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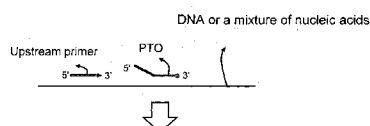
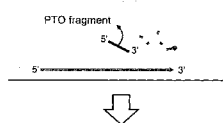
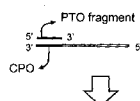
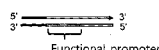
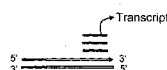
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(54) **Title:** DETECTION OF TARGET NUCLEIC ACID SEQUENCE BY PTO CLEAVAGE AND EXTENSION-DEPENDENT TRANSCRIPTION

A. Hybridization**B. Primer extension & Cleavage of PTO****C. Hybridization of PTO fragment to CPO****D. Extension of PTO fragment & Formation of functional promoter****E. Transcription**

(57) **Abstract:** The present invention relates to the detection of a target nucleic acid sequence by a PTO Cleavage and Extension-Dependent Transcription (PCET). According to the present invention, the target detection is accomplished using enzymatic reactions such as 5' nucleolytic reaction and extension, and extension-dependent transcription as well as probe hybridization, contributing to improvements in the target specificity and sensitivity, process convenience and workability in multiplex detection.

DETECTION OF TARGET NUCLEIC ACID SEQUENCE BY PTO CLEAVAGE AND EXTENSION-DEPENDENT TRANSCRIPTION

CROSS-REFERENCE TO RELATED APPLICATIONS

5 This application claims priority from Korean Patent Application No. 2012-0062300, filed on June 11, 2012, in the Korean Intellectual Property Office, the disclosure of which is incorporated herein by reference.

BACKGROUND OF THE INVENTION

10 FIELD OF THE INVENTION

 The present invention relates to the detection of a target nucleic acid sequence by a PTO Cleavage and Extension-Dependent Transcription (PCET).

DESCRIPTION OF THE RELATED ART

15 A target nucleic acid amplification process is prevalently involved in most of technologies for detecting target nucleic acid sequences. Nucleic acid amplification is a pivotal process for a wide variety of methods in molecular biology, such that various amplification methods have been proposed.

 The most predominant process for nucleic acid amplification known as
20 polymerase chain reaction (hereinafter referred to as "PCR") is based on repeated cycles of denaturation of double-stranded DNA, followed by oligonucleotide primer annealing to the DNA template, and primer extension by a DNA polymerase (Mullis et al. U.S. Pat. Nos. 4,683,195, 4,683,202, and 4,800,159; Saiki et al., (1985) *Science* 230, 1350-1354).

25 In addition, nucleic acid amplification methods involving transcript amplification have been reported (Kwoh, D. et al., Proc. Natl. Acad. Sci. U.S.A., 86:1173(1989); and Gingeras T.R. et al., WO 88/10315, US 5,130,238, US 5,409,818, US 5,554,517, US 6,063,603 and WO 90/06995).

As a representative of nucleic acid amplification-based target detection methods, a multitude of methods using probe hybridization have been suggested, including Molecular beacon method (Indian J Med Res 124: 385-398(2006) and Tyagi et al, Nature Biotechnology v.14 MARCH 1996) and Hybridization probe method (385-398, Indian J Med Res 124, review article October 2006 and 303-308, and Bernad et al, 147-148 Clin Chem 2000; 46). However, the methods in which probe hybridization is likely to be a sole determinant factor in target specificity, have serious drawbacks such as generation of false positives due to non-specific hybridization of probes with non-target sequences. Therefore, such methods are strongly required to improve fidelity of hybridization signals.

Besides probe hybridization processes, several approaches using additional enzymatic reactions, for example, TaqMan™ probe method, have been suggested.

In TaqMan™ probe method, the labeled probe hybridized with a target nucleic acid sequence is cleaved by a 5' nuclease activity of an upstream primer-dependent DNA polymerase, generating a signal indicating the presence of a target sequence (U.S. Pat. Nos. 5,210,015, 5,538,848 and 6,326,145). The TaqMan™ probe method suggests two approaches for signal generation: polymerization-dependent cleavage and polymerization-independent cleavage. In polymerization-dependent cleavage, extension of the upstream primer must occur before a nucleic acid polymerase encounters the 5'-end of the labeled probe. As the extension reaction continues, the polymerase progressively cleaves the 5'-end of the labeled probe. In polymerization-independent cleavage, the upstream primer and the labeled probe are hybridized with a target nucleic acid sequence in close proximity such that binding of the nucleic acid polymerase to the 3'-end of the upstream primer puts it in contact with the 5'-end of the labeled probe to release the label. In addition, the TaqMan™ probe method discloses that the labeled probe at its 5'-end having a 5'-tail region not-hybridizable with a target sequence is also cleaved to form a fragment comprising the 5'-tail region.

There have been reported some methods in which a probe having a 5'-tail region non-complementary to a target sequence is cleaved by 5' nuclease to release a

fragment comprising the 5'-tail region.

For instance, U.S. Pat. No. 5,691,142 discloses a cleavage structure to be digested by 5' nuclease activity of DNA polymerase. The cleavage structure is exemplified in which an oligonucleotide comprising a 5' portion non-complementary to
5 and a 3' portion complementary to a template is hybridized with the template and an upstream oligonucleotide is hybridized with the template in close proximity. The cleavage structure is cleaved by DNA polymerase having 5' nuclease activity or modified DNA polymerase with reduced synthetic activity to release the 5' portion non-complementary to the template. The released 5' portion is then hybridized with
10 an oligonucleotide having a hairpin structure to form a cleavage structure, thereby inducing progressive cleavage reactions to detect a target sequence.

U.S. Pat. No. 7,381,532 discloses a process in which the cleavage structure having the upstream oligonucleotide with blocked 3'-end is cleaved by DNA polymerase having 5' nuclease activity or FEN nuclease to release non-complementary
15 5' flap region and the released 5' flap region is detected by size analysis or interactive dual label. U.S. Pat. No. 6,893,819 discloses that detectable released flaps are produced by a nucleic acid synthesis dependent, flap-mediated sequential amplification method. In this method, a released flap from a first cleavage structure cleaves, in a nucleic acid synthesis dependent manner, a second cleavage structure to
20 release a flap from the second cleavage structure and the release flaps are detected. U.S. Pat. No. 7,309,573 disclose a method including formation of a released flap produced by a nucleic acid synthesis; extension of the released flap; cleavage of an oligonucleotide during extension of the flap; and detection of a signal generated by the cleavage of the oligonucleotide.

25 By hybridization of fluorescence-labeled probes in a liquid phase, a plurality of target nucleic acid sequences may be simultaneously detected using even a single type of a fluorescent label by melting curve analysis. However, the conventional technologies for detection of target sequences by 5' nuclease-mediated cleavage of interactive-dual labeled probes require different types of fluorescent labels for

different target sequences in multiplex target detection, which limits the number of target sequences to be detected due to limitation of the number of types of fluorescent labels.

U.S. Pat. Appln. Pub. 2008-0241838 discloses a target detection method using
5 cleavage of a probe having a 5' portion non-complementary to a target nucleic acid sequence and hybridization of a capture probe. A label is positioned on the non-complementary 5' portion. The labeled probe hybridized with the target sequence is cleaved to release a fragment, after which the fragment is then hybridized with the capture probe to detect the presence of the target sequence. In this method, it is
10 necessary that an uncleaved/intact probe is not hybridized with the capture probe. For that, the capture probe having a shorter length has to be immobilized onto a solid substrate. However, such a limitation results in lower efficiency of hybridization on a solid substrate and also in difficulties in optimization of reaction conditions.

Most of previous methods for target detection using the 5'-tail region generate
15 a target signal whose intensity is proportional to the number of the 5'-tail region fragment, which is likely to be limitation in target detection.

Meanwhile, as nucleic acid amplification methods involving transcript amplification, TMA (Transcription Mediated Amplification), NASBA (Nucleic Acid Sequence Based Amplification) and 3SR (Self-sustained Sequence Replication)
20 methods are carried out in such a manner that double-stranded DNA templates containing T7-promoter are produced using primers containing T7-promoter sequence and then RNA molecules are amplified using the DNA templates and T7 RNA polymerase. The amplification methods have some advantages in which RNA molecules can be amplified using double-stranded DNA templates containing
25 promoter sequence under isothermal conditions. However, where targets to be amplified is RNA, the methods requires modifications of intramolecular structures in the target RNA in order to increase primer binding efficiency, and additional enzymes such as DNA polymerase (*e.g.*, reverse transcriptase) and RNase H besides RNA polymerase.

As another example of nucleic acid amplification methods involving transcript amplification, SMART (Signal Mediated Amplification of RNA Technology; US 6,287,770) method has been reported to detect target RNA molecules by amplifying signaling RNA other than target RNA. In particular, SMART method involves formation of 3WJ (three-way junction) structure by hybridization of target sequence, extension probe and template probe. For formation of 3WJ structure, the extension probe has a complementary sequence to the target sequence at its 5'-end and a complementary sequence to the template probe at its 3'-end, and the template probe comprises in 5' to 3' direction 5'-transcription template sequence (*i.e.*, sequence to be transcribed to signaling RNA)-T7 promoter-complementary sequence to the 3'-end of the extension probe-complementary sequence to a portion of the target sequence adjacent to hybridization site of the extension probe-3'. 3WJ structure is formed by hybridization of the extension probe and template probe with adjacent positions on the target sequence and then hybridization between the two probes. Following the formation of 3WJ structure, the signaling RNA is amplified by extension and transcription using DNA polymerase and T7 RNA polymerase, and then the presence of the target sequence is determined by detection of the signaling RNA amplified. However, SMART method has serious shortcomings: difficulties in primer design and poor applicability in multiplex amplification.

Accordingly, there remain long-felt needs in the art to develop novel approaches using transcript amplification for detection of a target sequence, preferably multiple target sequences, in a liquid phase and on a solid phase by not only hybridization but also enzymatic reactions such as 5' nucleolytic reaction in a more convenient, reliable and reproducible 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 entities are hereby incorporated by references into this application in order to more fully describe this invention and the state of the art to

which this invention pertains.

SUMMARY OF THE INVENTION

The present inventors have made intensive researches to develop novel approaches to detect target sequences with more improved accuracy and convenience, *inter alia*, in a multiplex manner. As a result, we have established novel protocols for detection of target sequences, in which target detection is accomplished using not only probe hybridization but also enzymatic reactions such as 5' nucleolytic reaction and extension, and extension-dependent transcription, contributing to improvements in the target specificity and sensitivity, process convenience, and workability in multiplex detection. Also, we have found that nucleic acid molecules may be produced being dependent on the presence of target sequences during the novel detection protocol described above. In the present invention, a nucleic acid molecule (*e.g.* RNA) is produced by transcription reaction dependent on the presence of the target nucleic acid.

Accordingly, it is an object of this invention to provide a method for producing a nucleic acid molecule dependent on a target nucleic acid sequence.

It is another object of this invention to provide a method for detecting a target nucleic acid sequence from a DNA or a mixture of nucleic acids using a target-dependent transcription.

It is still another object of this invention to provide a kit for producing a nucleic acid molecule dependent on a target nucleic acid sequence.

It is further object of this invention to provide a kit for detecting a target nucleic acid sequence from a DNA or a mixture of nucleic acids using a target-dependent transcription.

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. 1A shows the schematic structures of PTO (Probing and Tagging Oligonucleotide) and CPO (Capturing and Promoter Oligonucleotide).

Fig. 1B shows the schematic structure of TCPO (Transcript-Capturing and Promoter Oligonucleotide).

Fig. 2 schematically represents one embodiment of the present invention for target-dependent production of nucleic acid molecules (*e.g.*, RNA).

Fig. 3 schematically represents one embodiment of the present invention using the TCPO for target-dependent production of nucleic acid molecules (*e.g.*, RNA).

Fig. 4 schematically represents one embodiment of the present invention for target-dependent production of nucleic acid molecules (*e.g.*, RNA) in a cyclic manner.

Fig. 5 schematically represents one embodiment of the present invention for detection of produced nucleic acid molecules. The hybridization of the produced nucleic acid molecule with the dual-labeled detection oligonucleotide comprising a reporter molecule and quencher molecule induces signal change.

Fig. 6 schematically represents one embodiment of the present invention for detection of produced nucleic acid molecules. The hybridization of the produced nucleic acid molecule with the dual-labeled detection oligonucleotide comprising a reporter molecule and quencher molecule, and the extension of the produced nucleic acid molecule induce signal change.

Fig. 7 schematically represents one embodiment of the present invention for detection of produced nucleic acid molecules by use of the fluorescent-labeled nucleic acid molecule and the detection oligonucleotide immobilized on a solid substrate.

Fig. 8 schematically represents one embodiment of the present invention for detection of produced nucleic acid molecules. The hybridization of the produced nucleic acid molecule with the dual-labeled CPO (serving as the detection oligonucleotide) comprising a reporter molecule and quencher molecule induces signal change.

Fig. 9 schematically represents one embodiment of the present invention for

detection of produced nucleic acid molecules. The hybridization of the produced nucleic acid molecule with the dual-labeled CPO (serving as the detection oligonucleotide) comprising a reporter molecule and quencher molecule, and the extension of the produced nucleic acid molecule induces signal change.

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DETAILED DESCRIPTION OF THIS INVENTION

In one aspect of the present invention, there is provided a method for producing a nucleic acid molecule dependent on a target nucleic acid sequence, comprising:

10 (a) hybridizing the target nucleic acid sequence with an upstream oligonucleotide and a PTO (Probing and Tagging Oligonucleotide); wherein the upstream oligonucleotide comprises a hybridizing nucleotide sequence complementary to the target nucleic acid sequence; the PTO comprises (i) a 3'-targeting portion comprising a hybridizing nucleotide sequence complementary to the target nucleic acid sequence and (ii) a 5'-tagging portion comprising a nucleotide sequence non-complementary to the target nucleic acid sequence; wherein the 3'-targeting portion is hybridized with the target nucleic acid sequence and the 5'-tagging portion is not hybridized with the target nucleic acid sequence; the upstream oligonucleotide is located upstream of the PTO;

20 (b) contacting the resultant of the step (a) to an enzyme having a 5' nuclease activity under conditions for cleavage of the PTO; wherein the upstream oligonucleotide or its extended strand induces cleavage of the PTO by the enzyme having a 5' nuclease activity such that the cleavage releases a fragment comprising the 5'-tagging portion or a part of the 5'-tagging portion of the PTO;

25 (c) hybridizing the fragment released from the PTO with a CPO (Capturing and Promoter Oligonucleotide); wherein the CPO comprises in a 3' to 5' direction (i) a capturing portion comprising a nucleotide sequence complementary to the 5'-tagging portion or a part of the 5'-tagging portion of the PTO, (ii) a promoter portion inducing transcription; and (iii) a transcription portion; wherein the fragment released from the

PTO is hybridized with the capturing portion of the CPO;

(d) performing an extension reaction using the resultant of the step (c) and a template-dependent nucleic acid polymerase, wherein the fragment hybridized with the capturing portion of the CPO is extended to form an extended strand comprising an extended sequence complementary to the promoter portion of the CPO, thereby
5 activating the promoter portion; and

(e) producing the nucleic acid molecule complementary to the transcription portion by use of a polymerase recognizing the activated promoter portion.

10 In another aspect of the present invention, there is provided a method for detecting a target nucleic acid sequence from a DNA or a mixture of nucleic acids using a target-dependent transcription, comprising:

(a) hybridizing the target nucleic acid sequence with an upstream oligonucleotide and a PTO (Probing and Tagging Oligonucleotide); wherein the
15 upstream oligonucleotide comprises a hybridizing nucleotide sequence complementary to the target nucleic acid sequence; the PTO comprises (i) a 3'-targeting portion comprising a hybridizing nucleotide sequence complementary to the target nucleic acid sequence and (ii) a 5'-tagging portion comprising a nucleotide sequence non-complementary to the target nucleic acid sequence; wherein the 3'-targeting portion
20 is hybridized with the target nucleic acid sequence and the 5'-tagging portion is not hybridized with the target nucleic acid sequence; the upstream oligonucleotide is located upstream of the PTO;

(b) contacting the resultant of the step (a) to an enzyme having a 5' nuclease activity under conditions for cleavage of the PTO; wherein the upstream
25 oligonucleotide or its extended strand induces cleavage of the PTO by the enzyme having a 5' nuclease activity such that the cleavage releases a fragment comprising the 5'-tagging portion or a part of the 5'-tagging portion of the PTO;

(c) hybridizing the fragment released from the PTO with a CPO (Capturing and Promoter Oligonucleotide); wherein the CPO comprises in a 3' to 5' direction (i) a

capturing portion comprising a nucleotide sequence complementary to the 5'-tagging portion or a part of the 5'-tagging portion of the PTO, (ii) a promoter portion inducing transcription; and (iii) a transcription portion; wherein the fragment released from the PTO is hybridized with the capturing portion of the CPO;

5 (d) performing an extension reaction using the resultant of the step (c) and a template-dependent nucleic acid polymerase, wherein the fragment hybridized with the capturing portion of the CPO is extended to form an extended strand comprising an extended sequence complementary to the promoter portion of the CPO, thereby activating the promoter portion;

10 (e) producing the nucleic acid molecule complementary to the transcription portion by use of a polymerase recognizing the activated promoter portion; and

 (f) detecting the nucleic acid molecule, whereby the presence of the nucleic acid molecule indicates the presence of the target nucleic acid sequence.

15 The present inventors have made intensive researches to develop novel approaches to detect target sequences with more improved accuracy and convenience, *inter alia*, in a multiplex manner. As a result, we have established novel protocols for detection of target sequences, in which target detection is accomplished using not only probe hybridization but also enzymatic reactions such as 5' nucleolytic
20 reaction and extension, and extension-dependent transcription, contributing to improvements in the target specificity and sensitivity, process convenience, and workability in multiplex detection. Also, we have found that additional nucleic acid molecules may be produced being dependent on the presence of target sequences during the novel detection protocol described above.

25 The present invention employs successive events following probe hybridization, including the cleavage and extension of a PTO (Probing and Tagging Oligonucleotide), and the extension-dependent nucleic acid production reaction (*e. g.*, transcription); therefore, it is named as a PCET (PTO Cleavage and Extension-Dependent Transcription) assay.

The present invention is directed to not only target-dependent nucleic acid molecule production but also target detection using target-dependent transcription. Except for a detection step, the nucleic acid molecule production and the target detection commonly comprises all the steps. In this regard, the detail descriptions for the nucleic acid production method and the target detection method will be commonly done.

The present invention will be described with reference to each step as follows:

Step (a): Hybridization of an upstream oligonucleotide and a PTO with a target nucleic acid sequence

According to the present invention, a target nucleic acid sequence is first hybridized with an upstream oligonucleotide and a PTO (Probing and Tagging Oligonucleotide).

The term used herein "target nucleic acid", "target nucleic acid sequence" or "target sequence" refers to a nucleic acid sequence of interest for detection, which is annealed to or hybridized with a probe or primer under hybridization, annealing or amplifying conditions.

