{End-Ratio}_{L/M}$ represents a difference, particularly a ratio, between the signal detected at the relatively low detection temperature and the signal detected at the relatively

middle detection temperature, which corresponds to one example of the signal difference described above. Since the $\text{End-Ratio}_{L/M}$ is used in a similar manner to the above-described signal difference, the detailed description thereof will be referred to the description of the signal difference as described above. Although the $\text{End-Ratio}_{L/M}$ is described as an example of the signal difference in step **432** of this embodiment **400**, it will be appreciated by those skilled in the art that various other signal differences may be used as well by reference to the embodiment of the present invention **100**. Further, the $\text{RT}_{L/M}$ is a threshold for distinguishing a sample containing a third target nucleic acid sequence from a sample containing no third target nucleic acid sequence, which corresponds to one example of the aforementioned signal-difference threshold (SDT). Since the $\text{RT}_{L/M}$ is used in a similar manner to the above-described SDT, a detailed description thereof will be referred to the description of the SDT value. Although the $\text{RT}_{L/M}$ is described as an example of the SDT in step **432** of this embodiment **400**, it will be appreciated by those skilled in the art that various other signal-difference thresholds may be used as well by reference to the embodiment of the present invention **100**. Specifically, the SDT may be $\text{RT}_{L/M}$, and the $\text{RT}_{L/M}$ may be any value between the ratio of the signal at a relatively low detection temperature to the signal at the relatively middle detection temperature for a control sample containing only a first target nucleic acid sequence and the ratio of the signal at the relatively low detection temperature to the signal at the relatively middle detection temperature for a control sample containing a first target nucleic acid sequence and a third target nucleic acid sequence. In an embodiment of the present invention, when the second target nucleic acid sequence is determined to be absent in a sample, $\text{End-Ratio}_{L/H}$ is used instead of $\text{End-Ratio}_{L/M}$, and $\text{RT}_{L/H}$ is used instead of $\text{RT}_{L/M}$. In this case, $\text{RT}_{L/H}$ may be any value between the ratio of the signal at a relatively low detection temperature to the signal at the relatively high detection temperature for a control sample containing only a first target nucleic acid sequence and the ratio of the signal at the relatively low detection temperature to the signal at the relatively high detection temperature for a control sample containing a first target

nucleic acid sequence and a third target nucleic acid sequence. The $RT_{L/M}$ used in the step **432** may or may not be identical to the $RT_{L/M}$ used in the step **440**.

When the $End-Ratio_{L/M}$ is not less than the $RT_{L/M}$, it can be determined that the third target nucleic acid sequence is present in a sample; whereas when the $End-Ratio_{L/M}$ is less than the $RT_{L/M}$, it can be determined that the third target nucleic acid sequence is absent in a sample.

When the step **420** is satisfied, the step **430** is not satisfied, and the step **432** is satisfied, the signal detected at the relatively high detection temperature or the signal detected at the relatively middle detection temperature is provided without signal extraction as the signal for the first target nucleic acid sequence, any signal indicative of the absence of a target nucleic acid sequence is provided without signal extraction as the signal for the absence of the second target nucleic acid sequence, and a signal for the third target nucleic acid sequence is provided by signal extraction process of eliminating the signal for the first target nucleic acid sequence from the signal detected at the relatively low detection temperature **434** (E-type of sample). In this case, the signal for the third target nucleic acid sequence can be extracted using a reference value calculated by the signal detected at the middle detection temperature and the signal detected at the low detection temperature for a control sample containing only a first target nucleic acid sequence.

When the step **420** is satisfied, the step **430** is not satisfied, and the step **432** is not satisfied, the signal detected at the relatively high detection temperature, the signal detected at the relatively middle detection temperature, or the signal detected at the relatively low detection temperature is provided without signal extraction as the signal for the first target nucleic acid sequence, and any signal indicative of the absence of target nucleic acid sequence is provided as the signal for the second target nucleic acid sequence or a signal for the absence of the third target nucleic acid sequence **436** (A-type of sample).

Meanwhile, when the step **420** is not satisfied, it is performed to identify whether the signal detected at the relatively middle detection temperature is not less than a middle-temperature threshold (MTT) in order to determine whether the second target nucleic acid sequence is present in a sample **450**. When the signal detected at the relatively middle detection temperature is not less than the MTT, the next step **460** proceeds; while when the signal detected at the relatively middle detection temperature is less than the MTT, the step **470** proceeds. This step **450** corresponds to step **120** of identifying whether the first criterion defined by the HTT is satisfied according to the embodiment of the present invention **100**. The MTT is a threshold for determining the significance of the signal detected at the relatively middle detection temperature, which corresponds to one example of the above-described HTT. Since the MTT is used in a same manner to the above-mentioned HTT, a detailed description thereof will be referred to the description of the HTT. When the signal detected at the relatively middle detection temperature is not less than the MTT, it can be determined that the second target nucleic acid sequence is present in a sample; while when the signal detected at the relatively middle detection temperature has signal values less than the MTT, it can be determined that the second target nucleic acid sequence is absent in a sample.

When step **420** is not satisfied but step **450** is satisfied, it is performed to identify whether the $\text{End-Ratio}_{L/M}$ is not less than the $\text{RT}_{L/M}$ in order to determine whether the third target nucleic acid sequence is present in a sample **460**. The $\text{End-Ratio}_{L/M}$ represents a difference, specifically a ratio, between the signal detected at the relatively low detection temperature and the signal detected at the relatively middle temperature detection temperature, and corresponds to one example of the signal difference described above. Since the $\text{End-Ratio}_{L/M}$ is used for a similar purpose to the above-described signal difference, the description of the above-described signal difference is specifically described. Although the $\text{End-Ratio}_{L/M}$ is described as an example of the signal difference in step **460** of this embodiment **400**, it will be appreciated by those skilled in the art that various other signal differences may be

used as well by reference to the embodiment of the present invention **100**. Further, the $RT_{L/M}$ is a threshold for distinguishing a sample containing a third target nucleic acid sequence from a sample containing no third target nucleic acid sequence, which corresponds to one example of the aforementioned SDT. Since the $RT_{L/M}$ is used in a similar manner to the above-described SDT, a detailed description thereof will be referred to the description of the SDT. Although the $RT_{L/M}$ is described as an example of the SDT in step **460** of this embodiment **400**, it will be appreciated by those skilled in the art the various signal-difference thresholds may be used as well by reference to the embodiment of the present invention **100**. Specifically, the SDT may be $RT_{L/M}$, and the $RT_{L/M}$ is any value between the ratio of the signal at the relatively low detection temperature to the signal at the relatively middle detection temperature for the control sample containing only the second target nucleic acid sequence and the ratio of the signal at the relatively low detection temperature to the signal at the relatively middle detection temperature for the control sample containing the second target nucleic acid sequence and the third target nucleic acid sequence to the signal at the relatively middle detection temperature. According to an embodiment of the present invention, the $RT_{L/M}$ used in the step **432**, the step **440** or the step **460** is a value determined by the signal values from the controls in consideration of the combination of the target nucleic acid sequences present in the sample. According to another embodiment of the present invention, some or all of the $RT_{L/M}$ used in the step **432**, the step **440** or the step **460** is a same value. The selection of the value can be made in consideration of the error ratio of the result interpretation process and the values determined from the controls containing only a combinations of the target nucleic acid sequences present in the sample. The $RT_{L/M}$ used in the step **460** may be the same as or different from the $RT_{L/M}$ used in the step **440** or **432**.

When the $End-Ratio_{L/M}$ is not less than the $RT_{L/M}$, it can be determined that the third target nucleic acid sequence is present in a sample; while when the $End-Ratio_{L/M}$ is less than the $RT_{L/M}$, it can be determined that the third target nucleic acid sequence is absent in a sample.

When the step **420** is not satisfied, the step **450** is satisfied and the step **460** is satisfied, any signal indicative of the absence of a target nucleic acid sequence (or the signal detected at the relatively high detection temperature) is provided, without signal extraction, as the signal for the absence of the first target nucleic acid sequence; the signal detected at the relatively middle detection temperature is provided, without signal extraction, as the signal for the second target nucleic acid sequence; and the signal for the third target nucleic acid sequence is provided by signal extraction process of eliminating the second target nucleic acid sequence from the signal detected at the relatively low detection temperature **462** (F-type of sample). In this case, the signal for the third target nucleic acid sequence can be extracted using a reference value (RV) for the second target nucleic acid sequence which is determined from signals at the relatively middle detection temperature and the relatively low detection temperature.

When the step **420** is not satisfied, the step **450** is satisfied and the step **460** is not satisfied, any signal indicative of the absence of a target nucleic acid sequence (or the signal detected at the relatively high detection temperature) is provided, without signal extraction, as the signal for the absence of the first target nucleic acid sequence; the signal detected at the relatively middle detection temperature is provided, without signal extraction, as the signal for the second target nucleic acid sequence; and any signal indicative of the absence of a target nucleic acid sequence may be provided, without signal extraction, as the signal for the absence of the third target nucleic acid sequence (B-type of sample) **464**.

Meanwhile, when the step **420** is not satisfied and the step **450** is not satisfied, it is identified whether the signal detected at the relatively low detection temperature is not less than a low-temperature threshold (LTT) in order to determine whether the third target nucleic acid sequence is present in a sample **470**. This step **470** corresponds to the step **120** of identifying whether the first criterion defined by the HTT is satisfied according to the embodiment of the present invention **100**. The

LTT is a threshold for determining the significance of the signal detected at the relatively low detection temperature. Since the LTT is used in a same manner as the above-mentioned LTT, a detailed description will be referred to the description of the LTT. When the signal detected at the relatively high detection temperature is not less than the LTT, it can be determined that the third target nucleic acid sequence is present in a sample; while when the signal detected at the relatively high detection temperature has signal values less than the LTT, it can be determined that the third target nucleic acid sequence is absent in a sample.

