METHOD FOR EXTRACTING OF NUCLEIC ACID FROM BIOLOGICAL MATERIAL

FIELD OF THE INVENTION

The present invention relates to a method of extracting nucleic acid to be subjected to a nucleic acid amplification reaction from biological material in a biological sample in a simple and quick method, and can be applied to medical fields such as infection testing, research fields such as molecular biology, industrial fields such as food safety management, and the like. Specifically the present invention permits the simple and efficient extraction of nucleic acid from biological materials such as *Mycobacterium* having a rigid cell wall, and subsequent genetic diagnosis using a nucleic acid amplification reaction.

BACKGROUND OF THE INVENTION

Conventionally, when nucleic acid is prepared from a sample of interest, it is commonly performed in two steps of extraction and purification, wherein suitable extraction and purification steps are selected depending on the properties of the sample.

As general methods of extracting nucleic acid from biological materials such as viruses, microorganisms, protozoa, plant and animal tissues, there are mechanical methods using a homogenizer etc., chemical methods using a denaturing agent etc., and biological methods using an enzyme etc. There are also methods in which a particulate solid is mixed with a frozen sample, and the sample is agitated to disrupt the cells. Among them, extraction methods using denaturing agents such as guanidine hydrochloride and guanidine thiocyanate (see Molecular Cloning: A Laboratory Manual, Appendix 7.23-7.25, New York Laboratory, 1989) contain a denaturing agent in the extract, and the agent per se inhibits the amplification reaction, and thus a purification step after extraction is required. Nucleic acid purification

by enzyme treatment is time-consuming, and specifically when it is applied to materials having rigid cell walls such as bacteria of the genus *Mycobacterium* and spores of molds such as fungi, cells cannot be easily disrupted and extraction efficiency of nucleic acid is extremely poor.

Thus, in order to overcome the problem of a cell wall that is rigid and thus cell disruption is difficult, a method of adding microparticles and conducting ultrasonication using an ultrasonic bath to release nucleic acid has been disclosed (see U.S. Pat. No. 5,374,522). This releasing method is simple and has a good disruption efficiency. However, when a test sample such as sputum or feces is used to extract nucleic acid, and the extracted nucleic acid is used for gene amplification, a further purification process is required to remove inhibitors to the amplification reaction.

Thus, in order to perform the rapid processing of a large number of test samples, a nucleic acid extraction method has been sought, which permits a simple and efficient separation of nucleic acid from biological materials and renders the extracts available for gene amplification reaction.

DISCLOSURE OF THE INVENTION

As test samples to be used in gene testing, there can be mentioned blood, urine, sputum, pus, blood culture, swab, colonies and the like. Nucleic acid extracted from these test samples may be subjected to direct testing. However, in recent years in nucleic acid testing, it is more common to amplify nucleic acid extracted from test samples using various amplification reactions prior to measurement in order to enable the qualification and quantitation of minute amounts of nucleic acid. Examples of such nucleic acid amplification reactions include RNA amplification methods such as the Nucleic Acid Sequence Based Amplification (NASBA) method as described in Japanese Patent No. 2650159, the so-called Transcription-Mediated

Amplification (TMA) method as described in Japanese Unexamined Patent Publication (Kohyo) No. 4-500759, and the Transcription-Reverse transcription Concerted reaction (TRC) as described in Japanese Unexamined Patent Publication (Kokai) No. 2000-14400 (Patent application No. 10-186434) which is the method of Examples of the present invention, and the DNA amplification methods such as the polymerase chain reaction (PCR).

