

(1) Preparation of RNA

RAW264.7 cells (ATCC TIB 71) were suspended in Dulbecco's modified Eagle's medium (Bio Whittaker) 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/ml 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, 74104) according to the instructions attached to the kit. As a 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, 60 U of Ribonuclease Inhibitor (Takara Shuzo) and 15 U of Reverse Transcriptase XL (AMV) (Takara Shuzo) 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 NS5 and NS6 having nucleotide sequences



represented by SEQ ID NOS:92 and 93 were synthesized based on the nucleotide sequence of the mRNA for mouse inducible NO synthase (iNOS). Furthermore, primers NS3 and NS4 for PCR represented by SEQ ID NOS:88 and 89 were also synthesized.

50 µl of a mixture containing 50 pmol each of the primers NS5 and NS6, 1 µl of the cDNA solution synthesized as described above (corresponding to 50 ng of the RNA) or a 10-, 100-, 1000- or 10000-fold dilution with water thereof as a template, 0.5 mM each of dNTPs, 32 mM HEPES-potassium hydroxide buffer (pH 7.8), 100 mM potassium acetate, 4.0 mM magnesium acetate, 0.01% BSA, 1% DMSO, 0.0156 µg of Pfu RNase H and 0.66 U of BcaBEST DNA polymerase was incubated at 60°C for 1 hour in a thermal cycler. The reacted samples were stored by freezing at -20°C until analysis.

On the other hand, a PCR was carried out as a control. 50 µl of a reaction system containing 50 pmol each of the primers NS3 and NS4, 1 µl of the cDNA solution (corresponding to 50 ng of the RNA) or a 10-, 100-, 1000- or 10000-fold dilution with water thereof, 5 µl of 10 x Ex Taq buffer (Takara Shuzo), 1.25 U of TaKaRa Ex Taq polymerase (Takara Shuzo) and 0.2 mM each of dNTPs was reacted using a thermal cycler. The program was as follows: 1 cycle of 94°C for 2 minutes; 35 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.

5 µl each of the reaction mixtures (ICAN or PCR) was analyzed by electrophoresis on 3.0% agarose gel. The results are shown in Figure 12. Figure 12 shows the results of the detection of iNOS gene according to the ICAN using Pfu RNase H or the PCR. Lane 1: a lane for 100 bp DNA ladder marker; lane 2: the 10000-fold dilution of the negative control cDNA; lane 3: the 1000-fold dilution of the negative control cDNA; lane 4: the 100-fold dilution of the negative control cDNA; lane 5: the 10-fold dilution of the negative control cDNA; lane 6: the original solution of the negative control cDNA; lane 7: the 10000-fold dilution of the cDNA from the group with the addition of LPS and IFN-γ; lane 8: the 1000-fold dilution of the cDNA from the group with the addition of LPS and IFN-γ; lane 9: the 100-fold dilution of the cDNA from the group with the addition of LPS and IFN-γ; lane 10: the 10-fold dilution of the cDNA from the group with the addition of LPS and IFN-γ; and lane 11: the original solution of the cDNA from the group with the addition of LPS and IFN-γ.

As shown in Figure 12, the amplification products were observed for both of the ICAN and the PCR only when the cDNA prepared from cells treated with LPS and IFN-γ was used as a template. For the ICAN, the increase in

amplification product was observed using the 1000-fold dilution of the cDNA. For the PCR, the increase in amplification product was observed using the 100-fold dilution of the cDNA.

Example 15

(1) Oligonucleotide primers 4 and 5 represented by SEQ ID NOS:90 and 91 were synthesized based on the nucleotide sequence of λ DNA. The oligonucleotide primer 4 is a sense primer having 75% of a GC content. The oligonucleotide primer 5 is an antisense primer having 80% of a GC content.

10 µl of a reaction system containing 120 pmol each of the primers 4 and 5, 2 µl of a 0.05% propylenediamine solution and 10 ng of a template was heat-denatured at 98°C for 2 minutes, and then rapidly cooled on ice to anneal the primers to the template. The PCR product (1005 bp) purified using Suprec02 as described in Example 2 was used as the template.

(2) After annealing, 40 µl of a mixture containing 0.625 mM each of dNTPs, 42.5 mM Bicine-potassium hydroxide buffer (pH 8.3), 5.0 mM magnesium acetate, 0.0125% BSA, 1.25% DMSO, 0.5 µl of Thermotoga maritima RNase HII (0.58 µg/ml) and 2.2 U of BcaBEST DNA polymerase was added to the mixture to carry out the ICAN at 60, 65 or 70°C for 1 hour. 3 µl each of the reaction mixtures after

the ICAN was confirmed by electrophoresis on 3.0% agarose gel. The results are shown in Figure 13. Figure 13 shows the results of the ICAN using Thermotoga maritima RNase HII. Lane 1: molecular weight marker (100 bp); lane 2: reaction temperature of 60°C; lane 3: reaction temperature of 65°C; and lane 4: reaction temperature of 70°C.

As shown in Figure 13, the amplification products of interest were observed for the respective reaction temperatures.

Example 16

(1) Amplification of a DNA fragment according to the ICAN using a PCR product as a template (alkali-denatured) was examined. 1 µl of a solution containing 10 fg to 10 pg of a template and 1 µl of 0.4 N NaOH were mixed together. The mixture was incubated at 37°C for 5 minutes to denature the template. The PCR-amplified iNOS cDNA (741 bp) purified using Suprec02 (Takara Shuzo) as described in Example 13 was used as a template. Each of the denatured templates was neutralized using 1 µl of 0.4 N HCl. 47 µl of a reaction mixture containing 50 pmol each of the primers NS1 and NS2, 0.5 mM each of dNTPs, 32 mM HEPES-potassium hydroxide buffer (pH 7.8), 100 mM potassium acetate, 4.0 mM magnesium acetate, 0.01% BSA, 1.0% DMSO, 0.0156 µg of Pfu RNase H and 0.66 U of BcaBEST DNA polymerase was then added thereto. The mixture was

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 14. Figure 14 shows the results of the ICAN using an alkali-denatured template. 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 14, the amplification product was clearly increased using 1 pg of the template.

Example 17

(1) Amplification of a DNA fragment according to the ICAN without denaturing a template was examined. Primers NS5 and NS6 represented by SEQ ID NOS:92 and 93 were used as primers. The template as prepared in Example 13 was used as a template DNA.

50 µl of a reaction mixture containing 10 fg to 100 pg of the template or water for a negative control, 50 pmol each of the primers NS5 and NS6, 0.5 mM each of dNTPs, 32 mM HEPES-potassium hydroxide buffer (pH 7.8), 100 mM potassium acetate, 4.0 mM magnesium acetate, 0.01% BSA, 1.0% DMSO, 0.0156 µg of Pfu RNase H and 1 U of BcaBEST DNA polymerase (Takara Shuzo) was incubated at 60°C for 1 hour in a thermal cycler. After reaction, 5 µl each of the reaction mixture was analyzed by electrophoresis on 3.0%

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agarose gel. Results of the electrophoresis are shown in Figure 15. Figure 15 illustrates the results of electrophoresis for the amplification method of the present invention without denaturing a template DNA. Lane 1: 100 bp DNA ladder marker; lane 2: negative control (water); lane 3: 10 fg of the template; lane 4: 100 fg of the template; lane 5: 1 pg of the template; lane 6: 10 pg of the template; and lane 7: 100 pg of the template.

As shown in Figure 15, the amplification product of interest was observed using 1 pg of the template.

Example 18

(1) Primers pDON-AI-1 and pDON-AI-2 represented by SEQ ID NOS:94 and 95 were synthesized based the nucleotide sequence of the packaging region in a vector plasmid pDON-AI DNA (Takara Shuzo).

(2) Amplification of DNA fragment according to ICAN without denaturing template

50 µl of a reaction mixture containing 1 µl of a solution containing 10 fg to 1 ng of pDON-AI DNA or water for a negative control, 50 pmol each of the primers, 0.5 mM each of dNTPs, 32 mM HEPES-potassium hydroxide buffer (pH 7.8), 100 mM potassium acetate, 4.0 mM magnesium acetate, 0.01% BSA, 1.0% DMSO, 0.0156 µg of Pfu RNase H as prepared in Referential Example 4 and 1 U of BcaBEST DNA polymerase was 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 16. Figure 16 illustrates the results of electrophoresis for the method of the present invention using a circular double-stranded DNA as a template without denaturation. Lane 1: 100 bp DNA ladder marker; lane 2: negative control (water); lane 3: 10 fg of the template; lane 4: 100 fg of the template; lane 5: 1 pg of the template; lane 6: 10 pg of the template; lane 7: 100 pg of the template; and lane 8: 1 ng of the template.

As shown in Figure 16, it was confirmed that the amplification product of interest was obtained using 10 fg of the template.

Example 19

Detection of human papilloma virus 16 gene utilizing the method of the present invention was examined. A DNA from cells infected with human papilloma virus 16, CaSki cells (Dainippon Pharmaceutical; containing 500 copies of human papilloma virus 16 in a cell), was used as a template. Primers HPV16 S3 and HPV16 A2 having nucleotide sequences represented by SEQ ID NOS:96 and 97 were used as primers for detecting HPV16. The expected size of the amplification product obtained using the primer pair was about 120 bp. The reaction was carried out as follows.

10 µl of a mixture containing 1 pg, 3 pg, 30 pg, 100 pg, 300 pg, 1 ng, 3 ng or 10 ng of the template DNA, 50 pmol each of the primers HPV16 S3 and HPV16 A2 and propylenediamine at a final concentration of 0.01% was prepared. The mixtures were incubated at 98°C for 2 minutes and at 55°C for 1 minute in Thermal Cycler Personal, and then placed on ice. 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. The reaction mixtures were placed in a thermal cycler which had been set at 55°C and reacted for 60 minutes. As a control, a PCR was carried out using Human Papillomavirus Primers HPVp16 (forward, reverse) (Takara Shuzo) according to the manual in Thermal Cycler Personal. The expected size of the amplification product was 140 bp.

After reaction, 3 µl each of the reaction mixtures was subjected to electrophoresis on 4% NuSieve 3:1 agarose. The results are shown in Figure 17A. Figure 17A shows the results of the detection of HPV16 gene utilizing the ICAN and the PCR. Lane M1: molecular weight marker (100 bp ladder); lane M2: molecular weight marker (50-2000 bp); lane 1: no template; lane 2: 1 pg of the template;

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lane 3: 3 pg of the template; lane 4: 30 pg of the template; lane 5: 100 pg of the template; lane 6: 300 pg of the template; lane 7: 1 ng of the template; lane 8: 3 ng of the template; and lane 9: 10 ng of the template.

As shown in Figure 17A, it was confirmed that the amplification products of interest were obtained using 3 pg of the template DNA for the ICAN and 1 pg of the template DNA for the PCR, respectively.

Furthermore, dot blot hybridization for the reaction products was carried out using an oligonucleotide HPV16 probe having a nucleotide sequence represented by SEQ ID NO:98. Hybridization was carried out as described in Example 9. The results are shown in Figure 17B. Figure 17B shows the results of dot blot hybridization detection of HPV16 gene according to the PCR and the ICAN. Lane 1: no template; lane 2: 1 pg of the template; lane 3: 3 pg of the template; lane 4: 30 pg of the template; lane 5: 100 pg of the template; lane 6: 300 pg of the template; lane 7: 1 ng of the template; lane 8: 3 ng of the template; and lane 9: 10 ng of the template.

As shown in Figure 17B, the detection sensitivities of the ICAN and the PCR were almost equivalent. Thus, it was confirmed that these methods were effective for detecting a virus or the like.

Example 20

Detection of human papilloma virus 16 gene from a clinical specimen DNA sample was examined. DNAs prepared according to a conventional method from 6 clinical specimens obtained with informed consent were used as templates. The types of the infecting HPVs in the samples prepared from these clinical specimens had been proved by the PCR. The primers HPV16 S3 and HPV16 A2 as described in Example 19 were used as primers for detection. The concentrations of the DNA samples from the clinical specimens to be used as templates were adjusted to 100 ng/μl with TE buffer. The composition of the reaction mixture and the reaction conditions as described in Example 19 were used except for the amount of the template. In addition, similar reactions were carried out using a reaction mixture without the addition of the template DNA as a negative control and a reaction mixture containing 500 pg of the DNA from CaSki cells infected with HPV 16 as a positive control. After reaction, 3 μl each of the reaction mixtures was subjected to electrophoresis on 4% NuSieve 3:1 agarose. The results are shown in Figure 18A. Figure 18A shows the results of the detection of HPV16 gene from clinical specimens. Lane M: molecular weight marker; lanes 1 to 6: clinical specimens; lane 7: negative control; and lane 8: positive control.

As shown in Figure 18A, the amplification

products of about 120 bp were observed according to the ICAN for the samples which had been proved to be infected with HPV16 by the conventional PCR. No amplification was observed for samples infected with other types of HPVs or non-infected samples.

Furthermore, dot blot hybridization for the amplification products was carried out as described in Example 9. The results are shown in Figure 18B and Table 8. Figure 18B shows the results of dot blot hybridization detection of HPV16 gene from the clinical specimens. Lanes 1 to 6: clinical specimens; lane 7: negative control; and lane 8: positive control.

As shown in Figure 18, results consistent with those obtained by the electrophoresis were obtained, confirming that similar results with those of the PCR could be obtained by using electrophoresis as well as dot blot hybridization. Thus, it was confirmed that HPV16 could be detected from practical clinical specimens according to the method of the present invention and that the method was effective for detecting a virus or the like.

Table 8

Sample	Typing by PCR	ICAN amplification using HPV16 detection primer
No.3	Non-infected	-
No.4	Non-infected	-
No.6	Type 18	-
No.7	Type 16	+
No.8	Type 67	-
No.9	Type 16	+
No template	*	-
Positive control	*	+

-: no amplification; +: amplification observed.

Example 21

Detection of HCV from clinical specimens was examined. Specimen samples were prepared from 300 µl each of 5 serum specimens from patients with HCV obtained with informed consent using TRIzol reagent (Life Technologies) according to the instructions attached to the reagent and finally dissolved in 6 µl of injectable water (Otsuka Pharmaceutical) to obtain RNA samples. An RNA similarly extracted from 300 µl of a serum from a healthy individual was used as a negative control. First, 4 µl of a reaction mixture for reverse transcription containing 1 x RNA PCR Buffer, 5 mM $MgCl_2$, 1 mM dNTPs, 1 U of AMV Reverse Transcriptase XL, 10 pmol each of primers HCV-F and HCV-R represented by SEQ ID NOS:99 and 100, and 2 µl of one the RNA samples was prepared using RNA PCR kit (AMW) ver 2.1 (Takara Shuzo). The mixtures were warmed at 30°C for 10

minutes and then reacted at 50°C for 30 minutes. After reverse transcription, the ICAN was carried out. Primers HCV-F2 and HCV-R1 having nucleotide sequences represented by SEQ ID NOS:101 and 102 were used for the ICAN. The reaction was carried as follows.

10 µl of a mixture containing 50 pmol each of the primers, 3 µl one of the reverse transcription reaction mixtures and propylenediamine at a final concentration of 0.01% was prepared. 3 µl of sterile water was used for a blank. The mixtures were heated at 98°C for 2 minutes, rapidly cooled to 60°C and incubated at the temperature for 1 minute in Thermal Cycler Personal, and stored 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 MP 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. The results are shown in Figure 19A. Figure 19A shows the results of HCV detection from clinical specimens. Lane B: sterile water as a template; lane 1: a sample from a healthy individual; lanes 2 to 6:

samples from patients with HCV; and lane M: molecular weight marker (50-2000 bp).