When the step **420** is not satisfied, the step **450** is not satisfied and the step **470** is satisfied, any signal indicative of the absence of a target nucleic acid sequence (or the signal detected at the relatively high detection temperature) is provided, without signal extraction, as the signal for the absence of the first target nucleic acid sequence; any signal indicative of the absence of a target nucleic acid sequence (or the signal detected at the relatively middle detection temperature) is provided, without signal extraction, as the signal for the absence of the second target nucleic acid sequence; and the signal detected at the relatively low detection temperature is provided, without signal extraction, as the signal for the third target nucleic acid sequence (C-type of sample) **472**.

When the step **420** is not satisfied, the step **450** is not satisfied and the step **470** is not satisfied, any signal indicative of the absence of a target nucleic acid sequence (or the signal detected at the relatively high detection temperature, the signal detected at the relatively high detection temperature, and the signal detected at the relatively low detection temperature) is provided, without signal extraction as the signal for the absence of the first target nucleic acid sequence, the signal for the absence of the second target nucleic acid sequence, or the signal for the absence of the third target nucleic acid sequence, respectively (H-type of sample) **474**.

As described above, each signal for three or more target nucleic acid sequences can be accurately provided by applying the method of the present

invention. The method of the present invention employs a signal extraction process only when particular criteria are satisfied, it can remarkably reduce the false positive results compared with the conventional method in which all samples are subjected to the signal extraction process.

5 The method described above is merely an example for providing a signal for at least one target nucleic acid sequence for a sample containing up to three target nucleic acid sequences. It should be understood by those skilled in the art that various modifications in sequence thereof are within the scope of the present invention.

10 Meanwhile, practicing the method for providing a signal for at least one target nucleic acid sequence for a sample containing up to three target nucleic acid sequences may comprise practicing the method for providing a signal for at least one target nucleic acid sequence for a sample containing up to two target nucleic acid sequences.

15 For example, where signals are detected at a first detection temperature, a second detection temperature and a third detection temperature (wherein the detection temperatures are high in the order of the first detection temperature, the second detection temperature, the third detection temperature) for a sample containing up to three target nucleic acid sequences, the process of processing the
20 signals detected at the first detection temperature and the second detection temperature may be carried out by using a process of the signals detected at the first detection temperature and the second detection temperature.

 Likewise, the process of processing the signals detected at a first detection temperature, a second detection temperature and a third detection temperature for a
25 sample containing up to four target nucleic acid sequences may be carried out by using a process of processing the signals detected at the first detection temperature, the second detection temperature and the third detection temperature. The above method can be applied in a similar manner even when the number of the target nucleic acid sequences that may be present is increased.

In the method of the present invention, the n signal generating means (n is an integer) used for detecting n target nucleic acid sequences (n is an integer) have the sequentially decreasing temperature ranges for providing signals, permitting n detection temperatures to be used.

When signals are measured at n detection temperatures, the present invention includes sequentially selecting two detection temperatures among the detection temperatures and analyzing the signals detected at the two detection temperatures. Particularly, the present invention comprises extracting a signal for a specific target nucleic acid sequence by using the signals obtained at the two detection temperatures and a reference value that can be selected in the step, only when the signal difference between the signals of the two selected detection temperatures satisfies a second criterion defined by a LTT.

A combination of target nucleic acid sequences to be considered in each step is logically derived in the course of sequentially selecting and analyzing two detection temperatures among the detection temperatures, and thereby, a SDT or a reference value to be applied at the corresponding step can be determined.

The method of the present invention provides a signal for a target nucleic acid sequence with a signal extraction process only when particular criteria are satisfied, and provides a signal for a target nucleic acid sequence without a signal extraction process when a particular criterion is not satisfied. The method of the present invention can minimize the signal extraction process, thereby significantly reducing false positive results which may occur due to undertaking a signal extraction process undesirable or unnecessary. Accordingly, the method of the present invention can provide a signal for a target nucleic acid sequence using two detection temperatures in a more accurate and efficient manner, from which the presence or absence of a target nucleic acid sequence can be determined.

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.

5

EXAMPLES

Example 1: Preparation of templates and oligonucleotides

The PTOCE method (WO 2012/096523) was used as a real-time PCR approach
10 for detecting signals in a real-time manner at different detection temperatures.

Taq DNA polymerase having a 5' nuclease activity was used for the extension of upstream primers and downstream primers, the cleavage of PTO (Probing and Tagging Oligonucleotide), and the extension of PTO fragment. Genomic DNA of *Chlamydia trachomatis* (CT) and genomic DNA of *Neisseria gonorrhoeae* (NG) were
15 used as first and second target nucleic acid sequences, respectively.

The PTOs for detection of CT and NG by using the PTOCE method as signal-generating means comprise (i) a 5'-tagging portion comprising a nucleotide sequence non-complementary to the target nucleic acid sequence and (ii) a 3'-targeting portion comprising a hybridizing nucleotide sequence complementary to the target nucleic
20 acid sequence. The CTOs (Capturing and Templating Oligonucleotides) used in the detection of CT and NG comprise, 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 PTOs and (ii) a templating portion comprising a nucleotide sequence non-complementary to the 5'-tagging portion and the 3'-
25 targeting portion of the PTOs. The CTOs were labeled with a quencher molecule (BHQ-2) at their 5'-ends and a fluorescent reporter molecule (CAL Fluor Red 610) in their templating portions. The PTOs and CTOs are blocked with a carbon spacer at their 3'-ends to prohibit their extension.

Because the PTOCE real-time method uses a single type of fluorescence label

(CAL Fluor Red 610), signals for the target nucleic acid sequences cannot be differentiated from each other in a single reaction vessel by a single detector.

The sequences of upstream primers, downstream primers, PTOs, and CTOs used in this Example are shown in Table 1.

5 **TABLE 1**

Name	Type	Sequence (5' → 3')	SEQ ID
NG_F	Primer	TACGCCTGCTACTTTCACGCTIIIIIGTAATCAGATG	SEQ ID NO:1
NG_R	Primer	CAATGGATCGGTATCACTCGCIIIIICGAGCAAGAAC	SEQ ID NO:2
NG_PTO	PTO	<u>GTACGCGATACGGGCCCTCATTGGCGTGTTCG</u> [C3 spacer]	SEQ ID NO:3
NG_CTO	CTO	[BHQ-2]TTTTTTTTTTTTTTTTTTG[T(Cal Fluor Red 610)]ACTGCCCGTATCGCGTAC[C3 spacer]	SEQ ID NO:4
CT_F	Primer	GAGTTTTAAATGGGAAATTCTGGTIIIIITTTGTATAAC	SEQ ID NO:5
CT_R	Primer	CCAATTGTAATAGAAGCATTGGTTGIIIIITTTATTGGAGA	SEQ ID NO:6
CT_PTO	PTO	<u>GATTACGCGACCGCATCAGAAGCTGTCATTTTGGCTGCG</u> [C3 spacer]	SEQ ID NO:7
CT_CTO	CTO	[BHQ-2]GCGCTGGATACCCTGGACGA[T(Cal Fluor Red 610)]ATGTGCGGTGCGTAATC[C3 spacer]	SEQ ID NO:8

I : Deoxyinosine

PTO : Probing and Tagging Oligonucleotide

CTO : Capturing and Templating Oligonucleotide

BHQ : Quencher (Black Hole Quencher)

10 Underlined letter : 5'-tagging portion of PTO

Example 2: Provision of signals for two target nucleic acid sequences from signals detected at different temperatures

15 After detecting signals at different detection temperatures using a single

detection channel in a single-reaction vessel, the signals were used to obtain signals for the target nucleic acid sequences in accordance with an embodiment of the present invention.

In this example, signals were detected at two different detection temperatures using the PTOCE real-time PCR method. According to the PTOCE real-time PCR method, when a target sequence is present, the PTO hybridized with the target sequence 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 the 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, the sequence and length of each extended duplex was designed such that the extended duplex for CT can be formed at 72°C and 60°C and the extended duplex for NG can be formed at 60°C not at 72°C.

Accordingly, at the detection temperature of 72°C, only the signal for CT is detected, whereas at the detection temperature of 60°C, the signal for NG as well as the signal for CT is detected.

Four types of samples were prepared and analyzed:

- (a) a sample containing only CT (A-type);
- (b) a sample containing only NG (B-type);
- (c) a sample containing both CT and NG (C-type); and
- (d) a sample containing no CT and NG (D-type; no target control).

<2-1> Preparation of templates and oligonucleotides

Genomic DNAs of CT and NG, and the primers (SEQ ID NOS: 1, 2, 5 and 6), PTOs (SEQ ID Nos: 3 and 7), and CTOs (SEQ ID NOS: 4 and 8) prepared in Example 1 were used.

<2-2> Real-time PCR and signal detection at different temperatures

The real-time PCR was conducted in the final volume of 20 μ l 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), 5 pmole of downstream primer (SEQ ID NO: 2), 3 pmole of PTO (SEQ ID NO: 3) and 1 pmole of CTO (SEQ ID NO: 4) for NG target amplification, 5 pmole of upstream primer (SEQ ID NO: 5) and 5 pmole of downstream primer (SEQ ID NO: 6), 3 pmole of PTO (SEQ ID NO: 7) and 1 pmole of CTO (SEQ ID NO: 8) for CT target amplification, and 10 μ l of 2X Master Mix [final, 200 μ M 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 signals was performed at 60°C and 72°C at each cycle. The results are represented by Fig. 1.

