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(54) METHOD FOR DETECTING OR QUANTIFYING DNA

(57) The present invention relates to a method for quantifying or detecting DNA having a target DNA region, and so on.

EP 2 305 806 A1

Description

TECHNICAL FIELD

[0001] The present invention relates to a method for quantifying or detecting DNA having a target DNA region, and so on.

BACKGROUND ART

[0002] Known as a method for quantifying or detecting DNA having a target DNA region contained in a specimen are, for example, a method of detecting DNA having a target DNA region amplified by a chain reaction of DNA synthesis by DNA polymerase (Polymerase Chain Reaction; hereinafter, sometimes referred to as PCR) after extraction of DNA from a specimen, a method of detecting DNA by hybridization of a fluorescent-labeled oligonucleotide with a target DNA region possessed by DNA in a specimen, and so on (see, for example, J. Cataract. Refract. Surg., 2007;33(4):635-641, Environ. Mol. Mutagen., 1991;18(4):259-262).

DISCLOSURE OF THE INVENTION

[0003] It is an object of the present invention to provide a method for quantifying or detecting DNA having a target DNA region in a simple and convenient manner.

[0004] Specifically, the present invention provides:

[Invention 1]

[0005] A method for quantifying or detecting DNA comprising a target DNA region contained in a specimen comprising:

(1) First step of preparing from a specimen DNA for which the target DNA region is to be detected;
(2) Second step of treating the DNA prepared in First step with a DNA methylation enzyme,
(3) Third step of preparing single-stranded methylated DNA from the DNA treated in Second step,
(4) Fourth step of forming a complex of a single-stranded methylated DNA comprising a methylated target DNA region, a methylated DNA antibody, and a specific oligonucleotide by mixing the single-stranded methylated DNA prepared in Third step, the methylated DNA antibody, and the specific oligonucleotide comprising a nucleotide sequence that does not inhibit binding between one or more methylated bases in the target DNA region in the single-stranded methylated DNA and the methylated DNA antibody, and that is capable of binding with the single-stranded DNA comprising the target DNA region by complementation, and
(5) Fifth step of quantifying or detecting the DNA comprising the target DNA region in the single-stranded methylated DNA by quantifying or detecting the methylated DNA antibody contained in the complex formed in Fourth step by its identification function (hereinafter, sometimes referred to as the present method);

[Invention 2]

[0006] The method according to Invention 1, wherein the complex is formed in a reaction system containing a divalent cation in Fourth step;

[Invention 3]

[0007] The method according to Invention 2, wherein the divalent cation is a magnesium ion;

[Invention 4]

[0008] The method according to any one of Inventions 1 to 3, wherein the antibody contained in the complex formed in Fourth step has been bound to a support before starting of Fifth step;

[Invention 5]

[0009] The method according to any one of Inventions 1 to 3, wherein the specific oligonucleotide contained in the complex formed in Fourth step has been bound to a support before starting of Fifth step;

[Invention 6]

[0010] The method according to any one of Inventions 1 to 5, wherein the DNA methylation enzyme is a cytosine methylation enzyme;

[Invention 7]

[0011] The method according to any one of Inventions 1 to 6, wherein the DNA methylation enzyme is SssI methylase;

[Invention 8]

[0012] The method according to any one of Inventions 1 to 7, wherein the methylated DNA antibody is a methylcytosine antibody;

[Invention 9]

[0013] The method according to any one of Inventions 1 to 8, wherein the specimen is any of the following specimen:

(a) mammalian blood, body fluid, excreta, body secretion, cell lysate, or tissue lysate,
(b) DNA extracted from one selected from the group consisting of mammalian blood, body fluid, excreta, body secretion, cell lysate, and tissue lysate,
(c) DNA prepared by using as a template RNA extracted from one selected from the group consisting of mammalian tissue, cell, tissue lysate and cell lysate,
(e) DNA extracted from cell, fungus or virus, or
(f) DNA prepared by using as a template RNA extracted from cell, fungus or virus;

[Invention 10]

[0014] The method according to any one of Inventions 1 to 9, wherein DNA for which the target DNA region is to be detected is any of the following DNAs (a) to (e):

(a) DNA digested in advance with a restriction enzyme recognition cleavage site for which is not present in the target DNA region,
(b) DNA purified in advance,
(c) free DNA in blood,
(d) DNA derived from microbial genome, or
(e) DNA generated from RNA by a reverse transcriptase;

[Invention 11]

[0015] The method according to any one of Inventions 1 to 10, wherein a counter oligonucleotide is added in forming the complex in Fourth step;

[Invention 12]

[0016] The method according to any one of Inventions 1 to 11, wherein concentration of a sodium salt in a solution used in a DNA extracting operation for preparing DNA from a specimen in First step is 100 mM or more and 1000 mM or less;

[Invention 13]

[0017] The method according to any one of Inventions 1 to 11, wherein concentration of a sodium salt in a solution used in a DNA extracting operation for preparing DNA from a specimen in First step is 100 mM or more and 200 mM or less;

[Invention 14]

[0018] A method for selecting a specimen from a cancer patient comprising the step of evaluating that a specimen from a test subject is a specimen from a cancer patient when there is significant difference between a quantification

result or a detection result of DNA quantified or detected by using the specimen from the test subject according to the method of any one of Inventions 1 to 13 and a quantification result or a detection result of DNA quantified or detected by using a specimen from a healthy subject according to the same method, and identifying a specimen from a cancer patient based on a result of the evaluation;

[Invention 15]

[0019] The method according to Invention 14, wherein the specimen is mammalian serum; and

[Invention 16]

[0020] The method according to Invention 14 or 15, wherein DNA comprising a target DNA region is free DNA comprising the target DNA region in a mammalian serum; and so on.

BRIEF DESCRIPTION OF THE DRAWINGS

[0021] Fig. 1 is a drawing showing a result obtained by using 0.5 μg/mL of methylcytosine antibody and 5'-end biotin-labeled oligonucleotide B1 in Example 1. Values of DNA amounts measured at excitation 340 nm/fluorescence 612 nm are shown for Solution A (10 ng/20 μL TE buffer solution), Solution B (1 ng/20 μL TE buffer solution), Solution C (0.1 ng/20 μL TE buffer solution), and Solution D (0 ng/20 μL TE buffer solution (negative control solution)), respectively in this order from right.

[0022] Fig. 2 is a drawing showing a result obtained by using 0.5 μg/mL of methylcytosine antibody and 5'-end biotin-labeled oligonucleotide B2 in Example 2. Values of DNA amounts measured at excitation 340 nm/fluorescence 612 nm are shown for Solution A (10 ng/20 μL TE buffer solution), Solution B (1 ng/20 μL TE buffer solution), Solution C (0.1 ng/20 μL TE buffer solution), and Solution D (0 ng/20 μL TE buffer solution (negative control solution)), respectively in this order from right.

[0023] Fig. 3 is a drawing showing a result obtained by using 0.5 μg/mL of methylcytosine antibody and 5'-end biotin-labeled oligonucleotide B1 in Example 3. Values of DNA amounts measured at excitation 340 nm/fluorescence 612 nm are shown for Solution MA (10 ng each/20 μL TE buffer solution), Solution MB (1 ng each/20 μL TE buffer solution), Solution MC (0.1 ng each/20 μL TE buffer solution), and Solution MD (0 ng each/20 μL TE buffer solution (negative control solution)), respectively in this order from right.

[0024] Fig. 4 is a drawing showing a result obtained by using 0.5 μg/mL of methylcytosine antibody and 5'-end biotin-labeled oligonucleotide B2 in Example 3. Values of DNA amounts measured at excitation 340 nm/fluorescence 612 nm are shown for Solution MA (10 ng each/20 μL TE buffer solution), Solution MB (1 ng each/20 μL TE buffer solution), Solution MC (0.1 ng each/20 μL TE buffer solution), and Solution MD (0 ng each/20 μL TE buffer solution (negative control solution)), respectively in this order from right.

[0025] Fig. 5 is a drawing showing a result obtained by using 0.5 μg/mL of methylcytosine antibody, 5'-end biotin-labeled oligonucleotide B1 and 5'-end biotin-labeled oligonucleotide B2 in Example 3. Values of DNA amounts measured at excitation 340 nm/fluorescence 612 nm are shown for Solution MA (10 ng each/20 μL TE buffer solution), Solution MB (1 ng each/20 μL TE buffer solution), Solution MC (0.1 ng each/20 μL TE buffer solution), Solution MD (0 ng each/20 μL TE buffer solution (negative control solution)), respectively in this order from right.

[0026] Fig. 6 is a drawing showing a result obtained by using 0.5 μg/mL of methylcytosine antibody and 5'-end biotin-labeled oligonucleotide B1 in Example 4. Values of DNA amounts measured at excitation 340 nm/fluorescence 612 nm are shown for Solution A (1000 ng each/30 μL TE buffer solution), Solution B (500 ng each/30 μL TE buffer solution), Solution C (200 ng each/30 μL TE buffer solution), and Solution D (0 ng each/30 μL TE buffer solution (negative control solution)), respectively in this order from right.

[0027] Fig. 7 is a drawing showing a result obtained by using 0.5 μg/mL of methylcytosine antibody and 5'-end biotin-labeled oligonucleotide B3 in Example 5. Values of DNA amounts measured at excitation 340 nm/fluorescence 612 nm are shown for Solution A (1000 ng each/30 μL TE buffer solution), Solution B (500 ng each/30 μL TE buffer solution), Solution C (200 ng each/30 μL TE buffer solution), and Solution D (0 ng each/30 μL TE buffer solution (negative control solution)), respectively in this order from right.

[0028] Fig. 8 is a drawing showing a result obtained by using 0.5 μg/mL of methylcytosine antibody and 5'-end biotin-labeled oligonucleotide B3 in Example 6. Values of DNA amounts measured at excitation 340 nm/fluorescence 612 nm are shown for Solution A (500 ng each/20 μL TE buffer solution), Solution B (50 ng each/20 μL TE buffer solution), Solution C (5 ng each/20 μL TE buffer solution), and Solution D (0 ng each/20 μL TE buffer solution (negative control solution)), respectively in this order from right.

[0029] Fig. 9 is a drawing showing a result obtained by using 0.5 μg/mL of methylcytosine antibody and 5'-end biotin-labeled oligonucleotide B4 in Example 7. Values of DNA amounts measured at excitation 340 nm/fluorescence 612 nm

are shown for Solution A (10 ng/20 μL TE buffer solution), Solution B (1 ng/20 μL TE buffer solution), Solution C (0.1 ng/20 μL TE buffer solution), and Solution D (0 ng/20 μL TE buffer solution (negative control solution)), respectively in this order from right.

[0030] Fig. 10 is a drawing showing a result obtained by using 0.5 μg/mL of methylcytosine antibody and 5'-end biotin-labeled oligonucleotide B5 in Example 8. Values of DNA amounts measured at excitation 340 nm/fluorescence 612 nm are shown for Solution A (10 ng/20 μL TE buffer solution), Solution B (1 ng/20 μL TE buffer solution), Solution C (0.1 ng/20 μL TE buffer solution), and Solution D (0 ng/20 μL TE buffer solution (negative control solution)), respectively in this order from right.

[0031] Fig. 11 is a drawing showing a result obtained by using 0.5 μg/mL of methylcytosine antibody and 5'-end biotin-labeled oligonucleotide B4 in Example 9. Values of DNA amounts measured at excitation 340 nm/fluorescence 612 nm are shown for Solution MA (10 ng each/20 μL TE buffer solution), Solution MB (1 ng each/20 μL TE buffer solution), Solution MC (0.1 ng each/20 μL TE buffer solution), and Solution MD (0 ng each/20 μL TE buffer solution (negative control solution)), respectively in this order from right.

[0032] Fig. 12 is a drawing showing a result obtained by using 0.5 μg/mL of methylcytosine antibody and 5'-end biotin-labeled oligonucleotide B5 in Example 9. Values of DNA amounts measured at excitation 340 nm/fluorescence 612 nm are shown for Solution MA (10 ng each/20 μL TE buffer solution), Solution MB (1 ng each/20 μL TE buffer solution), Solution MC (0.1 ng each/20 μL TE buffer solution), and Solution MD (0 ng each/20 μL TE buffer solution (negative control solution)), respectively in this order from right.

[0033] Fig. 13 is a drawing showing a result obtained by using 0.5 μg/mL of methylcytosine antibody and 5'-end biotin-labeled oligonucleotide B4 and 5'-end biotin-labeled oligonucleotide B5 in Example 9. Values of DNA amounts measured at excitation 340 nm/fluorescence 612 nm are shown for Solution MA (10 ng each/20 μL TE buffer solution), Solution MB (1 ng each/20 μL TE buffer solution), Solution MC (0.1 ng each/20 μL TE buffer solution), and Solution MD (0 ng each/20 μL TE buffer solution (negative control solution)), respectively in this order from right.

[0034] Fig. 14 is a drawing showing a result obtained by using 0.5 μg/mL of methylcytosine antibody and 5'-end biotin-labeled oligonucleotide B4 in Example 10. Values of DNA amounts measured at excitation 340 nm/fluorescence 612 nm are shown for Solution A (100 ng each/20 μL TE buffer solution), Solution B (10 ng each/20 μL TE buffer solution), Solution C (1 ng each/20 μL TE buffer solution), and Solution D (0 ng each/20 μL TE buffer solution (negative control solution)), respectively in this order from right.

[0035] Fig. 15 is a drawing showing a result obtained by using 0.5 ug/mL of methylcytosine antibody and 5'-end biotin-labeled oligonucleotide B5 in Example 11. Values of DNA amounts measured at excitation 340 nm/fluorescence 612 nm are shown for Solution A (100 ng each/20 μL TE buffer solution), Solution B (10 ng each/20 μL TE buffer solution), Solution C (1 ng each/20 μL TE buffer solution), and Solution D (0 ng each/20 μL TE buffer solution (negative control solution)), respectively in this order from right.

[0036] Fig. 16 is a drawing showing a result obtained by using 0.5 μg/mL of methylcytosine antibody and 5'-end biotin-labeled oligonucleotide B4 in Example 12. Values of DNA amounts measured at excitation 340 nm/fluorescence 612 nm are shown for Solution A (10 ng/20 μL TE buffer solution), Solution B (1 ng/20 μL TE buffer solution), Solution C (0.1 ng/20 μL TE buffer solution), and Solution D (0 ng/20 μL TE buffer solution (negative control solution)), respectively in this order from right.

[0037] Fig. 17 is a drawing showing a result obtained by using 0.5 μg/mL of methylcytosine antibody and 5'-end biotin-labeled oligonucleotide B5 in Example 13. Values of DNA amounts measured at excitation 340 nm/fluorescence 612 nm are shown for Solution A (10 ng/20 μL TE buffer solution), Solution B (1 ng/20 μL TE buffer solution), Solution C (0.1 ng/20 μL TE buffer solution), and Solution D (0 ng/20 μL TE buffer solution (negative control solution)), respectively in this order from right.

[0038] Fig. 18 is a drawing showing a result obtained by using 0.5 μg/mL of methylcytosine antibody and 5'-end biotin-labeled oligonucleotide B4 in Example 14. Values of DNA amounts measured at excitation 340 nm/fluorescence 612 nm are shown for Solution MA (10 ng each/20 μL TE buffer solution), Solution MB (1 ng each/20 μL TE buffer solution), Solution MC (0.1 ng each/20 μL TE buffer solution), and Solution MD (0 ng each/20 μL TE buffer solution (negative control solution)), respectively in this order from right.

[0039] Fig. 19 is a drawing showing a result obtained by using 0.5 μg/mL of methylcytosine antibody and 5'-end biotin-labeled oligonucleotide B5 in Example 14. Values of DNA amounts measured at excitation 340 nm/fluorescence 612 nm are shown for Solution MA (10 ng each/20 μL TE buffer solution), Solution MB (1 ng each/20 μL TE buffer solution), Solution MC (0.1 ng each/20 μL TE buffer solution), and Solution MD (0 ng each/20 μL TE buffer solution (negative control solution)), respectively in this order from right.

[0040] Fig. 20 is a drawing showing a result obtained by using 0.5 μg/mL of methylcytosine antibody and 5'-end biotin-labeled oligonucleotide B4 and 5'-end biotin-labeled oligonucleotide B5 in Example 14. Values of DNA amounts measured at excitation 340 nm/fluorescence 612 nm are shown for Solution MA (10 ng each/20 μL TE buffer solution), Solution MB (1 ng each/20 μL TE buffer solution), Solution MC (0.1 ng each/20 μL TE buffer solution), and Solution MD (0 ng each/20 μL TE buffer solution (negative control solution)), respectively in this order from right.

[0041] Fig. 21 is a drawing showing a result obtained by using 0.5 μg/mL of methylcytosine antibody and 5'-end biotin-labeled oligonucleotide B4 in Example 15. Values of DNA amounts measured at excitation 340 nm/fluorescence 612 nm are shown for Solution A (10 ng/20 μL TE buffer solution), Solution B (1 ng/20 μL TE buffer solution), Solution C (0.1 ng/20 μL TE buffer solution), and Solution D (0 ng/20 μL TE buffer solution (negative control solution)), respectively in this order from right.

[0042] Fig. 22 is a drawing showing a result obtained by using 0.5 μg/mL of methylcytosine antibody and 5'-end biotin-labeled oligonucleotide B5 in Example 16. Values of DNA amounts measured at excitation 340 nm/fluorescence 612 nm are shown for Solution A (10 ng/20 μL TE buffer solution), Solution B (1 ng/20 μL TE buffer solution), Solution C (0.1 ng/20 μL TE buffer solution), and Solution D (0 ng/20 μL TE buffer solution (negative control solution)), respectively in this order from right.

[0043] Fig. 23 is a drawing showing a result obtained by using 0.5 μg/mL of methylcytosine antibody and 5'-end biotin-labeled oligonucleotide B4 in Example 17. Values of DNA amounts measured at excitation 340 nm/fluorescence 612 nm are shown for Solution A (10 ng/20 μL TE buffer solution), Solution B (1 ng/20 μL TE buffer solution), Solution C (0.1 ng/20 μL TE buffer solution), and Solution D (0 ng/20 μL TE buffer solution (negative control solution)), respectively in this order from right.

[0044] Fig. 24 is a drawing showing a result obtained by using 0.5 μg/mL of methylcytosine antibody and 5'-end biotin-labeled oligonucleotide B5 in Example 18. Values of DNA amounts measured at excitation 340 nm/fluorescence 612 nm are shown for Solution A (10 ng/20 μL TE buffer solution), Solution B (1 ng/20 μL TE buffer solution), Solution C (0.1 ng/20 μL TE buffer solution), and Solution D (0 ng/20 μL TE buffer solution (negative control solution)), respectively in this order from right.

[0045] Fig. 25 is a drawing showing a result obtained by using 0.5 μg/mL of methylcytosine antibody and 5'-end biotin-labeled oligonucleotide B4 in Example 19. Values of DNA amounts measured at excitation 340 nm/fluorescence 612 nm are shown for Solution MA (10 ng each/20 μL TE buffer solution), Solution MB (1 ng each/20 μL TE buffer solution), Solution MC (0.1 ng each/20 μL TE buffer solution), and Solution MD (0 ng each/20 μL TE buffer solution (negative control solution)), respectively in this order from right.

[0046] Fig. 26 is a drawing showing a result obtained by using 0.5 μg/mL of methylcytosine antibody and 5'-end biotin-labeled oligonucleotide B5 in Example 19. Values of DNA amounts measured at excitation 340 nm/fluorescence 612 nm are shown for Solution MA (10 ng each/20 μL TE buffer solution), Solution MB (1 ng each/20 μL TE buffer solution), Solution MC (0.1 ng each/20 μL TE buffer solution), and Solution MD (0 ng each/20 μL TE buffer solution (negative control solution)), respectively in this order from right.

[0047] Fig. 27 is a drawing showing a result obtained by using 0.5 μg/mL of methylcytosine antibody and 5'-end biotin-labeled oligonucleotide B4 and 5'-end biotin-labeled oligonucleotide B5 in Example 19. Values of DNA amounts measured at excitation 340 nm/fluorescence 612 nm are shown for Solution MA (10 ng each/20 μL TE buffer solution), Solution MB (1 ng each/20 μL TE buffer solution), Solution MC (0.1 ng each/20 μL TE buffer solution), and Solution MD (0 ng each/20 μL TE buffer solution (negative control solution)), respectively in this order from right.

[0048] Fig. 28 is a drawing showing a result of an experiment for detecting a target DNA region W by Treatment 1 in Example 20. In Fig. 28, Solution A represents a measurement of fluorescent intensity of a sample subjected to an operation including Treatment 1 for Serum sample A using biotin-labeled oligonucleotide B6. Solution B represents a measurement of fluorescent intensity of a sample subjected to an operation including Treatment 1 for Serum sample B using biotin-labeled oligonucleotide B6. Solution C represents a measurement of fluorescent intensity of a sample subjected to an operation including Treatment 1 for Serum sample C (negative control solution) using biotin-labeled oligonucleotide B6.

[0049] Fig. 29 is a drawing showing a result of an experiment for detecting a target DNA region W by Treatment 2 in Example 20. In Fig. 29, Solution A represents a measurement of fluorescent intensity of a sample subjected to an operation including Treatment 2 for Serum sample A using biotin-labeled oligonucleotide B6. Solution B represents a measurement of fluorescent intensity of a sample subjected to an operation including Treatment 2 for Serum sample B using biotin-labeled oligonucleotide B6. Solution C represents a measurement of fluorescent intensity of a sample subjected to an operation including Treatment 2 for Serum sample C (negative control solution) using biotin-labeled oligonucleotide B6.