It is also possible to extract nucleic acid from the various test samples described above by conventional nucleic acid-extraction methods as described above. However, as the frequency of subjecting nucleic acid extracts to amplification reactions as described above increases, there are more chances that nucleic acid extracts obtained by a conventional nucleic acid extraction method, contain nucleic acid amplification reaction-inhibiting substances that inhibit the activity or reaction of enzymes, such as reverse transcriptase and DNA polymerase for use in nucleic acid amplification reactions as described above. It has been pointed out that sputum, among the above-mentioned test samples is more likely to contain nucleic acid amplification reaction-inhibiting substances derived from biological components such as airway mucus, various nucleic acids and cells, and when they contaminate the nucleic acid extract, nucleic acid amplification reaction is inhibited, resulting in accurate testing.

In gene testing, internal standards are usually used and false negatives can be prevented. However, it is clear that the most desirable solution is to remove nucleic acid amplification reaction-inhibiting substances during the nucleic acid extraction process.

Thus, it is an object of the present invention to provide a method of extracting nucleic acid, simply and efficiently, from bacteria having a rigid cell wall, such as *Mycobacterium*, which is carried out after removing substances that inhibit nucleic acid amplification

reactions from biological materials from which the nucleic acid is extracted, and a kit for conducting the method.

The first invention of the present application to achieve the above objective is a method of extracting biological material-derived nucleic acid from samples containing biological material, the method comprising mixing biological material with an aqueous solution containing at least a surfactant, subjecting the mixture to a heating treatment and then removing the aqueous solution to separate the biological material, adding a solid powder suspension to the biological material, agitating or sonicating it to disrupt the biological material, and extracting nucleic acid from the biological material. The second invention of the present application is as claimed in the above first invention, wherein the surfactant is an anionic surfactant having a steroid backbone or an ampholytic surfactant. The third invention of the present application is as claimed in the above second invention, wherein the anion surfactant or ampholytic surfactant is selected from the group consisting of bile acid, cholic acid, deoxycholic acid, 3-[(3-cholamidopropyl)dimethylammonio]propane sulfonic acid (CHAPS) and salts thereof. In a fourth invention of the present application, the concentration of the surfactant is in the range of 0.1 mM to 50 mM. In a fifth invention of the present application, the temperature of the heating treatment is 60°C to 90°C. In a sixth invention of the present application, the above solid powder is amorphous, its long diameter is 32 μm or less, its specific gravity is 3.5 or greater, and its hardness (Hv10) is 600 or greater. In a seventh invention of the present application, the above amorphous powder is zirconia. An eighth invention of the present application is a method of extracting biological material-derived nucleic acid from samples containing the biological material, the method comprising the steps of:

(1) separating the biological material from the above sample, (2) mixing the biological material with an aqueous solution containing, at least a cholic acid at a concentration of 0.1 to 50 mM, (3) subjecting the mixture to 60°C to 90°C and then removing the aqueous solution to separate the above biological material, (4) adding to the biological material a suspension containing zirconia powder, wherein the powder has an amorphous shape and has a major diameter of 20 μm or less, a specific gravity of 4.5 to 6.5, and a hardness (Hv10) of 800 or greater, (5) disrupting the above biological material by ultrasonication, and (6) obtaining the nucleic acid of interest in the supernatant of the disrupted product. In a ninth invention of the present application, the above ultrasonication is conducted by treating simultaneously or alternately with ultrasonic waves of two wavelengths. In a tenth invention of the present application, the above biological material is a virus, a microorganism, a protozoa, a plant or an animal tissue. In an eleventh invention of the present application, the above microorganism is one that belongs to the genus *Mycobacterium*. In a twelfth invention of the present application, the sample containing the biological material is an organism-derived from a sample, such as sputum, gastric juice, urine, pus, ascites, pleural effusion, pericardial fluid, blood and tissue, tissue washing liquid such as a bronchial lavage and an alveolar lavage, culture medium, and an environmental material such as soil, water and air. A thirteenth invention of the present application is a reagent kit for conducting the method of nucleic acid extraction as claimed in the above inventions 1-12, wherein the kit comprises at least a reagent containing the above surfactant and a reagent containing the above solid powder as a constituent. The present invention will now be explained in detail below.