As shown in Fig. 1, for the sample containing only CT (A-type) and the sample containing both CT and NG (C-type), significant signals were detected both at 60°C and at 72°C; for the sample containing only NG (B-type), a significant signal was detected at 60°C but not at 72°C; and for the sample containing no CT and NG (D-type), no significant signals were detected both at 60°C and at 72°C.

By analyzing the signals detected at different detection temperatures in Fig. 1, the signal information about each target nucleic acid sequence can be obtained, which can be used to determine the presence or absence of each target nucleic acid sequence. Usually, a signal for the first target nucleic acid sequence (CT) can be directly taken from the signal detected at the relatively high detection temperature, since the signal detected at the relatively high detection temperature consists of the signal for the first target nucleic acid sequence. In contrast, a signal for the second target nucleic acid sequence (NG) is not detected at the relatively high detection temperature but at the relatively low detection temperature together with a signal for the first target nucleic acid sequence (CT), and the signal for the second target nucleic acid sequence (NG) can be obtained by eliminating the signal for the first

target nucleic acid sequence (CT) from the signal detected at the relatively low detection temperature. The process is called as a signal extraction process and can be performed by the mathematical equation I as follows:

<Equation I>

5 Signal for the second target nucleic acid sequence = [signal at the relatively low detection temperature in the step (a)] - [(signal at the relatively high detection temperature in the step (a)) x (the reference value for the first target nucleic acid sequence)]

10 The application of the signal extraction process to all samples can increase possibility of false positive results.

The present method provides a signal for each target nucleic acid sequence by applying the signal extraction process only to a sample satisfying a particular criterion(s).

15 <2-3> Signal acquisition for target nucleic acid sequences according to an embodiment

In order to provide signals for two target nucleic acid sequences from signals detected at different temperatures, the signals detected were analyzed according to an embodiment as depicted in Fig. 2A.

20 Prior to this analysis, a threshold and a reference value were set as described below.

- High-temperature threshold (HTT) = RFU 500
- Signal-difference threshold (SDT) = Ratio 1.50
- Reference value for the first target nucleic acid sequence = 1.15

25 The HTT is a value for determining the significance of the signal detected at 72°C, which was set with reference to two signals detected at 72°C in the presence and absence of CT in Fig. 1.

The SDT is a value for distinguishing a sample containing only the first target nucleic acid sequence (A-type) from a sample containing both the first target nucleic

acid sequence and the second target nucleic acid sequence, which was set as follows.

First, for each of the sample containing only CT and the sample containing both CT and NG, a ratio (End-Ratio) of RFUs at end cycles was obtained from the signals detected at 60°C and 72°C. The results are shown in Table 2.

5 **TABLE 2**

Target	End-RFU		End-Ratio
	72°C	60°C	
CT (A-type)	1610	1844	1.15
CT + NG (C-type)	1548	3241	2.09

As shown in Table 2, the End-ratio was 1.15 for the sample containing only CT (A-type) and 2.09 for the sample containing both CT and NG (C-type). Thus, a SDT was set to 1.50, a value between 1.15 and 2.09, for distinguishing the two samples.

10 Meanwhile, the reference value is the value used to extract a signal for NG by eliminating a signal for CT from the signal detected at 60°C, which was set to 1.15 corresponding to the End-Ratio calculated from the sample containing only CT (A-type).

15 <Identification step 1> Identifying whether the signal detected at the relatively high detection temperature satisfies the first criterion defined by the high-temperature threshold (HTT)

In this step, it was identified whether the signal detected at the relatively high detection temperature obtained from the unknown sample satisfies the first criterion defined by the HTT. The first criterion was as follows:

(i) Satisfied: the signal detected at 72°C $\geq$ HTT (=RFU 500)

(ii) Unsatisfied: the signal detected at 72°C $<$ HTT (=RFU 500)

If the first criterion was unsatisfied, the signal detected at 72°C was provided without signal extraction as the signal for CT and the signal detected at 60°C was provided without signal extraction as the signal for NG. On the other hand, if the

first criterion was satisfied, the process proceeded to the subsequent step.

<Identification step 2> Identifying whether a signal difference between the signal detected at the relatively low detection temperature and the signal detected at the relatively high detection temperature satisfies the second criterion defined by the signal-difference threshold (SDT)

In this step, it was identified whether a signal difference between the signal detected at the relatively low detection temperature and the signal detected at the relatively high detection temperature satisfies the second criterion defined by the SDT.

10 The second criterion was as follows:

(i) Satisfied: End-Ratio $\geq$ SDT (= Ratio 1.50)

(ii) Unsatisfied: End-Ratio $<$ SDT (= Ratio 1.50)

If the second criterion was unsatisfied, the signal detected at 72°C was provided without signal extraction as the signal for CT and any signal indicative of the absence of a target nucleic acid sequence, *e.g.*, a signal of approximately RFU 0, was provided without signal extraction as the signal for the absence of NG. In this case, the signal detected at both 60°C and 72°C cannot be adopted as the signal for NG, because these signals are both significant. Instead, any other signal indicative of the absence of a target nucleic acid sequence was provided as the signal for the absence of NG.

On the other hand, if the second criterion was satisfied, the process proceeded to the subsequent step.

<Signal extraction step>

25 The signal for NG was extracted eliminating the signal for CT from the signal detected at 60°C as follows:

The signal for NG = signal detected at 60°C - (signal detected at 72°C x the reference value for CT (RV 1.15))

The above process was also referred to as the signal extraction process.

The results are shown in Figure 2B.

As shown in Fig. 2B, for the sample containing only NG (B-type) and the sample containing no CT and NG (D-type), the signal for CT and the signal for NG can be provided without signal extraction process from <Identification step 1>. And, for the sample containing only CT (A-type), the signal for CT and the signal for NG can be provided without signal extraction process from <Identification step 2>. Further, for sample containing both the CT and NG (C-type), the signal for CT and the signal for NG can be provided from <Signal extraction step>.

As described above, it was confirmed that the signal for each target nucleic acid sequence can be accurately provided by the method of the present invention. The signals for CT and NG provided can be used to determine the presence or absence of CT and NG, respectively.

As such, the method as described herein can (i) provide a signal for each target nucleic acid sequence from the signals detected at different temperatures, (ii) minimize the signal extraction process for detecting the presence of the target nucleic acid sequence having the relatively low detection temperature by sequential steps, and (iii) therefore reduce errors that may occur during signal extraction.

Having described a preferred embodiment of the present invention, it is to 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.

What is claimed is:

1. A method for providing a signal for at least one target nucleic acid sequence in a sample from signals detected at different temperatures, which comprises the steps of:

(a) incubating the sample with a first signal-generating means capable of
5 generating a signal for a first target nucleic acid sequence and a second signal-generating means capable of generating a signal for a second target nucleic acid sequence in a single reaction vessel, and detecting signals at a relatively high detection temperature and a relatively low detection temperature by a single type of detector; wherein the first signal-generating means generates signals at both the
10 relatively high detection temperature and the relatively low detection temperature in the presence of the first target nucleic acid sequence in the sample and the second signal-generating means generates a signal at the relatively low detection temperature in the presence of the second target nucleic acid sequence in the sample; wherein the two signals to be generated by the two signal-generating means are not
15 differentiated by the single type of detector;

(b) identifying whether the signal detected at the relatively high detection temperature satisfies a first criterion defined by a high-temperature threshold (HTT); when the signal detected at the relatively high detection temperature satisfies the first criterion, the next step (c) proceeds;

20 (c) identifying whether a signal difference between the signal detected at the relatively low detection temperature and the signal detected at the relatively high detection temperature satisfies a second criterion defined by a signal-difference threshold (SDT); when the signal difference satisfies the second criterion, the next step (d) proceeds; and

25 (d) extracting the signal for the second target nucleic acid sequence by using the signal detected at the relatively high detection temperature in the step (a), the signal detected at the relatively low detection temperature in the step (a) and a reference value for the first target nucleic acid sequence, thereby providing the signal for the second target nucleic acid sequence; wherein the reference value for the first

target nucleic acid sequence is a value representing a relationship of change in signals provided by the first signal-generating means at the relatively high detection temperature and the relatively low detection temperature; wherein the reference value for the first target nucleic acid sequence is determined from a control reaction
5 using the first target nucleic acid sequence and the first signal-generating means; wherein the steps (b) and (c) may be performed in reverse order.

2. The method of claim 1, wherein when the signal detected at the relatively high detection temperature does not satisfy the first criterion in the step (b), the signal
10 detected at the relatively low detection temperature in the step (a) is provided as the signal for the second target nucleic acid sequence.

3. The method of claim 1, wherein when the signal detected at the relatively high detection temperature does not satisfy the first criterion in the step (b), the method
15 further comprises a step of identifying whether the signal detected at the relatively low detection temperature satisfies a third criterion defined by a low-temperature threshold (LTT).

4. The method of claim 3, wherein when the signal detected at the relatively low detection temperature does not satisfy the third criterion, any signal indicative of the
20 absence of a target nucleic acid sequence is provided as the signal for the absence of the second target nucleic acid sequence.

5. The method of claim 3, wherein when the signal detected at the relatively low detection temperature satisfies the third criterion, the signal detected at the relatively
25 low detection temperature in the step (a) is provided as the signal for the second target nucleic acid sequence.