As shown in Figure 19A, the amplification products of about 107 bp expected from the nucleotide sequence of HCV genome were observed only for the RNA samples from patients with HCV, whereas such an amplification product was not observed for the serum from the healthy individual and the blank. Furthermore, dot blot hybridization for the ICAN-amplified products was carried out as described in Example 9 using a probe for HCV represented by SEQ ID NO:103 biotinylated at 5'-terminus. The results are shown in Figure 19B. The lanes for the samples in Figure 19B are as those indicated for the electrophoresis.

As shown in Figure 19, it was confirmed that the results from the electrophoresis were consistent with those from the dot blot hybridization. These results confirms that the method of the present invention can be used to detect HCV from practical clinical specimens and is effective for detecting a virus or the like.

Example 22

A method for detecting adenovirus was examined.

Primers for amplifying E1A (tumor gene), E1A-1 (sense), E1A-2 (antisense) and E1A-3 (antisense), represented by SEQ ID NOS:104 to 106 were constructed based

on the nucleotide sequence of adenovirus (GenBank accession no. J01917). Adenovirus (ATCC accession no. VR-5) was used. A template was prepared as follows. 100 µl of a solution containing adenovirus at a concentration of 8.73×10^{10} PFU/ml was incubated in the presence of SDS at a final concentration of 0.1% and proteinase K at a final concentration of 0.2 mg/ml at 37°C for 1 hour. The DNA was purified by adsorption to silica gel. Adenoviral DNAs corresponding to 10^3, 10^4, 10^5 or 10^6 PFU prepared by diluting the purified DNA with sterile water were used. The reaction was carried out as follows. Briefly, 10 µl of a reaction system containing 60 pmol each of the primers E1A-1 and E1A-2 (chain length to be amplified: 112 bp) or E1A-1 and E1A-3 (chain length to be amplified: 91 bp), 2 µl of 0.05% propylenediamine and the template was heat-denature 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 5.5 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. As a control, a detection by a PCR was carried out using the same template as the above and

primers constructed for PCR-amplifying E1A (tumor gene), E1A-1P (sense), E1A-2P (antisense) and E1A-3P (antisense) having nucleotide sequences represented by SEQ ID NOS:107, 108 and 142. The PCR was carried out as follows. Briefly, 50 µl of a PCR solution containing 60 pmol each of the primers E1A-1P and E1A-2P (chain length to be amplified: 112 bp) or E1A-1P and E1A-3P (chain length to be amplified: 91 bp), 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 conditions for the PCR were as follows: 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds.

After reaction, 3 µl each of the reaction mixtures of the ICAN and the PCR was subjected to electrophoresis on 3.0% agarose gel. The results are shown in Figure 20 and Table 9. Figure 20 shows the results of the detection of viral E1A gene from adenoviral particles. Lanes 1 to 10 show the results obtained using the combination of the primers E1A-1 and E1A-2. Lanes 11 to 20 show the results obtained using the combination of the primers E1A-1 and E1A-3. Lane 1: molecular weight marker (100 bp ladder); lane 2: ICAN using DNA corresponding to 10^6 PFU; lane 3: ICAN using DNA corresponding to 10^5 PFU; lane 4: ICAN using DNA corresponding to 10^4 PFU; lane 5: ICAN using DNA corresponding to 10^3 PFU; lane 6: molecular

weight marker (100 bp ladder); lane 7: PCR using DNA corresponding to 10^6 PFU; lane 8: PCR using DNA corresponding to 10^5 PFU; lane 9: PCR using DNA corresponding to 10^4 PFU; and lane 10: PCR using DNA corresponding to 10^3 PFU. In addition, lane 11: molecular weight marker (100 bp ladder); lane 12: ICAN using DNA corresponding to 10^6 PFU; lane 13: ICAN using DNA corresponding to 10^5 PFU; lane 14: ICAN using DNA corresponding to 10^4 PFU; lane 15: ICAN using DNA corresponding to 10^3 PFU; lane 16: molecular weight marker (100 bp ladder); lane 17: PCR using DNA corresponding to 10^6 PFU; lane 18: PCR using DNA corresponding to 10^5 PFU; lane 19: PCR using DNA corresponding to 10^4 PFU; and lane 20: PCR using DNA corresponding to 10^3 PFU.

Table 9

Amplification size (bp)	Detection limit	
	ICAN	PCR
112	10^4	10^4
91	10^4	10^4

As shown in Figure 20 and Table 9, it was confirmed that the detection sensitivity for the detection of adenovirus E1A gene by the ICAN was equivalent to that by the PCR.

Example 23

Detection of an integrated viral gene from cells infected with a retrovirus vector was examined. Cells infected with a retrovirus and a genomic DNA were prepared as follows. Briefly, a vector plasmid pDON-AI (Takara Shuzo) was introduced into packaging cells GPE+86 according to the calcium phosphate method. An ecotropic vector was prepared from the culture supernatant of the introduced cells. Cells infected with a virus vector were prepared by infecting NIH/3T3 cells with the ecotropic vector and culturing the infected cells for 14 days in a medium containing G418. 27 µg of a genomic DNA was obtained according to a conventional method from 4 x 10^4 of the prepared cells infected with the retrovirus. The primers pDON-AI-1 and pDON-AI-2 as described in Example 18(1) were used as primers. The reaction was carried out as follows. Briefly, 10 µl of a reaction system containing 60 pmol each of the primers, 2 µl of a 0.25% aqueous solution of propylenediamine and 0.1 ng to 1000 ng of the genomic DNA as a template was heated at 98°C for 2 minutes and then at 60°C in a thermal cycler (Takara Shuzo) to anneal the primers to the template.

After annealing, 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 mM magnesium acetate, 0.0125% BSA, 1.25% DMSO, 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. The reaction mixtures were incubated at 60°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. In addition, a PCR was carried out using primers pDON-AI-3 and pDON-AI-4 represented by SEQ ID NOS:111 and 112 in order to compare the sensitivities of detecting a DNA by the ICAN and the PCR. The PCR was carried out as follows. 50 µl of a reaction mixture containing 0.1 ng to 100 ng of the template, 60 pmol each of the primers, 5 µl of 10 x Ex Taq buffer, 1.25 U of TaKaRa Ex Taq polymerase and 0.2 mM each of dNTPs was prepared. The mixtures were subjected to reactions using Thermal Cycler Personal as follows: 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds. After reaction, 5 µl each of the reaction mixtures was subjected to electrophoresis on 3.0% agarose gel. The results are shown in Figure 21. Figure 21 shows the results of the detection of an integrated viral gene from cells infected with a retrovirus vector according to the ICAN and the PCR. Lane 1: molecular weight marker (100 bp ladder); lane 2: 1000 ng of the template; lane 3: 100 ng of the template; lane 4: 10 ng of the template; lane 5: 1 ng of the template; and lane 6: 0.1 ng of the template.

As shown in Figure 21, the amplification products of interest were observed for the ICAN using 1 ng of the template DNA and for the PCR of 35 cycles using 1 ng of the template.

Example 24

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 for the detection of Escherichia coli O-157 vero toxin I gene. Vero toxin I gene from enterohemorrhagic Escherichia coli O-157 was selected as a target. The template DNA was prepared as described in Example 8(1). A region of about 80 bp having a GC content of about 40% was selected as a region to be amplified. Primers VT1-IF4 and VT1-IR1 having nucleotide sequences represented by SEQ ID NOS:113 and 114 were used as primers. The reaction was carried out as follows. Briefly, 5 µl of a mixture containing 60 pmol each of the primers VT1-IF4 and VT1-IR1, propylenediamine at a final concentration of 0.01%, one of hot water-extracts corresponding to 0 to 10^5 cells and sterile water was prepared. The mixtures were heat-denatured at 98°C for 2 minutes, rapidly cooled to 55°C and incubated at the temperature for 1 minute in Thermal Cycler Personal, 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, 15 U of E. coli RNase H and 2.75 U of BcaBEST DNA polymerase were added to the mixture to make the final volume to 25 µl with sterile water. The reaction mixtures were placed in Thermal Cycler Personal which had been set at 55°C and incubated at the temperature for 60 minutes. As a control, a PCR for the hot water-extract was carried out using O-157 Typing Set (Takara Shuzo) according to the manual in 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. The total time required for the reaction was about 145 minutes. The expected size of the amplification product was 349 bp. After reaction, 3 µl each of the reaction mixtures was subjected to electrophoresis on 3% NuSieve 3:1 agarose. The results for the ICAN are shown in Figure 22. Figure 22 shows the results of the detection of O-157 vero toxin I gene. Lane M: molecular weight marker (50-2000 bp); lane N: sterile water as template; 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; and lane 4: the template corresponding to 10^3 cells. Furthermore, the results of the detection by the ICAN and the PCR are shown in Table 10.

Table 10

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

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

As shown in Table 10, the amplification products of interest were obtained for the reaction systems in which the hot water-extract corresponding to 1 cell was used for both of the ICAN and the PCR. Furthermore, dot blot hybridization for the amplification products was carried out using an oligonucleotide probe VT1 having a nucleotide sequence represented by SEQ ID NO:115 labeled with biotin at the 5'-terminus. The hybridization was carried out as described in Example 19. The results were consistent with those obtained by the electrophoresis. Thus, it was confirmed that the detection sensitivities of the ICAN and the PCR were equivalent. In addition, 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 pathogenic bacterium and the like.

Example 25

A method for detecting a gene for botulinum toxin type A was examined. A DNA prepared from Clostridium botulinum, a strain from a food poisoning case, type A-190 was used as a template. This strain is preserved in Department of Hygiene, Kagawa Nutrition University. Primers BotA S2 and BotA A2 having nucleotide sequences represented by SEQ IOD NOS:116 and 117 were synthesized as primers for detection. The size of the amplification product obtained using the primer pair was about 150 bp. Solutions containing 100 fg, 1 pg, 10 pg or 100 pg of the DNA from type A toxin-producing Clostridium botulinum in 1 µl of sterile water to be used as templates were prepared. The reaction was carried out as follows.

10 µl of a mixture containing 50 pmol each of the primers, propylenediamine at a final concentration of 0.01% and 1 µl of one of the DNA solutions as a template was prepared. The mixtures were subjected to the ICAN using the composition of the reaction mixture and the reaction conditions as described in Example 19. As a control, a PCR was carried out in using a primer set for detecting botulinum toxin type A gene, BAS-1 and BAS-2 (Takara Shuzo), according to the manual in Thermal Cycler Personal. The

expected size of the amplification product was 284 bp.

After reaction, 3 µl each of the reaction mixtures was subjected to electrophoresis on 4% NuSieve 3:1 agarose. The results are shown in Figure 23A. Figure 23A shows the results of the detection of the gene for botulinum toxin type A according to the ICAN and the PCR. Lane M1: molecular weight marker (100 bp ladder); lane M2: molecular weight marker (50-2000 bp marker); lane 1: no template; lane 2: 100 fg of the template; lane 3: 10 pg of the template; and lane 4: 100 pg of the template.

As shown in Figure 23A, the amplification product of interest was observed for the reaction in which 100 fg of the template DNA was used according to the ICAN. On the other hand, the amplification product of interest was not observed for the reaction in which 100 fg of the template DNA was used according to the PCR. Furthermore, dot blot hybridization for the reaction products was carried out using BotA probe having a nucleotide sequence represented by SEQ ID NO:118. Dot blot hybridization was carried out as described in Example 9. The results are shown in Figure 23B. As shown in Figure 23B, the signals were observed using 100 fg of the template for the ICAN and 10 pg of the template for the PCR, respectively. These results were consistent with those of the electrophoresis.

Example 26

Detection of chrysanthemum viroid was examined. 10-fold serial dilutions of low molecular weight RNA obtained according to the method for extracting low molecular weight RNAs from chrysanthemum infected with chrysanthemum stunt viroid (CSVd) as described in Example 1 of JP-A 9-140383 were prepared. A reverse transcription reaction was carried out using RNA PCR kit (AMW) ver 2.1 (Takara Shuzo). Specifically, 20 µl of a reaction mixture for reverse transcription containing 1 x RNA PCR Buffer, 5 mM $MgCl_2$, 1 mM each of dNTPs, 20 U of RNase Inhibitor, 5 U of AMV Reverse Transcriptase XL, 50 pmol of Random 9mers, 1 µl of one of the serial dilutions of the RNA was prepared. The mixtures were warmed at 30°C for 10 minutes, reacted at 55°C for 30 minutes and then heated at 99°C for 5 minutes for inactivating the reverse transcriptase. After cooling, the ICAN was carried out. 1 µl of the reverse transcription reaction mixture was used as a template for 50 µl of a reaction mixture for the ICAN. Primers CSVD-F4 and CSVD-R3 having nucleotide sequences represented by SEQ ID NOS:119 and 120 were used as primers in this Example. The reaction was carried out as described in Example 19 except that reaction temperature was 60°C and that Thermal Cycler MP was used. After reaction, 3 µl each of the reaction mixtures was subjected to electrophoresis on 3% NuSieve 3:1 agarose.

On the other hand, PCR amplification was carried out in a reaction system of 50 µl using 1 µl of the same reverse transcription reaction mixture as a template. Primers F94 and R264 represented by SEQ ID NOS:109 and 110 were used as primers. The reaction was carried out as follows. Briefly, a reaction mixture was prepared using TaKaRa PCR Amplification kit according to the protocol. 10 pmol each of the primers and 1 µl of one of the reverse transcription reaction mixture were added thereto to make the total volume to 50 µl. An amplification reaction was carried out using Thermal Cycler MP. The reaction was carried out as follows: 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds. After reaction, 5 µl each of the reaction mixtures was subjected to electrophoresis on 3% NuSieve 3:1 agarose. The results are shown in Table 11.

Table 11

Dilution rate of RNA as template	x 10^2	x 10^3
RT-ICAN	++	+
RT-PCR	+	-

-: not amplified; +: amplified; ++: amplified well.

As shown in Table 11, the amplification product was observed for the reaction in which the 10^3-fold-diluted

RNA sample was used as a template according to the ICAN. On the other hand, the amplification product was observed for the reaction in which the 10^2-fold-diluted RNA sample was used as a template according to the PCR.

Furthermore, the ICAN-amplified products and the PCR-amplified products were confirmed to be the products of interest by dot blot hybridization. Dot blot hybridization was carried out using CSVd probe having a nucleotide sequence represented by SEQ ID NO:121 labeled with biotin at the 5'-terminus. Hybridization was carried out as described in Example 9. The results were consistent with those of the electrophoresis. The signals were observed using the 10^3-fold-diluted RNA sample for the ICAN and the 10^2-fold-diluted RNA sample for the PCR, respectively, demonstrating that the ICAN was more sensitive than the PCR.

Example 27

Detection of a viroid gene from chrysanthemum infected with chrysanthemum dwarfing viroid (CSVd) using Pfu RNase H was examined. 60 µl of a mixture containing 3 µl of one of the 10-fold serial dilutions of the RNA as prepared in Example 26, 10 mM tris-hydrochloride buffer (pH 8.3), 50 mM potassium chloride, 5 mM magnesium chloride, 1 mM each of dNTPs, 150 pmol of Random 6mers, 60 U of Ribonuclease Inhibitor (Takara Shuzo) and 15 U of Reverse Transcriptase XL (AMV) (Takara Shuzo) was incubated at 30°C

for 10 minutes and at 42°C for 1 hour and then heated at 99°C for 5 minutes for inactivating the enzyme using a thermal cycler (GeneAmp PCR System 9600, Applied Biosystems) to prepare a cDNA. Primers Vd1, Vd2, Vd3 and Vd4 represented by SEQ ID NOS:122 to 125 were synthesized based on the nucleotide sequence of the mRNA for the viroid.

