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DESCRIPTION

METHOD OF AMPLIFYING NUCLEIC ACID

Technical Field

The present invention relates to a method for detecting a target nucleic acid which is useful in a field of clinical medicine and a method for synthesizing a DNA which is useful in a field of genetic engineering. It relates to a method for amplifying a nucleic acid as a template and a method for detecting a target nucleic acid amplified by said method.

Background Art

DNA synthesis is used for various purposes in studies in a field of genetic engineering. Most of the DNA synthesis with the exception of that of a short-chain DNA such as an oligonucleotide is carried out by enzymatic methods in which a DNA polymerase is utilized. An example of the methods is the polymerase chain reaction (PCR) method as described in United States Patent Nos. 4,683,195, 4,683,202 and 4,800,159 in detail. Another example is the reverse transcription-PCR (RT-PCR) method, which is a combination of the PCR and a reverse transcriptase reaction, as described in Trends in Biotechnology, 10:146-152 (1992).

The development of the above-mentioned methods has enabled the amplification of a region of interest from a DNA or an RNA.

The above-mentioned DNA synthesis methods are conducted, for example, using a reaction that consists of three steps. The three steps are a step of dissociating (denaturing) a double-stranded DNA into single-stranded DNAs, a step of annealing a primer to the single-stranded DNA and a step of synthesizing (extending) a complementary strand from the primer in order to amplify a region of a DNA of interest. Alternatively, they are conducted using a reaction designated as "the shuttle PCR" ("PCR hou saizensen" (Recent advances in PCR methodology), Tanpakushitsu Kakusan Kouso, Bessatsu, (Protein, Nucleic Acid and Enzyme, Supplement), 41(5):425-428 (1996)) in which two of the three steps, that is, the step of annealing the primer and the step of extending are carried out at the same temperature.

Alternatively, the ligase chain reaction (LCR) method as described in EP 320,308 published on June 14, 1989 or the transcription-based amplification system (TAS) method as described in PCR Protocols, Academic Press Inc., 1990, pp. 245-252 may be used. The four methods as mentioned above require repeating a reaction at a high temperature and that at a low temperature several times in

order to regenerate a single-stranded target molecule for the next amplification cycle. The reaction system should be conducted using discontinuous phases or cycles because the reaction is restricted by temperature as described above.

Thus, the methods require the use of an expensive thermal cycler that can strictly adjust a wide range of temperature over time. Furthermore, the reaction requires time for adjusting the temperature to the two or three predetermined ones. The loss of time increases in proportion to the cycle number.

Nucleic acid amplification methods that can be carried out isothermally have been developed in order to solve the problems. Examples thereof include the strand displacement amplification (SDA) method as described in JP-B 7-114718, the self-sustained sequence replication (3SR) method, the nucleic acid sequence based amplification (NASBA) method as described in Japanese Patent No. 2650159, the transcription-mediated amplification (TMA) method, the Qβ replicase method as described in Japanese Patent No. 2710159 and the various modified SDA methods as described in United States Patent No. 5,824,517, WO 99/09211, WO 95/25180 and WO 99/49081. A method of isothermal enzymatic synthesis of an oligonucleotide is described in United States Patent No. 5,916,777. Extension from a primer

and/or annealing of a primer to a single-stranded extension product (or to an original target sequence) followed by extension from the primer take place in parallel in a reaction mixture incubated at a constant temperature in the reaction of these methods of isothermal nucleic acid amplification or synthesis of an oligonucleotide.

Among the isothermal nucleic acid amplification methods, the SDA method is an example of systems in which a DNA is finally amplified. The SDA method is a method for amplifying a target nucleic acid sequence (and a complementary strand thereof) in a sample by displacement of double strands using a DNA polymerase and a restriction endonuclease. The method requires four primers used for the amplification, two of which should be designed to contain a recognition site for the restriction endonuclease. The method requires the use of a modified deoxyribonucleotide triphosphate as a substrate for DNA synthesis in large quantities. An example of the modified deoxyribonucleotide triphosphates is an (α-S) deoxyribonucleotide triphosphate in which the oxygen atom of the phosphate group at the α-position is replaced by a sulfur atom (S). The problem of running cost associated with the use of the modified deoxyribonucleotide triphosphate becomes serious if the reaction is routinely conducted, for example, for genetic test. Furthermore, the

incorporation of the modified nucleotide such as the (α-S) deoxyribonucleotide into the amplified DNA fragment in the method may abolish the cleavability of the amplified DNA fragment with a restriction enzyme, for example, when it is subjected to a restriction enzyme fragment length polymorphism (RFLP) analysis.

The modified SDA method as described in United States Patent No. 5,824,517 is a DNA amplification method that uses a chimeric primer that is composed of an RNA and a DNA and has as an essential element a structure in which DNA is positioned at least at the 3'-terminus. The modified SDA method as described in WO 99/09211 requires the use of a restriction enzyme that generates a 3'-protruding end. The modified SDA method as described in WO 95/25180 requires the use of at least two pairs of primers. The modified SDA method as described in WO 99/49081 requires the use of at least two pairs of primers and at least one modified deoxyribonucleotide triphosphate. On the other hand, the method for synthesizing an oligonucleotide as described in United States Patent No. 5,916,777 comprises synthesizing a DNA using a primer having a ribonucleotide at the 3'-terminus, completing a reaction using the primer, introducing a nick between the primer and an extended strand in an primer-extended strand with an endonuclease to separate them, digesting a template

and recovering the primer to reuse it. It is required to isolate the primer from the reaction system and then anneal it to the template again in order to reuse the primer in the method. Additionally, the Loop-mediated Isothermal Amplification (LAMP) method as described in WO 00/28082 requires four primers for amplification and the products amplified using the method are DNAs having varying size in which the target regions for the amplification are repeated.

As described above, the conventional isothermal nucleic acid amplification methods still have various problems. Thus, a method for amplifying a nucleic acid at low running cost by which a DNA fragment that can be further genetically engineered is obtained has been desired.

Objects of Invention

The main object of the present invention is to provide a method for amplifying a target nucleic acid which specifically amplifies with high sensitivity a target nucleic acid in a sample by DNA synthesis reaction using a chimeric oligonucleotide primer, a method for detecting a amplified fragment obtained by said method, a method for producing a target nucleic acid using said amplification method and a chimeric oligonucleotide primer used for these methods.

Summary of Invention

As a result of intensive studies, the present inventors have constructed an excellent system for gene amplification reaction. The construction was accomplished by developing a method in which a region of a DNA of interest is amplified in the presence of a chimeric oligonucleotide primer having a ribonucleotide positioned at the 3'-terminus or on the 3'-terminal side, an endonuclease and a DNA polymerase. Thus, the present invention has been completed. The method is a method for amplifying a nucleic acid in which a chimeric oligonucleotide primer is used and herein referred to as ICAN (Isothermal and Chimeric primer-initiated Amplification of Nucleic acids) method.

The first aspect of the present invention relates to a method for amplifying a target nucleic acid, the method comprising:

(a) preparing a reaction mixture by mixing a nucleic acid as a template, a deoxyribonucleotide triphosphate, a DNA polymerase having a strand displacement activity, at least one primer and an RNase H, wherein the primer is a chimeric oligonucleotide primer that is substantially complementary to the nucleotide sequence of the nucleic acid as the template and contains a ribonucleotide as well as at least one selected from the

group consisting of a deoxyribonucleotide and a nucleotide analog, the ribonucleotide being positioned at the 3'-terminus or on the 3'-terminal side of the primer; and

(b) incubating the reaction mixture for a sufficient time to generate a reaction product.

In the first aspect of the present invention, a reaction mixture that further contains a chimeric oligonucleotide primer having a sequence that is substantially homologous to the nucleotide sequence of the nucleic acid as the template can be used.

The second aspect of the present invention relates to a method for amplifying a nucleic acid, the method comprising:

(a) treating a nucleic acid as a template with at least one primer that is substantially complementary to the nucleotide sequence of the nucleic acid and a DNA polymerase to synthesize a primer-extended strand that is complementary to the template and synthesize a double-stranded nucleic acid, wherein the primer is a chimeric oligonucleotide primer containing a ribonucleotide as well as at least one selected from the group consisting of a deoxyribonucleotide and a nucleotide analog, the ribonucleotide being positioned at the 3'-terminus or on the 3'-terminal side of the primer;

(b) extending a nucleotide sequence that is

complementary to the double-stranded nucleic acid as a template obtained in the previous step using a DNA polymerase having a strand displacement activity in the presence of an RNase H to effect a strand displacement and synthesize a displaced strand and a double-stranded nucleic acid; and

(c) reusing in step (b) the double-stranded nucleic acid obtained in step (b) as a template.

The third aspect of the present invention relates to a method for amplifying a nucleic acid using at least two primers, the method comprising:

(a) treating a nucleic acid as a template with at least one primer that is substantially complementary to the nucleotide sequence of the nucleic acid and a DNA polymerase to synthesize a primer-extended strand that is complementary to the template, wherein the primer is a chimeric oligonucleotide primer containing a ribonucleotide as well as at least one selected from the group consisting of a deoxyribonucleotide and a nucleotide analog, the ribonucleotide being positioned at the 3'-terminus or on the 3'-terminal side of the primer;

(b) extending a nucleotide sequence that is complementary to the double-stranded nucleic acid as a template obtained in the previous step using a DNA polymerase having a strand displacement activity in the

presence of an RNase H to effect a strand displacement and synthesize a displaced strand and a double-stranded nucleic acid;

(c) reusing in step (b) the double-stranded nucleic acid obtained in step (b) as a template;

(d) treating a displaced strand obtained in step (b) as a template with at least one primer that is different from that used in step (a) and a DNA polymerase to synthesize a primer-extended strand that is complementary to the displaced strand, wherein the primer that is different from that used in step (a) is a chimeric oligonucleotide primer that is substantially complementary to the nucleotide sequence of the displaced strand and contains a ribonucleotide as well as at least one selected from the group consisting of a deoxyribonucleotide and a nucleotide analog, the ribonucleotide being positioned at the 3'-terminus or on the 3'-terminal side of the primer;

(e) extending a nucleotide sequence that is complementary to the double-stranded nucleic acid as a template obtained in the previous step using a DNA polymerase having a strand displacement activity in the presence of an RNase H to effect a strand displacement and synthesize a displaced strand and a double-stranded nucleic acid; and

(f) reusing in step (e) the double-stranded

nucleic acid obtained in step (e) as a template.

In the second or third aspect of the present invention, a DNA polymerase having a strand displacement activity can be used as the DNA polymerase.

The fourth aspect of the present invention relates to method for amplifying a nucleic acid, the method comprising:

(a) treating a double-stranded nucleic acid as a template with two primers that are substantially complementary to the nucleotide sequences of the respective strands of the double-stranded nucleic acid and a DNA polymerase having a strand displacement activity to synthesize primer-extended strands that are complementary to the template and obtain a double-stranded nucleic acid consisting of the synthesized primer-extended strands being annealed each other, wherein each primer is a chimeric oligonucleotide primer containing a ribonucleotide as well as at least one selected from the group consisting of a deoxyribonucleotide and a nucleotide analog, the ribonucleotide being positioned at the 3'-terminus or on the 3'-terminal side of the primer;

(b) cleaving the double-stranded nucleic acid consisting of the primer-extended strands obtained in step (a) with the endonuclease at sites that contain the ribonucleotides; and

(c) extending nucleotide sequences that are complementary to the template using a DNA polymerase having a strand displacement activity from the 3'-termini of the respective primer portions of the double-stranded nucleic acid in which the primer-extended strands are cleaved obtained in step (b) to effect strand displacements and obtain a double-stranded nucleic acid consisting of the template and the primer-extended strand.

The fifth aspect of the present invention relates to a method for amplifying a nucleic acid, the method comprising:

(a) treating a double-stranded nucleic acid as a template with two primers that are substantially complementary to the nucleotide sequences of the respective strands of the double-stranded nucleic acid and a DNA polymerase having a strand displacement activity to synthesize primer-extended strands that are complementary to the template and obtain a double-stranded nucleic acid consisting of the synthesized primer-extended strands being annealed each other, wherein each primer is a chimeric oligonucleotide primer containing a ribonucleotide as well as at least one selected from the group consisting of a deoxyribonucleotide and a nucleotide analog, the ribonucleotide being positioned at the 3'-terminus or on the 3'-terminal side of the primer;

(b) cleaving the double-stranded nucleic acid consisting of the primer-extended strands obtained in step (a) with the endonuclease at sites that contain the ribonucleotides; and

(c) extending nucleotide sequences that are complementary to the template using a DNA polymerase having a strand displacement activity from the 3'-termini of the respective primer portions of the double-stranded nucleic acid in which the primer-extended strands are cleaved obtained in step (b) to effect strand displacements and obtain a double-stranded nucleic acid consisting of the primer-extended strands being annealed each other.

The sixth aspect of the present invention relates to a method for amplifying a nucleic acid, the method comprising:

(a) treating a double-stranded nucleic acid as a template with two primers that are substantially complementary to the nucleotide sequences of the respective strands of the double-stranded nucleic acid and a DNA polymerase having a strand displacement activity to synthesize primer-extended strands that are complementary to the template and obtain a double-stranded nucleic acid consisting of the synthesized primer-extended strands being annealed each other, wherein each primer is a chimeric oligonucleotide primer containing a ribonucleotide as well

as at least one selected from the group consisting of a deoxyribonucleotide and a nucleotide analog, the ribonucleotide being positioned at the 3'-terminus or on the 3'-terminal side of the primer;

(b) cleaving the double-stranded nucleic acid consisting of the primer-extended strands obtained in step (a) with the endonuclease at sites that contain the ribonucleotides;

(c) extending nucleotide sequences that are complementary to the template using a DNA polymerase having a strand displacement activity from the 3'-termini of the respective primer portions of the double-stranded nucleic acid in which the primer-extended strands are cleaved obtained in step (b) to effect strand displacements and obtain a double-stranded nucleic acid consisting of the primer-extended strands being annealed each other and a double-stranded nucleic acid consisting of the templates being annealed each other to which the two primers in step (a) are annealed;

(d) extending nucleotide sequences that are complementary to the template using a DNA polymerase having a strand displacement activity from the 3'-termini of the respective primer portions of the double-stranded nucleic acid to which the two primers are annealed obtained in step (c) to effect strand displacements and obtain a double-

stranded nucleic acid consisting of the primer-extended strands being annealed each other and a double-stranded nucleic acid consisting of the templates being annealed each other to which the two primers in step (a) are annealed; and

(e) reusing in step (d) the double-stranded nucleic acid to which the two primers are annealed obtained in step (d).

The seventh aspect of the present invention relates to a method for amplifying a nucleic acid, the method comprising:

(a) treating a double-stranded nucleic acid as a template with two primers that are substantially complementary to the nucleotide sequences of the respective strands of the double-stranded nucleic acid and a DNA polymerase having a strand displacement activity to synthesize primer-extended strands that are complementary to the template and obtain a double-stranded nucleic acid consisting of the synthesized primer-extended strands being annealed each other, wherein each primer is a chimeric oligonucleotide primer containing a ribonucleotide as well as at least one selected from the group consisting of a deoxyribonucleotide and a nucleotide analog, the ribonucleotide being positioned at the 3'-terminus or on the 3'-terminal side of the primer;

(b) cleaving the double-stranded nucleic acid consisting of the primer-extended strands obtained in step (a) with the endonuclease at sites that contain the ribonucleotides;

(c) extending nucleotide sequences that are complementary to the template using a DNA polymerase having a strand displacement activity from the 3'-termini of the respective primer portions of the double-stranded nucleic acid in which the primer-extended strands are cleaved obtained in step (b) to effect strand displacements and obtain a double-stranded nucleic acid consisting of the primer-extended strands being annealed each other and a double-stranded nucleic acid consisting of the templates being annealed each other to which the two primers in step (a) are annealed;

(d) extending nucleotide sequences that are complementary to the template using a DNA polymerase having a strand displacement activity from the 3'-termini of the respective primer portions of the double-stranded nucleic acid to which the two primers are annealed obtained in step (c) to effect a strand displacement and obtain a double-stranded nucleic acid consisting of the template and the primer-extended strand;

(e) cleaving the double-stranded nucleic acid consisting of the template and the primer-extended strand

obtained in step (d) with the endonuclease at a site that contains the ribonucleotide; and

(f) extending a nucleotide sequence that is complementary to the template using a DNA polymerase having a strand displacement activity from the 3'-terminus of the primer portion of the double-stranded nucleic acid in which the primer-extended strand is cleaved obtained in step (e) to synthesize a displaced strand.

In the fourth to seventh aspect, an endoribonuclease such as an RNase H can be used as the endonuclease.

In the first to seventh aspect wherein an RNase H is used, an RNase H from Escherichia coli, a bacterium of genus Thermotoga, a bacterium of genus Thermus, a bacterium of genus Pyrococcus, a bacterium of genus Archaeoglobus, a bacterium of genus Bacillus or the like can be used.

In the first to seventh aspect, an example of the suitable length of the region to be specifically amplified in the nucleotide sequence of the target nucleic acid is 200 bp or shorter.

For the first to seventh aspect of the present invention, a chimeric oligonucleotide primer represented by general formula below can be used:

General formula: 5'-dNa-Nb-dNc-3'

(a: an integer of 11 or more; b: an integer of 1 or more;

c: 0 or an integer of 1 or more; dN: deoxyribonucleotide and/or nucleotide analog; N: unmodified ribonucleotide and/or modified ribonucleotide, wherein some of dNs in dNa may be replaced by Ns, and the nucleotide at the 3'-terminus may be modified such that extension from the 3'-terminus by the action of the DNA polymerase does not take place).

Such chimeric oligonucleotide primers are exemplified by a primer in which c is 0 and a primer in which the nucleotide analog is deoxyriboinosine nucleotide or deoxyribouracil nucleotide, and the modified ribonucleotide is (α-S) ribonucleotide. Furthermore, when such a chimeric oligonucleotide primer is used, the DNA extension reaction is conducted at a DNA extension reaction temperature suitable for the primer.

The amplification method of the first to seventh aspect can comprise annealing the nucleic acid as the template to the chimeric oligonucleotide primer that is substantially complementary to the nucleotide sequence of the nucleic acid in an annealing solution containing a substance that enhances the annealing of the nucleic acid to the primer. For example, the annealing solution may contain spermidine and/or propylenediamine. The annealing can be conducted by incubating the annealing solution containing the nucleic acid as the template and the

chimeric oligonucleotide primer that is substantially complementary to the nucleotide sequence of the nucleic acid at 90°C or above and then cooling the solution to a temperature at which the amplification reaction is conducted or below.

The amplification reaction of the first to seventh aspect can be conducted in a buffer containing a buffering component selected from the group consisting of Bicine and HEPES.

In the first to seventh aspect, for example, a DNA polymerase selected from the group consisting of Klenow fragment of DNA polymerase I from Escherichia coli, Bst DNA polymerase lacking 5'→3' exonuclease from Bacillus stearothermophilus and Bca DNA polymerase lacking 5'→3' exonuclease from Bacillus caldotenax can be used as the DNA polymerase having a strand displacement activity.

In one embodiment of the first to seventh aspect, Bca DNA polymerase lacking 5'→3' exonuclease from Bacillus caldotenax is used as the DNA polymerase having a strand displacement activity and an RNase H from Escherichia coli, a bacterium of genus Pyrococcus or a bacterium of genus Archaeoglobus is used as the endonuclease. The RNase H is exemplified by type I RNase H from Escherichia coli, or type II RNase H from a bacterium of genus Pyrococcus or a bacterium of genus Archaeoglobus.

In the first to seventh aspect, a DNA polymerase having an endonuclease activity can be used. Bca DNA polymerase lacking 5'→3' exonuclease from Bacillus caldotenax can be used as such a DNA polymerase and the amplification reaction can be conducted in the presence of a substance that allows the endonuclease activity of the DNA polymerase to express. The substance that allows the endonuclease activity of the Bca DNA polymerase to express is exemplified by a manganese ion.

The method for amplifying a target nucleic acid of the first to seventh aspect can be conducted in the presence of a substance that inhibits the reverse transcription activity of the DNA polymerase. The substance that inhibits the reverse transcription activity of the DNA polymerase is exemplified by phosphonoformic acid.

The first to seventh aspect of the present invention can be conducted using a single-stranded DNA or a double-stranded DNA as the template. If the nucleic acid as the template is a double-stranded DNA, the amplification reaction can be conducted after converting it into single-stranded DNAs.

The nucleic acid as the template may be a cDNA obtained by a reverse transcription reaction using an RNA as a template. In one aspect, the amplification reaction

is conducted after synthesizing a cDNA by a reverse transcription reaction using an RNA as a template. A DNA polymerase having a reverse transcriptase activity can be used for the reverse transcription reaction. For example, the reverse transcription reaction and the synthesis of the extended strand that is complementary to the template can be conducted using one DNA polymerase having a reverse transcriptase activity and a strand displacement activity. Such a DNA polymerase is exemplified by Bst DNA polymerase lacking 5'→3' exonuclease from Bacillus stearothermophilus or Bca DNA polymerase lacking 5'→3' exonuclease from Bacillus caldotenax.

In the first to seventh aspect, the amplification reaction can be conducted under isothermal conditions. It can be conducted in the presence of a deoxynucleotide triphosphate analog such as deoxyuridine triphosphate or a derivative thereof.

The eighth aspect of the present invention relates to a composition for amplifying a nucleic acid which contains:

(a) at least one primer that is substantially complementary to a nucleotide sequence of a nucleic acid as a template, wherein the primer is a chimeric oligonucleotide primer that contains a ribonucleotide as well as at least one selected from the group consisting of

a deoxyribonucleotide and a nucleotide analog, the ribonucleotide being positioned at the 3'-terminus or on the 3'-terminal side of the primer;

(b) an endonuclease; and

(c) a DNA polymerase having a strand displacement activity.

The ninth aspect of the present invention relates to a composition for amplifying a nucleic acid which contains:

(a) at least two primers that are substantially complementary to nucleotide sequences of respective strands of a double-stranded nucleic acid as a template, wherein each primer is a chimeric oligonucleotide primer that contains a ribonucleotide as well as at least one selected from the group consisting of a deoxyribonucleotide and a nucleotide analog, the ribonucleotide being positioned at the 3'-terminus or on the 3'-terminal side of the primer;

(b) an endonuclease; and

(c) a DNA polymerase having a strand displacement activity.

The tenth aspect of the present invention relates to a composition for amplifying a nucleic acid obtained by mixing a nucleic acid as a template, a deoxyribonucleotide triphosphate, a DNA polymerase having a strand displacement activity, at least one primer and an endonuclease, wherein

the primer is a chimeric oligonucleotide primer that is substantially complementary to the nucleotide sequence of the nucleic acid as the template and contains a ribonucleotide as well as at least one selected from the group consisting of a deoxyribonucleotide and a nucleotide analog, the ribonucleotide being positioned at the 3'-terminus or on the 3'-terminal side of the primer.

The eleventh aspect of the present invention relates to a composition for amplifying a nucleic acid obtained by mixing a nucleic acid as a template, a deoxyribonucleotide triphosphate, a DNA polymerase having a strand displacement activity, at least two primers and an endonuclease, wherein each primer is a chimeric oligonucleotide primer that is substantially complementary to the nucleotide sequence of each strand of the double-stranded nucleic acid as the template and contains a ribonucleotide as well as at least one selected from the group consisting of a deoxyribonucleotide and a nucleotide analog, the ribonucleotide being positioned at the 3'-terminus or on the 3'-terminal side of the primer.

The primer contained in the composition of the eighth to eleventh aspect of the present invention is exemplified by a chimeric oligonucleotide primer represented by general formula below:

General formula: 5'-dNa-Nb-dNc-3'

(a: an integer of 11 or more; b: an integer of 1 or more; c: 0 or an integer of 1 or more; dN: deoxyribonucleotide and/or nucleotide analog; N: unmodified ribonucleotide and/or modified ribonucleotide, wherein some of dNs in dNa may be replaced by Ns, and the nucleotide at the 3'-terminus may be modified such that extension from the 3'-terminus by the action of the DNA polymerase does not take place).

Such chimeric oligonucleotide primers are exemplified by a primer in which c is 0 and a primer in which the nucleotide analog is deoxyriboinosine nucleotide or deoxyribouracil nucleotide, and the modified ribonucleotide is (α-S) ribonucleotide.

The composition of the eighth to eleventh aspect can contain a buffering component suitable for a nucleic acid amplification reaction. For example, it can contain a buffering component selected from the group consisting of Bicine and HEPES.

The eighth to eleventh aspect is exemplified by a composition containing a DNA polymerase selected from the group consisting of Klenow fragment of DNA polymerase I from Escherichia coli, Bst DNA polymerase lacking 5'→3' exonuclease from Bacillus stearothermophilus and Bca DNA polymerase lacking 5'→3' exonuclease from Bacillus caldotenax as the DNA polymerase having a strand

displacement activity. An endoribonuclease such as an RNase H can be used as the endonuclease. The RNase H is exemplified by an RNase H from Escherichia coli, a bacterium of genus Thermotoga, a bacterium of genus Thermus, a bacterium of genus Pyrococcus, a bacterium of genus Archaeoglobus or a bacterium of genus Bacillus.

In one embodiment, the composition of the eighth to eleventh aspect contains Bca DNA polymerase lacking 5'→3' exonuclease from Bacillus caldotenax as the DNA polymerase having a strand displacement activity and an RNase H from Escherichia coli, a bacterium of genus Pyrococcus or a bacterium of genus Archaeoglobus as the endonuclease. The RNase H is exemplified by type I RNase H from Escherichia coli, or type II RNase H from a bacterium of genus Pyrococcus or a bacterium of genus Archaeoglobus.

The composition of the eighth to eleventh aspect may contain a DNA polymerase having an endonuclease activity. Bca DNA polymerase lacking 5'→3' exonuclease from Bacillus caldotenax can be used as such a DNA polymerase, which can be used in the presence of a substance that allows the endonuclease activity of the DNA polymerase to express. The substance that allows the endonuclease activity of the Bca DNA polymerase to express is exemplified by a manganese ion.

The composition of the eighth to eleventh aspect

can contain a substance that inhibits the reverse transcription activity of the DNA polymerase. The substance that inhibits the reverse transcription activity of the DNA polymerase is exemplified by phosphonoformic acid. Furthermore, the composition may contain a deoxynucleotide triphosphate analog such as deoxyuridine triphosphate or a derivative thereof.

The twelfth aspect of the present invention relates to a composition for amplifying a nucleic acid used for the method for amplifying a nucleic acid of the first to third aspect, which contains:

(a) an RNase H; and

(b) a DNA polymerase having a strand displacement activity.

The thirteenth aspect of the present invention relates to a composition for amplifying a nucleic acid used for the method for amplifying a nucleic acid of the fourth to seventh aspect, which contains:

(a) an endonuclease; and

(b) a DNA polymerase having a strand displacement activity.

An endoribonuclease such as an RNase H can be used as the endonuclease for the composition of the thirteenth aspect.

An RNase H selected from those from Escherichia

coli, a bacterium of genus Thermotoga, a bacterium of genus Thermus, a bacterium of genus Pyrococcus, a bacterium of genus Archaeoglobus and a bacterium of genus Bacillus can be used as the RNase H for the composition of the twelfth or thirteenth aspect which contains an RNase H.

The composition of the twelfth or thirteenth aspect can further contain a buffering component suitable for a nucleic acid amplification reaction. For example, the composition may contain a buffering component selected from the group consisting of Bicine and HEPES.

The twelfth or thirteenth aspect is exemplified by a composition that contains a DNA polymerase selected from the group consisting of Klenow fragment of DNA polymerase I from Escherichia coli, Bst DNA polymerase lacking 5'→3' exonuclease from Bacillus stearothermophilus and Bca DNA polymerase lacking 5'→3' exonuclease from Bacillus caldotenax as the DNA polymerase having a strand displacement activity. An endoribonuclease such as an RNase H can be used as the endonuclease. The RNase H is exemplified by an RNase H from Escherichia coli, a bacterium of genus Thermotoga, a bacterium of genus Thermus, a bacterium of genus Pyrococcus, a bacterium of genus Archaeoglobus or a bacterium of genus Bacillus.

In one embodiment, the composition of the twelfth or thirteenth aspect contains Bca DNA polymerase lacking

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5'→3' exonuclease from Bacillus caldotenax as the DNA polymerase having a strand displacement activity and an RNase H from Escherichia coli, a bacterium of genus Pyrococcus or a bacterium of genus Archaeoglobus as the endonuclease. The RNase H is exemplified by type I RNase H from Escherichia coli, or type II RNase H from a bacterium of genus Pyrococcus or a bacterium of genus Archaeoglobus.

The composition of the twelfth or thirteenth aspect may contain a DNA polymerase having an endonuclease activity. Bca DNA polymerase lacking 5'→3' exonuclease from Bacillus caldotenax can be used as such a DNA polymerase, which can be used in the presence of a substance that allows the endonuclease activity of the DNA polymerase to express. The substance that allows the endonuclease activity of the Bca DNA polymerase to express is exemplified by a manganese ion.