[0050] Fig. 30 is a drawing showing a result of an experiment for detecting a target DNA region W in Example 21. In Fig. 30, Solution A represents a measurement of fluorescent intensity of a sample subjected to an operation including Treatment 1 for Serum sample A using biotin-labeled oligonucleotide B6. Solution B represents a measurement of fluorescent intensity of a sample subjected to an operation including Treatment 1 for Serum sample B using biotin-labeled oligonucleotide B6. Solution C represents a measurement of fluorescent intensity of a sample subjected to an operation including Treatment 1 for Serum sample C (negative control solution) using biotin-labeled oligonucleotide B6.

[0051] Fig. 31 is a drawing showing a result of an experiment for detecting a target DNA region W in Example 21. In Fig. 31, Solution A represents a measurement of fluorescent intensity of a sample subjected to an operation including Treatment 2 for Serum sample A using biotin-labeled oligonucleotide B6. Solution B represents a measurement of

fluorescent intensity of a sample subjected to an operation including Treatment 2 for Serum sample B using biotin-labeled oligonucleotide B6. Solution C represents a measurement of fluorescent intensity of a sample subjected to an operation including Treatment 2 for Serum sample C (negative control solution) using biotin-labeled oligonucleotide B6.

[0052] Fig. 32 is a drawing showing a result of an experiment for detecting a target DNA region W contained in human serum in Example 22. The drawing shows comparison between a result detected by using methylcytosine antibody and 5'-end biotin-labeled oligonucleotide B6 and a result quantified by real-time PCR. The detection result is plotted on the vertical axis, and the quantification result by real-time PCR is plotted on the horizontal axis. The straight lines in the graph represent regression line (thick line) and standard error range (thin line).

[0053] Fig. 33 is a drawing showing a result of an experiment for detecting a target DNA region W contained in human serum in Example 22. For serum samples of human beings at age 59 or younger, DNA was detected using methylcytosine antibody and 5'-end biotin-labeled oligonucleotide B6, and plotted separately for cancer patients and healthy subjects, together with respective average values and standard deviations.

MODE FOR CARRYING OUT THE INVENTION

[0054] Examples of the "specimen" in the present method include (a) mammalian blood, body fluid, excreta, body secretion, cell lysate, or tissue lysate, (b) DNA extracted from one selected from the group consisting of mammalian blood, body fluid, excreta, body secretion, cell lysate and tissue lysate, (c) DNA prepared by using as a template RNA extracted from one selected from the group consisting of mammalian tissue, cell, tissue lysate and cell lysate, (e) DNA extracted from cell, fungus or virus, and (f) DNA prepared by using as a template RNA extracted from cell, fungus or virus. The term "tissue" means broadly including blood and lymph node.

[0055] The term "mammal" means animals classified into animal kingdom, Chordata, Chordate subphylum, and Mammalia, and concrete examples include human being, monkey, marmoset, guinea pig, rat, mouse, cattle, sheep, dog, and cat.

[0056] The term "body fluid" means a liquid existing between cells constituting an individual body, and concretely, plasma and interstitial fluid are recited, and it often functions to maintain homeostasis of an individual body. More concrete examples include lymph, tissue fluid (interinstitutional fluid, intercellular fluid, interstitial fluid), celomic fluid, serous cavity fluid, pleural effusion, ascetic fluid, pericardial fluid, cerebral fluid (spinal fluid), joint fluid (spinal fluid), eye aqueous fluid (aqueous fluid), and cerebrospinal fluid.

[0057] The term "body secretion" is a secretion from an exocrine gland, and concrete examples include saliva, gastric juice, bile, pancreatic juice, intestinal juice, sweat, tear, runny nose, semen, vaginal lubricant, amniotic fluid, and milk.

[0058] When the specimen is blood, body fluid or body secretion of a human being, a sample collected for a clinical test in a regular health check of human may be utilized.

[0059] Examples of the "cell lysate" include lysates containing intracellular fluids obtained by grinding cells, such as cell strains, primary cultured cells or blood cells, cultured in a plate for cell culture. As a method of grinding cells, a method based on sonication, a method using a surfactant, a method of using an alkaline solution and the like are recited. For lysing cells, a commercially available kit and the like may be used.

[0060] For example, after culturing cells to be confluent in a 10 cm plate, the culture solution is removed, and 0.6 mL of a RIPA buffer (1x TBS, 1% nonidet P-40, 0.5% sodium deoxysholate, 0.1% SDS, 0.004% sodium azide) is added to the plate. After shaking slowly the plate at 4°C for 15 minutes, cells adhered on the plate are removed by using a scraper or the like, and the liquid on the plate is transferred to a microtube. After adding 10 mg/mL PMSF in an amount of 1/10 volume of the liquid, the tube is left still on ice for 30 to 60 minutes, the solution is centrifuged at 4°C for 10 minutes at 10,000xg, to obtain the supernatant as a cell lysate.

[0061] As the "tissue lysate", lysates containing intracellular fluids obtained by grinding cells in tissues collected from animals such as mammals can be recited.

[0062] Concretely, after measuring the weight of a tissue obtained from an animal, the tissue is cut into small pieces with the use of a razor or the like. When a frozen tissue is used, it is necessary to make a smaller piece. After cutting, an ice-cooled RIPA buffer is added in a rate of 3 mL per 1 g of tissue, and homogenized at 4°C. Here, as the RIPA buffer, a protease inhibitor, a phosphatase inhibitor and the like may be added, and for example, 10 mg/mL PMSF in an amount of 1/10 volume of the RIPA buffer may be added. For homogenization, a sonicator or a pressurized cell grinder is used. In an operation of homogenization, a homogenized liquid is constantly kept at 4°C for preventing heat generation. The homogenized liquid is transferred to a microtube, and centrifuged at 4°C for 10 minutes at 10,000xg, and the supernatant is obtained as a tissue lysate.

[0063] Examples of the "specimen" in the present method include samples and surface adhered matters collected from foods, rivers, soils or general commercial products, and microorganisms such as fungi, cells, viruses and nucleic acids thereof can be contained.

[0064] Examples of the DNA used as a specimen include genomic DNA obtained by extraction from the biological sample or the microorganism, and DNA fragment or RNA derived from genomic DNA. For obtaining genomic DNA from

a sample derived from a mammal, for example, a commercially available DNA extraction kit and the like may be used. For obtaining DNA from RNA, a reverse transcriptase such as a commercially available cDNA preparation kit and the like may be used. As the specimen, artificially synthesized DNA may be used.

[0065] The term "target DNA region" (hereinafter, sometimes referred to as a target region) in the present method means a DNA region intended to be detected or quantified by the present method in DNA contained in a specimen. The target DNA region is represented by a nucleotide sequence on DNA when the specimen is DNA. When the specimen is RNA, the target DNA region is represented by a nucleotide sequence on DNA prepared from RNA by a reverse transcriptase, and is a complementary nucleotide sequence of a prescribed nucleotide sequence to be detected on RNA. In the present method, when cytosine is methylated and detected or quantified, a target region desirably contains a region abundantly containing cytosine or CpG as will be described later.

[0066] First step is a step of preparing from a specimen DNA for which a target DNA region is to be detected.

[0067] Examples of DNA prepared in First step include a DNA sample digested in advance with a restriction enzyme recognition cleavage site for which in not present in the target DNA region possessed by the DNA, a DNA sample purified in advance, free DNA in blood, DNA derived from microbial genome, and DNA prepared from RNA in a specimen by a reverse transcriptase. As DNA prepared in First step, for example, DNA that is designed based on gene information of the specimen and artificially synthesized may be recited.

[0068] When blood is used as a specimen, plasma or serum is prepared from blood by a routine method, and the prepared plasma or serum is used as a specimen, and free DNA (containing DNA derived from cancer cells such as gastric cancer cells) contained therein is analyzed, and thus DNA derived from cancer cells such as gastric cancer cells can be analyzed away from DNA derived from hemocytes, and sensitivity of detecting cancer cells such as gastric cancer cells, and tissues containing the same can be improved.

[0069] Examples of DNA prepared in First step include DNA derived from microorganisms such as gram-positive bacteria, gram-negative bacteria, fungi, viruses and pathogenic protozoans, and DNA obtained from RNA derived from such microorganisms by a reverse transcriptase. For example, genomic DNA or DNA prepared by a reverse transcriptase from RNA of Mycoplasma genitalium, Mycoplasma pneumoniae, Borrelia burgdorferi B31, Rickettsia prowazekii, Treponema pallidum, Chlamydia pneumoniae, Chlamydia trachomatis, Helicobacter pylori J99, Helicobacter pylori 26695, Haemophilus influenzae Rd, Mycobacterium tuberculosis H37Rv, Pseudomonas aeruginosa, Legionella pneumophila, Serratia marcescens, Escherichia coli, Listeria monocytogenes, Salmonella enterica, Campylobacter jejuni subsp. Jejuni, Staphylococcus aureus, Vibrio parahaemolyticus, Bacillusu cereus, Clostridium botulinum, Clostridium perfringens, Yersinia enterocolitica, Yersinia pseudotuberuculosis, Trichophyton ruburum, Trichophyton mentagrophytes, Candida albicans, Cryptococcus neoformans, Aspergillus fumigatus, Pneumocystis carinii, Coccidioides immitis, Cytomegalovirus, human herpesvirus 5, Epstein-Barr virus, Human Immunodeficiency Virus, Human Papilloma Virus, Enterovirus, Norovirus Influenza Virus, Toxoplasma gondii, Cryptosporidium parvum, or Entamoeba histolytica may be used for detection of a microorganism responsible for an infection in a specimen, or a microorganism responsible for a food poisoning in food.

[0070] For preparing genomic DNA, for example, when the specimen is a sample derived from a mammal, a commercially available DNA extraction kit (Genfind v2 Kit (available from BECKMAN COULTER), FastPure DNA Kit (available from TAKARA BIO INC.)) and the like may be used.

[0071] When the specimen is a microorganism such as fungus, genomic DNA may be prepared by a general preparation method of yeast genome or the like as described in Methods in Yeast Genetics (Cold Spring Harbor Laboratory Press), and when the specimen is a prokaryote such as Escherichia coli, a general preparation method of microorganism genome or the like as described in Molecular Cloning -A Laboratory Manual- (Cold Spring Harbor Laboratory Press) may be used.

[0072] When the specimen is a food sample, DNA may be prepared after separating a microorganism or the like from the food, and genomic DNA of non-microorganism and genome derived from a microorganism contained in the food may be obtained at the same time. When the specimen is a tissue derived from a mammal, and the target DNA region is DNA derived from a virus, RNA may be extracted from the tissue using such as a commercially available RNA extraction kit (ISOGEN(311-02501)(available from NIPPON GENE CO., LTD.), or FastRNA Pro Green Kit (available from Funakoshi Corporation), FastRNA Pro Blue Kit (available from Funakoshi Corporation), FastRNA Pro Red Kit (available from Funakoshi Corporation), and the like), and DNA may be obtained by a reverse transcriptase. When the specimen is a specimen derived from a mammal, viral DNA may be extracted after extracting virus particles, or after extracting virus particles, viral RNA may be extracted using a commercially available kit (QuickGene RNA tissue kit SII, available FUJIFILM Corporation) or the like, and DNA derived from the virus may be obtained by a reverse transcriptase. RNA may be extracted from a tissue infected by a virus, and DNA derived from the virus may be obtained by a reverse transcriptase, or DNA may be obtained from a tissue infected by a virus, and DNA derived from the virus may be obtained. When DNA is obtained from RNA by a reverse transcriptase, a commercially available kit (Transcripter high fidelity cDNA synthesis kit, available from Roche Diagnostics K.K.) and the like may be used.

[0073] In "methylated DNA", any of four kinds of bases constituting gene (genomic DNA) is methylated. For example, in a mammal is known a phenomenon that only cytosine in a nucleotide sequence represented by 5'-CG-3' (C represents

cytosine, and G represents guanine. Hereinafter, the nucleotide sequence is occasionally denoted by "CpG") is methylated. A methylation site of cytosine is position 5. In DNA duplication antecedent to cell division, only cytosine in "CpG" in a template chain derived from a parent cell is methylated in nascent double-stranded DNA, and cytosine in "CpG" in a nascent DNA chain is also methylated rapidly by the action of a methyltransferase. In this cytosine methylation is methylated cytosine in CpG in a nascent DNA chain that complementarily binds to CpG containing methylated cytosine in a DNA chain derived from a parent cell. Therefore, the methylation condition of DNA of the parent cell is taken over as it is to new two sets of DNA after DNA duplication. The term "CpG pair" means a double-stranded DNA in which a nucleotide sequence represented by CpG binds to CpG complementary to the sequence.

[0074] The term "single-stranded methylated DNA" means single-stranded DNA in which is methylated cytosine at a position 5 in a nucleotide sequence represented by 5'-CG-3' in a nucleotide sequence of the single-stranded DNA.

[0075] Examples of the "target DNA region" include promoter regions, untranslated regions or translated regions (coding regions) of useful protein genes such as Lysyl oxidase, HRAS-like suppressor, bA305P22.2.1, Gamma filamin, HAND1, Homologue of RIKEN 2210016F16, FLJ32130, PPARG angiopoietin-related protein, Thrombomodulin, p53-responsive gene 2, Fibrillin 2, Neurofilament 3, disintegrin and metalloproteinase domain 23, G protein-coupled receptor 7, G-protein coupled somatostatin and angiotensin-like peptide receptor, and Solute carrier family 6 neurotransmitter transporter noradrenalin member 2, and preferably include DNA regions containing one or more CpG present in these nucleotide sequences. In the present method, methylated DNA of "target DNA region" may be detected or quantified individually, and, for example, when more methylated DNA of "target DNA region" is detected in one detection system, the quantification accuracy and detection sensitivity are improved correspondingly.

[0076] To be more specific, when the useful protein gene is a Lysyl oxidase gene, as a nucleotide sequence that includes at least one nucleotide sequence represented by CpG present in a nucleotide sequence of its promoter region, untranslated region or translated region (coding region), a nucleotide sequence of a genomic DNA containing exon 1 of a Lysyl oxidase gene derived from human, and a promoter region located 5' upstream of the same can be recited, and more concretely, the nucleotide sequence of SEQ ID NO: 1 (corresponding to a nucleotide sequence represented by base No. 16001 to 18661 in the nucleotide sequence described in Genbank Accession No. AF270645) can be recited. In the nucleotide sequence of SEQ ID NO: 1, ATG codon encoding methionine at amino terminal of Lysyl oxidase protein derived from human is represented in base No. 2031 to 2033, and a nucleotide sequence of the above exon 1 is represented in base No. 1957 to 2661.

[0077] To be more specific, when the useful protein gene is a HRAS-like suppressor gene, as a nucleotide sequence that includes at least one nucleotide sequence represented by CpG present in a nucleotide sequence of its promoter region, untranslated region or translated region (coding region), a nucleotide sequence of a genomic DNA containing exon 1 of a HRAS-like suppressor gene derived from human, and a promoter region located 5' upstream of the same can be recited, and more concretely, the nucleotide sequence of SEQ ID NO: 2 (corresponding to a nucleotide sequence represented by base No. 172001 to 173953 in the nucleotide sequence described in Genbank Accession No. AC068162) can be recited. In the nucleotide sequence of SEQ ID NO: 2, the nucleotide sequence of exon 1 of a HRAS-like suppressor gene derived from human is represented in base No. 1743 to 1953.

[0078] To be more specific, when the useful protein gene is a bA305P22.2.1 gene, as a nucleotide sequence that includes at least one nucleotide sequence represented by CpG present in a nucleotide sequence of its promoter region, untranslated region or translated region (coding region), a nucleotide sequence of a genomic DNA containing exon 1 of a bA305P22.2.1 gene derived from human, and a promoter region located 5' upstream of the same can be recited, and more concretely, the nucleotide sequence of SEQ ID NO: 3 (corresponding to a nucleotide sequence represented by base No. 13001 to 13889 in the nucleotide sequence described in Genbank Accession No. AL121673) can be recited. In the nucleotide sequence of SEQ ID NO: 3, ATG codon encoding methionine at amino terminal of bA305P22.2.1 protein derived from human is represented in base No. 849 to 851, and a nucleotide sequence of the above exon 1 is represented in base No. 663 to 889.

[0079] To be more specific, when the useful protein gene is a Gamma filamin gene, as a nucleotide sequence that includes at least one nucleotide sequence represented by CpG present in a nucleotide sequence of its promoter region, untranslated region or translated region (coding region), a nucleotide sequence of a genomic DNA containing exon 1 of a Gamma filamin gene derived from human, and a promoter region located 5' upstream of the same can be recited, and more concretely, the nucleotide sequence of SEQ ID NO: 4 (corresponding to a complementary sequence to a nucleotide sequence represented by base No. 63528 to 64390 in the nucleotide sequence described in Genbank Accession No. AC074373) can be recited. In the nucleotide sequence of SEQ ID NO: 4, ATG codon encoding methionine at amino terminal of Gamma filamin protein derived from human is represented in base No. 572 to 574, and a nucleotide sequence of the above exon 1 is represented in base No. 463 to 863.

[0080] To be more specific, when the useful protein gene is a HAND1 gene, as a nucleotide sequence that includes at least one nucleotide sequence represented by CpG present in a nucleotide sequence of its promoter region, untranslated region or translated region (coding region), a nucleotide sequence of a genomic DNA containing exon 1 of a HAND1 gene derived from human, and a promoter region located 5' upstream of the same can be recited, and more concretely,

the nucleotide sequence of SEQ ID NO: 5 (corresponding to a complementary sequence to a nucleotide sequence represented by base No. 24303 to 26500 in the nucleotide sequence described in Genbank Accession No. AC026688) can be recited. In the nucleotide sequence of SEQ ID NO: 5, ATG codon encoding methionine at amino terminal of HAND1 protein derived from human is represented in base No. 1656 to 1658, and a nucleotide sequence of the above exon 1 is represented in base No. 1400 to 2198.

[0081] To be more specific, when the useful protein gene is a Homologue of RIKEN 2210016F16 gene, as a nucleotide sequence that includes at least one nucleotide sequence represented by CpG present in a nucleotide sequence of its promoter region, untranslated region or translated region (coding region), a nucleotide sequence of a genomic DNA containing exon 1 of a Homologue of RIKEN 2210016F16 gene derived from human, and a promoter region located 5' upstream of the same can be recited, and more concretely, the nucleotide sequence of SEQ ID NO: 6 (corresponding to a complementary nucleotide sequence to a nucleotide sequence represented by base No. 157056 to 159000 in the nucleotide sequence described in Genbank Accession No. AL354733) can be recited. In the nucleotide sequence of SEQ ID NO: 6, a nucleotide sequence of exon 1 of a Homologue of a RIKEN 2210016F16 gene derived from human is represented in base No. 1392 to 1945.

[0082] To be more specific, when the useful protein gene is a FLJ32130 gene, as a nucleotide sequence that includes at least one nucleotide sequence represented by CpG present in a nucleotide sequence of its promoter region, untranslated region or translated region (coding region), a nucleotide sequence of a genomic DNA containing exon 1 of a FLJ32130 gene derived from human, and a promoter region located 5' upstream of the same can be recited, and more concretely, the nucleotide sequence of SEQ ID NO: 7 (corresponding to a complementary nucleotide sequence to a nucleotide sequence represented by base No. 1 to 2379 in the nucleotide sequence described in Genbank Accession No. AC002310) can be recited. In the nucleotide sequence of SEQ ID NO: 7, ATG codon encoding methionine at amino terminal of FLJ32130 protein derived from human is represented in base No. 2136 to 2138, and a nucleotide sequence assumed to be the above exon 1 is represented in base No. 2136 to 2379.

[0083] To be more specific, when the useful protein gene is a PPARG angiopoietin-related protein gene, as a nucleotide sequence that includes at least one nucleotide sequence represented by CpG present in a nucleotide sequence of its promoter region, untranslated region or translated region (coding region), a nucleotide sequence of a genomic DNA containing exon 1 of a PPARG angiopoietin-related protein gene derived from human, and a promoter region located 5' upstream of the same can be recited, and more concretely, the nucleotide sequence of SEQ ID NO: 8 can be recited. In the nucleotide sequence of SEQ ID NO: 8, ATG codon encoding methionine at amino terminal of PPARG angiopoietin-related protein derived from human is represented in base No. 717 to 719, and a nucleotide sequence of the 5' side part of the above exon 1 is represented in base No. 1957 to 2661.

[0084] To be more specific, when the useful protein gene is a Thrombomodulin gene, as a nucleotide sequence that includes at least one nucleotide sequence represented by CpG present in a nucleotide sequence of its promoter region, untranslated region or translated region (coding region), a nucleotide sequence of a genomic DNA containing exon 1 of a Thrombomodulin gene derived from human, and a promoter region located 5' upstream of the same can be recited, and more concretely, the nucleotide sequence of SEQ ID NO: 9 (corresponding to a nucleotide sequence represented by base No. 1 to 6096 in the nucleotide sequence described in Genbank Accession No. AF495471) can be recited. In the nucleotide sequence of SEQ ID NO: 9, ATG codon encoding methionine at amino terminal of Thrombomodulin protein derived from human is represented in base No. 2590 to 2592, and a nucleotide sequence of the above exon 1 is represented in base No. 2048 to 6096.

[0085] To be more specific, when the useful protein gene is a p53-responsive gene 2 gene, as a nucleotide sequence that includes at least one nucleotide sequence represented by CpG present in a nucleotide sequence of its promoter region, untranslated region or translated region (coding region), a nucleotide sequence of a genomic DNA containing exon 1 of a p53-responsive gene 2 gene derived from human, and a promoter region located 5' upstream of the same can be recited, and more concretely, the nucleotide sequence of SEQ ID NO: 10 (corresponding to a complementary sequence to a nucleotide sequence represented by base No. 113501 to 116000 in the nucleotide sequence described in Genbank Accession No. AC009471) can be recited. In the nucleotide sequence of SEQ ID NO: 10, a nucleotide sequence of exon 1 of a p53-responsive gene 2 gene derived from human is represented in base No. 1558 to 1808.