50 µl of a mixture containing 50 pmol each of the primers Vd1 and Vd2, 1 µl of the cDNA solution synthesized as described above, a 10-, 100-, 1000- or 10000-fold dilution thereof with water or water for a negative control as a template, 0.5 mM each of dNTPs, 32 mM HEPES-potassium hydroxide buffer (pH 7.8), 100 mM potassium acetate, 4.0 mM magnesium acetate, 0.01% BSA, 1% DMSO, 0.0156 µg of Pfu RNase H and 1 U of BcaBEST DNA polymerase was incubated at 57°C for 1 hour in a thermal cycler. The reacted samples were stored by freezing at -20°C until analysis.

A PCR was carried out as a control. Briefly, 50 µl of a reaction system containing 50 pmol each of the primers Vd3 and Vd4, 1 µl of the cDNA solution, a 10-, 100-, 1000- or 10000-fold dilution thereof with water or water for a negative control, 5 µl of 10 x Ex Taq buffer, 1.25 U of TaKaRa Ex Taq polymerase and 0.2 mM each of dNTPs was subjected to a reaction in a thermal cycler. The program was as follows: 1 cycle of 94°C for 2 minute; 35 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.

5 µl each of the reaction mixtures of the ICAN and the PCR was subjected to electrophoresis on 3.0% agarose gel. The results are shown in Figure 24. Figure 24 shows the results of the detection of a viroid according to the ICAN using Pfu RNase H and the PCR. Lane 1: 100 bp DNA ladder marker; lane 2: negative control; lane 3: 10000-fold dilution of the cDNA; lane 4: 1000-fold dilution of the cDNA; lane 5: 100-fold dilution of the cDNA; lane 6: 10-fold dilution of the cDNA; and lane 7: the original solution of the cDNA.

As shown in Figure 24, the amplification products of interest were observed using the 100-fold dilution of the cDNA for both of the ICAN and the PCR.

Example 28

Detection of K-ras gene was examined.

(1) Detection from genomic DNA

Primers c-Ki-ras-1 and c-Ki-ras-2 represented by SEQ ID NOS:126 and 127 were constructed based on the nucleotide sequence of human c-Ki-ras.

10 µl of a mixture containing 60 pmol each of the primers, 2 µl of a 0.25% aqueous solution of propylenediamine and 1 ng to 100 ng of human genomic DNA (Clontech) as a template was prepared. The mixtures were

heated at 98°C for 2 minutes and then 53°C in Thermal Cycler Personal to anneal the primers to the template.

After annealing, 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 mM magnesium acetate, 0.0125% BSA, 1.25% DMSO, 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. The reaction mixtures were incubated at 53°C for 1 hour. After reaction, 5 µl each of the reaction mixtures was subjected to electrophoresis on 3.0% agarose gel.

On the other hand, a PCR was carried out as a control. Primers c-Ki-ras-3 and c-Ki-ras-4 represented by SEQ ID NOS:128 and 129 were used as primers. 50 µl of a solution containing 60 pmol each of the primers, 0.1 ng to 100 ng of the template, 5 µl of 10 x Ex Taq buffer, 1.25 U of TaKaRa Ex Taq polymerase and 0.2 mM each of dNTPs was prepared. The mixtures were subjected to reactions using Thermal Cycler Personal as follows: 30 or 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds. After reaction, 5 µl each of the reaction mixtures was subjected to electrophoresis on 3.0% agarose gel. The results are shown in Figure 25. Figure 25 shows the results of the detection of c-Ki-ras gene from human genomic DNA according to the ICAN and the PCR. For the

ICAN, lane 1: molecular weight marker; lane 2: 100 ng of the template; lane 3: 10 ng of the template; lane 4: 1 ng of the template; and lane 5: no template. For the PCR, lane 1: 100 ng of the template; lane 2: 10 ng of the template; lane 3: 1 ng of the template; and lane 4: no template.

As shown in Figure 25, the amplified products of interest were observed using 1 ng of the template for the ICAN and 10 ng of the template for the PCR of 30 cycles. The comparative results for the amounts of the amplified products obtained by the ICAN and the PCR using 1 ng to 100 ng of the template are shown in Figure 30. In the figure, the shaded bar represents the results for the PCR of 30 cycles, the dotted bar represents the results for the PCR of 35 cycles, and the closed bar represents the results for the ICAN, respectively. As shown in Figure 30, it was confirmed that the amount of the amplified product obtained by the ICAN was greater than that by the PCR.

(2) Detection from blood sample

Genomic DNAs were prepared using Dr. GenTLE™ (for Whole Blood) (Takara Shuzo) from 100 µl each of blood samples collected from a healthy individual using sodium citrate or heparin as an anticoagulating agent. Detection of c-Ki-ras gene by the ICAN was carried out as described in (1) above using the prepared DNA corresponding to 0.04

to 5 µl of the blood. Furthermore, detection using the DNA from 0.04 to 5 µl of the blood sample was carried out as described in (1) above in order to compare the sensitivities for detecting a DNA by the ICAN and the PCR. The results are shown in Figure 26. Figure 26 shows the results of the detection of c-Ki-ras gene from blood samples according to the ICAN and the PCR. For the ICAN, lane 1: molecular weight marker; lane 2: 5 µl of citrated blood; lane 3: 1 µl of citrated blood; lane 4: 0.2 µl of citrated blood; lane 5: 0.04 µl of citrated blood; lane 6: 5 µl of heparinized blood; lane 7: 1 µl of heparinized blood; lane 8: 0.2 µl of heparinized blood; and lane 9: 0.04 µl of heparinized blood. For the PCR, lane 1: molecular weight marker; lane 2: 5 µl of citrated blood, 30 cycles; lane 3: 1 µl of citrated blood, 30 cycles; lane 4: 0.2 µl of citrated blood, 30 cycles; lane 5: 0.04 µl of citrated blood, 30 cycles; lane 6: 5 µl of citrated blood, 35 cycles; lane 7: 1 µl of citrated blood, 35 cycles; lane 8: 0.2 µl of citrated blood, 35 cycles; lane 9: 0.04 µl of citrated blood, 35 cycles; lane 10: 5 µl of heparinized blood, 30 cycles; lane 11: 1 µl of heparinized blood, 30 cycles; lane 12: 0.2 µl of heparinized blood, 30 cycles; lane 13: 0.04 µl of heparinized blood, 30 cycles; lane 14: 5 µl of heparinized blood, 35 cycles; lane 15: 1 µl of heparinized blood, 35 cycles; lane 16: 0.2 µl of

heparinized blood, 35 cycles; and lane 17: 0.04 µl of heparinized blood, 35 cycles.

As shown in Figure 26, the amplification products of interest were observed using the genomic DNA corresponding to 0.2 µl of either of the blood samples for the ICAN, and the genomic DNA corresponding to 0.2 µl of either of the blood samples (citrated or heparinized) for the PCR of 30 cycles, respectively.

Example 29

Detection of Escherichia coli O-157 vero toxin 2 (VT-2) gene using Bca RNase HIII was examined. 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. Dilutions corresponding to 0, 1, 10, 10^2 or 10^3 cells in sterile water were prepared and used as templates. Primers VT-2 IF4 and VT-2 IR3 having nucleotide sequences represented by SEQ ID NOS:130 and 131 were synthesized as primers for detection. The size of the amplification product obtained using the primer pair was about 146 bp. 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% and one of the hot water-extracts was prepared. The mixtures were heated at 98°C for 2 minutes and incubated at 55°C for 1 minute in Thermal

Cycler Personal, and then placed on ice. At final concentrations, 34 mM Tricine buffer (pH 8.7), 10 mM potassium chloride, 10 mM ammonium sulfate, 1% DMSO, 0.01% BSA, 4 mM magnesium acetate, 500 µM each of dNTPs, 32 U of Bca RNase HIII as prepared in Referential Example 3(5) and 5.5 U of BcaBEST DNA polymerase were added to the mixture to make the final volume to 50 µl. The reaction mixtures were placed in a thermal cycler which had been set at 55°C and reacted at the temperature for 60 minutes. After reaction, 3 µl each of the reaction mixtures was subjected to electrophoresis on 4% NuSieve 3:1 agarose. The results are shown in Figure 27. Figure 27 shows the results of the detection of Escherichia coli O-157 vero toxin II (VT2) gene using Bca RNase HIII. Lane M: molecular weight marker (100 bp ladder); lane N: sterile water as template; 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; and lane 4: the template corresponding to 10^3 cells.

As shown in Figure 27, VT2 gene was detected using the hot water-extract corresponding to 1 cell according to the ICAN. These results were equivalent to those of the detection reactions according to the ICAN using E. coli RNase H and the PCR as described in Example 9. Thus, it was confirmed that the ICAN using a heat-resistant

RNase H, Bca RNase HIII, was also effective in detecting a virus, a bacterium and the like.

Example 30

Detection of Staphylococcus aureus enterotoxin A gene was examined. Primers SEA-1 and SEA-2 represented by SEQ ID NOS:136 and 137 were synthesized based on the nucleotide sequence of the enterotoxin A gene region of Staphylococcus aureus. 50 µl of a reaction mixture containing 1 µl of a solution containing 115 pg or 1.15 ng of a genomic DNA from Staphylococcus aureus (ATCC accession no. 13565) or 1 µl of water for a negative control, 50 pmol each of the primers, 0.5 mM each of dNTPs, 32 mM HEPES-potassium hydroxide buffer (pH 7.8), 100 mM potassium acetate, 4.0 mM magnesium acetate, 0.01% BSA, 1.0% DMSO, 0.0156 µg of Pfu RNase H and 1 U of BcaBEST DNA polymerase was incubated at 58°C for 1 hour in a thermal cycler. After reaction, 5 µl each of the reaction mixtures was analyzed by electrophoresis on 3.0% agarose gel. The results are shown in Figure 29. Figure 29 shows the results of electrophoresis for the detection of Staphylococcus aureus enterotoxin A gene. Lane 1: molecular weight marker (100 bp ladder); lane 2: negative control (sterile water); lane 3: 115 pg of the template; and lane 4: 1.15 ng of the template.

As shown in Figure 29, the amplification product

of interest was observed using about 1.15 ng of the template.

Example 31

Detection of hepatitis C virus (HCV) was examined. Primers HCV-F3 and HCV-R1 having nucleotide sequences represented by SEQ ID NOS:138 and 139 were synthesized based on the nucleotide sequence of HCV. A template DNA was prepared as follows. Briefly, 4 µl of a mixture containing RNA prepared as described in Example 21 from 100 µl of a serum from a healthy individual or a patient with HCV, 10 mM tris-hydrochloride buffer (pH 8.3), 5 mM $MgCl_2$, 1 mM each of dNTPs, 10 pmol of Random 6mers and 10 U of Reverse Transcriptase XL (Takara Shuzo) was incubated at 30°C for 10 minutes and at 42°C for 1 hour and then heated at 99°C for 5 minutes for inactivating the enzyme using a thermal cycler (Gene Amp PCR System 9600, Applied Biosystems) to prepare a cDNA.

An ICAN was carried out at 55°C for 1 hour as described in Example 13 except that 1 µl of the cDNA reaction mixture and 100 pmol each of the primers HCV-F3 and HCV-R1 were used. After reaction, 2.5 µl each of the reaction mixtures was subjected to electrophoresis on 3.0% agarose gel. The results are shown in Figure 31. Figure 31 shows the results of electrophoresis for the detection of hepatitis C virus. Lane 1: molecular weight marker (100

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bp); lane 2: the template prepared from a serum from a healthy individual; and lanes 3 to 6: the templates prepared from sera from patients infected with HCV.

As shown in Figure 31, it was confirmed that HCV could be specifically detected from the serum samples from patients infected with HCV.

Example 32

The amplification method of the present invention was examined.

(1) A PCR was carried out using the pUC19-150 plasmid DNA as prepared in Example 2(2) as a template and primers MCS-F and MCS-R represented by SEQ ID NOS:35 and 36. A PCR-amplified fragment of 534 bp was obtained by purifying the reaction mixture using Microcon-100 (Millipore). A reaction mixture containing 15 ng of the PCR fragment and 30 pmol of a primer MR2 having a nucleotide sequence represented by SEQ ID NO:140 labeled with [γ-^{32}P]ATP by phosphorylation at the 5'-terminus and sterile distilled water to 5 µl and a reaction mixture further containing 30 pmol of a primer MR1 having a nucleotide sequence represented by SEQ ID NO:141 were prepared. The reaction mixtures were heat-denatured at 98°C for 2 minutes and then cooled to 55°C. 20 µl of a reaction mixture (42.5 mM Tricine buffer (pH 8.7), 12.5 mM potassium chloride, 12.5 mM ammonium sulfate, 0.0125% BSA,

1.25% DMSO, 5 mM magnesium acetate, 0.625 mM each of dNTPs) containing 1 U of BcaBEST DNA polymerase was added to the reaction mixture. The resulting mixture was reacted at 55°C for 15 minutes. After reaction, 2.5 µl of a reaction termination solution (95% formamide, 20 mM EDTA, 0.05% Bromophenol Blue, 0.5% xylene cyanol) was added to 5 µl of the reaction mixture. The mixture was heat-denatured at 94°C for 3 minutes. 1.6 µl each of the reaction mixtures was subjected to electrophoresis on 6% polyacrylamide gel containing 8 M urea and the signals were read using BAS2000 (Fujix) to detect products extended from the primer MR1. The results are shown in Figure 32A. The sequence ladder in Figure 32A was prepared by sequencing M13mp18 single strand DNA (Takara Shuzo) using the primer MF2 labeled with [γ-^{32}P]ATP by phosphorylation and used for the determination of the length of the extension product. Lane 1: a combination of the primers MF2 and MR1; and lane 2: MR1.

As shown in Figure 32A, a band of 448 bp extended from the primer MR1 to the end of the template was detected when the extension reaction was carried out by adding only the primer MR1 to the template. On the other hand, a band of 373 bp bounded by the primers MR1 and MF2 was detected in addition to the above-mentioned band by further adding the primer MF2. Thus, it was confirmed that the extension

from the MR1 primer using the PCR-amplified fragment as a template by the action of BcaBEST DNA polymerase was switched due to template switching to the extension using a strand extended from the primer MF2 as a template. Furthermore, template switching was observed when Klenow DNA polymerase was used as a mesophilic DNA polymerase having a strand displacement activity under similar conditions. On the other hand, template switching was not observed when TaKaRa Taq DNA polymerase (Takara Shuzo) or PyroBEST DNA polymerase (Takara Shuzo) without a strand displacement activity was used.

(2) The template switching reaction was examined using a template DNA strand with a primer being annealed thereto. DNA fragments to which the primers MF2 and MR1 could be annealed were prepared as follows. PCRs were carried out using the plasmid pUC19 as a template and primers MCSF and RV (Takara Shuzo) or primers M4 (Takara Shuzo) and MCSR. The reaction mixtures were purified using Microcon-100 to obtain PCR-amplified fragments MSCF-RV (236 bp) and M4-MCSR (271 bp). The region bounded by the primers M4 and RV was commonly present in the two PCR-amplified fragments.

Next, a template-primer (1) in which template DNA strands with primers being annealed thereto were not annealed each other, and a template-primer (2) in which

template DNA strands with primers being annealed thereto were annealed each other were prepared as follows.