The term used herein "probe" refers to a single-stranded nucleic acid molecule comprising a portion or portions that are substantially complementary to a target nucleic acid sequence.

The term "primer" as used herein refers to an oligonucleotide, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of primer extension product which is complementary to a nucleic acid strand (template) is induced, *i.e.*, in the presence of nucleotides and an agent for polymerization, such as DNA polymerase, and at a suitable temperature and pH.

In a certain embodiment, the probe and primer are single-stranded deoxyribonucleotide molecules. The probes or primers used in this invention may be comprised of naturally occurring dNMP (*i.e.*, dAMP, dGM, dCMP and dTMP), modified nucleotide, or non-natural nucleotide. The probes or primers may also include

ribonucleotides.

The primer must be sufficiently long to prime the synthesis of extension products in the presence of the agent for polymerization. The exact length of the primers will depend on many factors, including temperature, application, and source of primer. The term "annealing" or "priming" as used herein refers to the apposition of an oligodeoxynucleotide or nucleic acid to a template nucleic acid, whereby the apposition enables the polymerase to polymerize nucleotides into a nucleic acid molecule which is complementary to the template nucleic acid or a portion thereof.

The term used "hybridizing" used herein refers to the formation of a double-stranded nucleic acid from complementary single stranded nucleic acids. The hybridization may occur between two nucleic acid strands perfectly matched or substantially matched with some mismatches. The complementarity for hybridization may depend on hybridization conditions, particularly temperature.

The hybridization of a target nucleic acid sequence with the upstream oligonucleotide and the PTO may be carried out under suitable hybridization conditions routinely determined by optimization procedures. Conditions such as temperature, concentration of components, hybridization and washing times, buffer components, and their pH and ionic strength may be varied depending on various factors, including the length and GC content of oligonucleotide (upstream oligonucleotide and PTO) and the target nucleotide sequence. For instance, when a relatively short oligonucleotide is used, it is suitable that low stringent conditions are adopted. The detailed conditions for hybridization can be found in Joseph Sambrook, et al., *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.(2001); and M.L.M. Anderson, *Nucleic Acid Hybridization*, Springer-Verlag New York Inc. N.Y.(1999).

There is no intended distinction between the terms "annealing" and "hybridizing", and these terms will be used interchangeably.

The upstream oligonucleotide and PTO have hybridizing nucleotide sequences complementary to the target nucleic acid sequence. The term "complementary" is

used herein to mean that primers or probes are sufficiently complementary to hybridize selectively to a target nucleic acid sequence under the designated annealing conditions or stringent conditions, encompassing the terms "substantially complementary" and "perfectly complementary", for instance, perfectly
5 complementary.

The 5'-tagging portion of the PTO comprises a nucleotide sequence non-complementary to the target nucleic acid sequence. The term "non-complementary" is used herein to mean that primers or probes are sufficiently non-complementary not to hybridize selectively to a target nucleic acid sequence under the designated annealing
10 conditions or stringent conditions, encompassing the terms "substantially non-complementary" and "perfectly non-complementary", for instance, perfectly non-complementary.

For example, the term "non-complementary" in conjunction with the 5'-tagging portion of the PTO means that the 5'-tagging portion is sufficiently non-complementary not to hybridize selectively to a target nucleic acid sequence under
15 the designated annealing conditions or stringent conditions, encompassing the terms "substantially non-complementary" and "perfectly non-complementary", for instance, perfectly non-complementary.

The term used herein "PTO (Probing and Tagging Oligonucleotide)" means an
20 oligonucleotide comprising (i) a 3'-targeting portion serving as a probe and (ii) a 5'-tagging portion with a nucleotide sequence non-complementary to the target nucleic acid sequence, which is nucleolytically released from the PTO after hybridization with the target nucleic acid sequence. The 5'-tagging portion and the 3'-targeting portion in the PTO have to be positioned in a 5' to 3' order. The PTO is schematically
25 illustrated in Fig. 1.

In an embodiment, the hybridization in step (a) is preformed under stringent conditions that the 3'-targeting portion is hybridized with the target nucleic acid sequence and the 5'-tagging portion is not hybridized with the target nucleic acid sequence.

The PTO does not require any specific lengths. For example, the length of the PTO may be 15-150 nucleotides, 15-100 nucleotides, 15-80 nucleotides, 15-60 nucleotides, 15-40 nucleotides, 20-150 nucleotides, 20-100 nucleotides, 20-80 nucleotides, 20-60 nucleotides, 20-50 nucleotides, 30-150 nucleotides, 30-100 nucleotides, 30-80 nucleotides, 30-60 nucleotides, 30-50 nucleotides, 35-100 nucleotides, 35-80 nucleotides, 35-60 nucleotides, or 35-50 nucleotides. The 3'-targeting portion of the PTO may be in any lengths so long as it is specifically hybridized with target nucleic acid sequences. For example, the 3'-targeting portion of the PTO may be 10-100 nucleotides, 10-80 nucleotides, 10-50 nucleotides, 10-40 nucleotides, 10-30 nucleotides, 15-100 nucleotides, 15-80 nucleotides, 15-50 nucleotides, 15-40 nucleotides, 15-30 nucleotides, 20-100 nucleotides, 20-80 nucleotides, 20-50 nucleotides, 20-40 nucleotides or 20-30 nucleotides in length. The 5'-tagging portion may be in any lengths so long as it is specifically hybridized with the capturing portion of the CPO and then extended. For instance, the 5'-tagging portion of the PTO may be 5-50 nucleotides, 5-40 nucleotides, 5-30 nucleotides, 5-20 nucleotides, 10-50 nucleotides, 10-40 nucleotides, 10-30 nucleotides, 10-20 nucleotides, 15-50 nucleotides, 15-40 nucleotides, 15-30 nucleotides or 15-20 nucleotides in length.

The 3'-end of the PTO may have a 3'-OH terminal. In certain embodiment, the 3'-end of the PTO is "blocked" to prohibit its extension.

The blocking may be achieved in accordance with conventional methods. For instance, the blocking may be performed by adding to the 3'-hydroxyl group of the last nucleotide a chemical moiety such as biotin, labels, a phosphate group, alkyl group, non-nucleotide linker, phosphorothioate or alkane-diol. Alternatively, the blocking may be carried out by removing the 3'-hydroxyl group of the last nucleotide or using a nucleotide with no 3'-hydroxyl group such as dideoxynucleotide.

Alternatively, the PTO may be designed to have a hairpin structure.

The non-hybridization between the 5'-tagging portion of the PTO and the target nucleic acid sequence refers to non-formation of a stable double-strand

between them under certain hybridization conditions. According to an embodiment of this invention, the 5'-tagging portion of the PTO not involved in the hybridization with the target nucleic acid sequence forms a single-strand.

The upstream oligonucleotide is located upstream of the PTO.

5 In addition, the upstream oligonucleotide or its extended strand hybridized with the target nucleic acid sequence induces cleavage of the PTO by an enzyme having a 5' nuclease activity.

The induction of the PTO cleavage by the upstream oligonucleotide may be accomplished by two fashions: (i) upstream oligonucleotide extension-independent
10 cleavage induction; and (ii) upstream oligonucleotide extension-dependent cleavage induction.

Where the upstream oligonucleotide is positioned adjacently to the PTO sufficient to induce the PTO cleavage by an enzyme having a 5' nuclease activity, the enzyme bound to the upstream oligonucleotide digests the PTO with no extension
15 reaction. In contrast, where the upstream oligonucleotide is positioned distantly to the PTO, an enzyme having a polymerase activity (*e.g.*, template-dependent polymerase) catalyzes extension of the upstream oligonucleotide (*e.g.*, upstream primer) and an enzyme having a 5' nuclease activity bound to the extended product digests the PTO.

Therefore, the upstream oligonucleotide may be located relatively to the PTO in
20 two fashions. The upstream oligonucleotide may be located adjacently to the PTO sufficient to induce the PTO cleavage in an extension-independent manner. Alternatively, the upstream oligonucleotide may be located distantly to the PTO sufficient to induce the PTO cleavage in an extension-dependent manner.

The term used herein "adjacent" with referring to positions or locations means
25 that the upstream oligonucleotide is located adjacently to the 3'-targeting portion of the PTO to form a nick. Also, the term means that the upstream oligonucleotide is located 1-30 nucleotides, 1-20 nucleotides or 1-15 nucleotides apart from the 3'-targeting portion of the PTO.

The term used herein "distant" with referring to positions or locations includes

any positions or locations sufficient to ensure extension reactions.

According to an embodiment, the upstream oligonucleotide is located distantly to the PTO sufficient to induce the PTO cleavage in an extension-dependent manner.

According to an embodiment, the upstream oligonucleotide is an upstream
5 primer or an upstream probe. The upstream primer is suitable in an extension-independent cleavage induction or an extension-dependent cleavage, and the upstream probe is suitable in an extension-independent cleavage induction.

Alternatively, the upstream oligonucleotide may have a partial-overlapped sequence with the 5'-part of the 3'-targeting portion of the PTO. In certain
10 embodiment, the overlapped sequence is 1-10 nucleotides, 1-5 nucleotides or 1-3 nucleotides in length. Where the upstream oligonucleotide has a partial-overlapped sequence with the 5'-part of the 3'-targeting portion of the PTO, the 3'-targeting portion is partially digested along with the 5'-tagging portion in the cleavage reaction of the step (b). In addition, the overlapped sequence permits to cleave a desired site
15 of the 3'-targeting portion.

According to an embodiment, the upstream primer induces through its extended strand the cleavage of the PTO by the enzyme having the 5' nuclease activity.

The conventional technologies for cleavage reactions by upstream
20 oligonucleotides may be applied to the present invention, so long as the upstream oligonucleotide induces cleavage of the PTO hybridized with the target nucleic acid sequence to release a fragment comprising the 5'-tagging portion or a part of the 5'-tagging portion of the PTO. For example, U.S. Pat. Nos. 5,210,015, 5,487,972, 5,691,142, 5,994,069 and 7,381,532 and U.S. Appl. Pub. No. 2008-0241838 may be
25 applied to the present invention.

According to an embodiment, the method is performed in the presence of a downstream primer. The downstream primer generates additionally a target nucleic acid sequence to be hybridized with the PTO, enhancing sensitivity in target detection.

According to an embodiment, when the upstream primer and the downstream

primer are used, a template-dependent nucleic acid polymerase is additionally employed for extension of the primers.

According to an embodiment, the upstream oligonucleotide (upstream primer or upstream probe), the downstream primer and/or 5'-tagging portion of the PTO have a dual priming oligonucleotide (DPO) structure developed by the present inventor. The oligonucleotides having the DPO structure show significantly improved target specificity compared with conventional primers and probes (see WO 2006/095981; Chun et al., Dual priming oligonucleotide system for the multiplex detection of respiratory viruses and SNP genotyping of CYP2C19 gene, *Nucleic Acid Research*, 35:6e40(2007)).

According to an embodiment, the 3'-targeting portion of the PTO has a modified dual specificity oligonucleotide (mDSO) structure developed by the present inventor. The modified dual specificity oligonucleotide (mDSO) structure shows significantly improved target specificity compared with conventional probes (see WO 2011/028041).

Step (b): Release of a fragment from the PTO cleavage

Afterwards, the resultant of the step (a) is contacted to an enzyme having a 5' nuclease activity under conditions for cleavage of the PTO. The PTO hybridized with the target nucleic acid sequence is digested by the enzyme having the 5' nuclease activity to release a fragment comprising the 5'-tagging portion or a part of the 5'-tagging portion of the PTO.

The term used herein "conditions for cleavage of the PTO" means conditions sufficient to digest the PTO hybridized with the target nucleic acid sequence by the enzyme having the 5' nuclease activity, such as temperature, pH, ionic strength, buffer, length and sequence of oligonucleotides and enzymes. For example, when *Taq* DNA polymerase is used as the enzyme having the 5' nuclease activity, the conditions for cleavage of the PTO include Tris-HCl buffer, KCl, MgCl₂ and temperature.

When the PTO is hybridized with the target nucleic acid sequence, its 3'-

targeting portion is involved in the hybridization and the 5'-tagging portion forms a single-strand with no hybridization with the target nucleic acid sequence (see Fig. 2). As such, an oligonucleotide comprising both single-stranded and double-stranded structures may be digested using an enzyme having a 5' nuclease activity by a variety of technologies known to one of skill in the art.

The cleavage sites of the PTO are varied depending on the type of upstream oligonucleotides (upstream probe or upstream primer), hybridization sites of upstream oligonucleotides and cleavage conditions (see U.S. Pat. Nos. 5,210,015, 5,487,972, 5,691,142, 5,994,069 and 7,381,532 and U.S. Appln. Pub. No. 2008-0241838).

A multitude of conventional technologies may be employed for the cleavage reaction of the PTO, releasing a fragment comprising the 5'-tagging portion or a part of the 5'-tagging portion.

Briefly, there may be three sites of cleavage in the step (b). Firstly, the cleavage site is a junction site between a hybridization portion of the PTO (3'-targeting portion) and a non-hybridization portion (5'-tagging portion). The second cleavage site is a site located several nucleotides in a 3'-direction apart from the 3'-end of the 5'-tagging portion of the PTO. The second cleavage site is located at the 5'-end part of the 3'-targeting portion of the PTO. The third cleavage site is a site located several nucleotides in a 5'-direction apart from the 3'-end of the 5'-tagging portion of the PTO.

According to an embodiment, the initial site for the cleavage of the PTO by the template-dependent polymerase having the 5' nuclease activity upon extension of the upstream primer is a starting point of the double strand between the PTO and the target nucleic acid sequence or a site 1-3 nucleotides apart from the starting point.

In this regard, the term used herein "a fragment comprising the 5'-tagging portion or a part of the 5'-tagging portion of the PTO" in conjunction with cleavage of the PTO by the enzyme having the 5' nuclease activity is used to encompass (i) the 5'-tagging portion, (ii) the 5'-tagging portion and the 5'-end part of the 3'-targeting portion and (iii) a part of the 5'-tagging portion. In this application, the term "a

fragment comprising the 5'-tagging portion or a part of the 5'-tagging portion of the PTO" may be also described as "PTO fragment".

According to an embodiment, the PTO has a blocker portion containing a blocker resistant to cleavage by the enzyme having 5' nuclease activity and the
5 blocker portion is used to control an initial cleavage site and/or successive cleavages.

According to an embodiment, the PTO has a blocker portion containing as a blocker at least one nucleotide resistant to cleavage by the enzyme having 5' nuclease activity.

For example, to induce cleavage at the junction site between a hybridization
10 portion of the PTO (3'-targeting portion) and a non-hybridization portion (5'-tagging portion), the 5'-end part of 3'-targeting portion of PTO may be blocked with blockers.

The number of blockers contained in the blocker portion may be not limited, including 1-10, 2-10, 3-8 or 3-6 blockers. The blockers present in the PTO may be in a continuous or intermittent manner, suitably a continuous manner. The nucleotides as
15 blockers with a backbone resistant to the 5' to 3' exonuclease activity include any one known to one of skill in the art. For example, it includes various phosphorothioate linkages, phosphonate linkages, phosphoroamidate linkages and 2'-carbohydrates modifications. According to an embodiment, nucleotides having a backbone resistant to the 5' to 3' exonuclease include phosphorothioate linkage, alkyl phosphotriester
20 linkage, aryl phosphotriester linkage, alkyl phosphonate linkage, aryl phosphonate linkage, hydrogen phosphonate linkage, alkyl phosphoroamidate linkage, aryl phosphoroamidate linkage, phosphoroselenate linkage, 2'-O-aminopropyl modification, 2'-O-alkyl modification, 2'-O-allyl modification, 2'-O-butyl modification, α -anomeric oligodeoxynucleotide and 1-(4'-thio- β -D-ribofuranosyl) modification.

25 According to an embodiment, a nucleotide as a blocker includes LNA(locked nucleic acid).

The term "part" used in conjunction with the PTO or CPO such as the part of the 5'-tagging portion of the PTO, the 5'-end part of the 3'-targeting portion of the PTO and the 5'-end part of the capturing portion of the CPO refers to a nucleotide

sequence composed of 1-40, 1-30, 1-20, 1-15, 1-10 or 1-5 nucleotides, suitably 1, 2, 3 or 4 nucleotides.

According to an embodiment, the enzyme having the 5' nuclease activity is DNA polymerase having a 5' nuclease activity or FEN nuclease, suitably a thermostable DNA polymerase having a 5' nuclease activity or FEN nuclease.

A suitable DNA polymerase having a 5' nuclease activity in this invention is a thermostable DNA polymerase obtained from a variety of bacterial species, including *Thermus aquaticus* (Taq), *Thermus thermophilus* (Tth), *Thermus filiformis*, *Thermis flavus*, *Thermococcus litoralis*, *Thermus antranikianii*, *Thermus caldophilus*, *Thermus chliarophilus*, *Thermus flavus*, *Thermus igniterrae*, *Thermus lacteus*, *Thermus oshimai*, *Thermus ruber*, *Thermus rubens*, *Thermus scotoductus*, *Thermus silvanus*, *Thermus species Z05*, *Thermus species sps 17*, *Thermus thermophilus*, *Thermotoga maritima*, *Thermotoga neapolitana*, *Thermosipho africanus*, *Thermococcus litoralis*, *Thermococcus barossi*, *Thermococcus gorgonarius*, *Thermotoga maritima*, *Thermotoga neapolitana*, *Thermosipho africanus*, *Pyrococcus woesei*, *Pyrococcus horikoshii*, *Pyrococcus abyssi*, *Pyrodictium occultum*, *Aquifex pyrophilus* and *Aquifex aeolicus*. In certain embodiment, the thermostable DNA polymerase is Taq polymerase.

Alternatively, the present invention may employ DNA polymerases having a 5' nuclease activity modified to have less polymerase activities.

The FEN (flap endonuclease) nuclease used is a 5' flap-specific nuclease.

The FEN nuclease suitable in the present invention comprises FEN nucleases obtained from a variety of bacterial species, including *Sulfolobus solfataricus*, *Pyrobaculum aerophilum*, *Thermococcus litoralis*, *Archaeoglobus veneficus*, *Archaeoglobus profundus*, *Acidianus brierlyi*, *Acidianus ambivalens*, *Desulfurococcus amylolyticus*, *Desulfurococcus mobilis*, *Pyrodictium brockii*, *Thermococcus gorgonarius*, *Thermococcus zilligii*, *Methanopyrus kandleri*, *Methanococcus igneus*, *Pyrococcus horikoshii*, *Aeropyrum pernix*, and *Archaeoglobus veneficus*.

Where the upstream primer is used in the step (a), the conditions for cleavage

of the PTO may comprise extension reaction of the upstream primer.

According to an embodiment, the upstream primer is used in the step (a), a template-dependent polymerase is used for extension of the upstream primer and the template-dependent polymerase is identical to the enzyme having the 5' nuclease activity.

Optionally, the upstream primer is used in the step (a), a template-dependent polymerase is used for extension of the upstream primer and the template-dependent polymerase is different from the enzyme having the 5' nuclease activity.