The composition of the twelfth or thirteenth aspect can contain a substance that inhibits the reverse transcription activity of the DNA polymerase. The substance that inhibits the reverse transcription activity of the DNA polymerase is exemplified by phosphonoformic acid. Furthermore, the composition may contain a deoxynucleotide triphosphate analog such as deoxyuridine triphosphate or a derivative thereof.

The fourteenth aspect of the present invention

relates to a kit for amplifying a nucleic acid used for the method for amplifying a nucleic acid of the first to third aspect of the present invention, which contains:

(a) an RNase H; and

(b) a DNA polymerase having a strand displacement activity.

The fifteenth aspect of the present invention relates to a kit for amplifying a nucleic acid used for the method for amplifying a nucleic acid of the fourth to seventh aspect of the present invention, which contains:

(a) an endonuclease; and

(b) a DNA polymerase having a strand displacement activity.

An endoribonuclease such as an RNase H can be used as the endonuclease for the kit of the fifteenth aspect.

The kit of the fourteenth or fifteenth aspect which contains an RNase H is exemplified by a kit containing an RNase H selected from the group consisting of an RNase H from Escherichia coli, an RNase H from a bacterium of genus Thermotoga, an RNase H from a bacterium of genus Thermus, an RNase H from a bacterium of genus Pyrococcus, an RNase H from a bacterium of genus Archaeoglobus and an RNase H from a bacterium of genus Bacillus.

The kit of the fourteenth or fifteenth aspect may further contain a buffering component suitable for a nucleic acid amplification reaction. For example, the composition can contain a buffering component selected from the group consisting of Bicine and HEPES. The kit may contain an annealing solution containing a substance that enhances the annealing of the nucleic acid as the template to the primer that is substantially complementary to the nucleotide sequence of the nucleic acid. For example, the annealing solution may contain spermidine and/or propylenediamine.

The DNA polymerase having a strand displacement activity contained in the kit of the fourteenth or fifteenth aspect of the present invention is exemplified by a DNA polymerase selected from the group consisting of Klenow fragment of DNA polymerase I from Escherichia coli, Bst DNA polymerase lacking 5'→3' exonuclease from Bacillus stearothermophilus and Bca DNA polymerase lacking 5'→3' exonuclease from Bacillus caldotenax.

In one embodiment, the kit of the fourteenth or fifteenth aspect contains Bca DNA polymerase lacking 5'→3' exonuclease from Bacillus caldotenax and an RNase H from Escherichia coli, a bacterium of genus Pyrococcus or a bacterium of genus Archaeoglobus. The RNase H is exemplified by type I RNase H from Escherichia coli, or

type II RNase H from a bacterium of genus Pyrococcus or a bacterium of genus Archaeoglobus.

The kit of the fourteenth or fifteenth aspect of the present invention may contain a DNA polymerase having an endonuclease activity. Bca DNA polymerase lacking 5'→3' exonuclease from Bacillus caldotenax can be used as such a DNA polymerase. The kit can contain a substance that allows the endonuclease activity of the DNA polymerase to express. The substance that allows the endonuclease activity of the Bca DNA polymerase to express is exemplified by a manganese ion.

The kit of the fourteenth or fifteenth aspect can contain a substance that inhibits the reverse transcription activity of the DNA polymerase. The substance that inhibits the reverse transcription activity of the DNA polymerase is exemplified by phosphonoformic acid. Furthermore, the kit may contain a deoxynucleotide triphosphate analog such as deoxyuridine triphosphate or a derivative thereof.

The sixteenth aspect of the present invention relates to a kit for amplifying a nucleic acid used for the method for amplifying a nucleic acid of the first to third aspect of the present invention, which is in a packaged form and contains instructions that direct the use of a DNA polymerase having a strand displacement activity and an

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RNase H.

The seventeenth aspect of the present invention relates to a kit for amplifying a nucleic acid used for the method for amplifying a nucleic acid of the fourth to seventh aspect of the present invention, which is in a packaged form and contains instructions that direct the use of a DNA polymerase having a strand displacement activity and an endonuclease.

The eighteenth aspect of the present invention relates to a product of a reagent for amplifying a nucleic acid consisting of a packing material and a reagent for amplifying a nucleic acid enclosed in the packing material, wherein the reagent for amplifying a nucleic acid contains a DNA polymerase having a strand displacement activity and/or an RNase H, and description that the reagent for amplifying a nucleic acid can be used for nucleic acid amplification under isothermal conditions is indicated in a label stuck to the packaging material or instructions attached to the packaging material.

The nineteenth aspect of the present invention relates to a product of a reagent for amplifying a nucleic acid consisting of a packing material and a reagent for amplifying a nucleic acid enclosed in the packing material, wherein the reagent for amplifying a nucleic acid contains a DNA polymerase having a strand displacement activity

and/or an endonuclease, and description that the reagent for amplifying a nucleic acid can be used for nucleic acid amplification under isothermal conditions is indicated in a label stuck to the packaging material or instructions attached to the packaging material.

The twentieth aspect of the present invention relates to a method for detecting a target nucleic acid in a sample, the method comprising:

(a) amplifying a nucleic acid by the method for amplifying a nucleic acid of the first to seventh aspect of the present invention; and

(b) detecting a target nucleic acid amplified in step (a).

The detection method of the twentieth aspect of the present invention can comprise detecting the amplified nucleic acid using a probe for detection. The probe may be a probe that has been labeled with a labeling substance. For example, an RNA probe labeled with two or more fluorescent substances positioned at a distance that results in a quenching state can be used.

The twenty-first aspect of the present invention relates to a chimeric oligonucleotide primer used for the twentieth aspect. The chimeric oligonucleotide primer is exemplified by a chimeric oligonucleotide primer represented by general formula below:

General formula: 5'-dNa-Nb-dNc-3'

(a: an integer of 11 or more; b: an integer of 1 or more; c: 0 or an integer of 1 or more; dN: deoxyribonucleotide and/or nucleotide analog; N: unmodified ribonucleotide and/or modified ribonucleotide, wherein some of dNs in dNa may be replaced by Ns, and the nucleotide at the 3'-terminus may be modified such that extension from the 3'-terminus by the action of the DNA polymerase does not take place).

Such chimeric oligonucleotide primers are exemplified by a primer in which c is 0 and a primer in which the nucleotide analog is deoxyriboinosine nucleotide or deoxyribouracil nucleotide, and the modified ribonucleotide is (α-S) ribonucleotide.

The primer of the twenty-first aspect of the present invention is exemplified by a primer for detecting a pathogenic microorganism or a disease-related gene. A chimeric oligonucleotide primer for detecting a pathogenic microorganism such as enterohemorrhagic Escherichia coli, Clostridium botulinum, Staphylococcus aureus, Mycobacterium tuberculosis, Chlamydia, papilloma virus, hepatitis C virus or a viroid is encompassed by the present invention.

The twenty-second aspect of the present invention relates to a chimeric oligonucleotide primer for detecting enterohemorrhagic Escherichia coli having a nucleotide

sequence selected from the group consisting of SEQ ID NOS: 31-34, 47, 48, 51-53, 64-72, 84, 85, 113, 114, 130 and 131.

The twenty-third aspect of the present invention relates to a chimeric oligonucleotide primer for detecting a viroid having a nucleotide sequence selected from the group consisting of SEQ ID NOS: 59, 60, 119, 120, 122 and 123.

The twenty-fourth aspect of the present invention relates to a chimeric oligonucleotide primer for detecting Clostridium botulinum having a nucleotide sequence represented by SEQ ID NO: 116 or 117.

The twenty-fifth aspect of the present invention relates to chimeric oligonucleotide primer for detecting papilloma virus having a nucleotide sequence represented by SEQ ID NO: 96 or 97.

The twenty-sixth aspect of the present invention relates to a chimeric oligonucleotide primer for detecting hepatitis C virus having a nucleotide sequence selected from the group consisting of SEQ ID NOS: 101, 102, 138, 139, 200, 201, 205 and 206.

The twenty-seventh aspect of the present invention relates to a chimeric oligonucleotide primer for detecting Staphylococcus aureus having a nucleotide sequence represented by SEQ ID NO: 136 or 137.

The twenty-eighth aspect of the present invention

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relates to a chimeric oligonucleotide primer for detecting Mycobacterium tuberculosis having a nucleotide sequence selected from the group consisting of SEQ ID NOS: 155, 156, 159 to 162, 194 and 195.

The twenty-ninth aspect of the present invention relates to a chimeric oligonucleotide primer for detecting Chlamydia having a nucleotide sequence selected from the group consisting of SEQ ID NOS: 157, 158, 203 and 204.

The thirtieth aspect of the present invention relates to a kit for amplifying a nucleic acid used for the method for amplifying a nucleic acid of the first to fifth aspect of the present invention, which contains the chimeric oligonucleotide primer of the twenty-first to twenty-ninth aspect.

The thirty-first aspect of the present invention relates to a kit for detecting a target nucleic acid used for the method for detecting a target nucleic acid of the twentieth aspect of the present invention, which contains the chimeric oligonucleotide primer of the twenty-first to twenty-ninth aspect.

The thirty-second aspect of the present invention relates to a probe used in the method of the twentieth aspect.

The thirty-third aspect of the present invention relates to a probe which hybridizes to the nucleic acid

amplified by the method of the first to seventh aspect.

The thirty-fourth aspect of the present invention relates to a probe which hybridizes to a region amplified using the chimeric oligonucleotide primer of the twenty-first to twenty-ninth aspect.

The probe of the thirty-second to thirty-fourth aspect may be a probe which has been labeled with a labeling substance, for example, an RNA probe labeled with two or more fluorescent substances positioned at a distance that results in a quenching state.

The thirty-fifth aspect of the present invention relates to a kit used for the detection of the target nucleic acid in the method of the twentieth aspect, which contains the probe of the thirty-second to thirty-fourth aspect.

The thirty-sixth aspect of the present invention relates to a method for amplifying a nucleic acid, which comprises using a DNA polymerase having a strand displacement activity to effect a template switching reaction.

The DNA polymerase having a strand displacement activity used in the thirty-sixth aspect is exemplified by Klenow fragment of DNA polymerase I from Escherichia coli, Bst DNA polymerase lacking 5'→3' exonuclease from Bacillus stearothermophilus and Bca DNA polymerase lacking 5'→3'

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exonuclease from Bacillus caldotenax.

The thirty-seventh aspect of the present invention relates to a method for producing a material having an immobilized nucleic acid in which the nucleic acid is arrayed in a predefined region, the method comprising:

(a) amplifying a nucleic acid to be immobilized by the method for amplifying a nucleic acid of the first to seventh aspect of the present invention; and

(b) arraying and immobilizing the nucleic acid amplified in step (a) in a predefined region.

The thirty-eighth aspect of the present invention relates to a material having an immobilized nucleic acid in which the nucleic acid is arrayed in a predefined region produced by the method of the thirty-seventh aspect.

The thirty-ninth aspect of the present invention relates to a method for producing a nucleic acid in large quantities, the method comprising:

(a) amplifying a nucleic acid by the method for amplifying a nucleic acid of the first to seventh aspect of the present invention; and

(b) collecting the nucleic acid amplified in step (a).

The fortieth aspect of the present invention relates to a method for amplifying a nucleic acid, the

method comprising:

(a) duplicating a DNA or an RNA containing a sequence to be amplified to prepare a nucleic acid as a template; and

(b) amplifying the nucleic acid as the template obtained in step (a) by the method for amplifying a nucleic acid of the first to seventh aspect.

The forty-first aspect of the present invention relates to a method for determining a nucleotide sequence of a nucleic acid, the method comprising amplifying a nucleic acid according to the method of the first to seventh, thirty-ninth or fortieth aspect.

Brief Description of Drawings

Figure 1 illustrates agarose gel electrophoresis pattern of amplified DNA fragments amplified according to the method of the present invention.

Figure 2: illustrates agarose gel electrophoresis pattern of amplified DNA fragments amplified according to the method of the present invention.

Figure 3 illustrates agarose gel electrophoresis pattern of amplified DNA fragments amplified according to the method of the present invention.

Figure 4 illustrates agarose gel electrophoresis pattern of amplified DNA fragments amplified according to

the method of the present invention.

Figure 5 illustrates agarose gel electrophoresis pattern of amplified DNA fragments amplified according to the method of the present invention.

Figure 6 illustrates agarose gel electrophoresis pattern of amplified DNA fragments amplified according to the method of the present invention.

Figure 7 illustrates agarose gel electrophoresis pattern of amplified DNA fragments amplified according to the method of the present invention.

Figure 8 illustrates agarose gel electrophoresis pattern of amplified DNA fragments amplified according to the method of the present invention.

Figure 9 illustrates agarose gel electrophoresis pattern of amplified DNA fragments amplified according to the method of the present invention.

Figure 10 illustrates agarose gel electrophoresis pattern of amplified DNA fragments amplified according to the method of the present invention. A: ICAN; B: PCR.

Figure 11 illustrates agarose gel electrophoresis pattern of amplified DNA fragments amplified according to the method of the present invention.

Figure 12 illustrates agarose gel electrophoresis pattern of amplified DNA fragments amplified according to the method of the present invention.

Figure 13 illustrates agarose gel electrophoresis pattern of amplified DNA fragments amplified according to the method of the present invention.

Figure 14 illustrates agarose gel electrophoresis pattern of amplified DNA fragments amplified according to the method of the present invention.

Figure 15 illustrates agarose gel electrophoresis pattern of amplified DNA fragments amplified according to the method of the present invention.

Figure 16 illustrates agarose gel electrophoresis pattern of amplified DNA fragments amplified according to the method of the present invention.

Figure 17 illustrates agarose gel electrophoresis pattern of amplified DNA fragments amplified according to the method of the present invention.

Figure 18 illustrates agarose gel electrophoresis pattern of amplified DNA fragments amplified according to the method of the present invention.

Figure 19 illustrates agarose gel electrophoresis pattern of amplified DNA fragments amplified according to the method of the present invention.

Figure 20 illustrates agarose gel electrophoresis pattern of amplified DNA fragments amplified according to the method of the present invention.

Figure 21 illustrates agarose gel electrophoresis

pattern of amplified DNA fragments amplified according to the method of the present invention.

Figure 22 illustrates agarose gel electrophoresis pattern of amplified DNA fragments amplified according to the method of the present invention.

Figure 23 illustrates agarose gel electrophoresis pattern of amplified DNA fragments amplified according to the method of the present invention.

Figure 24 illustrates agarose gel electrophoresis pattern of amplified DNA fragments amplified according to the method of the present invention.

Figure 25 illustrates agarose gel electrophoresis pattern of amplified DNA fragments amplified according to the method of the present invention.

Figure 26 illustrates agarose gel electrophoresis pattern of amplified DNA fragments amplified according to the method of the present invention.

Figure 27 illustrates agarose gel electrophoresis pattern of amplified DNA fragments amplified according to the method of the present invention.

Figure 28 illustrates agarose gel electrophoresis pattern of amplified DNA fragments amplified according to the method of the present invention and dot blot hybridization pattern.

Figure 29 illustrates agarose gel electrophoresis

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pattern of amplified DNA fragments amplified according to the method of the present invention.

Figure 30 is a graph which compares the amounts of amplification products amplified according to the method of the present invention and the PCR.

Figure 31 illustrates agarose gel electrophoresis pattern of amplified DNA fragments amplified according to the method of the present invention.

Figure 32 illustrates polyacrylamide gel electrophoresis pattern of amplified DNA fragments amplified according to the method of the present invention.

Figure 33 illustrates one aspect of the method for amplifying a nucleic acid of the present invention.

Figure 34 illustrates one aspect of the method for amplifying a nucleic acid of the present invention.

Figure 35 illustrates one aspect of the method for amplifying a nucleic acid of the present invention.

Figure 36 illustrates one aspect of the method for amplifying a nucleic acid of the present invention.

Figure 37 illustrates agarose gel electrophoresis pattern of amplified DNA fragments amplified according to the method of the present invention.

Figure 38 illustrates agarose gel electrophoresis pattern of amplified DNA fragments amplified according to the method of the present invention.

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Figure 39 illustrates agarose gel electrophoresis pattern of amplified DNA fragments amplified according to the method of the present invention.

Detailed Description of the Invention

As used herein, a deoxyribonucleotide (also referred to as a dN) refers to a nucleotide of which the sugar portion is composed of D-2-deoxyribose. The deoxyribonucleotides include, for example, ones having adenine, cytosine, guanine or thymine as the base portion. Furthermore, the deoxyribonucleotides also include a deoxyribonucleotide having a modified base such as 7-deazaguanosine and a deoxyribonucleotide analog such as deoxyinosine nucleotide.

As used herein, a ribonucleotide (also referred to as an N) refers to a nucleotide of which the sugar portion is composed of D-ribose. The ribonucleotides include ones having adenine, cytosine, guanine or uracil as the base portion. The ribonucleotides also include modified ribonucleotides such as a modified ribonucleotide in which the oxygen atom of the phosphate group at the α-position is replaced by a sulfur atom (also referred to as an (α-S) ribonucleotide or an (α-S) N) or other derivatives.

As used herein, a chimeric oligonucleotide primer refers to a primer that contains a deoxyribonucleotide and

a ribonucleotide. The primer may contain a nucleotide analog and/or a modified ribonucleotide.

The chimeric oligonucleotide primers used in the present invention include any chimeric oligonucleotide primer that has a ribonucleotide being positioned at the 3'-terminus or on the 3'-terminal side of the primer, can be used to extend a nucleic acid strand in the method of the present invention, can be cleaved with an endonuclease, and can be used to effect a strand displacement reaction.

As used herein, 3'-terminal side refers to a portion from the center to the 3'-terminus of a nucleic acid such as a primer. Likewise, 5'-terminal side refers to a portion from the center to the 5'-terminus of a nucleic acid.

As used herein, an endonuclease may be any one that acts on a double-stranded DNA generated by extending a DNA from the chimeric oligonucleotide primer which have been annealed to a nucleic acid as a template, and specifically cleaves it at a portion of the primer that contains a ribonucleotide.

As used herein, a DNA polymerase refers to an enzyme that synthesizes a DNA strand de novo using a DNA strand as a template. The DNA polymerases include naturally occurring DNA polymerases and variant enzymes having the above-mentioned activity. For example, such

enzymes include a DNA polymerase having a strand displacement activity, a DNA polymerase lacking a 5'→3' exonuclease activity and a DNA polymerase having a reverse transcriptase activity or an endonuclease activity.

As used herein, "a strand displacement activity" refers to an activity that can effect a strand displacement, that is, that can proceed DNA duplication on the basis of the sequence of the nucleic acid as the template while displacing the DNA strand to release the complementary strand that has been annealed to the template strand. In addition, a DNA strand released from a nucleic acid as a template as a result of a strand displacement is referred to as "a displaced strand" herein.

Hereinafter, the present invention will be described in detail.

(1) Chimeric oligonucleotide primer used in the present invention.

The primer used in the method of the present invention is a chimeric oligonucleotide primer that contains a ribonucleotide as well as at least one selected from the group consisting of a deoxyribonucleotide and a nucleotide analog. Such primers also include an oligoribonucleotide primer that contains an unmodified ribonucleotide and/or a modified ribonucleotide.

A chimeric oligonucleotide primer used in the

method of the present invention is a chimeric oligonucleotide primer that has a nucleotide sequence substantially complementary to a part of the nucleotide sequence of a nucleic acid as a template. It can contribute to extension of a DNA strand under conditions used. Furthermore, a ribonucleotide is positioned at the 3'-terminus or on the 3'-terminal side of the chimeric oligonucleotide primer. The primer is usually designed such that it is complementary to a portion upstream of the region to be amplified, that is, a portion 3' to the nucleotide sequence corresponding to a region to be amplified in a nucleic acid as a template. As used herein, "a substantially complementary nucleotide sequence" means a nucleotide sequence that can anneal to a DNA as a template under reaction conditions used.

The chimeric oligonucleotide primer used in the method of the present invention may contain one or more modified ribonucleotide. As used herein, a ribonucleotide may be an unmodified ribonucleotide and/or a modified ribonucleotide that can be positioned at the 3'-terminus or on the 3'-terminal side of a chimeric oligonucleotide primer and that is recognized by or cleaved with an endonuclease. The ribonucleotides include both of the unmodified ribonucleotide and the modified ribonucleotide as described above. An unmodified ribonucleotide, a

modified ribonucleotide or a combination thereof can be used for the chimeric oligonucleotide primer of the present invention as long as it does not abolish the function of the primer. Examples of the modified ribonucleotides include, but are not limited to, an (α-S) ribonucleotide in which the oxygen atom bound to the phosphate group is replaced by a sulfur atom, and a ribonucleotide in which the hydroxy group at the 2-position of the ribose is replaced by a methoxy group. Such a chimeric oligonucleotide primer containing a modified ribonucleotide can be produced by using, for example, an (α-S) ribonucleotide triphosphate, which is prepared by a method using a sulfuration reaction reagent (Glen Research) as described in United States Patent No. 5,003,097, or a 2-OMe-RNA-CE phosphoramidite reagent (Glen Research).

A chimeric oligonucleotide primer that can be used in the amplification method of the present invention may be designed to contain a modified ribonucleotide that confers resistance to the cleavage with an endonuclease. Such a primer is useful in that one can control the cleavage site with an endonuclease during amplification reaction steps.

One or two chimeric oligonucleotide primer may be used in the method of the present invention depending on the desired form of a DNA fragment after amplification

(single-stranded or double-stranded). Specifically, one chimeric oligonucleotide primer is used when a single-stranded DNA is desired, whereas two primers are used when a double-stranded DNA is desired.

The length of the chimeric oligonucleotide primer used in the method of the present invention is not specifically limited, but is preferably about 12 nucleotides to about 100 nucleotides, more preferably about 15 nucleotides to about 40 nucleotides. It is preferable that the nucleotide sequence of the chimeric oligonucleotide is substantially complementary to a nucleic acid as a template such that it anneals to the nucleic acid as the template under reaction conditions used. The primer contains a sequence recognized by an endonuclease, which is utilized in a step as described below, at the 3'-terminus or on the 3'-terminal side.

For example, an oligonucleotide having a structure represented by the following general formula can be used in the DNA synthesis method of the present invention as a primer, although it is not intended to limit the present invention:

General formula: 5'-dNa-Nb-dNc-3'

(a: an integer of 11 or more; b: an integer of 1 or more; c: 0 or an integer of 1 or more; dN: deoxyribonucleotide and/or nucleotide analog; N: unmodified ribonucleotide

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and/or modified ribonucleotide, wherein some of dNs in dNa may be replaced by Ns, and the nucleotide at the 3'-terminus may be modified such that extension from the 3'-terminus by the action of the DNA polymerase does not take place).

For example, a chimeric oligonucleotide primer represented by the general formula in which a = an integer of 11 or more; and b = 1 and c =0, b = 2 and c = 0, b = 3-5 and c = 0, or b = 2 and c = 0-5 can be preferably used in the present invention. The length of the ribonucleotides at the 3'-terminus or on the 3'-termunal side of the chimeric oligonucleotide primer used in the method of the present invention is preferably 1-mer to 15-mer, more preferably 1-mer to 10-mer, most preferably 1-mer to 5-mer. The number of c in the general formula is not specifically limited, but any number that can be used in the method of the present invention may be selected. Usually, 5 or less is preferably. Better results are obtained in a reaction by selecting 3 rather than 4, 2 rather than 3, and 1 rather than 2 for c. In particular, the most efficient reaction can be accomplished in case of c = 0.

The chimeric oligonucleotide primer used in the present invention has a structure in which an endonuclease recognizes or cleaves a DNA strand extended from the primer using a DNA polymerase (a primer-extended strand) at a site

that contains a ribonucleotide, which ribonucleotide being positioned at the 3'-terminus or on the 3'-terminal side of the chimeric oligonucleotide primer. Although it is not intended to limit the present invention, for example, when an RNase H acts on a double-stranded DNA generated by extending a DNA from a chimeric oligonucleotide primer represented by the general formula that has been annealed to a nucleic acid as a template, the chimeric oligonucleotide primer is cleaved at the ribonucleotide portion. A double-stranded DNA in which a nick is introduced between the oligonucleotide primer and the DNA strand synthesized by the extension is then generated. Then, a strand displacement reaction with a DNA polymerase proceeds from the nicked site. Thus, any chimeric oligonucleotide primer that can be used to extend a nucleic acid strand from the 3'-terminus of the primer, that can be cleaved with an endonuclease, and with which a DNA polymerase can effect a strand displacement reaction can be used in the method of the present invention. Furthermore, the chimeric oligonucleotide primers of the present invention include one whose 3'-terminus is modified such that extension by the action of the DNA polymerase can not take place, and DNA extension takes place from a 3'-terminus generated upon cleavage by the endonuclease.

In addition, a promoter sequence for an RNA

polymerase may be included on the 5'-terminal side of the chimeric oligonucleotide primer. Such RNA polymerases are exemplified by T7 RNA polymerase and SP6 RNA polymerase.

Furthermore, the chimeric oligonucleotide primer used in the method of the present invention may contain a nucleotide analog or other substances. That is, one or more nucleotide analog(s) can be contained in the chimeric oligonucleotide primer of the present invention as long as the function of the primer for effecting a polymerization extension reaction from the 3'-terminus by the action of a DNA polymerase is not abolished. Plural types of the nucleotide analogs can be used in combination. Examples of the nucleotide analogs include, but are not limited to, deoxyinosine nucleotide, deoxyuracil nucleotide, a nucleotide analog having a modified base such as 7-deazaguanine, a nucleotide analog having a ribose derivative and the like. Furthermore, the chimeric oligonucleotide primers used in the present invention may contain deoxynucleotides, ribonucleotides or nucleotide analogs having various modifications such as addition of labeled compounds as long as they retain the functions as described above.

Incorporation of a nucleotide analog into a primer is effective for suppressing the formation of high-order structure of the primer itself and stabilization of

annealing formation with the template. A ribonucleotide may be incorporated into a primer for the same purpose. Although it is not intended to limit the present invention, a modified ribonucleotide such as (α-S) ribonucleotide can be preferably used in order to prevent the digestion of the primer by a non-specific endonuclease (RNase).

The chimeric oligonucleotide primer can be synthesized to have desired nucleotide sequence using, for example, the 394 type DNA synthesizer from Applied Biosystems Inc. (ABI) according to a phosphoramidite method. Alternatively, any methods including a phosphate triester method, an H-phosphonate method and a thiophosphonate method may be used to synthesize the chimeric oligonucleotide primer.

(2) Endonuclease used in the present invention.

Any endonuclease that can act on a double-stranded DNA generated by DNA extension from the chimeric oligonucleotide primer as described above in (1) that has been annealed to a nucleic acid as a template and cleaves the extended strand to effect a strand displacement reaction may be used in the present invention. That is, the endonuclease is an enzyme that can generate a nick in the chimeric oligonucleotide primer portion of the double-stranded DNA. Examples of endonucleases that can be used in the present invention include, but are not limited to,

ribonucleases. Among these, endoribonuclease H (RNase H) that acts on an RNA portion of a double-stranded nucleic acid composed of a DNA and an RNA can be preferably used. Any ribonuclease that has the above-mentioned activities can be preferably used in the present invention, including mesophilic and heat-resistant ones. For example, an RNase H from E. coli can be used for a reaction at about 50°C to about 70°C in the method of the present invention as described below in Examples. A heat-resistant ribonuclease can be preferably used in the method of the present invention. Examples of the heat-resistant ribonucleases which can be preferably used include, but are not limited to, a commercially available ribonuclease, Hybridase™ Thermostable RNase H (Epicenter Technologies) as well as an RNase H from a thermophilic bacterium of genus Bacillus, a bacterium of genus Thermus, a bacterium of genus Pyrococcus, a bacterium of genus Thermotoga, a bacterium of genus Archaeoglobus or the like. Furthermore, both of naturally occurring ribonucleases and variants can be preferably used. The enzymatic unit of RNase H indicated herein is a value expressed according to a method of measuring an enzymatic unit as described in Referential Examples.

The RNase H is not limited to a specific one as long as it can be used in the method of the present invention. For example, the RNase H may be derived from

various viruses, phages, prokaryotes or eukaryotes. It may be either a cellular RNase H or a viral RNase H. The cellular RNase H is exemplified by Escherichia coli RNase HI and the viral RNase H is exemplified by HIV-1. Type I, type II or type III RNase H can be used in the method of the present invention. For example, RNase HI from Escherichia coli, or RNase HII from a bacterium of genus Pyrococcus or a bacterium of genus Archaeoglobus is preferable, without limitation.

The efficiency of the cleavage reaction with an endonuclease such as RNase H used in the method of the present invention may vary depending on the nucleotide sequence around the 3'-terminus of the primer and influence the amplification efficiency of the desired DNA. Therefore, it is natural to design the optimal primer for the RNase H used.

As used herein, the term "introducing a nick" or "nicking" means internally cleaving one of the two strands of a double-stranded nucleic acid. For example, an RNase H acts on a hybrid double-stranded nucleic acid composed of a DNA and a ribonucleotide-containing DNA to selectively cleave the ribonucleotide-containing strand among the two strands at the ribonucleotide portion, thereby introducing a nick into the hybrid double-stranded nucleic acid.

(3) DNA polymerase used in the present invention.

A DNA polymerase having a strand displacement activity on a DNA can be used in the present invention. Particularly, a DNA polymerase substantially lacking a 5'→3' exonuclease activity can be preferably used.