6. The method of claim 1, which further comprises identifying whether the signal

detected at the relatively low detection temperature satisfies a third criterion defined by a low-temperature threshold (LTT) prior to the step (b).

5 7. The method of claim 6, wherein when the signal detected at the relatively low detection temperature does not satisfy the third criterion, any signal indicative of the absence of a target nucleic acid sequence is provided as the signal for the absence of the second target nucleic acid sequence.

10 8. The method of claim 6, wherein when the signal detected at the relatively low detection temperature satisfies the third criterion, the next step (b) proceeds.

15 9. The method of claim 1, wherein the signal difference in the step (c) is calculated by mathematically processing the signals detected at the relatively low detection temperature and the relatively high detection temperature.

10. The method of claim 9, wherein the mathematical processing is a subtraction or ratio between the signals detected at the relatively low detection temperature and the relatively high detection temperature.

20 11. The method of claim 1, wherein when the signal difference in step (c) does not satisfy the second criterion, any signal indicative of the absence of a target nucleic acid sequence is provided as the signal for the absence of the second target nucleic acid sequence.

25 12. The method of claim 1, wherein the signal for the second target nucleic acid sequence in the step (d) is provided by eliminating a signal generated by the first signal-generating means from the signal detected at the relatively low detection temperature or the relatively high detection temperature in the step (a), by using the reference value for the first target nucleic acid sequence.

13. The method of claim 1, wherein the signal for the second target nucleic acid sequence in the step (d) is provided by the following mathematical equation I:

<Equation I>

5 Signal for the second target nucleic acid sequence = [signal detected at the relatively low detection temperature in the step (a)] - [(signal detected at the relatively high detection temperature in the step (a)) × (the reference value for the first target nucleic acid sequence)]

10 wherein the reference value for the first target nucleic acid sequence is a ratio of a signal provided by the first signal-generating means at the relatively low detection temperature to a signal provided by the first signal-generating means at the relatively high detection temperature.

14. The method of claim 1, wherein the signal for the second target nucleic acid sequence in the step (d) is provided by the following mathematical equation II:

<Equation II>

15 Signal for the second target nucleic acid sequence = [signal detected at the relatively high detection temperature in the step (a)] - [(signal detected at the relatively low detection temperature in the step (a)) ÷ (the reference value for the first target nucleic acid sequence)]

20 wherein the reference value for the first target nucleic acid sequence is a ratio of a signal provided by the first signal-generating means at the relatively low detection temperature to a signal provided by the first signal-generating means at the relatively high detection temperature.

25

15. The method of claim 1, which further comprises: (e) identifying whether the signal for the second target nucleic acid sequence provided in the step (d) satisfies a fourth criterion defined by a verification threshold; when the signal for the second target nucleic acid sequence does not satisfy the fourth criterion, any signal indicative

of the absence of a target nucleic acid sequence is provided as the signal for the absence of the second target nucleic acid sequence.

16. The method of any one of claims 1 to 15, wherein the signal detected at the relatively high detection temperature in the step (a) is provided as the signal for the first target nucleic acid sequence.

17. The method of any one of claims 2, 4, 5 and 7, wherein any signal indicative of the absence of a target nucleic acid sequence is provided as the signal for the absence of the first target nucleic acid sequence.

18. The method of claim 11 or 15, wherein the signal detected at the relatively high detection temperature in the step (a) or the signal detected at the relatively low detection temperature in the step (a) is provided as the signal for the first target nucleic acid sequence.

19. The method of claim 1, wherein the incubation in the step (a) is performed by using the first signal-generating means, the second signal-generating means and at least one additional signal-generating means capable of generating a signal for at least one additional target nucleic acid sequence; and wherein the detection by the single type of detector is performed for detecting signals at the relatively high detection temperature, the relatively low detection temperature and at least one additional detection temperature lower than the relatively high detection temperature and the relatively low detection temperature.

20. A computer readable storage medium containing instructions to configure a processor to perform a method for providing a signal for at least one target nucleic acid sequence in a sample from signals detected at different temperatures, which comprises the steps of:

(a) incubating the sample with a first signal-generating means capable of generating a signal for a first target nucleic acid sequence and a second signal-generating means capable of generating a signal for a second target nucleic acid sequence in a single reaction vessel, and receiving signals detected at a relatively high detection temperature and a relatively low detection temperature by a single type of detector; wherein the first signal-generating means generates signals at both the relatively high detection temperature and the relatively low detection temperature in the presence of the first target nucleic acid sequence in the sample and the second signal-generating means generates a signal at the relatively low detection temperature in the presence of the second target nucleic acid sequence in the sample; wherein the two signals to be generated by the two signal-generating means are not differentiated by the single type of detector;

(b) identifying whether the signal detected at the relatively high detection temperature satisfies a first criterion defined by a high-temperature threshold (HTT); when the signal detected at the relatively high detection temperature satisfies the first criterion, the next step (c) proceeds;

(c) identifying whether a signal difference between the signal detected at the relatively low detection temperature and the signal detected at the relatively high detection temperature satisfies a second criterion defined by a signal-difference threshold (SDT); when the signal difference satisfies the second criterion, the next step (d) proceeds; and

(d) extracting the signal for the second target nucleic acid sequence by using the signal detected at the relatively high detection temperature in the step (a), the signal detected at the relatively low detection temperature in the step (a) and a reference value for the first target nucleic acid sequence, thereby providing the signal for the second target nucleic acid sequence; wherein the reference value for the first target nucleic acid sequence is a value representing a relationship of change in signals provided by the first signal-generating means at the relatively high detection temperature and the relatively low detection temperature; wherein the reference

value for the first target nucleic acid sequence is determined from a control reaction using the first target nucleic acid sequence and the first signal-generating means; wherein the steps (b) and (c) may be performed in reverse order.

5 21. A device for providing a signal for at least one target nucleic acid sequence in a sample from signals detected at different temperatures, comprising: (a) a computer processor and (b) the computer readable storage medium of claim 20 coupled to the computer processor.

10 22. A computer program to be stored on a computer readable storage medium to configure a processor to perform a method for providing a signal for at least one target nucleic acid sequence in a sample from signals detected at different temperatures, which comprises the steps of:

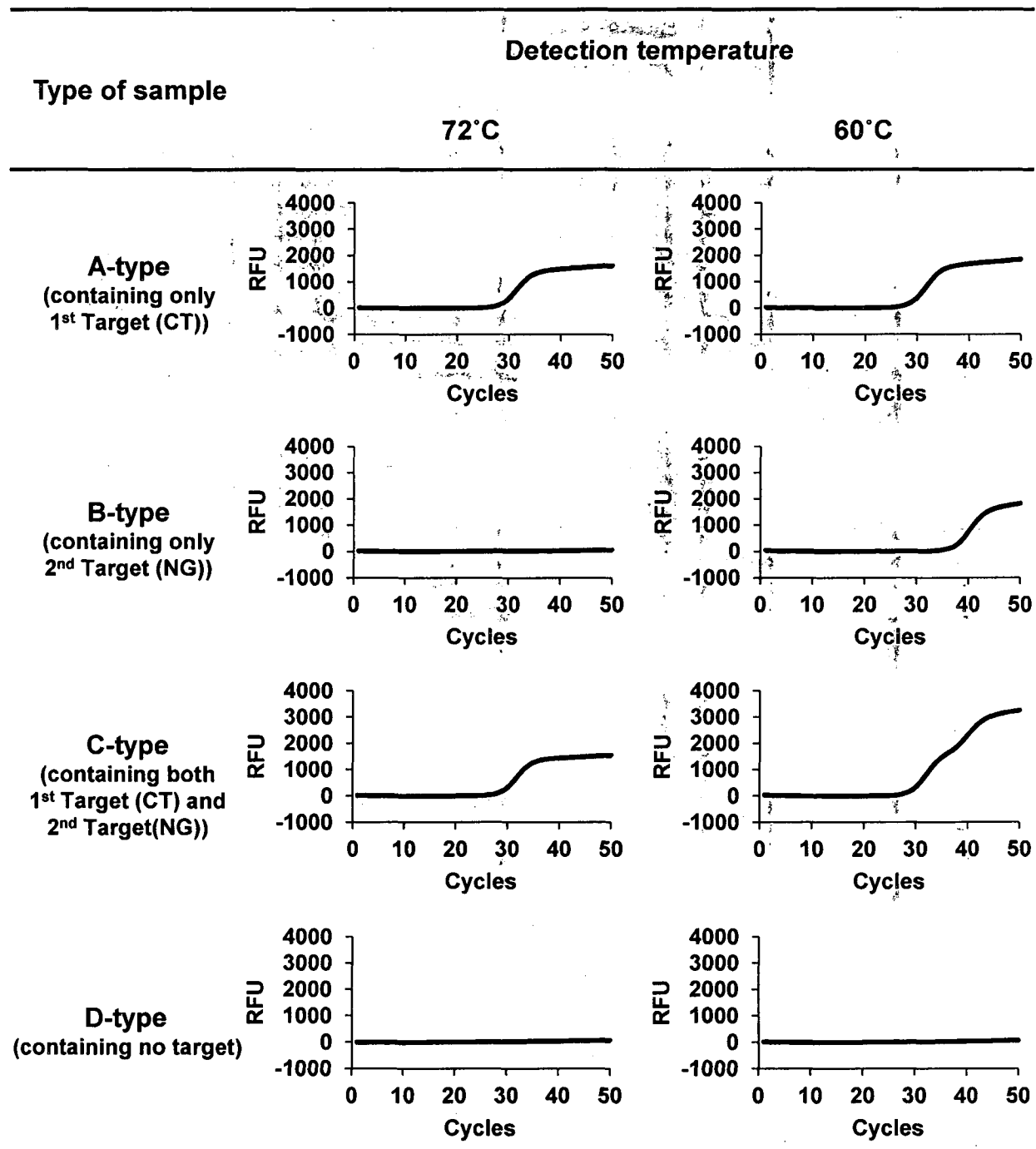
(a) incubating the sample with a first signal-generating means capable of
15 generating a signal for a first target nucleic acid sequence and a second signal-generating means capable of generating a signal for a second target nucleic acid sequence in a single reaction vessel, and receiving signals detected at a relatively high detection temperature and a relatively low detection temperature by a single type of detector; wherein the first signal-generating means generates signals at both
20 the relatively high detection temperature and the relatively low detection temperature in the presence of the first target nucleic acid sequence in the sample and the second signal-generating means generates a signal at the relatively low detection temperature in the presence of the second target nucleic acid sequence in the sample; wherein the two signals to be generated by the two signal-generating means are not
25 differentiated by the single type of detector;