[0086] To be more specific, when the useful protein gene is a Fibrillin2 gene, as a nucleotide sequence that includes at least one nucleotide sequence represented by CpG present in a nucleotide sequence of its promoter region, untranslated region or translated region (coding region), a nucleotide sequence of a genomic DNA containing exon 1 of a Fibrillin2 gene derived from human, and a promoter region located 5' upstream of the same can be recited, and more concretely, the nucleotide sequence of SEQ ID NO: 11 (corresponding to a complementary sequence to a nucleotide sequence represented by base No. 118801 to 121000 in the nucleotide sequence described in Genbank Accession No. AC113387) can be recited. In the nucleotide sequence of SEQ ID NO: 11, a nucleotide sequence of exon 1 of a Fibrillin2 gene derived from human is represented in base No. 1091 to 1345.

[0087] To be more specific, when the useful protein gene is a Neurofilament3 gene, as a nucleotide sequence that includes at least one nucleotide sequence represented by CpG present in a nucleotide sequence of its promoter region,

untranslated region or translated region (coding region), a nucleotide sequence of a genomic DNA containing exon 1 of a Neurofilament3 gene derived from human, and a promoter region located 5' upstream of the same can be recited, and more concretely, the nucleotide sequence of SEQ ID NO: 12 (corresponding to a complementary sequence to a nucleotide sequence represented by base No. 28001 to 30000 in the nucleotide sequence described in Genbank Accession No. AF106564) can be recited. In the nucleotide sequence of SEQ ID NO: 12, a nucleotide sequence of exon 1 of a Neurofilament3 gene derived from human is represented in base No. 614 to 1694.

[0088] To be more specific, when the useful protein gene is a disintegrin and metalloproteinase domain 23 gene, as a nucleotide sequence that includes at least one nucleotide sequence represented by CpG present in a nucleotide sequence of its promoter region, untranslated region or translated region (coding region), a nucleotide sequence of a genomic DNA containing exon 1 of a disintegrin and metalloproteinase domain 23 gene derived from human, and a promoter region located 5' upstream of the same can be recited, and more concretely, the nucleotide sequence of SEQ ID NO: 13 (corresponding to a nucleotide sequence represented by base No. 21001 to 23300 in the nucleotide sequence described in Genbank Accession No. AC009225) can be recited. In the nucleotide sequence of SEQ ID NO: 13, a nucleotide sequence of exon 1 of a disintegrin and metalloproteinase domain 23 gene derived from human is represented in base No. 1194 to 1630.

[0089] To be more specific, when the useful protein gene is a G protein-coupled receptor 7 gene, as a nucleotide sequence that includes at least one nucleotide sequence represented by CpG present in a nucleotide sequence of its promoter region, untranslated region or translated region (coding region), a nucleotide sequence of a genomic DNA containing exon 1 of a G protein-coupled receptor 7 gene derived from human, and a promoter region located 5' upstream of the same can be recited, and more concretely, the nucleotide sequence of SEQ ID NO: 14 (corresponding to a nucleotide sequence represented by base No. 75001 to 78000 in the nucleotide sequence described in Genbank Accession No. AC009800) can be recited. In the nucleotide sequence of SEQ ID NO: 14, a nucleotide sequence of exon 1 of a G protein-coupled receptor 7 gene derived from human is represented in base No. 1666 to 2652.

[0090] To be more specific, when the useful protein gene is a G-protein coupled somatostatin and angiotensin-like peptide receptor gene, as a nucleotide sequence that includes at least one nucleotide sequence represented by CpG present in a nucleotide sequence of its promoter region, untranslated region or translated region (coding region), a nucleotide sequence of a genomic DNA containing exon 1 of a G-protein coupled somatostatin and angiotensin-like peptide receptor gene derived from human, and a promoter region located 5' upstream of the same can be recited, and more concretely, the nucleotide sequence of SEQ ID NO: 15 (corresponding to a complementary sequence to a nucleotide sequence represented by base No. 57001 to 60000 in the nucleotide sequence described in Genbank Accession No. AC008971) can be recited. In the nucleotide sequence of SEQ ID NO: 15, a nucleotide sequence of exon 1 of a G-protein coupled somatostatin and angiotensin-like peptide receptor gene derived from human is represented in base No. 776 to 2632.

[0091] To be more specific, when the useful protein gene is a Solute carrier family 6 neurotransmitter transporter noradrenalin member 2 gene, as a nucleotide sequence that includes at least one nucleotide sequence represented by CpG present in a nucleotide sequence of its promoter region, untranslated region or translated region (coding region), a nucleotide sequence of a genomic DNA containing exon 1 of a Solute carrier family 6 neurotransmitter transporter noradrenalin member 2 gene derived from human, and a promoter region located 5' upstream of the same can be recited, and more concretely, the nucleotide sequence of SEQ ID NO: 16 (corresponding to a complementary sequence to a nucleotide sequence represented by base No. 78801 to 81000 in the nucleotide sequence described in Genbank Accession No. AC026802) can be recited. In the nucleotide sequence of SEQ ID NO: 16, a nucleotide sequence of exon 1 of a Solute carrier family 6 neurotransmitter transporter noradrenalin member 2 gene derived from human is represented in base No. 1479 to 1804.

[0092] Second step is a step of treating the DNA prepared in First step with a DNA methylation enzyme.

[0093] The "DNA methylation enzyme" means an enzyme that methylates a base in DNA, and various kinds DNA methylation enzymes are isolated from mammalian cells, bacteria and the like. DNA methylation enzymes are classified into several kinds such as adenine methylation enzymes, and cytosine methylation enzymes according to the kind of the base of a substrate. A cytosine methylation enzyme is an enzyme that recognizes a specific sequence in a DNA nucleotide sequence, and methylates cytosine near the sequence, and different cytosine methylation enzymes are known according to the recognized nucleotide sequences.

[0094] A number of methylation reactions of DNA catalyzed by a DNA methylation enzyme are found from a primitive immune system called a restriction-modification system. The restriction-modification system is a function that digests foreign DNA (in particular, bacteriophage) with a restriction enzyme after regularly methylating the entire genome functioning in bacteria to protect it from being digested by a restriction enzyme (restriction endonuclease) that recognizes a specific sequence, and is a system for protecting a microbial genome from bacteriophage infection. Enzymes functioning in methylation of genome are known to methylate cytosine or adenine, and often known to methylate nitrogen at position 6 (N6) or carbon at position 5 (C5) of a purine residue. Among these enzymes, known as a cytosine methylation enzyme that methylates C5 of cytosine are SssI (M.SssI) methylase, AluI methylase, HhaI methylase, HpaII methylase, MspI

methylase, HaeIII methylase, and so on. These enzymes that methylate position C5 of cytosine recognize different nucleotide sequences, and a cytosine methylation enzyme that recognizes CpG is only SssI.

[0095] As a methylation reaction of DNA in human genome, methylation at position 5 (C5) of cytosine in CpG is known as epigenetics (the mechanism generating diversity of gene expression independent of gene sequence), and as such a cytosine methylation enzyme, DNA methyltransferase is known. As a DNA methyltransferase, DnmtI methyltransferase is known.

[0096] In human cells, since position C5 of cytosine in a CpG sequence is methylated, for methylating genome artificially, the same position of the same cytosine in the same sequence (CpG) with methylation in a human cell can be methylated by using SssI.

[0097] For methylating DNA by a cytosine methylation enzyme, concretely, for example, a DNA sample is added with 5 μL of an optimum 10× buffer (NEBuffer2 (available from NEB Inc.)), 0.5 μL of S-adenosyl methionine (3.2 mM, available from NEB Inc.) and 0.5 μL of cytosine methylation enzyme SssI(available from NEB Inc.), and then the resultant mixture is added with sterilized ultrapure water to make the liquid amount 50 μL, and then incubated at 37°C for 30 minutes.

[0098] Third step is a step of preparing single-stranded methylated DNA from the DNA treated with a DNA methylation enzyme in Second step.

[0099] For example, when the methylated DNA is double-stranded DNA, the double-stranded DNA is divided into single-stranded DNA. Concretely, DNA that is methylated by a DNA methylation enzyme in Second step is prepared into a solution of 1 ng/μL with Tris-HCl buffer (10 mM), and 10 μL of a buffer (330 mM Tris-Acetate pH 7.9, 660 mM KOAc, 100 mM $MgOAc_2$, 5 mM Dithiothreitol), 10 μL of 100 mM $MgCl_2$ solution, and 10 μL of 1 mg/mL BSA solution are mixed, and the resultant mixture is added with sterilized ultrapure water to make the liquid amount 100 μL. Thereafter the mixture is heated at 95°C for 10 minutes, and then rapidly cooled on ice-cooled water for several minutes. For example, when the DNA prepared from a specimen is free DNA contained in blood or the like, the DNA methylated in Second step can be single-stranded DNA. Since such single-stranded DNA sometimes forms a higher order structure, it is advisable to conduct a treatment similar to the case of the double-stranded DNA.

[0100] Fourth step is a step of forming a complex of a single-stranded methylated DNA comprising a methylated target DNA region, a methylated DNA antibody, and a specific oligonucleotide by mixing the single-stranded methylated DNA prepared in Third step, the methylated DNA antibody, and the specific oligonucleotide comprising a nucleotide sequence that does not inhibit binding between one or more methylated bases in the target DNA region in the single-stranded methylated DNA and the methylated DNA antibody, and that is capable of binding with the single-stranded DNA by complementation.

[0101] "Methylated DNA antibody" is an antibody that binds with a methylated base in DNA as an antigen. Concretely, methylcytosine antibody can be recited, and an antibody having the property of recognizing cytosine whose position 5 is methylated in single-stranded DNA and binding thereto can be recited. Commercially available methylated DNA antibodies may also be used as far as they specifically recognize DNA in a methylation state described in the present specification, and are capable of specifically binding thereto. Such a methylated DNA antibody can be prepared in a conventional method using a methylated base, methylated DNA or the like as an antigen. For concretely preparing a methylcytosine antibody, selection is made according to specific binding to methylcytosine in DNA as an indicator from antibodies prepared using DNA containing 5-methylcytidine, 5-methylcytosine, or 5-methylcytosine as an antigen. Considering the property of the methylated DNA antibody, namely, the fact that one antibody binds to one methylated base (cytosine), improvements in quantification accuracy and detection sensitivity are expected by selecting the region where a number of methylated bases (cytosine), namely CpG, are present, as the target DNA region.

[0102] Known as antibodies that can be obtained by immunizing an animal with an antigen are an antibody of IgG fraction (polyclonal antibody), an antibody producing a single clone (monoclonal antibody) and the like that can be obtained by immunizing an animal with an antigen. In the present invention, since an antibody capable of specifically recognizing methylated DNA or methylcytosine is preferred, use of a monoclonal antibody is advisable.

[0103] As a method of preparing a monoclonal antibody, a procedure based on a cell fusion method can be recited. For example, in the cell fusion method, a hybridoma is prepared by allowing cell fusion between a spleen cell (B cell) derived from an immunized mouse and a myeloma cell, and an antibody produced by the hybridoma is selected for preparation of a methyl cytosine antibody (monoclonal antibody). When a monoclonal antibody is prepared by a cell fusion method, it is not necessary to purify an antigen, and for example, a mixture of 5-methyl cytidine, 5-methyl cytosine or DNA or the like containing 5-methyl cytosine may be administered as an antigen to an animal used for immunization. As an administration method, 5-methyl cytidine, 5-methyl cytosine or DNA or the like containing 5-methyl cytosine is directly administered to a mouse for production of an antibody. When an antibody is difficult to be produced, an antigen bound to a support may be used for immunization. Also, by thoroughly mixing an adjuvant solution (prepared, for example, by mixing liquid paraffin and Aracel A, and mixing killed tubercle bacilli as an adjuvant) and an antigen and administering the same, or immunizing via liposome incorporating the same, immunity of an antigen can be improved. Also a method involving adding equivalent amounts of a solution containing an antigen and an adjuvant solution, fully emulsifying them, and subcutaneously or intraperitoneally injecting the resultant mixture to a mouse, and a method of adding killed Bordetella

pertussis as an adjuvant after mixing well with alum water are known. A mouse may be boosted intraperitoneally or intravenously after an appropriate term from initial immunization. When the amount of an antigen is small, a solution in which the antigen is suspended may be directly injected into a mouse spleen to effect immunization.

[0104] After exenterating a spleen and peeling an adipose tissue off after several days from the final immunization, a spleen cell suspension is prepared. The spleen cell is fused, for example, with an HGPRT-deficient myeloma cell to prepare a hybridoma. As a cell fusion agent, any means capable of efficiently fusing a spleen cell (B cell) and a myeloma cell is applicable, and for example, a method of using a hemagglutinating virus of Japan (HVJ), polyethyleneglycol (PEG) and the like are recited. Cell fusion may be conducted by a method using a high voltage pulse.

[0105] After the cell fusion operation, cells are cultured in an HAT medium, a clone of a hybridoma in which a spleen cell and a myeloma cell are fused is selected, and the cell is allowed to grow until screening becomes possible. In a method of detecting an antibody for selecting a hybridoma that produces an intended antibody, or a method of measuring a titer of an antibody, an antigen-antibody reaction system may be used. Concretely, as a method of measuring an antibody against a soluble antigen, a radioisotope immune assay (RIA), an enzyme-linked immunosorbent assay (ELISA) and the like can be recited.

[0106] Single-stranded DNA is able to bind with an anti-methylation antibody as far as CpG existing therein is methylated at least at one site. Therefore, the term "methylated" in the present method means DNA in which CpG existing therein is methylated at least at one site, and is not limited to DNA in which every CpG existing therein is methylated.

[0107] The term "specific oligonucleotide" is an oligonucleotide comprising a nucleotide sequence capable of binding with single-stranded DNA comprising a target DNA region by complementation, and has a function of binding to a support as will be described later. Here, "nucleotide sequence capable of binding with single-stranded DNA comprising a target DNA region by complementation" is called a specific adhesion sequence.

[0108] The term "nucleotide sequence capable of binding with single-stranded DNA comprising a target DNA region by complementation" means a nucleotide sequence capable of complementarily binding with a part of single-stranded DNA comprising a target DNA region, having a nucleotide sequence that is complementary to a part of a nucleotide sequence of single-stranded DNA comprising a target DNA region, or to a part of a nucleotide sequence of the DNA region which is located further 5'-end side from 5'-end of the target DNA region, or to a part of a nucleotide sequence located further 3'-end side from 3'-end of the target DNA region.

[0109] The wording "not inhibiting binding between one or more methylated bases in a target DNA region in single-stranded methylated DNA and the methylated DNA antibody" in Fourth step of the present method means that complementary binding between the specific oligonucleotide and the single-stranded DNA does not occur in an occupied space required for the methylated DNA antibody to bind with the methylated single-stranded DNA. It is supposed that for the methylated DNA antibody to bind with the methylated base (cytosine), not only the directly-binding methylated base (cytosine), but also the peripheral space where the methylated base (cytosine) exists would be occupied. Therefore, the specific oligonucleotide should be a nucleotide sequence that fails to complementarily bind with the single-stranded DNA (DNA comprising a target DNA region) in an occupied space required for the methylated DNA antibody to bind on the DNA having a target DNA region. The specific oligonucleotide to be bound with the single-stranded DNA is not necessarily one kind, but two or more kinds may be used unless binding of the methylated DNA antibody is inhibited. By using a plurality of specific oligonucleotides, quantification accuracy and detection sensitivity can be improved.

[0110] The wording "forming a complex of a single-stranded methylated DNA comprising a methylated target DNA region, a methylated DNA antibody, and a specific oligonucleotide by mixing the single-stranded methylated DNA, the methylated DNA antibody, and the specific oligonucleotide comprising a nucleotide sequence that does not inhibit binding between one or more methylated bases in the target DNA region in the single-stranded methylated DNA and the methylated DNA antibody, and that is capable of binding with the single-stranded DNA by complementation" in Fourth step means allowing the single-stranded methylated DNA comprising a target DNA region to complementarily bind with the specific oligonucleotide, and further allowing the DNA comprising a target DNA region to bind with the methylated DNA antibody, thereby forming a complex formed of the methylated DNA comprising a target DNA region, the specific oligonucleotide, and the methylated DNA. Here, by immobilizing the specific oligonucleotide to a support, it is possible to immobilize the complex to the support.

[0111] In Fourth step, for binding "the single-stranded methylated DNA comprising a target DNA region and the specific oligonucleotide complementarily", for example, the single-stranded methylated DNA comprising a methylated target DNA region may be allowed to complementarily bind with the specific oligonucleotide, and the methylated DNA antibody may be allowed to bind with the methylated DNA comprising a target DNA region after immobilizing the specific oligonucleotide to a support. For "complementarily binding the single-stranded methylated DNA comprising a target DNA region and the specific oligonucleotide", for example, a solution of the DNA comprising a target DNA region and the specific oligonucleotide capable of complementarily binding with the DNA are prepared into solutions of 0.02μM with Tris-HCl buffer (10 mM), and each 10 μL of these solutions and a buffer liquid (330 mM Tris-Acetate pH 7.9, 660 mM KOAc, 100 mM $MgOAc_2$, 5 mM Dithiothreitol), 10 μL of 100 mM $MgCl_2$ solution, and 10 μL of 1 mg/mL BSA solution are mixed, and further the mixture is added with sterilized ultrapure water to make the liquid amount 100 μL. Then the

mixture is heated at 95°C for 10 minutes, and rapidly cooled to 70°C and retained at this temperature for 10 minutes, and cooled to 50°C and retained for 10 minutes, and further retained at 37°C for 10 minutes, and returned to room temperature. In this manner, formation of a conjugate of the DNA comprising a target DNA region and the specific oligonucleotide can be promoted.

[0112] The wording "complementarily bind" means that double-stranded DNA is formed by base-pairing through a hydrogen bond between bases. For example, it means that respective bases constituting single-stranded DNA of a double strand forming DNA form a double strand by base-pairing between purine and pyrimidine, and more concretely, double-stranded DNA is formed by base-pairing through hydrogen bonds between a plurality of sequential thymine and adenine, and guanine and cytosine. Binding based on complementation may be sometimes expressed by "complementarily binding". "Complementarily binding" may be sometimes expressed by "complementary binding", "binding by complementation", "complementary binding (by base-pairing)", "complementary base-pairing" or "capable of complementarily base-pairing". Nucleotide sequences that are capable of complementarily binding may be sometimes expressed by "having complementation" or "complementary" each other. Binding of inosine contained in an artificially prepared oligonucleotide with cytosine, or adenine, or thymine through hydrogen bonding is also implied in complementary binding. The wording "single-stranded DNA comprising a nucleotide sequence complementary to the target DNA region" means that it is a nucleotide sequence comprising a nucleotide sequence that is necessary for formation of a conjugate (double-strand) with the single-stranded DNA comprising a target DNA region, namely a nucleotide sequence comprising a nucleotide sequence complementary to a part of a nucleotide sequence of the target DNA region, and is also expressed by "complementary nucleotide sequence". The complementary nucleotide sequence may be sometimes expressed by "complementary", "nucleotide sequence capable of binding by complementation" or "complementary sequence".

[0113] In the present method, when the DNA comprising a target DNA region and the specific oligonucleotide "bind by complementation", it also includes the case where a part of the nucleotide sequence constituting the specific adhesion sequence of the specific oligonucleotide fails to base-pair with the DNA comprising a target DNA region. For example, the cases are also included where among bases constituting the specific adhesion sequence, at least 75%, preferably 80% or more bases base-pair with the DNA comprising a target DNA region, and the oligonucleotide having a homology of at least 75% or more, preferably 80% or more with the DNA comprising a target DNA region is able to bind with the specific adhesion sequence.

[0114] When the DNA comprising a target DNA region is a repetitive sequence in genome as will be described later, the repetitive sequence is a group of nucleotide sequences having homology, so that there is a possibility that a part of the specific adhesion sequence fails to base-pair with the DNA comprising a target DNA region. In other words, in the present method, when the DNA comprising a target DNA region is a repetitive sequence such as LINE sequence or SINE (Alu) sequence, a specific adhesion sequence capable of binding with a nucleotide sequence having a homology of 80% or more by complementation may be used.

[0115] As a preferred aspect in forming a conjugate of the single-stranded DNA comprising a target DNA region and the specific oligonucleotide, formation in a reaction system containing a divalent cation can be recited. More preferably, the divalent cation is a magnesium ion. Here, the wording "reaction system containing a divalent cation" means a reaction system containing a divalent cation in an annealing buffer used for binding between the single-stranded DNA comprising a target DNA region and the specific oligonucleotide, and concretely, for example, a system containing a salt composed of a magnesium ion (for example, $MgOAc_2$, $MgCl_2$ and the like) in a concentration of 1 mM to 600 mM can be recited.

[0116] In Fourth step of the present method, a specific oligonucleotide for two or more kinds of target DNA regions may be mixed to form a complex, or two or more kinds of specific oligonucleotides for one kind of target region may be mixed to form a complex.

[0117] In the present method, when "a complex is formed", the complex formed of the single-stranded methylated DNA comprising a target DNA region, the specific oligonucleotide and the methylated DNA antibody is bound and immobilized to the support as will be described later.

[0118] For "forming a complex", concretely, for example, it may be practiced in the following manner using "biotinylated specific oligonucleotide" whose terminal is labeled with biotin as a specific oligonucleotide that is immobilizable to a support.