As used herein, "a strand displacement activity" refers to an activity that can effect a strand displacement, that is, that can proceed DNA duplication on the basis of a sequence of a nucleic acid as a template while displacing a DNA strand to release a complementary strand that has been annealed to the template strand. Additionally, a DNA strand released from a nucleic acid as a template as a result of a strand displacement is referred to as "a displaced strand" herein.

Any DNA polymerases having the strand displacement activity can be used in the present invention. Examples thereof include variants of DNA polymerases lacking their 5'→3' exonuclease activities derived from thermophilic bacteria of genus Bacillus such as Bacillus caldotenax (hereinafter referred to as B. ca) and Bacillus stearothermophilus (hereinafter referred to as B. st), as well as large fragment (Klenow fragment) of DNA polymerase I from Escherichia coli (E. coli). Both of mesophilic and heat-resistant DNA polymerases can be preferably used in the present invention.

B. ca is a thermophilic bacterium having an

optimal growth temperature of about 70°C. Bca DNA polymerase from this bacterium is known to have a DNA-dependent DNA polymerase activity, an RNA-dependent DNA polymerase activity (a reverse transcription activity), a 5'→3' exonuclease activity and a 3'→5' exonuclease activity. The enzyme may be either an enzyme purified from its original source or a recombinant protein produced by using genetic engineering techniques. The enzyme may be subjected to modification such as substitution, deletion, addition or insertion by using genetic engineering techniques or other means. Examples of such enzymes include BcaBEST DNA polymerase (Takara Shuzo), which is Bca DNA polymerase lacking its 5'→3' exonuclease activity.

It is known that some DNA polymerases have an endonuclease activity such as an RNase H activity under specific conditions. Such a DNA polymerase can be used in the method of the present invention. In one aspect, the DNA polymerase may be used under conditions that allow the RNase H activity to express, e.g., in the presence of Mn^{2+}. In this case, the method of the present invention can be conducted without the addition of an RNase H. The present inventors have demonstrated that the Bca DNA polymerase exhibits an RNase activity in a buffer containing Mn^{2+} for the first time, and that the method for amplifying a nucleic acid of the present invention can be carried out in

a reaction mixture containing no enzyme other than the Bca DNA polymerase. The above-mentioned aspect is not limited to the use of the Bca DNA polymerase. DNA polymerases that are known to have an RNase H activity such as Tth DNA polymerase from Thermus thermophilus can be used in the present invention.

(4) Composition of reaction buffer used in the present invention.

A reaction buffer that contains a buffering component, a magnesium salt or another metal salt and dNTPs is used in the present invention. Naturally, the type and the concentration of the salt are optimized depending on the metal requirement or the like of the enzyme to be used. Examples of the buffering components that can be preferably used include, but are not limited to, Bicine, Tricine, HEPES, tris and a phosphate (such as sodium phosphate and potassium phosphate). Among these, a buffer that contains Bicine, Tricine, HEPES or a phosphate as a buffering component is preferable for the present invention. Although it is not intended to limit the present invention, for example, when the reaction temperature is high, a Bicine buffer of which the change in pH due to the change in temperature is little is preferably used. A HEPES buffer may be preferable depending on the type of RNase H used. Thus, the optimal buffer may be selected depending

on the reaction temperature, the endonuclease or the DNA polymerase to be used and the like. The final concentration of the buffering component ranges 5-100 mM, preferably 20-50 mM. The pH ranges 6.0-9.5, preferably 7.0-9.2. For example, a buffer containing 22-46 mM Tricine at pH 7.5-9.2 or a buffer containing 25-50 mM potassium phosphate at pH 7.0-8.0 is preferably used. Examples of magnesium salts that can be preferably used include, but are not limited to, magnesium chloride, magnesium acetate or magnesium sulfate. The final concentration of the magnesium salt ranges 1-20 mM, preferably 2-10 mM. The final concentrations of dNTPs (a mixture of dATP, dCTP, dGTP and dTTP) in a mixture as substrates for a DNA extension reaction range 0.1-3.0 mM, preferably 0.2-1.2 mM. The amount of the primers used in a reaction volume of 50 µl ranges 1-1000 pmol, preferably 10-150 pmol. Additionally, the reaction mixture may contain an additive, for example, in order to stabilize the amplification reaction. Bovine serum albumin (BSA) at a final concentration of 0.1% or less, dimethyl sulfoxide (DMSO) at a final concentration of 10% or less, putrescine dihydrochloride at a final concentration of 4 mM or less, or propylenediamine at a final concentration of 0.01% or less may be added. Additionally, NMP (1-methyl-2-pyrrolidinone), glycerol, polyethylene glycol, dimethyl

sulfoxide and/or formamide may be contained. It is expected that the addition of such an organic solvent reduces the non-specific annealing of oligonucleotide primers.

The method of the present invention may be carried out by adding a substance that inhibits the reverse transcription activity of the DNA polymerase such as phosphonoformic acid (PFA). If a substance that inhibits the reverse transcription activity is added, the amplification of non-specific products other than the target nucleic acid is reduced.

In another aspect, annealing of the nucleic acid as the template to the chimeric oligonucleotide primer used in the present invention before the amplification reaction is effective to reduce non-specific annealing of an oligonucleotide primer in the detection, amplification or production method of the present invention. It is preferable to conduct the annealing using an annealing solution that contains a substance that enhances the annealing such as a polyamine (e.g., spermine or spermidine) or propylenediamine. Preferably, the annealing solution containing the polyamine also contains a salt. For example, without limitation, the annealing solution may contain sodium chloride, potassium chloride, potassium acetate, sodium acetate or the like and a polyamine.

The annealing is usually conducted by incubating the annealing solution containing the primer and the nucleic acid as the template at a temperature at which a double-stranded nucleic acid is denatured (e.g., 90°C or above) and then cooling the solution to a reaction temperature used for the method of the present invention or below.

After annealing, the nucleic acid amplification reaction of the present invention is initiated by further adding other necessary components such as a DNA polymerase, an RNase H and dNTPs to the mixture.

The amount of an RNase H from Escherichia coli as an example of endonucleases in a reaction volume of 50 µl ranges preferably 3-200 U, more preferably 15-60 U. Similarly, the amount of an RNase H from a bacterium of genus Pyrococcus or a bacterium of genus Archaeoglobus as an example of endonuclease in a reaction volume of 50 µl ranges preferably 3-200 U, more preferably 4-40 U. The amount of BcaBEST DNA polymerase (Takara Shuzo) as an example of DNA polymerases in a reaction volume of 50 µl ranges preferably 0.5-100 U, more preferably 1-22 U.

When an endonuclease and a DNA polymerase is used in combination in the method of the present invention, for example, without limitation, a combination of an RNase H from Escherichia coli, a bacterium of genus Pyrococcus or a

bacterium of genus Archaeoglobus and BcaBEST DNA polymerase is preferably used. It is considered that the preferable units of the endonuclease and the DNA polymerase may vary depending on the types the enzymes. In such a case, the composition of the buffer used and the amount of the enzymes added may be adjusted using the increase in detection sensitivity or the amount of amplification product as an index. In either case, it is natural to optimize the composition of the reaction buffer and the like depending on the type of the enzyme to be used.

(5) Method for amplifying nucleic acid of the present invention.

The method of the present invention can be conducted by using at least one oligonucleotide primer as described above in (1) in combination with the endonuclease as described above in (2) and the DNA polymerase as described above in (3). Alternatively, a DNA polymerase having an RNase H activity can be used under conditions that allow the RNase H activity to express as described above.

dNTPs used for the PCR or the like (a mixture of dATP, dCTP, dGTP and dTTP) can be preferably used as nucleotide triphosphates that serve as substrates in the extension reaction in the method. In addition, dUTP may be used as a substrate. The dNTPs may contain a dNTP

(deoxyribonucleotide triphosphate) analog such as 7-deaza-dGTP, triphosphate of dITP or the like as long as it serves as a substrate for the DNA polymerase used. A derivative of a dNTP or a dNTP analog may be used. A derivative having a functional group such as a dUTP having an amino group may be contained. A chimeric oligonucleotide primer is used in the method. The primer can be prepared, for example, using a DNA synthesizer according to a conventional synthesis method. A combination of the chimeric oligonucleotide primer and a normal oligonucleotide primer can be used in the method of the present invention.

If the activity of the enzyme used may be decreased in the course of the reaction, the enzyme can be further added during the reaction in the method of the present invention. Although it is not intended to limit the present invention, for example, an RNase H from Escherichia coli may be further added during a reaction in which the RNase H is used. The added enzyme may be the same as that contained in the reaction mixture at the beginning of the reaction or it may be a different enzyme that exhibits the same activity. Thus, the type or the property of the enzyme to be added is not limited to a specific one as long as the addition during the reaction provides effects such as increase in the detection

sensitivity or increase in the amount of amplification product.

The nucleic acid (DNA or RNA) used as a template in the method of the present invention may be prepared or isolated from any sample that may contain the nucleic acid. Alternatively, the sample may be used directly in the nucleic acid amplification reaction according to the present invention. Examples of the samples that may contain the nucleic acid include, but are not limited to, samples from organisms such as a whole blood, a serum, a buffy coat, a urine, feces, a cerebrospinal fluid, a seminal fluid, a saliva, a tissue (e.g., a cancerous tissue or a lymph node) and a cell culture (e.g., a mammalian cell culture or a bacterial cell culture), samples that contain a nucleic acid such as a viroid, a virus, a bacterium, a fungi, a yeast, a plant and an animal, samples suspected to be contaminated or infected with a microorganism such as a virus or a bacterium (e.g., a food or a biological formulation), and samples that may contain an organism such as a soil and a waste water. The sample may be a preparation containing a nucleic acid obtained by processing the above-mentioned samples according to a known method. Examples of the preparations that can be used in the present invention include a cell destruction product or a sample obtained by fractionating the product, the nucleic

acid in the sample, or a sample in which specific nucleic acid molecules such as mRNAs are enriched. Furthermore, a nucleic acid such as a DNA or an RNA obtained amplifying a nucleic acid contained in the sample by a known method can be preferably used.

The preparation containing a nucleic acid can be prepared from the above-mentioned materials by using, for example, lysis with a detergent, sonication, shaking/stirring using glass beads or a French press, without limitation. In some cases, it is advantageous to further process the preparation to purify the nucleic acid (e.g., in case where an endogenous nuclease exists). In such cases, the nucleic acid is purified by a know method such as phenol extraction, chromatography, ion exchange, gel electrophoresis or density-gradient centrifugation.

When it is desired to amplify a nucleic acid having a sequence derived from an RNA, the method of the present invention may be conducted using as a template a cDNA synthesized by a reverse transcription reaction that uses the RNA as a template. Any RNA for which one can make a primer to be used in a reverse transcription reaction can be applied to the method of the present invention, including total RNA in a sample, RNA molecules such as, mRNA, tRNA and rRNA as well as specific RNA molecular species.

Any primer that anneals to an RNA as a template under reaction conditions used can be used in the reverse transcription reaction. The primer may be a primer having a nucleotide sequence that is complementary to a specific RNA as a template (a specific primer), an oligo-dT (deoxythymine) primer and a primer having a random sequence (a random primer). In view of specific annealing, the length of the primer for reverse transcription is preferably 6 nucleotides or more, more preferably 9 nucleotides or more. In view of oligonucleotide synthesis, the length is preferably 100 nucleotides or less, more preferably 30 nucleotides or less. A chimeric oligonucleotide primer can be used as a primer for reverse transcription. The chimeric oligonucleotide primer can also be utilized as a primer for a strand displacement reaction in the method for amplifying a nucleic acid of the present invention using a cDNA obtained after reverse transcription as a template. Such a primer may be any one that has the properties as described above in (1) and that can be used in a reverse transcription reaction from an RNA.

Any enzyme that has an activity of synthesizing a cDNA using an RNA as a template can be used in the reverse transcription reaction. Examples thereof include reverse transcriptases originating from various sources such as avian myeloblastosis virus-derived reverse transcriptase

(AMV RTase), Molony murine leukemia virus-derived reverse transcriptase (MMLV RTase) and Rous-associated virus 2 reverse transcriptase (RAV-2 RTase). In addition, a DNA polymerase that also has a reverse transcription activity can be used. An enzyme having a reverse transcription activity at a high temperature such as a DNA polymerase from a bacterium of genus Thermus (e.g., Tth DNA polymerase) and a DNA polymerase from a thermophilic bacterium of genus Bacillus is preferable for the present invention. For example, DNA polymerases from thermophilic bacteria of genus Bacillus such as a DNA polymerase from B. st (Bst DNA polymerase) and Bca DNA polymerase are preferable, although it is not intended to limit the present invention. For example, Bca DNA polymerase does not require a manganese ion for the reverse transcription reaction. Furthermore, it can synthesize a cDNA while suppressing the formation of a secondary structure of an RNA as a template under high-temperature conditions. Both a naturally occurring one and a variant of the enzyme having a reverse transcriptase activity can be used as long as they have the activity.

In another aspect, after duplicating a DNA or an RNA containing a nucleotide sequence to be amplified beforehand, the duplicated product may be used as a nucleic acid as a template in the method of the present invention.

Examples of the methods for duplication include, but are not limited to, a method in which an appropriate host is transformed with a vector into which the nucleic acid fragment containing the nucleotide sequence to be amplified is inserted, the resulting transformant is cultured, the vector into which the nucleic acid fragment containing the nucleotide sequence to be amplified is inserted is extracted therefrom and used. Any vectors can be used as long as they are stably replicated in the host. For example, pUC series, pBluescript series, pGEM series, cosmid type vectors and phage type vectors are preferably used. Any hosts can be used as long as they can maintain the vectors used. For example, Escherichia coli, which is readily cultured, is exemplified.

In another aspect of the duplication method, after an RNA having a nucleotide sequence to be amplified is transcribed using an RNA polymerase and a nucleic acid fragment containing the nucleotide sequence as a template, the RNA may be used as a template for the method of the present invention directly or after converting it into a cDNA by reverse transcription reaction. The nucleic acid fragment containing the nucleotide sequence to be amplified is not limited to a specific one as long as it has a promoter sequence for an RNA polymerase. It may be inserted into a vector having a promoter sequence for an

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RNA polymerase, ligated with an adapter or a cassette having a promoter sequence for an RNA polymerase at its end or enzymatically synthesized using a primer having a promoter sequence for an RNA polymerase and an appropriate template. Thus, a nucleic acid fragment containing a nucleotide sequence to be amplified can be duplicated or amplified in a form of an RNA using a promoter sequence for an RNA polymerase being positioned as described above. Any vectors can be used as long as they have promoter sequences for RNA polymerases. For example, pUC series, pBluescript series, pGEM series, cosmid type vectors and phage type vectors are preferably used. The vector can be preferably used in its circular form or after being linearized. Any RNA polymerases can be used for the duplication or amplification method. For example, SP6 RNA polymerase, T7 RNA polymerase, T3 RNA polymerase or the like can be preferably used.

Both of a double-stranded DNA such as a genomic DNA isolated as described above or a PCR fragment and a single-stranded DNA such as a cDNA prepared by a reverse transcription reaction from a total RNA or an mRNA can be preferably used as a template DNA in the present invention. The double-stranded DNA is preferably used after denaturing it into single-stranded DNAs or without such denaturation.

The denaturing step may be eliminated in the

nucleic acid amplification method of the present invention if a linear double-stranded DNA such as a PCR amplification product is used as a template. The elimination may be accomplished by locating the annealing site for the primer used in the method of the present invention about 50 bases inside from the terminus of the DNA. If a nucleic acid having a sequence from an RNA is to be amplified, amplification reaction can be initiated by adding an RNA-cDNA double-stranded nucleic acid obtained by reverse transcription reaction using an RNA as a template to the amplification reaction mixture of the present invention containing an RNase H to digest the RNA strand and convert the nucleic acid into a single-stranded cDNA. Furthermore, a reverse transcription reaction using an RNA as a template and a DNA amplification reaction using a cDNA generated by the reverse transcription reaction as a template can be conducted with one DNA polymerase in the DNA synthesis method of the present invention. Such a DNA polymerase has a reverse transcriptase activity and a strand displacement activity.

The suitable length of the template is one that provides a sufficient binding of the primer sequence due to the presence of the whole target sequence or at least a sufficient part of the target sequence in the fragment.

Without limitation, if a DNA as a template is a

double-stranded DNA, the DNA can be denatured into single-stranded DNAs to allow a primer to bind to the DNA strand as the template in the method of the present invention. Incubating the double-stranded DNA at a temperature at which it is denatured (e.g., about 95°C) is preferable for the denaturation. Other processes include one in which an elevated pH is used. In this case, the pH should be lowered upon the amplification reaction in order to allow an oligonucleotide primer to bind to a target. A nucleic acid is successively amplified under isothermal conditions after denaturing a double-stranded DNA into single-stranded DNAs or, if an RNA is used as a template, preparing a cDNA (a single-stranded DNA) by a reverse transcription reaction.

"Successively" means that a reaction proceeds without a change in the reaction temperature or the composition of the reaction mixture. As used herein, "isothermal" means conditions of a substantially constant temperature under which an enzyme and a nucleic acid strand function in each step.

The nucleic acid amplification reaction of the present invention may be conducted at a normal temperature (e.g., 37°C) by using a mesophilic DNA polymerase such as Klenow fragment. It can also be conducted at a high temperature (e.g., 50°C or higher, or 60°C or higher) by using heat-resistant enzymes (an endonuclease and a DNA

polymerase). In this case, non-specific annealing of a primer is suppressed, resulting in increase in the specificity of DNA amplification. Furthermore, the problem of forming secondary structure of a DNA as a template is solved, resulting in increase in the ability of extension of a DNA polymerase. A reverse transcription reaction and the nucleic acid amplification can be successively conducted in the method. In this case, a DNA having a sequence derived from an RNA can be amplified by combining the use of a reverse transcriptase with the above-mentioned reaction or by using a DNA polymerase having a reverse transcription activity.

In each aspect of the present invention, preferably, a chimeric oligonucleotide primer that is complementary to a single-stranded DNA as a template is first annealed to the DNA, although it is not intended to limit the present invention. A DNA that is complementary to the DNA as the template (a primer-extended strand) is then extended along the remained sequence of the DNA as the template from the 3'-terminus of the primer by the action of a DNA polymerase to synthesize a double-stranded DNA. An endonuclease acts on the double-stranded DNA and begins to reextend a DNA from the primer portion of the primer-extended strand de novo. In one aspect of the present invention, the endonuclease acts as a nicking enzyme that

introduces a nick into the double-stranded DNA or it alters the structure of the double-stranded DNA composed of the chimeric oligonucleotide primer and the DNA as the template, although the present invention is not restricted by a theory. A DNA polymerase having a strand displacement activity re-extends a DNA strand from the 3'-terminus of the nick introduced in the double-stranded DNA to generate a new primer-extended strand while releasing the DNA downstream from the 3'-terminus of the nick. Thus, the new primer-extended strand replaces the previously synthesized primer-extended strand.

The method for amplifying a nucleic acid of the present invention can be carried out using two primers, i.e., a chimeric oligonucleotide primer that is complementary to a nucleic acid as a template and another chimeric oligonucleotide primer that is complementary to a displaced strand. In this case, one primer binds to a DNA strand as a template to cause a strand displacement reaction, whereas another primer binds to a displaced strand released as a result of the strand displacement reaction to initiate another strand displacement reaction. It is clear that a reaction product with one primer can function as a template for another primer if this aspect is used. Thus, the amount of amplification product increases in a non-linear manner as the amount of the template

increases.

When the method for amplifying a nucleic acid of the present invention is conducted using a double-stranded DNA as a template, both strands can serve as templates in the amplification reaction by using chimeric oligonucleotide primers that anneal to the respective two strands. If the reaction is initiated after denaturing the double-stranded DNA, a chimeric oligonucleotide primer, four deoxyribonucleotide triphosphates (dNTPs), a DNA polymerase and an endonuclease are added to a reaction mixture before or after the double-stranded DNA is denatured. If heat treatment is used for denaturing the double-stranded DNA and a heat-resistant enzyme is not used, it is preferable to add the enzyme after the denaturation.

In the aspect of the nucleic acid amplification method of the present invention in which a double-stranded DNA as a template and two chimeric oligonucleotide primers are used, switching of templates may occur among the template-extended strand intermediates during the extension reaction from the primers to generate a double-stranded nucleic acid consisting of the synthesized primer-extended strands being annealed each other, although it depends on the reaction conditions or the like. The double-stranded nucleic acid has chimeric oligonucleotide primers at both ends. Then, reaction of extending complementary strands

comprising strand displacement can be initiated from both of the ends again. As a result of the reaction, an amplification product having the primer sequence at one end is generated. Furthermore, if switching of templates occurs during the reaction, a double-stranded nucleic acid similar to one that described above is generated again.

The present invention provides a method for amplifying a nucleic acid which comprises using a DNA polymerase having a strand displacement activity to effect a template switching reaction. In the template switching reaction in the presence of a double-stranded nucleic acid as a template, two chimeric oligonucleotide primers substantially complementary to the nucleotide sequences of the respective strands and a DNA polymerase having a strand displacement activity, two primer-extended strands complementary to the template are synthesized. Template switching of each of the primer-extended strands from the template to the other primer-extended strand takes place during the synthesis of the primer-extended strands.

As used herein, a template switching reaction refers to a reaction in which when complementary strands are synthesized by strand displacement reactions from the both sides of a double-stranded nucleic acid, a DNA polymerase switches the template and synthesizes a complementary strand thereafter using the other

complementary strand newly synthesized by another DNA polymerase as a template. In other words, a template switching reaction refers to a reaction in which a double-stranded nucleic acid as a template is treated with primers and a DNA polymerase having a strand displacement activity to generate extended strands complementary to the template, wherein a DNA polymerase that synthesized the primer-extended strands actively switches the template from the original templates to the other primer-extended strands during the synthesis of the extended strands. The ability of the DNA polymerase to effect a template switching reaction can be determined, for example, according to the method as described in Example 32 below, although it is not intended to limit the present invention.

A DNA polymerase capable of an effect the template switching reaction during strand displacement reaction can be preferably used for the present invention. For example, a variant enzyme of Bca DNA polymerase lacking a 5'→3' exonuclease activity is preferably used in particular. Such an enzyme is commercially available as BcaBEST DNA polymerase (Takara Shuzo). It can also be prepared from Escherichia coli HB101/pU1205 (FERM BP-3720) which contains the gene for the enzyme according to the method as described in Japanese Patent No. 2978001. Escherichia coli HB101/pU1205 (FERM BP-3720) has been

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deposited at International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, AIST Tsukuba Central 6, 1-1, Higashi 1-chome, Tsukuba-shi, Ibaraki 305-8566, Japan since May 10, 1991 (date of original deposit).

Although it is not intended to limit the present invention, the mode of reaction of the method for amplifying a nucleic acid of the present invention is considered as follows, for example.

In the method for amplifying a nucleic acid of the present invention, a double-stranded nucleic acid as a template is treated in the presence of an RNase H with two chimeric oligonucleotide primers that are substantially complementary to the nucleotide sequences of the respective strands and a DNA polymerase having a strand displacement activity to synthesize primer-extended strands that are complementary to the template. A double-stranded nucleic acid consisting of the synthesized primer-extended strands being annealed each other and a double-stranded nucleic acid consisting of the templates being annealed each other to which the two primers are annealed as a result of template switching reaction can be obtained. The latter double-stranded nucleic acid is reused as a template.

The double-stranded nucleic acid consisting of the primer-extended strands being annealed each other is

cleaved with an RNase H at sites that contain the ribonucleotides. Nucleic acids that are complementary to the template are extended using a DNA polymerase having a strand displacement activity from the 3'-termini of the respective primer portions of the double-stranded nucleic acid to effect strand displacements. A double-stranded nucleic acid consisting of the primer-extended strands being annealed each other and a double-stranded nucleic acid consisting of the templates being annealed each other to which the two primers are annealed as a result of template switching reaction can be obtained.

If the template switching reaction does not take place, two types of double-stranded nucleic acids each consisting of the template and the primer-extended strand can be obtained.

Nucleic acids that are complementary to the template are extended to effect strand displacements using a DNA polymerase having a strand displacement activity from the 3'-termini of the respective primer portions of the double-stranded nucleic acid to which the two primers are annealed. As a result of template switching reaction, a double-stranded nucleic acid consisting of the primer-extended strands being annealed each other and a double-stranded nucleic acid consisting of the templates being annealed each other to which the two primers are annealed

can be obtained. The double-stranded nucleic acid to which the two primers are annealed is reused as a template.

If the template switching reaction does not take place, two types of double-stranded nucleic acids each consisting of the template and the primer-extended strand can be obtained.

The two types of double-stranded nucleic acids are cleaved with an RNase H at sites that contain the ribonucleotides. Nucleic acids that are complementary to the template are extended using a DNA polymerase having a strand displacement activity from the 3'-termini of the respective primer portions of the double-stranded nucleic acid to effect strand displacements.

In the method for amplifying a nucleic acid of the present invention, a double-stranded nucleic acid as a template is treated in the presence of an RNase H with two chimeric oligonucleotide primers that are substantially complementary to the nucleotide sequences of the respective strands and a DNA polymerase having a strand displacement activity, and primer-extended strands that are complementary to the template are synthesized. If the template switching reaction does not take place, two types of double-stranded nucleic acids each consisting of the template and the primer-extended strand can be obtained.

In the amplification method of the present

invention, the chimeric oligonucleotide primer-extended strand may be cleaved at a site that contains the ribonucleotide such that the 5' fragment (primer portion) resulting from the cleavage does not contain the ribonucleotide. A primer-extended strand extended from the thus generated primer portion is no longer cleaved by an endonuclease. As a result, an amplification product having the primer sequence at its end is generated.

As described above, an amplification product without the primer sequence and a product having the primer sequence(s) at one or both of the ends may be generated in the nucleic acid amplification method of the present invention. These products are included in the amplification products herein.

An example of the method for amplifying a nucleic acid of the present invention is illustrated in Figures 33 to 36. In other words, Figures 33 to 36 illustrate an exemplary nucleic acid amplification in the nucleic acid amplification method of the present invention.

Figures 33 to 36 illustrate an exemplary nucleic acid amplification in the presence of a DNA as a template which is a double-stranded nucleic acid, a pair of chimeric oligonucleotide primers synthesized on the basis of the nucleotide sequence information of the DNA as the template (in the figures, the chimeric oligonucleotide

primers have three ribonucleotides at their 3'-termini; open circles in the figures represent the ribonucleotides), a strand displacement type DNA synthetase (DNA polymerase) having a strand displacement activity, an RNase H which is a ribonuclease that cleaves at a DNA-RNA hybrid site, and dNTPs which are substrates to be incorporated into the extended strands.

As shown in Figure 33, the pair of the chimeric oligonucleotide primers synthesized on the basis of the nucleotide sequence information of the DNA as the template are annealed to the specific portions of the DNA as the template. DNA strands are extended from the 3'-termini of the respective chimeric oligonucleotide primers as a result of the strand displacement reaction as indicated with Step 1.

Next, as shown in Figure 34, some of the primer-extended strands extended from upstream and downstream are released from the original templates as a result of the template switching reaction as indicated with Step 2. The primer-extended strands are annealed each other at their 3' portions. Complementary strands are extended from the annealed extended strands, forming a double-stranded DNA consisting of the primer-extended strands being annealed each other. Additionally, a double-stranded DNA consisting of the displaced strands being annealed each other to which

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the above-mentioned pair of the chimeric oligonucleotide primers are annealed is generated. This is utilized as the starting material in Figure 34.

As indicated with Step 3 in Figure 35, only one strand containing an RNA derived from the chimeric oligonucleotide primer of the double-stranded DNA in Figure 34 consisting of the primer-extended strands being annealed each other is cleaved by the action of an RNase H at the DNA/RNA hybrid site of the double-stranded DNA, resulting in introduction of a nick in the double-stranded DNA.

Subsequently, the strand displacement reaction takes place from the nick in the double-stranded DNA and a DNA is extend as indicated with Step 4 in Figure 35. Next, a template switching reaction like that in Step 2 in Figure 34 takes place in some degree or at some ratio as indicated with Step 5 in Figure 35, resulting in a double-stranded DNA consisting of the amplification products, i.e., the primer-extended strands being annealed each other.

In addition, a double-stranded DNA consisting of the displaced strands being annealed each other to which the above-mentioned pair of chimeric oligonucleotide primers are annealed are generated.

Next, as shown in Figure 36, DNA strands are extended as a result of the strand displacement reaction from the 3'-termini of the respective chimeric

oligonucleotide primers in the double-stranded DNA in Figure 35 which consists of the displaced strands being annealed each other to which the pair of the chimeric oligonucleotide primers are annealed. A template switching reaction similar to that in Step 2 and Step 5 takes place in some degree, resulting in a double-stranded DNA consisting of the primer-extended strands being annealed each other. This double-stranded DNA is brought back to Step 3 in Figure 35. The reaction starts on Step 3 again. A double-stranded DNA which consists of the displaced strands being annealed each other to which the pair of the chimeric oligonucleotide primers are annealed is generated and utilized as a starting material in Figure 36. As a result, a chain reaction, in which these double-stranded nucleic acids are repeatedly generated, takes place to specifically amplify and produce a region bounded by a pair of chimeric oligonucleotide primers.