10 **Step (c): Hybridization of the fragment released from the PTO with CPO**

The fragment released from the PTO is hybridized with a CPO (Capturing and Promoter Oligonucleotide).

The CPO comprises in a 3' to 5' direction (i) a capturing portion comprising a nucleotide sequence complementary to the 5'-tagging portion or a part of the 5'-tagging portion of the PTO, (ii) a promoter portion inducing transcription; and (iii) a transcription portion.

The CPO acts as a template for extension of the fragment released from the PTO. The fragment serving as a primer is hybridized with the CPO and extended to form an extended duplex.

20 As described above, when the fragment having the 5'-tagging portion of the PTO is released, the capturing portion of the CPO may be designed to comprise a nucleotide sequence complementary to the 5'-tagging portion. When the fragment having the 5'-tagging portion and a 5'-end part of the 3'-targeting portion is released, the capturing portion of the CPO may be designed to comprise a nucleotide sequence complementary to the 5'-tagging portion and the 5'-end part of the 3'-targeting portion. When the fragment having a part of the 5'-tagging portion of the PTO is released, the capturing portion of the CPO may be designed to comprise a nucleotide sequence complementary to the part of the 5'-tagging portion.

Moreover, it is possible to design the capturing portion of the CPO with

anticipating cleavage sites of the PTO. For example, where the capturing portion of the CPO is designed to comprise a nucleotide sequence complementary to the 5'-tagging portion, either the fragment having a part of the 5'-tagging portion or the fragment having the 5'-tagging portion can be hybridized with the capturing portion and then extended. Where the fragment comprising the 5'-tagging portion and a 5'-end part of the 3'-targeting portion is released, it may be hybridized with the capturing portion of the CPO designed to comprise a nucleotide sequence complementary to the 5'-tagging portion and then successfully extended although mismatch nucleotides are present at the 3'-end portion of the fragment. That is because primers can be extended depending on reaction conditions although its 3'-end contains some mismatch nucleotides (*e.g.* 1-3 mismatch nucleotides).

When the fragment comprising the 5'-tagging portion and a 5'-end part of the 3'-targeting portion is released, the 5'-end part of the capturing portion of the CPO may be designed to have a nucleotide sequence complementary to the cleaved 5'-end part of the 3'-targeting portion, overcoming problems associated with mismatch nucleotides.

In an embodiment, the nucleotide sequence of the 5'-end part of the capturing portion of the CPO complementary to the cleaved 5'-end part of the 3'-targeting portion may be selected depending on anticipated cleavage sites on the 3'-targeting portion of the PTO. The nucleotide sequence of the 5'-end part of the capturing portion of the CPO complementary to the cleaved 5'-end part of the 3'-targeting portion may be 1-10 nucleotides, 1-5 nucleotides or 1-3 nucleotides in length.

The 3'-end of the CPO may comprise additional nucleotides not involved in hybridization with the fragment. Moreover, the capturing portion of the CPO may comprise a nucleotide sequence complementary only to a part of the fragment (*e.g.*, a part of the fragment containing its 3'-end portion) so long as it is stably hybridized with the fragment.

The term used "capturing portion comprising a nucleotide sequence complementary to the 5'-tagging portion or a part of the 5'-tagging portion" is

described herein to encompass various designs and compositions of the capturing portion of the CPO as discussed above.

According to an embodiment, the transcription portion of the CPO comprises a nucleotide sequence non-complementary to the 5'-tagging portion of the PTO, the 3'-
5 targeting portion of the PTO or both of them.

The CPO may be designed to have a hairpin structure.

The promoter portion of the CPO includes a wide variety of promoters inducing transcription. In an embodiment, the promoter portion of the CPO comprises a RNA polymerase promoter.

10 Generally, functional promoters recognized by RNA polymerase are in double strand. In this regard, the term "promoter", "promoter sequence" or "promoter portion" in conjunction with the CPO refers to a promoter sequence in single strand which enables to be a functional promoter in the double-stranded form, particularly meaning a sense promoter sequence connected to a template sequence in
15 transcription.

The promoters for RNA polymerases in this invention comprises any promoter, particularly including promoters recognizable with T7 RNA polymerase, T3 RNA polymerase, SP6 RNA polymerase, *Tth* RNA polymerase, *E. coli* RNA polymerase or their varieties, more particularly T7 RNA polymerase, T3 RNA polymerase, SP6 RNA
20 polymerase or their varieties, still more particularly T7 RNA polymerase, T3 RNA polymerase or SP6 RNA polymerase. The detailed descriptions for T7 RNA polymerase, T3 RNA polymerase or SP6 RNA polymerase, and promoters recognizable with them may be found in Cheetham, et al., *Curr. Op. In Struc. Biol.* 10:117-123(2000) and Rong, et al., *Proc. Natl. Acad. Sci. USA* 95:515-519(1998). The varieties of RNA
25 polymerases are discussed in U.S. Pat. No. 5,849,546, Padilla, R. et al., *Nucleic Acids Res.*, 15: e138(2002) and Sousa, R. et al., *Prog Nucleic Acid Res Mol. Biol.*, 73:1-41(2003).

In certain embodiment, the promoter portion of the CPO comprises a minimum sequence necessary to be recognized by polymerases and induce transcription.

The promoter portion of the CPO is located in the 5'-direction of the capturing portion. The capturing portion and the promoter portion of the CPO are located in a non-overlapped manner or overlapped manner to each other, particularly, a non-overlapped manner.

5 According to an embodiment, 1-50 nucleotides may exist between the capturing portion and the promoter portion.

In the event of the overlapped manner, a part of the PTO fragment may be hybridized with the promoter portion.

Where the capturing portion and the promoter portion are located in an overlapped manner to each other, they are overlapped to the extent that hybridization of an uncleaved PTO with the capturing portion does not induce the activation of the promoter. Therefore, the promoter portion has no activity to promote transcription unless the PTO fragment is extended to generate a complementary sequence to the promoter portion.

15 The transcription portion may comprise any sequence so long as it serves as templates in transcription by polymerases recognizing activated promoters. In an embodiment, the transcription portion comprises non-complementary sequence to the 5'-tagging portion of the PTO, 3'-targeting portion or both of them. As described below, where the present invention is cyclically performed (see Fig. 4), the transcription portion may comprise a complementary sequence to the 5'-tagging portion of the PTO.

The length of the CPO may be widely varied. For example, the CPO is 20-1000 nucleotides, 20-500 nucleotides, 20-300 nucleotides, 20-100 nucleotides, 20-80 nucleotides, 20-60 nucleotides, 20-50 nucleotides, 25-1000 nucleotides, 25-500 nucleotides, 25-300 nucleotides, 25-100 nucleotides, 25-80 nucleotides, 25-60 nucleotides, 25-50 nucleotides, 30-1000 nucleotides, 30-500 nucleotides, 30-300 nucleotides, 30-100 nucleotides, 30-80 nucleotides, 30-60 nucleotides, 30-50 nucleotides, 35-1000 nucleotides, 35-500 nucleotides, 35-300 nucleotides, 35-100 nucleotides, 35-80 nucleotides, 35-60 nucleotides or 35-50 nucleotides in length. The

capturing portion of the CPO may have any length so long as it is specifically hybridized with the fragment released from the PTO. For example, the capturing portion of the CPO is 5-100 nucleotides, 5-60 nucleotides, 5-40 nucleotides, 5-30 nucleotides, 5-20 nucleotides, 10-100 nucleotides, 10-60 nucleotides, 10-40 nucleotides, 10-30 nucleotides, 10-20 nucleotides, 15-100 nucleotides, 15-60 nucleotides, 15-40 nucleotides, 15-30 nucleotides or 15-20 nucleotides in length. The promoter portion of the CPO may have any length so long as it is capable of inducing polymerization (particularly, transcription) by polymerases (particularly, RNA polymerases) recognizing promoters. For example, the promoter portion of the CPO is 10-100 nucleotides, 10-80 nucleotides, 10-60 nucleotides, 10-50 nucleotides, 10-40 nucleotides, 10-30 nucleotides, 10-20 nucleotides, 13-100 nucleotides, 13-80 nucleotides, 13-60 nucleotides, 13-50 nucleotides, 13-40 nucleotides, 13-30 nucleotides, 13-20 nucleotides, 15-100 nucleotides, 15-80 nucleotides, 15-60 nucleotides, 15-50 nucleotides, 15-40 nucleotides, 15-30 nucleotides or 15-20 nucleotides in length. The transcription portion may have any length so long as it can serve as templates in transcription by polymerases recognizing activated promoters. For instance, the transcription portion is 1-900 nucleotides, 1-400 nucleotides, 1-300 nucleotides, 1-100 nucleotides, 1-80 nucleotides, 1-60 nucleotides, 1-40 nucleotides, 1-20 nucleotides, 2-900 nucleotides, 2-400 nucleotides, 2-300 nucleotides, 2-100 nucleotides, 2-80 nucleotides, 2-60 nucleotides, 2-40 nucleotides, 2-20 nucleotides, 5-900 nucleotides, 5-400 nucleotides, 5-300 nucleotides, 5-100 nucleotides, 5-80 nucleotides, 5-60 nucleotides, 5-40 nucleotides, 5-30 nucleotides, 10-900 nucleotides, 10-400 nucleotides, 10-300 nucleotides, 15-900 nucleotides, 15-100 nucleotides, 15-80 nucleotides, 15-60 nucleotides, 15-40 nucleotides or 15-20 nucleotides in length.

The 3'-end of the CPO may have a 3'-OH terminal. In certain embodiment, the 3'-end of the CPO is blocked to prohibit its extension. The non-extendible blocking of the CPO may be achieved in accordance with conventional methods. For instance, the blocking may be performed by adding to the 3'-hydroxyl group of the last nucleotide of the CPO a chemical moiety such as biotin, labels, a phosphate group, alkyl group,

non-nucleotide linker, phosphorothioate or alkane-diol. Alternatively, the blocking may be carried out by removing the 3'-hydroxyl group of the last nucleotide or using a nucleotide with no 3'-hydroxyl group such as dideoxynucleotide.

5 The fragment released from the PTO is hybridized with the CPO, providing a form suitable in extension of the fragment.

The hybridization in the step (c) can be described in detail with referring to descriptions in the step (a).

10 **Step (d): Extension of the fragment by template-dependent nucleic acid polymerase**

The extension reaction is carried out using the resultant of the step (c) and a template-dependent nucleic acid polymerase. The fragment hybridized with the capturing portion of the CPO is extended to form an extended strand comprising an extended sequence complementary to the promoter portion of the CPO, thereby
15 activating the promoter portion. In contrast, uncleaved PTO hybridized with the capturing portion of the CPO is not extended such that no activation of the promoter portion occurs.

The term used herein "extended strand" in conjunction with the fragment means a sequence composed of the fragment and its extended sequence. The term
20 used herein "extended sequence" in conjunction with the fragment means only a newly extended sequence which is a portion of the extended strand except the fragment.

In certain embodiment, the promoter portion of the CPO is recognized by transcription-mediating polymerases (particularly, RNA polymerases) only when it is in
25 double strand.

The term used herein "activation of promoter portion" or "promoter activation" means that the promoter portion of the CPO is allowed to be in double strand and rendered recognizable to transcription-mediating polymerases.

The promoter portion of the CPO becomes activated or functional promoter by

the activation.

In an embodiment, the fragment hybridized with the capturing portion of the CPO is extended to form an extended strand comprising an extended sequence complementary to the promoter portion and transcription portion of the CPO, thereby activating the promoter portion and forming an extended duplex.

The term used herein "extended duplex" means a duplex formed by extension reaction in which the fragment hybridized with the capturing portion of the CPO is extended using the promoter portion and the transcription portion of the CPO as a template and the template-dependent nucleic acid polymerase.

In certain embodiment, the extension of the fragment may be adjusted to the extent that the extension forms an extended strand consisting of an extended sequence complementary only to the promoter portion of the CPO, thereby activating the promoter portion.

The template-dependent nucleic acid polymerase used in the step (d) may include any nucleic acid polymerases, for example, Klenow fragment of *E. coli* DNA polymerase I, a thermostable DNA polymerase and bacteriophage T7 DNA polymerase. Preferably, the polymerase is a thermostable DNA polymerase which may be obtained from a variety of bacterial species, including *Thermus aquaticus* (Taq), *Thermus thermophilus* (Tth), *Thermus filiformis*, *Thermis flavus*, *Thermococcus litoralis*, *Thermus antranikianii*, *Thermus caldophilus*, *Thermus chliarophilus*, *Thermus flavus*, *Thermus igniterrae*, *Thermus lacteus*, *Thermus oshimai*, *Thermus ruber*, *Thermus rubens*, *Thermus scotoductus*, *Thermus silvanus*, *Thermus species Z05*, *Thermus species sps 17*, *Thermus thermophilus*, *Thermotoga maritima*, *Thermotoga neapolitana*, *Thermosipho africanus*, *Thermococcus litoralis*, *Thermococcus barossi*, *Thermococcus gorgonarius*, *Thermotoga maritima*, *Thermotoga neapolitana*, *Thermosipho africanus*, *Pyrococcus furiosus* (Pfu), *Pyrococcus woesei*, *Pyrococcus horikoshii*, *Pyrococcus abyssi*, *Pyrodictium occultum*, *Aquifex pyrophilus* and *Aquifex aeolicus*. Particularly, the template-dependent nucleic acid polymerase is Taq polymerase.

In certain embodiment, the template-dependent nucleic acid polymerase used in the step (d) is reverse transcriptase.

According to an embodiment, the enzyme having the 5' nuclease activity used in the step (b) is identical to the template-dependent nucleic acid polymerase used in the step (d). More particularly, the enzyme having the 5' nuclease activity used in the step (b), the template-dependent nucleic acid polymerase used for extension of the upstream primer and the template-dependent nucleic acid polymerase used in the step (d) are identical to one another.

10 **Step (e): Production of nucleic acid molecules by activated promoter-recognizing polymerase**

Afterwards, the nucleic acid molecule complementary to the transcription portion is produced by use of a polymerase recognizing the activated promoter portion.

15 The polymerase recognizing the activated promoter portion used in this invention comprises a variety of polymerases known to one of skill in the art, particularly including T7 RNA polymerase, T3 RNA polymerase, SP6 RNA polymerase, *Tth* RNA polymerase, *E. coli* RNA polymerase or their varieties, more particularly T7 RNA polymerase, T3 RNA polymerase, SP6 RNA polymerase or their varieties, still
20 more particularly T7 RNA polymerase, T3 RNA polymerase or SP6 RNA polymerase.

In certain embodiment, the polymerase recognizing the activated promoter is a thermostable polymerase.

The transcription may be under isothermal or non-isothermal conditions, particularly isothermal conditions.

25 In certain embodiment, the nucleic acid molecule produced in the step (e) is RNA. Depending on reaction conditions and type of polymerases, DNA may be produced in the step (e).

According to the present invention, copies of plural nucleic acid molecules may be produced from one PTO fragment. Because the nucleic acid molecule to be

produced may be arbitrarily selected, nucleic molecules with various sequences may be produced.

The production of the nucleic acid molecule is dependent on the presence of a target nucleic acid sequence and is therefore capable of indicating the presence of a target nucleic acid sequence.

The term used herein "transcription" refers to production of DNA or RNA using templates (including DNA and RNA) by polymerases recognizing promoters, particularly production of RNA using DNA templates by RNA polymerases recognizing promoters.

10

One of the striking features of the present invention lies in the fact in which the present invention may be carried out in a cyclic manner. By such a cyclic performance, the present invention enables to amplify not only nucleic acid molecules (particularly, RNA) in a target-dependent manner but also signal indicating the presence of a target nucleic acid sequence.

In the cyclic embodiment, the nucleic acid molecule produced in the step (e) comprises a complementary nucleotide sequence to a portion positioned in the 3'-direction of the transcription portion of the CPO (particularly, the capturing portion or the promoter portion of the CPO) and hybridization of the nucleic acid molecule with the CPO causes additional production of the nucleic acid molecule.

In certain embodiment, the method further comprises after the step (e) the following steps (e-1) to (e-3): (e-1) hybridizing the nucleic acid molecule with the CPO; wherein the nucleic acid molecule comprises a complementary nucleotide sequence to a portion positioned in the 3'-direction of the transcription portion of the CPO; (e-2) performing an extension reaction using the resultant of the step (e-1) and a template-dependent nucleic acid polymerase, wherein the nucleic acid molecule hybridized with the CPO is extended to form an extended strand comprising an extended sequence complementary to the promoter portion of the CPO, thereby activating the promoter portion; and (e-3) producing the nucleic acid molecule

complementary to the transcription portion by use of a polymerase recognizing the activated promoter portion (see Fig. 4).

In certain embodiment, the steps (e-1) to (e-3) are repeated at least twice, in order to amplify either nucleic acid molecules (particularly, RNA) in a target-dependent manner or signal to a target nucleic acid sequence.

In certain embodiment, the nucleic acid molecule produced in the step (e) comprises a complementary nucleotide sequence to the capturing portion of the CPO and the nucleic acid molecule hybridized with the capturing portion of the CPO in the step (e-1) is extended to form an extended strand comprising an extended sequence complementary to the promoter portion and the transcription portion of the CPO, thereby forming an extended duplex.

In an embodiment, the method further comprises after the step (e) the following steps (e-1) to (e-2): (e-1) hybridizing the nucleic acid molecule with the CPO; wherein the nucleic acid molecule comprises a complementary nucleotide sequence to the promoter portion of the CPO; hybridization of the nucleic acid molecule with the promoter portion induces the activation of the promoter portion; and (e-2) producing the nucleic acid molecule complementary to the transcription portion by use of recognizing the activated promoter portion.

In certain embodiment, the steps (e-1) to (e-2) are repeated at least twice, in order to amplify either nucleic acid molecules (particularly, RNA) in a target-dependent manner or signal to a target nucleic acid sequence.

In certain embodiment, the fragment released from the PTO and the nucleic acid molecule produced in the step (e) is hybridized with the same site or different sites from each other on the CPO.

Moreover, the present invention may produce a further nucleic acid molecule by hybridizing the produced nucleic acid molecule with additional oligonucleotide (see Fig. 3). In certain embodiment, the method further comprises after the step (e) hybridizing the nucleic acid molecule produced in the step (e) with a TCPO (Transcript-Capturing and Promoter Oligonucleotide); wherein the TCPO comprises in

a 3' to 5' direction (i) a transcript-capturing portion comprising a nucleotide sequence complementary to the nucleic acid molecule, (ii) a promoter portion inducing transcription, and (iii) a transcription portion; wherein hybridization of the nucleic acid molecule with the TCPO causes production of a nucleic acid molecule complementary to the transcription portion.

In an embodiment, the transcript-capturing portion and the promoter portion of the TCPO are located in a non-overlapped manner or overlapped manner to each other.

The detailed descriptions for the TCPO may be done with reference to those of the CPO except for the fact that the TCPO is designed to have the transcript-capturing portion comprising a nucleotide sequence complementary to the produced nucleic acid molecule other than the PTO fragment.