(a) A DNA sample derived from genomic DNA is added with an annealing buffer (for example, 33 mM Tris-Acetate pH 7.9, 66 mM KOAc, 10 mM $MgOAc_2$, 0.5 mM Dithothreitol) and a biotinylated specific oligonucleotide to obtain a mixture. Then the obtained mixture is heated at 95°C, for example, for several minutes to make the double-stranded DNA derived from genomic DNA into single-stranded DNA. Then for forming a conjugate of the single-stranded DNA comprising a target DNA region and the biotinylated specific oligonucleotide, the mixture is cooled rapidly to a temperature lower than the Tm value of the biotinylated specific oligonucleotide by about 10 to 20°C, and retained at this temperature, for example, for several minutes, and then returned to room temperature.

(b) The mixture obtained in the above (a) is added to a support coated with streptavidin, and further retained it at 37°C, for example, for several minutes, to immobilize the conjugate of the single-stranded DNA comprising a target

DNA region and the biotinylated specific oligonucleotide to the support coated with streptavidin. Thereafter, the remaining solution is removed and washed. A washing buffer (for example, 0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4)) is added, for example, in an amount of 300 μL/well, and the solution is removed. This washing operation is repeated several times, to leave the conjugate of the biotinylated specific oligonucleotide and the single-stranded DNA comprising a target DNA region that is immobilized to the support, on the well.

(c) An appropriate amount (for example, 100 μL/well of 4 μg/mL solution) of a methylated DNA antibody is added to a well, and then left still, for example, for three hours at room temperature, to promote formation of a complex of the methylated DNA antibody, the methylated single-stranded DNA comprising a target DNA region from the single-stranded DNA, and the biotinylated specific oligonucleotide (formation of complex). Then the remaining solution is removed and washed. A washing buffer (for example, 0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4)) is added, for example, in an amount of 300 μL/well, and the solution is removed. This washing operation is repeated several times, to leave the complex on the well (selection of complex).

[0119] As the annealing buffer used in (a), those suited for binding the specific oligonucleotide and the single-stranded DNA comprising a target DNA region may be used without limited to the aforementioned annealing buffer. Use of a buffer in which a divalent ion, preferably a magnesium ion is dissolved in a concentration of 1 to 600 mM improves the binding stability.

[0120] The washing operation in (b) and (c) is important for removing the DNA that is not bound to the specific oligonucleotide, the methylated DNA antibody that is not immobilized to the support, or the DNA digested with a later-described restriction enzyme and thus suspended in the solution, from the reaction solution. The washing buffer is not limited to the washing buffer described above, insofar as it is suited for removing the free methylated DNA antibody, single-stranded DNA suspended in the solution and the like, and a DELFIA buffer (available from PerkinElmer, Tris-HCl pH 7.8 with Tween 80), a TE buffer and the like may be used.

[0121] In the above (a) to (c), binding between the single-stranded DNA comprising a target DNA region and the biotinylated specific oligonucleotide is executed in a stage previous to immobilization of the biotinylated specific oligonucleotide to the support coated with streptavidin, however, this order may be inverted. For example, by adding a DNA sample derived from genomic DNA to the biotinylated specific oligonucleotide immobilized to the support coated with streptavidin, a mixture is obtained. For making double-stranded DNA comprising a target DNA region possessed by genomic DNA into single strands, the obtained mixture is heated at 95°C, for example, for several minutes, and then for allowing formation of a conjugate with the biotinylated specific oligonucleotide, the mixture is rapidly cooled to a temperature lower than the Tm value of the biotinylated specific oligonucleotide by about 10 to 20°C, and retained at this temperature, for example, for several minutes. Thereafter, the operation of (c) may be executed, to form and select a complex. In this stage, a conjugate of the unmethylated single-stranded DNA comprising a target DNA region and the specific oligonucleotide fails to form a complex.

[0122] The operations of the above (a) to (c) may be conducted using a chromatostrip. In this case, concretely, the operations are conducted in the following manner. A solution in which the conjugate of the single-stranded DNA comprising a target DNA region possessed by genomic DNA and the biotinylated specific oligonucleotide is formed is developed by a chromatostrip partially coated with streptavidin. By this operation, the conjugate of the single-stranded DNA comprising a target DNA region possessed by genomic DNA and the biotinylated specific oligonucleotide is immobilized to the part coated with streptavidin. Then an appropriate amount of the methylated DNA antibody is developed by the chromatostrip as described above. Through these operations, the complex of the methylated single-stranded DNA comprising a target DNA region possessed by genomic DNA, the biotinylated specific oligonucleotide, and the methylated DNA antibody is immobilized to the part coated with streptavidin (formation and selection of complex). Also for these operations, the order of formation of complex may be inverted. For example, after forming a complex made up of a methylated single-stranded DNA comprising a target DNA region, a biotinylated specific oligonucleotide, and a methylated DNA antibody, the complex may be developed by a chromatostrip, and immobilized to the part coated with streptavidin. In these operations, unnecessary components can be removed by developing the solution by a chromatostrip, and a washing operation can be omitted. A washing operation (development of a chromatostrip by a washing buffer (for example, a 0.05% Tween 20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4) may be conducted between these operations without causing any problem.

[0123] As the "support", the material and form thereof are not particularly limited as far as a complex can bind thereto. For example, any form suited for use purpose may be employed, including the forms of tube, test plate, filter, disc, bead and the like. As the material, those used as supports for a usual immune measuring method, for example, synthetic resins such as polystyrene, polypropylene, polyacrylamide, polymethylmethacrylate, polysulfone, polyacrylonitrile and nylon, or those obtained by incorporating a reactive functional group such as sulfonic group, amino group or the like to the synthetic resins can be recited. Also, glass, polysaccharides or derivatives thereof (cellulose, nitrocellulose and the

like), silica gel, porous ceramics, metal oxides and the like may be used.

[0124] When the complex is immobilized to a support, it suffices that the complex is eventually immobilized to the support in the condition that the complex of the methylated single-stranded DNA comprising a target DNA region, the specific oligonucleotide, and the methylated DNA antibody is formed, and

(1) the specific oligonucleotide may be immobilized in the stage where the single-stranded DNA and the specific oligonucleotide complementarily bind, and then the methylated DNA antibody may be bound to the single-stranded DNA, and

(2) the specific oligonucleotide may be immobilized to the support after the single-stranded DNA, the specific oligonucleotide and the methylated DNA antibody form a complex.

[0125] For immobilizing a specific oligonucleotide to a support, concretely a method of immobilizing a biotinylated oligonucleotide obtained by biotinylating 5'-end or 3'-end of the specific oligonucleotide to a support coated with streptavidin (for example, a PCR tube coated with streptavidin, magnetic beads coated with streptavidin, a chromatostrip partially coated with streptavidin and the like) is recited. Also there is a method of letting 5'-end or 3'-end of the specific oligonucleotide covalently bind with a molecule having an active functional group such as an amino group, a thiol group, an aldehyde group or the like, and then letting it covalently bind to a support made of glass, a polysaccharide derivative, silica gel or the synthetic resin or a thermostable plastic whose surface is activated by a silane coupling agent or the like. Covalent binding is achieved, for example, by a spacer formed by serially connecting five triglycerides, a cross linker or the like. Also there is a method of chemically synthesizing from the terminal side of the specific oligonucleotide directly on a support made of glass or silicon.

[0126] In the case of "the specific oligonucleotide is immobilized in the stage where the single-stranded DNA and the specific oligonucleotide complementarily bind, and then the methylated DNA antibody is bound to the single-stranded DNA", the methylated DNA antibody and a masking oligonucleotide may be added at the same time.

[0127] The "masking oligonucleotide" in the present method is an oligonucleotide capable of complementarily binding with the specific oligonucleotide, and indicates the oligonucleotide added for preventing the methylated DNA antibody from nonspecifically binding with the specific oligonucleotide existing in a single-stranded state by complementarily binding with the specific oligonucleotide in a single-stranded state immobilized to the support without binding with the DNA comprising a target DNA region which is single-stranded DNA in the stage where "the specific oligonucleotide is immobilized to the support in the stage where the single-stranded DNA and the specific oligonucleotide complementarily bind". As the masking oligonucleotide, any oligonucleotide that has a nucleotide sequence complementary to the specific oligonucleotide, and does not generate a part which is to be a single strand as a result of complementary binding with the specific oligonucleotide is employed.

[0128] Here, the masking oligonucleotide may be added before formation of a complex by causing the methylated DNA antibody to bind with the conjugate of the DNA comprising a target DNA region and the specific oligonucleotide, or may be added concurrently with the methylated DNA antibody in forming a complex by causing the methylated DNA antibody to bind with the conjugate of the DNA comprising a target DNA region and the specific oligonucleotide.

[0129] Fifth step is a step of quantifying or detecting the DNA comprising a target DNA region in the single-stranded methylated DNA by quantifying or detecting the methylated DNA antibody contained in the complex formed in Fourth step based on its identification function.

[0130] The term "detection" in Fifth step means that discrimination is possible by the identification function of the methylated DNA antibody, when a total amount of the methylated DNA obtained in First step and the methylated DNA methylated by the DNA methylation enzyme treatment in Second step exceeds a detection limit. When methylated DNA is not detected, it indicates that the methylated DNA obtained in First step and the methylated DNA methylated by the DNA methylation enzyme treatment in Second step is less than a detection limit in the target DNA region in the specimen.

[0131] "Quantification" in Fifth step means a quantified detection amount detected by the identification function of the methylated DNA antibody. That is, it means that a value correlated with a total amount of the methylated DNA obtained in First step and the methylated DNA methylated by the DNA methylation enzyme treatment in Second step is obtained. For example, the quantified value of an amount of the methylated DNA antibody as the identification function is a value correlated with an amount of DNA in the target DNA region in the specimen, and for example, when the specimen is 1 mL of serum, it means that a value correlated with a total amount of the methylated DNA obtained in First step and the methylated DNA methylated by the DNA methylation enzyme treatment in Second step of the DNA of the target region contained in 1 mL of serum is acquired.

[0132] The term "identification function" in Fifth step may be a function capable of detecting or quantifying a methylated DNA antibody. The identification function may be any function possessed by the methylated DNA antibody, and for example, an identification function based on labeling of the methylated DNA antibody, and an identification function imparted to the methylated DNA antibody by a detection molecule binding to the methylated DNA antibody can be recited. Concretely, fluorescent and chromogenic characteristics of a methylated DNA antibody labeled with europium, gold

colloid, latex bead, radioisotope, fluorescent substance (FITC or the like), horseradish Peroxidase (HRP), alkaline phosphatase, biotin and the like are recited. These labels are fluorescent, chromogenic functions, and such a labeled molecule may be bound to the methylated DNA antibody by the characteristic of the antibody itself (the characteristic of binding with a secondary antibody of the antibody itself). The antibody binding with the methylated DNA antibody (also called secondary antibody) may be labeled with europium, gold colloid, latex bead, radioisotope, fluorescent substance (FITC or the like), horseradish Peroxidase (HRP), alkaline phosphatase, biotin and the like. As to the secondary antibody, when the methylated DNA antibody is not labeled, an antibody that recognizes the methylated DNA antibody as an antigen may be used as a secondary antibody. When the methylated DNA antibody is labeled, an antibody against the label may be used as a secondary antibody. Concretely, for a FITC-labeled methylated DNA antibody, a FITC antibody is applicable as a detection molecule secondary antibody. As a means for quantifying or detecting these functions, for example, measurement by a radiation detector, a spectrophotometer and the like, or visual observation and the like are recited.

[0133] In the present method, the support to which the specific oligonucleotide is immobilized may be a microparticle, and a microparticle as same as the support may be bound to the methylated DNA antibody. As the microparticles, latex beads, gold colloids (gold nanoparticles) and the like are recited.

[0134] In the present method, when the same kinds of microparticles are bound to the support and the methylated DNA antibody, the microparticle serving as a support, and the microparticle bound to the methylated DNA antibody can be detected as aggregation of microparticles by formation of a complex of the methylated DNA immobilized to the microparticle, the DNA comprising a target DNA region, and the methylated DNA antibody. In this case, when the microparticle is a latex bead, the aggregate can be detected by change in turbidity. When the microparticle is a gold colloid (gold nanoparticle), the aggregate can be detected by change in color tone (pink to purple).

[0135] Further, in the present method, aggregation of microparticles can be detected also when a plurality of methylated DNA antibodies to which the microparticles are bound bind at the same time on one DNA comprising a target DNA region. When the microparticle bound to the methylated DNA antibody is a latex bead, an aggregate can be detected by change in turbidity, and when the microparticle bound to the methylated DNA antibody is a gold colloid (gold nanoparticle), an aggregate can be detected by color tone change (from pink to purple). In this case, an equivalent result to that obtained by adding the methylated DNA antibody can be achieved even when the specific oligonucleotide is not added.

[0136] That is, it means that by formation of a detection complex as a result of binding of the methylated DNA antibody, the specific oligonucleotide, and the single-stranded methylated DNA comprising a methylated target DNA region, the microparticle which is a support for binding of the specific oligonucleotide, and the microparticle binding to the methylated DNA antibody form an aggregate. When the degree of methylation is detected by aggregation of the microparticles in this manner, an equivalent result to that obtained by adding the specific oligonucleotide can be achieved even when the specific oligonucleotide is not added.

[0137] An amount detected by aggregation of microparticles is correlated with a sum of DNA methylated by Second step. When DNA obtained in First step is DNA contained in a cell of a tissue, the degree of methylation of DNA contained in the cell of the tissue differs depending on the tissue, so that the degree of methylation of DNA obtained in Second step includes both the degree of methylation in the cell of the tissue, and the degree of methylation in Second step. That is, an amount detected by an aggregate of microparticles when a DNA methylation enzyme treatment is not executed in Second step is a value correlated with the degree of methylation in the cell of the tissue.

[0138] In the present method, one preferred aspect is that "DNA for which a target DNA region is to be detected contained in a specimen" is a DNA sample that is digested in advance with a restriction enzyme recognition cleaving site for which is not present in the target DNA region possessed by the genomic DNA. In forming a conjugate of the single-stranded DNA comprising a target DNA region possessed by genomic DNA and the specific oligonucleotide, the shorter the single-stranded DNA is, the better the operability is and the more easily a complex is formed as far as it contains the target DNA region. To shorten the single-stranded DNA, it is efficient to make it short when it is in original genomic DNA. Therefore, a digestion treatment may be conducted by making a restriction enzyme recognition cleaving site for which is not present in the target DNA region directly act on a DNA sample derived from genomic DNA. As a method of the digestion treatment by the restriction enzyme recognition cleaving site for which is not present in the target DNA region, a generally known restriction enzyme treatment method may be used. When the specimen is a DNA sample purified in advance, the treatment may be executed using an amount of a restriction enzyme generally used, whereas when the specimen is a tissue lysate, a cell lysate or the like, the treatment may be conducted with a large excess of a restriction enzyme, namely using an amount of 500 times or more the DNA amount of a restriction enzyme.

[0139] As a method of quantifying or detecting minor substances contained in a biological sample such as blood or urine, immunological measuring methods are generally used. Among such methods, what is called immuno chromatography using chromatography is currently widely used in various situations including, for example, clinical examinations in hospitals, assays in laboratories and the like because of its simple operation and short time required for assay. In recent years, what is called hybrid chromatography has been utilized in which labeled DNA (gene) is developed on a

chromatostrip, and target DNA (gene) is detected by hybridization using a probe capable of capturing the target DNA (gene). Also this method is now coming to be widely used in situations including, for example, clinical examinations in hospitals, assays in laboratories and the like because of its simple operation and short required time for assay. The present method conceptually enables a combined method of the immuno chromatography and the hybrid chromatography. In the present method, since the order of formation of a complex and selection of a complex is not particularly limited, various methods are possible. Concretely, such methods may be executed in the following manner.

[0140] Method 1: Methylated DNA (DNA in which a target DNA region has a cytosine methylated by a DNA methylation enzyme) is added with a biotinylated specific oligonucleotide which is a specific oligonucleotide to which biotin is bound as a function of immobilization to a support, to cause formation of a conjugate of the single-stranded methylated DNA comprising a target DNA region and the biotinylated specific oligonucleotide, and then added with a methylated antibody having an identification function, to cause formation of a complex in which the single-stranded DNA comprising a methylated target DNA region, the biotinylated specific oligonucleotide, and the methylated DNA antibody having an identification function are bound. As the obtained sample is dropped (introduced) into an introduction part of a chromatostrip, the complex migrates in a development part by a capillary phenomenon, and is trapped in the part coated in advance with streptavidin. Thereafter, by quantifying or detecting the methylated DNA antibody forming the obtained complex according to its identification function, methylated DNA in the target DNA region can be quantified or detected.

[0141] Method 2: Methylated DNA (DNA in which a target DNA region has a cytosine methylated by a DNA methylation enzyme) is added with a biotinylated specific oligonucleotide which is a specific oligonucleotide to which biotin is bound as a function of immobilization to a support, to cause formation of a conjugate of the single-stranded DNA comprising a target DNA region and the biotinylated specific oligonucleotide. As the obtained sample is dropped (introduced) into an introduction part of a chromatostrip, the conjugate migrates in a development part by a capillary phenomenon, and is trapped in the part preliminarily coated with streptavidin. Then, as a methylated antibody having an identification function is dropped (introduced) into an introduction part, it migrates in a development part and binds to methylated cytosine of the conjugate, to form a complex of the single-stranded DNA comprising a methylated target DNA region, the biotinylated specific oligonucleotide, and the methylated DNA antibody having an identification function. By quantifying or detecting the methylated DNA antibody forming the obtained complex according to its identification function, methylated DNA in the target DNA region can be quantified or detected.

[0142] Method 3: As a biotinylated specific oligonucleotide is dropped (introduced) into an introduction part of a chromatostrip, the oligonucleotide migrates in a development part by a capillary phenomenon, and is trapped in the part coated in advance with streptavidin. Then, as a single-stranded methylated DNA (single-stranded DNA having methylated cytosine in a target DNA region) is dropped (introduced) into an introduction part, it migrates in a development part, and is trapped by the biotinylated specific oligonucleotide that has been already trapped in the condition that the single-stranded DNA comprising a target DNA region forms a conjugate (the conjugate formed in this stage includes not only a conjugate of the single-stranded DNA comprising a methylated target DNA region and the specific oligonucleotide, but also a conjugate of a single-stranded DNA comprising an unmethylated target DNA region and the specific oligonucleotide). Then, as the methylated antibody having an identification function is dropped (introduced) into an introduction part, it migrates in a development part, and binds to the methylated cytosine of the conjugate, to form a complex of the single-stranded DNA comprising a methylated target DNA region, the biotinylated specific oligonucleotide, and the methylated DNA antibody having an identification function (in this stage, the conjugate of the single-stranded DNA comprising a unmethylated target DNA region and the specific oligonucleotide fails to form a complex). By quantifying or detecting the methylated DNA antibody forming the obtained complex according to its identification function, methylated DNA in the target DNA region can be quantified or detected.

[0143] Method 4: As a biotinylated specific oligonucleotide is dropped (introduced) into an introduction part of a chromatostrip, the oligonucleotide migrates in a development part by a capillary phenomenon, and is trapped in the part preliminarily coated with streptavidin. Single-stranded methylated DNA (single-stranded DNA having methylated cytosine in a target DNA region) is added with a methylated DNA antibody having an identification function, to cause formation of a conjugate of the single-stranded DNA having methylated cytosine (in which single-stranded DNA comprising a target DNA region and single-stranded DNA other than the target exist) and the methylated DNA antibody having an identification function (the conjugate formed in this stage includes not only a conjugate of the single-stranded DNA comprising a methylated target DNA region and the methylated antibody, but also a conjugate of methylated single-stranded DNA other than the target DNA region and the methylated antibody). As the obtained conjugate is dropped (introduced) into an introduction part, it migrates in a development part, and the single-stranded DNA comprising a methylated target DNA region binds to the biotinylated specific oligonucleotide that has been already trapped, to form a complex of the single-stranded DNA containing a methylated target DNA region, the biotinylated specific oligonucleotide, and the methylated DNA antibody having an identification function (in this stage, the conjugate of methylated single-stranded DNA other than in the target DNA region and the methylated antibody fails to form a complex). By quantifying or detecting the methylated DNA antibody forming the obtained complex according to its identification function, methylated DNA in the target DNA region can be quantified or detected.

[0144] Also a plurality of detection sites may be provided on a single chromatostrip (specific oligonucleotides capable of trapping different target DNA regions are immobilized to a support), and each target DNA region may be sequentially quantified or detected, and by enabling one detection site to trap a plurality of target DNA regions, or by immobilizing a number of the specific oligonucleotides capable of trapping a plurality of target DNA regions on one detection site, it is possible to dramatically improve the detection sensitivity.

[0145] In Fourth step of the present invention; a counter oligonucleotide may be added in forming a complex.

[0146] The term "counter oligonucleotide" is an oligonucleotide prepared for facilitating binding of a methylated DNA antibody to a methylated base which is a methylated base in double-stranded DNA. Usually, a methylated base is a base methylated in double-stranded DNA, however, the methylated DNA antibody is difficult to bind when DNA is in a double-stranded state. That is, for making the region where the methylated DNA antibody can bind into single-stranded DNA, an oligonucleotide (plus strand) having a nucleotide sequence which is the same as a target sequence (plus strand) is designed, and caused to bind with a nucleotide sequence (minus strand) that pairs with a target region, to make the target region into single-stranded DNA. This makes the methylated DNA antibody easy to bind with a methylated DNA base.