BRIEF EXPLANATION OF THE DRAWINGS

Fig. 1 shows the result of a TRC reaction conducted in the method of Example 1, in which various

concentrations of standard RNA of *Mycobacterium tuberculosis* 16S rRNA were added to a nucleic acid extract from a NALC-treated sputum and then subjected to the TRC reaction.

Fig. 2 shows a graph showing the reaction time and the fluorescence intensity ratio of an RNA amplification reaction conducted in Example 2, wherein test samples in which 10 to 160 cfu (colony forming unit) BCG were dispersed in 200 µl of a tuberculosis-negative NALC-treated product, from which nucleic acid was extracted, and the extract obtained was subjected to the RNA amplification reaction. Test samples up to bacteria 10 cfu/200 µl were detected.

Fig. 3 shows a graph showing the reaction time and the fluorescence intensity ratio of a RNA amplification reaction conducted in Example 3 wherein test samples in which 10 to 160 cfu of *Mycobacterium avium* were dispersed in 200 µl of a tuberculosis-negative NALC-treated product, from which nucleic was acid extracted, and the extract obtained was subjected to the RNA amplification reaction. Test samples up to bacteria 10 cfu/200 µl were detected.

Fig. 4 shows a graph showing the reaction time and the fluorescence intensity ratio of an RNA amplification reaction conducted in Example 4 wherein test samples in which 10 to 160 cfu of *Mycobacterium intracellulare* were dispersed in 200 µl of a tuberculosis-negative NALC-treated product, from which nucleic acid was extracted, and the extract obtained was subjected to the RNA amplification reaction. Test samples up to bacteria 10 cfu/200 µl were detected.

Fig. 5 shows a graph showing the reaction time and the fluorescence intensity ratio of an RNA amplification reaction conducted in Example 5 wherein test samples in which 10 to 160 cfu of *Mycobacterium kansasii* were dispersed in 200 µl of a sham sputum (mucin 2.1 mg/ml,

peptone 4.2% solution), from which nucleic acid was extracted, and the extract obtained was subjected to the RNA amplification reaction. Test samples with up to 10 cfu/200 µl of bacteria were detected.

BEST MODE FOR CARRYING OUT THE INVENTION

As test samples in the present invention, there can be mentioned sputum, gastric juice, urine, pus, alveolar lavage, ascites, pleural effusion, pericardial fluid, feces, tissue, blood, serum, colonies, swabs or other body fluids, and sample suspensions, such as homogenized food samples. When the sample is a sputum, a pretreatment such as a NALC treatment to lower the viscosity of the sample may be carried out to obtain a more preferred result. There can also be mentioned environmental water, waste water and soil in the environmental analysis.

As biological materials in the present invention, there can be mentioned, but not limited to, viruses, bacteria, fungi, protozoa, plants or animals. The present invention is useful for biological materials, such as fungi and *Mycobacterium* for which cell disruption is difficult.

While, in the nucleic acid extraction method of the present invention, substances that inhibit the nucleic acid amplification reaction are eluted with a surfactant and removed during the extraction of nucleic acid from test samples, the present inventors believe that one of the substances that inhibit the nucleic acid amplification reaction is glycoprotein, and thus by solubilizing glycoprotein with a surfactant, the effect of removing substances that inhibit the nucleic acid amplification reaction may be exhibited. As used herein, examples of such glycoproteins include airway mucus glycoprotein (called mucin) contained in sputum.