In an embodiment, the transcription portion of the TCPO is the same as or different from that of the CPO.

The CPO and TCPO are typically designed to hybridize with the PTO fragment and the produced nucleic acid molecule, respectively. Alternatively, the CPO may be designed to play a role as the TCPO, and *vice versa* for the TCPO.

The second format of the cyclic embodiment using the TCPO may be carried out in the same manner as the first format using the CPO.

The third format of the cyclic embodiment employs both the CPO and the TCPO. For instance, the nucleic acid molecule produced by use of the CPO is hybridized with the TCPO and allows to produce a nucleic acid molecule by use of the TCPO, after which the nucleic acid molecule produced by use of the TCPO is hybridized with the CPO and allows to produce a nucleic acid molecule by use of the CPO.

The cyclic embodiments of the present invention may be provided with various combinations of the CPO and the TCPO.

In certain embodiment, the method further comprises after the step (e) the following steps (e-1) to (e-3): (e-1) hybridizing the nucleic acid molecule with the

TCPO; wherein the nucleic acid molecule comprises a complementary nucleotide sequence to the transcript-capturing portion of the TCPO; (e-2) performing an extension reaction using the resultant of the step (e-1) and a template-dependent nucleic acid polymerase, wherein the nucleic acid molecule hybridized with the TCPO is extended to form an extended strand comprising an extended sequence complementary to the promoter portion of the TCPO, thereby activating the promoter portion; and (e-3) producing the nucleic acid molecule complementary to the transcription portion by use of a polymerase recognizing the activated promoter portion.

10 In an embodiment, the steps (e-1) to (e-3) are repeated at least twice, in order to amplify either nucleic acid molecules (particularly, RNA) in a target-dependent manner or signal to a target nucleic acid sequence.

In certain embodiment, the method further comprises after the step (e) the following steps (e-1) to (e-2): (e-1) hybridizing the nucleic acid molecule with the TCPO; wherein the nucleic acid molecule comprises a complementary nucleotide sequence to the promoter portion of the TCPO; hybridization of the nucleic acid molecule with the promoter portion induces the activation of the promoter portion; and (e-2) producing the nucleic acid molecule complementary to the transcription portion by use of a polymerase recognizing the activated promoter portion.

20 In certain embodiment, the steps (e-1) to (e-2) are repeated at least twice, in order to amplify either nucleic acid molecules (particularly, RNA) in a target-dependent manner or signal to a target nucleic acid sequence.

According to an embodiment, the template-dependent nucleic acid polymerases used for extension of the upstream primer on the target nucleic acid, PTO fragment on CPO and nucleic acid molecule on CPO, TCPO or detection oligonucleotide are identical to each another.

According to an embodiment, the template-dependent nucleic acid polymerases used for extension reactions in the present invention may be a combination of at least two types.

According to an embodiment, the polymerases recognizing the activated promoter portion used for transcription reactions in the present invention is a single type or combination of at least two types.

5 **Step (f): Detection of nucleic acid molecules**

Finally, the produced nucleic acid molecule is detected. The presence of the nucleic acid molecule indicates the presence of the target nucleic acid sequence.

The produced nucleic acid molecule may be detected by a multitude of electrophoresis techniques such as gel electrophoresis, polyacrylamide gel electrophoresis and capillary electrophoresis.

The produced nucleic acid molecule may be detected using various labeling systems known to those of skill in the art.

In certain embodiment, the detection of the nucleic acid molecule in the step (f) is performed using a detection oligonucleotide to be hybridized with the nucleic acid molecule; wherein the detection oligonucleotide, the nucleic acid molecule or each of them has at least one label; and the detection of the nucleic acid molecule is performed by measuring a signal provided from the label.

The detection oligonucleotide may be a new oligonucleotide or CPO. The CPO may play a role as the detection oligonucleotide (see Figs. 8 and 9). In addition, TCPO may play a role as the detection oligonucleotide.

In certain embodiment, the detection oligonucleotide has a single label or an interactive dual label.

In an embodiment, the nucleic acid molecule has a single label or a plurality of the single label.

In an embodiment, each of the detection oligonucleotide and the nucleic acid molecule has a label, the label on the detection oligonucleotide and the label on the nucleic acid molecule form an interactive dual label.

(i) Single label

The present invention may provide signal using a single label for the production of the nucleic acid molecule indicating the presence of the target nucleic acid sequence.

The single label includes, but not limited to, a chemical label (*e.g.*, biotin), an enzymatic label (*e.g.*, alkaline phosphatase, peroxidase, β -galactosidase and β -glucosidase), a radioisotope label (*e.g.*, I^{125} and C^{14}), a fluorescent label, a luminescent label, a chemiluminescent label, and a metal label (*e.g.*, gold).

In certain embodiment, the single label is linked to either the detection oligonucleotide or the nucleic acid molecule.

In certain embodiment, the nucleic acid molecule has the label by performing the step (e) using a labeled dNTP or NTP. In this case, a plurality of single-typed label is incorporated into the nucleic acid molecule. The single label may be incorporated into a specific site of the nucleic acid molecule by use of a non-natural base capable of base-pairing with certain non-natural base, which is disclosed in U.S. Pat. No. 7,422,850.

In certain embodiment, the single label used in this invention is a label capable of providing a detectable signal change (*e.g.*, intensity change) upon hybridization between the nucleic acid molecule and the detection oligonucleotide.

In certain embodiment, the single label is a fluorescent label which generates signals with different intensities depending on whether the nucleic acid molecule and the detection oligonucleotide are hybridized to form a double strand or not hybridized to form a single strand.

The production of the nucleic acid molecule (*i.e.*, the presence of the target nucleic acid sequence) may be detected by measuring intensity changes (increase or decrease) in a fluorescent signal from the fluorescent label.

The types and positions of the fluorescent label are disclosed in U.S. Pat. Nos. 7,537,886 and 7,348,141. Particularly, the fluorescent label includes JOE, FAM, TAMRA, ROX and fluorescein-based label.

In certain embodiment, the single label on the detection oligonucleotide is

located at the 5'-end or at 1-15 nucleotides, 1-10 nucleotides or 1-5 nucleotides apart from the 5'-end. Alternatively, the single label is located at the 3'-end or at 1-15 nucleotides, 1-10 nucleotides or 1-5 nucleotides apart from the 3'-end of the detection oligonucleotide. Alternatively, the single label is located around the center of the detection oligonucleotide.

In certain embodiment, the nucleic acid molecule has a single label. As illustrated in Fig. 7, the nucleic acid molecule is allowed to have a fluorescent label (a plurality of single-typed label depending on reactions) by performing the step (e) using dNTP or NTP with fluorescent label. The signal for the target nucleic acid sequence may be obtained by hybridization of the fluorescent label-nucleic acid molecule with the detection oligonucleotide immobilized on a solid substrate.

The label may be linked to the detection oligonucleotide or the nucleic acid molecule by conventional methods. For instance, the label is linked to the detection oligonucleotide or the nucleic acid molecule through a spacer containing carbon atoms (*e.g.*, 3-carbon spacer, 6-carbon spacer or 12-carbon spacer).

(ii) Interactive dual label

The present invention may provide signal using an interactive dual label for the production of the nucleic acid molecule indicating the presence of the target nucleic acid sequence.

The interactive label system is a signal generating system in which energy is passed non-radioactively between a donor molecule and an acceptor molecule. As a representative of the interactive label system, the FRET (fluorescence resonance energy transfer) label system includes a fluorescent reporter molecule (donor molecule) and a quencher molecule (acceptor molecule). In FRET, the energy donor is fluorescent, but the energy acceptor may be fluorescent or non-fluorescent. In another form of interactive label systems, the energy donor is non-fluorescent, *e.g.*, a chromophore, and the energy acceptor is fluorescent. In yet another form of interactive label systems, the energy donor is luminescent, *e.g.* bioluminescent,

chemiluminescent, electrochemiluminescent, and the acceptor is fluorescent. The donor molecule and the acceptor molecule may be described as a reporter molecular and a quencher molecule in the present invention, respectively.

According to an embodiment of this invention, the signal for the production of the nucleic acid molecule (*i.e.*, the presence of the target nucleic acid sequence) is generated by interactive label systems, particularly the FRET label system (*i.e.*, interactive dual label system).

The reporter molecule and the quencher molecule useful in the present invention may include any molecules known in the art. Examples of those are: Cy2™ (506), YO-PRO™-1 (509), YOYO™-1 (509), Calcein (517), FITC (518), FluorX™ (519), Alexa™ (520), Rhodamine 110 (520), Oregon Green™ 500 (522), Oregon Green™ 488 (524), RiboGreen™ (525), Rhodamine Green™ (527), Rhodamine 123 (529), Magnesium Green™ (531), Calcium Green™ (533), TO-PRO™-1 (533), TOTO1 (533), JOE (548), BODIPY530/550 (550), DiI (565), BODIPY TMR (568), BODIPY558/568 (568), BODIPY564/570 (570), Cy3™ (570), Alexa™ 546 (570), TRITC (572), Magnesium Orange™ (575), Phycoerythrin R&B (575), Rhodamine Phalloidin (575), Calcium Orange™ (576), Pyronin Y (580), Rhodamine B (580), TAMRA (582), Rhodamine Red™ (590), Cy3.5™ (596), ROX (608), Calcium Crimson™ (615), Alexa™ 594 (615), Texas Red (615), Nile Red (628), YO-PRO™-3 (631), YOYO™-3 (631), R-phycoerythrin (642), C-Phycocyanin (648), TO-PRO™-3 (660), TOTO3 (660), DiD DiI (665), Cy5™ (670), Thiadicarbocyanine (671), Cy5.5 (694), HEX (556), TET (536), Biosearch Blue (447), CAL Fluor Gold 540 (544), CAL Fluor Orange 560 (559), CAL Fluor Red 590 (591), CAL Fluor Red 610 (610), CAL Fluor Red 635 (637), FAM (520), Fluorescein (520), Fluorescein-C3 (520), Pulsar 650 (566), Quasar 570 (667), Quasar 670 (705) and Quasar 705 (610). The numeric in parenthesis is a maximum emission wavelength in nanometer. For example, the reporter molecule and the quencher molecule include JOE, FAM, TAMRA, ROX and fluorescein-based label.

Suitable pairs of reporter-quencher are disclosed in a variety of publications as follows: Pesce et al., editors, Fluorescence Spectroscopy (Marcel Dekker, New York,

1971); White et al., Fluorescence Analysis: A Practical Approach (Marcel Dekker, New York, 1970); Berlman, Handbook of Fluorescence Spectra of Aromatic Molecules, 2nd Edition (Academic Press, New York, 1971); Griffiths, Color AND Constitution of Organic Molecules (Academic Press, New York, 1976); Bishop, editor, Indicators (Pergamon Press, Oxford, 1972); Haugland, Handbook of Fluorescent Probes and Research Chemicals (Molecular Probes, Eugene, 1992); Pringsheim, Fluorescence and Phosphorescence (Interscience Publishers, New York, 1949); Haugland, R. P., Handbook of Fluorescent Probes and Research Chemicals, 6th Edition (Molecular Probes, Eugene, Oreg., 1996) U.S. Pat. Nos. 3,996,345 and 4,351,760.

10 It is noteworthy that a non-fluorescent black quencher molecule (or dark quencher molecule) capable of quenching a fluorescence of a wide range of wavelengths or a specific wavelength may be used in the present invention. Examples of those are BHQ and DABCYL.

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

(ii-a) Intrastrand interactive dual label

20 In certain embodiment, the detection oligonucleotide has an interactive dual label comprised of a reporter molecule and quencher molecule, and hybridization of the detection oligonucleotide with the nucleic acid molecule causes signal change from the interactive dual label to provide a detectable signal. The nucleic acid molecule may be detected using the dual-labeled detection oligonucleotide by the
25 molecular beacon method (Tyagi et al, Nature Biotechnology v.14 MARCH 1996) or the self quenching probe method (U.S. Pat. No. 5,876,930) (see Fig. 5).

(ii-b) Interstrand interactive dual label

The interactive dual label system comprised of the labeled nucleic acid

molecule and the labeled detection oligonucleotide may be used to detect the nucleic acid molecule. In interstrand interactive dual label system, the detection oligonucleotide is labeled by one of a reporter molecule and quencher molecule and the nucleic acid molecule by the other of a reporter molecule and quencher molecule, and hybridization of the detection oligonucleotide with the nucleic acid molecule causes signal change from the interactive dual label to provide a detectable signal.

In certain embodiment, the nucleic acid molecule has the label by performing the step (e) using a labeled dNTP or NTP. In this case, a plurality of single-typed label is incorporated into the nucleic acid molecule. The single label may be incorporated into a specific site of the nucleic acid molecule by use of a non-natural base capable of base-pairing with certain non-natural base, which is disclosed in U.S. Pat. No. 7,422,850.

In an embodiment, the interactive dual label comprises two detection oligonucleotides each of which is labeled with one of a reporter molecule and quencher molecule. The two detection oligonucleotides are hybridized adjacently to each other with the nucleic acid molecule, providing signals for the nucleic acid molecule.

Alternatively, the detection of the nucleic acid molecule may be synchronized with extension of the nucleic acid molecule on the detection oligonucleotide.

In certain embodiment, the nucleic acid molecule is hybridized with the detection oligonucleotide and then extended on the detection oligonucleotide as templates to form an extended duplex, thereby providing a detectable signal change. In certain embodiment, the detection oligonucleotide comprises in a 3' to 5' direction (i) a capturing portion comprising a nucleotide sequence complementary to the nucleic acid molecule; and (ii) a templating portion.

As illustrated in Fig. 6, where the target nucleic acid sequence is present, the produced nucleic acid molecule is hybridized with the detection oligonucleotide and then extended, and the reporter molecule and the quencher molecule on the

detection oligonucleotide are conformationally separated to allow the quencher molecule to unquench the signal from the reporter molecule, thereby inducing signal change (*e.g.*, signal increase from the reporter molecule) to provide a detectable signal.

5 In certain embodiment, the dual label is linked to both ends of the detection oligonucleotide.

The expression used herein "the reporter molecule and the quencher molecule are conformationally adjacent" means that the reporter molecule and the quencher molecule are three-dimensionally adjacent to each other by a conformational structure of the detection oligonucleotide such as random coil and hairpin structure.

The expression used herein "the reporter molecule and the quencher molecule are conformationally separated" means that the reporter molecule and the quencher molecule are three-dimensionally separated by change of a conformational structure of the detection oligonucleotide upon the formation of a double strand.

15 Fig. 9 illustrates detection of the nucleic acid molecule using the CPO as the detection oligonucleotide and formation of the extended duplex. TCPO can be used as a detection oligonucleotide.

The detection of the nucleic acid molecule using formation of the extended duplex may employ various labeling approaches as well as the dual label. As described above, the extended duplex is rendered to have a single label or a dual label by use of single or dual labels linked to (i) the detection oligonucleotide, (ii) the nucleic acid molecule or (iii) the detection oligonucleotide and the nucleic acid molecule, or their combinations. Furthermore, labels may be incorporated into the extended duplex by use of labeled dNTP or NTP during formation of the extended duplex.

25 In certain embodiment, the labels used in this invention provide a detectable signal during formation of the extended duplex or melting (or hybridization) of the extended duplex.

The detection method using formation of the extended duplex is characterized

in that the T_m value of the extended duplex is adjustable by sequence and length of the nucleic acid molecule and/or the detection oligonucleotide.

The detection method using formation of the extended duplex will be described with reference to disclosures of WO2012/096523, which is hereby incorporated by
5 references into this application.

Where the nucleic acid molecule is detected by forming the its extended strand, oligonucleotides to be hybridized with the extended strand may be used (see PCT/KR2012/005281).

As described above, the signal for the formation of the nucleic acid molecule
10 (*i.e.*, the presence of the target nucleic acid sequence) may be obtained using various labels. Furthermore, the generation of the signal may be associated with hybridization of the nucleic acid molecule with the detection oligonucleotide or the hybridization plus extension.

The method using hybridization of the detection oligonucleotide and the nucleic
15 acid molecule, or the detection oligonucleotide and the extended strand of the nucleic acid molecule may provide a detectable signal by a melting analysis or hybridization analysis.

The term used herein "melting analysis" means a method in which a target signal indicative of the presence of the produced nucleic acid molecule is obtained by
20 melting of a duplex, including a method to measure signals at two different temperatures, melting curve analysis, melting pattern analysis and melting peak analysis.

The term used herein "hybridization analysis" (or "annealing analysis") means a method in which a target signal indicative of the presence of the produced nucleic
25 acid molecule is obtained during the formation of a duplex, including a method to measure signals at two different temperatures, hybridization curve analysis, hybridization pattern analysis and hybridization peak analysis.

In general, where a target signal can be generated by the melting analysis, it also may be obtained by the hybridization analysis; and *vice versa*. Unless otherwise

indicated herein, the term "melting analysis" is intended to encompass the hybridization analysis.

The melting curve or hybridization curve may be obtained by conventional technologies, for example, as described in U.S. Pat Nos. 6,174,670 and 5,789,167, 5 Drobyshev et al, *Gene* 188: 45(1997); Kochinsky and Mirzabekov *Human Mutation* 19:343(2002); Livehits et al *J. Biomol. Structure Dynam.* 11:783(1994); and Howell et al *Nature Biotechnology* 17:87(1999). For example, a melting curve or hybridization curve may consist of a graphic plot or display of the variation of the output signal with the parameter of hybridization stringency. Output signal may be plotted directly 10 against the hybridization parameter. Typically, a melting curve or hybridization curve will have the output signal, for example fluorescence, which indicates the degree of duplex structure (*i.e.* the extent of hybridization), plotted on the Y-axis and the hybridization parameter on the X axis.

In certain embodiment, the melting analysis is performed at least twice for 15 quantitative analysis. The area or height of melting peaks obtained in melting analysis is affected by the extended duplex, providing information as to the initial amount of target nucleic acid sequences. The cycle number of melting analysis at which the melting peak area or height crosses a threshold value is measured to quantify the amount of target nucleic acid sequences.

20 Alternatively, melting analysis results (*e.g.*, the melting peak area or height) are plotted against each cycle number (or cumulative cycle number) of melting analysis and compared, thereby quantifying the amount of target nucleic acid sequences.

Besides the methods discussed above, numerous target detection methods 25 known to one of skill in the art may be applied for detection of the nucleic acid molecule.

The primer, PTO, CPO, TCPO and detection oligonucleotide may be comprised of naturally occurring dNMPs and/or NMPs. Alternatively, the primer, PTO, CPO, TCPO and detection oligonucleotide may be comprised of modified nucleotide or non-natural

nucleotide such as PNA (peptide nucleic acid, see PCT Publication No. WO 92/20702) and LNA (locked nucleic acid, see PCT Publication Nos. WO 98/22489, WO 98/39352 and WO 99/14226). The primer, PTO, CPO, TCPO and detection oligonucleotide may comprise universal bases such as deoxyinosine, inosine, 1-(2'-deoxy-beta-D-ribofuranosyl)-3-nitropyrrole and 5-nitroindole. The term "universal base" refers to one capable of forming base pairs with each of the natural DNA/RNA bases with little discrimination between them.