In the nucleic acid amplification method of the present invention using a chimeric oligonucleotide, a polymer in which the regions to be amplified are connected each other may be generated. The polymer has a structure in which plural regions to be amplified are repeated in the same direction. The polymers are observed upon electrophoretic analysis of amplification products as laddered bands. It is considered that the generation of

such polymers is influenced by the region to be amplified, the size of the region, the flanking regions, the nucleotide sequence of the chimeric oligonucleotide primer to be used, the reaction conditions and the like.

The polymer as described above contains plural regions to be amplified. For example, the polymer is useful when detection of a nucleic acid containing a region to be amplified is intended because it hybridizes to a number of probes upon hybridization using an appropriate probe and generates a intensive signal. The region to be amplified or a portion thereof can be obtained from the polymer as a monomer by using digestion with a restriction enzyme or the like in combination.

The DNA polymerase used in the present invention should synthesize an extended strand from the 3'-terminus of the primer portion towards the downstream while displacing a previously extended DNA strand. It is important that the DNA polymerase should not exhibit a 5'→3' exonuclease activity that may digest the displaced strand. For example, Klenow fragment, which is an exonuclease-deficient variant of DNA polymerase I from Escherichia coli, a similar fragment derived from Bst DNA polymerase (New England Biolabs), and BcaBEST DNA polymerase from B. ca (Takara Shuzo) are useful as such a DNA polymerase. Sequenase 1.0 and Sequenase 2.0 (United

States Biochemical), and T5 DNA polymerase and φ29 DNA polymerase as described in Gene, 97:13-19 (1991) can also be used. A polymerase that normally has a 5'→3' exonuclease activity can be used in the DNA synthesis method of the present invention if addition of an appropriate inhibitor can inhibit the activity.

The method for amplifying a nucleic acid of the present invention may be conducted at varying temperatures or it may be conducted isothermally. Varying temperatures means that the reaction temperatures are changed for respective steps such that the change does not interfere with the reactions in the steps. Specifically, varying temperatures refers to change in temperature to that suitable for, for example, each of annealing of a primer, synthesis reaction of a complementary strand, nicking of a complementary strand and a displacement reaction.

On the other hand, isothermal means that the reaction temperature for each step is unchanged and each step is conducted at a substantially constant temperature. It is natural to select the temperature to optimize the reaction conditions in both cases.

One feature of the method for amplifying a nucleic acid of the present invention is that the method does not require adjusting the temperature up and down during the nucleic acid synthesis. Thus, the present

invention provides a method for isothermally synthesizing a nucleic acid. Many of conventional nucleic acid amplification methods require adjusting the temperature up and down to dissociate a target from a synthesized strand. These methods require a special reaction equipment such as a thermal cycler for this purpose. However, the method of the present invention can be conducted only using an equipment that can keep a constant temperature. As described above, the method of the present invention can be conducted at a single temperature. Preferably, it is conducted by selecting the reaction temperature and the stringency level such that non-specific annealing of a primer is reduced and such that the primer specifically anneals to a nucleic acid as a template. Although it is not intended to limit the present invention, the method of the present invention can be conducted under high-temperature conditions by using a heat-resistant enzyme as described above. In addition, it is preferable to conduct the method of the present invention at an appropriate temperature for sufficiently retaining the activity of the enzyme used in order to maintain the reaction efficiency at high level. Thus, the reaction temperature is preferably about 20°C to about 80°C, more preferably about 30°C to about 75°C, most preferably about 50°C to about 70°C although it varies depending on the enzyme used. When the

reaction is conducted under high-temperature conditions in particular, it is preferable to use a longer primer than that for a reaction at a normal temperature. The sequence and the length of the primer appropriate for the reaction temperature may be determined, for example, with reference to its Tm value. Alternatively, a commercially available software for designing a primer such as OLIGO™ Primer Analysis software (Takara Shuzo) may be used. For example, when a reaction temperature of 55°C to 60°C or 65°C is used, the primer used for the method of the present invention may be, for example, without limitation, 12-100 nucleotides in length, preferably 14-50 nucleotides in length, more preferably 15-40 nucleotides in length. An example of effects brought by the elevated reaction temperature is the solution of a problem of forming secondary structure of a DNA as a template. The elevated reaction temperature enables amplification of a desired nucleic acid even if a nucleic acid having a high GC content is used as a template. Furthermore, it is similarly effective in amplifying a region of a long chain length. Such effect is observed in a range between about 60 bp and about 20 kbp, specifically between about 110 bp and about 4.3 kbp, more specifically about 130 bp and about 1500 bp.

The amplification efficiency can be increased by adjusting the reaction temperature in accordance with the

GC content of the nucleic acid as the template. For example, if a nucleic acid having a low GC content is used as a template, the amplification reaction of the present invention can be conducted at 50 to 55°C, although the temperature depends on the chain length to be amplified and the Tm value of the primer.

Use of a DNA polymerase having a reverse transcriptase activity (e.g., BcaBEST DNA polymerase) in the method of the present invention can make the amplification of a nucleic acid from an RNA, which comprises a step of preparing a cDNA from an RNA (a reverse transcription reaction), be conveniently conducted. Alternatively, a product obtained by independently conducting a step of preparing a cDNA from an RNA, i.e., a cDNA, can be used in the method of the present invention as the DNA as a template.

In each case, the reaction in the method of the present invention is repeated until it is terminated by appropriate means, for example, by inactivating the enzyme or by lowering the reaction temperature, or until the reaction is deprived of one of the substrates.

The method for amplifying a nucleic acid of the present invention can be used for various experimental procedures that utilize amplification of a nucleic acid including detection, labeling and sequencing of a nucleic

acid.

Furthermore, the method for amplifying a nucleic acid of the present invention can be used for an in situ nucleic acid amplification method, a method for amplifying a nucleic acid on a solid substrate such as a DNA chip, or a multiplex nucleic acid amplification method in which plural regions are simultaneously amplified.

One of the features of the method for amplifying a nucleic acid of the present invention is its ability to prepare a single-stranded DNA. One or two chimeric oligonucleotide primers can be used in the method for this purpose. For example, if two oligonucleotide primers are used, the method of the present invention can be conducted applying a similar primer ratio to the so-called asymmetric-PCR in which an amplification reaction is carried out by using an excess amount of one oligonucleotide primer relative to another. The primer ratio is, without limitation, preferably in a range of 1:10 to 1:500, more preferably in a range of 1:10 to 1:100. As a result, the amount of the replacement product from one strand becomes excessive relative to that from another.

According to the method for amplifying a nucleic acid of the present invention, a single-stranded DNA substantially free of a complementary strand thereto can be prepared. For example, a single-stranded DNA for producing

a material having an immobilized nucleic acid such as a DNA chip, a single-stranded DNA probe for detecting a target nucleic acid, or a mega-primer for the long-chain PCR can be readily produced in a short time. Only a sense sequence or an antisense sequence can be selectively amplified by using the method of the present invention. Thus, the present invention is also useful as a method for producing a nucleic acid having a sense sequence or a antisense sequence.

A region of a nucleic acid of interest can be amplified even from a trace amount of a nucleic acid as a template by conducting the method of the present invention in a buffer of Bicine, Tricine, HEPES, phosphate or tris.

Furthermore, the methods for amplifying a nucleic acid of the present invention does not require the specific reaction equipment that can adjust a temperature with the passage of time. Therefore, an amplification reaction can be conducted in a large volume of reaction mixture. Thus, a nucleic acid (e.g., for medical use) can be industrially produced in large quantities.

The utilization efficiency of the primer in the method for amplifying a nucleic acid of the present invention is about 100%, which may be 5- to 10-fold higher than that in a conventional method such as the PCR.

The nucleic acid amplification method of the

present invention can produce an amplification product with high fidelity to the nucleotide sequence of the template nucleic acid. When the frequency of error in the DNA synthesis in the method of the present invention was confirmed by analyzing the nucleotide sequences of resulting amplification products, the frequency of error found in amplification products obtained by the method of the present invention was equivalent to that by LA-PCR which is known to be able to amplify a nucleic acid with high fidelity. In other words, the method of the present invention has fidelity equivalent to that of the LA-PCR.

(6) Method for detecting target nucleic acid of the present invention and kit for the method.

A target nucleic acid in a sample can be detected by using the method for amplifying a nucleic acid of the present invention. The detection method comprises:

(a) amplifying a target nucleic acid by the method for amplifying a nucleic acid of the present invention as described above; and

(b) detecting the target nucleic acid amplified in the step above.

In step (a) above, if an RNA is used as a template, the reverse transcription reaction and the nucleic acid amplification reaction may be conducted in one step. Although it is not intended to limit the present

invention, for example, a combination of AMV RTase, MMLV RTase or RAV-2 RTase and Bca DNA polymerase can be preferably used as a combination of a reverse transcriptase and a strand displacement-type DNA polymerase.

The method can be utilized to detect or quantify a specific gene in a sample. In other words, a specific gene can be detected or quantified from all samples suspected to contain a nucleic acid such as a DNA or an RNA. Examples of the samples from which a specific gene can be detected or quantified include, but are not limited to, samples from organisms such as a whole blood, a serum, a buffy coat, a urine, feces, a cerebrospinal fluid, a seminal fluid, a saliva, a tissue (e.g., a cancerous tissue or a lymph node) and a cell culture (e.g., a mammalian cell culture or a bacterial cell culture), samples that contain a nucleic acid such as a viroid, a virus, a bacterium, a fungi, a yeast, a plant and an animal, samples suspected to be contaminated or infected with a microorganism such as a virus or a bacterium (e.g., a food or a biological formulation), and samples that may contain an organism such as a soil and a waste water. For example, a viroid, a virus, a fungi, a bacterium or other microorganisms in a sample can be detected or quantified on the basis of the presence or the content of a specific gene derived from these microorganisms as a target. Particularly, a method

for detecting a pathogenic microorganism is useful in fields of sanitation and environment. Furthermore, the method of the present invention can be used to distinguish a genotype of an organism or to determine the expression level of a gene. Particularly, detection or confirmation of the expression state of a disease-related gene, e.g., a gene related to canceration of cells is useful in a field of medicine. Both of an RNA and a DNA can be preferably used as the nucleic acid as the template in the detection.

The method for detecting a target nucleic acid of the present invention can be used to distinguish the difference in the nucleotide sequence of the target nucleic acid. In this aspect, the chimeric oligonucleotide primer to be used is designed such that the 3'-terminal portion of the primer is positioned close to the specific base of the target nucleotide sequence to be distinguished. For example, it is designed such that a hydrogen bond is formed between the base and the 3'-terminal base of the primer. If a mismatch exists between the nucleotide sequence of the 3'-terminal portion of the primer and the nucleotide sequence of the template, amplification from the target nucleic acid does not take place and no amplification product is generated using the above-mentioned chimeric oligonucleotide primer for amplification reaction. Information concerning a specific base in a gene such as a

point mutation or a single nucleotide polymorphism (SNP) can be obtained using the method.

The method for detecting a target nucleic acid of the present invention can be conducted by amplifying the target nucleic acid directly from a sample containing the nucleic acid. In this case, the chain length of the target nucleic acid to be amplified is not limited to a specific one. For example, a region of 200 bp or shorter, preferably 150 bp or shorter is effective for sensitive detection of the target nucleic acid. The target nucleic acid in the sample can be detected with high sensitivity by designing the chimeric oligonucleotide primers of the present invention to result in the chain length to be amplified as described above.

In addition, a target nucleic acid can be detected with more sensitivity even from a trace amount of a nucleic acid sample in the detection method of the present invention by using a reaction buffer containing Bicine, Tricine, HEPES, phosphate or tris as a buffering component and an annealing solution containing spermidine or propylenediamine as exemplified in (4) above. In this case, the endonuclease and the DNA polymerase to be used are not limited to specific ones. For example, a combination of an RNase H from Escherichia coli, a bacterium of genus Pyrococcus or a bacterium of genus

Archaeoglobus and BcaBEST DNA polymerase is preferable. It is considered that the preferable units of the endonuclease and the DNA polymerase may vary depending on the types the enzymes. In such a case, the composition of the buffer and the amount of the enzymes added may be adjusted using the increase in detection sensitivity or the amount of amplification product as an index.

In the detection method of the present invention, dUTP may be incorporated as a substrate during amplification of a target nucleic acid. Thus, if dUTP is used as a substrate, it is possible to prevent carry-over contamination of amplification products by degrading amplification products utilizing uracil N-glycosidase (UNG).

Known methods for detecting a nucleic acid can be used for step (b). Examples of such methods include detection of a reaction product having a specific size by electrophoresis, and detection by hybridization with a probe. Furthermore, a detection method in which magnetic beads are combined can be preferably used. A fluorescent substance such as ethidium bromide is usually used in the detection by electrophoresis. The hybridization with a probe may be combined with the detection by electrophoresis. The probe may be labeled with a radioisotope or with a non-radioactive substance such as biotin or a fluorescent substance. Additionally, use of a labeled nucleotide in

step (a) may facilitate the detection of amplification product into which the labeled nucleotide is incorporated, or may enhance the signal for detection utilizing the label. A fluorescence polarization method, a fluorescence energy transition or the like can also be utilized for the detection. The target nucleic acid can be detected automatically or quantified by constructing a suitable detection system. In addition, detection with naked eyes by a hybrid chromatography method can be preferably used.

A ribonucleotide (RNA) probe labeled with two or more fluorescent substances positioned at a distance that results in a quenching state can be used in the detection method of the present invention. The probe does not emit fluorescence. When it is annealed to a DNA amplified from a target nucleic acid that is complementary to the probe, RNase H digests the probe. The distance between the fluorescent substances on the probe then increases, resulting in the emission of fluorescence. Thus, the emission reveals the presence of the target nucleic acid. If RNase H is used in the method for amplifying a nucleic acid of the present invention, a target nucleic acid can be detected only by adding the probe to the reaction mixture. For example, a combination of fluorescent substances, 6-carboxyfluorescein (6-FAM) and N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA), can be preferably used for

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labeling the probe.

The present invention further provides a probe used in the above-mentioned method for detecting a target nucleic acid. The probe of the present invention is not limited to specific one as long as it can hybridize to a target nucleic acid amplified by the nucleic acid amplification method of the present invention under normal hybridization conditions. In view of specific detection of amplification product, a probe that hybridizes under conditions, for example, known to those skilled in the art as being stringent is preferable. The stringent hybridization conditions are described in, for example, T. Maniatis et al. (eds.), Molecular Cloning: A Laboratory Manual 2nd ed., 1989, Cold Spring Harbor Laboratory. Specifically, the stringent conditions are exemplified by the following: incubation at a temperature about 25°C lower than the Tm of the probe to be used for 4 hours to overnight in 6 x SSC (1 x SSC: 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) containing 0.5% SDS, 5 x Denhardt's (0.1% bovine serum albumin (BSA), 0.1% polyvinylpyrrolidone, 0.1% Ficoll 400) and 100 µg/ml salmon sperm DNA. A probe having a label as described above may be used as the probe for facilitating the detection of the target nucleic acid.

The method for amplifying a nucleic acid under isothermal conditions of the present invention does not

require the use of an equipment such as a thermal cycler. The number of primers used in the amplification method of the present invention can be one or two, which is less than that used in a conventional method. Since reagents such as dNTPs used for PCR and the like can be applied to the method of the present invention, the running cost can be reduced as compared with a conventional method. Therefore, the method of the present invention can be preferably used, for example, in a field of genetic test in which the detection is routinely conducted. The method of the present invention provides a greater amount of an amplification product in a shorter time than the PCR. Therefore, the method of the present invention can be utilized as a convenient, rapid and sensitive method for detecting a gene.

In genetic analysis on a genomic level, attempts are made to make the reaction system small and to increase the degree of integration in order to analyze a large amount of nucleotide sequences. An system has been developed for this purpose by utilizing the latest hyperfine processing techniques in which basic processes for genome analysis (e.g., extraction of DNA from cells, DNA amplification reaction, electrophoresis, hybridization, detection of the DNA of interest, etc.) are integrated on a microchip of several square centimeters to fingertip size.

Such a system is called as a microchip or a nanochip.

Application of the PCR to the system as a gene amplification reaction is currently considered. However, since the PCRs requires means for repeatedly controlling temperature up and down over time, the system would become complicated. By contrast, since the system can be simplified using the method of the present invention which can amplify a nucleic acid under isothermal conditions, the method is quite suitably utilized for the integrated system as described above. A highly integrated system can be constructed by utilizing the techniques of the present invention.

(7) Kit of the present invention

The present invention provides a kit used for the method for amplifying or detecting a nucleic acid of present invention as described above. In one embodiment, the kit is in a packaged form and contains instructions regarding the use of a DNA polymerase and an endonuclease in a strand displacement reaction. Also, a kit that contains a DNA polymerase having a strand displacement activity, an endonuclease, and a buffer for a strand displacement reaction is preferably used for the method of the present invention. Alternatively, a commercially available DNA polymerase having a strand displacement activity and/or endonuclease may be selected and used

according to the instructions. Additionally, the kit may contain a reagent for a reverse transcription reaction that is used when an RNA is used as a template. The DNA polymerase can be selected from the DNA polymerases to be used in the present invention as described above in (3). The endonuclease can be selected from the endonucleases as described above in (2). One having the reaction buffer composition as described above in (4) can be preferably used as the buffer for the strand displacement reaction.

"Instructions" are printed matters describing a method of using the kit, e.g., a method for preparing a reagent solution for a strand displacement reaction, recommended reaction conditions and the like. The instructions include an instruction manual in a form of a pamphlet or a leaflet, a label stuck to the kit, and description on the surface of the package containing the kit. The instructions also include information disclosed or provided through electronic media such as the Internet.

The kit of the present invention may further contain a reaction buffer containing Bicine, Tricine, HEPES, phosphate or tris as a buffering component and an annealing solution as exemplified in (4) above. Additionally, it may contain a DNA polymerase having a strand displacement activity and an RNase H. Furthermore, the kit may contain a modified deoxyribonucleotide or a deoxynucleotide

triphosphate analog.

The kit used in the method for detecting a target nucleic acid may further contain an appropriate chimeric oligonucleotide primer for amplifying the target nucleic acid and a reagent for detecting the amplified target nucleic acid such as a probe in addition to the instructions and the reagents for amplification reaction as described above.

In addition, the kits of the present invention include a kit containing the chimeric oligonucleotide primer used in the present invention and/or the probe of the present invention as described above.

(8) Composition of the present invention.

The present invention provides a composition used for the method for amplifying a nucleic acid of the present invention or the method for detecting a nucleic acid of the present invention as described above. For example, the composition may contain the endonuclease as described in (2) above and the DNA polymerase as described in (3) above. The composition may further contain a buffering component, a magnesium salt, dNTPs and the like as components for conducting an amplification reaction. Furthermore, it may contain a modified deoxyribonucleotide or a deoxynucleotide triphosphate analog. Those as described in (4) above can be used as the buffering component and other additives.

In a particularly preferable aspect, the composition may contain suitable amounts of the various components as listed above for the nucleic acid amplification method of the present invention. Amplification reaction can be conducted only by adding an appropriate template and chimeric oligonucleotide primer(s) to the composition. If the object to be amplified is known beforehand, the composition preferably contains chimeric oligonucleotide primer(s) suitable for amplifying the object.

(9) Material having immobilized nucleic acid arrayed in predefined region of the present invention and method for producing the same.

A DNA chip (also referred to as a DNA microarray or a DNA array) is a material having an immobilized nucleic acid in which various fragments of genes or DNAs are arrayed and immobilized in a predefined region or at a predefined position on a solid substrate such as a slide glass. The DNA chip is used for examining the presence of a nucleic acid in a nucleic acid sample that has a sequence complementary to an arrayed and immobilized DNA in a predefined region on the DNA chip. The examination is carried out by contacting the DNA chip with the nucleic acid sample prepared from a sample, preferably a labeled nucleic acid sample, for hybridization. Since the DNA chip

can be used to detect or quantify a number of nucleic acids in a sample in one procedure, it is a very useful means that dramatically promotes the analysis of gene expression, or the analysis of a mutation or polymorphism. A DNA chip in which a double-stranded nucleic acid is arrayed and immobilized in a predefined region is used for hybridization after it is subjected to appropriate denaturation. A DNA chip in which a single-stranded DNA complementary to a target nucleic acid to be detected is arrayed and immobilized in a predefined region is particularly preferable for the detection of a target nucleic acid.

As described above, a desired DNA can be amplified in a single-stranded form by the method of the present invention. Although any method for purifying an amplification product can be used, purification using isopropanol precipitation is preferable. The thus obtained DNA, preferably a single-stranded DNA substantially free of a complementary strand thereto, can be preferably used as a DNA fragment to be immobilized onto a DNA chip. Thus, the method of the present invention is preferably used as a method for preparing a DNA to be arrayed and immobilized in a predefined region for producing a DNA chip. Any insoluble substrate can be used as a substrate onto which the thus obtained DNA is arrayed and immobilized in a

predefined region, but a plate-shaped substrate made from glass or plastic, and a membrane-shaped substrate made from nitrocellulose or nylon are preferably used. A known method for immobilizing a nucleic acid can be used for the immobilization. The DNA may be directly immobilized onto a substrate. Alternatively, the DNA may be immobilized through a suitable linker or after ligating plural DNA molecules. Furthermore, since a modified deoxyribonucleotide can be incorporated into an amplified nucleic acid by the production method of the present invention, the material having an immobilized nucleic acid can be produced utilizing the modification group.

A target nucleic acid that hybridizes with a nucleic acid on a material having an immobilized nucleic acid in which a DNA amplified by the method of the present invention is arrayed and immobilized in a predefined region (e.g., a DNA chip) can be detected or quantified. Such detection or quantification can be accomplished by contacting the material with a nucleic acid sample prepared from a sample suspected to contain the target nucleic acid for hybridization. Among these, a DNA chip in which a single-stranded DNA amplified by the method of the present invention is arrayed and immobilized in a predefined region allows the detection of a target nucleic acid with more convenient operation, higher sensitivity and higher

reproducibility as compared with a conventional material.

(10) Method for producing nucleic acid in large quantities of the present invention.

As described above, one aspect of the present invention provides a method for amplifying a nucleic acid that can be carried out under isothermal conditions. A desired nucleic acid can be produced in the method by mixing a nucleic acid as a template for the nucleic acid to be amplified and various components required for a reaction and reacting the mixture under isothermal conditions. Since the PCR requires changing the temperature of the reaction mixture over time, the reaction volume is limited to one in which the temperature can be controlled (usually, 200 µl or less). Therefore, it is difficult to scale up the volume. On the other hand, there is no such limitation in the method of the present invention. A large amount of nucleic acid can be produced by increasing the volume of the reaction mixture. In the method of the present invention, the volume is, for example, preferably more than 200 µl, more preferably 300 µl or more, and most preferably 500 µl or more, although it is not intended to limit the present invention. In the method of the present invention, a number of complementary strand molecules are synthesized from one template molecule. Furthermore, nucleic acids can be synthesized using these complementary strand molecules

as templates. Thus, a desired nucleic acid can be efficiently produced in large quantities by suitably selecting the template and the primer. Additionally, the fact that, unlike the PCR, the method of the present invention does not require a special equipment or a complicated temperature change makes it advantageous in view of the cost of equipment and energy. Therefore, the method is an excellent industrial method for producing a nucleic acid in large quantities.

The nucleic acid of interest can be amplified or produced even from a trace amount of a nucleic acid as a template in the production method of the present invention by using the reaction buffer and the annealing solution as exemplified in (4) above. In this case, the endonuclease and the DNA polymerase to be used are not limited to specific ones. For example, a combination of an RNase H from Escherichia coli and BcaBEST DNA polymerase is preferable. It is considered that the preferable units of the endonuclease and the DNA polymerase may vary depending on the types of the enzymes. In such a case, the composition of the buffer and the amount of the enzymes added may be adjusted using the maximal amount of amplification product as an index.

Furthermore, the method of the present invention is useful as a method for supplying a variety of DNA

fragments in large quantities, such as those to be immobilized onto the DNA chip. Specifically, DNA fragments can be obtained in large quantities in simple reaction steps in one embodiment. In another embodiment, a limited number of primers can be used to obtain a variety of DNA fragments. A step of amplifying the nucleic acid that serves as the template in the method of the present invention beforehand by a known nucleic acid amplification method such as the PCR can be incorporated in the latter embodiment. All kinds of nucleic acids as templates can be amplified using a limited number of primers, for example, based on the method for amplifying a nucleic acid using a random primer having a tag sequence (Nucleic Acids Research, 24(19):3778-3783 (1996)) or the degenerate oligonucleotide-primed PCR (DOP-PCR; Genomics, 13:718-725 (1992)), which uses a degenerate primer. The nucleic acid amplification method of the present invention can be conducted using one or several primers for all of the nucleic acids as templates produced in the above-mentioned step. This can be accomplished by designing the primer used in the nucleic acid amplification method of the present invention such that it corresponds to the tag sequence added to the random or degenerate primer. Thus, a combination of a suitable step for preparing a nucleic acid as a template and the method of the present invention can supply a variety of DNA

fragments in larger quantities and at a lower cost as compared with a conventional method.

A pharmaceutical composition containing a nucleic acid may contain a double-stranded DNA for expressing a useful polypeptide in a cell or a single-stranded antisense DNA for suppressing the expression of a gene of interest. Such a nucleic acid is administered into an organism using suitable means, for example, a carrier for gene transfer such as liposome. The method for producing a nucleic acid of the present invention is preferable for producing a single-stranded or double-stranded nucleic acid for medical use in large quantities. Additionally, a nucleic acid containing a dNTP analog that, for example, suppresses the degradation of the nucleic acid in vivo can be readily produced by the method of the present invention.

Since the DNA fragment amplified in the present invention is composed of normal nucleotides, the amplified DNA can be, for example, subcloned into a suitable vector utilizing a restriction enzyme site in the DNA. Furthermore, the DNA can be treated with a restriction enzyme for RFLP without a problem, for example. Therefore, the DNA can be widely utilized in a field of genetic test. In addition, a promoter sequence for an RNA polymerase can be incorporated into the amplified fragment. The amplified fragment can be used as a template to synthesize an RNA,

which can be used as a probe, for example. Of course, a fluorescence-labeled DNA probe can be produced by conducting the method for amplifying a nucleic acid of the present invention using a fluorescence-labeled dNTP instead of a normal dNTP.

Features of the method for amplifying a nucleic acid of the present invention are listed below.

1. It can amplify a large amount of a nucleic acid from a small amount of a template. The amplification product increases quadratically when two primers are used.

2. It can be conducted isothermally. In this case, it does not require the use of an equipment such as a thermal cycler. Therefore, the reaction volume can be readily scaled up.

3. Usually, the amplification reaction is conducted using one or two chimeric oligonucleotide primer and two enzymes (a DNA polymerase and an endonuclease).

4. Since a number of DNA strands are synthesized from one molecule of a primer, the amount of the primer does not restrict the amount of the amplification product. Furthermore, the primer utilization efficiency is about 100%, which is very higher than that of the PCR.

5. A single-stranded or double-stranded DNA can be selectively amplified depending on the purpose.

6. Since it does not require a dNTP analog such as an (α-S) dNTP for the amplification reaction, the cost of reagents is low. Furthermore, a nucleic acid in a natural form without a dNTP analog can be obtained.

7. It can supply an amplified DNA fragment at low cost and in large quantities by combining the method of the present invention with another nucleic acid duplication method.

8. The detection method of the present invention has an equal or higher detection sensitivity as compared with that of a conventional method. The detection method of the present invention can be conducted in a shorter time than that required for a conventional method having the equal sensitivity.

9. The method is suitable for amplification of a nucleic acid, automated detection, detection in a small amount and highly integrated detection on a microchip or a nanochip.

As described above, the method of the present invention is suitable for detection of a gene and industrial production of a nucleic acid.

Examples

The following Examples illustrate the present invention in more detail, but are not to be construed to limit the scope thereof.

Referential Example 1: Preparation of RNase H from thermophile Bacillus caldotenax

Bacillus caldotenax YT-G (purchased from Deutsche Sammlung von Mikroorganismen; DSM406) was inoculated into 100 ml of a medium containing 0.2% Tryptone (Difco Laboratories) and 1.5% yeast extract (Difco Laboratories) (pH 6.5), cultured at 60°C for 140 minutes with shaking and used as a pre-culture. 30 ml of the pre-culture was inoculated into 3 L of a medium having the same composition and cultured with aeration at 2.5 L/minute and stirring at 250 rpm at a temperature of 60°C for 5 hours.

The cells were collected by centrifuging the culture at 5000 x g for 15 minutes. 402 g (wet weight) of the cells were suspended in 1000 ml of 50 mM tris-HCl buffer (pH 7.5) containing 10 mM mercaptoethanol, 0.5 M NaCl, 1 mM EDTA and 20 μM PAPMSF and disrupted using MINI-Lab (APV GAULIN/RANNIE). Cell debris were removed by centrifugation to recover a supernatant.