Examples of the surfactant for use in the present invention are not specifically limited as long as they are anionic surfactants having a steroid backbone or

ampholytic surfactants, and preferably at least one surfactant selected from bile acid, cholic acid, deoxycholic acid, CHAPS and salts thereof. When cholic acid is used, concentrations of 0.1 mM to 50 mM, preferably 1 mM to 20 mM are used. The temperature of the heating treatment is preferably, but not limited to, 60°C to 90°C, more preferably 60°C to 80°C, and heating time is, but not limited to, preferably 2 minutes to 20 minutes, more preferably 2 minutes to 5 minutes. The heating treatment as used herein may preferably be conducted by being left at rest. The number of times of surfactant treatment preferably is, but not limited to, one to about five times. In order to mix nucleic acid-containing test samples with a surfactant and then selectively removing the eluent, a centrifugation process, for example, may be employed. The nucleic acid-containing test samples and the surfactant-eluent containing substances that inhibit nucleic acid amplification reactions can be separated by centrifuging at, but not limited to, 6,000×g for 5 minutes. In the process of the present invention of mixing a surfactant and then selectively removing the eluent, it is important to establish specific conditions taking into account the nucleic acid to be extracted and test samples subjected to extraction and conducting preliminary experiments.

The material of the solid powder of the present invention includes for example, but not limited to, zirconia, diamond, alumina (aluminum oxide), iron, an alloy and the like. However, from the viewpoint of extraction efficiency and cost, zirconia is preferred.

The shape of the above solid powder is not specifically limited as long as it can physically disrupt the biological material, but preferably is amorphous. Amorphous solid powder refers to a solid powder comprising particles that do not have smooth surfaces like a sphere or an ellipsoid, but have sharp convexo-concave surfaces. The size of the above particles should

be at least 32 μm at the longest portion (long diameter) of the particle diameter, and preferably a mean of 20 μm or less (measuring instrument: COULTER LS130). Its specific gravity is preferably as large as 3.0 or greater, since it can disrupt tissues using the kinetic energy of the powder by ultrasound waves or agitation. The supernatant containing extracted nucleic acid and the above solid powder can be separated by centrifugation or being left at rest. By including magnetic bodies in the solid powders in advance, magnetic separation may be effected. The greater the hardness of the solid powder is, the better, and it is preferably 600 or greater in terms of Vickers' hardness (Hv10). In a preferred embodiment of the present invention, an amorphous solid powder of zirconia having a long diameter of 20 μm or less, a specific gravity of 5.7 to 6.2, and a hardness (Hv10) of 900 or greater is used. Examples of an amorphous powder of zirconia include, but not limited to, fused zirconia ZCO-E6 (manufactured by ASTRON, mean long diameter of 6 μm, specific gravity of 5.8, Vickers' hardness (Hv10) 1200), fused zirconia NST 8H, F350 (manufactured by SAINT-GOBAIN, mean long diameter of 24 μm, specific gravity of 6.0, Vickers' hardness (Hv10) 1200), fused zirconia #400 (manufactured by PACIFIC-RUNDUM Co., Ltd., mean long diameter of 18 μm) and the like. The amount of the solid powder added to the sample is determined as appropriate depending on the type of the sample, the method of agitation, and the time of agitation. After adding the solid powder, agitation and/or ultrasonication may be conducted to physically disrupt the cells.

The method of agitation may be manual reciprocating motion of a container having a sample therein, and the direction of the motion may be either vertical or horizontal, and preferably conducted in a vertical reciprocating motion. Alternatively, it may be agitated

with a vortex mixer. For the disruption of cells, ultrasonic disruption is more preferred, and in order to eliminate the effect of a stationary wave, alternate or simultaneous ultrasonication with 2 frequencies or more is most preferred. For ultrasonic treatment, a container holding the sample and the powdery solid is immersed in or floated on the liquid in the washing vessel of the ultrasonic cleaner. The agitating time or ultrasonication time may be until the cell membrane of the microorganism or the tissue is disrupted, and may be 2 minutes or longer. The time may be longer than this, when the tissue has a tenacious and rigid cell wall such as plant tissue.