[0147] For example, for making a methylcytosine antibody bind with methylcytosine in a target region, a counter oligonucleotide having the same nucleotide sequence as that of the target region is added. In the present method, the counter oligonucleotide has a nucleotide sequence that does not pair with the specific oligonucleotide, and is characterized by not inhibiting binding between the specific oligonucleotide and a DNA fragment comprising a target region. Concretely, the counter oligonucleotide is an oligonucleotide comprising 5 to 100 bases, and preferably an oligonucleotide comprising 10 to 50 bases. The counter oligonucleotide having a nucleotide sequence of a target region is usually used in mixture of several kinds.

[0148] By comparing an amount of the methylated DNA measured in Fifth step without conducting methylation of DNA by a DNA methylation enzyme in Second step (total amount of methylated DNA), with an amount of the DNA measured in Fifth step after methylating DNA in Second step by a DNA methylation enzyme (total amount of methylated DNA and unmethylated DNA), it is possible to calculate a rate of methylated DNA in the target DNA region (hereinafter, sometimes referred to as the present methylation rate measuring method).

[0149] The present methylation rate measuring method may be used in the following situations.

[0150] It is known that DNA methylation abnormality occurs in various diseases (for example, cancer), and it is supposed that the level (degree) of various diseases can be measured by detecting this DNA methylation abnormality. For example, when there is a DNA region where methylation occurs at 100% in genomic DNA contained in a biological specimen derived from disease, and the present method or the present methylation rate measuring method is executed for the DNA region, the amount of methylated DNA that is detected or quantified will be large. On the other hand, when there is a DNA region where methylation does not occur at 100% in genomic DNA contained in a biological specimen derived from disease, and the present method or the present methylation rate measuring method is executed for the DNA region, the amount of methylated DNA that is detected or quantified would be approximately 0. For example, when there is a DNA region where a methylation rate is low in genomic DNA contained in a biological specimen derived from a healthy subject, and a methylation rate is high in genomic DNA contained in a biological specimen derived from a disease subject, and the present method or the present methylation rate measuring method is executed for the DNA region, the amount of methylated DNA would be approximately 0 for the healthy subject. On the other hand, since a value significantly higher than that of the healthy subject is shown in the disease patient, "level (degree) of disease" can be determined based on this difference in value.

[0151] "Level of disease" used herein has the same meaning commonly used in this field of art, and concretely means, for example, malignancy of a cell when the biological specimen is the cell, and means, for example, abundance of a disease cell in a tissue when the biological specimen is the tissue. Therefore, the present method or the present methylation rate measuring method makes it possible to diagnose degrees of various diseases by examining the degree (level) of methylation abnormality.

[0152] The restriction enzyme, specific oligonucleotide, or methylated DNA antibody that can be used in the present method is useful as a reagent of a detection kit. The present method makes it possible to provide a blood free DNA detection kit and a kit for detecting a microorganism in a sample, which contain the restriction enzyme, specific oligonucleotide, or methylated DNA antibody as a regent.

[0153] The DNA comprising a target DNA region is selected by complementary binding with the specific oligonucleotide. When cytosine in CpG in the target DNA region is methylated, the cytosine is an object detected by binding of the methylcytosine antibody, so that the specific oligonucleotide is advisable not to base-pair with cytosine or CpG in the target DNA region. Therefore, the target DNA region is advisable to have a nucleotide sequence capable of specifically base-pairing with the specific oligonucleotide in the vicinity. When the specific oligonucleotide is caused to bind inside the target region by complementary base-pairing, it is advisable that the nucleotide sequence in the target DNA region which binds with the specific oligonucleotide by complementation does not include CpG.

[0154] When the target region is a nucleotide sequence derived from a microorganism, DNA for which a target DNA

region is to be detected may be genomic DNA extracted from a specimen, a DNA fragment, or a nucleotide sequence of DNA that is prepared by a reverse transcriptase from RNA extracted from a specimen. As a nucleotide sequence capable of complementarily binding with a specific oligonucleotide, a region specific to the microorganism may be selected. For example, when the target region in the present invention is a microbial nucleotide sequence, for selectively extracting the target region from the specimen, a nucleotide sequence that is peculiar to the microorganism near the target region may be selected as a nucleotide sequence that specifically binds with the specific oligonucleotide among microbial genomic DNA, or nucleotide sequences of DNA that is prepared by a reverse transcriptase from RNA extracted from the specimen.

[0155] Generally, for examining presence or absence of a pathogenic microorganism contained in a biopsy sample or food, presence or absence of such a pathogenic microorganism is examined, or such a pathogenic microorganism is identified by a test based on immunization method for each microbial antigen. However, preparation of an antibody used for such a immunization method is not easy, and for detecting a plurality of pathogenic microorganisms, it is necessary to prepare antibodies against respective antigens of the pathogenic microorganisms. By using the present method, it is possible to realize a simple test for pathogenic microorganisms without conducting such complicated antibody preparation. Further, according to the present method, since nucleotide sequences of different pathogenic microorganisms can be tested at the same time, it is possible to detect several kinds of pathogenic microorganisms contained in one specimen at the same time. Concretely, food poisoning bacteria such as Listeria monocytogenes, Salmonella enterica, Campylobacter jejuni subsp. Jejuni, Staphylococcus aureus, Vibrio parahaemolyticus, Bacillusu cereus, Clostridium botulinum, Yersinia enterocolitica, Yersinia pseudotuberuculosis and Clostridium perfringens are known, however, a technique of detecting several kinds of these food poisoning bacteria at the same time is not known. However, by using the present method, it is possible to detect nucleotide sequences of several kinds of food poisoning bacteria at the same time. Further, when a nucleotide sequence found plurally in genome such as CRISPR (Clustered regularly interspaced short palindromic repeats) region is selected as a nucleotide sequence to be detected by a specific oligonucleotide, detection at higher sensitivity is realized compared to the case of detecting one gene in one genome. Such a technique is useful also for diagnosis of infection and rapid detection of food poisoning bacteria. Further, the present method may be used for identification of an industrially useful bacterium, or for a simple test of a microbial community in soil, river or lake sediments and the like by detecting genomes of microorganisms in such environments. Of the microorganisms in such environments, inhabitation of, for example, Methanococcus jannaschii, Methanobacterium thermoautotrophicum deltaH, Aquifex aeolicus, Pyrococcus horikoshii OT3, Archaeoglobus fulgidus, Thermotoga maritima MSB8, Aeropyrum pernix K1, and Haloferax mediterranei can be verified. It is also possible to detect and identify industrially available bacteria such as Geobacter sulfurreducens and microorganisms used for fermentation such as Streptococcus thermophilus.

[0156] For example, applicable as a region where the target region for detecting genome in a microorganism and the specific oligonucleotide complementarily bind is, concretely, a nucleotide sequence not encoding a gene such as a region corresponding to the nucleotide number 271743-272083 of yeast chromosome VII (SEQ ID NO: 38) shown, for example, in Genbank Accession No. NC_001139, or the nucleotide number 384569-384685 of yeast chromosome VII (SEQ ID NO: 65) shown, for example, in Genbank Accession No. NC_001139. It is also useful to detect a nucleotide sequence conserved among pathogenic microorganisms in a characteristic gene that is common in various pathogenic microorganisms because a method of detecting a plurality of pathogenic microorganisms at the same time can be provided. Concretely, since mce-family gene (Micobacterium tuberculosis), tRNA-Tyrnucleotide sequence on 13th chromosome (Cryptococcus neoformans), and chitin synthase activator (Chs3) have a nucleotide sequence peculiar to Aspergillus fumigatus and genus Neosartorya, they can be used for assay of infection by a microorganism, by assaying whether DNA derived from these microorganisms is contained in DNA extracted from a biopsy sample of human expectoration or lung.

Further, since actA (Listeria monocytogenes), pyrG (NC 002163, Campylobacter jejuni subsp. jejuni) and the like are common genes peculiar to food poisoning bacteria, these genes may be used for a microbial assay in food poisoning. ThrA has a sequence that is conserved among Salmonella enterica, Yersinia enterocolitica, and Escherichia coli, so that a plurality of microorganisms can be detected by one gene.

[0157] Of nucleotide sequences published on a database, a nucleotide sequence peculiar to a microorganism may be retrieved, and a nucleotide sequence peculiar to a microorganism may be searched. For example, a nucleotide sequence on a published database such as PubMed may be obtained through regular procedure, and the obtained nucleotide sequence can be examined whether it is a peculiar nucleotide sequence by Blast search through regular procedure. The peculiar nucleotide sequence means that a nucleotide sequence in a detection object does not include a nucleotide sequence having homology with a nucleotide sequence derived from an organism other than the genomic nucleotide sequence of the microorganism to be detected.

[0158] In particular, when the specimen is a human biopsy sample, it is important to design a specific oligonucleotide that would not complementarily bind with human genes. Similarly, when the specimen is food, it is important to design a specific oligonucleotide that would not complementarily bind with a nucleotide sequence derived from an organism

other than the detection object contained in the food.

[0159] For detecting free DNA in blood, the target DNA region may be a region correlated with an amount of free DNA, and when it is intended to quantify or detect free DNA, what is called repetitive sequence where the same sequence in genome appears repetitively, several or more times, is preferred, and a simple repetitive sequence (called tandem repetitive sequence, or tandem repeat), and an interspersed repeat sequence are more preferred.

[0160] The simple repetitive sequence is **characterized in that** the same sequences neighbor in the same orientation, and a series of nucleotide sequences such as satellite DNA, minisatellite, microsatellite, centromere, telomere, kinetochore, and ribosome group genes are known.

[0161] The interspersed repetitive sequence is **characterized in that** the same sequences are interspersed without neighboring each other, and is believed to be DNA derived from retrotransposon. Interspersed repetitive sequences are classified into SINE (Short Interspersed Repetitive Element: short chain interspersed repetitive sequence) and LINE (Long Interspersed Elements: long chain interspersed repetitive sequence) depending on the length of the nucleotide sequence, and as a human nucleotide sequence, Alu sequence and LINE-1 sequence are respectively known as representative repetitive sequences. Also an inactive processed pseudogene that is reverse transcribed from RNA or protein, and a gene sequence amplified by gene duplication are also known.

[0162] The term dupulicated gene indicates the case where a plurality of genes having high homology exist on one genome, and is, in many cases, a nucleotide sequence that exists in tandem near one gene. It is often the case where a pseudogene is one of duplicated genes.

[0163] As concrete examples of the repetitive sequence, such sequences as (A)n, (T)n, (GA)n, (CA)n, (TAA)n, (GGA)n, (CAGC)n, (CATA)n, (GAAA)n, (TATG)n, (TTTG)n, (TTTA)n, (TTTC)n, (TAAA)n, (TTCA)n, (TATAA)n, (TCTCC)n, (TTTCC)n, (TTTAA)n, (TTTTC)n, (TTTTA)n, (TTTTG)n, (CAAAA)n, (CACCC)n, (TATATG)n, (CATATA)n, (TCTCTG)n, (AGGGGG)n, (CCCCCA)n, and (TGGGGG)n (n means a number of repetition) are known as repetition comprising a relatively short nucleotide sequence, and as a sequence derived from a transcription factor, MER1-Charlie, and Zaphod of hAT group, and MER2-Tigger, Tc-1, and Mariner of Tc-1 group can be recited. As others, concretely, Tigger1, Tigger2a, Tigger5, Charlie4a, Charlie7 and the like are known. These sequences are generally short and simple nucleotide sequences, and are difficult to set the specific adhesion sequence as will be described later, however, these sequences can be used in the present method as far as they have a sequence that can be set into setting objects of the specific adhesion sequence and a detection adhesion sequence as will be described later. Therefore, it is not necessarily excluded as an object of the present method. Further, satellite DNA, minisatellite, microsatellite and the like are repetitive sequences classified into simple repetitive sequences.

[0164] Further, as a sequence having multi-copies in gene, ALR6 as a sequence existing in centromere, U2 and U6 as snRNA, as well as the genes such as tRNA and rRNA that are generally known to have multi-copies in genome, and the genes that have plural copies in genome as a result of gene duplication are recited.

[0165] It is also known that a retrovirus, a retrotransposon having LTR (Long terminal repeat) in its terminal, an endogenous sequence such as MaLRs (Mammalian apparent LTR-Retrotransposons) considered to be derived from viruses, and LTR derived from a retrovirus exist in multicopy in one genome.

[0166] For example, as the LTR derived from a retrovirus, concretely, subfamilies such as LTR1, LTR1B, LTR5, LTR7, LTR8, LTR16A1, LTR16A1, LTR16C, LTR26, LTR26E, MER48, and MLT2CB are known. The LTRs derived from a retrotransposon are classified into classes of ERV, ERVK and ERVL, and concrete examples include subfamilies such as LTR8A, LTR28, MER21B, MER83, MER31B, MER49, MER66B, HERVH, ERVL, LTR16A1, LTR33A, LTR50, LTR52, MLT2A1, MLT2E, MER11C, and MER11C. Further, MaLRs indicate DNA factors including LTRs in both ends likewise a typical retrotransposon, wherein an internal sequence sandwiched between LTRs is not derived from a retrovirus. For example, subfamilies such as MLT1A1, MLT1A2, MLT1B, MLT1C, MLT1D, MLT1F, MLT1G, MLT1H, MLT1J, MLT1K, MLT1I, MLT2CB, MSTA, MSTA-int, MSTB, THE1A, THE1B, THE1B-internal, and THE1 can be recited.

[0167] The interspersed repetitive sequences are **characterized in that** the same sequences are interspersed without neighboring each other, and are considered to be derived from a retrotransposon. Further, the interspersed repetitive sequences are classified into SINE (Short Interspersed Repetitive Element: short chain interspersed repetitive sequences) and LINE (Long Interspersed Elements: long-chain interspersed repetitive sequences) according to the length. Most of SINEs are sequences belonging to the Alu family. A common feature is that it has a sequence of 3'-side or a sequence of 5'-side of 7SL RNA, and that it has an AT-Rich region sandwiched between a Left-monomer and a Right-monomer. As subfamilies of the Alu family, Alu, AluJb, AluJo, AluSc, AluSg, AluSp, AluSq, AluSx, AluY, and FAM (Fossil Alu Monomer), FLAM (Free Left Alu Monomer) having a sequence of FAM, and FRAM (Free Right Alu Monomer) can be recited. As SINEs other than the Alu family, MIR, and Ther/MIR3 are known, and MIR and MIR3 are known as respective subfamilies. As subfamilies of the Alu family including other biological species, B1, B2, B4, PB1, PB1D and so on are known. As LINEs, subfamilies of LINE1 to Line23 are reported, and it is known that subfamilies such as LINE-1, LINE2, and LINE3 broadly exist in a genome. As for LINE-1, for example, L1M1, L1M2, L1M3, L1M3d, L1M4, L1M4c, L1MA2, L1MA7, L1MA8, L1MA9, L1MB1, L1MB1, L1MB3, L1MB4, L1MB5, L1MB6, L1MB7, L1MCa, L1MCb, L1MC2, L1MC3, L1MC4, L1MC4a, L1MC5, L1MDa, L1ME, L1MEc, L1MEd, L1MEg, L1ME1, L1ME2, L1ME3, L1ME3A, L1ME3B,

L1ME4a, L1PB3, L1P4, L1PA2, L1PA3, L1PA4, L1PA5, L1PA6, L1PA7, L1PA10, L1PA12, L1PA13, L1PA14, L1PA16, L1PB1, L1PB3, L1PB4, L1PREC2, and HAL1 are known, and as LINE-2, subfamilies such as L2 and L2c are known. For example, if the later-described specific adhesion sequence and the detection adhesion sequence can be set, for a sequence common to the Alu family or subfamilies of Alu, or the LINE-1 family or subfamilies of LINE-1, a plurality of detection objects can be set in one genome, so that sensitivity of genome detection can be improved.

[0168] As a target DNA region, concretely, for example, a partial sequence of LINE-1 (the nucleotide sequence of SEQ ID NO: 28, SEQ ID NO: 62, or SEQ ID NO: 63), a partial sequence of Alu (the nucleotide sequence of SEQ ID NO: 64) or nucleotide sequences having homology to these sequences can be recited.

[0169] For example, when a repetitive sequence in a certain region needs to be examined, databases such as Repbase (http://www.girinst.org/repbase/) and RepeatMasker (http://www.repeatmasker.org/) may be used because it is difficult to retrieve a general sequence retrieving database such as PuMed. If a specific adhesion sequence of the present method can be set, the detection sensitivity can be improved. Measuring these repetitive sequences can be treated, for example, as a surrogate marker of a free DNA amount in blood, and can be utilized for identification of an organism species when an organism species-specific repetitive sequence is noted.

[0170] In the present method, by measuring a repetitive sequence, a nucleotide sequence existing plurally in one genome can be measured concurrently. For example, a nucleotide sequence having a sequence homology of 80% or higher with the nucleotide sequence of SEQ ID NO: 28 has about 280 copies in a human genome, and a nucleotide sequence having a sequence identity of 80% or higher with the nucleotide sequence of SEQ ID NO: 64 has about 820 copies in a human genome. Therefore, if a specific adhesion sequence can be set in each nucleotide sequence, the detection sensitivity of one genome can be improved to 280 to 820 folds theoretically, compared to the case where a specific adhesion sequence is set for a sequence having just one kind in genome.

[0171] A duplicated gene means a gene or a gene fragment that is generated by doubling of a specific gene or gene fragment in genome due to gene duplication. Gene duplication is a phenomenon that a certain region of DNA including a gene is overlapped. As a cause of gene duplication, abnormality of gene recombination, translocation of retrotransposon, duplication of the entire chromosome and the like are recited.

For example, it means that one gene is copied and inserted into genomic DNA, and the copy is inserted to a different chromosome site in some cases, and inserted near the original gene in the other cases. The site where copied genes are aligned as a result of insertion near the original gene is called a tandem repeat, and a group of genes generated by gene duplication is called a gene family.

[0172] A pseudogene means a gene having a characteristic nucleotide sequence that is assumable to have encoded a gene product (particularly protein) in a sequence of DNA, but currently loosing the function. It is assumed that it is generated as a result of mutation of the original functioning sequence. For example, there is the case where a stop codon arises by mutation and a peptide chain of a protein is shortened, so that the function as a protein is no longer effective, and there is the case where a function of a regulatory sequence required for normal transcription is impaired due to mutation such as single nucleotide substitution. In many pseudogenes, the original normal genes are remained separately, however, those becoming pseudogenes by themselves are also known.

[0173] Pseudogenes are classified into three types according to the characteristic of the gene sequence. There are known the case where DNA prepared from mRNA by a reverse transcriptase of retrotransposon is inserted into genome (processed pseudogene), the case where an original gene sequence is duplicated in genome, and a part of the copies looses the function due to mutation or the like to become a pseudogene (duplicated pseudogene or non-processed pseudogene), and the case where gene in genome (in the condition of single gene with no duplicated gene) looses the function to become a pseudogene.

[0174] Currently, among the genes known as pseudogenes, transcribed examples, examples having a gene function (whether it is called a pseudogene is not determined) and the like also have been known, so that the term "pseudogene" in the present method means the "processed pseudogene" or "duplicated pseudogene (non-processed pseudogene)" rather than presence or absence of gene function or whether it is transcribed or not.

[0175] In First step of the present method, it is preferred to extract DNA from a specimen by a system containing a sodium salt at high concentration. Concretely, as a concentration of sodium salt in a solution (for example, buffer) used in a DNA extraction operation for obtaining DNA from a specimen in First step of the present method, at least 50 mM or more, and preferably 100 mM or more can be recited. More concretely, 50 mM or more and 1000 mM or less, preferably 100 mM or more and 1000 mM or less, more preferably 100 mM or more and 200 mM or less can be recited. Any salts including NaCl, $NaCO_3$, Na_2SO_4 and the like are applied as far as it is a salt containing a sodium ion, and preferably means NaCl.

[0176] The present invention is a method of selecting a specimen derived from a cancer patient, and includes the steps of evaluating a specimen derived from a test subject as a specimen derived from a cancer patient when there is a significant difference between a DNA quantification result or detection result quantified or detected using a specimen derived from a test subject by the method according to any one of Inventions 1 to 13, and a DNA quantification result or detection result quantified or detected using a specimen derived from a healthy subject by the method, and identifying

the specimen derived from a cancer patient based on the evaluation result. As a preferred aspect of the present invention, the invention in which the specimen is a serum derived from a mammal, and the invention in which the DNA comprising a target DNA region is free DNA comprising a target DNA region in serum derived from a mammal can be recited. Use of these inventions will make it possible to identify a cancer patient in a simple and convenient manner by a blood test.

[0177] Here, the "cancer patient" is a test subject developing a cancer, and as the cancer, solid cancers developing in organs of human and mammals, and non-solid cancers developing in blood of human and mammals such as lung cancer (non-small-cell lung cancer, small-cell lung cancer), esophageal cancer, gastric cancer, duodenal cancer, colon cancer, rectal cancer, hepatic cancer (hepatocarcinoma, cholangiocellular carcinoma), gallbladder cancer, bile duct cancer, pancreatic cancer, colon cancer, anal cancer, breast cancer, cervical cancer, uterine cancer, ovarian cancer, vulvar cancer, vaginal cancer, prostate cancer, kidney cancer, ureter cancer, bladder cancer, prostate cancer, penile cancer, testicular (testis) cancer, maxillary cancer, tongue cancer, (naso-, oro-, hypo-) pharyngeal cancer, acute myeloid leukemia, chronic myelogenous leukemia, acute lymphoblastic leukemia, chronic lymphoblastic leukemia, malignant lymphoma, myelodysplastic syndrome, thyroid cancer, brain tumor, osteosarcoma and skin cancer (basal cell cancer, squamous cell cancer) are included.

EXAMPLES

[0178] In the following, the present invention will be described in detail by way of examples, however, the present invention is not limited to these examples.