(1) A reaction mixture containing 30 ng of the fragment MCSF-RV, 40 pmol of the primer MF2 labeled with [γ-^{32}P]ATP by phosphorylation at the 5'-terminus, propylenediamine at a final concentration of 0.01% and sterile distilled water to 5 µl, and a mixture containing 30 ng of the fragment M4-MCSR, 40 pmol of the primer MR1, propylenediamine at a final concentration of 0.01% and sterile distilled water to 5 µl were separately heat-denatured at 98°C for 2 minutes and then cooled to 55°C. 2.5 µl each of the reaction mixtures were mixed together to prepare a template-primer.

(2) A reaction mixture containing 15 ng of the fragment MCSF-RV, 15 ng of the fragment M4-MCSR, 20 pmol of the primer MF2 labeled with [γ-^{32}P]ATP by phosphorylation at the 5'-terminus, 20 pmol of the primer MR1, propylenediamine at a final concentration of 0.01% and sterile distilled water to 5 µl was heat-denatured at 98°C for 2 minutes and then cooled to 55°C to prepare a template-primer.

20 µl of a reaction mixture (42.5 mM Tricine buffer (pH 8.7), 12.5 mM potassium chloride, 12.5 mM ammonium sulfate, 0.0125% BSA, 1.25% DMSO, 5 mM magnesium acetate, 0.625 mM each of dNTPs) containing 1 U of BcaBEST

DNA polymerase was added to 5 µl of the template-primer reaction mixture. The resulting mixture was reacted at 55°C for 15 minutes. After reaction, 2.5 µl of a reaction termination solution (95% formamide, 20 mM EDTA, 0.05% Bromophenol Blue, 0.5% xylene cyanol) was added to 5 µl of the reaction mixture. The mixture was heat-denatured at 94°C for 3 minutes. 1.6 µl each of the reaction mixtures was subjected to electrophoresis on 6% polyacrylamide gel containing 8 M urea and the signals were read using BAS2000 (Fujix) to detect products extended from the primer MF2. The results are shown in Figure 32B. The sequence ladder in Figure 32B was prepared by sequencing M13mp18 single strand DNA using the primer MR1 labeled with [γ-^{32}P]ATP by phosphorylation and used for the determination of the length of the extension product. Lane 1: template DNA strands not being annealed each other; and lane 2: template DNA strands being annealed each other.

As shown in Figure 32B, only a band of 161 bp extended from the primer MF2 to the end of the template was detected for the template-primer in which template DNA strands with primers being annealed thereto were not annealed each other. On the other hand, a band of 223 bp bounded by the primers MF2 and MR1 in addition to the above-mentioned band was detected for the template-primer in which template DNA strands with primers being annealed

thereto were annealed each other. Thus, it was confirmed that a template switching reaction took place if template DNA strands with primers being annealed thereto were annealed each other.

Example 33

(1) Detection of Mycobacterium tuberculosis was examined using the RNase H derived from Archaeoglobus fulgidus (Afu) described in Referential Example 7. Primers MTIS2F (SEQ ID NO:155) and MTIS2R (SEQ ID NO:156) were synthesized on the basis of the nucleotide sequence of the Mycobacterium tuberculosis genome registered in GenBank under accession number AL123456. The length of the region bordered by the primer pair including the primer portions is 103 bp. Mycobacterium tuberculosis genomic DNA as a template was extracted from dried BCG vaccine (Nippon BCG Seizo) according to a conventional method. Solutions containing 100 pg, 10 pg, 1 pg, 100 fg, 10 fg or 1 fg of the genomic DNA per µl of sterile water were prepared. Reactions were carried out as follows. Briefly, at final concentrations, 32 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, 50 pmol each of the primers MTIS2F and MTIS2R, 8.75 U of RNase H from Afu, 8 U of BcaBEST DNA polymerase and 1 µl of one of the templates were mixed and the final volume was made to 50 µl

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with sterile water. The reaction mixtures were placed in Thermal Cycler Personal which had been set at 60°C and incubated for 60 minutes. After reaction, 3 µl each of the reaction mixtures was subjected to electrophoresis on 3.0% agarose gel.

On the other hand, a PCR was carried out as a control. Primers MTIS PCR-F and MTIS PCR-R as described in Rinsho Byori (the Japanese Journal of Clinical Pathology), 43(9):941-947 (1995) were used as primers. An amplification product of 276 bp is obtained using the primer pair. A reaction mixture of 50 µl was prepared using 10 pmol each of the primers according to the instruction manual attached to Ex Taq DNA polymerase (Takara Shuzo). The reaction mixture was placed in a thermal cycler and subjected to a reaction of 40 cycles each cycle consisting of 94°C for 30 seconds, 50°C for 30 seconds and 72°C for 30 seconds. After reaction, 3 µl each of the reaction mixtures was subjected to electrophoresis on 3.0% agarose gel.

As a result, the amplification products of interest were observed in either case using 100 fg of the template.

(2) Detection of Chlamydia trachomatis was examined using RNase H from Afu or RNase H from Pyrococcus horikoshii (Pho). Primers CT2F (SEQ ID NO:157) and CT2R

(SEQ ID NO:158) were synthesized on the basis of the nucleotide sequence of the Chlamydia trachomatis plasmid registered in GenBank under accession number X06707. The length of the region bordered by the primer pair including the primer portions is 109 bp. A samples as a template DNA was prepared by subjecting a clinical specimen obtained from a patient agreed with the informed consent to phenol-chloroform treatment and ethanol precipitation. Reactions were carried out as follows. Briefly, at final concentrations, 32 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, 50 pmol each of the primers CT2F and CT2R, 46.4 U of RNase H from Pho or 8.75 U of RNase H from Afu, 8 U of BcaBEST DNA polymerase and 1 µl of the sample were mixed and the final volume was made to 50 µl with sterile water. The reaction mixtures were placed in Thermal Cycler Personal which had been set at 55°C and incubated for 60 minutes. After reaction, 3 µl each of the reaction mixtures was subjected to electrophoresis on 3.0% agarose gel. As a result, the amplification products of interest was observed. These results demonstrate that Chlamydia trachomatis can be detected using RNase H from Afu or Pho in the method of the present invention.

(3) Furthermore, detection using magnetic beads

in a commercially available detection instrument was examined. Briefly, an amplification reaction was carried out as described in (1) above using the primer MTIS2R used in (1) above labeled at its 5'-terminus with biotin as a primer and 100 ng of the Mycobacterium tuberculosis genomic DNA as a template. The resulting amplified fragment was diluted 30-fold, 300-fold or 3000-fold and subjected to detection using streptoavidin-coated magnetic beads (Pierce) in an automated detection instrument Lumipuls (Fujirebio).

Magnetic beads having streptoavidin capable of binding 100 pmol of biotin being immobilized was reacted with the biotinylated amplified fragment for 5 minutes on the first layer of a cuvette. 0.1 N NaOH was then added thereto. Hybridization with a FITC-labeled probe MTISBF was carried out for 5 minutes. After washing, a POD-labeled anti-FITC antibody was added thereto. After reacting for 5 minutes and washing, a luminescent substrate was added thereto. As a result, it was shown that detection can be carried out semi-quantitatively in a short time (20 minutes) using magnetic beads in a conventional automated detection instrument. The detection was carried out by measuring the luminescence level by photocounting. The results are shown in Table 12.

Table 12

Mycobacterium tuberculosis amplicon	Photocounting	S/N ratio
x 30	3.55×10^7	29.6
x 300	1.21×10^7	10.0
x 3000	0.21×10^7	1.75
0	0.12×10^7	-

The results in Table 12 demonstrate that detection can be carried out with sensitivity equivalent to that of the conventional plate luminescence method.

(4) A hybrid chromatography method was examined as a method for detecting the above-mentioned amplified fragment. Streptoavidin (Nacalai Tesque) was immobilized onto a nitrocellulose membrane. A water-absorptive pad was connected therewith to construct a hybrid chromatography strip. This strip was used to detect the amplified fragment used in (3) above according to a hybrid chromatography method. Detection was carried out by color development using 1-step TMB-Blotting (Pierce). Specifically, a reaction mixture containing the amplified fragment was developed on the nitrocellulose membrane. Then, 0.1 N NaOH solution, FITC-labeled probe, washing solution and color development solution were developed in this order. As a result, a blue band was detected for the amplified fragment derived from a Mycobacterium tuberculosis-positive sample. It was demonstrated that

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this method is useful as a rapid gene examination method because, using this method, results are obtained with naked eyes in 5 to 10 minutes by the method of the present invention.

Example 34

(1) Southern hybridization analysis of amplified products containing laddered bands

In some cases, plural high molecular weight laddered bands other than three bands of interest may be observed in the amplification method of the present invention. The laddered bands were examined. Enterohemorrhagic E. coli O-157 was selected as a target. A template DNA, chimeric primers and reaction conditions for ICAN were as described in Example 9. After reaction, 5 µl of the reaction mixture was subjected to electrophoresis on 3% NuSieve 3:1 agarose gel. The results are shown in Figure 37. In Figure 37, the lanes represent results for the following: lane M: 100 bp DNA ladder marker; lane 1: negative control; lane 2: the heated extract corresponding to 1 cell; lane 3: the heated extract corresponding to 10 cells; lane 4: the heated extract corresponding to 10^2 cells; lane 5: the heated extract corresponding to 10^3 cells; lane 6: the heated extract corresponding to 10^4 cells; and lane 7: the heated extract corresponding to 10^5 cells. Ladder bands were observed as shown in Figure 37.

(2) Analysis of laddered amplified fragments

The nucleotide sequences of the laddered bands obtained in (1) above were analyzed. Briefly, 50 µl of the reaction mixture prepared in (1) was subjected to electrophoresis on 3% agarose gel. After electrophoresis, the laddered bands were excised from the gel. The amplified DNA fragments were then recovered from the gel using EASYTRAP Ver.2 (Takara Shuzo). The recovered amplified fragments were blunt-ended using DNA Blunting kit (Takara Shuzo).

The blunt-ended DNA fragments were ligated with a vector pGEM-3Z (Promega) digested with a restriction enzyme HincII (Takara Shuzo) using DNA ligation Kit (Takara Shuzo). The ligation mixtures were used to transform competent cells of JM109 (Takara Shuzo). After transformation, the cells were cultured on LB agar plate containing 0.1 mM ampicillin, 1 mM IPTG and 0.02% X-gal at 37°C overnight.

After cultivation, several white colonies were selected from the plates. Colony PCRs were carried out using primers M13-M4 and M13-RV (both from Takara Shuzo) to determine the presence of an insert. Colonies having inserts were cultured with shaking in LB liquid medium containing 0.1 mM ampicillin at 37°C overnight. After cultivation, plasmids were purified from cells using QIAGEN plasmid mini Kit (Qiagen). The sequences of fragments

cloned at the HincII sites of the plasmids were analyzed in both directions using primers M13-M4 and M13-RV according to a conventional method.

As a result, it was demonstrated that the laddered fragment obtained by the method of the present invention has a structure in which the region to be amplified is repeated. Furthermore, it was confirmed that the repetition is in the same direction from 5' to 3'.

(3) The laddered amplified fragments other than three amplified fragments of interest formed in the method of the present invention for detecting Mycobacterium tuberculosis, HCV or Chlamydia trachomatis were examined. HCV was detected under conditions as described in Example 31. Mycobacterium tuberculosis or Chlamydia trachomatis was detected under conditions as described in Example 33. The resulting laddered amplified fragments were subcloned and sequenced as described in (2) above. As a result, it was demonstrated that the laddered fragment obtained by the method of the present invention has a structure in which the region to be amplified is repeated. Furthermore, it was confirmed that the repetition is in the same direction from 5' to 3'.

Example 35

(1) The detection method of the present invention for Mycobacterium tuberculosis was examined. First,

primers K-F-1033(60) (SEQ ID NO:159) and K-F-1133(62) (SEQ ID NO:160) were synthesized for amplifying a region with relatively low GC content in the Mycobacterium tuberculosis genome. Serial dilutions containing 100 fg to 10 pg of the Mycobacterium tuberculosis genomic DNA as described in Example 33(1) were used as templates. The reaction was carried our as follows. Briefly, at final concentrations, 32 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, 50 pmol each of the primers K-F-1033(60) and K-F-1133(62), 9.375 U of RNase HII from Pfu or 4.375 U of RNase H from Afu, 2.75 U of BcaBEST DNA polymerase and 1 µl of one of the templates were mixed and the final volume was made to 25 µl with sterile water. The reaction mixtures were placed in Thermal Cycler Personal which had been set at 62°C and incubated for 60 minutes. After reaction, 3 µl each of the reaction mixtures was subjected to electrophoresis on 3.0% agarose gel. As a result, it was demonstrated that the amplification products could be detected using 100 fg to 10 pg of the genomic DNA as a template and either RNase H.

(2) The method as described in (1) above was examined using primers having higher Tm values. First, primers K-F-1033(68) (SEQ ID NO:161) and K-F-1133(68) (SEQ ID NO:162) were synthesized. The amplification reaction

was carried out under the same conditions as those described in (1) above except that the reaction temperature was 63°C. After reaction, 3 µl each of the reaction mixtures was subjected to electrophoresis on 3.0% agarose gel. The results are shown in Figure 38. Figure 38 illustrates results of electrophoresis of Mycobacterium tuberculosis genome amplified using RNase H from Pfu or RNase H from Afu. Lanes 1 to 4 represent results obtained using RNase HII from Pfu and the following amount of template DNA: lane 1: 10 pg; lane 2: 1 pg; lane 3: 100 fg; and lane 4: negative control. Lanes 5 to 8 represent results obtained using RNase HII from Afu and the following amount of template DNA: lane 5: 10 pg; lane 6: 1 pg; lane 7: 100 fg; and lane 8: negative control. Lane M represents 100 bp DNA ladder marker.

As shown in Figure 38, it was demonstrated that the amplification fragment of interest could be detected using 100 fg of the template DNA and either RNase H. It was shown that more amplification product is obtained when RNase H from Afu is used. In addition, it was shown that more stable detection sensitivity is accomplished when RNase H from Afu is used.

(3) Amplification using a combination of the primers K-F-1033(68) and K-F-1133(68) and a plasmid containing a region to be amplified as a template was

examined. First, primers F26 (SEQ ID NO:163) and R1310 (SEQ ID NO:164) were synthesized in order to prepare a plasmid containing a region to be amplified. A PCR was carried out using these primers and BCG vaccine strain. The resulting amplification product was then introduced into pT7-Blue-T vector (Takara Shuzo) to prepare the plasmid. The composition of the reaction mixture was as described in (2) above except that 4 U of Bca DNA polymerase was used. As a result, it was confirmed that detection can be carried out using 1 fg of the template.

(4) The detection sensitivity accomplished when a combination of primers MTIS2F and MTIS2R, which results in the formation of laddered amplified fragments containing the three amplified fragments of interest, was used was compared with that accomplished when a combination of primers K-F-1033(68) and K-F-1133(68), which results in the three amplified fragments of interest, was used. Mycobacterium tuberculosis was selected as a target. Reactions were carried out as described in Example 33(2) when the combination of the primers MTIS2F and MTIS2R was used or in (2) above when the combination of the primers K-F-1033(68) and K-F-1133(68) was used. As a result, the same detection sensitivity was observed in either case.

Example 36

The amplification method of the present invention

that does not comprise denaturation of a genomic DNA as a template was examined.

(1) Primers pDON-AI-68-1 (SEQ ID NO:165) and pDON-AI-68-2 (SEQ ID NO:166) were synthesized in accordance with the nucleotide sequence of the packaging region in a plasmid pDON-AI (Takara Shuzo).