In accordance with the present invention, nucleic acid may be harvested from cells in a purified form to the extent that the subject organism may be identified and detected by a nucleic acid amplification reaction. The present invention is also useful for various test samples that contain as the subject organism microorganisms having a rigid cell wall and various nucleic acid-inhibiting substances. For example, nucleic acid may be efficiently extracted from *Mycobacterium*, as well as contained in sputum, and a sensitive and rapid gene testing may be conducted.

EXAMPLES

The present invention will now be explained in detail with reference to Examples below. It should be noted, however, that the present invention is not limited to these examples.

Example 1

An inhibiting substance present in nucleic acid-extracted samples from sputum

Two hundred microliters of a test sample prepared by subjecting sputum (hereinafter referred to as "tuberculosis-negative sputum") obtained with consent from a patient infected with nontuberculous acid-fast bacteria to a NALC-NaOH treatment (trade name: BBL

Mycoprep, Nippon Becton Dickinson Co., Ltd) were used in the following two treatments. Condition 1: The sample was added to 1 ml of PBS, agitated, and then centrifuged at 16,000 × g for 5 minutes to remove the supernatant. Condition 2: The sample was added to 1 ml of 50 mM glycine-NaOH buffer (hereinafter referred to as "washing reagent") adjusted to pH 9 containing 10 mM sodium cholate and 1 mM EDTA, allowed to stand on a dry heat block at 80°C for 2 minutes, and then centrifuged at 16,000 × g for 5 minutes to remove the supernatant. To each of the precipitates obtained under above Conditions 1 and 2, 50 µl of a TE solution (manufactured by WAKO, 10 mM Tris-HCl, 1 mM EDTA, pH 8) (hereinafter referred to as "lysis reagent") containing 0.005% of yeast RNA (manufactured by SIGMA) in which 0.5 g/ml fused zirconia ZCO-E6 (manufactured by ASTRON, mean diameter of 6 µm) had been suspended was added, and the tube was floated on the washing vessel of the ultrasonic cleaner WT-70ST (manufactured by HONDA ELECTRONICS Co., Ltd., frequency 40 kHz, output power 70 W), and then subjected to an ultrasonic treatment for 5 minutes. It was then centrifuged at 16,000 × g for 3 minutes, and 30 µl of the supernatant was transferred to another tube to obtain a nucleic acid extract.

(1) Nucleic acid extracts obtained as above from a plurality of tuberculosis-negative sputa were mixed to prepare a negative sputum-extracted sample. 4.5 µl of this negative sputum-extracted sample and 0.5 µl of each solution of varying concentrations of standard RNA [a standard RNA containing the base number 243-1843 (the base number was attached in conformity to Z83862 registered in GenBank), which was synthesized by *in vitro* transcription using double stranded DNA containing the sequence of *Mycobacterium tuberculosis* 16S rRNA as the template, and then purified, 1601 bases; the standard RNA was dissolved in a 10 mM Tris-HCl buffer containing 1 mM

EDTA and RNase inhibitor; this buffer will be hereinafter be referred to as a "RNA dilution buffer" were mixed, and was subjected to a nucleic acid amplification reaction according to the method described in Japanese Unexamined Patent Publication (Kokai) No. 2004-194583. In the Control (Nega), the negative sputum-extracted sample was replaced and mixed with 4.5 μl of the RNA dilution buffer.

(2) Twenty microliters of a reaction mixture having the following composition was dispensed into a PCR tube (volume 0.5 ml; Gene Amp Thin-Walled Reaction Tube, manufactured by Perkin-Elmer), and the above mixed sample was added thereto.