(b) identifying whether the signal detected at the relatively high detection temperature satisfies a first criterion defined by a high-temperature threshold (HTT); when the signal detected at the relatively high detection temperature satisfies the first criterion, the next step (c) proceeds;

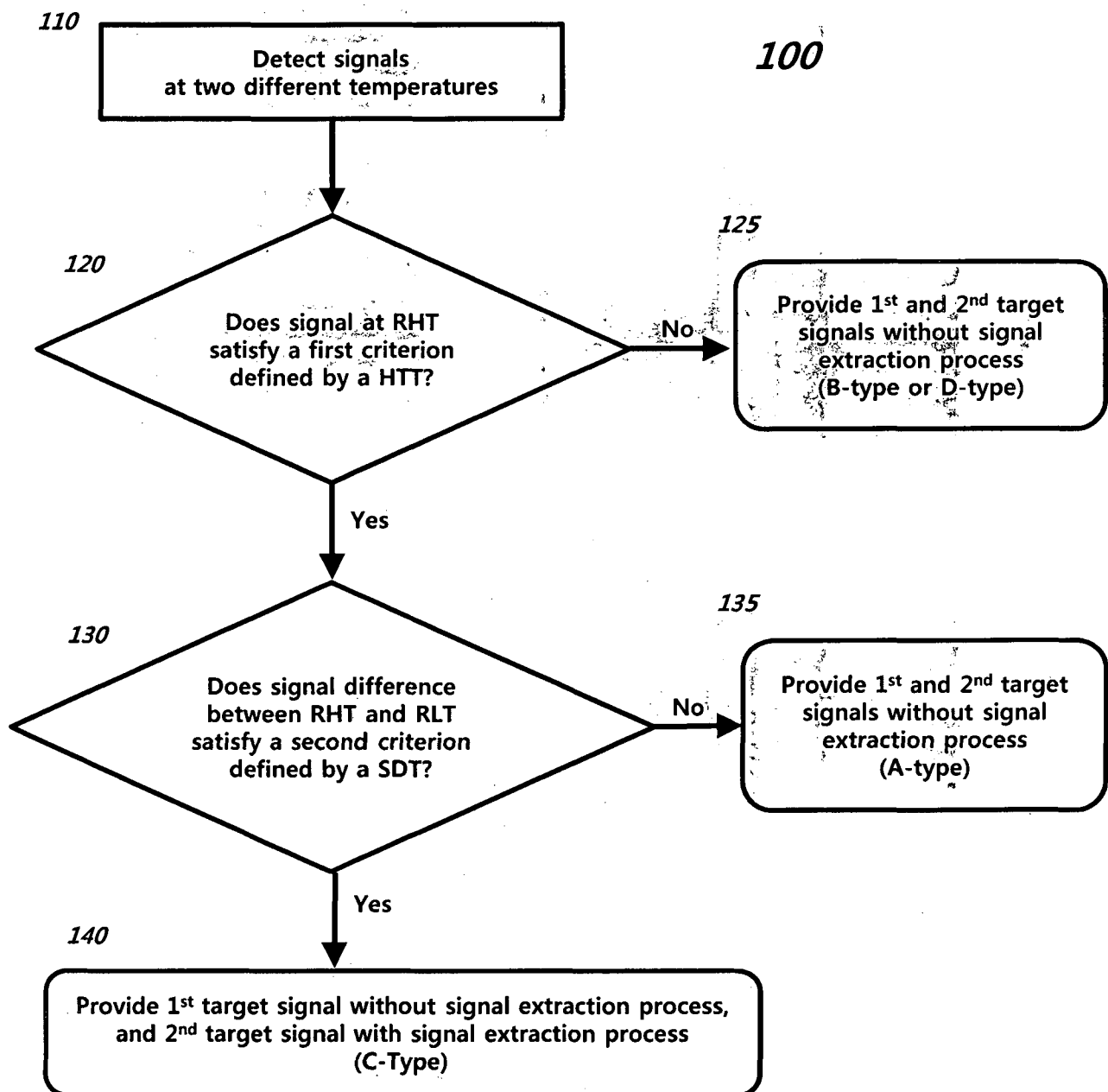
(c) identifying whether a signal difference between the signal detected at the relatively low detection temperature and the signal detected at the relatively high detection temperature satisfies a second criterion defined by a signal-difference threshold (SDT); when the signal difference satisfies the second criterion, the next
5 step (d) proceeds; and

(d) extracting the signal for the second target nucleic acid sequence by using the signal detected at the relatively high detection temperature in the step (a), the signal detected at the relatively low detection temperature in the step (a) and a reference value for the first target nucleic acid sequence, thereby providing the signal
10 for the second target nucleic acid sequence; wherein the reference value for the first target nucleic acid sequence is a value representing a relationship of change in signals provided by the first signal-generating means at the relatively high detection temperature and the relatively low detection temperature; wherein the reference
15 value for the first target nucleic acid sequence is determined from a control reaction using the first target nucleic acid sequence and the first signal-generating means; wherein the steps (b) and (c) may be performed in reverse order.