(2) A reaction mixture of a total volume of 50 µl containing 1 µl of a solution containing 10 fg or 1 pg of pDON-AI, 1 µl of a solution containing 1 ng, 10 ng or 100 ng of the genomic DNA derived from NIH/3T3 cells having pDON-AI being incorporated prepared in Example 23, or 1 µl of water as a negative control, 50 pmol each of the primers as described in (1) above, 0.5 mM each of dNTPs, 32 mM HEPES-potassium hydroxide buffer (pH 7.8), 100 mM potassium acetate, 4 mM magnesium acetate, 0.01% BSA, 1% DMSO, 18.5 U of RNase HII from Pfu and 4 U of BcaBEST DNA polymerase was prepared. The reaction mixtures were placed in a thermal cycler and incubated at 64°C for 1 hour. After reaction, 5 µl each of the reaction mixtures was subjected to electrophoresis on 3% agarose gel to observe amplification products. The results are shown in Figure 39. In Figure 39, the lanes represent results for the following templates: lane M: 100 bp DNA ladder marker; lane 1: negative control; lane 2: 1 ng of genomic DNA having pDON-AI being incorporated; lane 3: 10 ng of genomic DNA having

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pDON-AI being incorporated; lane 4: 100 ng of genomic DNA having pDON-AI being incorporated; lane 5: 10 fg of pDNA-AI DNA; and lane 6: 1 pg of pDON-AI DNA.

As shown in Figure 39, amplification of specific DNA fragments was observed for either pDON-AI or the genomic DNA having pDON-AI being incorporated. Thus, it was confirmed that a DNA fragment of interest can be amplified without denaturing the DNA as a template prior to the reaction even if a genomic DNA is used as a template.

Example 37

The fidelity (accuracy) of the present invention was compared with that of a PCR utilizing LA technology in which TaKaRa Ex Taq polymerase (Takara Shuzo) was used. First, a plasmid as a template was prepared as follows.

Specifically, the regions each consisting of 300 bp represented by SEQ ID NOS:167 to 170 were amplified by PCR from human proto-oncogene, Wnt-5a gene (GenBank accession no. L20861), ribosomal protein S5 gene (GenBank accession no. XM_009371), human NADH gene (GenBank accession no. NM_000903) and human protocadherin 43 gene (GenBank accession no. AU077347). In this case, the PCRs were carried out using specific primers each having a site for a restriction enzyme SfiI at the 5'-terminus of the primer. After reaction, the amplified fragments were digested with a restriction enzyme SfiI (Takara Shuzo).

pUC19 (Takara Shuzo) was digested with restriction enzymes AflIII (NEB) and NdeI (Takara Shuzo) and subjected to agarose gel electrophoresis. A fragment of about 2 kbp was excised from the gel and recovered using EASYTRAP (Takara Shuzo). A DNA having the sequence of SEQ ID NO:171 and a DNA complementary thereto were synthesized using a DNA synthesizer. These DNAs were heat-denatured and then annealed each other to form a double-stranded DNA. This double-stranded DNA has cohesive ends for restriction enzymes AflIII and NdeI at its termini. The double-stranded synthetic DNA was inserted into the fragment obtained by digesting pUC19 with restriction enzymes using DNA Ligation kit ver. 2 (Takara Shuzo). The resulting plasmid was designated as pIC62. pIC62 has sequences to which primers ICAN2 (SEQ ID NO:172) and ICAN6 (SEQ ID NO:173) anneal as well as a site for a restriction enzyme SfiI. The plasmid pIC62 was digested with the restriction enzyme SfiI. The above-mentioned PCR-amplified fragment digested with the restriction enzyme SfiI was ligated with the plasmid using Ligation kit ver. 2 (Takara Shuzo). The ligation mixture was used to transform Escherichia coli JM109 (Takara Shuzo). The resulting transformants were cultured, plasmids having the about 300-bp DNAs being inserted were obtained, and the sequences of the inserted DNAs were determined. The plasmids were then used as

templates in this Example.

(2) ICAN amplification products were prepared as follows. Briefly, a solution of 10 µl containing 10 ng of one of the plasmids as a template, 50 pmol each of chimeric primers ICAN2 (SEQ ID NO:172) and ICAN6 (SEQ ID NO:173) and 0.01% propylenediamine was prepared. The solution was denatured at 98°C for 2 minutes and incubated at 60°C for 1 minute in Thermal Cycler Personal, and then transferred onto ice. Next, 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 RNase H from E. coli and 5.5 U of BcaBEST DNA polymerase were added thereto and the final volume was made to 50 µl with sterile water. The reaction mixtures were placed in Thermal Cycler Personal which had been set at 60°C and incubated for 60 minutes. After reaction, the reaction mixtures were subjected to electrophoresis on 2% SeaKem GTG agarose gel (Takara Shuzo). After electrophoresis, bands for the amplification products of interest were recovered by excision from the agarose gel, recovery using SUPREC-01 (Takara Shuzo), treatment with phenol-chloroform and ethanol precipitation.

(3) PCR amplification products were prepared as follows using TaKaRa Ex Taq DNA polymerase. Briefly, a reaction mixture was prepared using 10 ng of the above-

mentioned plasmid as a template as well as 10 pmol each of DNA primers ICAN2 (SEQ ID NO:174) and ICAN6 (SEQ ID NO:175) according to the instruction manual attached to TaKaRa Ex Taq DNA polymerase (Takara Shuzo). The reaction mixture was placed in a thermal cycler and subjected to a reaction of 30 cycles, each cycle consisting 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 as described in (2) above. The amplification products of interest were recovered by recovery using Microcon-100 (Takara Shuzo), treatment with phenol-chloroform and ethanol precipitation.

(4) The amplification products obtained in (2) and (3) above were subcloned as follows. Briefly, the ICAN amplification products and the PCR amplification products were introduced into a vector pT7 Blue (Takara Shuzo) using Perfectly Blunt Cloning kit (Takara Shuzo) according to the instruction manual. The ligation mixture was used to transform NovaBlue Singles Competent Cell (Takara Shuzo). For each clone, 10 colonies were selected from the resulting transformants and cultured to obtain plasmids each having about 0.4-kb DNA being inserted. The fragments inserted in the plasmids were sequenced using T7 promoter primer (Takara Shuzo) and M3 primer (Takara Shuzo).

A total of about 16,000 bases were analyzed by

sequencing as described above. As a result, one mutation was found in about 2500 bases for both of the fragments amplified according to the method of the present invention and the fragments amplified by PCR using TaKaRa Ex Taq DNA polymerase. Thus, it was confirmed that the fidelity (accuracy) of the method of the present invention is equivalent to that of LA-PCR whose fidelity is high.

Example 38

(1) Preparation of template for ICAN reaction by PCR

A double-stranded cDNA was prepared from polyA+ RNA derived from mouse brain (OriGene) using cDNA synthesis kit (Takara Shuzo). PCR fragments were amplified using the double-stranded cDNA as a template and combinations of the primers having nucleotide sequences of SEQ ID NOS:176-189. The fragments were introduced into pT7 Blue T-vector (Takara Shuzo) by TA cloning to obtain plasmid clones. A reaction mixture of a total volume of 50 µl containing 1 µl (1 ng) of one the plasmid clones, 10 pmol each of primers MCS-F (SEQ ID NO:190) and MCS-R (SEQ ID NO:191), 1.25 U of Ex Taq (Takara Shuzo), 5 µl of 10 x Ex Taq buffer (Takara Shuzo) and 0.2 mM each of dNTPs was subjected to the following reaction using TaKaRa PCR Thermal Cycler Personal (Takara Shuzo): 94°C for 2 minutes; 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute. The

resulting amplified DNA fragment was used as a template for an ICAN reaction.

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

An ICAN reaction was carried out using Aminoallyl dUTP (Sigma) in order to introduce an amino group into an ICAN amplification product. The ratio of amino group introduced into an ICAN amplification product was examined by changing the ratio of the amount of dTTP to the amount of Aminoallyl dUTP in the ICAN reaction as follows: 10 : 0, 9 : 1, 8 : 2, 7 : 3 and 6 : 4. The reaction was carried out as follows.

First, a solution of a total volume of 10 µl containing 1 µl of the PCR reaction mixture prepared in (1) above, 50 pmol each of primers MF2N3(24) (SEQ ID NO:192) and MR1N3(24) (SEQ ID NO:193) and 2 µl of 0.05% aqueous solution of propylenediamine was heated at 98°C for 2 minutes followed by 65°C for 30 seconds in TaKaRa PCR Thermal Cycler Personal and rapidly cooled on ice to anneal the primers to the template. A reaction mixture of a total volume of 40 µl containing 0.625 mM each of dATP, dCTP and dGTP, 0.625 mM of a dTTP + Aminoallyl dUTP mixture, 32 mM HEPES-potassium hydroxide buffer (pH 7.8), 5.0 mM magnesium acetate, 0.6 U of RNase H from E. coli (Takara Shuzo) and 2.75 U of BcaBEST DNA polymerase was added to the heated

solution. The resulting mixture was incubated in Thermal Cycler at 65°C for 1 hour.

50 µl of isopropanol and 5 µl of 3 M sodium acetate solution (pH 5.2) were added to the reaction mixture of 50 µl. The resulting mixture was cooled at -80°C for 20 minutes and then centrifuged to remove a supernatant. 200 µl of 70% ethanol solution was added thereto. The supernatant was removed by centrifugation. The precipitate was air-dried. The resulting DNA was re-dissolve in water. $OD_{260/280}$ was measured to determined the amount of the product.

(3) Confirmation of introduction of Aminoallyl dUTP into ICAN amplification product

Introduction of an amino group into an ICAN product was confirmed by fluorescence-label the amino group in the ICAN product using 5-carboxyfluorecein succinimidil ester (Molecular Probe). The above-mentioned DNA solution was diluted to make the concentration to 2 µg/50 µl. 20 µl of 1 M sodium carbonate buffer (pH 9.0) was added thereto. Then, 4 µl of a solution of FITC (Nacalai Tesque) in N,N-dimethylformamide at a concentration of 10 mM was further added. The resulting mixture was reacted at 20°C for 16 hours. After removing excess FITC using a commercially available spin column, 10 µl of the reaction mixture was applied onto 2.0% agarose gel and electrophoresed. After

electrophoresis, fluorescent dye was detected using FM-BIO. Furthermore, the ICAN-amplified fragment was detected by staining with EtBr. As a result, it was confirmed that an amino group can be introduced into an ICAN amplification product by conducting an ICAN using Aminoallyl dUTP. It was also confirmed that the detection sensitivity can be further increased by using a modified nucleotide having a functional group and fluorescent label for an amplification product in combination.

(4) The amplification method of the present invention was examined using deoxyUTP. Mycobacterium tuberculosis was selected as a target. First, primers MTIS2F-16 (SEQ ID NO:194) and MTIS2R-ACC (SEQ ID NO:195) were synthesized in accordance with the nucleotide sequence of Mycobacterium tuberculosis genome registered in GenBank under accession no. AL123456. The length of the region to be amplified using this primer pair including the primer portions is 98 bp. A template was prepared as follows. Briefly, a product obtained by PCR-amplifying the Mycobacterium tuberculosis genome using primers MTIS-PCR-F-2 (SEQ ID NO:196) and MTIS-PCR-R-2 (SEQ ID NO:197) was inserted into pT7 Blue T-Vector (Takara Shuzo) using DNA Ligation kit Ver. 2. The ligated plasmid was used to transform Escherichia coli JM109. The resulting transformants were cultured to obtain a plasmid having an

about 400-bp DNA being introduced. A solution containing 10^3 copies of the plasmid per µl was prepared based on the concentration determined by measuring OD260.

At final concentrations, 32 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 dATP, dCTP and dGTP, a dTTP/dUTP mixture (500/0, 400/100, 300/200, 200/300, 100/400 or 0/500 µM), 50 pmol each of the primers MTIS 2F-16 and MTIS 2R-AAC, 8.75 U of RNase H from Afu, 8 U of BcaBEST DNA polymerase and 1 µl of the template (10^3 copies) were mixed and the final volume was made to 50 µl with sterile water. The reaction mixtures were placed in Thermal Cycler Personal which had been set at 60°C and incubated for 60 minutes. After reaction, 3 µl each of the reaction mixtures was subjected to electrophoresis on 3% agarose gel.

As a result, the amplification product of interest was observed for each of the dTTP/dUTP ratios. Based on these results, it was confirmed that a modified nucleotide can be used as a substrate in the method of the present invention.

Example 39

Application of the method of the present invention to a one-step amplification method was examined. HCV was selected as a target.

(1) Preparation of transcript RNA

A transcript RNA as a template was prepared. Preparation was carried out from 300 µl of a serum derived from a patient with hepatitis C virus agreed with the informed consent using TRIzol reagent (Life Technologies) according to the instructions attached to the reagent followed by final dissolution in 20 µl of injectable water (Otsuka Pharmaceutical). An RT-PCR was carried out using the RNA sample above as a template. The reaction was carried out as follows. A reaction mixture of 50 µl was prepared using 2 µl of the RNA sample and 20 pmol each of primers SP6-HCV-F (SEQ ID NO:198) and T7-HCV-R (SEQ ID NO:199) according to a manual attached to One-Step RNA PCR kit (Takara Shuzo). The reaction mixture was placed in Thermal Cycler Personal and subjected to the following reaction: 50°C for 15 minutes; 94°C for 2 minutes; and 40 cycles of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds. After reaction, the reaction mixture was subjected to electrophoresis on 2% SeaPlaque GTG agarose gel. A 350-bp amplification product of interest was excised from the gel. The DNA was then recovered using EASYTRAP Ver. 2 according to the instructions attached to the kit. A transcript RNA was synthesized using the recovered DNA as a template and Competitive RNA Transcription kit (Takara Shuzo) according to the

instructions attached to the kit. The transcript RNA was used as a template for examination of a one-step RT-ICAN.

(2) Examination of one-step RT-ICAN

The concentration of the transcript RNA prepared in (1) above was determined based on the OD_{260} value and dilutions containing 10^4, 10^5, 10^6 or 10^7 copies per µl were prepared. A reaction mixture of 50 µl containing, at final concentrations, 32 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, 50 pmol each of the primers HCV-A S (SEQ ID NO:200) and HCV-A A (SEQ ID NO:201), 30 U of RNase H from Pfu, 8 U of BcaBEST DNA polymerase, 20 U of RNase inhibitor, 0, 1, 2.5, 3 or 5 U of AMV RTase XL (Takara Shuzo) and 1 µl of one of the dilutions containing varying copies of the transcript RNA was prepared. The reaction mixtures were placed in Thermal Cycler Personal which had been set at 60°C and incubated for 60 minutes. After reaction, 2 µl each of the reaction mixtures was subjected to electrophoresis on 3% agarose gel.

As a result, no amplification product of interest was observed using each of the dilutions of the template when AMV RTase was not added. On the other hand, the amplification product of interest was observed using the dilution containing 10^7 copies (1 U of AMV RTase XL added), 10^6 copies (2.5 U of AMV RTase XL added), 10^6 copies (3 U

of AMV RTase XL added) or 10^6 copies (5 U of AMV RTase XL added). When the reaction was carried out at a temperature of 57°C, the amplification product of interest was observed using the dilution containing 10^5 copies and 2.5 U of AMV RTase XL. Furthermore, when 1 U of BcaBEST DNA polymerase and 10 U of RNase H from Pfu were used, the amplification product of interest was observed using the dilution containing 10^6 copies even if no AMV RTase was added.