A polyethylene imine solution was added to the resulting supernatant at a final concentration of 0.1%. After stirring, the mixture was allowed to stand for 1 hour. A supernatant was then recovered by centrifugation. Ammonium sulfate was added to the supernatant to 50% saturation. A precipitate obtained by centrifugation was dissolved in 20 mM tris-HCl buffer (pH 7.5) containing 10

mM mercaptoethanol, 0.1 mM EDTA, 50 mM NaCl and 10% glycerol. The solution was dialyzed against the same buffer. The dialyzed sample was loaded onto a 280-ml DE52 column (Whatman) equilibrated with the same buffer and non-adsorptive fractions were collected.

The column was further washed with 420 ml of the buffer used for the equilibration and washing fractions were collected. The non-adsorptive fractions and the washing fractions from the DE52 column chromatography were mixed together and loaded onto a 240-ml P-11 column (Whatman) equilibrated with 20 mM tris-HCl buffer (pH 7.5) containing 10 mM mercaptoethanol, 0.1 mM EDTA, 50 mM NaCl and 10% glycerol. Elution was then carried out using the equilibration buffer containing 0 to 0.5 M NaCl.

The resulting active fractions were placed in a dialysis tube. The tube was placed on solid polyethylene glycol 20000 for dehydration-concentration at 4°C. The enzyme concentrate was then loaded onto a 300-ml Superdex G-200 column (Amersham Pharmacia Biotech) equilibrated with 25 mM tris-HCl buffer (pH 7.5) containing 5 mM mercaptoethanol, 0.5 mM EDTA, 30 mM NaCl and 50% glycerol. Elution was carried out using the buffer used for equilibration to obtain active fractions. The active fractions were loaded onto a 15-ml Heparin-Sepharose column (Amersham Pharmacia Biotech) equilibrated with 20 mM tris-

HCl buffer (pH 7.5) containing 10 mM mercaptoethanol, 0.1 mM EDTA, 50 mM NaCl and 10% glycerol. Elution was carried out using the equilibration buffer containing 0 to 0.5 M NaCl.

The resulting active fractions were loaded onto a 5-ml Hitrap-SP column (Amersham Pharmacia Biotech) equilibrated with 20 mM tris-HCl buffer (pH 7.5) containing 10 mM mercaptoethanol, 0.1 mM EDTA, 50 mM NaCl and 10% glycerol. Elution was carried out using the equilibration buffer containing 0 to 0.5 M NaCl. The resulting active fractions were loaded onto a 300-ml Superdex G-200 column (Amersham Pharmacia Biotech) equilibrated with 25 mM tris-HCl buffer (pH 7.5) containing 5 mM mercaptoethanol, 0.5 mM EDTA, 30 mM NaCl and 50% glycerol again. The resulting active fractions were used as an RNase H preparation (an enzyme solution).

A heat-resistant RNase H activity was measured as follows.

1 mg of poly(rA) or poly(dT) (both from Amersham Pharmacia Biotech) was dissolved in 1 ml of 40 mM tris-HCl (pH 7.7) containing 1 mM EDTA to prepare a poly(rA) solution and a poly(dT) solution.

The poly(rA) solution (to a final concentration of 20 µg/ml) and the poly(dT) solution (to a final concentration of 30 µg/ml) were then added to 40 mM tris-

HCl (pH 7.7) containing 4 mM $MgCl_2$, 1 mM DTT, 0.003% BSA and 4% glycerol. The mixture was reacted at 37°C for 10 minutes and then cooled to 4°C at prepare a poly(rA)-poly(dT) solution.

1 µl of an enzyme solution was added to 100 µl of the poly(rA)-poly(dT) solution. The mixture was reacted at 40°C for 10 minutes. 10 µl of 0.5 M EDTA was added thereto to terminate the reaction. Absorbance at 260 nm was then measured. As a control, 10 µl of 0.5 M EDTA was added to the reaction mixture, the resulting mixture was reacted at 40°C for 10 minutes, and the absorbance was then measured. A value (difference in absorbance) was obtained by subtracting the absorbance for the control from the absorbance for the reaction in the absence of EDTA. Thus, the concentration of nucleotide released from poly(rA)-poly(dT) hybrid by the enzymatic reaction was determined on the basis of the difference in absorbance. One unit of an RNase H was defined as an amount of enzyme that increases A_{260} corresponding to release of 1 nmol of ribonucleotide in 10 minutes calculated according to the following equation. If a diluted enzyme solution is used, the value obtained using the following equation was corrected based on the dilution rate:

Unit = [Difference in Absorbance x Reaction Volume (ml)] / 0.0152

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Referential Example 2: Cloning of Bacillus caldotenax RNase HII gene

(1) Preparation of genomic DNA from Bacillus caldotenax

Bacillus caldotenax YT-G (DSM406) was inoculated into 60 ml of LB medium (1% Tryptone, 0.5% yeast extract and 0.5% NaCl, pH 7.2) and cultured at 65°C for 20 hours. After culturing, the culture was centrifuged to collect cells. The cells were suspended in 2 ml of 25% sucrose and 50 mM tris-HCl (pH 8.0). 0.2 ml of 10 mg/ml lysozyme chloride (Nacalai Tesque) in water was added thereto. The mixture was reacted at 20°C for 1 hour. After reaction, 12 ml of a mixture containing 150 mM NaCl, 1 mM EDTA and 20 mM tris-HCl (pH 8.0), 0.1 ml of 20 mg/ml proteinase K (Takara Shuzo) and 1 ml of a 10% aqueous solution of sodium lauryl sulfate were added to the reaction mixture. The mixture was incubated at 37°C for 1 hour.

2.1 ml of 5 M NaCl and 2 ml of a CTAB-NaCl solution [10% cetyltrimethylammonium bromide (Nacalai Tesque) and 0.7 M NaCl] were then added to the mixture and the resulting mixture was mixed thoroughly and incubated at 65°C for 10 minutes. An equal volume of a mixture of chloroform/isoamyl alcohol (24 : 1, v/v) was added thereto. The resulting mixture was gently mixed for 10 minutes and then centrifuged for 10 minutes at 10000 x g. After

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centrifugation, an equal volume of a mixture of phenol saturated with 100 mM tris-HCl (pH 8.0)/chloroform/isoamyl alcohol (25 : 24: 1, v/v) was added to the resulting supernatant. The resulting mixture was gently mixed for 10 minutes and then centrifuged for 10 minutes at 10000 x g. After centrifugation, a 0.6 volume of 2-propanol was added to the resulting supernatant. The resulting fibrous precipitate was wound using a glass bar, washed with 70% ethanol in water, air-dried and then dissolved in 0.5 ml of TE buffer to obtain a genomic DNA solution.

(2) Cloning of middle portion of RNase HII gene

Oligonucleotides BsuII-3 and BsuII-6 represented by SEQ ID NOS:1 and 2 were synthesized on the basis of Motif I and Motif III, portions conserved among amino acid sequences of RNase HIIs from various organisms (Biochemistry, 38:605-608 (1999)).

A PCR was carried out in a volume of 100 µl using 1 µl of the Bacillus caldotenax genomic DNA solution as prepared in Referential Example 2-(1) as a template, and 100 pmol of BsuII-3 and 100 pmol of BsuII-6 as primers. TaKaRa Taq polymerase (Takara Shuzo) was used as a DNA polymerase for the PCR according to the attached protocol. The PCR was carried out as follows: 50 cycles of 94°C for 30 seconds, 45°C for 30 seconds and 72°C for 1 minute. After reaction, the reaction mixture was subjected to

phenol treatment followed by ethanol precipitation to purify a DNA. The resulting DNA was blunt-ended using T4 DNA polymerase (Takara Shuzo) and then subjected to agarose gel electrophoresis to recover an amplified DNA fragment of about 0.4 kb from the gel. The about 0.4-kb DNA fragment was ligated with pUC119 (Takara Shuzo) digested with Sma I (Takara Shuzo) using T4 DNA ligase (Takara Shuzo). The ligation mixture was used to transform Escherichia coli JM109. The resulting transformants were cultured to obtain a plasmid 21-12 into which the about 0.4-kb DNA was inserted.

(3) Cloning of upstream portion of RNase II gene

The nucleotide sequence of the inserted fragment of about 0.4 kb in the plasmid 21-12 obtained in Referential Example 2-(2) was determined. Oligonucleotides RNII-S1 and RNII-S2 represented by SEQ ID NOS:3 and 4 were synthesized on the basis of the determined nucleotide sequence.

The Bacillus caldotenax genomic DNA as prepared in Referential Example 2-(1) was digested with BamHI (Takara Shuzo) and ligated with a Sau3AI cassette (Takara Shuzo) using T4 DNA ligase. A procedure was carried out according to the protocol attached to TaKaRa LA PCR in vitro cloning kit (Takara Shuzo) using the ligation mixture as a template, RNII-S2 as a primer for a primary PCR and

RNII-S1 as a primer for a secondary PCR. A DNA was purified from the solution after the secondary PCR by phenol extraction followed by ethanol precipitation. The DNA was blunt-ended using T4 DNA polymerase and then subjected to agarose gel electrophoresis to recover an amplified DNA fragment of about 1.5 kb from the gel. The about 1.5-kb DNA fragment was ligated with pUC119 digested with Sma I using T4 DNA ligase. The ligation mixture was used to transform Escherichia coli JM109.

The resulting transformants were cultured to obtain a plasmid B25N16 into which the about 1.5-kb DNA was inserted.

(4) Cloning of entire RNase II gene

Oligonucleotides RNII-S5 and RNII-S6 represented by SEQ ID NOS:5 and 6 were synthesized on the basis of the nucleotide sequence of the inserted fragment of about 0.4 kb in the plasmid 21-12 as determined in Referential Example 2-(3).

A PCR was carried out using the plasmid 21-12 as prepared in Referential Example 2-(2) as a template, and RNII-S5 and RNII-S6 as primers. TaKaRa Ex Taq polymerase (Takara Shuzo) was used as a DNA polymerase for the PCR according to the attached protocol. The PCR was carried out as follows: 25 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds. After reaction, the

reaction mixture was subjected to agarose gel electrophoresis. An amplified DNA fragment of about 0.3 kb was recovered from the gel. The about 0.3-kb DNA fragment was labeled with digoxigenin using DIG High-prime (Roche Diagnostics).

Southern hybridization was carried out for digests of the Bacillus caldotenax genomic DNA as prepared in Referential Example 2-(1) with HindIII (Takara Shuzo), Sac I (Takara Shuzo), or HindIII and SacI using the digoxigenin-labeled DNA as a probe. Hybridization and detection were carried out using DIG Luminescent Detection Kit (Roche Diagnostics) according to the protocol attached thereto. As a result, DNA fragments of about 4.5 kb, about 5.8 kb and about 1.3 kb were hybridized with the probe for the digests with HindIII, SacI, and HindIII and SacI, respectively.

Based on these results, the Bacillus caldotenax genomic DNA was digested with HindIII and subjected to agarose gel electrophoresis to recover DNAs of about 4.5 kb from the gel. The resulting DNA fragments were digested with SacI and subjected to agarose gel electrophoresis to recover DNAs of about 1.3 kb from the gel. The resulting DNAs were ligated with pUC19 (Takara Shuzo) digested with HindIII and SacI using T4 DNA ligase. The ligation mixture was used to transform Escherichia coli HB101.

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The resulting transformants were replica-plated onto Hybond-N™ (Amersham Pharmacia Biotech). Colony hybridization was then carried out using the above-mentioned digoxigenin-labeled probe according to a conventional method. A plasmid pRHB1 was prepared from the thus obtained positive clone.

The nucleotide sequence of the DNA inserted into pRHB1 was then determined. Comparison of the amino acid sequence deduced from the nucleotide sequence with the amino acid sequence of the RNase HII from Bacillus subtilis suggested that a region of about 40 bp from the initiation codon was missing in the DNA in pRHB1. Then, the full-length RNase H gene was constructed as follows.

B25N16 as prepared in Referential Example 2-(3) was digested with HindIII and subjected to agarose gel electrophoresis to recover a DNA fragment of about 160 bp from the gel. The about 160-bp DNA fragment was ligated with pRHB1 digested with HindIII using T4 DNA ligase. The ligation mixture was used to transform Escherichia coli HB101. Plasmids were prepared from the resulting transformants.

Next, an oligonucleotide RNII-Nde represented by SEQ ID NO:7 was synthesized on the basis of the presumed nucleotide sequence around the initiation codon. PCRs were carried out using the plasmids prepared from the

transformants as templates, and RNII-Nde and RNII-S6 as primers. A plasmid from which a DNA fragment of about 0.7 kb was amplified was selected and designated as pRHB11.

The nucleotide sequence of the DNA fragment inserted into the thus obtained plasmid pRHB11 was determined. Analysis of the results revealed an open reading frame presumably encoding RNase HII. This nucleotide sequence is shown in SEQ ID NO:8. The amino acid sequence of RNase HII deduced from the nucleotide sequence is shown in SEQ ID NO:9.

Escherichia coli HB101 transformed with the plasmid pRHB11 is designated and indicated as Escherichia coli JM109/pRHB11, and deposited on September 5, 2000 (date of original deposit) at International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, AIST Tsukuba Central 6, 1-1, Higashi 1-chome, Tsukuba-shi, Ibaraki 305-8566, Japan under accession number FERM BP-7655.

(5) Expression of Bacillus caldotenax RNase HII gene

Escherichia coli HB101 transformed with pRHB11 or pRHB1 was inoculated into 5 ml of LB medium containing 100 μg/ml of ampicillin and cultured with shaking at 37°C overnight. After cultivation, cells collected by centrifugation were suspended in 0.5 ml of TE buffer and

sonicated. A supernatant obtained by centrifugation was used as a crude cell extract.

10 mM tris-HCl (pH 8.0), 1 mM dithiothreitol (Nacalai Tesque), 0.003% bovine serum albumin (fraction V, Sigma), 4% glycerol, 20 µg/ml poly(dT) (Amersham Pharmacia Biotech) and 30 µg/ml poly(rA) (Amersham Pharmacia Biotech) were mixed together. The mixture was incubated at 37°C for 10 minutes and used as a substrate solution for measuring an RNase H activity.

1 µl of 1 M $MnCl_2$ was added to 100 µl of the substrate solution. The mixture was incubated at 40°C. 10 µl of a 10-fold dilution of the crude cell extract was added to the mixture to initiate a reaction. After reacting at 40°C for 30 minutes, 10 µl of 0.5 M EDTA was added thereto to terminate the reaction. Absorbance at 260 nm was then measured. As a result, the absorbance at 260 nm from a reaction in which a crude cell extract prepared from Escherichia coli HB101 harboring pRHB11 was used was clearly higher than that from a reaction in which a crude cell extract prepared from Escherichia coli HB101 harboring pRHB1 was used. Thus, it was demonstrated that pRHB11 contained an RNase H gene and that Escherichia coli harboring pRHB11 expressed an RNase H activity.

(6) Preparation of purified RNase HII preparation

Escherichia coli HB101 transformed with pRHB11

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obtained in Referential Example 2-(4) was inoculated into 1 L of LB medium containing 100 μg/ml of ampicillin and cultured with shaking at 37°C for 16 hours. After cultivation, cells collected by centrifugation were suspended in 52.3 ml of a sonication buffer [50 mM tris-HCl (pH 8.0), 2 mM 2-mercaptoethanol, 10% glycerol, 2 mM phenylmethanesulfonyl fluoride] and sonicated. A supernatant obtained by centrifuging the sonicated suspension at 12000 rpm for 10 minutes was heated at 60°C for 15 minutes. It was then centrifuged at 12000 rpm for 10 minutes again to collect a supernatant. Thus, 50.0 ml of a heated supernatant was obtained.

The solution was subjected to RESOURSE Q column (Amersham Pharmacia Biotech) equilibrated with Buffer C [50 mM tris-HCl (pH 8.0), 2 mM 2-mercaptoethanol, 10% glycerol] and chromatographed using FPLC system (Amersham Pharmacia Biotech). As a result, RNase HII flowed through the RESOURSE Q column. 51 ml of the flow-through RNase HII fraction was subjected to RESOURSE S column (Amersham Pharmacia Biotech) equilibrated with Buffer C and eluted with a linear gradient of 0 to 500 mM NaCl using FPLC system. A fraction containing RNase II eluted with about 240 mM NaCl was obtained. 3.0 ml of the RNase II fraction was subjected in two portions to PD-10 column (Amersham Pharmacia Biotech) equilibrated with Buffer C containing 50

mM NaCl. 7.0 ml the resulting eluate was subjected to HiTrap-heparin column (Amersham Pharmacia Biotech) equilibrated with Buffer C containing 50 mM NaCl and eluted with a linear gradient of 50 to 550 mM NaCl using FPLC system. A fraction containing RNase II eluted with about 310 mM NaCl was obtained. 4.4 ml of the RNase II fraction was concentrated by ultrafiltration using Centricon-10 (Amicon). 280 µl of the concentrate was subjected to Superdex 200 gel filtration column (Amersham Pharmacia Biotech) equilibrated with 50 mM tris-HCl (pH 8.0) containing 100 mM NaCl and 0.1 mM EDTA and eluted with the same buffer. As a result, RNase HII was eluted at a position corresponding to a molecular weight of 35 kilodalton. This molecular weight corresponds to that of the RNase HII in the monomeric form. The thus eluted RNase HII was used as Bca RNase HII preparation.

The enzymatic activity of the thus obtained Bca RNase HII preparation was measured as follows.

100 µl of a reaction mixture [20 mM HEPES-potassium hydroxide (pH 7.8), 0.01% bovine serum albumin (Takara Shuzo), 1% dimethyl sulfoxide, 10 mM manganese chloride, 20 µg/ml poly(dT) (Amersham Pharmacia Biotech), 30 µg/ml poly(rA) (Amersham Pharmacia Biotech)] which had been incubated at 40°C was added to 1 µl of the Bca RNase HII preparation. The mixture was reacted at 40°C for 10

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minutes. The reaction was then terminated by adding 10 µl of 0.5 M EDTA (pH 8.0). Absorbance at 260 nm was then measured.

As a result, an RNase H activity was observed for the Bca RNase HII preparation.

Referential Example 3: Cloning of Bacillus caldotenax RNase HIII gene

(1) Cloning of fragment of RNase HIII gene

Primers BsuIII-1, BsuIII-3, BsuIII-6 and BsuIII-8 represented by SEQ ID NOS:10 to 13 for screening a gene encoding RNase HIII were synthesized based on the amino acid sequences of regions well conserved among Bacillus subtilis and other organisms determined on the basis of the homology among the amino acid sequences of RNase HIIIs from Bacillus subtilis [Biochemistry, 38:605-608 (1999)] and other organisms.

A first PCR was carried out in a volume of 50 µl using 200 ng of the Bacillus caldotenax genomic DNA as prepared in Referential Example 2-(1) as a template, and 100 pmol of BsuIII-1 and 100 pmol of BsuIII-8 as primers. A second PCR was then carried out in a volume of 100 µl using 1 µl of the reaction mixture as a template, and 100 pmol of BsuIII-3 and 100 pmol of BsuIII-6 as primers. TaKaRa Taq polymerase (Takara Shuzo) was used as a DNA polymerase for the two PCRs according to the attached

protocol. The PCRs were carried out as follows: 25 (the first PCR) or 30 (the second PCR) cycles of 94°C for 30 seconds, 45°C for 30 seconds and 72°C for 1 minute.

An amplified DNA fragment of about 450 bp was blunt-ended using T4 DNA polymerase (Takara Shuzo) and then subjected to agarose gel electrophoresis to recover the amplified DNA fragment of about 450 bp. The about 450-bp DNA fragment was ligated with pUC119 (Takara Shuzo) digested with Sma I (Takara Shuzo) using T4 DNA ligase (Takara Shuzo). The ligation mixture was used to transform Escherichia coli JM109. The resulting transformants were cultured to obtain a plasmid pBCA3204 into which the about 450-bp DNA fragment was inserted.

(2) Cloning of RNase HIII gene using Southern hybridization method

The nucleotide sequence of the DNA fragment inserted in pBCA3204 obtained in Referential Example 3-(1) was determined. Primers RNIII-S3 and BcaRNIII-3 represented by SEQ ID NOS:14 and 15 were synthesized on the basis of the determined nucleotide sequence. A PCR was carried out in a volume of 100 µl using RNIII-S3 and BcaRNIII-3 as primers and pBCA3204 as a template. TaKaRa Z-Taq (Takara Shuzo) was used as a DNA polymerase for the PCR according to the attached protocol. The PCR was carried out as follows: 30 cycles of 98°C for 0 second,

55°C for 0 second and 72°C for 20 seconds. After reaction, the reaction mixture was subjected to phenol-chloroform extraction, ethanol precipitation and agarose gel electrophoresis to recover a DNA fragment of about 0.4 kb from the gel. The about 0.4-kb DNA fragment was labeled using DIG DNA Labeling Kit (Boehringer Mannheim) to prepare a probe.

20 µg of the Bacillus caldotenax genomic DNA prepared in Referential Example 2-(1) was completely digested with BamHI, EcoRI, HindIII, PstI or XbaI (all from Takara Shuzo). The half of each of the digests was then subjected to agarose gel electrophoresis. The DNAs were transferred from the agarose gel to a nylon membrane using 0.4 N sodium hydroxide and fixed at 120°C for 30 minutes. The membrane was pre-incubated in a sealed bag containing 30 ml of a hybridization buffer [43.4 g/L sodium chloride, 17.6 g/L sodium citrate, 1% blocking agent (Boehringer Mannheim), 0.1% N-lauroyl sarcosine, 0.02% sodium lauryl sulfate (SDS)] at 60°C for 4 hours and then incubated in a sealed bag containing 5 ml of a hybridization buffer containing the probe at 60°C for 16 hours.

The membrane was washed twice in 50 ml of 2 x SSC (17.5 g/L NaCl, 8.8 g/L sodium citrate) containing 0.1% SDS at room temperature, and twice in 50 ml of 0.5 x SSC (4.3 g/L sodium chloride, 1.9 g/L sodium citrate) containing

0.1% SDS at 45°C. Then, an EcoRI fragment of about 8 kb, a PstI fragment of about 4.5 kb and a HindIII fragment of about 1 kb which have sequences complementary to the probe were detected using DIG nucleic acid detection kit (Boehringer Mannheim).

The remaining half of the Bacillus caldotenax genomic DNA completely digested with PstI was subjected to agarose gel electrophoresis. PstI fragments of about 4.5 kb were recovered from the gel. The DNA fragments were then ligated with a plasmid vector pTV119N, which had been digested with PstI and dephosphorylated with alkaline phosphatase (Takara Shuzo). The ligation mixture was used to transform Escherichia coli JM109.

A PCR was carried out in a volume of 50 µl using RNIII-S3 and BcaRNIII-3 as primers, and one of the colonies as a template to select a colony presumably harboring an RNase HIII gene. TaKaRa-Z Taq (Takara Shuzo) was used for the PCR according to the attached protocol. The PCR was carried out as follows: 30 cycles of 98°C for 0 second, 55°C for 0 second and 72°C for 20 seconds. As a result, it was found that the gene of interest was contained in the colony No. 88.

A PCR was carried out using a plasmid prepared from the colony No. 88 as a template, and a primer pair RN-N (Takara Shuzo) and BcaRNIII-3 or a primer pair M4 (Takara

Shuzo) and RNIII-S3 to examine whether or not the entire RNase HIII gene was contained in the plasmid. As a result, it was found that the entire RNase HIII gene was contained in the plasmid, which was designated as pBCA3P88.

(3) Determination of nucleotide sequence of DNA fragment containing RNase HIII gene

The nucleotide sequence of the DNA fragment inserted into the plasmid pBCA3P88 obtained in Referential Example 3-(2) was determined according to a dideoxy method.

Analysis of the determined nucleotide sequence revealed the existence of an open reading frame encoding an amino acid sequence including the N-terminal amino acid sequence of RNase HIII. The nucleotide sequence of the open reading frame and the amino acid sequence of RNase HIII deduced from the nucleotide sequence are shown in SEQ ID NO:16 and SEQ ID NO:17, respectively.

(4) Construction of plasmid for expressing RNase HIII

A PCR was carried out in a volume of 100 µl using the plasmid pBCA3P88 as described in Referential Example 3-(2) as a template, BcaRNIIINde represented by SEQ ID NO:18 designed with reference to the sequence around the above-mentioned open reading frame for RNase HIII and M13 primer M4 (Takara Shuzo). Pyrobest DNA polymerase (Takara Shuzo) was used as a DNA polymerase for the PCR according to the

attached protocol. The PCR was carried out as follows: 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 3 minutes. An amplified DNA fragment of about 4 kb was digested with NdeI (Takara Shuzo) and subjected to agarose gel electrophoresis to recover an NdeI fragment of about 1.4 kb from the gel. The about 1.4-kb DNA fragment was ligated with pTV119Nd (a plasmid in which the NcoI site in pTV119N is converted into a NdeI site) which had been digested with NdeI and dephosphorylated with alkaline phosphatase (Takara Shuzo). The ligation mixture was used to transform Escherichia coli JM109.

Next, a PCR was carried out in a volume of 50 µl using one of the colonies as a template, and RN-N (Takara Shuzo) and BcaRNIII-3 as primers in order to screen for a plasmid in which the RNase HIII gene in the NdeI fragment was linked downstream from the lac promoter in the vector pTV119Nd. A colony presumably harboring the RNase HIII gene was then selected. TaKaRa-Z Taq (Takara Shuzo) was used as a DNA polymerase for the PCR according to the attached protocol. The PCR was carried out as follows: 30 cycles of 98°C for 0 second, 55°C for 0 second and 72°C for 20 seconds. As a result, it was found that the colony No. 2 contained a plasmid in which the RNase HIII gene in the NdeI fragment was linked downstream from the lac promoter in the vector pTV119Nd. This plasmid was designated as

pBCA3Nd2.

The determination of the nucleotide sequence of the DNA fragment inserted into the plasmid by a dideoxy method revealed that there was no mutation due to the PCR except that the initiation codon GTG was changed to ATG.

Escherichia coli JM109 transformed with the plasmid pBCA3Nd2 is designated and indicated as Escherichia coli JM109/pBCA3Nd2, and deposited on September 5, 2000 (date of original deposit) at International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, AIST Tsukuba Central 6, 1-1, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken 305-8566, Japan under accession number FERM BP-7653.

(5) Preparation of purified RNase HIII preparation

Escherichia coli JM109 transformed with pBCA3Nd2 obtained in Referential Example 3-(4) was inoculated into 2 L of LB medium containing 100 μg/ml of ampicillin and cultured with shaking at 37°C for 16 hours. After cultivation, cells collected by centrifugation were suspended in 39.6 ml of a sonication buffer [50 mM tris-HCl (pH 8.0), 1 mM EDTA, 2 mM phenylmethanesulfonyl fluoride] and sonicated. A supernatant obtained by centrifuging the sonicated suspension at 12000 rpm for 10 minutes was heated at 60°C for 15 minutes. It was then centrifuged at 12000

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rpm for 10 minutes again to collect a supernatant. Thus, 39.8 ml of a heated supernatant was obtained.

The heated supernatant was subjected to RESOURSE Q column (Amersham Pharmacia Biotech) equilibrated with Buffer A [50 mM tris-HCl (pH 8.0), 1 mM EDTA] and chromatographed using FPLC system (Amersham Pharmacia Biotech). As a result, RNase HIII flowed through the RESOURSE Q column.

45 ml of the flow-through RNase HIII fraction was dialyzed against 2 L of Buffer B [50 mM tris-HCl (pH 7.0), 1 mM EDTA] for 2 hours. The dialysis was repeated for two more times under the same conditions. 55.8 ml of the dialyzed enzyme solution was subjected to RESOURSE S column (Amersham Pharmacia Biotech) equilibrated with Buffer B and eluted with a linear gradient of 0 to 500 mM NaCl using FPLC system. A fraction containing RNase HIII eluted with about 105 mM NaCl was obtained.

Buffer B containing 1 M NaCl was added to 7.0 ml of the fraction to make the NaCl concentration to 150 mM. The mixture was subjected to HiTrap-heparin column (Amersham Pharmacia Biotech) equilibrated with Buffer B containing 150 mM NaCl. As a result, RNase HIII flowed through the HiTrap-heparin column.

7.5 ml of the flow-through RNase HIII fraction was concentrated by ultrafiltration using Centricon-10

(Amicon). 190 µl of the concentrate was subjected to Superdex 200 gel filtration column (Amersham Pharmacia Biotech) equilibrated with 50 mM tris-HCl (pH 7.0) containing 100 mM NaCl and 0.1 mM EDTA and eluted with the same buffer. As a result, RNase HIII was eluted at a position corresponding to a molecular weight of 33 kilodalton. This molecular weight corresponds to that of the RNase HIII in the monomeric form.

The thus eluted RNase HIII was used as Bca RNase HIII preparation.

The enzymatic activity of the thus obtained Bca RNase HIII preparation was measured as follows.

100 µl of a reaction mixture [20 mM HEPES-potassium hydroxide (pH 7.8), 0.01% bovine serum albumin (Takara Shuzo), 1% dimethyl sulfoxide, 4 mM magnesium acetate, 20 µg/ml poly(dT) (Amersham Pharmacia Biotech), 30 µg/ml poly(rA) (Amersham Pharmacia Biotech)] which had been incubated at 40°C was added to 1 µl of the Bca RNase HIII preparation. The mixture was reacted at 40°C for 10 minutes. The reaction was terminated by adding 10 µl of 0.5 M EDTA (pH 8.0). Absorbance at 260 nm was then measured. As a result, an RNase H activity was observed for the Bca RNase HIII preparation.