As described above, the PTO may be cleaved at a site located in a 3'-direction apart from the 3'-end of the 5'-tagging portion of the PTO. The cleavage site may be located at the 5'-end part of the 3'-targeting portion of the PTO. Where the PTO fragment comprises the 5'-end part of the 3'-targeting portion of the PTO, a site of the CPO hybridized with the 5'-end part of the 3'-targeting portion may comprise a universal base, degenerate sequence or their combination. For instance, if the PTO is cleaved at a site located one nucleotide in a 3'-direction apart from the 3'-end of the 5'-tagging portion of the PTO, it is advantageous that the 5'-end part of the capturing portion of the CPO comprises a universal base for hybridization with the nucleotide. If the PTO is cleaved at a site located two nucleotides in a 3'-direction apart from the 3'-end of the 5'-tagging portion of the PTO, it is advantageous that the 5'-end of the capturing portion of the CPO comprises a degenerate sequence and its 3'-direction-adjacent nucleotide comprises a universal base. As such, where the cleavage of the PTO occurs at various sites of the 5'-end part of the 3'-targeting portion, the utilization of universal bases and degenerate sequences in the CPO is useful. In addition, where the PTOs having the same 5'-tagging portion are used for screening multiple target nucleic acid sequences under upstream primer extension-dependent cleavage induction, the PTO fragments having different 5'-end parts of the 3'-targeting portion may be generated. In such cases, universal bases and degenerate sequences are usefully employed in the CPO. The strategies using universal bases and degenerate sequences in the CPO ensure to use one type or minimal types of the CPO for screening multiple target nucleic acid sequences.

In certain embodiment, the present method is performed under isothermal conditions. If necessary, the temperature may be changed in ranges of $\pm 10^{\circ}\text{C}$ or $\pm 5^{\circ}\text{C}$.

In certain embodiment, some of the steps (a)-(f) are performed under non-isothermal conditions and some performed under isothermal conditions.

5 According to an embodiment, the present method further comprises repeating all or some of the steps (a)-(f) with denaturation between repeating cycles. This repetition permits to amplify the target nucleic acid sequence and/or the target signal. According to an embodiment, the steps (a)-(b), (a)-(d), (a)-(e) or (a)-(f) may be repeated with denaturation. The steps (c)-(e) may be repeated. The denaturation
10 may be carried out by conventional technologies, including, but not limited to, heating, alkali, formamide, urea and glyoxal treatment, enzymatic methods (e.g., helicase action), and binding proteins. For instance, the melting 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., *Molecular Cloning, A Laboratory*
15 *Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.(2001).

 According to an embodiment, the steps (a)-(f) are performed in a single reaction vessel or some of the steps (a)-(f) are performed in separate vessels. For example, the steps (a)-(b), (a)-(d), (c)-(e) and (f) may be performed in a single reaction vessel or separate reaction vessels. For example, where the sequences of the
20 PTO and CPO, and the reaction conditions are determined such that the hybridization between the 3'-targeting portion of the PTO and the target nucleic acid sequence may be performed under higher stringent conditions than the hybridization between the PTO fragment and the CPO, the steps (a)-(b) may be repeated with no undertaking the steps (c)-(f). Following the repetition of the steps (a)-(b), the steps (c)-(f) may be
25 performed.

In certain embodiment, the transcription reaction is performed in a separate vessel.

It would be appreciated by one of skill in the art that repetition of certain steps, intervention of denaturation in repetition, separate performance of certain

step(s) and time point of detection may be widely varied.

According to an embodiment, where the repetition is performed with denaturation using the upstream primer to the PTO, the repetition is carried out in the presence of a downstream primer, particularly according to PCR. The use of the
5 upstream primer and downstream primer to the PTO can amplify the target nucleic acid sequence.

According to an embodiment, where the repetition is performed with denaturation using the upstream probe to the PTO, the repetition is carried out in the presence of a downstream primer to the PTO.

10 The present invention does not require that target nucleic acid sequences to be detected and/or amplified have any particular sequence or length, including any DNA (gDNA and cDNA) and RNA molecules. The target nucleic acid sequence may be in a single- or double-strand.

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

20 The target nucleic acid sequences which may be detected and/or amplified include any naturally occurring prokaryotic, eukaryotic (for example, protozoans and parasites, fungi, yeast, higher plants, lower and higher animals, including mammals and humans) or viral (for example, Herpes viruses, HIV, influenza virus, Epstein-Barr virus, hepatitis virus, polio virus, etc.) or viroid nucleic acid.

25 The target nucleic acid sequence to be detected by the present invention includes a wide variety of nucleic acid sequences, *e.g.*, sequences in a genome, artificially isolated or fragmented sequences and synthesized sequences (*e.g.*, cDNA sequences and barcode sequences). For instance, the target nucleic acid sequence includes nucleic acid marker sequences for Immuno-PCR (IPCR). IPCR employs

conjugates between nucleic acid marker sequences and antibodies together with PCR, which is widely applied for detecting various types of targets including proteins (see Sano et al., Science 258 pp:120-122(1992), U.S. Pat. No. 5,665,539, Niemeyer et al., Trends in Biotechnology 23 pp:208-216(2005), U.S. Pat. Pub. No. 2005/0239108 and
5 Ye et al., Journal of Environmental Science 22 pp:796-800(2010)).

The target nucleic acid molecule of the present invention includes nucleic acid markers as used in IPCR method and the present invention may be applied to detect nucleic acid markers in IPCR method.

The present invention is also useful in detection of a nucleotide variation.
10 Preferably, the target nucleic acid sequence comprises a 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
15 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),
20 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.

25 In the present invention for detection of a nucleotide variation in a target nucleic acid sequence, where primers or probes used have a complementary sequence to the nucleotide variation in the target nucleic acid sequence, the target nucleic acid sequence containing the nucleotide variation is described herein as a matching template. Where primers or probes used have a non-complementary

sequence to the nucleotide variation in the target nucleic acid sequence, the target nucleic acid sequence containing the nucleotide variation is described herein as a mismatching template.

For detection of nucleotide variations, the 3'-end of the upstream primer may
5 be designed to be opposite to a site of a nucleotide variation in a target nucleic acid sequence. According to an embodiment, the 3'-end of the upstream primer has a complementary sequence to the nucleotide variation in a target nucleic acid sequence. The 3'-end of the upstream primer having a complementary sequence to the nucleotide variation in the target nucleic acid sequence is annealed to the matching
10 template and extended to induce cleavage of the PTO. The resultant PTO fragment is hybridized with the CPO and extended, and the nucleic acid molecule is produced to provide the target signal. In contrast, where the 3'-end of the upstream primer is mismatched to a nucleotide variation in a mismatching template, it is not extended under conditions that annealing of the 3'-end of primers is essential for extension
15 even when the upstream primer is hybridized with the mismatching template, thereby resulting in no generation of the target signal.

Alternatively, it is possible to use PTO cleavage depending on the hybridization of PTO having a complementary sequence to a nucleotide variation in a target nucleic acid sequence. For example, under controlled conditions, a PTO having a
20 complementary sequence to the nucleotide variation in the target nucleic acid sequence is hybridized with the matching template and then cleaved. The resultant PTO fragment is hybridized with the CPO and extended, and the nucleic acid molecule is produced to provide the target signal. While, under the controlled conditions, the PTO is not hybridized with a mismatching template having non-complementary
25 sequence in the nucleotide variation position and not cleaved. Preferably, in this case, the complementary sequence to the nucleotide variation in the PTO is positioned at its middle of the 3'-targeting portion of the PTO.

According to an embodiment, the use of an artificial mismatch nucleotide enhances discrimination potential of the PTO to nucleotide variations.

Alternatively, the present invention uses the PTO having the nucleotide variation discrimination site positioned on the 5'-end part of the 3'-targeting portion for selectivity of the PTO to a specific nucleotide variation. The 5'-end part of the 3'-targeting portion of the PTO is positioned to a nucleotide variation in a target nucleic acid sequence for the detection of the nucleotide variation and the 5'-end part of the 3'-targeting portion of the PTO has a complementary sequence to the nucleotide variation in a target nucleic acid sequence.

Where the PTO is hybridized with the target nucleic acid sequence (*i.e.*, match template) having the nucleotide variation complementary to the nucleotide variation discrimination site, the 5'-end part of the 3'-targeting portion forms a double strand with the match template; however, where the PTO is hybridized with a target nucleic acid sequence (*i.e.*, mismatch template) having a nucleotide variation non-complementary to the nucleotide variation discrimination site, the 5'-end part of the 3'-targeting portion does not form a double strand with the mismatch template.

The term used herein "nucleotide variation discrimination site" with reference to the PTO is a complementary sequence on the 5'-end part of the 3'-targeting portion of the PTO to a nucleotide variation in a target nucleic acid sequence.

It is noteworthy that such distinct hybridization patterns on the nucleotide variation of interest are responsible for differences in initial cleavage sites of the PTO, thereby producing two types of PTO fragments to give signal differentiation depending on the presence of the nucleotide variation of interest.

In the presence of the nucleotide variation of interest, a first fragment is generated by cleavage of hybrid between the PTO and matching template, and in the absence of the nucleotide variation of interest, a second fragment is generated by cleavage of hybrid between the PTO and mismatching template. The second fragment comprises an additional 3'-end portion rendering the second fragment to be different from the first fragment.

In an embodiment for the detection of a single nucleotide variation, the 5'-end of the 3'-targeting portion of the PTO has a complementary sequence to the single

nucleotide variation in a target nucleic acid sequence. As described above, the cleavage of the PTO hybridized with a matching template may be induced at a site immediately adjacent in a 3'-direction to the 5'-end of the 3'-targeting portion of the PTO, for example, under upstream primer extension-dependent cleavage induction.

5 The 3'-end of the PTO fragment has the complementary nucleotide to the single nucleotide variation. The PTO fragment is hybridized with a CPO having a capturing portion comprising a sequence corresponding to the nucleotide variation and then extended to form the extended duplex, providing the target signal. If the same PTO is hybridized with a mismatching template having the identical sequence to the

10 matching template except for the single nucleotide variation, the cleavage of the PTO may occur at a site two nucleotides apart in a 3'-direction from the 5'-end of the 3'-targeting portion of the PTO. The 3'-end of the PTO fragment has the further cleaved nucleotide than the complementary nucleotide to the single nucleotide variation. Where the site of the CPO hybridized with the additional-cleaved nucleotide is

15 designed to have a non-complementary sequence to the further cleaved nucleotide, the 3'-end of the PTO fragment is not hybridized with the CPO, resulting in no extension of the PTO fragment in a controlled condition.

According to an embodiment, a cleavage site of the PTO having a complementary sequence to the nucleotide variation at its 5'-end part of the 3'-

20 targeting portion is different depending on hybridization with a matching template or with a mismatching template, such that the PTO fragment released from either hybridization event has different sequence preferably, in its 3'-end part, more preferably, in its 3'-end.

According to an embodiment, the selection of the nucleotide sequence of CPO

25 in consideration of the difference in 3'-end parts of the PTO fragments allows to discriminate the matching template from the mismatching template.

According to an embodiment, the production of either the PTO fragments may be distinctly detected by an extension reaction on the CPO.

According to an embodiment, the CPO has a sequence selected such that the

CPO is not hybridized with the additional 3'-end portion of the second fragment to prevent the second fragment from extension when the second fragment is hybridized with the capturing portion of the CPO.

According to an embodiment, the 5'-end part of the 3'-targeting portion of the PTO comprises a non-base pairing moiety located within 1-10 nucleotides (more preferably 1-5 nucleotides) apart from the nucleotide variation discrimination site.

The non-base pairing moiety prevents the 5'-end part of the 3'-targeting portion from formation of a double strand with the target nucleotide sequence when the PTO is hybridized with the target nucleic acid sequence having the nucleotide variation non-complementary to the variation discrimination site.

The use of the non-base pairing moiety (*e.g.*, artificial mismatch nucleotide) enhances discrimination potential of the PTO to nucleotide variations.

According to an embodiment, the non-base pairing moiety does not inhibit the formation of a double strand between the 5'-end part and the target nucleic acid sequence when the PTO is hybridized with the target nucleic acid sequence having the nucleotide variation complementary to the nucleotide variation discrimination site.

According to an embodiment, the non-base pairing moiety widens the distance between the initial cleavage site on the hybrid of the PTO and the matching template and the initial cleavage site on the hybrid of the PTO and the mismatching template.

According to an embodiment, the introduction of a non-base pairing moiety sequence enables the initial cleavage site to be adjusted, particularly the initial cleavage site on the hybrid of the PTO and the mismatching template.

According to an embodiment, the non-base pairing moiety is located downstream of the nucleotide variation discrimination site.

The non-base pairing moiety includes any moieties not forming a base pair between target nucleic acid sequences. Preferably, the non-base pairing moiety is (i) a nucleotide comprising an artificial mismatch base, a natural/non-natural base incapable of base-pairing, a base modified to be incapable of base pairing or a universal base, (ii) a non-base pairing nucleotide modified to be incapable of base

pairing, or (iii) a non-base pairing chemical compound.

For example, the non-base pairing moiety includes alkylene group, ribofuranosyl naphthalene, deoxy ribofuranosyl naphthalene, metaphosphate, phosphorothioate linkage, alkyl phosphotriester linkage, aryl phosphotriester linkage, 5 alkyl phosphonate linkage, aryl phosphonate linkage, hydrogen phosphonate linkage, alkyl phosphoroamidate linkage and aryl phosphoroamidate linkage. Conventional carbon spacers are also used as non-base pairing moieties. Universal bases as non-base pairing moieties are useful in adjusting cleavage sites of the PTO.

As base pairs containing universal bases such as deoxyinosine, 1-(2'-deoxy-10 beta-D-ribofuranosyl)-3-nitropyrrole and 5-nitroindole have a lower binding strength than those between natural bases, universal bases may be employed as non-base pairing moieties under certain hybridization conditions.

The non-base pairing moiety introduced into the 5'-end part has preferably 1-10, more preferably 1-5, still more preferably 1-2 moieties. A plurality of non-base 15 pairing moieties in the 5'-end part may be present in a consecutive or intermittent manner. Preferably, the non-base pairing moiety has 2-5 consecutive moieties.

Preferably, the non-base pairing moiety is a non-base pairing chemical compound.

According to an embodiment, the nucleotide variation discrimination site and 20 the non-base pairing moiety of the PTO are located within 10 nucleotides (more preferably 8 nucleotides, 7 nucleotides, 6 nucleotides, 5 nucleotides, 4 nucleotides, 3 nucleotides, 2 nucleotides or 1 nucleotide, still more preferably 1 nucleotide) apart from the 5'-end of the 3'-targeting portion.

According to an embodiment, the PTO has a blocker portion containing as a 25 blocker at least one nucleotide resistant to cleavage by the enzyme having 5' nuclease activity and the blocker portion is positioned to control the initial cleavage site or prevent the cleavage at a site or sites.

For improving detection efficiency of nucleotide variations, the present invention may be performed with the clamping method. The representative clamping

method using PNA is disclosed in Henrik et al., *Nucleic Acid Research* 21:5332-5336(1993) and Luo et al., *Nucleic Acid Research* Vol. 34, No 2 e12 (2006).

For instance, the clamping technology using PNA allows to amplify a nucleic acid sequence having a mutant type nucleotide variation but not to amplify a nucleic acid sequence having a wild type nucleotide variation, which is followed by the PCET assay, enabling more efficient detection of nucleotide variations. In particular, since the clamping technology permits to amplify only a nucleic acid sequence having a specific-typed nucleotide variation, its combination with the present method would allow for minority-variant detection in a more efficient manner.

10 In the present invention, the term "amplification blocker" means an oligonucleotide used for clamping.

In general, the amplification blockers for clamping are hybridized only with templates having perfectly complementary sequence to the amplification blockers under the same condition, which are designed not to be hybridized with templates having even single mismatch. The template hybridized with the amplification blocker inhibiting primer annealing or chain elongation is not amplified and only that not hybridized with the amplification blocker is amplified. Nucleic acid analogues such as PNA and LNA are useful as amplification blockers in the senses that they show significant T_m differences for even a single base difference.

20 According to an embodiment, the amplification blocker is further used in the present invention particularly for minority-variant detection.

According to an embodiment, the amplification blocker prevents the extension of the primer located upstream of the amplification blocker.

According to an embodiment, the amplification blocker and PTO used may be designed to be hybridized with the same strand in a double strand or different strands from each other.

According to an embodiment, an amplification blocker comprises nucleosides/nucleotides having a backbone resistant to the 5' nuclease activity.

According to an embodiment, the amplification blocker comprises peptide

nucleic acid (PNA), locked nucleic acid (LNA), Morpholino, glycol nucleic acid (GNA), threose nucleic acid (TNA), bridged nucleic acids (BNA), N3'-P5' phosphoramidate (NP) oligomers, minor groove binder-linked-oligonucleotides (MGB-linked oligonucleotides), phosphorothioate (PS) oligomers, C₁-C₄ alkylphosphonate oligomers, phosphoramidates, β -phosphodiester oligonucleotides, α -phosphodiester oligonucleotides or combination thereof.

Where a probe having at its 5'-end portion a nucleotide variation discrimination portion is hybridized with a mismatch template, its 5'-end portion may form a single strand under a certain condition. The probe may correspond to a PTO. The signal may be generated by PTO assay of the present invention. This approach may be useful in detection of a target nucleic acid sequence having a nucleotide variation non-complementary to the nucleotide variation discrimination site of probes.

According to an embodiment, the target nucleic acid sequence used in the present invention is a pre-amplified nucleic acid sequence. The utilization of the pre-amplified nucleic acid sequence permits to significantly increase the sensitivity and specificity of target detection of the present invention.

According to an embodiment, the method is performed in the presence of a downstream primer to the PTO.

The advantages of the present invention may be highlighted in the simultaneous (multiplex) detection of at least two target nucleic acid sequences.

According to an embodiment, the method is performed to detect at least two types (more preferably, at least three types, still more preferably at least five types) of target nucleic acid sequences.

According to an embodiment, the method is performed to detect at least two types (more preferably, at least three types, still more preferably at least five types) of target nucleic acid sequences; wherein the upstream oligonucleotide comprises at least two types (more preferably at least three types, still more preferably at least five types) of oligonucleotides, the PTO comprises at least two types (more preferably at

least three types, still more preferably at least five types) of the PTOs, and the CPO comprises at least two types (preferably at least three types, more preferably at least five types) of the CPO. In certain embodiment, the method uses at least two types of the detection oligonucleotides.

5 In certain embodiment, when the at least two types of target nucleic acid sequences are present, their corresponding at least two types of signals are provided.

Where the upstream oligonucleotide to the detection oligonucleotide is used in the method for detecting at least two target nucleic acid sequences, it comprises at least two types of upstream oligonucleotide to the detection oligonucleotide.