Industrial Applicability

The present invention provides a method for amplifying a target nucleic acid comprising amplifying a region suitable for specific amplification in the nucleotide sequence of the target nucleic acid by a DNA synthesis reaction using a chimeric oligonucleotide primer. The present invention also provides a method for detecting a target nucleic acid comprising detecting a fragment amplified from the target nucleic acid obtained by the method for amplifying a target nucleic acid. Furthermore, the amplification method of the present invention can be utilized as an efficient method for producing a nucleic acid by combining it with another method for amplifying a nucleic acid or a method for duplicating a nucleic acid. The present invention also provides a method for detecting a target nucleic acid for the specific detection and

quantification of a microorganism, particularly a pathogenic microorganism, such as a virus, a bacterium, a fungi, a yeast or the like with high sensitivity as well as a chimeric oligonucleotide primer for the method. The present invention further provides a system for amplifying and detecting a nucleic acid which is automated, in a small amount and highly integrated.

Sequence Listing Free Text

SEQ ID NO:1: PCR primer BsuII-3 for cloning a gene encoding a polypeptide having a RNaseHII activity from Bacillus caldotenax.

SEQ ID NO:2: PCR primer BsuII-6 for cloning a gene encoding a polypeptide having a RNaseHII activity from Bacillus caldotenax.

SEQ ID NO:3: PCR primer RNII-S1 for cloning a gene encoding a polypeptide having a RNaseHII activity from Bacillus caldotenax.

SEQ ID NO:4: PCR primer RNII-S2 for cloning a gene encoding a polypeptide having a RNaseHII activity from Bacillus caldotenax.

SEQ ID NO:5: PCR primer RNII-S5 for cloning a gene encoding a polypeptide having a RNaseHII activity from Bacillus caldotenax.

SEQ ID NO:6: PCR primer RNII-S6 for cloning a

gene encoding a polypeptide having a RNaseHII activity from Bacillus caldotenax.

SEQ ID NO:7: PCR primer RNII-Nde for cloning a gene encoding a polypeptide having a RNaseHII activity from Bacillus caldotenax.

SEQ ID NO:8: Nucleotide sequence of ORF in RNaseHII gene from Bacillus caldotenax.

SEQ ID NO:9: Amino acid sequence of RNaseHII from Bacillus caldotenax.

SEQ ID NO:10: PCR primer BsuIII-1 for cloning a gene encoding a polypeptide having a RNaseHIII activity from Bacillus caldotenax.

SEQ ID NO:11: PCR primer BsuIII-3 for cloning a gene encoding a polypeptide having a RNaseHIII activity from Bacillus caldotenax.

SEQ ID NO:12: PCR primer BsuIII-6 for cloning a gene encoding a polypeptide having a RNaseHIII activity from Bacillus caldotenax.

SEQ ID NO:13: PCR primer BsuIII-8 for cloning a gene encoding a polypeptide having a RNaseHIII activity from Bacillus caldotenax.

SEQ ID NO:14: PCR primer RNIII-S3 for cloning a gene encoding a polypeptide having a RNaseHIII activity from Bacillus caldotenax.

SEQ ID NO:15: PCR primer BcaRNIII-3 for cloning a

gene encoding a polypeptide having a RNaseHIII activity from Bacillus caldotenax.

SEQ ID NO:16: Nucleotide sequence of ORF in RNaseHIII from Bacillus caldotenax.

SEQ ID NO:17: Amino acid sequence of RNaseHIII from Bacillus caldotenax.

SEQ ID NO:18: PCR primer BcaRNIIINde for amplifying a gene encoding a polypeptide having a RNaseHIII activity from Bacillus caldotenax.

SEQ ID NO:19: Nucleotide sequence conserving between PH1650 and a portion of Pyrococcus furiosus genome sequence.

SEQ ID NO:20: PCR primer 1650Nde for cloning a gene encoding a polypeptide having a RNaseHII activity from Pyrococcus furiosus.

SEQ ID NO:21: PCR primer 1650Bam for cloning a gene encoding a polypeptide having a RNaseHII activity from Pyrococcus furiosus.

SEQ ID NO:22: Nucleotide sequence of ORF in RNaseHII from Pyrococcus furiosus.

SEQ ID NO:23: Amino acid sequence of RNaseHII from Pyrococcus furiosus.

SEQ ID NO:24: PCR primer 915-F1 for cloning a gene encoding a polypeptide having a RNaseHII activity from Thermotoga maritima.

SEQ ID NO:25: PCR primer 915-F2 for cloning a gene encoding a polypeptide having a RNaseHII activity from Thermotoga maritima.

SEQ ID NO:26: PCR primer 915-R1 for cloning a gene encoding a polypeptide having a RNaseHII activity from Thermotoga maritima.

SEQ ID NO:27: PCR primer 915-R2 for cloning a gene encoding a polypeptide having a RNaseHII activity from Thermotoga maritima.

SEQ ID NO:28: Designed chimeric oligonucleotide primer designated as pUC19 upper 150 to amplify a portion of plasmid pUC19. "nucleotides 24 to 25 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:29: Designed chimeric oligonucleotide primer designated as MR1N3 to amplify a portion of plasmid pUC19. "nucleotides 28 to 30 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:30: Designed oligonucleotide primer designated as M13M4

SEQ ID NO:31: Designed chimeric oligonucleotide primer to amplify a portion of vero toxin 1-encoding sequence from hemorrhagic Escherichia coli O-157. "nucleotides 16 to 18 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:32: Designed chimeric oligonucleotide

primer to amplify a portion of vero toxin 1-encoding sequence from hemorrhagic Escherichia coli O-157. "nucleotides 15 to 17 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:33: Designed chimeric oligonucleotide primer to amplify a portion of vero toxin 2-encoding sequence from hemorrhagic Escherichia coli O-157. "nucleotides 16 to 18 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:34: Designed chimeric oligonucleotide primer to amplify a portion of vero toxin 2-encoding sequence from hemorrhagic Escherichia coli O-157. "nucleotides 16 to 18 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:35: Designed oligonucleotide primer designated as MCR-F to amplify a long DNA fragment

SEQ ID NO:36: Designed oligonucleotide primer designated as MCR-R to amplify a long DNA fragment

SEQ ID NO:37: Designed chimeric oligonucleotide primer designated as MF2N3(24) to amplify a long DNA fragment. "nucleotides 22 to 24 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:38: Designed chimeric oligonucleotide primer designated as MR1N3(24) to amplify a long DNA fragment. "nucleotides 22 to 24 are ribonucleotides-other

nucleotides are deoxyribonucleotides"

SEQ ID NO:39: Designed oligonucleotide primer to amplify a portion of lambda DNA. "nucleotides 18 to 20 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:40: Designed chimeric oligonucleotide primer to amplify a portion of lambda DNA. "nucleotides 18 to 20 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:41: Designed oligonucleotide primer to amplify a portion of lambda DNA

SEQ ID NO:42: Designed oligonucleotide primer to amplify a portion of lambda DNA

SEQ ID NO:43: Designed chimeric oligonucleotide primer to amplify a portion of Flavobacterium species DNA. "nucleotides 18 to 20 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:44: Designed chimeric oligonucleotide primer to amplify a portion of Flavobacterium species DNA. "nucleotides 18 to 20 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:45: Designed oligonucleotide primer to amplify a portion of Flavobacterium species DNA.

SEQ ID NO:46: Designed oligonucleotide primer to amplify a portion of

Flavobacterium species DNA.

SEQ ID NO:47: Designed chimeric oligonucleotide primer to amplify a portion of vero toxin 2-encoding sequence from hemorrhagic Escherichia coli O-157. "nucleotides 19 to 21 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:48: Designed chimeric oligonucleotide primer to amplify a portion of vero toxin 2-encoding sequence from hemorrhagic Escherichia coli O-157. "nucleotides 18 to 20 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:49: Designed oligonucleotide primer to amplify a portion of vero toxin 2-encoding sequence from hemorrhagic Escherichia coli O-157.

SEQ ID NO:50: Designed oligonucleotide primer to amplify a portion of vero toxin 2-encoding sequence from hemorrhagic Escherichia coli O-157.

SEQ ID NO:51: Designed chimeric oligonucleotide primer to amplify a portion of vero toxin 2-encoding sequence from hemorrhagic Escherichia coli O-157. "nucleotides 18 to 20 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:52: Designed chimeric oligonucleotide primer to amplify a portion of vero toxin 2-encoding sequence from hemorrhagic Escherichia coli O-157.

"nucleotides 18 to 20 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:53: Designed chimeric oligonucleotide primer to amplify a portion of vero toxin 2-encoding sequence from hemorrhagic Escherichia coli O-157. "nucleotides 18 to 20 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:54: Designed oligonucleotide primer to amplify a portion of vero toxin 2-encoding sequence from hemorrhagic Escherichia coli O-157.

SEQ ID NO:55: Designed oligonucleotide primer to amplify a portion of vero toxin 2-encoding sequence from hemorrhagic Escherichia coli O-157.

SEQ ID NO:56: Designed chimeric oligonucleotide primer to amplify a portion of lambda DNA. "nucleotides 18 to 20 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:57: Designed oligonucleotide primer to amplify a portion of viroid CSVd.

SEQ ID NO:58: Designed oligonucleotide primer to amplify a portion of viroid CSVd.

SEQ ID NO:59: Designed chimeric oligonucleotide primer to amplify a portion of viroid CSVd. "nucleotides 16 to 18 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:60: Designed chimeric oligonucleotide primer to amplify a portion of viroid CSVd. "nucleotides 18 to 20 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:61: Designed chimeric oligonucleotide primer to amplify a portion of Flavobacterium species DNA. "nucleotides 18 to 20 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:62: Designed chimeric oligonucleotide primer to amplify a portion of Flavobacterium species DNA. "nucleotides 18 to 20 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:63: Designed chimeric oligonucleotide primer to amplify a portion of Flavobacterium species DNA. "nucleotides 18 to 20 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:64: Designed chimeric oligonucleotide primer to amplify a portion of vero toxin 2-encoding sequence from hemorrhagic Escherichia coli O-157. "nucleotides 19 to 21 are ribonucleotides-nucleotide 18 is inosine-other nucleotides are deoxyribonucleotides"

SEQ ID NO:65: Designed chimeric oligonucleotide primer to amplify a portion of vero toxin 2-encoding sequence from hemorrhagic Escherichia coli O-157. "nucleotides 19 to 21 are ribonucleotides-nucleotide 17 is

inosine other nucleotides are deoxyribonucleotides"

SEQ ID NO:66: Designed chimeric oligonucleotide primer to amplify a portion of vero toxin 2-encoding sequence from hemorrhagic Escherichia coli O-157. "nucleotides 19 to 21 are ribonucleotides-nucleotide 16 is inosine-other nucleotides are deoxyribonucleotides"

SEQ ID NO:67: Designed chimeric oligonucleotide primer to amplify a portion of vero toxin 2-encoding sequence from hemorrhagic Escherichia coli O-157. "nucleotides 18 to 20 are ribonucleotides-nucleotide 17 is inosine-other nucleotides are deoxyribonucleotides"

SEQ ID NO:68: Designed chimeric oligonucleotide primer to amplify a portion of vero toxin 2-encoding sequence from hemorrhagic Escherichia coli O-157. "nucleotides 18 to 20 are ribonucleotides-nucleotide 16 is inosine-other nucleotides are deoxyribonucleotides"

SEQ ID NO:69: Designed chimeric oligonucleotide primer to amplify a portion of vero toxin 2-encoding sequence from hemorrhagic Escherichia coli O-157. "nucleotides 18 to 20 are ribonucleotides-nucleotide 15 is inosine-other nucleotides are deoxyribonucleotides"

SEQ ID NO:70: Designed chimeric oligonucleotide primer to amplify a portion of vero toxin 2-encoding sequence from hemorrhagic Escherichia coli O-157. "nucleotides 9 to 11 and 19 to 21 are ribonucleotides-other

nucleotides are deoxyribonucleotides"

SEQ ID NO:71: Designed chimeric oligonucleotide primer to amplify a portion of vero toxin 2-encoding sequence from hemorrhagic Escherichia coli O-157. "nucleotides 8 to 10 and 18 to 20 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:72: Designed chimeric oligonucleotide primer to amplify a portion of vero toxin 2-encoding sequence from hemorrhagic Escherichia coli O-157. "nucleotides 18 to 20 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:73: Designed oligonucleotide probe to detect a DNA fragment amplifying a portion of vero toxin 2-encoding sequence from hemorrhagic Escherichia coli O-157.

SEQ ID NO:74: Designed chimeric oligonucleotide primer to amplify a portion of iNOS-encoding sequence from mouse. "nucleotides 18 to 20 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:75: Designed chimeric oligonucleotide primer to amplify a portion of iNOS-encoding sequence from mouse. "nucleotides 17 to 19 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:76: Designed oligonucleotide primer to amplify a portion of iNOS-encoding sequence from mouse.

SEQ ID NO:77: Designed oligonucleotide primer to

amplify a portion of iNOS-encoding sequence from mouse

SEQ ID NO:78: Designed oligonucleotide primer designated as GMO-PCR-F 20mer

SEQ ID NO:79: Designed oligonucleotide primer designated as GMO-PCR-R 20mer

SEQ ID NO:80: Designed chimeric oligonucleotide primer designated as GMO-S1 20mer. "nucleotides 19 to 20 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:81: Designed oligonucleotide primer designated as GMO-S2 20mer. "nucleotides 19 to 20 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:82: Designed oligonucleotide primer designated as GMO-A1 20mer. "nucleotides 19 to 20 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:83: Designed oligonucleotide primer designated as GMO-A2 20 mer. "nucleotides 19 to 20 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:84: Designed chimeric oligonucleotide primer to amplify a portion of vero toxin 2-encoding sequence from hemorrhagic Escherichia coli O-157. "nucleotides 18 to 20 are (alpha-thio)ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:85: Designed chimeric oligonucleotide primer to amplify a portion of vero toxin 2-encoding

sequence from hemorrhagic Escherichia coli O-157. "nucleotides 18 to 20 are (alpha-thio)ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:86: Designed chimeric oligonucleotide primer to amplify a portion of INOS-encoding sequence from mouse."nucleotides 20 to 22 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:87: Designed chimeric oligonucleotide primer to amplify a portion of INOS-encoding sequence from mouse."nucleotides 20 to 22 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:88: Designed oligonucleotide primer to amplify a portion of INOS-encoding sequence from mouse.

SEQ ID NO:89: Designed oligonucleotide primer to amplify a portion of INOS-encoding sequence from mouse.

SEQ ID NO:90: Designed chimeric oligonucleotide primer to amplify a portion of lambda DNA."nucleotides 18 to 20 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:91: Designed chimeric oligonucleotide primer to amplify a portion of lambda DNA."nucleotides 19 to 21 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:92: Designed chimeric oligonucleotide primer to amplify a portion of INOS-encoding sequence from

mouse."nucleotides 21 to 23 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:93: Designed chimeric oligonucleotide primer to amplify a portion of INOS-encoding sequence from mouse."nucleotides 20 to 22 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:94: Designed chimeric oligonucleotide primer to amplify a portion of pDON-AI DNA."nucleotides 17 to 19 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:95: Designed chimeric oligonucleotide primer to amplify a portion of pDON-AI DNA."nucleotides 19 to 21 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:96: Designed chimeric oligonucleotide primer to amplify a portion of HPV DNA."nucleotides 19 to 21 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:97: Designed chimeric oligonucleotide primer to amplify a portion of HPV DNA."nucleotides 19 to 21 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:98: Designed oligonucleotide probe to detect a DNA fragment amplifying a portion of HPV DNA.

SEQ ID NO:99: Designed oligonucleotide primer to

amplify a portion of HCV.

SEQ ID NO:100: Designed oligonucleotide primer to amplify a portion of HCV.