Example 1

[0179] Using genomic DNA derived from human blood purchased from Clontech as a template, PCR was conducted using an oligonucleotide primer PF1 of SEQ ID NO: 17 and an oligonucleotide primer PR1 of SEQ ID NO: 18 in the following reaction condition, to amplify a DNA fragment X (the region corresponding to the nucleotide numbers 25687390-25687775 shown in Genbank Accession No. NT_029419) of SEQ ID NO: 19.

<Oligonucleotide primers designed for PCR>

[0180]

PF1:5'- CTCAGCACCCAGGCGGCC -3' (SEQ ID NO: 17)
PR1:5'- CTGGCCAAACTGGAGATCGC -3' (SEQ ID NO: 18)

<DNA fragment>

[0181]

X:5'-

CTCAGCACCCAGGCGGCCGCGATCATGAGGCGCGAGCGGCGCGCGGGCTGTTGCAGAGTCTT

GAGCGGGTGGCACACCGCGATGTAGCGGTCGGCTGTCATGACTACCAGCATGTAGGCCGACG

CAAACATGCCGAACACCTGCAGGTGCTTCACCACGCGGCACAGCCAGTCGGGGCCGCGGAAG

CGGTAGGTGATGTCCCAGCACATTTGCGGCAGCACCTGGAAGAATGCCACGGCCAGGTCGGC

CAGGCTGAGGTGTCGGATGAAGAGGTGCATGCGGGACGTCTTGCGCGGCGTCCGGTGCAGAG

CCAGCAGTACGCTGCTGTTGCCCAGCACGGCCACCGCGAAAGTCACCGCCAGCACGGCGATC

TCCAGTTTGGCCAG -3'(SEQ ID NO: 19)

[0182] Ten (10) ng of genomic DNA as a template, each 3 μL of 5 μM of the above primer solutions, 5 μL of each 2 mM dNTP, and 5 μL of 10×buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% Gelatin) were mixed with 0.25 μL of 5U/μL thermostable DNA polymerase (AmpliTaq Gold), and added with sterilized ultrapure water to

prepare a reaction liquid having a liquid amount of 50 μL. After retaining the reaction liquid at 95°C for 10 minutes, PCR was conducted by 40 cycles each consisting of 30 seconds at 95°C, 30 seconds at 61°C and 45 seconds at 72°C.

[0183] The PCR reaction liquid was subjected to 2% agarose gel electrophoresis to check the amplified DNA, and the DNA was purified by Wizard SV Gel/PCR Kit (PROMEGA Corporation), to obtain the DNA fragment X.

[0184] For the DNA fragment X, the following solutions were prepared respectively in duplicate.

Solution A: DNA fragment X 10ng/20 μL TE buffer solution
Solution B: DNA fragment X 1ng/20 μL TE buffer solution
Solution C: DNA fragment X 0.1ng/20 μL TE buffer solution
Solution D: TE buffer solution (negative control solution)

[0185] Twenty (20) μL of each obtained solution, 0.5 μL of SssI methylase (available from NEB Inc.), 5 μL of 10xNEBuffer2 (available from NEB Inc.), and 0.5 μL of 3.2 mM S-adenosyl methionine (available from NEB Inc.) were mixed, and added with sterilized ultrapure water to prepare a reaction liquid having a liquid amount of 50 μL. The reaction liquid was incubated at 37°C for 30 minutes.

[0186] Synthesized was 5'-end biotin-labeled oligonucleotide B1 having the nucleotide sequence of SEQ ID NO: 20 capable of binding by complementation with a plus strand of the DNA fragment X comprising the target DNA region of SEQ ID NO: 19, and a 0.02 μM TE buffer solution was prepared.

<5'-end biotin-labeled oligonucleotide>

[0187]

B1:5'- CTGGCCAAACTGGAGAT -3' (SEQ ID NO: 20)

[0188] In a PCR tube, 40 μL of the reaction liquid prepared in the above, 10 μL of the 5'-end biotin-labeled oligonucleotide solution, 10 μL of a buffer (330 mM Tris-Acetate pH 7.9, 660 mM KOAc, 100 mM $MgOAc_2$, 5 mM Dithiothreitol), 10 μL of a 100 mM $MgCl_2$ solution and 10·μl of a 1 mg/mL BSA solution were added, and further the mixture was added with sterilized ultrapure water to make the liquid amount 100 μL, and mixed. Then the PCR tube was heated at 95°C for 10 minutes, cooled rapidly to 70°C, and retained for 10 minutes at this temperature. Then the PCR tube was cooled to 50°C and retained for 10 minutes, and further retained for 10 minutes at 37°C, and then the PCR tube was returned to room temperature, to promote formation of a conjugate of the 5'-end biotin-labeled oligonucleotide and the DNA fragment.

[0189] The entire obtained mixture was transferred to a well coated with streptavidin, and left still at room temperature for about 30 minutes, to immobilize the conjugate of the 5'-end biotin-labeled oligonucleotide and the DNA fragment to the well. Thereafter, the solution was removed by pipetting, and each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM Na_2HPO $7H_2O$, 154 mM NaCl pH7.4)].

[0190] Synthesized was a masking oligonucleotide M1 having the nucleotide sequence of SEQ ID NO: 21 capable of binding by complementation with 5'-end biotin-labeled oligonucleotide B1 having the nucleotide sequence of SEQ ID NO: 20, and 0.1 μM TE buffer solution was prepared.

<Masking oligonucleotide>

[0191]

M1:5'- ATCTCCAGTTTGGCCAG -3' (SEQ ID NO: 21)

[0192] Each well was added with 100 μL of a methylcytosine antibody [available from Aviva Systems Biology, 0.5 μg/mL 0.1% BSA-containing phosphate buffer (1 mM KH_2PO_4, 3 mM Na_2HPO $7H_2O$, 154 mM NaCl pH7.4) solution], and added with 1 μL of the masking oligonucleotide solution, and left still for 1 hour at room temperature. Then the solution was removed by pipetting, and each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM Na_2HPO $7H_2O$, 154 mM NaCl pH7.4)].

[0193] Then, each well was added with 100 μL of an Eu-N1-labeled mouse IgG antibody [available from Perkin Elmer, 0.05μg/mL 0.1% BSA-containing phosphate buffer (1 mM KH_2PO_4, 3 mM Na_2HPO $7H_2O$, 154 mM NaCl pH7.4) solution], and then left still at room temperature for 1 hour. After leaving still, the solution was removed by pipetting, and each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM Na_2HPO $7H_2O$, 154 mM NaCl pH7.4)].

[0194] Each well was added with 150 μL of Enhancement Solution (available from Perkin Elmer), stirred at room temperature for 5 minutes, and left still for 15 minutes at room temperature. Then fluorescence was measured at excitation 340 nm/fluorescence 612 nm, and an average value of duplicate was calculated for the obtained measurements.

[0195] The result is shown in Fig. 1. It was revealed that a DNA fragment is selected accurately by the immobilized 5'-end biotin-labeled oligonucleotide, and quantified and detected with excellent sensitivity.

[0196] In the present experiment, it was revealed that a DNA fragment can be quantified and detected by forming and selecting a complex of a methylcytosine antibody, a methylated DNA fragment, and an immobilized 5'-end biotin-labeled oligonucleotide, and quantifying and detecting the methylcytosine antibody in the complex according to its identification function.

Example 2

[0197] Using genomic DNA derived from human blood purchased from Clontech as a template, PCR was conducted using an oligonucleotide primer PF2 of SEQ ID NO: 22 and an oligonucleotide primer PR2 of SEQ ID NO: 23 in the following reaction condition, to amplify a DNA fragment Y (the region corresponding to the nucleotide number 76606-76726 shown in Genbank Accession No. ac009800) of SEQ ID NO: 24.

<Oligonucleotide primers designed for PCR>

[0198]

PF2:5'-TGAGCTCCGTAGGGCGTCC -3' (SEQ ID NO: 22)
PR2:5'-GCGCCGGGTCCGGGCCC-3' (SEQ ID NO: 23)

<DNA fragment>

[0199]

Y:5'-
GCGCCGGGTCCGGGCCCGATGCGTTGGCGGGCCAGGGCTCCGAGAACGAGGCGTTGTCCATC
TCAACGAGGGCAGAGGAGCCGGCGACCTGGCGTCCCCCAAGGACGCCCTACGGAGCTCA -
3'(SEQ ID NO: 24)

[0200] As a reaction liquid of PCR, 10 ng of genomic DNA as a template, each 3 μL of 5 μM of the above primer solutions, 5 μL of each 2 mM dNTP, and 5 μL of 10×buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% Gelatin) were mixed with 0.25 μL of 5U/μL thermostable DNA polymerase (AmpliTaq Gold), and added with sterilized ultrapure water to prepare a reaction liquid having a liquid amount of 50 μL. After retaining the reaction liquid at 95°C for 10 minutes, PCR was conducted by 50 cycles each consisting of 30 seconds at 95°C, 30 seconds at 60°C and 45 seconds at 72°C.

[0201] After conducting PCR, amplification was checked by 2% agarose gel electrophoresis, and the DNA fragment Y was purified by Wizard SV Gel/PCR Kit (PROMEGA Corporation).

[0202] For the DNA fragment Y, the following solutions were prepared respectively in duplicate.

Solution A: DNA fragment Y 10ng/20 μL TE buffer solution
Solution B: DNA fragment Y 1ng/20 μL TE buffer solution
Solution C: DNA fragment Y 0.1ng/20 μL TE buffer solution
Solution D: TE buffer solution (negative control solution)

[0203] Twenty (20) μL of each obtained solution, 0.5 μL of SssI methylase (available from NEB Inc.), 5 μL of 10xNEBuffer2 (available from NEB Inc.), and 0.5 μL of 3.2 mM S-adenosyl methionine (available from NEB Inc.) were mixed, and added with sterilized ultrapure water to prepare a reaction liquid having a liquid amount of 50 μL. The reaction liquid was incubated at 37°C for 30 minutes.

[0204] Synthesized was 5'-end biotin-labeled oligonucleotide B2 having the nucleotide sequence of SEQ ID NO: 25

capable of binding by complementation with a plus strand of the DNA fragment Y comprising the target DNA region of SEQ ID NO: 24, and a 0.02 μM TE buffer solution was prepared.

<5'-end biotin-labeled oligonucleotide>

[0205]

B2:5'- GACAACGCCTCGTTCTCGG -3' (SEQ ID NO: 25)

[0206] Each obtained reaction liquid was subjected to the following treatments.

[0207] In a PCR tube, 40 μL of the reaction liquid prepared in the above, 10 μL of the 5'-end biotin-labeled oligonucleotide solution, 10 μL of a buffer (330 mM Tris-Acetate pH 7.9, 660 mM KOAc, 100 mM $MgOAc_2$, 5 mM Dithiothreitol), 10 μL of a 100 mM $MgCl_2$ solution and 10 μL of a 1 mg/mL BSA solution were added, and further the mixture was added with sterilized ultrapure water to make the liquid amount 100 μL, and mixed. Then the PCR tube was heated at 95°C for 10 minutes, cooled rapidly to 70°C, and retained for 10 minutes at this temperature. Then the PCR tube was cooled to 50°C and retained for 10 minutes, and further retained for 10 minutes at 37°C, and then the PCR tube was returned to room temperature, to promote formation of a conjugate of the 5'-end biotin-labeled oligonucleotide and the DNA fragment.

[0208] The entire obtained mixture was transferred to a well coated with streptavidin, and left still at room temperature for about 30 minutes, to immobilize the conjugate of the 5'-end biotin-labeled oligonucleotide and the DNA fragment to the well. Thereafter, the solution was removed by pipetting, and each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4)].

[0209] Synthesized was masking oligonucleotide M2 having the nucleotide sequence of SEQ ID NO: 26 capable of binding by complementation with 5'-end biotin-labeled oligonucleotide B2 having the nucleotide sequence of SEQ ID NO: 25, and 0.1 μM TE buffer solution was prepared.

<Masking oligonucleotide>

[0210]

M2:5'- CCGAGAACGAGGCGTTGTCT -3' (SEQ ID NO: 26)

[0211] Each well was added with 100 μL of an methylcytosine antibody [available from Aviva Systems Biology, 0.5 μg/mL 0.1% BSA-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4) solution], and added with 1 μL of the masking oligonucleotide solution, and left still for 1 hour at room temperature. Then the solution was removed by pipetting, and each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4)].

[0212] Then, each well was added with 100 μL of an Eu-N1-labeled mouse IgG antibody [available from Perkin Elmer, 0.05μg/mL 0.1% BSA-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4) solution], and then left still at room temperature for 1 hour. After leaving still, the solution was removed by pipetting, and each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4)].

[0213] Each well was added with 150 μL of Enhancement Solution (available from Perkin Elmer), stirred at room temperature for 5 minutes, and left still for 15 minutes at room temperature. Then fluorescence was measured at excitation 340 nm/fluorescence 612 nm, and an average value of duplicate was calculated for the obtained measurements.

[0214] The result is shown in Fig. 2. It was revealed that a DNA fragment is selected accurately by the immobilized 5'-end biotin-labeled oligonucleotide, and quantified and detected with excellent sensitivity.

[0215] In the present experiment, it was revealed that a DNA fragment can be quantified and detected by forming and selecting a complex of a methylcytosine antibody, a methylated DNA fragment, and an immobilized 5'-end biotin-labeled oligonucleotide, and quantifying and detecting the methylcytosine antibody in the complex according to its identification function.

Example 3

[0216] Using genomic DNA derived from human blood purchased from Clontech as a template, PCR was conducted using the oligonucleotide primer PF1 of SEQ ID NO: 17 and the oligonucleotide primer PR1 of SEQ ID NO: 18 in the following reaction condition, to amplify the DNA fragment X (the region corresponding to the nucleotide number

25687390-25687775 shown in Genbank Accession No. NT_029419) of SEQ ID NO: 19.

[0217] As a reaction liquid of PCR, 10 ng of genomic DNA as a template, each 3 μL of 5 μM of the above primer solutions, 5 μL of each 2 mM dNTP, and 5 μL of 10×buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% Gelatin) were mixed with 0.25 μL of 5U/μL thermostable DNA polymerase (AmpliTaq Gold), and added with sterilized ultrapure water to prepare a reaction liquid having a liquid amount of 50 μL. After retaining the reaction liquid at 95°C for 10 minutes, PCR was conducted by 40 cycles each consisting of 30 seconds at 95°C, 30 seconds at 61°C and 45 seconds at 72°C.

[0218] After conducting PCR, amplification was checked by 2% agarose gel electrophoresis, and the DNA fragment X was purified by Wizard SV Gel/PCR Kit (PROMEGA Corporation).

[0219] Using genomic DNA derived from human blood purchased from Clontech as a template, PCR was conducted using the oligonucleotide primer PF2 of SEQ ID NO: 22 and the oligonucleotide primer PR2 of SEQ ID NO: 23 in the following reaction condition, to amplify the DNA fragment Y (the region corresponding to the nucleotide number 76606-76726 shown in Genbank Accession No. ac009800) of SEQ ID NO: 24.

[0220] As a reaction liquid of PCR, 10 ng of genomic DNA as a template, each 3 μL of 5 μM of the above primer solutions, 5 μL of each 2 mM dNTP, and 5 μL of 10×buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% Gelatin) were mixed with 0.25 μL of 5U/μL thermostable DNA polymerase (AmpliTaq Gold), and added with sterilized ultrapure water to prepare a reaction liquid having a liquid amount of 50 μL. After retaining the reaction liquid at 95°C for 10 minutes, PCR was conducted by 50 cycles each consisting of 30 seconds at 95°C, 30 seconds at 60°C and 45 seconds at 72°C.

[0221] After conducting PCR, amplification was checked by 2% agarose gel electrophoresis, and the DNA fragment Y was purified by Wizard SV Gel/PCR Kit (PROMEGA Corporation).

[0222] For each of the DNA fragment X and the DNA fragment Y, the following solutions were prepared.

Solution A: DNA fragment X or DNA fragment Y 10 ng/10 μL TE buffer solution
Solution B: DNA fragment X or DNA fragment Y 1 ng/10 μL TE buffer solution
Solution C: DNA fragment X or DNA fragment Y 0.1 ng/10 μL TE buffer solution
Solution D: TE buffer solution (negative control solution)

[0223] Equivalent amounts of Solution A of the DNA fragment X and Solution A of the DNA fragment Y were mixed, to prepare DNA fragment-mixed Solution MA, equivalent amounts of Solution B of the DNA fragment X and Solution B of the DNA fragment Y were mixed, to prepare DNA fragment-mixed Solution MB, equivalent amounts of Solution C of the DNA fragment X and Solution C of the DNA fragment Y were mixed, to prepare DNA fragment-mixed Solution MC, and equivalent amounts of Solution D of the DNA fragment X and Solution D of the DNA fragment Y were mixed, to prepare DNA fragment-mixed Solution MD. Three sets in duplicate were prepared respectively, for each of DNA fragment-mixed Solutions MA , MB, MC and MD.

[0224] Twenty (20) μL of each obtained solution, 0.5 μL of SssI methylase (available from NEB Inc.), 5 μL of 10xNEBuffer2 (available from NEB Inc.), and 0.5 μL of 3.2 mM S-adenosyl methionine (available from NEB Inc.) were mixed, and added with sterilized ultrapure water to prepare a reaction liquid having a liquid amount of 50 μL. The reaction liquid was incubated at 37°C for 30 minutes.

[0225] Further, prepared were 0.02 μM TE buffer solutions of 5'-end biotin-labeled oligonucleotide B1 having the nucleotide sequence of SEQ ID NO: 20 capable of binding by complementation with a plus strand of the DNA fragment X comprising the target DNA region of SEQ ID NO: 19 and 5'-end biotin-labeled oligonucleotide B2 having the nucleotide sequence of SEQ ID NO: 25 capable of binding by complementation with a plus strand of the DNA fragment Y comprising the target DNA region of SEQ ID NO: 24. Also prepared was a TE buffer solution (0.02 μM for each) mixing equivalent amounts of 5'-end biotin-labeled oligonucleotide B1 having the nucleotide sequence of SEQ ID NO: 20 and 5'-end biotin-labeled oligonucleotide B2 having the nucleotide sequence of SEQ ID NO: 25.

[0226] For each reaction liquid of the DNA fragment Solutions MA to MD, the following treatment was conducted using each of the 5'-end biotin-labeled oligonucleotide solutions.

[0227] In a PCR tube, 40 μL of the reaction liquid prepared in the above, 10 μL of the 5'-end biotin-labeled oligonucleotide solution, 10 μL of a buffer (330 mM Tris-Acetate pH 7.9, 660 mM KOAc, 100 mM $MgOAc_2$, 5 mM Dithiothreitol), 10 μL of a 100 mM $MgCl_2$ solution and 10 μL of a 1 mg/mL BSA solution were added, and further the mixture was added with sterilized ultrapure water to make the liquid amount 100 μL, and mixed. Then the PCR tube was heated at 95°C for 10 minutes, cooled rapidly to 70°C, and retained for 10 minutes at this temperature. Then the PCR tube was cooled to 50°C and retained for 10 minutes, and further retained for 10 minutes at 37°C, and then the PCR tube was returned to room temperature, to promote formation of a conjugate of the 5'-end biotin-labeled oligonucleotide and the DNA fragment.

[0228] The entire obtained mixture was transferred to a well coated with streptavidin, and left still at room temperature for about 30 minutes, to immobilize the conjugate of the 5'-end biotin-labeled oligonucleotide and the DNA fragment to

the well. Thereafter, the solution was removed by pipetting, and each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2 PO_4, 3 mM Na_2HPO $7H_2O$, 154 mM NaCl pH7.4)].

[0229] Synthesized were masking oligonucleotide M1 having the nucleotide sequence of SEQ ID NO: 21 capable of binding by complementation with 5'-end biotin-labeled oligonucleotide B1 having the nucleotide sequence of SEQ ID NO: 20 and masking oligonucleotide M2 having the nucleotide sequence of SEQ ID NO: 26 capable of binding by complementation with 5'-end biotin-labeled oligonucleotide B2 having the nucleotide sequence of SEQ ID NO: 25, and respective 0.1 μM TE buffer solutions were prepared. Prepared was a TE buffer solution (each 0.1 μM) mixing equivalent amounts of masking oligonucleotide M1 having the nucleotide sequence of SEQ ID NO: 21 capable of binding by complementation with 5'-end biotin-labeled oligonucleotide B1 having the nucleotide sequence of SEQ ID NO: 20 and masking oligonucleotide M2 having the nucleotide sequence of SEQ ID NO: 26 capable of binding by complementation with 5'-end biotin-labeled oligonucleotide B2 having the nucleotide sequence of SEQ ID NO: 25.

[0230] For respective reaction liquids of the DNA fragment Solutions MA to MD, the following treatment was conducted using each of the masking oligonucleotide solutions. The masking oligonucleotide solutions to be added were: masking oligonucleotide M1 solution for 5'-end biotin-labeled oligonucleotide B1 treatment solution, masking oligonucleotide M2 solution for 5'-end biotin-labeled oligonucleotide B2 treatment solution, and masking oligonucleotide M1 and masking oligonucleotide M2-mixed solution for 5'-end biotin-labeled oligonucleotide B1 and 5'-end biotin-labeled oligonucleotide B2-mixed treatment solution.

[0231] Each well was added with 100 μL of a methylcytosine antibody [available from Aviva Systems Biology, 0.5μg/mL 0.1% BSA-containing phosphate buffer (1 mM KH_2 PO_4, 3 mM Na_2HPO $7H_2O$, 154 mM NaCl pH7.4) solution], and added with 1 μL of the masking oligonucleotide solution, and left still for 1 hour at room temperature. Then the solution was removed by pipetting, and each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2 PO_4, 3 mM Na_2HPO $7H_2O$, 154 mM NaCl pH7.4)].