10 Where at least two types of target nucleic acid sequences are simultaneously detected by melting analysis, the nucleic acid molecule and detection oligonucleotide having optimized sequences permits to minimize the number of the detection oligonucleotides used. For example, where the method is performed to detect at least two types of target nucleic acid sequences, the sequences of the produced nucleic acid molecules corresponding to the target nucleic acid sequences are designed to have different T_m values upon hybridization with the detection oligonucleotide, enabling to detect at least two types of target nucleic acid sequences using even a single type of the detection oligonucleotide.

15 According to an embodiment, the present invention is performed using at least two types of downstream primers to the PTO.

The present invention may be carried out either in a liquid phase or on a solid phase.

25 Target Detection on a Solid Phase

According to an embodiment, the present invention is performed on the solid phase, and the CPO is immobilized through its 5'-end or 3'-end onto a solid substrate.

In certain embodiment, the present invention is performed on the solid phase and the detection oligonucleotide is immobilized through its 5'-end or 3'-end onto a

solid substrate.

In certain embodiment, the nucleic acid molecule has at least one label, the detection oligonucleotide is immobilized onto a solid substrate and the detection of the nucleic acid molecule in the step (f) is performed by measuring a signal from the label on the solid substrate (see Fig. 7).

For the solid phase reaction, the CPO or the detection oligonucleotide is immobilized directly or indirectly (preferably indirectly) through its 5'-end or 3'-end (particularly the 3'-end) onto the surface of the solid substrate. Furthermore, the CPO or the detection oligonucleotide may be immobilized on the surface of the solid substrate in a covalent or non-covalent manner. Where the immobilized the CPO or the detection oligonucleotide is immobilized indirectly onto the surface of the solid substrate, suitable linkers are used. The linkers useful in this invention may include any linkers utilized for probe immobilization on the surface of the solid substrate. For example, alkyl or aryl compounds with amine functionality, or alkyl or aryl compounds with thiol functionality serve as linkers for immobilization. In addition, poly (T) tail or poly (A) tail may serve as linkers and significantly decrease space hindrance that is an inhibitory factor to enzymatic actions (*e.g.*, enzymatic cleavage reactions), contributing to increase in hybridization efficiency. The poly (T) tail or poly (A) tail as linkers is not considered a sequence of probes.

According to an embodiment, the solid substrate used in the present invention is a microarray. The microarray to provide a reaction environment in this invention may include any those known to one of skill in the art. All processes of the present invention, *i.e.*, hybridization to target nucleic acid sequences, cleavage, extension, melting and fluorescence detection, are carried out on the microarray. The immobilized CPO or detection oligonucleotide on the microarray serves as hybridizable array elements. The solid substrate to fabricate microarray includes, but not limited to, metals (*e.g.*, gold, alloy of gold and copper, aluminum), metal oxide, glass, ceramic, quartz, silicon, semiconductor, Si/SiO₂ wafer, germanium, gallium arsenide, carbon, carbon nanotube, polymers (*e.g.*, polystyrene, polyethylene, polypropylene and

polyacrylamide), sepharose, agarose and colloids. A plurality of immobilized CPOs or detection oligonucleotides in this invention may be immobilized on an addressable region or two or more addressable regions on a solid substrate that may comprise 2-1,000,000 addressable regions. Immobilized CPOs or the detection oligonucleotides
5 may be fabricated to produce array or arrays for a given application by conventional fabrication technologies such as photolithography, ink-jetting, mechanical microspotting, and derivatives thereof.

The present invention performed on the solid phase can detect simultaneously a plurality of target nucleic acid sequences even using a single type of a label because
10 the labels on the detection oligonucleotides immobilized are physically separated. In this regard, the number of target nucleic acid sequences to be detected by the present invention on the solid phase is not limited.

According to an embodiment, the detection oligonucleotide is immobilized on the surface of a solid substrate via its 3'-end or 5'-end, the nucleic acid molecule has
15 a single label or a plurality of the single label, the hybridization of the detection oligonucleotide with the nucleic acid molecule causes a signal change on the solid substrate to detect the production of the nucleic acid molecule (*i.e.*, the presence of the target nucleic acid molecule).

Using confocal detection devices, the signal only on the solid substrate may be
20 detected without influence of labels suspended in a liquid phase.

It is also possible to provide additional fragments extendible on the CPO for enhancing the number of the extended strands by an additional 5' nuclease cleavage reaction using an additional PTO which comprises (i) a 3'-targeting portion comprising
25 a hybridizing nucleotide sequence complementary to the extended strand and (ii) a 5'-tagging portion comprising a nucleotide sequence non-complementary to the extended strand but complementary to the capturing portion of the CPO. It is preferable to use an additional upstream oligonucleotide comprising a hybridizing nucleotide sequence complementary to the extended strand and being located

upstream of the additional PTO for 5' nuclease cleavage reaction.

The above preferable embodiment has the feature that the formation of the additional fragments is dependent on the formation of an extended strand.

Alternatively, the additional fragments can be provided by using an additional
5 PTO which comprises (i) a 3'-targeting portion comprising a hybridizing nucleotide sequence complementary to the CPO (e.g. the promoter portion or transcription portion) of and (ii) a 5'-tagging portion comprising a nucleotide sequence non-complementary to the CPO but complementary to the capturing portion of the CPO.

According to an embodiment, additional extended duplexes are formed by
10 additional production of the extended strands, contributing to amplification of the target signal.

Nucleic Acid Production and Target Detection by a PCET Assay based on Upstream
Oligonucleotide-independent 5' nuclease activity

15 In a further aspect of the present invention, there is provided a method for producing a nucleic acid molecule dependent on a target nucleic acid sequence, comprising:

(a) hybridizing the target nucleic acid sequence with a PTO (Probing and Tagging Oligonucleotide); the PTO comprises (i) a 3'-targeting portion comprising a
20 hybridizing nucleotide sequence complementary to the target nucleic acid sequence and (ii) a 5'-tagging portion comprising a nucleotide sequence non-complementary to the target nucleic acid sequence; wherein the 3'-targeting portion is hybridized with the target nucleic acid sequence and the 5'-tagging portion is not hybridized with the target nucleic acid sequence;

25 (b) contacting the resultant of the step (a) to an enzyme having a 5' nuclease activity under conditions for cleavage of the PTO; wherein when the PTO is hybridized with the target nucleic acid sequence, it is then cleaved by the enzyme having a 5' nuclease activity such that the cleavage releases a fragment comprising the 5'-tagging portion or a part of the 5'-tagging portion of the PTO;

(c) hybridizing the fragment released from the PTO with a CPO (Capturing and Promoter Oligonucleotide); wherein the CPO comprises in a 3' to 5' direction (i) a capturing portion comprising a nucleotide sequence complementary to the 5'-tagging portion or a part of the 5'-tagging portion of the PTO, (ii) a promoter portion inducing transcription; and (iii) a transcription portion; wherein the fragment released from the PTO is hybridized with the capturing portion of the CPO;

(d) performing an extension reaction using the resultant of the step (c) and a template-dependent nucleic acid polymerase, wherein the fragment hybridized with the capturing portion of the CPO is extended to form an extended strand comprising an extended sequence complementary to the promoter portion of the CPO, thereby activating the promoter portion; and

(e) producing the nucleic acid molecule complementary to the transcription portion by use of a polymerase recognizing the activated promoter portion.

15 In still further aspect of the present invention, there is provided a method for detecting a target nucleic acid sequence from a DNA or a mixture of nucleic acids using a target-dependent transcription, comprising:

(a) hybridizing the target nucleic acid sequence with a PTO (Probing and Tagging Oligonucleotide); the PTO comprises (i) a 3'-targeting portion comprising a hybridizing nucleotide sequence complementary to the target nucleic acid sequence and (ii) a 5'-tagging portion comprising a nucleotide sequence non-complementary to the target nucleic acid sequence; wherein the 3'-targeting portion is hybridized with the target nucleic acid sequence and the 5'-tagging portion is not hybridized with the target nucleic acid sequence;

25 (b) contacting the resultant of the step (a) to an enzyme having a 5' nuclease activity under conditions for cleavage of the PTO; wherein when the PTO is hybridized with the target nucleic acid sequence, it is then cleaved by the enzyme having a 5' nuclease activity such that the cleavage releases a fragment comprising the 5'-tagging portion or a part of the 5'-tagging portion of the PTO;

(c) hybridizing the fragment released from the PTO with a CPO (Capturing and Promoter Oligonucleotide); wherein the CPO comprises in a 3' to 5' direction (i) a capturing portion comprising a nucleotide sequence complementary to the 5'-tagging portion or a part of the 5'-tagging portion of the PTO, (ii) a promoter portion inducing transcription; and (iii) a transcription portion; wherein the fragment released from the PTO is hybridized with the capturing portion of the CPO;

(d) performing an extension reaction using the resultant of the step (c) and a template-dependent nucleic acid polymerase, wherein the fragment hybridized with the capturing portion of the CPO is extended to form an extended strand comprising an extended sequence complementary to the promoter portion of the CPO, thereby activating the promoter portion;

(e) producing the nucleic acid molecule complementary to the transcription portion by use of a polymerase recognizing the activated promoter portion; and

(f) detecting the nucleic acid molecule, whereby the presence of the nucleic acid molecule indicates the presence of the target nucleic acid sequence.

Since the present method based on upstream oligonucleotide-independent 5' nuclease activity is the same as those by the PCET assay using upstream oligonucleotides except for no use of upstream oligonucleotides, the common descriptions between them are omitted in order to avoid undue redundancy leading to the complexity of this specification.

Interestingly, the present method based on upstream oligonucleotide-independent 5' nuclease activity practically provides target signals by the PCET assay even no use of upstream oligonucleotides.

For the present method, conventional enzymes having upstream oligonucleotide-independent 5' nuclease activity may be used. Among template-dependent polymerases having 5' nuclease activity, there are several enzymes having upstream oligonucleotide-independent 5' nuclease activity, *e.g.*, *Taq* DNA polymerase.

Considering amplification of target nucleic acid sequences and cleavage

efficiency of the PTO, the PCET assay of the present invention is preferably performed using upstream oligonucleotides.

Kits for Nucleic Acid Production or Target Detection

5 In still another aspect of this invention, there is provided a kit for producing a nucleic acid molecule dependent on a target nucleic acid sequence or a kit for detecting a target nucleic acid sequence from a DNA or a mixture of nucleic acids using a target-dependent transcription, comprising:

- (a) a PTO (Probing and Tagging Oligonucleotide); wherein the PTO comprises
 - 10 (i) a 3'-targeting portion comprising a hybridizing nucleotide sequence complementary to the target nucleic acid sequence and (ii) a 5'-tagging portion comprising a nucleotide sequence non-complementary to the target nucleic acid sequence; wherein the 3'-targeting portion is hybridized with the target nucleic acid sequence and the 5'-tagging portion is not hybridized with the target nucleic acid sequence;
- 15 (b) an upstream oligonucleotide; wherein the upstream oligonucleotide comprises a hybridizing nucleotide sequence complementary to the target nucleic acid sequence; the upstream oligonucleotide is located upstream of the PTO; wherein the upstream oligonucleotide or its extended strand induces cleavage of the PTO by an enzyme having a 5' nuclease activity such that the cleavage releases a fragment
 - 20 comprising the 5'-tagging portion or a part of the 5'-tagging portion of the PTO; and
- (c) a CPO (Capturing and Promoter Oligonucleotide); wherein the CPO comprises in a 3' to 5' direction (i) a capturing portion comprising a nucleotide sequence complementary to the 5'-tagging portion or a part of the 5'-tagging portion of the PTO, (ii) a promoter portion inducing transcription; and (iii) a transcription
 - 25 portion; wherein the fragment released from the PTO is hybridized with the capturing portion of the CPO.

Since the kit of this invention is constructed to perform the detection method of the present invention described above, the common descriptions between them are

omitted in order to avoid undue redundancy leading to the complexity of this specification.

In an embodiment of this invention, the kit further comprises an enzyme having a 5' nuclease activity.

5 In an embodiment of this invention, the kit further comprises a template-dependent nucleic acid polymerase.

In an embodiment of this invention, the kit further comprises a polymerase recognizing the promoter portion.

In an embodiment of this invention, the capturing portion and the promoter
10 portion of the CPO are located in a non-overlapped manner or overlapped manner to each other.

According to an embodiment, where the capturing portion and the promoter portion are located in an overlapped manner to each other, they are overlapped to the extent that hybridization of an uncleaved PTO with the capturing portion does not
15 induce the activation of the promoter.

According to an embodiment, the transcription portion of the CPO comprises a nucleotide sequence non-complementary to the 5'-tagging portion of the PTO, the 3'-targeting portion of the PTO or both of them.

In an embodiment of this invention, the PTO and/or CPO is blocked at its 3'-
20 end to prohibit its extension.

In an embodiment of this invention, the upstream oligonucleotide is an upstream primer or an upstream probe.

In an embodiment of this invention, the upstream oligonucleotide is located adjacently to the PTO to the extent that the upstream oligonucleotide induces
25 cleavage of the PTO by the enzyme having the 5' nuclease activity.

In an embodiment of this invention, the upstream primer induces through its extended strand the cleavage of the PTO by the enzyme having the 5' nuclease activity.

In an embodiment of this invention, the capturing portion of the CPO

comprises at its 5'-end part a nucleotide sequence complementary to a part of the 3'-targeting portion of the PTO.

In an embodiment of this invention, the promoter portion of the CPO comprises a RNA polymerase promoter. In an embodiment of this invention, the RNA polymerase promoter is a promoter recognizable with T7 RNA polymerase, T3 RNA polymerase, SP6 RNA polymerase, *Tth* RNA polymerase or *E. coli* RNA polymerase.

In an embodiment of this invention, the polymerase recognizing the promoter portion produces a nucleic acid molecule complementary to the transcription portion and the nucleic acid molecule produced is RNA.

10 In an embodiment of this invention, the polymerase recognizing the promoter portion produces a nucleic acid molecule comprising a complementary nucleotide sequence to a portion positioned in the 3'-direction of the transcription portion of the CPO.

In an embodiment of this invention, the kit further comprises a TCPO (Transcript-Capturing and Promoter Oligonucleotide) comprising a nucleotide sequence complementary to the produced nucleic acid molecule. In an embodiment of this invention, the TCPO comprises in a 3' to 5' direction (i) a transcript-capturing portion comprising a nucleotide sequence complementary to the nucleic acid molecule, (ii) a promoter portion inducing transcription, and (iii) a transcription portion.

20 In an embodiment of this invention, the transcript-capturing portion and the promoter portion of the TCPO are located in a non-overlapped manner or overlapped manner to each other. In an embodiment of this invention, the transcription portion of the CPO is the same as or different from the transcription portion of the TCPO.

25 In an embodiment of this invention, the kit further comprises a detection oligonucleotide comprising a nucleotide sequence complementary to the nucleic acid molecule. In an embodiment of this invention, the detection oligonucleotide, the nucleic acid molecule or each of them has at least one label.

In an embodiment of this invention, the nucleic acid molecule has at least one

label and the detection oligonucleotide is immobilized onto a solid substrate.

In an embodiment of this invention, the kit is used to detect at least two types of target nucleic acid sequences; wherein the upstream oligonucleotide comprises at least two types of oligonucleotides, the PTO comprises at least two types of the PTOs, the CPO comprises at least two types of the CPOs.

In an embodiment of this invention, the kit further comprises a downstream primer.

All of the present kits described hereinabove may optionally include the reagents required for performing target amplification PCR reactions (*e.g.*, PCR reactions) such as buffers, DNA polymerase cofactors, and deoxyribonucleotide-5-triphosphates. Optionally, the kits may also include various polynucleotide molecules, reverse transcriptase, various buffers and reagents, and antibodies that inhibit DNA polymerase activity. The kits may also include reagents necessary for performing positive and negative control reactions. Optimal amounts of reagents to be used in a given reaction can be readily determined by the skilled artisan having the benefit of the current disclosure. The kits, typically, are adopted to contain the constituents afore-described in separate packaging or compartments.

The features and advantages of this invention will be summarized as follows:

(a) Even though the present invention does not directly amplify target nucleic acid sequences, it produces a nucleic acid molecule (*e.g.*, RNA) with arbitrary sequences dependent on the presence of target nucleic acid sequences. The sequence of the nucleic acid molecule produced in this invention may be variously selected for interest. Some or all of a target nucleic acid sequence may be produced as the nucleic acid molecule by designing the sequence of the nucleic acid molecule.

(b) Since the production of the nucleic acid molecule is dependent on the presence of the target nucleic acid sequence, the present method permits to determine the presence of the target nucleic acid sequence.

(c) In the present method, the PTO fragment is generated by cleavage of the

PTO hybridized with the target nucleic acid sequence and then the amplification of the nucleic acid molecule (*e.g.*, RNA) is induced using the PTO fragment and promoter-containing template.

Unlike to conventional transcript amplification methods using promoter-containing promoters, the present invention requires no complicated process for
5 promoter activation (formation of double strand). Unlike to SMART method using two probes inducing transcription, the present invention has no limitation associated with hybrid formation and maintenance between probes and target sequences for transcription induction.

10 In this regard, the present method has convenience in terms of both the amplification of the nucleic acid molecule (*e.g.*, RNA) and the optimization of reaction conditions.

(d) In conventional methods to detect target sequences using cleavage of probes with 5'-tagging portion, a single probe is cleaved to generate a single tagging
15 portion fragment, and provide a single signal. Unlikely, even when a single tagging portion fragment (PTO fragment) is generated, the nucleic acid molecule (*e.g.*, RNA) is amplified by transcription reaction to provide signals, thereby amplifying target signals.

(e) The present invention may employ any method to detect nucleic acid
20 sequences for the detection of the produced nucleic acid molecule (*e.g.*, RNA). Interestingly, the promoter-containing template (*e.g.*, CPO) used in production of the nucleic acid molecule (*e.g.*, RNA) may be used to detect the production of the nucleic acid molecule (*e.g.*, RNA).

(f) By use of the produced nucleic acid molecule (*e.g.*, RNA), the present
25 method enables to re-amplify either the nucleic acid molecule (*e.g.*, RNA) or new nucleic acid molecule (*e.g.*, RNA) different from the former nucleic acid molecule in sequence. For example, the production of the nucleic acid molecule having a nucleic acid sequence same as that of the PTO fragment allows for cyclic re-amplification of the nucleic acid molecule. In other instance, the use of TCPO allows for the

production of a new nucleic acid molecule (*e.g.*, RNA) different from the former one in sequence.

The present invention will now be described in further detail by examples. It would be obvious to those skilled in the art that these examples are intended to be more concretely illustrative and the scope of the present invention as set forth in the appended claims is not limited to or by the examples.

EXAMPLES

10 **EXAMPLE 1: Preparation of Template and Oligonucleotides**

The template and oligonucleotides listed in Table 1 were prepared to evaluate workability of the PTO Cleavage and Extension-dependent Transcription (PCET) assay of the present invention for detection of target nucleic acid sequences.