SEQ ID NO:101: Designed chimeric oligonucleotide primer to amplify a portion of HCV."nucleotides 19 to 21 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:102: Designed chimeric oligonucleotide primer to amplify a portion of HCV."nucleotides 16 to 18 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:103: Designed oligonucleotide probe to detect a DNA fragment amplifying portion of HCV.

SEQ ID NO:104: Designed chimeric oligonucleotide primer to amplify a portion of adenovirus."nucleotides 19 to 21 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:105: Designed chimeric oligonucleotide primer to amplify a portion of adenovirus."nucleotides 19 to 21 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:106: Designed chimeric oligonucleotide primer to amplify a portion of adenovirus."nucleotides 19 to 21 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:107: Designed oligonucleotide primer to amplify a portion of adenovirus

SEQ ID NO:108: Designed oligonucleotide primer to amplify a portion of adenovirus.

SEQ ID NO:109: Designed oligonucleotide primer to amplify a portion of viroid CSVd.

SEQ ID NO:110: Designed oligonucleotide primer to amplify a portion of viroid CSVd.

SEQ ID NO:111: Designed oligonucleotide primer to amplify a portion of pDON-AI DNA.

SEQ ID NO:112: Designed oligonucleotide primer to amplify a portion of pDON-AI DNA.

SEQ ID NO:113: Designed chimeric oligonucleotide primer to amplify a portion of verotoxin-1 encoding sequence from hemorrhagic Escherichia coli 0-157."nucleotides 18 to 20 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:114: Designed chimeric oligonucleotide primer to amplify a portion of verotoxin-1 encoding sequence from hemorrhagic Escherichia coli 0-157."nucleotides 18 to 20 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:115: Designed oligonucleotide probe to detect a DNA fragment amplifying a portion of verotoxin-1 encoding sequence from hemorrhagic Escherichia coli 0-157.

SEQ ID NO:116: Designed chimeric oligonucleotide primer to amplify a portion of botulinum toxin A encoding sequence from Clostridium botulinum."nucleotides 19 to 21 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:117: Designed chimeric oligonucleotide primer to amplify a portion of botulinum toxin A encoding sequence from Clostridium botulinum."nucleotides 21 to 23 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:118: Designed oligonucleotide probe to detect a DNA fragment amplifying a portion of botulinum toxin A encoding sequence from Clostridium botulinum.

SEQ ID NO:119: Designed chimeric oligonucleotide primer to amplify a portion of viroid CSVd."nucleotides 19 to 21 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:120: Designed chimeric oligonucleotide primer to amplify a portion of viroid CSVd."nucleotides 18 to 20 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:121: Designed oligonucleotide probe to detect a DNA fragment amplifying a portion of viroid CSVd.

SEQ ID NO:122: Designed chimeric oligonucleotide primer to amplify a portion of viroid CSVd."nucleotides 19

to 21 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:123: Designed chimeric oligonucleotide primer to amplify a portion of viroid CSVd."nucleotides 19 to 21 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:124: Designed oligonucleotide primer to amplify a portion of viroid CSVd.

SEQ ID NO:125: Designed oligonucleotide primer to amplify a portion of viroid CSVd.

SEQ ID NO:126: Designed chimeric oligonucleotide primer to amplify a portion of c-ki-ras oncogene."nucleotides 18 to 20 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:127: Designed chimeric oligonucleotide primer to amplify a portion of c-ki-ras oncogene."nucleotides 18 to 20 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:128: Designed oligonucleotide primer to amplify a portion of c-ki-ras oncogene.

SEQ ID NO:129: Designed oligonucleotide primer to amplify a portion of c-ki-ras oncogene.

SEQ ID NO:130: Designed chimeric oligonucleotide primer to amplify a portion of verotoxin-1 encoding sequence from hemorrhagic Escherichia coli O-

157."nucleotides 18 to 20 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:131: Designed chimeric oligonucleotide primer to amplify a portion of verotoxin-1 encoding sequence from hemorrhagic Escherichia coli O-157."nucleotides 18 to 20 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:132: Designed oligonucleotide primer to amplify a portion of INOS-encoding sequence from mouse.

SEQ ID NO:133: Designed oligonucleotide primer to amplify a portion of INOS-encoding sequence from mouse.

SEQ ID NO:134: Designed oligonucleotide primer designated as pUC19 upper 150 to amplify a portion of plasmid pUC19.

SEQ ID NO:135: Designed oligonucleotide primer designated as pUC19 lower NN to amplify a portion of plasmid pUC19.

SEQ ID NO:136: Designed chimeric oligonucleotide primer designated as SEA-1 to amplify a portion of Staphylococcus aureus. "nucleotides 19 to 21 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:137: Designed chimeric oligonucleotide primer designated as SEA-2 to amplify a portion of Staphylococcus aureus. "nucleotides 19 to 21 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:138: Designed chimeric oligonucleotide primer designated as HCV-F3 to amplify a portion of HCV. "nucleotides 17 to 19 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:139: Designed chimeric oligonucleotide primer designated as HCV-R1 to amplify a portion of HCV. "nucleotides 16 to 18 are ribonucleotides-other nucleotides are deoxyribonucleotides"

SEQ ID NO:140: Designed oligonucleotide primer designated as MF2 to amplify a portion of pUC19 plasmid DNA.

SEQ ID NO:141: Designed oligonucleotide primer designated as MR1 to amplify a portion of pUC19 plasmid DNA.

SEQ ID NO:142: Designed oligonucleotide primer to amplify a portion of adenovirus.

SEQ ID NO:143: Nucleotide sequence of ORF in RNaseHII gene from Thermotoga maritima.

SEQ ID NO:144: Amino acid sequence of RNaseHII from Thermotoga maritima.

SEQ ID NO:145: Nucleotide sequence of PH1650 from Pyrococcus horikoshii.

SEQ ID NO:146: PCR primer PhoNde for cloning a gene encoding a polypeptide having a RNaseHII activity from Pyrococcus horikoshii

SEQ ID NO:147: PCR primer PhoBam for cloning a gene encoding a polypeptide having a RNaseHII activity from

Pyrococcus horikoshii

SEQ ID NO:148: Nucleotide sequence of ORF in RNaseHII gene from Pyrococcus horikoshii.

SEQ ID NO:149: Amino acid sequence of RNaseHII from Pyrococcus horikoshii.

SEQ ID NO:150: Nucleotide sequence of AF0621 from Archaeoglobus fulgidus.

SEQ ID NO:151: PCR primer AfuNde for cloning a gene encoding a polypeptide having a RNaseHII activity from Archaeoglobus fulgidus

SEQ ID NO:152: PCR primer AfuBam for cloning a gene encoding a polypeptide having a RNaseHII activity from Archaeoglobus fulgidus

SEQ ID NO:153: Nucleotide sequence of ORF in RNaseHII gene from Archaeoglobus fulgidus.

SEQ ID NO:154: Amino acid sequence of RNaseHII from Archaeoglobus fulgidus.

SEQ ID NO:155: Designed chimeric oligonucleotide primer designated as MTIS2F to amplify a portion of Mycobacterium tuberculosis DNA."nucleotides 16 to 18 are ribonucleotides-other nucleotides are deoxyribonucleotides.".

SEQ ID NO:156: Designed chimeric oligonucleotide primer designated as MTIS2R to amplify a portion of Mycobacterium tuberculosis DNA."nucleotides 19 to 21 are

ribonucleotides-other nucleotides are deoxyribonucleotides.".

SEQ ID NO:157: Designed chimeric oligonucleotide primer designated as CT2F to amplify a portion of Chlamydia trachomatis cryptic plasmid DNA."nucleotides 18 to 20 are ribonucleotides-other nucleotides are deoxyribonucleotides.".

SEQ ID NO:158: Designed chimeric oligonucleotide primer designated as CT2R to amplify a portion of Chlamydia trachomatis cryptic plasmid DNA."nucleotides 16 to 18 are ribonucleotides-other nucleotides are deoxyribonucleotides.".

SEQ ID NO:159: Designed chimeric oligonucleotide primer designated as K-F-1033(60) to amplify a portion of Mycobacterium tuberculosis DNA."nucleotides 17 to 19 are ribonucleotides-other nucleotides are deoxyribonucleotides.".

SEQ ID NO:160: Designed chimeric oligonucleotide primer designated as K-R-1133(62) to amplify a portion of Mycobacterium tuberculosis DNA."nucleotides 18 to 20 are ribonucleotides-other nucleotides are deoxyribonucleotides.".

SEQ ID NO:161: Designed chimeric oligonucleotide primer designated as K-F-1033(68) to amplify a portion of Mycobacterium tuberculosis DNA."nucleotides 20 to 22 are

ribonucleotides-other nucleotides are deoxyribonucleotides.".

SEQ ID NO:162: Designed chimeric oligonucleotide primer designated as K-R-1133(68) to amplify a portion of Mycobacterium tuberculosis DNA."nucleotides 20 to 22 are ribonucleotides-other nucleotides are deoxyribonucleotides.".

SEQ ID NO:163: Designed oligonucleotide primer designated as F26 to amplify a portion of Mycobacterium tuberculosis DNA.

SEQ ID NO:164: Designed oligonucleotide primer designated as R1310 to amplify a portion of Mycobacterium tuberculosis DNA.

SEQ ID NO:165: Designed chimeric oligonucleotide primer designated as pDON-AI-68-1 to amplify a portion of pDON-AI."nucleotides 20 to 22 are ribonucleotides-other nucleotides are deoxyribonucleotides.".

SEQ ID NO:166: Designed chimeric oligonucleotide primer designated as pDON-AI-68-2 to amplify a portion of pDON-AI."nucleotides 21 to 23 are ribonucleotides-other nucleotides are deoxyribonucleotides.".

SEQ ID NO:167: Nucleotide sequence of Homo sapiens proto-oncogene Wn t-5a

SEQ ID NO:168: Nucleotide sequence of Homo sapiens ribosomal protein S5

SEQ ID NO:169: Nucleotide sequence of Homo sapiens diaphorase

SEQ ID NO:170: Nucleotide sequence of Human protocadherin

SEQ ID NO:171: Designed oligonucleotide for making of pIC62.

SEQ ID NO:172: Designed chimeric oligonucleotide primer designated as ICAN2."nucleotides 19 to 20 are ribonucleotides-other nucleotides are deoxyribonucleotides.".

SEQ ID NO:173: Designed chimeric oligonucleotide primer designated as ICAN6."nucleotides 19 to 20 are ribonucleotides-other nucleotides are deoxyribonucleotides.".

SEQ ID NO:174: Designed oligonucleotide primer designated as ICAN2 DNA.

SEQ ID NO:175: Designed oligonucleotide primer designated as ICAN6 DNA.

SEQ ID NO:176: Designed oligonucleotide primer to amplify a portion of ribosomal protein S18-encoding sequence from mouse.

SEQ ID NO:177: Designed oligonucleotide primer to amplify a portion of ribosomal protein S18-encoding sequence from mouse.

SEQ ID NO:178: Designed oligonucleotide primer to

amplify a portion of transferrin receptor (TFR)-encoding sequence from mouse.

SEQ ID NO:179: Designed oligonucleotide primer to amplify a portion of transferrin receptor (TFR)-encoding sequence from mouse.

SEQ ID NO:180: Designed oligonucleotide primer to amplify a portion of stromal cell derived factor 4 (Sdf4)-encoding sequence from mouse.

SEQ ID NO:181: Designed oligonucleotide primer to amplify a portion of stromal cell derived factor 4 (Sdf4)-encoding sequence from mouse.

SEQ ID NO:182: Designed oligonucleotide primer to amplify a portion of cytoplasmic beta-actin encoding sequence from mouse.

SEQ ID NO:183: Designed oligonucleotide primer to amplify a portion of cytoplasmic beta-actin encoding sequence from mouse.

SEQ ID NO:184: Designed oligonucleotide primer to amplify a portion of ornithine decarboxylase-encoding sequence from mouse.

SEQ ID NO:185: Designed oligonucleotide primer to amplify a portion of ornithine decarboxylase-encoding sequence from mouse.

SEQ ID NO:186: Designed oligonucleotide primer to amplify a portion of hypoxanthine guanine phosphoribosyl

transferase (HPRT)-encoding sequence from mouse.

SEQ ID NO:187: Designed oligonucleotide primer to amplify a portion of hypoxanthine guanine phosphoribosyl transferase (HPRT)-encoding sequence from mouse.

SEQ ID NO:188: Designed oligonucleotide primer to amplify a portion of tyrosine 3-monooxygenase encoding sequence from mouse.

SEQ ID NO:189: Designed oligonucleotide primer to amplify a portion of tyrosine 3-monooxygenase encoding sequence from mouse.

SEQ ID NO:190: Designed oligonucleotide primer designated as MCS-F.

SEQ ID NO:191: Designed oligonucleotide primer designated as MCS-R

SEQ ID NO:192: Designed chimeric oligonucleotide primer designated as MF2N3(24). "nucleotides 22 to 24 are ribonucleoitdes-other nucleotides are deoxyribonucleotides"

SEQ ID NO:193: Designed chimeric oligonucleotide primer designated as MR1N3(24). "nucleotides 22 to 24 are ribonucleoitdes-other nucleotides are deoxyribonucleotides"

SEQ ID NO:194: Designed chimeric oligonucleotide primer designated as MTIS2F-16 to amplify a portion of Mycobacterium tuberculosis DNA."nucleotides 14 to 16 are ribonucleotides-other nucleotides are deoxyribonucleotides.

SEQ ID NO:195: Designed chimeric oligonucleotide

primer designated as MTIS2R-ACC to amplify a portion of Mycobacterium tuberculosis DNA."nucleotides 18 to 20 are ribonucleotides-other nucleotides are deoxyribonucleotides."

SEQ ID NO:196: Designed oligonucleotide primer designated as MTIS-PCR-F-2 to amplify a portion of Mycobacterium tuberculosis DNA

SEQ ID NO:197: Designed oligonucleotide primer designated as MTIS-PCR-R-2 to amplify a portion of Mycobacterium tuberculosis DNA

SEQ ID NO:198: Designed oligonucleotide primer designated as SP6-HCV-F to amplify a portion of HCV

SEQ ID NO:199: Designed oligonucleotide primer designated as SP6-HCV-R to amplify a portion of HCV

SEQ ID NO:200: Designed chimeric oligonucleotide primer designated as HCV-A S to amplify a portion of HCV. "nucleotides 18 to 20 are ribonucleotides-other nucleotides are deoxyribonucleotides."

SEQ ID NO:201: Designed chimeric oligonucleotide primer designated as HCV-A A to amplify a portion of HCV. "nucleotides 18 to 20 are ribonucleotides-other nucleotides are deoxyribonucleotides.

11 FEB 2005

CLAIMS

1. A method for amplifying a 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.

2. The method according to claim 1, wherein the reaction mixture further contains a chimeric oligonucleotide primer having a sequence substantially homologous to the nucleotide sequence of the nucleic acid as the template.

3. 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.

4. 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.

5. The method according to claim 3 or 4, wherein the DNA polymerase is at least one DNA polymerase having a strand displacement activity.

6. 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 sites that contain the ribonucleotide of the double-stranded nucleic acid consisting of the primer-extended strands obtained in step (a) with the endonuclease; 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.

7. 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 sites that contain the ribonucleotide of the double-stranded nucleic acid consisting of the primer-extended strands obtained in step (a) with the endonuclease; 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.

8. 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 sites that contain the ribonucleotide of the double-stranded nucleic acid consisting of the primer-extended strands obtained in step (a) with the endonuclease;

(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).

9. 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 sites that contain the ribonucleotide of the double-stranded nucleic acid consisting of the primer-extended strands obtained in step (a) with the endonuclease;

(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 sites that contain the ribonucleotide of the double-stranded nucleic acid consisting of the template and the primer-extended strand obtained in step (d) with the endonuclease; 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.