[0232] Then, each well was added with 100 μL of an Eu-N1-labeled mouse IgG antibody [available from Perkin Elmer, 0.05μg/mL 0.1% BSA-containing phosphate buffer (1 mM KH_2 PO_4, 3 mM Na_2HPO $7H_2O$, 154 mM NaCl pH7.4) solution], and then left still at room temperature for 1 hour. After leaving still, each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2 PO_4, 3 mM Na_2HPO $7H_2O$, 154 mM NaCl pH7.4)].

[0233] Each well was added with 150 μL of Enhancement Solution (available from Perkin Elmer), stirred at room temperature for 5 minutes, and left still for 15 minutes at room temperature. Then fluorescence was measured at excitation 340 nm/fluorescence 612 nm, and an average value of duplicate was calculated for the obtained measurements.

[0234] The results are shown in Figs. 3 to 5. It was revealed that the DNA fragment is selected by forming a complex with immobilized 5'-end biotin-labeled oligonucleotide B1 (Fig. 3), immobilized 5'-end biotin-labeled oligonucleotide B2 (Fig. 4), the 5'-end biotin-labeled oligonucleotides which are mixture of the foregoing two kinds (Fig. 5), and the methylcytosine antibody, and is quantified and detected with excellent sensitivity. It was revealed that when two kinds of the 5'-end biotin-labeled oligonucleotides are mixed (Fig. 5), in particular, quantification and detection with better sensitivity can be achieved compared with the case of detection by a single oligonucleotide (Fig. 3 and Fig. 4).

[0235] In the present experiment, it was revealed that a DNA fragment can be quantified and detected by forming and selecting a complex of a methylcytosine antibody, a methylated DNA fragment, and an immobilized 5'-end biotin-labeled oligonucleotide, and quantifying and detecting the methylcytosine antibody in the complex according to its identification function. It was also revealed that by using a plurality of immobilized 5'-end biotin-labeled oligonucleotides, more sensitive quantification and detection are realized than the case where one kind of immobilized 5'-end biotin-labeled oligonucleotide is used (that is, not only using one target DNA region but also using a plurality of target DNA regions at the same time).

Example 4

[0236] Seven (7) μg of genomic DNA derived from human blood purchased from Clontech, 48 U of restriction enzyme AluI, and 40 μL of 10x buffer optimum for AluI (100 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 10 mM Dithiothreitol, 500 mM NaCl) were mixed, and added with sterilized ultrapure water to prepare a reaction liquid having a liquid amount of 400 μL. The reaction liquid was incubated at 37°C for 4 hours. After conducting an enzyme treatment, cleavage was checked by 1.5% agarose gel electrophoresis, and enzyme-treated genomic DNA was purified by Wizard SV Gel/PCR Kit (PROMEGA Corporation).

[0237] The following solutions were prepared in duplicate using the obtained enzyme-treated genomic DNA.

Solution A: Enzyme-treated genomic DNA 1000 ng/30 μL TE buffer solution
Solution B: Enzyme-treated genomic DNA 500 ng/30 μL TE buffer solution
Solution C: Enzyme-treated genomic DNA 200 ng/30 μL TE buffer solution
Solution D: TE buffer solution (negative control solution)

[0238] Thirty (30) μL of the enzyme-treated genomic DNA solution prepared in the above, 0.5 μL of SssI methylase (available from NEB Inc.), 5 μL of 10xNEBuffer2 (available from NEB Inc.), and 0.5 μL of 3.2 mM S-adenosyl methionine (available from NEB Inc.) were mixed, and added with sterilized ultrapure water to prepare a reaction liquid having a liquid amount of 50 μL. The reaction liquid was incubated at 37°C for 30 minutes.

[0239] A 0.02 μM TE buffer solution of 5'-end biotin-labeled oligonucleotide B1 having the nucleotide sequence of SEQ ID NO: 20 capable of binding by complementation with a plus strand of the DNA fragment X' comprising the target DNA region of SEQ ID NO: 27 was prepared.

<DNA fragment comprising target DNA region>

[0240]

X':5'-

CTCAGCACCCAGGCGGCCGCGATCATGAGGCGCGAGCGGCGCGCGGGCTGTTGCAGAGTCTT

GAGCGGGTGGCACACCGCGATGTAGCGGTCGGCTGTCATGACTACCAGCATGTAGGCCGACG

CAAACATGCCGAACACCTGCAGGTGCTTCACCACGCGGCACAGCCAGTCGGGGCCGCGGAAG

CGGTAGGTGATGTCCCAGCACATTTGCGGCAGCACCTGGAAGAATGCCACGGCCAGGTCGGC

CAGGCTGAGGTGTCGGATGAAGAGGTGCATGCGGGACGTCTTGCGCGGCGTCCGGTGCAGAG

CCAGCAGTACGCTGCTGTTGCCCAGCACGGCCACCGCGAAAGTCACCGCCAGCACGGCGATC

TCCAGTTTGGCCAG -3' (SEQ ID NO: 27)

<5'-end biotin-labeled oligonucleotide>

[0241]

B1:5'- CTGGCCAAACTGGAGAT -3' (SEQ ID NO: 20)

[0242] For respective reaction liquids of enzyme-treated genomic DNA Solutions A to D, the following treatment was conducted.

[0243] In a PCR tube, 40 μL of the reaction liquid prepared in the above, 10 μL of the 5'-end biotin-labeled oligonucleotide solution, 10 μL of a buffer (330 mM Tris-Acetate pH 7.9, 660 mM KOAc, 100 mM $MgOAc_2$, 5 mM Dithiothreitol), 10 μL of a 100 mM $MgCl_2$ solution and 10 μL of a 1 mg/mL BSA solution were added, and further the mixture was added with sterilized ultrapure water to make the liquid amount 100 μL, and mixed. Then the PCR tube was heated at 95°C for 10 minutes, cooled rapidly to 70°C, and retained for 10 minutes at this temperature. Then the PCR tube was cooled to 50°C and retained for 10 minutes, and further retained for 10 minutes at 37°C, and then the PCR tube was returned to room temperature, to promote formation of a conjugate of the 5'-end biotin-labeled oligonucleotide and the DNA fragment.

[0244] The entire obtained mixture was transferred to a well coated with streptavidin, and left still at room temperature for about 30 minutes, to immobilize the conjugate of the 5'-end biotin-labeled oligonucleotide and the DNA fragment to the well. Thereafter, the solution was removed by pipetting, and each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4)].

[0245] Synthesized was masking oligonucleotide M1 having the nucleotide sequence of SEQ ID NO: 21 capable of binding by complementation with 5'-end biotin-labeled oligonucleotide B1 having the nucleotide sequence of SEQ ID NO: 20, and 0.1 μM TE buffer solution was prepared.

[0246] Each well was added with 100 μL of a methylcytosine antibody [available from Aviva Systems Biology, 0.5 μg/mL 0.1% BSA-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4) solution], and added with 1 μL of the masking oligonucleotide solution, and left still for 1 hour at room temperature. Then the solution was removed by pipetting, and each well was washed three times with 200 μL of a washing buffer [0.05%

Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4)].

[0247] Then, each well was added with 100 μL of an Eu-N1-labeled mouse IgG antibody [available from Perkin Elmer, 0.05 μg/mL 0.1% BSA-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4) solution], and then left still at room temperature for 1 hour. After leaving still, each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4)].

[0248] Each well was added with 150 μL of Enhancement Solution (available from Perkin Elmer), stirred at room temperature for 5 minutes, and left still for 15 minutes at room temperature. Then fluorescence was measured at excitation 340 nm/fluorescence 612 nm, and an average value of duplicate was calculated for the obtained measurements.

[0249] The result is shown in Fig. 6. It was revealed that in the enzyme-treated human genomic DNA solution, the DNA fragment is selected by forming a complex with immobilized 5'-end biotin-labeled oligonucleotide B1 and the methylcytosine antibody, and thus quantified and detected with excellent sensitivity.

[0250] In the present experiment, it was revealed that genomic DNA can be quantified and detected by forming and selecting a complex of a methylcytosine antibody, a methylated DNA fragment, and an immobilized 5'-end biotin-labeled oligonucleotide, and quantifying and detecting the methylcytosine antibody in the complex according to its identification function.

Example 5

[0251] Using genomic DNA derived from human blood purchased from Clontech, the following solutions were prepared respectively in duplicate.

Solution A: Genomic DNA derived from human blood 500 ng/20 μL TE buffer solution
Solution B: Genomic DNA derived from human blood 50 ng/20 μL TE buffer solution
Solution C: Genomic DNA derived from human blood 5 ng/20 μL TE buffer solution
Solution D: TE buffer solution (negative control solution)

[0252] Twenty (20) μL of each obtained solution, 10 U of restriction enzyme XspI, and 5 μL of 10x buffer optimum for XspI (200 mM Tris-HCl pH 8.5, 100 mM $MgCl_2$, 10 mM Dithiothreitol, 1000 mM KCl) were mixed, and added with sterilized ultrapure water to prepare a reaction liquid having a liquid amount of 50 μL. The reaction liquid was incubated at 37°C for 1 hour.

[0253] Twenty (20) μL of the enzyme-treated genomic DNA solution prepared in the above, 0.5 μL of SssI methylase (available from NEB Inc.), 5 μL of 10xNEBuffer2 (available from NEB Inc.), and 0.5 μL of 3.2 mM S-adenosyl methionine (available from NEB Inc.) were mixed, and added with sterilized ultrapure water to prepare a reaction liquid having a liquid amount of 50 μL. The reaction liquid was incubated at 37°C for 30 minutes.

[0254] Prepared was a 0.02 μM TE buffer solution of 5'-end biotin-labeled oligonucleotide B3 having the nucleotide sequence of SEQ ID NO: 29 capable of binding by complementation with a plus strand of DNA fragment Z (region corresponding to the nucleotide number 115-386 as shown in Genbank Accession No. M80340 or the like) comprising the target DNA region of SEQ ID NO: 28.

<DNA fragment>

[0255]

Z:5'-

TAGGGAGTGCCAGACAGTGGGCGCAGGCCAGTGTGTGTGCGCACCGTGCGCGAGCCGAAGCA

GGGCGAGGCATTGCCTCACCTGGGAAGCGCAAGGGGTCAGGGAGTTCCCTTTCTGAGTCAAA

GAAAGGGGTGACGGTCGCACCTGGAAAATCGGGTCACTCCCACCCGAATATTGCGCTTTTCA

GACCGGCTTAAGAAACGGCGCACCACGAGACTATATCCCACACCTGGCTCGGAGGGTCCTAC

GCCCACGGAATCTCGCTGATTGC -3' (SEQ ID NO: 28)

<5'-end biotin-labeled oligonucleotide>

[0256]

B3:5'- ATAGTCTCGTGGTGCGCCGT-3' (SEQ ID NO: 29)

[0257] For respective reaction liquids of enzyme-treated genomic DNA Solutions A to D, the following treatment was conducted.

[0258] In a PCR tube, 40 μL of the reaction liquid prepared in the above, 10 μL of the 5'-end biotin-labeled oligonucleotide solution, 10 μL of a buffer (330 mM Tris-Acetate pH 7.9, 660 mM KOAc, 100 mM $MgOAc_2$, 5 mM Dithiothreitol), 10 μL of a 100 mM $MgCl_2$ solution and 10 μL of a 1 mg/mL BSA solution were added, and further the mixture was added with sterilized ultrapure water to make the liquid amount 100 μL, and mixed. Then the PCR tube was heated at 95°C for 10 minutes, cooled rapidly to 70°C, and retained for 10 minutes at this temperature. Then the PCR tube was cooled to 50°C and retained for 10 minutes, and further retained for 10 minutes at 37°C, and then the PCR tube was returned to room temperature, to promote formation of a conjugate of the 5'-end biotin-labeled oligonucleotide and the DNA fragment.

[0259] The entire obtained mixture was transferred to a well coated with streptavidin, and left still at room temperature for about 30 minutes, to immobilize the conjugate of the 5'-end biotin-labeled oligonucleotide and the DNA fragment to the well. Thereafter, the solution was removed by pipetting, and each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4)].

[0260] Synthesized was masking oligonucleotide M3 having the nucleotide sequence of SEQ ID NO: 30 capable of binding by complementation with 5'-end biotin-labeled oligonucleotide B3 having the nucleotide sequence of SEQ ID NO: 29, and a 0.1 μM TE buffer solution was prepared.

<Masking oligonucleotide>

[0261]

M3:5'- ACGGCGCACCACGAGACTAT -3' (SEQ ID NO: 30)

[0262] Each well was added with 100 μL of a methylcytosine antibody [available from Aviva Systems Biology, 0.5 μg/mL 0.1% BSA-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4) solution], and added with 1 μL of the masking oligonucleotide solution, and left still for 1 hour at room temperature. Then the solution was removed by pipetting, and each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4)].

[0263] Then, each well was added with 100 μL of an Eu-N1-labeled mouse IgG antibody [available from Perkin Elmer, 0.05 μg/mL 0.1% BSA-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4) solution], and then left still at room temperature for 1 hour. After leaving still, each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4)].

[0264] Each well was added with 150 μL of Enhancement Solution (available from Perkin Elmer), stirred at room temperature for 5 minutes, and left still for 15 minutes at room temperature. Then fluorescence was measured at excitation 340 nm/fluorescence 612 nm, and an average value of duplicate was calculated for the obtained measurements.

[0265] The result is shown in Fig. 7, it was revealed that in the enzyme-treated human genomic DNA solution, the DNA fragment is selected by forming a complex with immobilized 5'-end biotin-labeled oligonucleotide B3 and the methylcytosine antibody, and thus quantified and detected with excellent sensitivity.

[0266] In the present experiment, it was revealed that genomic DNA can be quantified and detected by forming and selecting a complex of a methylcytosine antibody, a methylated DNA fragment, and an immobilized 5'-end biotin-labeled oligonucleotide, and quantifying and detecting the methylcytosine antibody in the complex according to its identification function.

Example 6

[0267] Using genomic DNA derived from human blood purchased from Clontech, the following solutions were prepared respectively in duplicate.

Solution A: Genomic DNA derived from human blood 500 ng/20 μL TE buffer solution

Solution B: Genomic DNA derived from human blood 50 ng/20 μL TE buffer solution
Solution C: Genomic DNA derived from human blood 5 ng/20 μL TE buffer solution
Solution D: TE buffer solution (negative control solution)

[0268] Twenty (20) μL of each obtained solution, 10 U of restriction enzyme XspI, and 5 μL of 10x buffer optimum for XspI (200 mM Tris-HCl pH 8.5, 100 mM $MgCl_2$, 10 mM Dithiothreitol, 1000 mM KC1) were mixed, and added with sterilized ultrapure water to prepare a reaction liquid having a liquid amount of 50 μL. The reaction liquid was incubated at 37°C for 1 hour.

[0269] Twenty (20) μL of the enzyme-treated genomic DNA solution prepared in the above, 0.5 μL of SssI methylase (available from NEB Inc.), 5 μL of 10xNEBuffer2 (available from NEB Inc.), and 0.5 μL of 3.2 mM S-adenosyl methionine (available from NEB Inc.) were mixed, and added with sterilized ultrapure water to prepare a reaction liquid having a liquid amount of 50 μL. The reaction liquid was incubated at 37°C for 30 minutes.

[0270] Prepared was a 0.02 μM TE buffer solution of 5'-end biotin-labeled oligonucleotide B3 having the nucleotide sequence of SEQ ID NO: 29 capable of binding by complementation with a plus strand of DNA fragment Z (region corresponding to the nucleotide number 115-386 as shown in Genbank Accession No. M80340 or the like) comprising the target DNA region of SEQ ID NO: 28.

[0271] Prepared were 0.01 μM TE buffer solutions of counter oligonucleotides C1, C2, C3, C4, and C5 having the nucleotide sequences of SEQ ID NO: 31, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, and SEQ ID NO: 35, respectively capable of binding by complementation with a minus strand (complementary chain) of DNA fragment Z comprising the target DNA region of SEQ ID NO: 28

<DNA fragment>

[0272]

Z:5'-

TAGGGAGTGCCAGACAGTGGGCGCAGGCCAGTGTGTGTGCGCACCGTGCGCGAGCCGAAGCA

GGGCGAGGCATTGCCTCACCTGGGAAGCGCAAGGGGTCAGGGAGTTCCCTTTCTGAGTCAAA

GAAAGGGGTGACGGTCGCACCTGGAAAATCGGGTCACTCCCACCCGAATATTGCGCTTTTCA

GACCGGCTTAAGAAACGGCGCACCACGAGACTATATCCCACACCTGGCTCGGAGGGTCCTAC

GCCCACGGAATCTCGCTGATTGC -3' (SEQ ID NO: 28)

<Counter oligonucleotides>

[0273]

C1:5'- CAGTGTGTGTGCGCACCGTGCGCGAGCCGA-3' (SEQ ID NO: 31)
C2:5'- GGCGAGGCATTGCCTCACCTGGGAAGCGCA-3' (SEQ ID NO: 32)
C3:5'- GGTGACGGTCGCACCTGGAAAATCGGGTCA-3' (SEQ ID NO: 33)
C4:5'- ACCCGAATATTGCGCTTTTCAGACCGGCTT-3' (SEQ ID NO: 34)
C5:5'- TCGGAGGGTCCTACGCCCACGGAATCTCGC-3' (SEQ ID NO: 35)

[0274] For respective reaction liquids of enzyme-treated genomic DNA Solutions A to D, the following treatment was conducted.

[0275] In a PCR tube, 40 μL of the reaction liquid prepared in the above, 10 μL of the 5'-end biotin-labeled oligonucleotide solution, 10 μL of the counter oligonucleotide solution, 10 μL of a buffer (330 mM Tris-Acetate pH 7.9, 660 mM KOAc, 100 mM $MgOAC_2$, 5 mM Dithiothreitol), 10 μL of a 100 mM $MgCl_2$ solution and 10 μL of a 1 mg/mL BSA solution were added, and further the mixture was added with sterilized ultrapure water to make the liquid amount 100 μL, and mixed. Then the PCR tube was heated at 95°C for 10 minutes, cooled rapidly to 70°C, and retained for 10 minutes at this temperature. Then the PCR tube was cooled to 50.°C and retained for 10 minutes, and further retained for 10 minutes at 37°C, and then the PCR tube was returned to room temperature, to promote formation of a conjugate of the 5'-end

biotin-labeled oligonucleotide and the DNA fragment.

[0276] The entire obtained mixture was transferred to a well coated with streptavidin, and left still at room temperature for about 30 minutes, to immobilize the conjugate of the 5'-end biotin-labeled oligonucleotide and the DNA fragment to the well. Thereafter, the solution was removed by pipetting, and each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4)].

[0277] Synthesized was a masking oligonucleotide M3 having the nucleotide sequence of SEQ ID NO: 30 capable of binding by complementation with 5'-end biotin-labeled oligonucleotide B3 having the nucleotide sequence of SEQ ID NO: 29, and 0.1 μM TE buffer solution was prepared.

[0278] Each well was added with 100 μL of a methylcytosine antibody [available from Aviva Systems Biology, 0.5 μg/mL 0.1% BSA-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4) solution], and added with 1 μL of the masking oligonucleotide solution, and left still for 1 hour at room temperature. Then the solution was removed by pipetting, and each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4)].

[0279] Then, each well was added with 100 μL of an Eu-N1-labeled mouse IgG antibody [available from Perkin Elmer, 0.05μg/mL 0.1% BSA-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4) solution], and then left still at room temperature for 1 hour. After leaving still, the solution was removed by pipetting, and each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4)].

[0280] Each well was added with 150 μL of Enhancement Solution (available from Perkin Elmer), stirred at room temperature for 5 minutes, and left still for 15 minutes at room temperature. Then fluorescence was measured at excitation 340 nm/fluorescence 612 nm, and an average value of duplicate was calculated for the obtained measurements.

[0281] The result is shown in Fig. 8. It was revealed that in the enzyme-treated human genomic DNA solution, the DNA fragment is selected by forming a complex with immobilized 5'-end biotin-labeled oligonucleotide B3 and the methylcytosine antibody, and thus quantified and detected with excellent sensitivity.

[0282] In the present experiment, it was revealed that genomic DNA can be quantified and detected by forming and selecting a complex of a methylcytosine antibody, a methylated DNA fragment, and an immobilized 5'-end biotin-labeled oligonucleotide, and quantifying and detecting the methylcytosine antibody in the complex according to its identification function.

Example 7

[0283] Yeast strain X2180-1A of baker's yeast was cultured in a YPD medium (1% Yeast extract, 2% Peptone, 2% Glucose, pH 5.6 to 6.0) to a turbidity of OD_{600} 0.6 to 1.0, and centrifuged at 10,000 g for 10 minutes, to prepare 1×10^7 of yeast cells. From the prepared yeast cells, a yeast genome was acquired using a generally used preparation method of a yeast genome as described in Methods in Yeast Genetics (Cold Spring Harbor Laboratory).