The synthetic oligonucleotide for *Neisseria gonorrhoeae* (NG) gene was used as a target template. The PTO was prepared to comprise a non-complementary sequence and a complementary sequence to the target template at its 5'-tagging portion and 3'-targeting portion, respectively. The 3'-end of the PTO was blocked with a carbon spacer to prevent extension by DNA polymerase. The CPO was prepared to comprise a complementary sequence to the 5'-tagging portion of the PTO at the capturing portion and a non-complementary sequence to the PTO at the promoter and transcription portions. The promoter portion of the CPO comprises a sequence recognizable by T7 RNA polymerase. The 3'-end of the CPO was blocked with a carbon spacer to prevent extension by DNA polymerase. The DO labeled at its 5'-end with a quencher molecule (BHQ-2) and at its 3'-end with a reporter molecule (CAL Fluor Red 610) was prepared to comprise the same sequence as the transcription portion of the CPO for detection of RNA that was produced dependent on the presence of the target nucleic acid sequence.

TABLE 1

Name	Sequence(5' → 3')	SEQ ID NOs
NG-T	AAATATGCGAAACACGCCAATGAGGGGCATGATGCTTTCTTTT GTTCTTGCTCGGCAGAGCGAGTGATACCGATCCATTGAAAAA	SEQ ID NO: 1
NG_R	CAATGGATCGGTATCACTCGC	SEQ ID NO: 2
PTO	<u>ACGACGGCTTGGCT</u> GCCCCTCATTGGCGTGTTCG[C3 spacer]	SEQ ID NO: 3
CPO	TGGATACCCTGGACGATATG <u>CCCTATAGTGAGTCGTATTA</u> TA TACAGCCAAGCCGTCGT[C3 spacer]	SEQ ID NO: 4
DO	[BHQ-2]TGGATACCCTGGACGATATGCC[CAL Red 610]	SEQ ID NO: 5

PTO : Probing and Tagging Oligonucleotide

CPO: Capturing and Promoter Oligonucleotide

DO: Detecting Oligonucleotide

BHQ : Quencher (Black Hole Quencher)

5 Underlined letter: 5'-tagging portion of PTO

Boxed letters: Promoter portion

EXAMPLE 2: Evaluation of PTO Cleavage and Extension-dependent Transcription (PCET) assay

10 The PCET assay was evaluated using the PTO, CPO and DO for detection of the target nucleic acid sequence. Where the target nucleic acid sequence is present, the PTO fragment is generated. The PTO fragment is hybridized with the capturing portion of the CPO and then extended on the promoter portion of the CPO, permitting the promoter portion to be in double strand. T7 RNA polymerase recognizes and binds

15 to the double-stranded promoter, and produces RNA through transcription reaction using the transcription portion of the CPO as templates. The produced RNA is hybridized with the dual-labeled DO to generate fluorescent signal. Finally, the fluorescent signal is measured for the detection of the presence of the target nucleic acid sequence.

20 *Taq* DNA polymerase having a 5' nuclease activity was used for the extension of upstream primer, the cleavage of PTO and the extension of PTO fragment. For transcription, T7 RNA polymerase was used.

Template DNA and oligonucleotides

The template (SEQ ID NO:1), upstream primer (SEQ ID NO:2), PTO (SEQ ID NO: 3), CPO (SEQ ID NO: 4) and DO (SEQ ID NO: 5) prepared in Example 1 were used.

PCET assay

The reaction was conducted in the final volume of 20 μ l containing 2 pmole of the synthetic template (SEQ ID NO: 1) for NG gene, 10 pmole of the upstream primer (SEQ ID NO: 2), 5 pmole of PTO (SEQ ID NO: 3), 0.5 pmole of CPO (SEQ ID NO: 4), 3 pmole of DO (SEQ ID NO: 5), 2.5 mM $MgCl_2$, 200 μ M of dNTPs, 1.6 units of *Taq* DNA polymerase (Solgent, Korea), 0.5 mM DTT, 200 μ M rNTPs and 20 units of TOP-T7 RNA polymerase (Enzynomics, Korea); and the tube containing the reaction mixture was placed on the real-time thermocycler (CFX96, Bio-Rad). The reaction was undertaken at 45°C with measuring the fluorescence signal in the interval of 10 sec (measurements of the total 100 times).

The fluorescent signal changes over the reaction time of the PCET assay were detected only when the template (*i.e.*, target nucleic acid sequence) was present. In contrast, in the absence of the template, no fluorescent signal change was observed. Furthermore, in the absence of the PTO, CPO or DO, no fluorescent signal change was also observed.

These results urge us to reason that the PCET assay of the present invention allows to detect a target nucleic acid sequence in a real-time manner.

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 producing a nucleic acid molecule dependent on a target nucleic acid sequence, comprising:

5 (a) hybridizing the target nucleic acid sequence with an upstream oligonucleotide and a PTO (Probing and Tagging Oligonucleotide); wherein the upstream oligonucleotide comprises a hybridizing nucleotide sequence complementary to the target nucleic acid sequence; the PTO comprises (i) a 3'-targeting portion comprising a hybridizing nucleotide sequence complementary to the target nucleic acid sequence and (ii) a 5'-tagging portion comprising a nucleotide sequence non-
10 complementary to the target nucleic acid sequence; wherein the 3'-targeting portion is hybridized with the target nucleic acid sequence and the 5'-tagging portion is not hybridized with the target nucleic acid sequence; the upstream oligonucleotide is located upstream of the PTO;

(b) contacting the resultant of the step (a) to an enzyme having a 5' nuclease activity under conditions for cleavage of the PTO; wherein the upstream
15 oligonucleotide or its extended strand induces cleavage of the PTO by the enzyme having a 5' nuclease activity such that the cleavage releases a fragment comprising the 5'-tagging portion or a part of the 5'-tagging portion of the PTO;

(c) hybridizing the fragment released from the PTO with a CPO (Capturing and Promoter Oligonucleotide); wherein the CPO comprises in a 3' to 5' direction (i) a capturing portion comprising a nucleotide sequence complementary to the 5'-
20 tagging portion or a part of the 5'-tagging portion of the PTO, (ii) a promoter portion inducing transcription; and (iii) a transcription portion; wherein the fragment released from the PTO is hybridized with the capturing portion of the
25 CPO;

(d) performing an extension reaction using the resultant of the step (c) and a template-dependent nucleic acid polymerase, wherein the fragment hybridized with the capturing portion of the CPO is extended to form an extended strand comprising an extended sequence complementary to the promoter portion of the

CPO, thereby activating the promoter portion; and

(e) producing the nucleic acid molecule complementary to the transcription portion by use of a polymerase recognizing the activated promoter portion.

- 5 2. A method for detecting a target nucleic acid sequence from a DNA or a mixture of nucleic acids using a target-dependent transcription, comprising:

(a) hybridizing the target nucleic acid sequence with an upstream oligonucleotide and a PTO (Probing and Tagging Oligonucleotide); wherein the upstream oligonucleotide comprises a hybridizing nucleotide sequence complementary to the
10 target nucleic acid sequence; the PTO comprises (i) a 3'-targeting portion comprising a hybridizing nucleotide sequence complementary to the target nucleic acid sequence and (ii) a 5'-tagging portion comprising a nucleotide sequence non-complementary to the target nucleic acid sequence; wherein the 3'-targeting portion is hybridized with the target nucleic acid sequence and the 5'-tagging
15 portion is not hybridized with the target nucleic acid sequence; the upstream oligonucleotide is located upstream of the PTO;

(b) contacting the resultant of the step (a) to an enzyme having a 5' nuclease activity under conditions for cleavage of the PTO; wherein the upstream oligonucleotide or its extended strand induces cleavage of the PTO by the enzyme
20 having a 5' nuclease activity such that the cleavage releases a fragment comprising the 5'-tagging portion or a part of the 5'-tagging portion of the PTO;

(c) hybridizing the fragment released from the PTO with a CPO (Capturing and Promoter Oligonucleotide); wherein the CPO comprises in a 3' to 5' direction (i) a capturing portion comprising a nucleotide sequence complementary to the 5'-
25 tagging portion or a part of the 5'-tagging portion of the PTO, (ii) a promoter portion inducing transcription; and (iii) a transcription portion; wherein the fragment released from the PTO is hybridized with the capturing portion of the CPO;

(d) performing an extension reaction using the resultant of the step (c) and a

- template-dependent nucleic acid polymerase, wherein the fragment hybridized with the capturing portion of the CPO is extended to form an extended strand comprising an extended sequence complementary to the promoter portion of the CPO, thereby activating the promoter portion;
- 5 (e) producing the nucleic acid molecule complementary to the transcription portion by use of a polymerase recognizing the activated promoter portion; and
- (f) detecting the nucleic acid molecule, whereby the presence of the nucleic acid molecule indicates the presence of the target nucleic acid sequence.
- 10 3. The method according to claim 1 or 2, wherein the capturing portion and the promoter portion of the CPO are located in a non-overlapped manner or overlapped manner to each other.
4. The method according to claim 1 or 2, wherein the PTO and/or CPO is blocked at
15 its 3'-end to prohibit its extension.
5. The method according to claim 1 or 2, wherein the upstream oligonucleotide is an upstream primer or an upstream probe.
- 20 6. The method according to claim 1 or 2, wherein the upstream oligonucleotide is located adjacently to the PTO to the extent that the upstream oligonucleotide induces cleavage of the PTO by the enzyme having the 5' nuclease activity.
7. The method according to claim 5, wherein the upstream primer induces through
25 its extended strand the cleavage of the PTO by the enzyme having the 5' nuclease activity.
8. The method according to claim 1 or 2, wherein the capturing portion of the CPO comprises at its 5'-end part a nucleotide sequence complementary to a part of the 3'-

targeting portion of the PTO.

9. The method according to claim 1 or 2, wherein the promoter portion of the CPO comprises a RNA polymerase promoter.

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10. The method according to claim 9, wherein the RNA polymerase promoter is a promoter recognizable with T7 RNA polymerase, T3 RNA polymerase, SP6 RNA polymerase, *Tth* RNA polymerase or *E. coli* RNA polymerase.

10 11. The method according to claim 1 or 2, wherein the nucleic acid molecule in the step (e) is RNA.

12. The method according to claim 1 or 2, wherein the nucleic acid molecule produced in the step (e) comprises a complementary nucleotide sequence to a portion
15 positioned in the 3'-direction of the transcription portion of the CPO and hybridization of the nucleic acid molecule with the CPO causes additional production of the nucleic acid molecule.

13. The method according to claim 12, wherein the method further comprises after
20 the step (e) the following steps (e-1) to (e-3):

(e-1) hybridizing the nucleic acid molecule with the CPO; wherein the nucleic acid molecule comprises a complementary nucleotide sequence to a portion positioned in the 3'-direction of the transcription portion of the CPO;

(e-2) performing an extension reaction using the resultant of the step (e-1)
25 and a template-dependent nucleic acid polymerase, wherein the nucleic acid molecule hybridized with the CPO is extended to form an extended strand comprising an extended sequence complementary to the promoter portion of the CPO, thereby activating the promoter portion; and

(e-3) producing the nucleic acid molecule complementary to the transcription

portion by use of a polymerase recognizing the activated promoter portion.

14. The method according to claim 13, wherein the nucleic acid molecule produced in the step (e) comprises a complementary nucleotide sequence to the capturing
5 portion of the CPO.

15. The method according to claim 12, wherein the method further comprises after the step (e) the following steps (e-1) to (e-2):

(e-1) hybridizing the nucleic acid molecule with the CPO; wherein the nucleic
10 acid molecule comprises a complementary nucleotide sequence to the promoter portion of the CPO; hybridization of the nucleic acid molecule with the promoter portion induces the activation of the promoter portion; and

(e-2) producing the nucleic acid molecule complementary to the transcription portion by use of a polymerase recognizing the activated promoter portion.

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16. The method according to claim 12, wherein the fragment released from the PTO and the nucleic acid molecule produced in the step (e) is hybridized with the same site or different sites from each other on the CPO.

20 17. The method according to claim 1 or 2, wherein the method further comprises after the step (e) hybridizing the nucleic acid molecule produced in the step (e) with a TCPO (Transcript-Capturing and Promoter Oligonucleotide); wherein the TCPO comprises in a 3' to 5' direction (i) a transcript-capturing portion comprising a nucleotide sequence complementary to the nucleic acid molecule, (ii) a promoter
25 portion inducing transcription, and (iii) a transcription portion; wherein hybridization of the nucleic acid molecule with the TCPO causes production of a nucleic acid molecule complementary to the transcription portion.

18. The method according to claim 17, wherein the transcript-capturing portion and

the promoter portion of the TCPO are located in a non-overlapped manner or overlapped manner to each other.

19. The method according to claim 17, wherein the method further comprises after
5 the step (e) the following steps (e-1) to (e-3):

(e-1) hybridizing the nucleic acid molecule with the TCPO; wherein the nucleic acid molecule comprises a complementary nucleotide sequence to the transcript-capturing portion of the TCPO;

(e-2) performing an extension reaction using the resultant of the step (e-1)
10 and a template-dependent nucleic acid polymerase, wherein the nucleic acid molecule hybridized with the TCPO is extended to form an extended strand comprising an extended sequence complementary to the promoter portion of the TCPO, thereby activating the promoter portion; and

(e-3) producing the nucleic acid molecule complementary to the transcription
15 portion by use of a polymerase recognizing the activated promoter portion.

20. The method according to claim 17, wherein the method further comprises after the step (e) the following steps (e-1) to (e-2):

(e-1) hybridizing the nucleic acid molecule with the TCPO; wherein the nucleic
20 acid molecule comprises a complementary nucleotide sequence to the promoter portion of the TCPO; hybridization of the nucleic acid molecule with the promoter portion induces the activation of the promoter portion; and

(e-2) producing the nucleic acid molecule complementary to the transcription
25 portion by use of a polymerase recognizing the activated promoter portion.

21. The method according to claim 17, wherein the transcription portion of the CPO is the same as or different from the transcription portion of the TCPO.

22. The method according to claim 2, wherein the detection of the nucleic acid

molecule in the step (f) is performed using a detection oligonucleotide to be hybridized with the nucleic acid molecule; wherein the detection oligonucleotide, the nucleic acid molecule or each of them has at least one label, and the detection of the nucleic acid molecule is performed by measuring a signal provided the label.

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23. The method according to claim 22, wherein the detection oligonucleotide has a single label or an interactive dual label.

24. The method according to claim 22, wherein the nucleic acid molecule has a
10 single label or a plurality of single labels.

25. The method according to claim 22, wherein each of the detection oligonucleotide and the nucleic acid molecule has a label, the label on the detection oligonucleotide and the label on the nucleic acid molecule form an interactive dual
15 label.

26. The method according to claim 22, wherein the nucleic acid molecule is hybridized with the detection oligonucleotide to provide a detectable signal change.

20 27. The method according to claim 22, wherein the nucleic acid molecule is hybridized with the detection oligonucleotide and extended using as a template the detection oligonucleotide to form an extended duplex, thereby providing a detectable signal change.

25 28. The method according to claim 22, wherein the nucleic acid molecule has the label by performing the step (e) using a dNTP or NTP with the label.

29. The method according to claim 22, wherein the nucleic acid molecule has at least one label, the detection oligonucleotide is immobilized onto a solid substrate and

the detection of the nucleic acid molecule in the step (f) is performed by measuring a signal from the label on the solid substrate.

30. The method according to claim 2, wherein the method is performed to detect at least two types of target nucleic acid sequences; wherein the upstream oligonucleotide comprises at least two types of oligonucleotides, the PTO comprises at least two types of the PTOs, the CPO comprises at least two types of the CPOs.

31. The method according to claim 1 or 2, wherein the target nucleic acid sequence comprises a nucleotide variation.

32. The method according to any one of claims 1 to 31, wherein the method is performed in the presence of a downstream primer.

33. A method for producing a nucleic acid molecule dependent on a target nucleic acid sequence, comprising:

(a) hybridizing the target nucleic acid sequence with a PTO (Probing and Tagging Oligonucleotide); the PTO comprises (i) a 3'-targeting portion comprising a hybridizing nucleotide sequence complementary to the target nucleic acid sequence and (ii) a 5'-tagging portion comprising a nucleotide sequence non-complementary to the target nucleic acid sequence; wherein the 3'-targeting portion is hybridized with the target nucleic acid sequence and the 5'-tagging portion is not hybridized with the target nucleic acid sequence;

(b) contacting the resultant of the step (a) to an enzyme having a 5' nuclease activity under conditions for cleavage of the PTO; wherein when the PTO is hybridized with the target nucleic acid sequence, it is then cleaved by the enzyme having a 5' nuclease activity such that the cleavage releases a fragment comprising the 5'-tagging portion or a part of the 5'-tagging portion of the PTO;

(c) hybridizing the fragment released from the PTO with a CPO (Capturing and

Promoter Oligonucleotide); wherein the CPO comprises in a 3' to 5' direction (i) a capturing portion comprising a nucleotide sequence complementary to the 5'-tagging portion or a part of the 5'-tagging portion of the PTO, (ii) a promoter portion inducing transcription; and (iii) a transcription portion; wherein the
5 fragment released from the PTO is hybridized with the capturing portion of the CPO;

(d) performing an extension reaction using the resultant of the step (c) and a template-dependent nucleic acid polymerase, wherein the fragment hybridized with the capturing portion of the CPO is extended to form an extended strand
10 comprising an extended sequence complementary to the promoter portion of the CPO, thereby activating the promoter portion; and

(e) producing the nucleic acid molecule complementary to the transcription portion by use of a polymerase recognizing the activated promoter portion.

15 34. A kit for producing a nucleic acid molecule dependent on a target nucleic acid sequence, comprising:

(a) a PTO (Probing and Tagging Oligonucleotide); wherein the PTO comprises (i) a 3'-targeting portion comprising a hybridizing nucleotide sequence complementary to the target nucleic acid sequence and (ii) a 5'-tagging portion
20 comprising a nucleotide sequence non-complementary to the target nucleic acid sequence; wherein the 3'-targeting portion is hybridized with the target nucleic acid sequence and the 5'-tagging portion is not hybridized with the target nucleic acid sequence;

(b) an upstream oligonucleotide; wherein the upstream oligonucleotide
25 comprises a hybridizing nucleotide sequence complementary to the target nucleic acid sequence; the upstream oligonucleotide is located upstream of the PTO; wherein the upstream oligonucleotide or its extended strand induces cleavage of the PTO by an enzyme having a 5' nuclease activity such that the cleavage releases a fragment comprising the 5'-tagging portion or a part of the 5'-tagging

portion of the PTO; and

(c) a CPO (Capturing and Promoter Oligonucleotide); wherein the CPO comprises in a 3' to 5' direction (i) a capturing portion comprising a nucleotide sequence complementary to the 5'-tagging portion or a part of the 5'-tagging portion of the PTO, (ii) a promoter portion inducing transcription; and (iii) a transcription portion; wherein the fragment released from the PTO is hybridized with the capturing portion of the CPO.