Composition of the reaction mixture (each concentration is the concentration in the final volume of 30 μl)

60 mM Tris-HCl buffer (pH 8.6)

17 mM magnesium chloride

100 mM potassium chloride

6 U RNase inhibitor (manufactured by TAKARA BIO INC.)

1 mM DTT

0.25 mM each of dATP, dCTP, dGTP, dTTP

3.6 mM ITP

3.0 mM each of ATP, CTP, GTP, UTP

0.16 μM of the first oligonucleotide (MYR-1S-10, SEQ ID NO: 1. The hydroxyl group at the 3'-end was amidated)

1.0 μM of the second oligonucleotide (MYR-1F-10, SEQ ID NO: 2. A region from "A" at No. 1 to "A" at No. 22 of the 5'-end is the region of T7 promoter, and an ensuing region from "G" at No. 23 to "A" at No. 28 is an enhancer region)

1.0 μM of the third oligonucleotide (MYR-3RT18, SEQ ID NO: 3)

25 nM of an oligonucleotide labelled with an intercalating fluorescent dye (YO-MYR-P2-2-S-G, SEQ ID

NO: 4. An intercalating dye was labelled in between "A" at No. 7 and "G" at No. 8 of the 5'-end. The hydroxyl group at the 3'-end was modified by glycolic acid)

13% DMSO

Distilled water for adjusting volume

(3) After keeping the temperature of the above reaction mixture at 43°C for 5 minutes, 5 µl of an enzyme solution that had the following composition and that was previously kept at 43°C for 2 minutes was added:

Composition of the enzyme solution (each concentration is the concentration in the final volume of 30 µl)

2.0% sorbitol

3.6 µg bovine serum albumin

142 U T7 RNA polymerase (manufactured by TAKARA BIO INC.)

6.4 U AMV reverse transcriptase (manufactured by TAKARA BIO INC.)

Distilled water for adjusting volume

(4) Then, while keeping the temperature at 43°C using a fluorescent photometer equipped with a temperature control function that permits direct measurement of the PCR tube, the reaction mixture was measured for a period of time with an excitation wavelength of 470 nm and an emission wavelength of 520 nm.

(5) The result of the rise time (time until a fluorescence increase ratio becomes equal to 1.2 times the value of the sum of the negative mean and three times the standard deviation) of each nucleic acid extract is shown in Fig. 1. These results show that under above condition 1, the inhibiting substances derived from tuberculosis-negative sputa delayed the rise time by 4 minutes or more for 10^3 copies of standard RNA, 3 minutes for 10^4 copies, and 2 minutes or more for 10^5 copies than that with no extract-addition. On the other hand, under above condition 2, it was improved to 3 minutes for 10^3 copies, 1 minute for 10^4 copies, and 1 minute for 10^5

copies.

Example 2

Pretreatment of tuberculosis-negative sputum and nucleic acid extraction therefrom - 1

To 200 µl of a test sample prepared by subjecting a tuberculosis-negative sputum to a NALC-NaOH treatment (trade name: BBL Mycoprep, Nippon Becton Dickinson Co., Ltd), BCG solutions with 10, 20, 40, 80 and 160 cfu were added and used as test samples.

One ml of the wash reagent was added thereto, allowed to stand on a dry heat block at 70°C for 3 minutes, and then centrifuged at 16,000 × g for 5 minutes to remove the supernatant. To the precipitate obtained, 50 µl of the lysis reagent was added, and the tube was floated on the washing vessel of ultrasonic cleaner VS-D100 (manufactured by HONDA ELECTRONICS Co., Ltd., frequency 24, 31 kHz, output power 110 W), and then subjected to an ultrasonic treatment for 5 minutes. Then it was centrifuged at 16,000 × g for 3 minutes, and 30 µl of the supernatant was transferred to another tube to obtain a nucleic acid extract. Five microliters of it was subjected to a nucleic acid amplification reaction as in Example 1 to measure BCG 16S rRNA.

The result of the rise time (time until a fluorescence increase ratio becomes equal to 1.2 times the value of the sum of the negative mean and three times the standard deviation) of each nucleic acid extract is shown in Fig. 2. These results show that the extraction method of the present invention can detect a BCG in sputum.

Example 3

It was examined to determine whether the extraction method of the present invention can be applied to a nontuberculous acid-fast bacterium *Mycobacterium avium*.