Referential Example 4: Cloning of Pyrococcus furiosus RNase HII gene

(1) Preparation of genomic DNA from Pyrococcus furiosus

2 L of a medium containing 1% Tryptone (Difco Laboratories), 0.5% yeast extract (Difco Laboratories), 1% soluble starch (Nacalai Tesque), 3.5% Jamarine S Solid (Jamarine Laboratory), 0.5% Jamarine S Liquid (Jamarine Laboratory), 0.003% $MgSO_4$, 0.001% NaCl, 0.0001% $FeSO_4 \cdot 7H_2O$, 0.0001% $CoSO_4$, 0.0001% $CaCl_2 \cdot 7H_2O$, 0.0001% $ZnSO_4$, 0.1 ppm $CuSO_4 \cdot 5H_2O$, 0.1 ppm $KAl(SO_4)_2$, 0.1 ppm H_3BO_4, 0.1 ppm $Na_2MoO_4 \cdot 2H_2O$ and 0.25 ppm $NiCl_2 \cdot 6H_2O$ was placed in a 2-L medium bottle, sterilized at 120°C for 20 minutes, bubbled with nitrogen gas to remove dissolved oxygen, then Pyrococcus furiosus (purchased from Deutsche Sammlung von Mikroorganismen; DSM3638) was inoculated into the medium and cultured at 95°C for 16 hours without shaking. After cultivation, cells were collected by centrifugation.

The resulting cells were then suspended in 4 ml of 25% sucrose, 50 mM tris-HCl (pH 8.0). 0.4 ml of 10 mg/ml lysozyme chloride (Nacalai Tesque) in water was added thereto. The mixture was reacted at 20°C for 1 hour. After reaction, 24 ml of a mixture containing 150 mM NaCl, 1 mM EDTA and 20 mM tris-HCl (pH 8.0), 0.2 ml of 20 mg/ml proteinase K (Takara Shuzo) and 2 ml of 10% aqueous solution of sodium lauryl sulfate were added to the reaction mixture. The mixture was incubated at 37°C for 1

hour. After reaction, the mixture was subjected to phenol-chloroform extraction followed by ethanol precipitation to prepare about 1 mg of genomic DNA.

(2) Cloning of RNase HII gene

The entire genomic sequence of Pyrococcus horikoshii was published [DNA Research, 5:55-76 (1998)]. The existence of one gene encoding a homologue of RNase HII (PH1650) in the genome was known (SEQ ID NO:19, the home page of National Institute of Technology and Evaluation: http://www.nite.go.jp/).

Homology between the PH1650 gene and the partially published genomic sequence of Pyrococcus furiosus (the home page of University of Utah, Utah Genome Center: http://www.genome.utah.edu/sequence.html) was searched. As a result, a highly homologous sequence was found. Primers 1650Nde (SEQ ID NO:20) and 1650Bam (SEQ ID NO:21) were synthesized on the basis of the homologous sequence.

A PCR was carried out in a volume of 100 µl using 200 ng of the Pyrococcus furiosus genomic DNA obtained in Referential Example 4-(1) as a template, and 20 pmol of 1650Nde and 20 pmol of 1650Bam as primers. TaKaRa Ex Taq (Takara Shuzo) was used as a DNA polymerase for the PCR according to the attached protocol. The PCR was carried out as follows: 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute. An amplified DNA

fragment of about 0.7 kb was digested with NdeI and BamHI (both from Takara Shuzo). The resulting DNA fragment was inserted between the NdeI site and the BamHI site in a plasmid vector pET3a (Novagen) to make a plasmid pPFU220.

(3) Determination of nucleotide sequence of DNA fragment containing RNase HII gene

The nucleotide sequence of the DNA fragment inserted into pPFU220 obtained in Referential Example 4-(2) was determined according to a dideoxy method.

Analysis of the determined nucleotide sequence revealed the existence of an open reading frame presumably encoding RNase HII. The nucleotide sequence of the open reading frame is shown in SEQ ID NO:22. The amino acid sequence of RNase HII deduced from the nucleotide sequence is shown in SEQ ID NO:23.

Escherichia coli JM109 transformed with the plasmid pPFU220 is designated and indicated as Escherichia coli JM109/pPFU220, and deposited on September 5, 2000 (date of original deposit) at International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, AIST Tsukuba Central 6, 1-1, Higashi 1-chome, Tsukuba-shi, Ibaraki 305-8566, Japan under accession number FERM BP-7654.

(4) Preparation of purified RNase HII preparation

Escherichia coli HMS174(DE3) (Novagen) was

transformed with pPFU220 obtained in Referential Example 4-(2). The resulting Escherichia coli HMS174(DE3) harboring pPFU220 was inoculated into 2 L of LB medium containing 100 µg/ml of ampicillin and cultured with shaking at 37°C for 16 hours. After cultivation, cells collected by centrifugation were suspended in 66.0 ml of a sonication buffer [50 mM tris-HCl (pH 8.0), 1 mM EDTA, 2 mM phenylmethanesulfonyl fluoride] and sonicated. A supernatant obtained by centrifuging the sonicated suspension at 12000 rpm for 10 minutes was heated at 60°C for 15 minutes. It was then centrifuged at 12000 rpm for 10 minutes again to collect a supernatant. Thus, 61.5 ml of a heated supernatant was obtained.

The heated supernatant was subjected to RESOURSE Q column (Amersham Pharmacia Biotech) equilibrated with Buffer A [50 mM tris-HCl (pH 8.0), 1 mM EDTA] and chromatographed using FPLC system (Amersham Pharmacia Biotech). As a result, RNase HII flowed through the RESOURSE Q column.

60.0 ml of the flow-through RNase HII fraction was subjected to RESOURSE S column (Amersham Pharmacia Biotech) equilibrated with Buffer A and eluted with a linear gradient of 0 to 500 mM NaCl using FPLC system. A fraction containing RNase HII eluted with about 150 mM NaCl was obtained. 2.0 ml of the RNase HII fraction was

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concentrated by ultrafiltration using Centricon-10 (Amicon). 250 µl of the concentrate was subjected to Superdex 200 gel filtration column (Amersham Pharmacia Biotech) equilibrated with 50 mM tris-HCl (pH 8.0) containing 100 mM NaCl and 0.1 mM EDTA and eluted with the same buffer. As a result, RNase HII was eluted at a position corresponding to a molecular weight of 17 kilodalton. This molecular weight corresponds to that of the RNase HII in the monomeric form.

The thus eluted RNase HII was used as Pfu RNase HII preparation.

The enzymatic activity of the thus obtained Pfu RNase HII preparation was measured as described in Referential Example 3-(5). As a result, an RNase H activity was observed for the Pfu RNase HII preparation.

Referential Example 5: Cloning of Thermotoga maritima RNase HII gene

(1) Preparation of genomic DNA from Thermotoga maritima

2 L of a medium containing 1% Tryptone, 0.5% yeast extract, 1% soluble starch, 3.5% Jamarine S Solid, 0.5% Jamarine S Liquid, 0.003% $MgSO_4$, 0.001% NaCl, 0.0001% $FeSO_4 \cdot 7H_2O$, 0.0001% $CoSO_4$, 0.0001% $CaCl_2 \cdot 7H_2O$, 0.0001% $ZnSO_4$, 0.1 ppm $CuSO_4 \cdot 5H_2O$, 0.1 ppm $KAl(SO_4)_2$, 0.1 ppm H_3BO_3, 0.1 ppm $Na_2MoO_4 \cdot 2H_2O$ and 0.25 ppm $NiCl_2 \cdot 6H_2O$ was placed in a 2-L medium bottle, sterilized at 120°C for 20 minutes,

bubbled with nitrogen gas to remove dissolved oxygen, then Thermotoga maritima (purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; DSM3109) was inoculated into the medium and cultured at 85°C for 16 hours without shaking.

Cells collected by centrifugation from 300 ml of the culture were then suspended in 3 ml of TE buffer [10 mM tris-HCl (pH 7.5), 1 mM EDTA]. 150 µl of 10% aqueous solution of sodium lauryl sulfate (Nacalai Tesque) and 15 µl of 20 mg/ml proteinase K (Takara Shuzo) were added thereto. The mixture was incubated at 37°C for 1 hour. After reaction, 0.5 ml of 5 M NaCl was added to the mixture. After thoroughly mixing, 0.4 ml of a CTAB-NaCl solution [10% cetyltrimethylammonium bromide (Nacalai Tesque), 0.7 M NaCl] was added to the mixture. After thoroughly mixing, the mixture was incubated at 65°C for 10 minutes. 1.5 ml of a mixture of chloroform/isoamyl alcohol (24:1, v/v) was added thereto. The mixture was gently mixed for 10 minutes and centrifuged at 20000 x g for 5 minutes. After centrifugation, an equal volume of a mixture of phenol saturated with 100 mM tris-HCl (pH 8.0)/chloroform/isoamyl alcohol (25:24:1, v/v) was added to the resulting supernatant. The mixture was gently mixed for 10 minutes and then centrifuged at 20000 x g for 5 minutes. After centrifugation, a 0.6 volume of 2-propanol was added to the

supernatant. The precipitate obtained by centrifugation at 10000 x g for 5 minutes was washed with 70% ethanol in water, air-dried and then dissolved in 200 µl of TE to obtain a genomic DNA solution.

(2) Cloning of RNase HII gene

Oligonucleotides 915-F1, 915-F2, 915-R1 and 915-R2 represented by SEQ ID NOS:24 to 27 were synthesized on the basis of the nucleotide sequence of a portion that had been identified as an RNase HII gene in the nucleotide sequence of the genomic DNA of Thermotoga maritima (http://www.tigr.org/tdb/CMR/btm/htmls/SplashPage.html) in order to obtain an amplified DNA fragment containing an RNase H gene by carrying out a PCR using the Thermotoga maritima genomic DNA as a template.

PCRs were carried out using the Thermotoga maritima genomic DNA as prepared in Referential Example 5-(1) as a template, and 915-F1 and 915-R1, 915-F1 and 915-R2, 915-F2 and 915-R1, or 915-F2 and 915-R2 as a primer pair. TaKaRa Ex Taq was used as a DNA polymerase for the PCRs according to the attached protocol. The PCRs were carried out as follows: 25 cycles of 95°C for 0.5 minute, 55°C for 0.5 minute and 72°C for 1.5 minute. After reactions, the respective PCR products were subjected to agarose gel electrophoresis to extract and purify amplified DNA fragments of about 0.7 kb. The DNAs amplified using a

primer pair 915-F1 and 915-R1 or 915-F1 and 915-R2 were digested with HindIII and XbaI (both from Takara Shuzo) and ligated with pUC19 digested with HindIII and XbaI using T4 DNA ligase. The ligation mixture was used to transform Escherichia coli JM109. The resulting transformants were cultured to prepare plasmid DNAs into which the about 0.7-kb DNAs were inserted. As a result, plasmids No. 1 and No. 2 having DNAs amplified using 915-F1 and 915-R1, and plasmids No. 3 and No. 4 having DNAs amplified using 915-F1 and 915-R2 were obtained.

In addition, the DNAs amplified using a primer pair 915-F2 and 915-R1 or 915-F2 and 915-R2 were doubly digested with NcoI (Takara Shuzo) and XbaI and ligated with pTV119N (Takara Shuzo) doubly digested with NcoI and XbaI using T4 DNA ligase. The ligation mixture was used to transform Escherichia coli JM109.

The resulting transformants were cultured to prepare plasmid DNAs into which the about 0.7-kb DNAs were inserted. As a result, plasmids No. 5 and No. 6 having DNAs amplified using 915-F2 and 915-R1, and a plasmid No. 7 having a DNA amplified using 915-F2 and 915-R2 were obtained.

Escherichia coli JM109 transformed with the plasmid No. 7 is designated and indicated as Escherichia coli JM109/pTM-RNH, and deposited on September 5, 2000

(date of original deposit) at International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, AIST Tsukuba Central 6, 1-1, Higashi 1-chome, Tsukuba-shi, Ibaraki 305-8566, Japan under accession number FERM BP-7652.

(3) Expression of Thermotoga maritima RNase HII gene

Escherichia coli JM109 transformed with one of the plasmids No. 1 to 7 or pUC19 was inoculated into 5 ml of LB medium (10 g/L Tryptone, 5 g/L yeast extract, 5 g/L NaCl, pH 7.2) containing 100 μg/ml of ampicillin and cultured with shaking at 37°C. When the absorbance at 660 nm reached 0.5, isopropyl-β-D-thiogalactopyranoside was added thereto to a final concentration of 1 mM and the cells were cultured overnight. After cultivation, cells collected by centrifugation were suspended in 1 ml of TE buffer and sonicated. The sonicated suspension was heated at 80°C for 10 minute. A supernatant obtained by centrifugation was used as a crude cell extract. Absorbance was measured using the crude cell extract as described in Referential Example 2-(5). As a result, when reactions were carried out in the presence of $MnCl_2$, the absorbance at 260 nm from each of the reactions in which crude cell extracts prepared from Escherichia coli JM109 harboring the plasmid No. 3, 5, 6 or 7 were used was

clearly higher than that from a reaction in which a crude extract prepared from Escherichia coli JM109 harboring pUC19 was used. Thus, it was demonstrated that the plasmids No. 3, 5, 6 and 7 contained RNase H genes and that Escherichia coli harboring one of these plasmids expressed an RNase H activity.

The partial nucleotide sequences of the DNA fragments inserted into the plasmids which were demonstrated to express RNase activities in Escherichia coli were determined. Analysis of the determined nucleotide sequences revealed an open reading frame presumably encoding RNase HII. The nucleotide sequence of the open reading frame is shown in SEQ ID NO:143. The amino acid sequence of RNase HII deduced from the nucleotide sequence is shown in SEQ ID NO:144. Then, it was found that one base substitution that was presumably generated upon the PCR was observed in a portion of the nucleotide sequence of the DNA fragment inserted in the plasmid No. 7, resulting in the change in the encoded amino acid residue.

(4) Preparation of purified RNase HII preparation

Escherichia coli JM109 was transformed with pTM-RNH obtained in Referential Example 5-(2). The resulting Escherichia coli JM109 harboring pTM-RNH was inoculated into 1 L of LB medium containing 100 μg/ml of ampicillin

and cultured with shaking at 37°C for 16 hours. After cultivation, cells collected by centrifugation were suspended in 31.0 ml of a sonication buffer [50 mM tris-HCl (pH 8.0), 2 mM 2-mercaptoethanol, 10% glycerol, 2 mM phenylmethanesulfonyl fluoride] and sonicated. A supernatant obtained by centrifuging the sonicated suspension at 12000 rpm for 10 minutes was heated at 70°C for 15 minutes. It was then centrifuged at 12000 rpm for 10 minutes again to collect a supernatant. Thus, 32.0 ml of a heated supernatant was obtained.

The heated supernatant was subjected to RESOURSE Q column (Amersham Pharmacia Biotech) equilibrated with Buffer C [50 mM tris-HCl (pH 8.0), 2 mM 2-mercaptoethanol, 10% glycerol] and chromatographed using FPLC system (Amersham Pharmacia Biotech). As a result, RNase HII flowed through the RESOURSE Q column. 32.5 ml of the flow-through RNase HII fraction was subjected to RESOURSE S column (Amersham Pharmacia Biotech) equilibrated with Buffer C and eluted with a linear gradient of 0 to 500 mM NaCl using FPLC system. A fraction containing RNase II eluted with about 240 mM NaCl was obtained. 2.0 ml of the RNase II fraction was subjected to PD-10 column (Amersham Pharmacia Biotech) equilibrated with Buffer C containing 50 mM NaCl. 3.5 ml of the resulting eluate was subjected to HiTrap-heparin column (Amersham Pharmacia Biotech)

equilibrated with Buffer C containing 50 mM NaCl and eluted with a linear gradient of 50 to 550 mM NaCl using FPLC system. As a result, a fraction containing RNase II eluted with about 295 mM NaCl was obtained. The thus eluted RNase HII was used as Tma RNase HII preparation.

The enzymatic activity of the thus obtained Tma RNase HII preparation was measured as described in Referential Example 2-(6). As a result, an RNase H activity was observed for the Tma RNase HII preparation.

Referential Example 6: Cloning of RNase HII gene from Pyrococcus horikoshii

(1) Preparation of genomic DNA from Pyrococcus horikoshii

2 L of a medium containing 1% Tryptone (Difco Laboratories), 0.5% yeast extract (Difco Laboratories), 1% soluble starch (Nacalai Tesque), 3.5% Jamarine S Solid (Jamarine Laboratory), 0.5% Jamarine S Liquid (Jamarine Laboratory), 0.003% $MgSO_4$, 0.001% NaCl, 0.0001% $FeSO_4 \cdot 7H_2O$, 0.0001% $CoSO_4$, 0.0001% $CaCl_2 \cdot 7H_2O$, 0.0001% $ZnSO_4$, 0.1 ppm $CuSO_4 \cdot 5H_2O$, 0.1 ppm $KAl(SO_4)_2$, 0.1 ppm H_3BO_4, 0.1 ppm $Na_2MoO_4 \cdot 2H_2O$ and 0.25 ppm $NiCl_2 \cdot 6H_2O$ was placed in a 2-L medium bottle, sterilized at 120°C for 20 minutes, bubbled with nitrogen gas to remove dissolved oxygen, then Pyrococcus horikoshii OT3 (purchased from the Institute of Physical and Chemical Research (RIKEN); JCM9974) was

inoculated into the medium and cultured at 95°C for 16 hours without shaking. After cultivation, cells were collected by centrifugation.

The cells were then suspended in 4 ml of 25% sucrose, 50 mM tris-HCl (pH 8.0). 0.4 ml of 10 mg/ml lysozyme chloride (Nacalai Tesque) in water was added thereto. The mixture was reacted at 20°C for 1 hour. After reaction, 24 ml of a mixture containing 150 mM NaCl, 1 mM EDTA and 20 mM tris-HCl (pH 8.0), 0.2 ml of 20 mg/ml proteinase K (Takara Shuzo) and 2 ml of 10% aqueous solution of sodium lauryl sulfate were added to the reaction mixture. The mixture was incubated at 37°C for 1 hour.

After reaction, the mixture was subjected to phenol-chloroform extraction followed by ethanol precipitation to prepare about 1 mg of genomic DNA.

(2) Cloning of RNase HII gene

The entire genomic sequence of the Pyrococcus horikoshii has been published [DNA Research, 5:55-76 (1998)]. The existence of one gene encoding a homologue of RNase HII (PH1650) was known (SEQ ID NO:145, the home page of National Institute of Technology and Evaluation: http://www.nite.go.jp/).

Primers PhoNde (SEQ ID NO:146) and PhoBam (SEQ ID NO:147) were synthesized on the basis of the sequence of

the PH1650 gene (SEQ ID NO:145).

A PCR was carried out using 100 ng of the Pyrococcus horikoshii genomic DNA prepared in Referential Example 6-(1) as a template, and 20 pmol each of PhoNde and PhoBam as primers in a volume of 100 µl. TaKaRa Ex Taq (Takara Shuzo) was used as a DNA polymerase for the PCR according to the attached protocol. The PCR was carried out as follows: 40 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute. An amplified DNA fragment of about 0.7 kb was digested with NdeI and BamHI (both from Takara Shuzo). Then, a plasmid pPHO238 was constructed by incorporating the resulting DNA fragment between NdeI and BamHI sites in a plasmid vector pET3a (Novagen).

(3) Determination of nucleotide sequence of DNA fragment containing RNase HII gene

The nucleotide sequence of the DNA fragment inserted into pPHO238 obtained in Referential Example 6-(2) was determined according to a dideoxy method.

Analysis of the determined nucleotide sequence revealed an open reading frame presumably encoding RNase HII. The nucleotide sequence of the open reading frame is shown in SEQ ID NO:148. The amino acid sequence of RNase HII deduced from the nucleotide sequence is shown in SEQ ID NO:149.

Escherichia coli JM109 transformed with the plasmid pPHO238 is designated and indicated as Escherichia coli JM109/pPHO238, and deposited on February 22, 2001 (date of original deposit) at International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, AIST Tsukuba Central 6, 1-1, Higashi 1-chome, Tsukuba-shi, Ibaraki 305-8566, Japan under accession number FERM BP-7692.

(4) Preparation of purified RNase HII preparation

Escherichia coli HMS174(DE3) (Novagen) was transformed with pPHO238 obtained in Referential Example 6-(2). The resulting Escherichia coli HMS174(DE3) harboring pPHO238 was inoculated into 1 L of LB medium containing 100 μg/ml of ampicillin and cultured with shaking at 37°C for 16 hours. After cultivation, cells collected by centrifugation were suspended in 34.3 ml of a sonication buffer [50 mM tris-HCl (pH 8.0), 1 mM EDTA, 2 mM phenylmethanesulfonyl fluoride] and sonicated. A supernatant obtained by centrifuging the sonicated suspension at 12000 rpm for 10 minutes was heated at 80°C for 15 minutes. It was then centrifuged at 12000 rpm for 10 minutes again to collect a supernatant. Thus, 33.5 ml of a heated supernatant was obtained.

The heated supernatant was subjected to RESOURSE Q column (Amersham Pharmacia Biotech) equilibrated with

Buffer A [50 mM tris-HCl (pH 8.0), 1 mM EDTA] and chromatographed using FPLC system (Amersham Pharmacia Biotech). As a result, RNase HII flowed through the RESOURSE Q column.

35.0 ml of the flow-through RNase HII fraction was dialyzed against 2 L of Buffer B (50 mM tris-HCl (pH 7.0), 1 mM EDTA) for 2 hours. The dialysis was repeated two more times. 34.5 ml of the dialyzed enzyme solution was subjected to RESOURSE S column (Amersham Pharmacia Biotech) equilibrated with Buffer B and eluted with a linear gradient of 0 to 500 mM NaCl using FPLC system. A fraction containing RNase HII eluted with about 155 mM NaCl was obtained.

Buffer B was added to 4.0 ml of the fraction to make the NaCl concentration to 50 mM. The mixture was subjected to HiTrap-heparin column (Amersham Pharmacia Biotech) equilibrated with Buffer B containing 50 mM NaCl and eluted with a linear gradient of 50 to 550 mM NaCl using FPLC system. As a result, a fraction containing RNase HII eluted with about 160 mM NaCl was obtained.

6.9 ml of the RNase HII fraction was concentrated by ultrafiltration using Centricon-10 (Amicon). Two portions each separated from 250 µl of the concentrate were subjected to Superose 6 gel filtration column (Amersham Pharmacia Biotech) equilibrated with 50 mM tris-HCl (pH

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7.0) containing 100 mM NaCl and 0.1 mM EDTA and eluted with the same buffer. As a result, RNase HII was eluted at a position corresponding to a molecular weight of 24.5 kilodalton. This molecular weight corresponds to that of the RNase HII in the monomeric form.

The RNase HII eluted as described above was used as Pho RNase HII preparation.

The enzymatic activity of the thus obtained Pho RNase HII preparation was measured as described in Referential Example 3-(5). As a result, an RNase H activity was observed for the Pho RNase HII preparation.

Referential Example 7: Cloning of RNase HII gene from Archaeoglobus fulgidus

(1) Preparation of genomic DNA from Archaeoglobus fulgidus

Cells of Archaeoglobus fulgidus (purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; DSM4139) collected from 8 ml of a culture was suspended in 100 µl of 25% sucrose, 50 mM tris-HCl (pH 8.0). 20 µl of 0.5 M EDTA and 10 µl of 10 mg/ml lysozyme chloride (Nacalai Tesque) in water was added thereto. The mixture was reacted at 20°C for 1 hour. After reaction, 800 µl of a mixture containing 150 mM NaCl, 1 mM EDTA and 20 mM tris-HCl (pH 8.0), 10 µl of 20 mg/ml proteinase K (Takara Shuzo) and 50 µl of 10% aqueous solution of sodium lauryl sulfate

were added to the reaction mixture. The mixture was incubated at 37°C for 1 hour. After reaction, the mixture was subjected to phenol-chloroform extraction, ethanol precipitation and air-drying, and then dissolved in 50 µl of TE to obtain a genomic DNA solution.

(2) Cloning of RNase HII gene

The entire genomic sequence of the Archaeoglobus fulgidus has been published [Nature, 390:364-370 (1997)]. The existence of one gene encoding a homologue of RNase HII (AF0621) was known (SEQ ID NO:150, http://www.tigr.org/tdb/CMR/btm/htmls/SplashPage.htlm).

Primers AfuNde (SEQ ID NO:151) and AfuBam (SEQ ID NO:152) were synthesized on the basis of the sequence of the AF0621 gene (SEQ ID NO:150).

A PCR was carried out using 30 ng of the Archaeoglobus fulgidus genomic DNA prepared in Referential Example 7-(1) as a template, and 20 pmol each of AfuNde and AfuBam as primers in a volume of 100 µl. Pyrobest DNA polymerase (Takara Shuzo) was used as a DNA polymerase for the PCR according to the attached protocol. The PCR was carried out as follows: 40 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute. An amplified DNA fragment of about 0.6 kb was digested with NdeI and BamHI (both from Takara Shuzo). Then, a plasmid pAFU204 was constructed by incorporating the resulting DNA fragment

between NdeI and BamHI sites in a plasmid vector pTV119Nd (a plasmid in which the NcoI site in pTV119N is converted into a NdeI site).

(3) Determination of nucleotide sequence of DNA fragment containing RNase HII gene

The nucleotide sequence of the DNA fragment inserted into pAFU204 obtained in Referential Example 7-(2) was determined according to a dideoxy method.

Analysis of the determined nucleotide sequence revealed an open reading frame presumably encoding RNase HII. The nucleotide sequence of the open reading frame is shown in SEQ ID NO:153. The amino acid sequence of RNase HII deduced from the nucleotide sequence is shown in SEQ ID NO:154.

Escherichia coli JM109 transformed with the plasmid pAFU204 is designated and indicated as Escherichia coli JM109/pAFU204, and deposited on February 22, 2001 (date of original deposit) at International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, AIST Tsukuba Central 6, 1-1, Higashi 1-chome, Tsukuba-shi, Ibaraki 305-8566, Japan under accession number FERM BP-7691.

(4) Preparation of purified RNase HII preparation

Escherichia coli JM109 was transformed with pAFU204 obtained in Referential Example 7-(2). The

resulting Escherichia coli JM109 harboring pAFU204 was inoculated into 2 L of LB medium containing 100 μg/ml of ampicillin and cultured with shaking at 37°C for 16 hours. After cultivation, cells collected by centrifugation were suspended in 37.1 ml of a sonication buffer [50 mM tris-HCl (pH 8.0), 1 mM EDTA, 2 mM phenylmethanesulfonyl fluoride] and sonicated. A supernatant obtained by centrifuging the sonicated suspension at 12000 rpm for 10 minutes was heated at 70°C for 15 minutes. It was then centrifuged at 12000 rpm for 10 minutes again to collect a supernatant. Thus, 40.3 ml of a heated supernatant was obtained.

The heated supernatant was subjected to RESOURSE Q column (Amersham Pharmacia Biotech) equilibrated with Buffer A [50 mM tris-HCl (pH 8.0), 1 mM EDTA] and chromatographed using FPLC system (Amersham Pharmacia Biotech). As a result, RNase HII flowed through the RESOURSE Q column.

The flow-through RNase HII fraction was subjected to RESOURSE S column (Amersham Pharmacia Biotech) equilibrated with Buffer A and chromatographed using FPLC system (Amersham Pharmacia Biotech). As a result, RNase HII flowed through the RESOURSE S column.

40.0 ml of the flow-through RNase HII fraction was dialyzed against 2 L of Buffer B (50 mM tris-HCl (pH 7.0), 1 mM EDTA) containing 50 mM NaCl for 2 hours. The

dialysis was repeated two more times. 40.2 ml of the dialyzed enzyme solution was subjected to HiTrap-heparin column (Amersham Pharmacia Biotech) equilibrated with Buffer B containing 50 mM NaCl and eluted with a linear gradient of 50 to 550 mM NaCl using FPLC system. As a result, a fraction containing RNase HII eluted with about 240 mM NaCl was obtained.

7.8 ml of the RNase HII fraction was concentrated by ultrafiltration using Centricon-10 (Amicon). Four portions each separated from about 600 µl of the concentrate were subjected to Superose 6 gel filtration column (Amersham Pharmacia Biotech) equilibrated with 50 mM tris-HCl (pH 7.0) containing 100 mM NaCl and 0.1 mM EDTA and eluted with the same buffer. As a result, RNase HII was eluted at a position corresponding to a molecular weight of 30.0 kilodalton. This molecular weight corresponds to that of the RNase HII in the monomeric form.

The RNase HII eluted as described above was used as Afu RNase HII preparation.

The enzymatic activity of the thus obtained Afu RNase HII preparation was measured as described in Referential Example 3-(5). As a result, an RNase H activity was observed for the Afu RNase HII preparation.