35. A kit for detecting a target nucleic acid sequence from a DNA or a mixture of nucleic acids using a target-dependent transcription, comprising:

(a) a PTO (Probing and Tagging Oligonucleotide); wherein the PTO comprises (i) a 3'-targeting portion comprising a hybridizing nucleotide sequence complementary to the target nucleic acid sequence and (ii) a 5'-tagging portion comprising a nucleotide sequence non-complementary to the target nucleic acid sequence; wherein the 3'-targeting portion is hybridized with the target nucleic acid sequence and the 5'-tagging portion is not hybridized with the target nucleic acid sequence;

(b) an upstream oligonucleotide; wherein the upstream oligonucleotide comprises a hybridizing nucleotide sequence complementary to the target nucleic acid sequence; the upstream oligonucleotide is located upstream of the PTO; wherein the upstream oligonucleotide or its extended strand induces cleavage of the PTO by an enzyme having a 5' nuclease activity such that the cleavage releases a fragment comprising the 5'-tagging portion or a part of the 5'-tagging portion of the PTO; and

(c) a CPO (Capturing and Promoter Oligonucleotide); wherein the CPO comprises in a 3' to 5' direction (i) a capturing portion comprising a nucleotide sequence complementary to the 5'-tagging portion or a part of the 5'-tagging portion of the PTO, (ii) a promoter portion inducing transcription; and (iii) a transcription portion; wherein the fragment released from the PTO is hybridized with the capturing

portion of the CPO.

36. The kit according to claim 34 or 35, wherein the kit further comprises an enzyme having a 5' nuclease activity.

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37. The kit according to claim 34 or 35, wherein the kit further comprises a template-dependent nucleic acid polymerase.

38. The kit according to claim 34 or 35, wherein the kit further comprises a polymerase recognizing the promoter portion.

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39. The kit according to claim 34 or 35, wherein the capturing portion and the promoter portion of the CPO are located in a non-overlapped manner or overlapped manner to each other.

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40. The kit according to claim 34 or 35, wherein the PTO and/or CPO is blocked at its 3'-end to prohibit its extension.

41. The kit according to claim 34 or 35, wherein the upstream oligonucleotide is an upstream primer or an upstream probe.

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42. The kit according to claim 34 or 35, wherein the upstream oligonucleotide is located adjacently to the PTO to the extent that the upstream oligonucleotide induces cleavage of the PTO by the enzyme having the 5' nuclease activity.

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43. The kit according to claim 41, wherein the upstream primer induces through its extended strand the cleavage of the PTO by the enzyme having the 5' nuclease activity.

44. The kit according to claim 34 or 35, wherein the capturing portion of the CPO comprises at its 5'-end part a nucleotide sequence complementary to a part of the 3'-targeting portion of the PTO.
- 5 45. The kit according to claim 34 or 35, wherein the promoter portion of the CPO comprises a RNA polymerase promoter.
46. The kit according to claim 45, wherein the RNA polymerase promoter is a promoter recognizable with T7 RNA polymerase, T3 RNA polymerase, SP6 RNA
10 polymerase, *Tth* RNA polymerase or *E. coli* RNA polymerase.
47. The kit according to claim 38, wherein the polymerase produces a nucleic acid molecule complementary to the transcription portion and the nucleic acid molecule produced is RNA.
- 15 48. The kit according to claim 38, wherein the polymerase produces a nucleic acid molecule comprising a complementary nucleotide sequence to a portion positioned in the 3'-direction of the transcription portion of the CPO.
- 20 49. The kit according to claim 34 or 35, wherein the kit further comprises a TCPO (Transcript-Capturing and Promoter Oligonucleotide) comprising a nucleotide sequence complementary to the produced nucleic acid molecule.
50. The kit according to claim 49, wherein the TCPO comprises in a 3' to 5' direction
25 (i) a transcript-capturing portion comprising a nucleotide sequence complementary to the nucleic acid molecule, (ii) a promoter portion inducing transcription, and (iii) a transcription portion.
51. The kit according to claim 50, wherein the transcript-capturing portion and the

promoter portion of the TCPO are located in a non-overlapped manner or overlapped manner to each other.

52. The kit according to claim 50, wherein the transcription portion of the CPO is the
5 same as or different from the transcription portion of the TCPO.

53. The kit according to claim 35, wherein the kit further comprises a detection
oligonucleotide comprising a nucleotide sequence complementary to the nucleic acid
molecule.

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54. The kit according to claim 53, wherein the detection oligonucleotide, the nucleic
acid molecule or each of them has at least one label.

55. The kit according to claim 54, wherein the nucleic acid molecule has at least one
15 label and the detection oligonucleotide is immobilized onto a solid substrate.

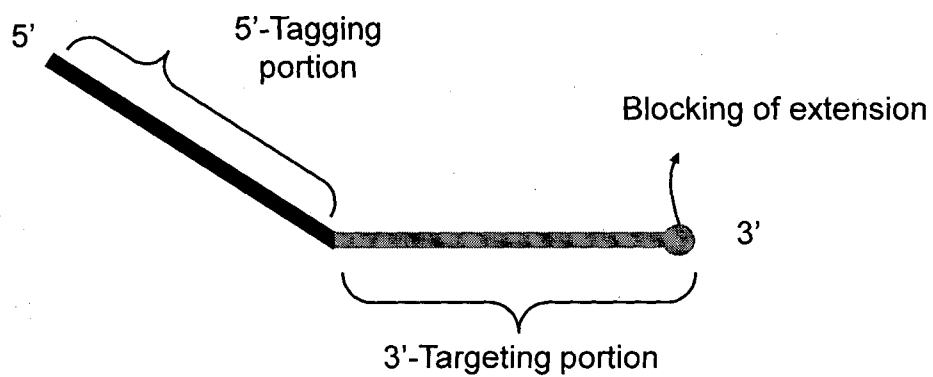
56. The kit according to claim 35, wherein the kit is used to detect at least two types
of target nucleic acid sequences; wherein the upstream oligonucleotide comprises at
least two types of oligonucleotides, the PTO comprises at least two types of the PTOs,
20 the CPO comprises at least two types of the CPOs.

57. The kit according to any one of claims 34 to 56, wherein the kit further
comprises a downstream primer.

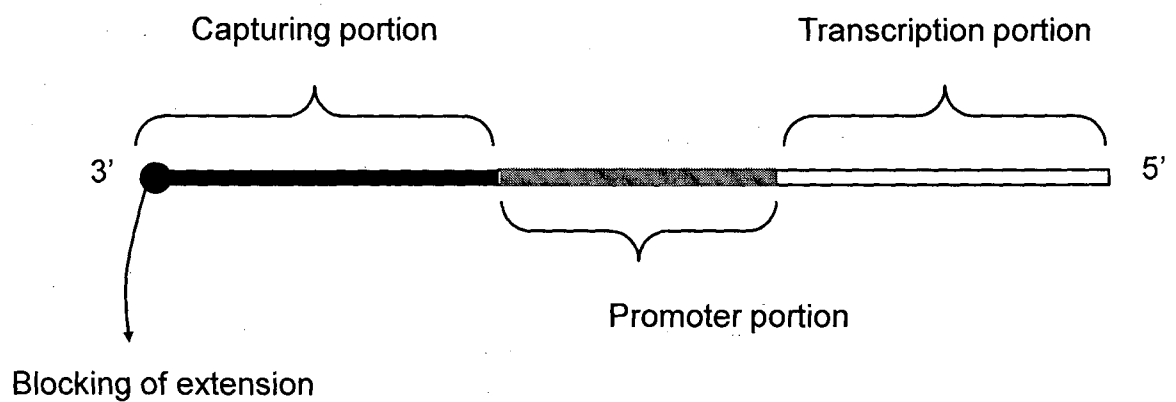
1/10

Fig. 1A

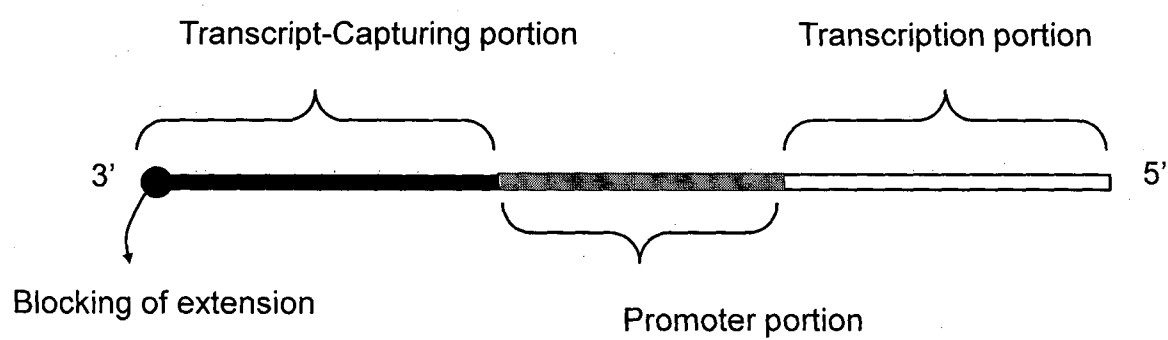
A. Probing and Tagging Oligonucleotide (PTO)



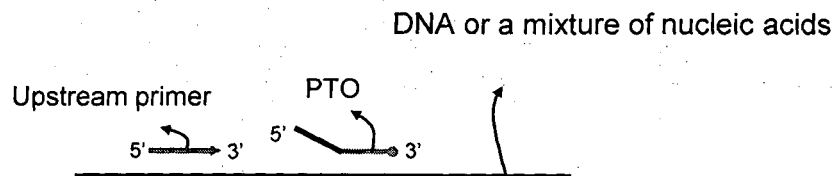
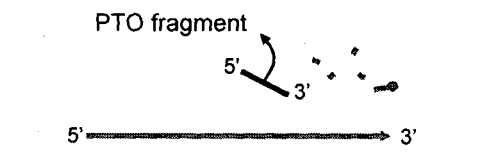
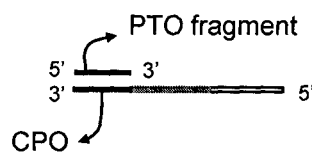
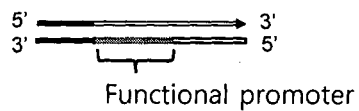
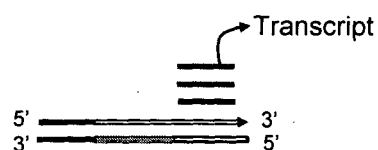
B. Capturing and Promoter Oligonucleotide (CPO)



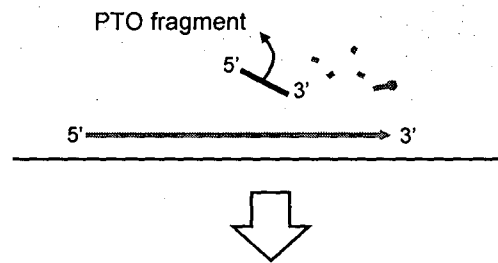
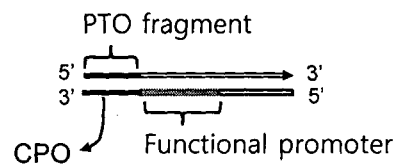
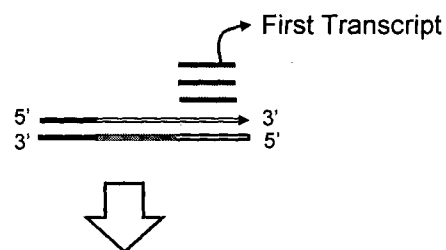
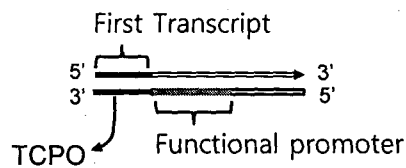
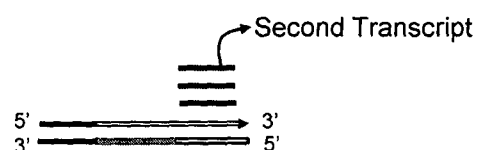
2/10

Fig. 1B**Transcript-Capturing and Promoter Oligonucleotide (TCPO)**

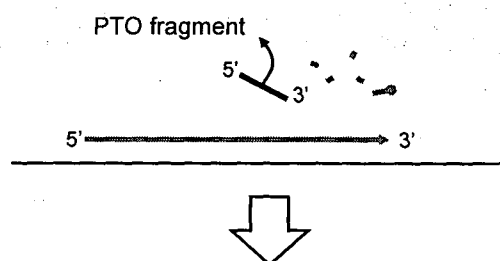
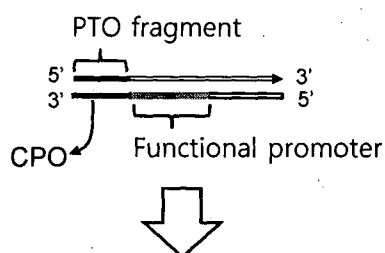
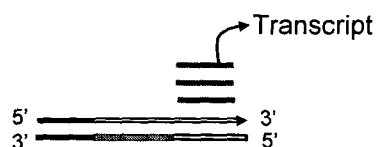
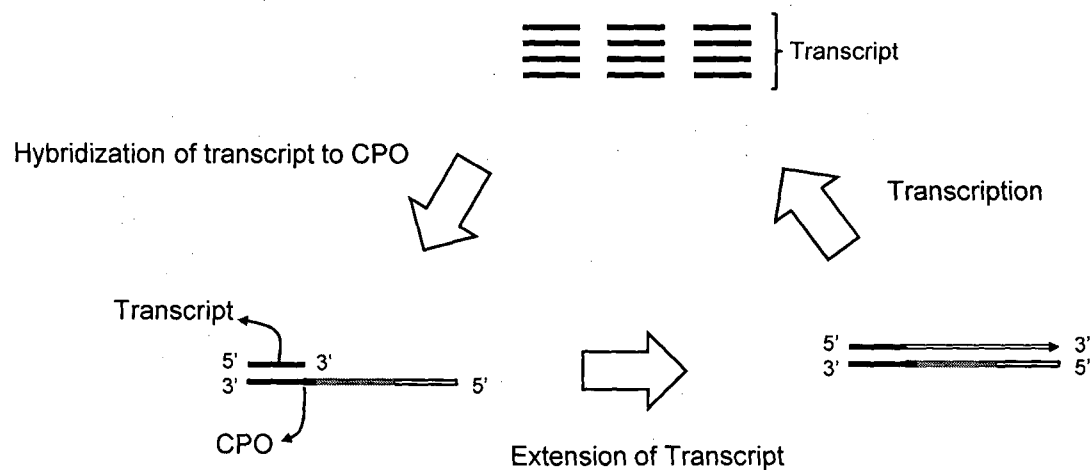
3/10

Fig. 2**A. Hybridization****B. Primer extension & Cleavage of PTO****C. Hybridization of PTO fragment to CPO****D. Extension of PTO fragment & Formation of functional promoter****E. Transcription**

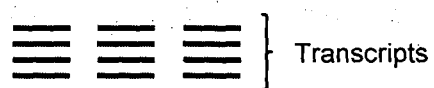
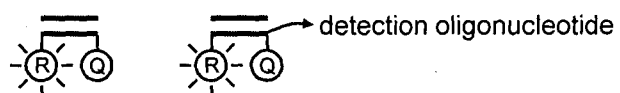
4/10

Fig. 3**A. Target-dependent PTO fragment release****B. Extension of PTO fragment & Formation of Functional Promoter****C. Transcription****D. Hybridization of Transcript to TCPO and Extension****E. Transcription**

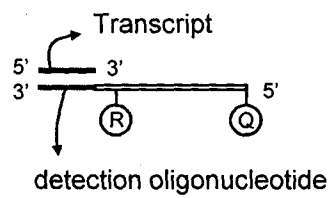
5/10

Fig. 4**A. Target-dependent PTO fragment release****B. Extension of PTO fragment & Formation of Functional Promoter****C. Transcription****D. Cyclic amplification using CPO**

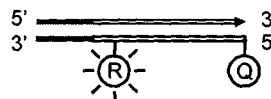
6/10

Fig. 5**A. Formation of transcript****B. Detection using detection oligonucleotide**

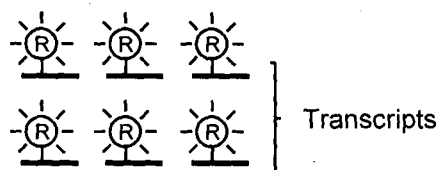
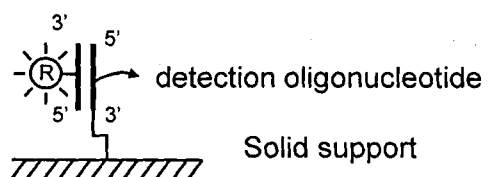
7/10

Fig. 6**A. Formation of transcript****B. Detection using detection oligonucleotide**

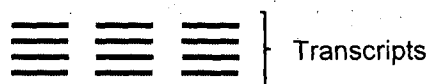
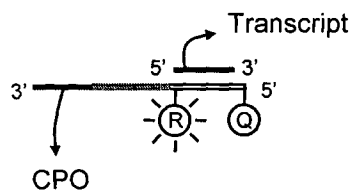
Extension of transcript



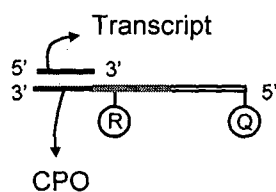
8/10

Fig. 7**A. Formation of labeled transcript****B. Detection of labeled transcript**

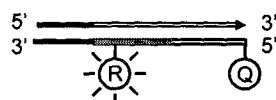
9/10

Fig. 8**A. Formation of transcript****B. Detection using labeled CPO as detection oligonucleotide**

10/10

Fig. 9**A. Formation of transcript****B. Detection using labeled CPO as detection oligonucleotide**

Extension of Transcript & Detection



A. CLASSIFICATION OF SUBJECT MATTER**C12N 15/10(2006.01)i, 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)

C12N 15/10; C07H 21/04; C07H 21/02; C12P 19/34; C12Q 1/68

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

Korean utility models and applications for utility models

Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKOMPASS(KIPO internal) & Keywords: detection, nucleic acid, PTO, CPO, probing and tagging oligonucleotide, capturing and promoter oligonucleotide, nucleic acid amplification**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2008-0241838 A1 (SCABOO, KRISTIAN et al.) 02 October 2008 See claim 1.	1-31,33-56
A	US 7381532 B2 (SORGE, JOSEPH A.) 03 June 2008 See claim 1.	1-31,33-56
A	US 5691142 A (DAHLBERG, JAMES E. et al.) 25 November 1997 See abstract.	1-31,33-56
A	US 6355431 B1 (CHEE, MARK S. et al.) 12 March 2002 See claim 1.	1-31,33-56
A	US 5422253 A (DAHLBERG, JAMES E. et al.) 06 June 1995 See claim 1.	1-31,33-56



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

12 September 2013 (12.09.2013)

Date of mailing of the international search report

12 September 2013 (12.09.2013)

Name and mailing address of the ISA/KR

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2013/004911

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

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

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

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

3. ☒ Claims Nos.: 32,57
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

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

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

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

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

Remark on Protest

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

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR2013/004911

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		US 2006-0110748 A1	25/05/2006
		US 2006-0246469 A1	02/11/2006
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