To 200 µl of test samples prepared by subjecting tuberculosis-negative sputum to a NALC-NaOH treatment

(trade name: BBL Mycoprep, Nippon Becton Dickinson Co., Ltd), 10, 20, 40, 80 and 160 cfu of *M. avium* were added and used as test samples.

One ml of the wash reagent was added to the test sample and after mixing, it was allowed to stand on a dry heat block at 70°C for 3 minutes, and then centrifuged at 16,000 x g for 5 minutes to remove the supernatant. To the precipitate obtained, 50 µl of the lysis reagent was added, and the tube was floated on the washing vessel of ultrasonic cleaner VS-D100 (manufactured by HONDA ELECTRONICS Co., Ltd., frequency 24, 31 kHz, output power 110 W), and then subjected to an ultrasonic treatment for 5 minutes. Then it was centrifuged at 16,000 xg for 3 minutes, and 30 µl of the supernatant was transferred to another tube to obtain a nucleic acid extract. Five microliters of it was subjected to a nucleic acid amplification reaction as in Example 1 to measure *M. avium* 16S rRNA. The combinations of the primers and probes used are as follows:

First oligonucleotide (MYR-1S-40, SEQ ID NO: 5)

Second oligonucleotide (MYR-1F-40, SEQ ID NO: 6)

Third oligonucleotide (MYR-3RA16-4, SEQ ID NO: 7)

An oligonucleotide labelled with an intercalating fluorescent dye (YO-MYR-P5-S-G, SEQ ID NO: 8)

The result of the rise time (time until a fluorescence increase ratio becomes equal to 1.2 times the value of the sum of the negative mean and three times the standard deviation) of each nucleic acid extract is shown in Fig. 3. These results show that the extraction method of the present invention can detect each cfu number of *M. avium* in sputum.

Example 4

It was examined to determine whether the extraction method of the present invention can be applied to a nontuberculous acid-fast bacterium *Mycobacterium intracellulare*.

To 200 µl of test samples prepared by subjecting tuberculosis-negative sputum to a NALC-NaOH treatment (trade name: BBL Mycoprep, Nippon Becton Dickinson Co., Ltd), 10, 20, 40, 80 and 160 cfu *M. intracellulare* were added and used as test samples.

One ml of the wash reagent was added to the test sample, a nucleic acid extract was obtained as in Example 3. Five microliters of it was subjected to a nucleic acid amplification reaction as in Example 3 to measure *M. intracellulare* 16S rRNA. The combinations of the primers and probes used are as follows:

First oligonucleotide (MYR-1S-40, SEQ ID NO: 5)

Second oligonucleotide (MYR-1F-40, SEQ ID NO: 6)

Third oligonucleotide (MYR-3RI18, SEQ ID NO: 9)

An oligonucleotide labelled with an intercalating fluorescent dye (YO-MYR-P5-S-G, SEQ ID NO: 8)

The result of the rise time (time until a fluorescence increase ratio becomes equal to 1.2 times the value of the sum of the negative mean and three times the standard deviation) of each nucleic acid extract is shown in Fig. 4. These results show that the extraction method of the present invention can detect each cell number of *M. intracellulare* in sputum.

Example 5

It was examined to determine whether the extraction method of the present invention can be applied to a nontuberculous acid-fast bacterium *Mycobacterium kansasii*.

To 200 µl of a mucin solution (mucin 2.1 mg/ml, peptone 4.2%, 10 mM Tris-HCl, 1 mM EDTA, pH 8) as a substitute for the NALC-NaOH-treated tuberculosis-negative sputum, 10, 20, 40, 80 and 160 cfu of *M. kansasii* were added and they were used as test samples.