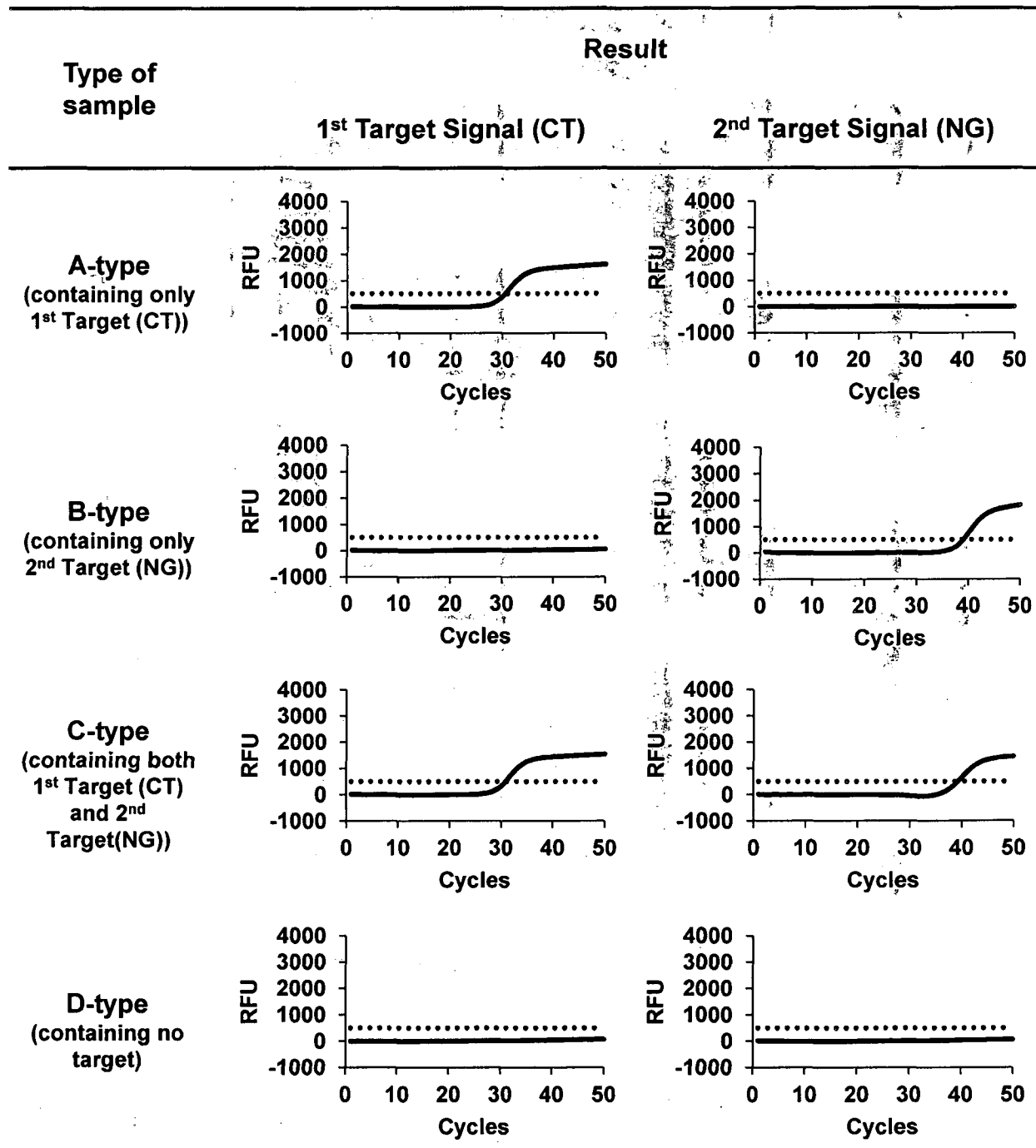
1/10

Fig. 1

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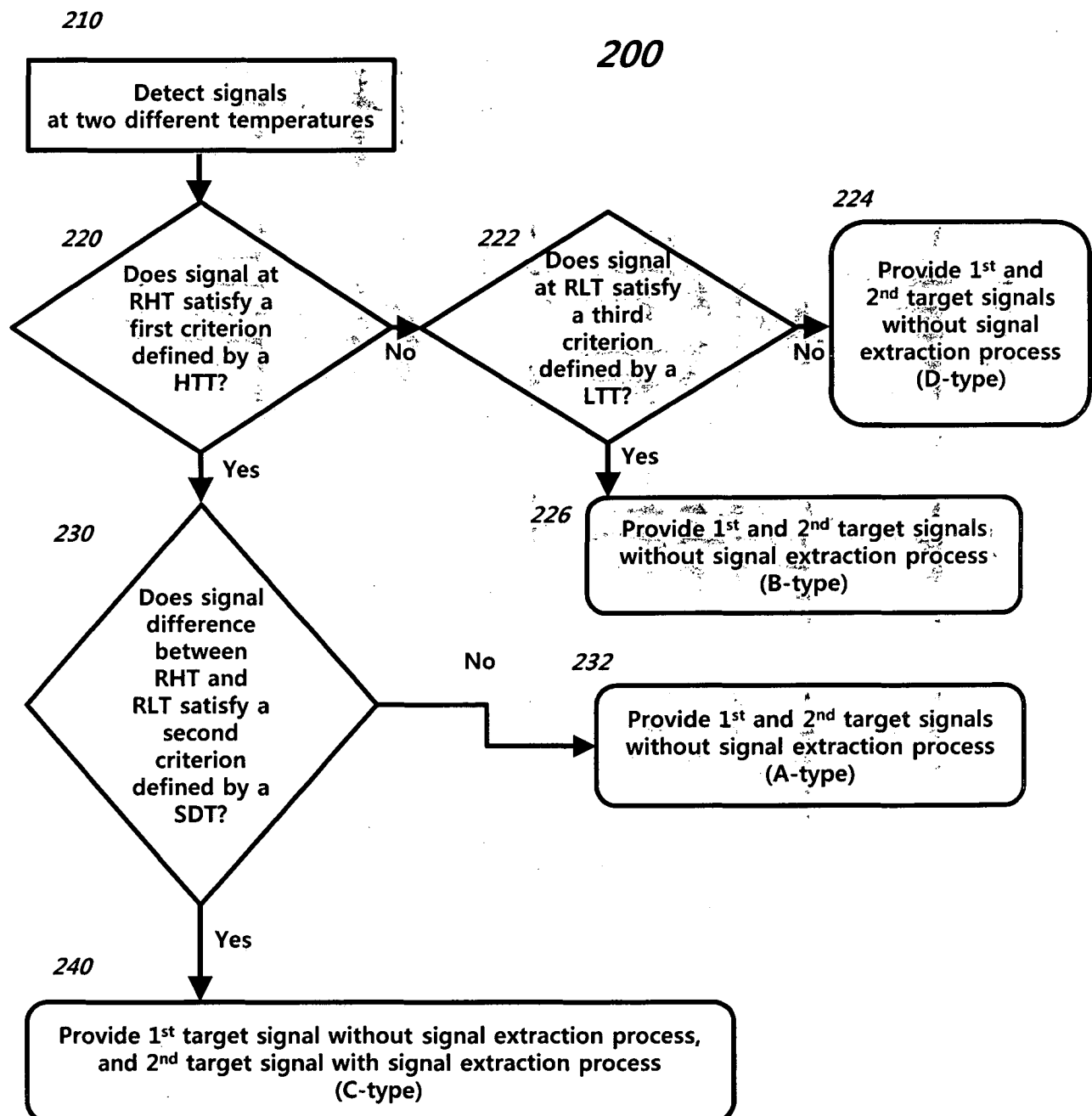
Fig. 2A

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Fig. 2B

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Fig. 3



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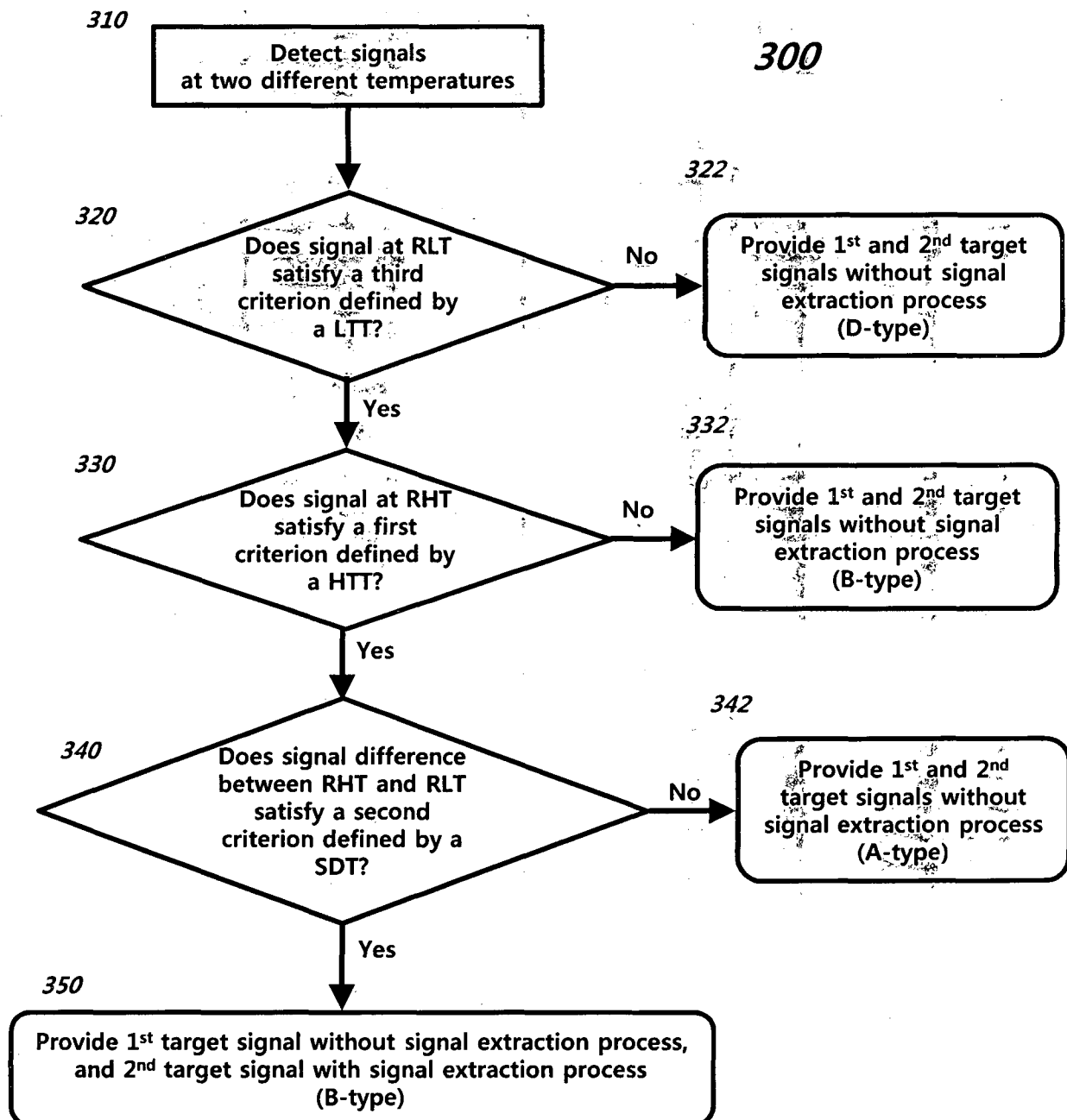
Fig. 4