Referential Example 8

Unit value of RNase H from Escherichia coli used

in the method of the present invention was measured according to the following method.

(1) Preparation of reagent solutions used

Reaction mixture for determining activity: The following substances at the indicated final concentrations were contained in sterile water: 40 mM tris-hydrochloride (pH 7.7 at 37°C), 4 mM magnesium chloride, 1 mM DTT, 0.003% BSA, 4% glycerol and 24 μM poly(dT).

Poly[8-^{3}H]adenylic acid solution: 370 kBq of a poly[8-^{3}H]adenylic acid solution was dissolved in 200 μl of sterile water.

Polyadenylic acid solution: Polyadenylic acid was diluted to a concentration of 3 mM with sterile ultrapure water.

Enzyme dilution solution: The following substances at the indicated final concentrations were contained in sterile water: 25 mM tris-hydrochloride (pH 7.5 at 37°C), 5 mM 2-mercaptoethanol, 0.5 mM EDTA (pH 7.5 at 37°C), 30 mM sodium chloride and 50% glycerol.

Preparation of heat-denatured calf thymus DNA: 200 mg of calf thymus DNA was suspended and allowed to swell in 100 ml of TE buffer. The solution was diluted to a concentration of 1 mg/ml with sterile ultrapure water based on the absorbance measured at UV 260 nm. The diluted solution was heated at 100°C for 10 minutes and then

rapidly cooled in an ice bath.

(2) Method for measuring activity

7 µl of the poly[8-^{3}H]adenylic acid solution was added to 985 µl of the reaction mixture for determining activity prepared in (1) above. The mixture was incubated at 37°C for 10 minutes. 8 µl of polyadenylic acid was added to the mixture to make the final concentration to 24 µM. The mixture was further incubated at 37°C for 5 minutes. Thus, 1000 µl of a poly[8-^{3}H]rA-poly-dT reaction mixture was prepared. 200 µl of the reaction mixture was then incubated at 30°C for 5 minutes. 1 µl of an appropriate serial dilution of an enzyme solution was added thereto. 50 µl each of samples was taken from the reaction mixture over time for use in subsequent measurement. The period of time in minutes from the addition of the enzyme to the sampling is defined as Y. 50 µl of a reaction mixture for total CPM or for blank was prepared by adding 1 µl of the enzyme dilution solution instead of an enzyme solution. 100 µl of 100 mM sodium pyrophosphate, 50 µl of the heat-denatured calf thymus DNA solution and 300 µl of 10% trichloroacetic acid (300 µl of ultrapure water for measuring total CPM) were added to the sample. The mixture was incubated at 0°C for 5 minutes, and then centrifuged at 10000 rpm for 10 minutes. After centrifugation, 250 µl of the resulting supernatant was placed in a vial. 10 ml of

Aquasol-2 (NEN Life Science Products) was added thereto. CPM was measured in a liquid scintillation counter.

(3) Calculation of units

Unit value for each enzyme was calculated according to the following equation.

Unit/ml = {(measured CPM - blank CPM) x 1.2* x 20 x 1000 x dilution rate} 200 (µl) / (total CPM x Y (min.) x 50 (µl) x 9**)

1.2*: Amount in nmol of poly[8-^{3}H]rA-poly-dT contained in total CPM per 50 µl.

9**: Correction coefficient.

Example 1

The method of the present invention was used to detect enterohemorrhagic E. coli O-157.

Sequences of the primers used in this Example are shown in SEQ ID NOS:31 to 34 of the Sequence Listing. A combination of primers each having a sequence of SEQ ID NO:31 or 32 were constructed for detecting a sequence encoding vero toxin (VT) 1 of O-157, and a combination of primers each having a sequence of SEQ ID NO:33 or 34 were constructed for detecting a sequence encoding vero toxin 2 of O-157. A hot water-extract prepared by culturing enterohemorrhagic E. coli O-157 (ATCC accession no. 43895), collecting cells, suspending the cells in sterile water at an appropriate cell density and treating it at 98°C for 10

minutes was used as a template. The composition of the reaction mixture was as follows.

27 mM phosphate buffer (pH 7.3), 0.01% bovine serum albumin (BSA), 5% dimethyl sulfoxide (DMSO), 1 mM each of dNTPs, 8 mM magnesium acetate, 60 pmol each of the primers, the DNA as the template (the hot water-extract) corresponding to 10^4-10^6 cells and sterile distilled water to a reaction volume of 48 µl. The reaction mixture was heat-denatured at 98°C for 1 minute, and then cooled to 55°C. 5.5 U of BcaBEST DNA polymerase and 60 U of E. coli RNase H were added thereto. The reaction mixture was incubated at 55°C for 60 minutes. Thereafter, the mixture was heated at 90°C for 2 minutes to inactivate the enzymes. 3 µl each of the reaction mixtures was subjected to electrophoresis on 4% NuSieve 3:1 agarose (Takara Shuzo) gel. As a result, O-157 vero toxin 1 and 2 could be detected using either one of the primer pairs and the DNA as the template corresponding to 10^4 cells, confirming that the method of the present invention can be utilized as a method for detecting a virulent bacterium.

Example 2

(1) The effect of the type of the buffer used in the method of the present invention was examined. Primers for amplifying λ DNA having sequences represented by SEQ ID NOS:39 and 40 were used in this Example. The reaction was

carried out as follows. Briefly, 10 µl of a mixture containing 120 pmol each of the primers, 0.01% aqueous solution of propylenediamine and 10 ng or 1 ng of a DNA as a template was heat-denatured at 98°C for 2 minutes, and then cooled on ice to anneal the primers to the template DNA. An amplified product (1005 bp) obtained by a PCR using λ DNA (Takara Shuzo) as a template and primers represented by SEQ ID NOS:41 and 42, which was then purified using Suprec02 (Takara Shuzo), was used as the template.

After annealing, 40 µl of one of three types of buffers for reaction (42.5 mM Tricine-potassium hydroxide buffer (pH 8.5), 42.5 mM Bicine-potassium hydroxide buffer (pH 8.3) and 42.5 mM HEPES-potassium hydroxide buffer (pH 7.8)) each containing 0.625 mM each of dNTPs, 5.0 mM magnesium acetate, 0.0125% bovine serum albumin (BSA), 1.25% dimethyl sulfoxide (DMSO), 30 U of E. coli RNase H and 11 U of BcaBEST DNA polymerase was added to the mixture to make the final volume to 50 µl. The reaction mixtures were incubated at 60°C for 1 hour. After reaction, 3 µl each of the reaction mixtures was subjected to electrophoresis on 3.0% agarose gel for confirmation. Then, the amplified fragments of interest were observed for both of the amounts of the template. In particular, a greater amount of amplification product was obtained for the

reaction system containing Bicine-potassium hydroxide buffer (pH 8.5).

(2) Improvement of reactivity by the use of HEPES-potassium hydroxide buffer was examined. pUC19 plasmid DNA with a DNA fragment of about 150 bp being inserted into the multi-cloning site was used as a template. This template was prepared as follows.

A PCR was carried out using pUC19 upper 150 PCR primer and pUC19 lower PCR primer having sequences represented by SEQ ID NOS:134 and 135, and 100 pg of pUC19 plasmid DNA as a template. The resulting amplified fragment was purified using Microcon-100, blunt-ended using DNA blunting kit (Takara Shuzo) and subcloned into a HincII site of the plasmid pUC19. The plasmid with the amplified fragment being inserted was used to transform Escherichia coli JM109. The transformant was cultured. The plasmid having the inserted DNA was purified from the cells using QIAGEN plasmid mini kit (Qiagen) and used as a template.

A DNA fragment PCR-amplified using the pUC19-150 plasmid DNA prepared as described above as a template, and primers MCS-F and MCS-R having nucleotide sequences represented by SEQ ID NOS:35 and 36 was used as a template. Primers MF2N3(24) and MR1N3(24) having nucleotide sequences represented by SEQ ID NOS:37 and 38 were used as chimeric oligonucleotide primers. The size of the amplified

fragment obtained using the combination of these primers was about 350 bp.

An HEPES-potassium hydroxide buffer system was selected as a buffer to be examined. A potassium phosphate buffer system and a Tricine buffer system were used as controls. The compositions of the reaction mixtures are shown below.

Reaction mixture A: 10 ng of the PCR-amplified fragment, 50 pmol each of the primers MF2N3(24) and MR1N3(24), 0.01% aqueous solution of propylenediamine and sterile distilled water to a reaction volume of 10 µl.

Reaction mixtures B: The following three types were prepared.

Potassium phosphate buffer system: 40 µl of a reaction mixture containing, at final concentrations, 35 mM potassium phosphate buffer (pH 7.5), 1.25% DMSO, 0.0125% BSA, 5 mM magnesium acetate, 0.625 mM each of dNTPs, 60 U of E. coli RNase H and 5.5 U of BcaBEST DNA polymerase was prepared.

Tricine buffer system: 40 µl of a reaction mixture containing, at final concentrations, 42.5 mM Tricine buffer (pH 8.7), 12.5 mM potassium chloride, 12.5 mM ammonium sulfate, 1.25% DMSO, 0.0125% BSA, 5 mM magnesium acetate, 0.625 mM each of dNTPs, 30 U of E. coli RNase H and 5.5 U of BcaBEST DNA polymerase was prepared.

HEPES-potassium hydroxide buffer system: 40 µl of a reaction mixture containing, at final concentrations, 25 mM HEPES-potassium hydroxide buffer (pH 7.8), 125 mM potassium acetate, 1.25% DMSO, 0.0125% BSA, 5 mM magnesium acetate, 0.625 mM each of dNTPs, 30 U of E. coli RNase H and 5.5 U of BcaBEST DNA polymerase was prepared.

The reaction mixture A was heat-denatured at 98°C for 2 minutes, cooled to 60°C or 65°C and then allowed to stand on ice. One of the reaction mixtures B was added to the reaction mixture A on ice and mixed to make the reaction volume to 50 µl. The reaction mixtures were incubated at 60°C or 65°C for 1 hour. After reaction, they were cooled to 4°C, and a 1/10 volume of 0.5 M EDTA was added to each of the mixtures to terminate the reaction. 3 µl each of the reaction mixtures was subjected to electrophoresis on 3% NuSieve 3:1 agarose gel. As a result, the amplified fragments of interest were observed for the three buffer systems regardless of the reaction temperature used. In particular, the largest amount of the amplification product and the highest reactivity were observed for the HEPES-potassium hydroxide buffer system in this Example.

Example 3

(1) Conditions used in the method of the present invention for annealing primers to a template were examined.

Primers having nucleotide sequences represented by SEQ ID NOS:43 and 44 based on the partial nucleotide sequence of a bacterium Flavobacterium sp. SA-0082 as described in WO 97/32010 (deposited at International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, AIST Tsukuba Central 6, 1-1, Higashi 1-chome, Tsukuba-shi, Ibaraki 305-8566, Japan on March 29, 1995 under accession number FERM P-14872, and deposited at International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology, AIST Tsukuba Central 6, 1-1, Higashi 1-chome, Tsukuba-shi, Ibaraki 305-8566, Japan under accession number FERM BP-5402 (date of request for transmission to international depositary authority: February 15, 1996)) were used. An amplified product (573 bp) obtained by a PCR using a genomic DNA from Flavobacterium sp. SA-0082 as a template and a combination of primers represented by SEQ ID NOS:45 and 46, which was then purified using Suprec02 (Takara Shuzo), was used as the DNA as the template in this Example. The reaction was carried out as follows. Briefly, 2 µl of one of two annealing solutions (500 mM potassium chloride and 8 µM spermidine, or 0.05% propylenediamine) was added to 120 pmol each of the primers. A final volume of 10 µl of each of the mixtures further containing 10 ng or 1 ng of the PCR-amplified fragment was heat-denatured at

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98°C for 2 minute. After denaturation, the mixtures were rapidly cooled on ice to anneal the primers to the template.

After annealing, 40 µl of one of three types of buffers (42.5 mM Tricine-potassium hydroxide buffer (pH 8.5), 42.5 mM Bicine-potassium hydroxide buffer (pH 8.3) and 42.5 mM HEPES-potassium hydroxide buffer (pH 7.8)) each containing 0.625 mM each of dNTPs, 5.0 mM magnesium acetate, 0.0125% bovine serum albumin (BSA), 1.25% dimethyl sulfoxide (DMSO), 30 U of E. coli RNase H and 11 U of BcaBEST DNA polymerase was added to the mixture to make the final volume to 50 µl. The reaction mixtures were incubated at 52°C for 1 hour. After reaction, 3 µl each of the reaction mixtures was subjected to electrophoresis on 3.0% agarose gel. The results are shown in Figure 1. Figure 1 illustrates the results of electrophoresis of reactions using the respective combinations of the annealing solutions and the buffers. Lane 1: molecular weight marker (100 bp ladder, Takara Shuzo); lane 2: propylenediamine/Tricine (template: 10 ng); lane 3: propylenediamine/HEPES (template: 10 ng); lane 4: propylenediamine/HEPES (template: 1 ng); lane 5: propylenediamine/Bicine (template: 10 ng); lane 6: propylenediamine/Bicine (template: 1 ng); lane 7: 500 mM potassium chloride and 8 µM spermidine/Bicine (template: 10 ng); lane 8: 500 mM potassium chloride and 8 µM

spermidine/Bicine (template: 1 ng); lane 9: molecular weight marker (100 bp ladder); lane 10: propylenediamine/Tricine (template: 1 ng); lane 11: 500 mM potassium chloride and 8 μM spermidine/Tricine (template: 1 ng); lane 12: propylenediamine/HEPES (template: 1 ng); lane 13: 500 mM potassium chloride and 8 μM spermidine/HEPES (template: 1 ng); lane 14: propylenediamine/Bicine (template: 1 ng); and lane 15: 500 mM potassium chloride and 8 μM spermidine/Bicine (template: 1 ng).

As shown in Figure 1, a greater amount of the amplification product of interest was obtained when the annealing solution containing 500 mM potassium chloride + 8 μM spermidine was used for the annealing of the primers to the template DNA for either of the three buffers regardless of the amount of the DNA as the template. In particular, the combination of the annealing solution containing 500 mM potassium chloride + 8 μM spermidine and the Bicine-potassium hydroxide buffer yielded good results in this Example.

(2) The effect of an annealing solution in case where a PCR-amplified fragment from λ DNA was used as a template was examined. The chimeric oligonucleotide primers as described in Example 2(1) were used in this Example. The PCR-amplified fragment as prepared in Example 2(1) or λ DNA was used as a template DNA. The reaction was

carried out as follows. Briefly, 2 µl of one of three types of annealing solutions (500 mM potassium chloride and 8 µM spermidine, 0.05% propylenediamine, or sterile water) was added to 120 pmol each of the primers. 10 µl of the mixtures further containing 10 ng or 1 ng of the PCR-amplified fragment were prepared. The mixtures were heat-denatured at 98°C for 2 minutes, and then rapidly cooled on ice to anneal the primers to the template.

After annealing, 40 µl of one of three types of buffers (42.5 mM Tricine-potassium hydroxide buffer (pH 8.5), 42.5 mM Bicine-potassium hydroxide buffer (pH 8.3) and 42.5 mM HEPES-potassium hydroxide buffer (pH 7.8)) each containing 0.625 mM each of dNTPs, 5.0 mM magnesium acetate, 0.0125% BSA, 1.25% DMSO, 30 U of E. coli RNase H and 11 U of BcaBEST DNA polymerase was added to the mixture to make the final volume to 50 µl. The reaction mixtures were incubated at 60°C for 1 hour. After reaction, 3 µl each of the reaction mixtures was subjected to electrophoresis on 3.0% agarose gel. The results are shown in Figure 2. Figure 2 illustrates the results of electrophoresis showing the results for the examination of combinations of the amounts of the template, the reaction buffers and the annealing solutions. Lane 1: marker (100 bp ladder); lane 2: 10 ng of the template, a combination of Tricine/500 mM potassium chloride and 8 µM spermidine; lane 3: 1 ng of the

template, a combination of Tricine/500 mM potassium chloride and 8 µM spermidine; lane 4: 10 ng of the template, a combination of Bicine/500 mM potassium chloride and 8 µM spermidine; lane 5: 1 ng of the template, a combination of Bicine/500 mM potassium chloride and 8 µM spermidine; lane 6: 10 ng of the template, a combination of HEPES/500 mM potassium chloride and 8 µM spermidine; lane 7: 1 ng of the template, a combination of HEPES/500 mM potassium chloride and 8 µM spermidine; lane 8: molecular weight marker (100 bp ladder); lane 9: 10 ng of the template, a combination of Tricine/propylenediamine; lane 10: 1 ng of the template, a combination of Tricine/propylenediamine; lane 11: 10 ng of the template, a combination of Bicine/propylenediamine; lane 12: 1 ng of the template, a combination of Bicine/propylenediamine; lane 13: 10 ng of the template, a combination of HEPES/propylenediamine; lane 14: 1 ng of the template, a combination of HEPES/propylenediamine; lane 15: molecular weight marker (100 bp ladder); lane 16: 10 ng of the template, a combination of Tricine/water; lane 17: 1 ng of the template, a combination of Tricine/water; lane 18: 10 ng of the template, a combination of Bicine/water; lane 19: 1 ng of the template, a combination of Bicine/water; lane 20: 10 ng of the template, a combination of HEPES/water; and lane 21: 1 ng of the template, a combination of HEPES/water.

As shown in Figure 2, the amplified fragments of interest were observed for the respective combinations of the three buffers and the three annealing solutions regardless of the amount of the template DNA. In particular, it was confirmed that a greater amount of the amplified fragment was obtained using the combination of the Bicine buffer and the annealing solution containing 500 mM potassium chloride and 8 μM spermidine.

Example 4

The effect of the presence of an inhibitor of reverse transcriptase (RTase) on the method of the present invention was examined. Phosphonoformic acid (PFA) was used as an inhibitor of RTase. Primers represented by SEQ ID NOS:47 and 48 were used in this Example. An amplified product (576 bp) obtained by a PCR using a genomic DNA from enterohemorrhagic Escherichia coli O-157 as a template and primers represented by SEQ ID NOS:49 and 50, which was then purified using Suprec02 (Takara Shuzo), was used as the template DNA. The reaction was carried out as follows. Briefly, 10 μl of a mixture prepared by adding 1 ng of the PCR-amplified fragment to 120 pmol each of the primers and 2 μl of an annealing solutions containing 500 mM potassium chloride and 8 μM spermidine was heat-denatured at 98°C for 2 minutes, and then rapidly cooled on ice to anneal the primers to the template.

After annealing, 40 µl of a mixture containing 0.625 mM each of dNTPs, 42.5 mM Tricine-potassium hydroxide buffer (pH 8.5), 5.0 mM magnesium acetate, 0.0125% bovine serum albumin (BSA), 1.25% dimethyl sulfoxide (DMSO), 30 U of E. coli RNase H and 11 U of BcaBEST DNA polymerase as well as PFA to a concentration of 500 µg/ml or 50 µg/ml were added to the annealing mixture to make the final volume to 50 µl. The reaction mixtures were incubated at 55°C for 1 hour. A system without the addition of PFA was also prepared as a control. After reaction, 9 µl each of the reaction mixtures was subjected to electrophoresis on 3.0% agarose gel. The results are shown in Figure 3. Figure 3 illustrates the results of electrophoresis which shows the effect of the inhibitor of reverse transcriptase activity. Lane 1: molecular weight marker (100 bp ladder); lane 2: without the addition of PFA; lane 3: with the addition of PFA at a concentration of 500 µg/ml; lane 4: with the addition of PFA at a concentration of 50 µg/ml.

As shown in Figure 3, when PFA was added, non-specific amplification was suppressed and the amplified fragment of interest was observed. In particular, it was confirmed that the non-specific amplification product which was observed in the system without the addition of PFA was not observed and the amplified fragment of interest was clearly amplified in the system to which PFA was added at a

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concentration of 500 µg/ml.

Example 5

The relationship between the length of a fragment to be amplified and the detection sensitivity in the method of the present invention was examined.

(1) Primers for amplifying Escherichia coli O-157 vero toxin represented by SEQ ID NOS:51 to 53 were synthesized. The chimeric oligonucleotide primers used in Example 4 were also used. The length of a fragment to be amplified using each of the combinations of primers was as follows: 247 bp (SEQ ID NOS:51 and 48); 168 bp (SEQ ID NOS:52 and 53); 206 bp (SEQ ID NOS:52 and 48); 135 bp (SEQ ID NOS:47 and 53); and 173 bp (SEQ ID NOS:47 and 48). The purified PCR-amplified 576-bp fragment prepared in Example 4 was used as a template DNA in this Example. The reaction was carried out as follows. Briefly, 10 µl of a mixture containing 60 pmol each of the primers, 2 µl of 0.05% aqueous solution of propylenediamine and 10 fg to 10 ng of the PCR-amplified fragment was heat-denatured at 98°C for 2 minutes and then cooled to 55°C in Thermal Cycler Personal (Takara Shuzo) to anneal the primers to the template.

After annealing, 40 µl of a reaction mixture containing 0.625 mM each of dNTPs, 42.5 mM Tricine-potassium hydroxide buffer (pH 8.5), 5.0 mM magnesium acetate, 0.0125% bovine serum albumin (BSA), 1.25% dimethyl

sulfoxide (DMSO), 30 U of E. coli RNase H, 5.5 U of BcaBEST DNA polymerase and sterile water was added to the mixture to make the final volume to 50 µl. The reaction mixtures were incubated at 55°C for 1 hour. After reaction, 5 µl each of the reaction mixtures was subjected to electrophoresis on 3.0% agarose gel. As a control, detection of 10 fg to 1 pg of the PCR-amplified fragment was carried out using primers represented by SEQ ID NOS:54 and 55. A 135-bp fragment is amplified using the combination of these primers. 50 µl of a PCR solution containing 60 pmol each of the primers, 5 µl of 10 x Ex Taq buffer (Takara Shuzo), 1.25 U of TaKaRa Ex Taq DNA polymerase (Takara Shuzo) and 0.2 mM each of dNTPs was prepared. The PCR was carried out as follows: 25 or 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds (2 min. 38 sec./cycle). After reaction, 1 µl (ICAN) or 5 µl (PCR) each of the reaction mixtures was subjected to electrophoresis on 3.0% agarose gel. The results are shown in Figure 4 and Table 1.

Table 1

Amplification size (bp)	Detection limit
ICAN (total time: 70 minutes)	
247	100 pg
168	100 fg
206	100 pg
135	10 fg
173	100 fg
PCR (25 cycles; total time: about 66 minutes)	
135	100 fg
PCR (30 cycles; total time: about 80 minutes)	
135	10 fg

Figure 4 illustrates the results of electrophoresis which shows the detection limits for amplifying a chain length of 135 bp accomplished by the ICAN (1/50 of the reaction mixture was loaded) and the PCR (1/10 of the reaction mixture was loaded). Lane 1: molecular weight marker (100 bp ladder); lane 2: ICAN using 1 pg of the template; lane 3: ICAN using 100 fg of the template; lane 4: ICAN using 10 fg of the template; lane 5: PCR (25 cycles) using 1 pg of the template; lane 6: PCR (25 cycles) using 100 fg of the template; lane 7: PCR (25 cycles) using 10 fg of the template; lane 8: PCR (30 cycles) using 1 pg of the template; lane 9: PCR (30 cycles) using 100 fg of the template; and lane 10: PCR (30 cycles) using 10 fg of the template.

As shown in Table 1, it was confirmed that almost

the same detection sensitivity as that of the PCR was accomplished by the ICAN. Furthermore, the total reaction time for the PCR was about 80 minutes, whereas the reaction time required for accomplishing the same detection sensitivity using the method of the present invention was 70 minutes, confirming that the reaction time can be shortened by using the method of the present invention.

(2) Primers for amplifying λ DNA having nucleotide sequences represented by SEQ ID NOS:39, 40 and 56 were synthesized. The length of a fragment to be amplified using each of the combinations of primers was as follows: 151 bp (SEQ ID NOS:39 and 40); and 125 bp (SEQ ID NOS:56 and 40). The template DNA prepared in Example 2(1) was used in this Example. The reaction was carried out as follows. Briefly, 2 µl of an annealing solution (500 mM potassium chloride and 8 µM spermidine), 1 fg to 1 ng of the template were added to 120 pmol each of the primers and fill up to 10 µl by sterile water. The mixture was heat-denatured at 98°C for 2 minutes, and then rapidly cooled on ice to anneal the primers to the template.

After annealing, 40 µl of a mixture containing 0.625 mM each of dNTPs, 42.5 mM Tricine-potassium hydroxide buffer (pH 8.5), 5.0 mM magnesium acetate, 0.0125% BSA, 1.25% DMSO, 30 U of E. coli RNase H and 11 U of BcaBEST DNA polymerase was added to the mixture to make the final

volume to 50 µl with sterile water. The reaction mixtures were incubated at 60°C for 1 hour. After reaction, 3 µl each of the reaction mixtures was subjected to electrophoresis on 3.0% agarose gel. The results are shown in Table 2.

Table 2

Amplification size (bp)	Detection limit
125	10 fg
151	100 fg

As shown in Table 2, it was confirmed that, when λ DNA was used as a template, a detection sensitivity of as low as 10 fg could be also accomplished by examining the optimal region.

(3) A plasmid was prepared by inserting an amplified fragment (length: 340 bp) into a plasmid T7Blue T-Vector (Takara Shuzo). The fragment was prepared as described in JP-A 9-140383 using synthetic primers for amplifying a chrysanthemum viroid gene having nucleotide sequences represented by SEQ ID NOS:57 and 58, and an RNA from chrysanthemum infected with the viroid as a template. The plasmid was used to transform Escherichia coli JM109 competent cells (Takara Shuzo). The transformant was cultured in 5 ml of LB medium at 37°C for 16 hour. The plasmid was purified from the collected cells using QIAGEN

Plasmid Mini Kit (Qiagen) according to the manual. Dilutions containing 0 fg, 1 fg, 10 fg, 100 fg, 1 pg, 10 pg, 100 pg or 1 ng of the plasmid in 1 µl of sterile water were prepared based on the concentration of the plasmid as measured using Beckman DU-600 (Beckman). 1 µl of one of the thus prepared plasmid solutions was used as a template for 50 µl each of ICAN reaction systems. Primers CSVD-F2 and CSVD-R6 having nucleotide sequences represented by SEQ ID NOS:59 and 60 were used in this Example. The reaction was carried out as follows. Briefly, 10 µl of a mixture containing 50 pmol each of the primers, 1 µl of the prepared plasmid solution and propylenediamine at a final concentration of 0.01% was prepared. The mixture was heated at 98°C for 2 minutes, cooled to 60°C, incubated at the temperature for 1 minute in Thermal Cycler Personal (Takara Shuzo), and then placed on ice.

After annealing, at final concentrations, 20 mM HEPES-potassium hydroxide buffer (pH 7.8), 100 mM potassium acetate, 1% DMSO, 0.01% BSA, 4 mM magnesium acetate, 500 µM each of dNTPs, 30 U of E. coli RNase H and 5.5 U of BcaBEST DNA polymerase were added to the mixture to make the final volume to 50 µl with sterile water. The reaction mixtures were placed in Thermal Cycler Personal which had been set at 60°C and reacted for 60 minutes. After reaction, 3 µl each of the reaction mixtures was subjected to

electrophoresis on 3% NuSieve 3:1 agarose. As a result, the amplification products of interest (about 90 bp, about 70 bp and about 50 bp) were observed using a template at a concentration of 10 fg.

Example 6

Primers to be used in the method of the present invention were examined.

(1) The Tm value of a primer and the reaction temperature were examined. Primers for amplifying Flavobacterium sp. SA-0082 having nucleotide sequences represented by SEQ ID NOS:43 and 61-63 were synthesized. These primers were constructed such that a region of 160 bp or shorter having a GC content of about 20% was amplified. The length of a fragment to be amplified using each of the combinations of primers was as follows: 126 bp (SEQ ID NOS:43 and 62); 158 bp (SEQ ID NOS:43 and 63); 91 bp (SEQ ID NOS:61 and 62); and 123 bp (SEQ ID NOS:61 and 63). The PCR-amplification product as prepared in Example 3(1) was used as a DNA as the template in this Example. The reaction was carried out as follows. Briefly, 10 µl of a mixture containing 120 pmol each of the primers, 2 µl of one of three types of annealing solutions (500 mM potassium chloride and 8 µM spermidine, 0.05% propylenediamine, or water) and 1 fg to 10 ng of the template was prepared. The mixture was heat-denatured at 98°C for 2 minutes, and then

cooled on ice to anneal the primers to the template.

After annealing, 40 µl of one of three types of buffers (17 mM Tricine-potassium hydroxide buffer (pH 8.5), 17 mM Bicine-potassium hydroxide buffer (pH 8.3) and 20 mM HEPES-potassium hydroxide buffer (pH 7.8)) containing 0.625 mM each of dNTPs, 5.0 mM magnesium acetate, 0.0125% bovine serum albumin (BSA), 1.25% dimethyl sulfoxide (DMSO), 30 U of E. coli RNase H and 11 U of BcaBEST DNA polymerase was added to the mixture to make the final volume to 50 µl. The reaction mixtures were incubated at 52, 55 or 60°C for 1 hour. After reaction, 3 µl each of the reaction mixtures was subjected to electrophoresis on 3.0% agarose gel. As a result, the amplified fragment of interest was observed using the reaction temperature of 52°C. In particular, a greater amount of amplified fragment of interest was obtained using the combination of the annealing solution containing 500 mM potassium chloride and 8 µM spermidine and Tricine or Bicine buffer. The primer pair, the length of the amplified fragment and the detection sensitivity for the reaction temperature of 52°C are shown in Figure 5 and Table 3.