One ml of the wash reagent was added to the test sample, and a nucleic acid extract was obtained as in Example 3. Five microliters of it was subjected to a nucleic acid amplification reaction as in Example 3 to

measure *M. kansasii* 16S rRNA. The combinations of the primers and probes used are as follows:

First oligonucleotide (MYR-1S-40, SEQ ID NO: 5)

Second oligonucleotide (MYR-1F-40, SEQ ID NO: 6)

Third oligonucleotide (MYR-3RK16-4, SEQ ID NO: 10)

An oligonucleotide labelled with an intercalating fluorescent dye (YO-MYR-P4-S-G, SEQ ID NO: 11)

The result of the rise time (time until a fluorescence increase ratio becomes equal to 1.2 times the value of the sum of the negative mean and three times the standard deviation) of each nucleic acid extract is shown in Fig. 5. These results show that the extraction method of the present invention can detect each cfu number of *M. kansasii* in sputum.

CLAIMS

1. A method of extracting biological material-derived nucleic acid from samples containing biological material, said method comprising mixing said biological material with an aqueous solution containing at least a surfactant, subjecting the mixture to a heating treatment and then removing said aqueous solution to separate the biological material, adding a solid powder suspension to said biological material, agitating or sonicating it to disrupt said biological material, and extracting nucleic acid from said biological material.

2. The method of extracting nucleic acid according to claim 1, wherein said surfactant is an anionic surfactant having a steroid backbone or an ampholytic surfactant.

3. The method of extracting nucleic acid according to claim 2, wherein said anion surfactant or ampholytic surfactant is selected from the group consisting of bile acid, cholic acid, deoxycholic acid, 3-[(3-cholamidopropyl)dimethylammonio]propane sulfonic acid (CHAPS) and salts thereof.

4. The method of extracting nucleic acid according to claim 3, wherein the concentration of said surfactant is in the range of 0.1 mM to 50 mM.

5. The method of extracting nucleic acid according to any of claims 1 to 4, wherein the temperature of the heating treatment is 60°C to 90°C.

6. The method of extracting nucleic acid according to any of claims 1 to 5, wherein the above solid powder is amorphous, its long diameter is 32 μm or less, its specific gravity is 3.0 or greater, and its hardness (Hv10) is 600 or greater.

7. The method of extracting nucleic acid according to claim 6, wherein the above amorphous powder is zirconia.

8. A method of extracting biological material-derived nucleic acid from samples containing the

biological material, said method comprising the steps of: (1) separating the biological material from the above sample, (2) mixing said biological material with an aqueous solution containing, at least, cholic acid at a concentration of 0.1 to 50 mM, (3) subjecting the mixture to a heating treatment at 60°C to 90°C and then removing said aqueous solution to separate the above biological material, (4) adding to the biological material a suspension containing zirconia powder, wherein said powder has an amorphous shape and a major diameter of 20 μm or less, a specific gravity of 4.5 to 6.5, and a hardness (Hv10) of 800 or greater, (5) disrupting the above biological material by ultrasonication, and (6) obtaining the nucleic acid of interest in the supernatant of the disrupted product.

9. The method of extracting nucleic acid according to claim 8, wherein the above ultrasonication is conducted by treating simultaneously or alternately with ultrasonic waves of two wavelengths.

10. The method of extracting nucleic acid according to any of claims 1 to 9, wherein the above biological material is a virus, microorganism, protozoa, plant or animal tissue.

11. The method of extracting nucleic acid according to claim 10, wherein the above microorganism is one that belongs to the genus *Mycobacterium*.

12. The method of extracting nucleic acid according to any of claims 1 to 11, wherein the sample containing the biological material is an organism-derived sample such as sputum, gastric juice, urine, pus, ascites, pleural effusion, pericardial fluid, blood and tissue, a tissue washing liquid such as a bronchial lavage and an alveolar lavage, culture medium, and an environmental material such as soil, water and air.

13. A reagent kit for conducting the method of nucleic acid extraction according to any of claims 1 to 12, wherein said kit comprises at least a reagent containing the above surfactant and a reagent containing

the above solid powder as a constituent.

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