Fig. 5A

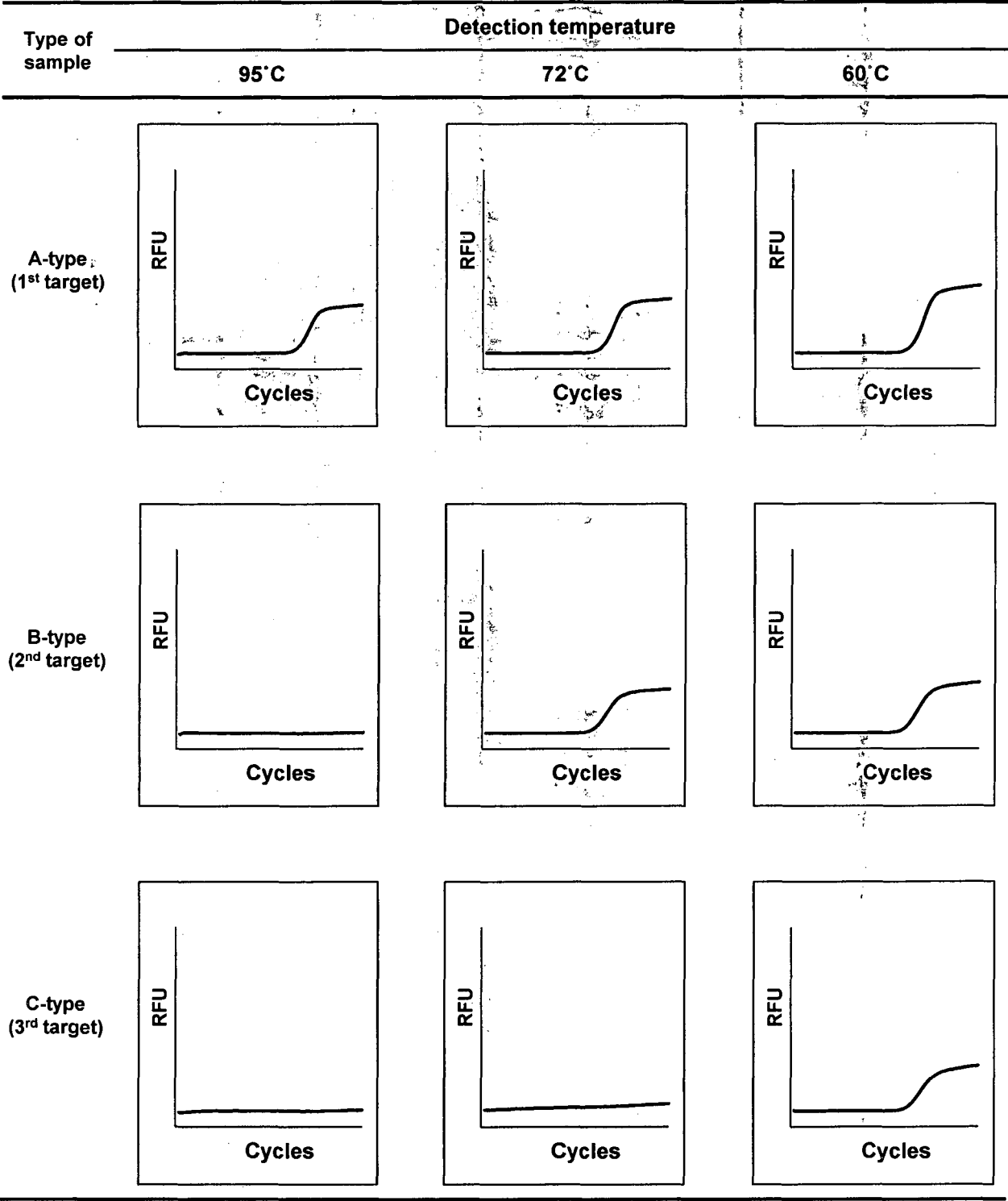
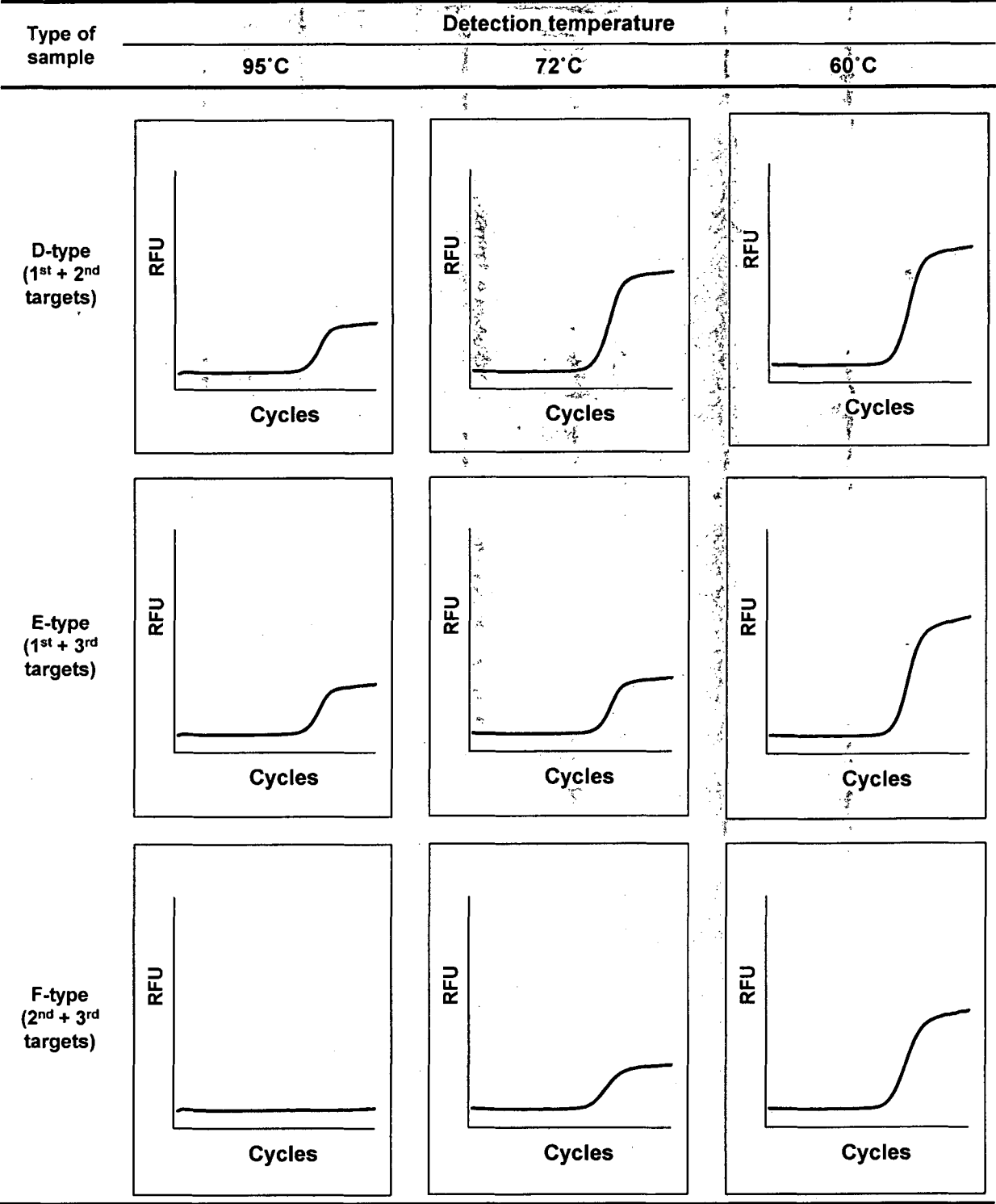
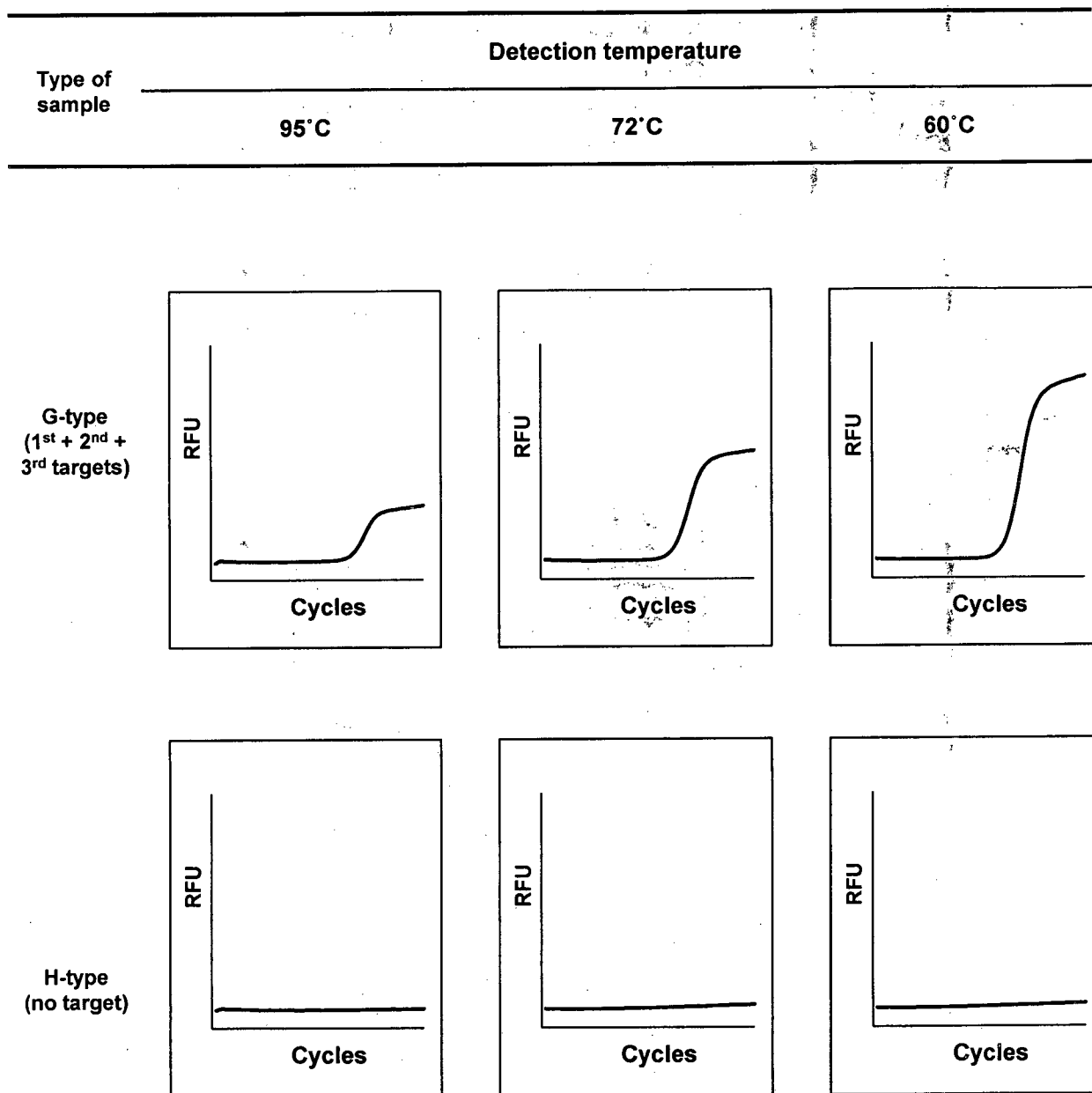