Table 3

Amplification size (bp)	Detection limit
126	100 fg
158	1 pg
91	1 fg
123	100 fg

Figure 5 illustrates the results of electrophoresis which shows the relationship between the length of the amplified fragment and the amount of the DNA as the template when an AT-rich region was amplified. Lane 1: molecular weight marker (100 bp ladder); lane 2: amplification of a fragment of 91 bp in length using 1 pg of the template; lane 3: amplification of a fragment of 91 bp in length using 100 fg of the template; lane 4: amplification of a fragment of 91 bp in length using 10 fg of the template; lane 5: amplification of a fragment of 91 bp in length using 1 fg of the template; lane 6: amplification of a fragment of 123 bp in length using 1 pg of the template; lane 7: amplification of a fragment of 123 bp in length using 100 fg of the template; lane 8: amplification of a fragment of 123 bp in length using 10 fg of the template; lane 9: amplification of a fragment of 126 bp in length using 1 pg of the template; lane 10: amplification of a fragment of 126 bp in length using 100 fg of the template; lane 11: amplification of a fragment of 126 bp in length using 10 fg of the template; lane 12:

amplification of a fragment of 158 bp in length using 1 pg of the template; lane 13: amplification of a fragment of 158 bp in length using 100 fg of the template; and lane 14: amplification of a fragment of 158 bp in length using 10 fg of the template.

As shown in Figure 5 and Table 3, it was demonstrated that good results were yielded by lower the reaction temperature depending on the Tm value of the primer when the method of the present invention was carried out using an AT-rich template and a set of AT-rich primers.

(2) The higher-order structure of the primer may influence the reactivity in the method of the present invention. Then, modification of the primer for avoiding the formation of the higher-order structure of the primer and for making the primer readily anneal to the objective template was examined. Primers represented by SEQ ID NOS:47, 48 and 64-69 were used. Specifically, a primer having a nucleotide sequence represented by SEQ ID NO:47, primers 120I4, 121I5 and 122I6 which are primers having nucleotide sequences represented by SEQ ID NOS:64 to 66 and having an inosine deoxynucleotide at the fourth, fifth or sixth base from the 3'-terminus, a primer having a nucleotide sequence represented by SEQ ID NO:104, and primers 123I4, 124I5 and 125I6 which are primers having nucleotide sequences represented by SEQ ID NOS:67 to 69 and

having an inosine deoxynucleotide at the fourth, fifth or sixth base from the 3'-terminus were used. The DNA as the template as prepared in Example 4 was used in this Example. The reaction was carried out as follows. Briefly, 10 µl of a mixture containing 50 pmol each of the primers, 2 µl of 0.05% aqueous solution of propylenediamine, 1 ng to 10 ng of the DNA as the template and sterile distilled water was heated at 98°C for 2 minutes, cooled to 55°C, and incubated at the temperature for 1 minute using a thermal cycler (GeneAmp PCR System 9600, Applied Biosystems).

After annealing, 0.625 mM each of dNTPs, 42.5 mM Tricine-potassium hydroxide buffer (pH 8.5), 5.0 mM magnesium acetate, 0.0125% BSA, 1.25% DMSO, 30 U of E. coli RNase H or 5 U of a heat-resistant RNase H from Thermus thermophilus (Tth) (Toyobo, hereinafter referred to as Tth RNase H) and 5.5 U of BcaBEST DNA polymerase were added to the mixture to make the final volume to 50 µl with sterile water. The reaction mixtures were incubated at 55°C for 1 hour. After reaction, 5 µl each of the reaction mixtures was subjected to electrophoresis on 3.0% agarose gel. The results are shown in Figure 6.

Figure 6 illustrates the results of electrophoresis which shows the effects of chimeric oligonucleotide primers containing inosine deoxynucleotides when E. coli RNase H and Tth RNase H were used. Lanes 2 to

9 represent the results obtained using E. coli RNase H. Lanes 10 to 17 represent the results obtained using Tth RNase H. Lane 1: molecular weight marker (100 bp ladder); lane 2: a pair of primers represented by SEQ ID NOS:47 and 48, 1 ng of the template; lane 3: a pair of primers 120I4 and 123I4, 1 ng of the template; lane 4: a pair of primers 121I5 and 124I5, 1 ng of the template; lane 5: a pair of primers 122I6 and 125I6, 1 ng of the template; lane 6: a pair of primers represented by SEQ ID NOS:47 and 48, 10 ng of the template; lane 7: a pair of primers 120I4 and 123I4, 10 ng of the template; lane 8: a pair of primers 121I5 and 124I5, 10 ng of the template; lane 9: a pair of primers 122I6 and 125I6, 1 ng of the template; lane 10: a pair of primers represented by SEQ ID NOS:47 and 48, 1 ng of the template; lane 11: a pair of primers 120I4 and 123I4, 1 ng of the template; lane 12: a pair of primers 121I5 and 124I5, 1 ng of the template; lane 13: a pair of primers 122I6 and 125I6, 1 ng of the template; lane 14: a pair of primers represented by SEQ ID NOS:47 and 48, 10 ng of the template; lane 15: a pair of primers 120I4 and 123I4, 10 ng of the template; lane 16: a pair of primers 121I5 and 124I5, 10 ng of the template; and lane 17: a pair of primers 122I6 and 125I6, 10 ng of the template.

As shown in Figure 6, when primers having inosines being incorporated at fourth or fifth base from

the 3'-termini of the primers were used, increase in the amplification product of interest was observed using either of E. coli RNase H and the heat-resistant RNase H from Thermus thermophilus regardless of the amount of the template. These results demonstrate that the reactivity of the ICAN is improved by incorporating inosine at an appropriate position.

(3) Primers were examined for the same purpose as that in (2) above. Oligonucleotide primers 1S and 4S having nucleotide sequences represented by SEQ ID NOS:84 and 85 in which the three bases at the 3'-terminus were α-S (or alpha-thio) ribonucleotides, i.e., which had 5'-phosphothioate bonds in the RNA moieties, were synthesized. In addition, oligonucleotide primers 1N3N3 and 4N3N3 having nucleotide sequences represented by SEQ ID NOS:70 and 71 and having ribonucleotides at the three bases from the 3'-terminus and in a portion of the sequence of the deoxyribonucleotide moiety, i.e., ribonucleotides at eleventh to thirteenth bases from the 3'-terminus of the primer, were synthesized. The template DNA as prepared in Example 4 was used. The reaction was carried out as follows. Briefly, 10 µl of a mixture containing 50 pmol each of the primers, 2 µl of 0.05% aqueous solution of propylenediamine, 10 ng of the DNA as the template and sterile water was heated at 98°C for 2 minutes using a

thermal cycler, and then placed on ice for cooling.

After annealing, 40 µl of a mixture containing 0.625 mM each of dNTPs, 42.5 mM Tricine-potassium hydroxide buffer (pH 8.5), 5.0 mM magnesium acetate, 0.0125% BSA, 1.25% DMSO, 30 U of E. coli RNase H or 5 U of Tth RNase H and 5.5 U of BcaBEST DNA polymerase was added to the mixture to make the final volume to 50 µl with sterile water. The reaction mixtures were incubated at 55°C for 1 hour in a thermal cycler.

After reaction, 5 µl each of the reaction mixtures was subjected to electrophoresis on 3.0% agarose gel. As a result, the amplification product was clearly observed at the expected position using the combination of primers 1S and 4S or 1N3N3 and 4N3N3 regardless of the type of the RNase H used. These results confirmed that modification of a primer at its 3'-terminus with 5'-phophothioate was effective for the method of the present invention. Additionally, it was confirmed that substitution with a ribonucleotide at an appropriate internal position in addition to the 3'-terminus of a primer was effective for improving the reactivity of the method of the present invention.

Example 7

Use of a DNA polymerase having E. coli RNase H activity in the presence of a specific metal ion in the

method of the present invention was examined. 10 µl of a mixture containing 120 pmol each of the chimeric oligonucleotide primers used in Example 2(1), 2 µl of an annealing solution containing 500 mM potassium chloride and 8 µM spermidine, 1 ng of the DNA as the template used in Example 2(1) and sterile water was heat-denatured at 98°C for 2 minutes, and then rapidly cooled on ice to anneal the primers to the template. After annealing, 40 µl of a mixture containing 0.625 mM each of dNTPs, 42.5 mM Tricine-potassium hydroxide buffer (pH 8.5), 5.0 mM magnesium acetate, 0.0125% BSA, 1.0% DMSO and 11 U of BcaBEST DNA polymerase, and manganese chloride (Nacalai Tesque) at a final concentration of 0.5, 2.5, 5.0 or 10 mM were added to the mixture to make the final volume to 50 µl with sterile water. The reaction mixtures were incubated at 60°C for 1 hour. In addition, a mixture without the addition of manganese chloride, and a mixture to which 30 U of E. coli RNase H was added but manganese chloride was not added were prepared as controls. After reaction, 3 µl each of the reaction mixtures was subjected to electrophoresis on 3.0% agarose gel. The results are shown in Figure 7.

Figure 7 illustrates electrophoresis which shows the results of the ICAN utilizing the RNase H activity of BcaBEST DNA polymerase. Lane 1: molecular weight marker (100 bp ladder); lane 2: without the addition of manganese

chloride/with the addition of E. coli RNase H; lane 3: without the addition of manganese chloride/without the addition of E. coli RNase H; lane 4: with the addition of 0.5 mM manganese chloride/without the addition of E. coli RNase H; lane 5: with the addition of 2.5 mM manganese chloride/without the addition of E. coli RNase H; lane 6: with the addition of 5.0 mM manganese chloride/without the addition of E. coli RNase H; and lane 7: with the addition of 10.0 mM manganese chloride/without the addition of E. coli RNase H.

As shown in Figure 7, the amplification product of interest was observed for a reaction system to which manganese chloride was added at a concentration of 2.5 mM in the absence of E. coli RNase H.

Example 8

The method of the present invention was examined using a practical biological sample.

(1) Detection was carried out using a hot water-extract prepared from a culture of enterohemorrhagic Escherichia coli O-157 (ATCC accession number 43895) as a template. Enterohemorrhagic Escherichia coli O-157 was cultured in mEC medium containing novobiocin at 42°C for 18 hours, and then heated at 95°C for 10 minutes. Hot water-extracts of O-157 corresponding to 0, 1, 10, 10^2, 10^3, 10^4 or 10^5 cells were prepared by diluting the extract with

sterile water. Vero toxin 2 (VT2) gene was amplified using one of these hot water-extracts of O-157 under the same conditions as those in Example 5(1). In addition, a PCR was carried out using the same template under the conditions as described in Example 5(1) as a control. After reaction, 1 µl (ICAN) or 5 µl (PCR) each of the reaction mixtures was subjected to electrophoresis on 3.0% agarose gel. The results are shown in Table 4 and Figure 8.

Table 4

Amplification size (bp)	Detection limit (cells)
ICAN (total time: 70 minutes)	
135	10^2
173	10^3
PCR (25 cycles; total time: about 66 minutes)	
135	10^3
PCR (30 cycles; total time: about 80 minutes)	
135	10^2

Figure 8 illustrates the results of electrophoresis which shows the detection of Escherichia coli O157 using ICAN or PCR. The chain length to be amplified was 135 bp. Lane 1: molecular weight marker (100 bp ladder); lane 2: ICAN for 10^4 cells; lane 3: ICAN for 10^3 cells; lane 4: ICAN for 10^2 cells; lane 5: PCR of 25 cycles for 10^4 cells; lane 6: PCR of 25 cycles for 10^3

cells; lane 7: PCR of 25 cycles for 10^2 cells; lane 8: PCR of 30 cycles for 10^4 cells; lane 9: PCR of 30 cycles for 10^3 cells; and lane 10: PCR of 30 cycles for 10^2 cells.

As shown in Table 4 and Figure 8, it was confirmed that the detection sensitivity of the detection method of the present invention was equivalent to that of the PCR, and that the method of the present invention required shorter time for detection as compared with that required for the PCR.

(2) λ DNA was detected using the primers represented by SEQ ID NOS:40 and 56 used in Examples 2 and 4. The reaction was carried out as follows. Briefly, 10 µl of a mixture containing 120 pmol each of the primers, 2 µl of an annealing solution containing 500 mM potassium chloride and 8 µM spermidine, 10 fg to 1 ng of λ DNA (Takara Shuzo) and sterile water was prepared. The mixture was heat-denatured at 98°C for 2 minutes, and then rapidly cooled on ice to anneal the primers to the template.

After annealing, 40 µl of a mixture containing 0.625 mM each of dNTPs, 42.5 mM Tricine-potassium hydroxide buffer (pH 8.5), 5.0 mM magnesium acetate, 0.0125% BSA, 1.25% DMSO, 30 U of E. coli RNase H and 11 U of BcaBEST DNA polymerase was added to the mixture to make the final volume to 50 µl with sterile water. The reaction mixtures were incubated at 60°C for 1 hour. After reaction, 3 µl

each of the reaction mixtures was subjected to electrophoresis on 3.0% agarose gel. The results are shown in Table 5.

Table 5

Amplification size (bp)	Detection limit
125	1 pg

As shown in Table 5, it was confirmed that the method of the present invention was effective in detecting λ DNA.

(3) Detection was carried out using a genomic DNA from a bacterium Flavobacterium sp. SA-0082 as a template and the primers represented by SEQ ID NOS:61 and 62 used in Example 6(1). The genomic DNA as a template was prepared according to a conventional method from the bacterium of genus Flavobacterium cultured as described in WO 97/32010. The reaction was carried out as follows. Briefly, 10 µl of a mixture containing 120 pmol each of the primers, 2 µl of an annealing solution containing 500 mM potassium chloride and 8 µM spermidine, 10 fg to 1 ng of the genomic DNA and sterile water was prepared. The mixture was heat-denatured at 98°C for 2 minutes, and then rapidly cooled on ice to anneal the primers to the template.

After annealing, 40 µl of a mixture containing

0.625 mM each of dNTPs, 42.5 mM Tricine-potassium hydroxide buffer (pH 8.5), 5.0 mM magnesium acetate, 0.0125% BSA, 1.25% DMSO, 30 U of E. coli RNase H and 11 U of BcaBEST DNA polymerase was added to the mixture to make the final volume to 50 µl with sterile water. The reaction mixtures were incubated at 52°C for 1 hour. After reaction, 3 µl each of the reaction mixtures was subjected to electrophoresis on 3.0% agarose gel. The results are shown in Table 6 and Figure 9.

Table 6

Amplification size (bp)	Detection limit
91	100 fg

Figure 9 illustrates the results of electrophoresis which shows the detection of a bacterium of genus Flavobacterium. Lane 1: molecular weight marker (100 bp ladder); lane 2: 1 ng of the template; lane 3: 10 pg of the template; lane 4: 1 pg of the template; lane 5: 100 fg of the template; and lane 6: 10 fg of the template.

As shown in Table 6 and Figure 9, it was confirmed that the method of the present invention was effective in detecting a bacterium.

Example 9

A method for detecting a target nucleic acid in

which the amplification method of the present invention and a hybridization method were combined was examined. Enterohemorrhagic Escherichia coli O-157 was selected as a target. The DNA as the template was prepared as described in Example 8(1). A region of about 100 bp having a GC content of about 40% was selected as a fragment to be amplified. Primers VT2-IF20 and VT2-IR20-2 having nucleotide sequences represented by SEQ ID NOS:51 and 72 were used as primers. The reaction was carried out as follows. Briefly, 10 µl of a mixture containing 50 pmol each of the primers VT2-IF20 and VT2-IR20-2, an annealing solution containing propylenediamine at a final concentration of 0.01%, one of the hot water-extracts corresponding to 0 to 10^4 cells and sterile water was prepared. The mixture was heat-denatured at 98°C for 2 minutes, cooled to 55°C and incubated at the temperature for 1 minute in Thermal Cycler Personal (Takara Shuzo), and then placed on ice for annealing.

After annealing, at final concentrations, 20 mM HEPES-potassium hydroxide buffer (pH 7.8), 100 mM potassium acetate, 1% DMSO, 0.01% BSA, 4 mM magnesium acetate, 500 µM each of dNTPs, 30 U of E. coli RNase H and 5.5 U of BcaBEST DNA polymerase was added to the mixture to make the final volume to 50 µl with sterile water. The reaction mixtures were placed on Thermal Cycler Personal which had been set

at 55°C and incubated at the temperature for 60 minutes. As a control, a PCR was carried out using O-157 Typing Set (Takara Shuzo) according to the manual using Thermal Cycler Personal. The PCR was carried out as follows: 35 cycles of 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute. This reaction required about 4 minutes per cycle and a total time of about 145 minutes. The expected size of the amplification product was 404 bp. After reaction, 3 µl each of the reaction mixtures was subjected to electrophoresis on 3% NuSieve 3:1 agarose. The results are shown in Figure 28A. Figure 28A shows the results of electrophoresis for the detection of enterohemorrhagic Escherichia coli O157 vero toxin II gene using the ICAN or the PCR. Lane M1: molecular weight marker (50-2000 bp); lane M2: molecular weight marker (100 bp ladder); lane N: negative control; lane 1: the template corresponding to 1 cell; lane 2: the template corresponding to 10 cells; lane 3: the template corresponding to 10^2 cells; lane 4: the template corresponding to 10^3 cells; and lane 5: the template corresponding to 10^4 cells. Furthermore, results of comparison between the amplification levels accomplished by the ICAN and the PCR for 1 cell or 10 cells are shown in Table 7.

Table 7

	Number of E. coli O-157 cells		
	0	1	10
ICAN	-	+	+++
PCR	-	+	++

-: no amplification; + to +++ indicate the degree of amplification in three grades.

As shown in Figure 28A and Table 7, the amplification products of interest were obtained for the reaction systems using a hot water-extract corresponding to 1 cell according to both the detection method of the present invention and the PCR. Dot blot hybridization was further carried out for the amplification product obtained according to the ICAN using an oligonucleotide VT2 having a nucleotide sequence represented by SEQ ID NO:73 labeled with biotin at the 5'-terminus as a probe. Hybridization was carried out under as follows. Briefly, 1 µl of a reaction mixture was denatured at 98°C for 5 minutes, rapidly cooled on ice, and spotted onto Hybond-N™ (Amersham Pharmacia Biotech). After exposure to UV, the membrane was placed in a hybridization bag. 10 ml of a hybridization solution containing 0.5 M disodium hydrogenphosphate (pH 7.2), 1 mM ethylenediaminetetraacetic

acid and 7% sodium lauryl sulfate was added thereto. Pre-hybridization was then carried out at 42°C for 30 minutes. 10 µl of the solution of the VT2 probe at a concentration of 100 ng/µl was heat-denatured and added to the pre-hybridization reaction system. After hybridization at 42°C for 60 minutes, the membrane was washed twice in a solution containing 66.6 mM sodium chloride, 66.6 mM sodium citrate and 0.1% sodium lauryl sulfate at room temperature for 5 minutes, incubated in 6 ml of a washing buffer (0.3 M sodium chloride, 17.3 mM disodium hydrogenphosphate dihydrate, 2.5 mM EDTA, 0.1% sodium lauryl sulfate) to which 2 µl of 5 mg/ml horseradish peroxidase streptoavidin conjugate (PIERCE) was added at 42°C for 12 minutes, and then washed twice in the washing buffer at room temperature. The membrane was then washed in 10 ml of 0.1 M citrate buffer (pH 5.0) at room temperature and reacted in a mixture of 5 ml of 0.1 M citrate buffer, 5 µl of 3% hydrogen peroxide and 250 µl of a solution of 2 mg/ml tetramethylbenzidine (TMB, Nacalai Tesque) in ethanol in the dark for about 10 minutes. After color development, the reaction was terminated with deionized water. The results are shown in Figure 28B. Figure 28B shows the results of dot blot hybridization for detecting a gene for vero toxin II from enterohemorrhagic Escherichia coli O-157 by the ICAN. The results were consistent with those

obtained for the above-mentioned electrophoresis. Thus, the detection sensitivity of the method of the present invention was equivalent to that of the PCR. The total time required for the amplification reaction using the ICAN of the present invention was 1/2 or shorter as compared with that required for the PCR. Thus, the ICAN of the present invention was confirmed to be effective as a method for detecting a pathogen and the like.

Example 10

(1) Combination of a reverse transcription reaction and the method of the preset invention was examined using an RNA from cultured cells as a template. The reaction was carried out as follows. Briefly, RAW264.7 cells (ATCC TIB 71) were suspended in Dulbecco's modified Eagle's medium (Bio Whittaker, 12-604F) containing 10% fetal calf serum (Gibco) at a concentration of 1.5×10^5 cells/ml. 5 ml of the suspension was added to each well of a 6-well microtiter plate and the plate was incubated at 37°C overnight in the presence of 5% CO_2. 50 µl of a 100 µg/ml solution of lipopolysaccharide (LPS, Sigma) in water and 50 µl of a 1000 U/µl solution of interferon-γ (IFN-γ, Genzyme Techne) in water were added to the well. The plate was incubated for additional 4 hours. An RNA was then prepared from the cells using RNeasy Mini Kit (Qiagen) according to the instructions attached to the kit. As a

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negative control, a group to which LPS or IFN-γ was not added was provided.

A cDNA was prepared by incubating 60 µl of a mixture containing 3 µg of the thus prepared RNA, 10 mM tris-hydrochloride buffer (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 1 mM each of dNTPs, 150 pmol of random 6mers primer, 60 U of ribonuclease inhibitor (Takara Shuzo) and 15 U of Reverse Transcriptase XL (AMV) (Takara Shuzo, 2620A) at 30°C for 10 minutes, 42°C for 1 hour and then 99°C for 5 minutes for inactivating the enzyme using a thermal cycler (GeneAmp PCR System 9600, Applied Biosystems).

Primers having nucleotide sequences represented by SEQ ID NOS:74 and 75 were synthesized based on the nucleotide sequence of the mRNA for mouse inducible NO synthase (iNOS) (GeneBank accession no. NM-010927). As a control, primers for PCR represented by SEQ ID NOS:76 and 77 were also synthesized.

10 µl of a mixture containing 50 pmol each of the primers, 2 µl of an aqueous solution of propylenediamine at a concentration of 0.05%, 1 µl of the cDNA as a template (corresponding to 50 ng of the RNA) and sterile water was prepared. The mixture was heat-denatured at 98°C for 2 minutes, cooled to 55°C and incubated at the temperature for 1 minute in a thermal cycler to anneal the primers to the template.

After annealing, 40 µl of a mixture containing 0.625 mM each of dNTPs, 42.5 mM Tricine-potassium hydroxide buffer (pH 8.5), 5.0 mM magnesium acetate, 0.0125% BSA, 1.25% DMSO, 15 U of E. coli RNase H and 11 U of BcaBEST DNA polymerase was added to the mixture to make the final volume to 50 µl with sterile water. The reaction mixtures were incubated at 55°C for 1 hour in a thermal cycler. The reacted samples were stored by freezing at -20°C until analysis. The PCR as a control was carried out as follows. Briefly, 50 µl of a reaction mixture containing 50 pmol each of the primers, 1 µl of the cDNA (corresponding to 50 ng of the RNA), 5 µl of 10 x Ex Taq buffer (Takara Shuzo), 1.25 U of TaKaRa Ex Taq DNA polymerase (Takara Shuzo) and 0.2 mM each of dNTPs was reacted in a thermal cycler. The program was as follows: 1 cycle of 94°C for 2 minutes; 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds; and 1 cycle of 72°C for 5 minutes. The reacted samples were stored by freezing at -20°C until analysis. After reaction, 5 µl each of the reaction mixtures was subjected to electrophoresis on 3.0% agarose gel. The results are shown in Figure 10.

Figure 10 illustrates the results of electrophoresis which shows the comparison between the RT-ICAN (A) and the RT-PCR (B). Lane 1: molecular weight marker (100 bp ladder); lane 2: negative control group; and

lane 3: a group treated with LPS and IFN-γ.

As shown in Figure 10, the amplification products were observed for both of the method of the present invention and the PCR only when the cDNA prepared from cells treated with LPS and IFN-γ was used as a template. Thus, it was confirmed that, since the method of the present invention required a shorter period of time for reaction as compared with the PCR, the method of the present invention was more effective as a DNA amplification method after reverse transcription.

Example 11

E. coli RNase H of which the optimal temperature is 37°C may become inactivated during amplification reaction of the present invention. Then, the effect of addition of E. coli RNase H to the reaction mixture during the amplification reaction was examined. An amplified fragment (1071 bp) obtained by a PCR using primers GMO-PCR-F and GMO-PCR-R represented by SEQ ID NOS:78 and 79 from a genomic DNA extracted from recombinant soybeans into which cauliflower mosaic virus 35S promoter and EPSPS gene had been introduced was used as a template DNA. In addition, primers GMO-S1, S2, A1 and A2 having nucleotide sequences represented by SEQ ID NOS:80 to 83 were used. The reaction was carried out as follows. Briefly, 10 μl of a mixture containing 50 pmol each of the primers, propylenediamine at

a final concentration of 0.01%, 1 pg to 10 ng of the DNA as the template and sterile water was prepared. The mixture was heat-denatured at 98°C for 2 minutes and cooled to 55°C for annealing.

After annealing, at final concentrations, 500 μM each of dNTPs, 34 mM Tricine-potassium hydroxide buffer (pH 8.7), 4.0 mM magnesium acetate, 0.01% BSA, 1% DMSO, 30 U of E. coli RNase H and 5.5 U of BcaBEST DNA polymerase were added to the mixture to make the final volume to 50 μl with sterile water. The reaction mixtures were incubated at 55°C for 25 minutes in a thermal cycler. 30 U of E. coli RNase H was further added thereto 25 minutes after the initiation of the reaction. The mixture was incubated at 55°C for 30 minutes. As a control, a reaction was carried out by incubating the mixture at 55°C for 55 minutes. After reaction, 3 μl each of the reaction mixtures was subjected to electrophoresis on 3% agarose. As a result, it was confirmed that the amplification efficiency was improved by the addition of E. coli RNase H during the reaction regardless of the concentration of the template DNA for either of the combinations of the primers, S1/A1, S1/A2, S2/A1 and S2/A2.

Example 12

The combination of a method for amplifying or duplicating a nucleic acid to be used as a template in the

present invention and the method of the present invention was examined. The reaction was carried out as follows. Briefly, in vitro transcription was carried out using a plasmid containing the chrysanthemum viroid gene as prepared in Example 5(3) which was duplicated in Escherichia coli as a template and T7 RNA polymerase (Takara Shuzo) to obtain a fragment duplicated from an RNA. A cDNA was synthesized using primers having nucleotide sequences represented by SEQ ID NOS:57 and 58 and cDNA synthesis kit (Takara Shuzo). An amplification reaction was carried out as described in Example 5(3) using the cDNA fragment or the duplicated plasmid as a template. As a result, it was confirmed that both a nucleic acid duplicated in a form of a plasmid and a nucleic acid in a form of a cDNA duplicated from an RNA using an RNA polymerase can be used as templates for the method of the present invention.

Example 13

(1) Synthesis of primer

Oligonucleotide primers NS1 and NS2 represented by SEQ ID NOS:86 and 87 were synthesized on the basis of the nucleotide sequence of the mRNA for mouse inducible NO synthase (iNOS).

(2) Amplification of DNA fragment according to ICAN using PCR product as template

10 µl of a mixture containing 50 pmol each of the synthetic oligonucleotide primers, 2 µl of a 0.05% aqueous solution of propylenediamine and 10 fg to 10 pg of the template was heated at 98°C for 2 minutes and then 60°C for 2 minutes using a thermal cycler (GeneAmp PCR System 9600, Applied Biosystems) to anneal the primers to the template. iNOS cDNA (741 bp) amplified using primers NS-PCR1 and NS-PCR2 represented by SEQ ID NOS:132 and 133, which was then purified using Suprec02 (Takara Shuzo), was used as the template. 40 µl of a reaction mixture containing 0.625 mM each of dNTPs, 40 mM HEPES-potassium hydroxide buffer (pH 7.8), 125 mM potassium acetate, 5.0 mM magnesium acetate, 0.0125% BSA, 1.25% DMSO, 0.0156 µg of Pfu RNase H and 0.66 U of BcaBEST DNA polymerase was added to the heated solution. The mixtures were at incubated at 60°C for 1 hour in a thermal cycler. 5 µl each of the reaction mixtures was analyzed by electrophoresis on 3.0% agarose gel. The results are shown in Figure 11. Figure 11 represents the results of the ICAN using Pfu RNase H. Lane 1: molecular weight marker (100 bp); lane 2: 10 fg of the template; lane 3: 100 fg of the template; lane 4: 1 pg of the template; and lane 5: 10 pg of the template.

As shown in Figure 11, the amplification product of interest was observed using 100 fg of the template.

Example 14