10. The method according to any one of claims 6 to 9, wherein the endonuclease is an endoribonuclease.

11. The method according to claim 10, wherein the endoribonuclease is an RNase H.

12. The method according to any one of claims 1 to 5 and 11, wherein the RNase H is 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.

13. The method according to any one of claims 1 to 12, wherein the length of the region of the nucleic acid to be amplified is 200 bp or shorter.

14. The method according to any one of claims 1 to 13, wherein a chimeric oligonucleotide primer represented by general formula below is 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).

15. The method according to claim 14, wherein c is 0.

16. The method according to claim 14 or 15, wherein the nucleotide analog is deoxyriboinosine nucleotide or deoxyribouracil nucleotide and the modified ribonucleotide is (α-S) ribonucleotide.

17. The method according to any one of claims 14 to 16, wherein the DNA extension reaction is conducted at a DNA extension reaction temperature suitable for the

chimeric oligonucleotide primer as defined in any one of claims 14 to 16.

18. The method according to any one of claims 1 to 17, which comprises 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.

19. The method according to claim 18, wherein the annealing solution contains spermidine and/or propylenediamine.

20. The method according to claim 18 or 19, wherein the annealing is 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.

21. The method according to any one of claims 1 to 20, wherein the amplification reaction is conducted in a buffer containing a buffering component selected from the group consisting of Bicine and HEPES.

22. The method according to any one of claims 1

to 21, wherein the DNA polymerase having a strand displacement activity is 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.

23. The method according to any one of claims 1 to 5 and 11 to 22, wherein the DNA polymerase having a strand displacement activity is Bca DNA polymerase lacking 5'→3' exonuclease from Bacillus caldotenax and the RNase H is selected from the group consisting of an RNase H from Escherichia coli, an RNase H from a bacterium of genus Pyrococcus and an RNase H from a bacterium of genus Archaeoglobus.

24. The method according to claim 23, wherein the RNase H is type I RNase H from Escherichia coli, or type II RNase H from a bacterium of genus Pyrococcus or a bacterium of genus Archaeoglobus.

25. The method according to any one of claims 1 to 24, wherein a DNA polymerase having an endonuclease activity is used.

26. The method according to claim 25, wherein the DNA polymerase is Bca DNA polymerase lacking 5'→3' exonuclease from Bacillus caldotenax and the Bca DNA polymerase is used in the presence of a substance that

allows the endonuclease activity of the Bca DNA polymerase to express.

27. The method according to claim 26, wherein the substance that allows the endonuclease activity of the DNA polymerase to express is a manganese ion.

28. The method according to any one of claims 1 to 27, wherein the amplification reaction is conducted in the presence of a substance that inhibits the reverse transcription activity of the DNA polymerase.

29. The method according to claim 28, wherein the substance that inhibits the reverse transcription activity of the DNA polymerase is phosphonoformic acid.

30. The method according any one of claims 1 to 29, wherein the nucleic acid as the template is a single-stranded DNA or a double-stranded DNA.

31. The method according to claim 30, which is conducted after converting a double-stranded DNA as the template into single-stranded DNAs.

32. The method according to claim 30 or 31, wherein the nucleic acid as the template is a cDNA obtained by a reverse transcription reaction using an RNA as a template.

33. The method according to claim 32, which is conducted after synthesizing a cDNA by a reverse transcription reaction using an RNA as a template.

34. The method according to claim 32 or 33, wherein a DNA polymerase having a reverse transcriptase activity is used as a reverse transcriptase.

35. The method according to any one of claims 31 to 34, wherein the reverse transcription reaction and the synthesis of the extended strand that is complementary to the template are conducted using one DNA polymerase having both a reverse transcriptase activity and a strand displacement activity.

36. The method according to claim 35, wherein the DNA polymerase is Bst DNA polymerase lacking 5'→3' exonuclease from Bacillus stearothermophilus or Bca DNA polymerase lacking 5'→3' exonuclease from Bacillus caldotenax.

37. The method according to any one of claims 1 to 36, wherein the nucleic acid amplification reaction is conducted under isothermal conditions.

38. The method according to any one of claims 1 to 37, which is conducted in the presence of a deoxyribonucleotide triphosphate analog.

39. The method according to claim 38, wherein the deoxyribonucleotide triphosphate analog is deoxyuridine triphosphate or a derivative thereof.

40. 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.

41. 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.

42. 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.

43. 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.

44. The composition according to any one of claims 40 to 43, wherein the primer is 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).

45. The composition according to claim 44, wherein c is 0.

46. The composition according to claim 44 or 45, wherein the nucleotide analog is deoxyriboinosine nucleotide or deoxyribouracil nucleotide and the modified ribonucleotide is (α-S) ribonucleotide.

47. The composition according to any one of claims 40 to 46, which contains a buffering component suitable for a nucleic acid amplification reaction.

48. The composition according to claim 47, which contains a buffering component selected from the group consisting of Bicine and HEPES.

49. The composition according to any one of claims 40 to 48, wherein 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 is used as the DNA polymerase having a strand displacement activity.

50. The composition according to any one of claims 40 to 49, wherein the endonuclease is an endoribonuclease.

51. The composition according to claim 50, wherein the endoribonuclease is an RNase H.

52. The composition according to claim 51, wherein the RNase H is 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.

53. The composition according to any one of claims 40 to 52, wherein the DNA polymerase having a strand displacement activity is Bca DNA polymerase lacking 5'→3' exonuclease from Bacillus caldotenax and the endonuclease is an RNase H selected from the group consisting of an RNase H from Escherichia coli, an RNase H from a bacterium

of genus Pyrococcus and an RNase H from a bacterium of genus Archaeoglobus.

54. The composition according to claim 53, wherein the RNase H is type I RNase H from Escherichia coli, or type II RNase H from a bacterium of genus Pyrococcus or a bacterium of genus Archaeoglobus.

55. The composition according to any one of claims 40 to 54, wherein a DNA polymerase having an endonuclease activity is used.

56. The composition according to claim 55, wherein the DNA polymerase is Bca DNA polymerase lacking 5'→3' exonuclease from Bacillus caldotenax, the composition containing a substance that allows the endonuclease activity of the Bca DNA polymerase to express.

57. The composition according to claim 56, wherein the substance that allows the endonuclease activity of the DNA polymerase to express is a manganese ion.

58. The composition according to any one of claims 40 to 57, which contains a substance that inhibits the reverse transcription activity of the DNA polymerase.

59. The composition according to claim 58, wherein the substance that inhibits the reverse transcription activity of the DNA polymerase is phosphonoformic acid.

60. The composition according to any one of

claims 40 to 59, which contains a deoxyribonucleotide triphosphate analog.

61. The composition according to claim 60, wherein the deoxyribonucleotide triphosphate analog is deoxyuridine triphosphate or a derivative thereof.

62. A composition for amplifying a nucleic acid used for the method for amplifying a nucleic acid defined by any one of claims 1 to 5, which contains:

(a) an RNase H; and

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

63. A composition for amplifying a nucleic acid used for the method for amplifying a nucleic acid defined by any one of claims 6 to 9, which contains:

(a) an endonuclease; and

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

64. The composition according to claim 63, wherein the endonuclease is an endoribonuclease.

65. The composition according to claim 64, wherein the endoribonuclease is an RNase H.

66. The composition according to claim 62 or 65, wherein the RNase H is 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.

67. The composition according to any one of claims 62 to 66, which contains a buffering component suitable for a nucleic acid amplification reaction.

68. The composition according to claim 67, which contains a buffering component selected from the group consisting of Bicine and HEPES.

69. The composition according to any one of claims 62 to 68, wherein 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 is used as the DNA polymerase having a strand displacement activity.

70. The composition according to claim 62, wherein the DNA polymerase having a strand displacement activity is Bca DNA polymerase lacking 5'→3' exonuclease from Bacillus caldotenax and the RNase H is an RNase H selected from the group consisting of an RNase H from Escherichia coli, an RNase H from a bacterium of genus Pyrococcus and an RNase H from a bacterium of genus

Archaeoglobus.

71. The composition according to claim 63, wherein the DNA polymerase having a strand displacement activity is Bca DNA polymerase lacking 5'→3' exonuclease from Bacillus caldotenax and the endonuclease is an RNase H selected from the group consisting of an RNase H from Escherichia coli, an RNase H from a bacterium of genus Pyrococcus and an RNase H from a bacterium of genus Archaeoglobus.

72. The composition according to any one of claims 62 to 71, wherein a DNA polymerase having an endonuclease activity is used.

73. The composition according to claim 72, wherein the DNA polymerase is Bca DNA polymerase lacking 5'→3' exonuclease from Bacillus caldotenax, the composition containing a substance that allows the endonuclease activity of the Bca DNA polymerase to express.

74. The composition according to claim 73, wherein the substance that allows the endonuclease activity of the DNA polymerase to express is a manganese ion.

75. The composition according to any one of claims 62 to 74, which contains a substance that inhibits the reverse transcription activity of the DNA polymerase.

76. The composition according to claim 75, wherein the substance that inhibits the reverse

transcription activity of the DNA polymerase is phosphonoformic acid.

77. The composition according to any one of claims 62 to 76, which contains a deoxynucleotide triphosphate analog.

78. The composition according to claim 77, wherein the deoxynucleotide triphosphate analog is deoxyuridine triphosphate or a derivative thereof.

79. A kit for amplifying a nucleic acid used for the method for amplifying a nucleic acid defined by any one of claims 1 to 5, which contains:

(a) an RNase H; and

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

80. A kit for amplifying a nucleic acid used for the method for amplifying a nucleic acid defined by any one of claims 6 to 9, which contains:

(a) an endonuclease; and

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

81. The kit according to claim 80, wherein the endonuclease is an endoribonuclease.

82. The kit according to claim 81, wherein the endoribonuclease is an RNase H.

83. The kit according to claim 79 or 82, wherein

the RNase H is 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.

84. The kit according to any one of claims 79 to 83, which contains a buffer suitable for a nucleic acid amplification reaction.

85. The kit according to claim 84, which contains a buffer for nucleic acid amplification containing a buffering component selected from the group consisting of Bicine and HEPES.

86. The kit according to any one of claims 79 to 85, which contains 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.

87. The kit according to claim 86, wherein the annealing solution contains spermidine and/or propylenediamine.

88. The kit according to any one of claims 79 to 87, wherein 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 is used as the DNA polymerase having a strand displacement activity.

89. The kit according to claim 79, wherein the DNA polymerase having a strand displacement activity is Bca DNA polymerase lacking 5'→3' exonuclease from Bacillus caldotenax and the RNase H is an RNase H selected from the group consisting of an RNase H from Escherichia coli, an RNase H from a bacterium of genus Pyrococcus and an RNase H from a bacterium of genus Archaeoglobus.

90. The kit according to claim 80, wherein the DNA polymerase having a strand displacement activity is Bca DNA polymerase lacking 5'→3' exonuclease from Bacillus caldotenax and the endonuclease is an RNase H selected from the group consisting of an RNase H from Escherichia coli, an RNase H from a bacterium of genus Pyrococcus and an RNase H from a bacterium of genus Archaeoglobus.

91. The kit according to claim 89 or 90, wherein the RNase H is type I RNase H from Escherichia coli, or type II RNase H from a bacterium of genus Pyrococcus or a bacterium of genus Archaeoglobus.

92. The kit according to any one of claims 79 to 91, wherein a DNA polymerase having an endonuclease

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activity is used.

93. The kit according to claim 92, wherein the DNA polymerase is Bca DNA polymerase lacking 5'→3' exonuclease from Bacillus caldotenax, the kit containing a substance that allows the endonuclease activity of the Bca DNA polymerase to express.

94. The kit according to claim 93, wherein the substance that allows the endonuclease activity of the DNA polymerase to express is a manganese ion.

95. The kit according to any one of claims 79 to 94, which contains a substance that inhibits the reverse transcription activity of the DNA polymerase.

96. The kit according to claim 95, wherein the substance that inhibits the reverse transcription activity of the DNA polymerase is phosphonoformic acid.

97. The kit according to any one of claims 79 to 96, which contains a deoxynucleotide triphosphate analog.

98. The kit according to claim 97, wherein the deoxynucleotide triphosphate analog is deoxyuridine triphosphate or a derivative thereof.

99. A kit for amplifying a nucleic acid used for the method for amplifying a nucleic acid defined by any one of claims 1 to 5, which is in a packaged form and contains instructions that direct the use of a DNA polymerase having a strand displacement activity and an RNase H.

100. A kit for amplifying a nucleic acid used for the method for amplifying a nucleic acid defined by any one of claims 6 to 9, 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.

101. 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.

102. 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.

103. 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 defined by any one of claims 1 to 39; and

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

104. The method according to claim 103, which comprises detecting the amplified nucleic acid using a probe for detection.

105. The method according to claim 104, wherein the probe for detection is a probe that has been labeled with a labeling substance.

106. The method according to claim 105, wherein the probe is an RNA probe labeled with two or more fluorescent substances positioned at a distance that results in a quenching state.

107. 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.

108. 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.

109. A chimeric oligonucleotide primer for detecting Clostridium botulinum having a nucleotide sequence represented by SEQ ID NO: 116 or 117.

110. A chimeric oligonucleotide primer for detecting papilloma virus having a nucleotide sequence represented by SEQ ID NO: 96 or 97.

111. 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 and 201.

112. A chimeric oligonucleotide primer for detecting Staphylococcus aureus having a nucleotide sequence represented by SEQ ID NO: 136 or 137.

113. A chimeric oligonucleotide primer for detecting Mycobacterium tuberculosis having a nucleotide sequence selected from the group consisting of SEQ ID NOS:

155, 156, 159-162, 194 and 195.

114. A chimeric oligonucleotide primer for detecting Chlamydia having a nucleotide sequence represented by SEQ ID NO: 157 or 158.

115. A kit for amplifying a nucleic acid used for the method for amplifying a nucleic acid defined by any one of claims 1 to 39, which contains the chimeric oligonucleotide primer defined by any one of claims 107 to 114.

116. A kit for detecting a target nucleic acid used for the method for detecting a target nucleic acid defined by any one of claims 103 to 106, which contains the chimeric oligonucleotide primer defined by any one of claims 107 to 114.

117. A probe which hybridizes to a region of the nucleic acid amplified by the method defined by any one of claims 1 to 39, using the chimeric oligonucleotide primer defined by any one of claims 107 to 114, wherein said probe has been labeled with a labeling substance.

118. The probe according to claim 117, which is an RNA probe labeled with two or more fluorescent substances positioned at a distance that results in a quenching state.

119. A kit used in the method defined by any one of claims 103 to 106, which contains the probe defined by any one of claims 117 to 118.

120. A method for amplifying a nucleic acid, which comprises using a DNA polymerase having a strand displacement activity to effect a template switching reaction.

121. The method according to claim 120, wherein the DNA polymerase having a strand displacement activity is 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.

122. 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 defined by any one of claims 1 to 39; and

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

123. A material having an immobilized nucleic acid in which the nucleic acid is arrayed in a predefined region produced by the method defined by claim 122.

124. 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 defined by any one of claims 1 to 39; and

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

125. 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 defined by any one of claims 1 to 39.

126. A method for determining a nucleotide sequence of a nucleic acid, the method comprising amplifying a nucleic acid according to the method defined by any one of claims 1 to 39, 124 and 125.

127. A method for preparing a single-stranded nucleic acid, the method comprising preparing a single-stranded nucleic acid using the method defined by any one of claims 1 to 39.

128. The method according to claim 127, wherein at least two primers at different concentrations are used.