Fig. 5B

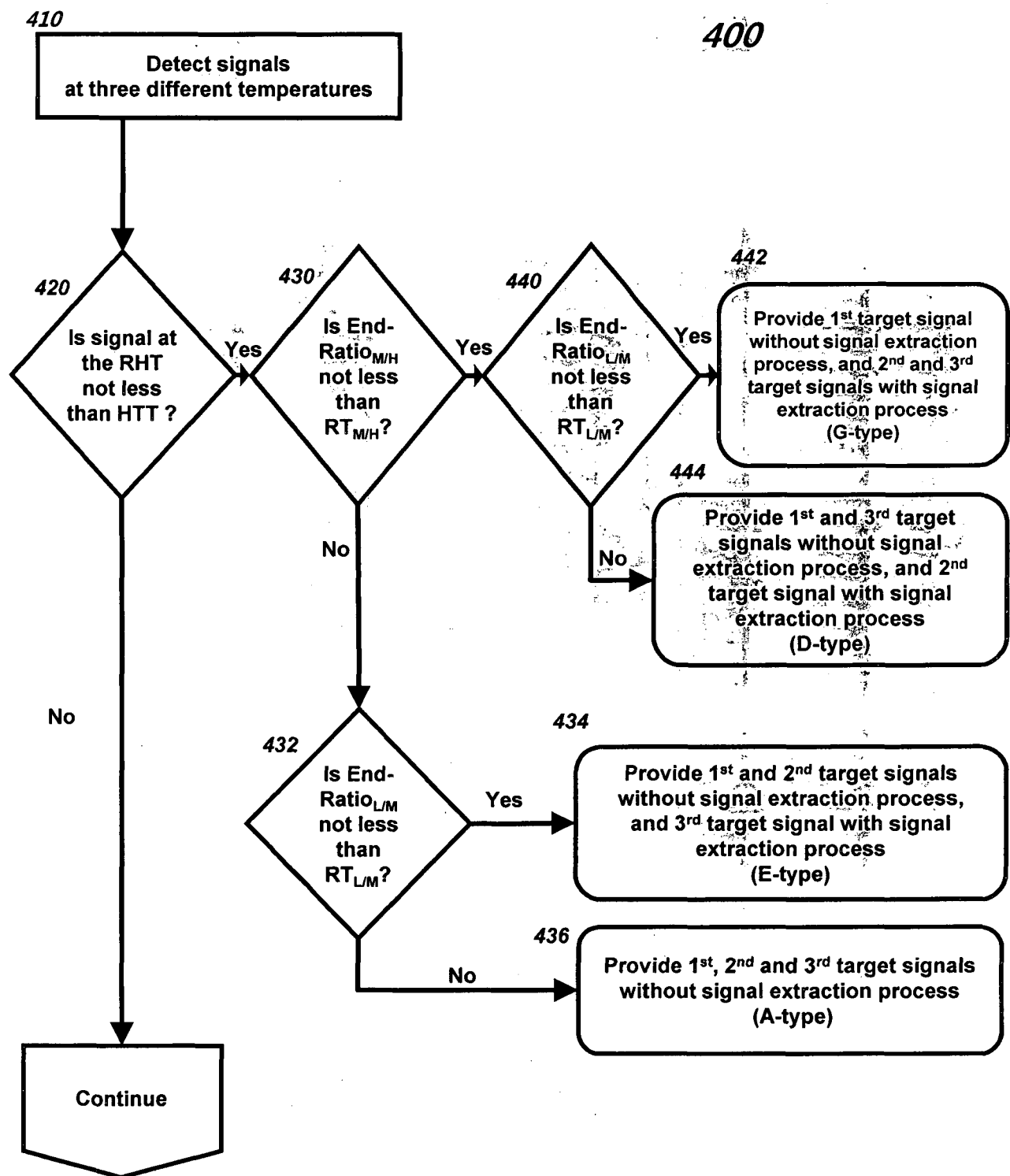


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Fig. 5C

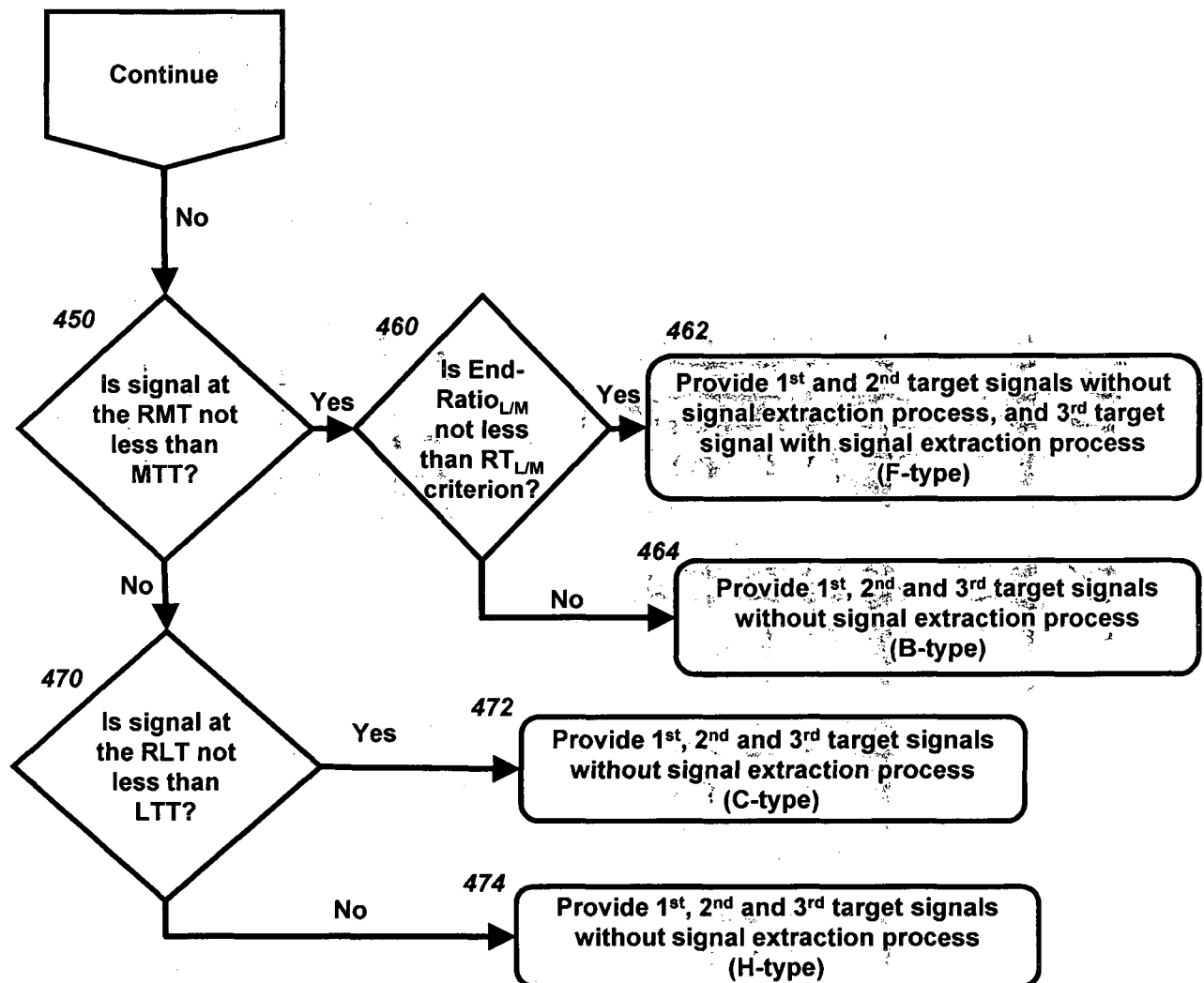
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Fig. 5D



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Fig. 5E



INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2017/000951**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; C12Q 1/25; C07H 21/00

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: target nucleic acid, signal, signal-generating means, high detection temperature, low detection temperature, single reaction vessel, high temperature threshold, signal-difference threshold

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2015-147412 A1 (SEEGENE, INC.) 01 October 2015 See claims 1, 55-57.	1-22
A	WO 2014-176575 A1 (FIREFLY BIOWORKS, INC.) 30 October 2014 See claim 1.	1-22
A	US 2015-0005198 A1 (FIREFLY BIOWORKS, INC.) 01 January 2015 See claim 1.	1-22
A	US 8486622 B2 (ERIKSON et al.) 16 July 2013 See claims 1, 61.	1-22
A	WO 01-32922 A2 (STRATAGENE) 10 May 2001 See claim 1.	1-22



Further documents are listed in the continuation of Box C.



See patent family annex.

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"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

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"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

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