[0284] The prepared yeast cells were suspended in Buffer A (1 M sorbitol, 0.1 M EDTA, pH 7.4), added with 2-mercaptoethanol (final concentration 14 mM) and 100 U zymolase (10 mg/ml), and incubated under stirring at 30°C for an hour until the solution became clear. After collecting a protoplast by centrifugation at 550 g for 10 minutes, it was suspended in Buffer B (50 mM Tris-HCl, pH 7.4, 20 mM EDTA), added with sodium dodecyl sulfate in 1% (w/v), and then incubated at 65°C for 30 minutes. Sequentially, 5 M CH_3COOK was added and mingled in a volume ratio of 2/5, and the mixture was cooled on ice for 30 minutes, and then centrifuged at 15,000 g for 30 minutes to collect the supernatant. The collected supernatant was added with 3 M CH_3COONa in a volume ratio of 1/10 and an equal amount of isopropanol and mingled well, and the precipitate obtained by centrifugation at 15,000 g at 4°C for 30 minutes was rinsed with 70% ethanol and collected. After drying, the precipitate was dissolved in 1 mL of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), and added with RNase A (available from Sigma) in a concentration of 40 μg/ml, incubated at 37°C for an hour, and then the mixture was added with proteinase K (available from Sigma) and sodium dodecyl sulfate in a concentrations of 500 μg/mL and 1% (w/v), respectively, and shaken at 55°C for about 16 hours. After end of the shaking, the mixture was extracted with phenol [saturated with 1 M Tris-HCl (pH 8.0)]·chloroform. An aqueous layer was collected, added with NaCl in a concentration of 0.5 N, and allowed to precipitate from ethanol, and the generated precipitate was collected. The collected precipitate was rinsed with 70% ethanol, to obtain genomic DNA.

[0285] From the obtained genomic DNA, a DNA fragment to be used as a test sample (S, SEQ ID NO: 38, the region corresponding to the nucleotide number 271743-272083 of yeast chromosome VII shown in Genbank Accession No. NC_001139) was amplified by conducting PCR using oligonucleotide primers (PF3 and PR3) designed for PCR of SEQ ID NO: 36 and SEQ ID NO: 37 and the following reaction condition.

<Oligonucleotide primers designed for PCR>

[0286]

PF3: 5'-AGGTGAGCTACGTGTGTTTGG-3' (SEQ ID NO: 36)
PR3: 5'-AGACATGTGCTCACGTACGGT-3' (SEQ ID NO: 37)

<DNA fragment>

[0287]

S: 5'-
AGGTGAGCTACGTGTGTTTGGGCGTCGTGCACTGGCTCACTTGTACGCGCAGAAATGGCAGC
TTGTACGATTGGTGACCCGCCTTTTCGACACTGGACCGCTATGGACGTGGCGGCGGTGTGGC
GGCGGCTCAATGACCTGTGGCGCCCGTTTGTGGCGTGCGATAGTCGAGCCGCCTGTCACGTG
CGCGGCCGCCCTGCTCCGTTTGACGCGATGCATAGCATGCGACCACCCAGTAATCATACTGC
TGACGCTATTGGTCACGTGGTTATGGCAGCTGCTGTTGACTGCGGTGGCGTCCCGTTTCCAC
ACCGTACGTGAGCACATGTCT-3' (SEQ ID NO: 38)

[0288] As a reaction liquid of PCR, 10 ng of genomic DNA as a template, each 3 μL of 5 μM of the above primer solutions, 5 μL of each 2 mM dNTP, and 5 μL of 10×buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% Gelatin) were mixed with 0.25 μL of 5U/μL thermostable DNA polymerase (AmpliTaq Gold), and added with sterilized ultrapure water to prepare a reaction liquid having a liquid amount of 50 μL. After retaining the reaction liquid at 95°C for 10 minutes, PCR was conducted by 40 cycles each consisting of 20 seconds at 95°C, 30 seconds at 58°C and 30 seconds at 72°C.

[0289] After conducting PCR, amplification was checked by 2% agarose gel electrophoresis, and the DNA fragment S was purified by Wizard SV Gel/PCR Kit (PROMEGA Corporation).

[0290] For the DNA fragment S, the following solutions were prepared respectively in duplicate.

Solution A: 10ng/20 μL TE buffer solution
Solution B: 1ng/20 μL TE buffer solution
Solution C: 0.1ng/20 μL TE buffer solution
Solution D: TE buffer solution (negative control solution)

[0291] Twenty (20) μL of each obtained solution, 0.5 μL of SssI methylase (available from NEB Inc.), 5 μL of 10xNEBuffer2 (available from NEB Inc.), and 0.5 μL of 3.2 mM S-adenosyl methionine (available from NEB Inc.) were mixed, and added with sterilized ultrapure water to prepare a reaction liquid having a liquid amount of 50 μL. The reaction liquid was incubated at 37°C for 30 minutes.

[0292] Prepared was 0.02 μM TE buffer solution of 5'-end biotin-labeled oligonucleotide B4 having the nucleotide sequence of SEQ ID NO: 39 capable of binding by complementation with a plus strand of the DNA fragment S comprising the target DNA region of SEQ ID NO: 38.

<5'-end biotin-labeled oligonucleotide>

[0293]

B4:5'- AGACATGTGCTCACGTACGGT -3' (SEQ ID NO: 39)

[0294] Each obtained reaction liquid was subjected to the following treatments.

[0295] In a PCR tube, 40 μL of the reaction liquid prepared in the above, 10 μL of the 5'-end biotin-labeled oligonu-

cleotide solution, 10 μL of a buffer (330 mM Tris-Acetate pH 7.9, 660 mM KOAc, 100 mM $MgOAc_2$, 5 mM Dithiothreitol), 10 μL of a 100 mM $MgCl_2$ solution and 10 μL of a 1 mg/mL BSA solution were added, and further the mixture was added with sterilized ultrapure water to make the liquid amount 100 μL, and mixed. Then the PCR tube was heated at 95°C for 10 minutes, cooled rapidly to 70°C, and retained for 10 minutes at this temperature. Then the PCR tube was cooled to 50°C and retained for 10 minutes, and further retained for 10 minutes at 37°C, and then the PCR tube was returned to room temperature, to promote formation of a conjugate of the 5'-end biotin-labeled oligonucleotide and the DNA fragment.

[0296] The entire obtained mixture was transferred to a well coated with streptavidin, and left still at room temperature for about 30 minutes, to immobilize the conjugate of the 5'-end biotin-labeled oligonucleotide and the DNA fragment to the well. Thereafter, the solution was removed by pipetting, and each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4)].

[0297] Synthesized was a masking oligonucleotide M4 having the nucleotide sequence of SEQ ID NO: 40 capable of binding by complementation with 5'-end biotin-labeled oligonucleotide B4 having the nucleotide sequence of SEQ ID NO: 39, and 0.1 μM TE buffer solution was prepared.

<Masking oligonucleotide>

[0298]

M4:5'- ACCGTACGTGAGCACATGTCT -3' (SEQ ID NO: 40)

[0299] Each well was added with 100 μL of a methylcytosine antibody [available from Aviva Systems Biology, 0.5 μg/mL 0.1% BSA-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4) solution], and added with 1 μL of the masking oligonucleotide solution, and left still for 1 hour at room temperature. Then the solution was removed by pipetting, and each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4)].

[0300] Then, each well was added with 100 μL of an Eu-N1-labeled mouse IgG antibody [available from Perkin Elmer, 0.05μg/mL 0.1% BSA-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4) solution], and then left still at room temperature for 1 hour. After leaving still, the solution was removed by pipetting, and each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4)].

[0301] Each well was added with 150 μL of Enhancement Solution (available from Perkin Elmer), stirred at room temperature for 5 minutes, and left still for 15 minutes at room temperature. Then fluorescence was measured at excitation 340 nm/fluorescence 612 nm, and an average value of duplicate was calculated for the obtained measurements.

[0302] The result is shown in Fig. 9. It was revealed that a DNA fragment is selected accurately by the immobilized 5'-end biotin-labeled oligonucleotide, and quantified and detected with excellent sensitivity.

[0303] In the present experiment, it was revealed that a DNA fragment can be quantified and detected by forming and selecting a complex of a methylcytosine antibody, a methylated DNA fragment, and an immobilized 5'-end biotin-labeled oligonucleotide, and quantifying and detecting the methylcytosine antibody in the complex according to its identification function.

Example 8

[0304] Yeast strain X2180-1A of baker's yeast was cultured in a YPD medium (1% Yeast extract, 2% Peptone, 2% Glucose, pH 5.6 to 6.0) to a turbidity of OD_{600} 0.6 to 1.0, and centrifuged at 10,000 g for 10 minutes, to prepare 1×10^7 of yeast cells. From the prepared yeast cells, a yeast genome was acquired using a generally used preparation method of a yeast genome as described in Methods in Yeast Genetics (Cold Spring Harbor Laboratory).

[0305] The prepared yeast cells were suspended in Buffer A (1 M sorbitol, 0.1 M EDTA, pH 7.4), added with 2-mercaptoethanol (final concentration 14 mM) and 100 U zymolase (10 mg/ml), and incubated under stirring at 30°C for an hour until the solution became clear. After collecting a protoplast by centrifugation at 550 g for 10 minutes, it was suspended in Buffer B (50 mM Tris-HCl, pH 7.4, 20 mM EDTA), added with sodium dodecyl sulfate in 1% (w/v), and then incubated at 65°C for 30 minutes. Sequentially, 5 M CH_3COOK was added and mingled in a volume ratio of 2/5, and the mixture was cooled on ice for 30 minutes, and then centrifuged at 15,000 g for 30 minutes to collect the supernatant. The collected supernatant was added with 3 M CH_3COONa in a volume ratio of 1/10 and an equal amount of isopropanol and mingled well, and the precipitate obtained by centrifugation at 15,000 g at 4°C for 30 minutes was rinsed with 70% ethanol and collected. After drying, the precipitate was dissolved in 1 mL of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), and added with RNase A (available from Sigma) in a concentration of 40 μg/ml, incubated at 37°C

for an hour, and then the mixture was added with proteinase K (available from Sigma) and sodium dodecyl sulfate in a concentrations of 500 μg/mL and 1% (w/v), respectively, and shaken at 55°C for about 16 hours. After end of the shaking, the mixture was extracted with phenol [saturated with 1 M Tris-HCl (pH 8.0)]·chloroform. An aqueous layer was collected, added with NaCl in a concentration of 0.5 N, and allowed to precipitate from ethanol, and the generated precipitate was collected. The collected precipitate was rinsed with 70% ethanol, to obtain genomic DNA.

[0306] From the obtained genomic DNA, a DNA fragment to be used as a test sample (T, SEQ ID NO: 43, the region corresponding to the nucleotide number 384523-384766 of yeast chromosome VII shown in Genbank Accession No. NC_001139) was amplified by conducting PCR using oligonucleotide primers (PF4 and PR4) designed for PCR of SEQ ID NO: 41 and SEQ ID NO: 42 and the following reaction condition.

<Oligonucleotide primers designed for PCR>

[0307]

PF4: 5'-GGACCTGTGTTTGACGGGTAT-3' (SEQ ID NO: 41)
PR4: 5'-AGTACAGATCTGGCGTTCTCG-3' (SEQ ID NO: 42)

<DNA fragment>

[0308]

T: 5'-

GGACCTGTGTTTGACGGGTATAACACTAAGTTGCGCAATTTGCTGTATTGCGAAATCCGCCC

GGACGATATCACTCTTGAGCGCATGTGCCGTTTCCGAGAACGCCAGATCTGTACT-3'

(SEQ ID NO: 43)

[0309] As a reaction liquid of PCR, 10 ng of genomic DNA as a template, each 3 μL of 5 μM of the above primer solutions, 5 μL of each 2 mM dNTP, and 5 μL of 10×buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% Gelatin) were mixed with 0.25 μL of 5U/μL thermostable DNA polymerase (AmpliTaq Gold), and added with sterilized ultrapure water to prepare a reaction liquid having a liquid amount of 50 μL. After retaining the reaction liquid at 95°C for 10 minutes, PCR was conducted by 40 cycles each consisting of 20 seconds at 95°C, 30 seconds at 58°C and 30 seconds at 72°C.

[0310] After conducting PCR, amplification was checked by 2% agarose gel electrophoresis, and the DNA fragment T was purified by Wizard SV Gel/PCR Kit (PROMEGA Corporation).

[0311] For the DNA fragment T, the following solutions were prepared respectively in duplicate.

Solution A: DNA fragment T 10ng/20 μL TE buffer solution
Solution B: DNA fragment T 1ng/20 μL TE buffer solution
Solution C: DNA fragment T 0.1ng/20 μL TE buffer solution
Solution D: TE buffer solution (negative control solution)

[0312] Twenty (20) μL of each obtained solution, 0.5 μL of SssI methylase (available from NEB Inc.), 5 μL of 10xNEBuffer2 (available from NEB Inc.), and 0.5 μL of 3.2 mM S-adenosyl methionine (available from NEB Inc.) were mixed, and added with sterilized ultrapure water to prepare a reaction liquid having a liquid amount of 50 μL. The reaction liquid was incubated at 37°C for 30 minutes.

[0313] Prepared was 0.02 μM TE buffer solution of 5'-end biotin-labeled oligonucleotide B5 having the nucleotide sequence of SEQ ID NO: 44 capable of binding by complementation with a plus strand of the DNA fragment T comprising the target DNA region of SEQ ID NO: 43.

<5'-end biotin-labeled oligonucleotide>

[0314]

B5:5'- AGTACAGATCTGGCGTTCTCG -3' (SEQ ID NO: 44)

[0315] Each obtained reaction liquid was subjected to the following treatments.

[0316] In a PCR tube, 40 μL of the reaction liquid prepared in the above, 10 μL of the 5'-end biotin-labeled oligonucleotide solution, 10 μL of a buffer (330 mM Tris-Acetate pH 7.9, 660 mM KOAc, 100 mM $MgOAc_2$, 5 mM Dithiothreitol), 10 μL of a 100 mM $MgCl_2$ solution and 10 μL of a 1 mg/mL BSA solution were added, and further the mixture was added with sterilized ultrapure water to make the liquid amount 100 μL, and mixed. Then the PCR tube was heated at 95°C for 10 minutes, cooled rapidly to 70°C, and retained for 10 minutes at this temperature. Then the PCR tube was cooled to 50°C and retained for 10 minutes, and further retained for 10 minutes at 37°C, and then the PCR tube was returned to room temperature, to promote formation of a conjugate of the 5'-end biotin-labeled oligonucleotide and the DNA fragment.

[0317] The entire obtained mixture was transferred to a well coated with streptavidin, and left still at room temperature for about 30 minutes, to immobilize the conjugate of the 5'-end biotin-labeled oligonucleotide and the DNA fragment to the well. Thereafter, the solution was removed by pipetting, and each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4)].

[0318] Synthesized was a masking oligonucleotide M5 having the nucleotide sequence of SEQ ID NO: 45 capable of binding by complementation with 5'-end biotin-labeled oligonucleotide B5 having the nucleotide sequence of SEQ ID NO: 44, and 0.1 μM TE buffer solution was prepared.

<Masking oligonucleotide>

[0319]

M5:5'- CGAGAACGCCAGATCTGTACT -3' (SEQ ID NO: 45)

[0320] Each well was added with 100 μL of a methylcytosine antibody [available from Aviva Systems Biology, 0.5 μg/mL 0.1% BSA-containing phosphate buffer (1 mM KH_2PO_4, 3. mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4) solution], and added with 1 μL of the masking oligonucleotide solution, and left still for 1 hour at room temperature. Then the solution was removed by pipetting, and each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4)].

[0321] Then, each well was added with 100 μL of an Eu-N1-labeled mouse IgG antibody [available from Perkin Elmer, 0.05μg/mL 0.1% BSA-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4) solution], and then left still at room temperature for 1 hour. After leaving still, the solution was removed by pipetting, and each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4)].

[0322] Each well was added with 150 μL of Enhancement Solution (available from Perkin Elmer), stirred at room temperature for 5 minutes, and left still for 15 minutes at room temperature. Then fluorescence was measured at excitation 340 nm/fluorescence 612 nm, and an average value of duplicate was calculated for the obtained measurements.

[0323] The result is shown in Fig. 10. It was revealed that a DNA fragment is selected accurately by the immobilized 5'-end biotin-labeled oligonucleotide, and quantified and detected with excellent sensitivity.

[0324] In the present experiment, it was revealed that a DNA fragment can be quantified and detected by forming and selecting a complex of a methylcytosine antibody, a methylated DNA fragment, and an immobilized 5'-end biotin-labeled oligonucleotide, and quantifying and detecting the methylcytosine antibody in the complex according to its identification function.

Example 9

[0325] Yeast strain X2180-1A of baker's yeast was cultured in a YPD medium (1% Yeast extract, 2% Peptone, 2% Glucose, pH 5.6 to 6.0) to a turbidity of OD_{600} 0.6 to 1.0, and centrifuged at 10,000 g for 10 minutes, to prepare 1×10^7 of yeast cells. From the prepared yeast cells, a yeast genome was acquired using a generally used preparation method of a yeast genome as described in Methods in Yeast Genetics (Cold Spring Harbor Laboratory).

[0326] The prepared yeast cells were suspended in Buffer A (1 M sorbitol, 0.1 M EDTA, pH 7.4), added with 2-mercaptoethanol (final concentration 14 mM) and 100 U zymolase (10 mg/ml), and incubated under stirring at 30°C for an hour until the solution became clear. After collecting a protoplast by centrifugation at 550 g for 10 minutes, it was suspended in Buffer B (50 mM Tris-HCl, pH 7.4, 20 mM EDTA), added with sodium dodecyl sulfate in 1% (w/v), and then incubated at 65°C for 30 minutes. Sequentially, 5 M CH_3COOK was added and mingled in a volume ratio of 2/5, and the mixture was cooled on ice for 30 minutes, and then centrifuged at 15,000 g for 30 minutes to collect the supernatant. The collected supernatant was added with 3 M CH_3COONa in a volume ratio of 1/10 and an equal amount of isopropanol and mingled well, and the precipitate obtained by centrifugation at 15,000 g at 4°C for 30 minutes was

rinsed with 70% ethanol and collected. After drying, the precipitate was dissolved in 1 mL of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), and added with RNase A (available from Sigma) in a concentration of 40 μg/ml, incubated at 37°C for an hour, and then the mixture was added with proteinase K (available from Sigma) and sodium dodecyl sulfate in a concentrations of 500 μg/mL and 1% (w/v), respectively, and shaken at 55°C for about 16 hours. After end of the shaking, the mixture was extracted with phenol [saturated with 1 M Tris-HCl (pH 8.0)]·chloroform. An aqueous layer was collected, added with NaCl in a concentration of 0.5 N, and allowed to precipitate from ethanol, and the generated precipitate was collected. The collected precipitate was rinsed with 70% ethanol, to obtain genomic DNA.

[0327] From the obtained genomic DNA, a DNA fragment to be used as a test sample (S, SEQ ID NO: 38, the region corresponding to the nucleotide number 271743-272083 of yeast chromosome VII shown in Genbank Accession No. NC_001139) was amplified by conducting PCR using oligonucleotide primers (PF3 and PR3) designed for PCR of SEQ ID NO: 36 and SEQ ID NO: 37 and the following reaction condition.

[0328] As a reaction liquid of PCR, 10 ng of genomic DNA as a template, each 3 μL of 5 μM of the above primer solutions, 5 μL of each 2 mM dNTP, and 5 μL of 10×buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% Gelatin) were mixed with 0.25 μL of 5U/μL thermostable DNA polymerase (AmpliTaq Gold), and added with sterilized ultrapure water to prepare a reaction liquid having a liquid amount of 50 μL. After retaining the reaction liquid at 95°C for 10 minutes, PCR was conducted by 40 cycles each consisting of 20 seconds at 95°C, 30 seconds at 58°C and 30 seconds at 72°C.

[0329] After conducting PCR, amplification was checked by 2% agarose gel electrophoresis, and the DNA fragment S was purified by Wizard SV Gel/PCR Kit (PROMEGA Corporation).

[0330] From the obtained genomic DNA, a DNA fragment to be used as a test sample (T, SEQ ID NO: 43, the region corresponding to the nucleotide number 384523-384766 of yeast chromosome VII shown in Genbank Accession No. NC_001139) was amplified by conducting PCR using oligonucleotide primers (PF4 and PR4) designed for PCR of SEQ ID NO: 41 and SEQ ID NO: 42 and the following reaction condition.

[0331] As a reaction liquid of PCR, 10 ng of genomic DNA as a template, each 3 μL of 5 μM of the above primer solutions, 5 μL of each 2 mM dNTP, and 5 μL of 10×buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% Gelatin) were mixed with 0.25 μL of 5U/μL thermostable DNA polymerase (AmpliTaq Gold), and added with sterilized ultrapure water to prepare a reaction liquid having a liquid amount of 50 μL. After retaining the reaction liquid at 95°C for 10 minutes, PCR was conducted by 40 cycles each consisting of 20 seconds at 95°C, 30 seconds at 58°C and 30 seconds at 72°C.

[0332] After conducting PCR, amplification was checked by 2% agarose gel electrophoresis, and the DNA fragment T was purified by Wizard SV Gel/PCR Kit (PROMEGA Corporation).

[0333] For each of the DNA fragment S and the DNA fragment T, the following solutions were prepared.

Solution A: DNA fragment S or DNA fragment T 10 ng/10 μL TE buffer solution
Solution B: DNA fragment S or DNA fragment T 1 ng/10 μL TE buffer solution
Solution.C: DNA fragment S or DNA fragment T 0.1 ng/10 μL TE buffer solution
Solution D: TE buffer solution (negative control solution)

[0334] Equivalent amounts of Solution A of the DNA fragment S and Solution A of the DNA fragment T were mixed, to prepare DNA fragment-mixed Solution MA, equivalent amounts of Solution B of the DNA fragment S and Solution B of the DNA fragment T were mixed, to prepare DNA fragment-mixed Solution MB, equivalent amounts of Solution C of the DNA fragment S and Solution C of the DNA fragment T were mixed, to prepare DNA fragment-mixed Solution MC, and equivalent amounts of Solution D of the DNA fragment S and Solution D of the DNA fragment T were mixed, to prepare DNA fragment-mixed Solution MD. Three sets in duplicate were prepared respectively, for each of DNA fragment-mixed Solutions MA , MB, MC and MD.

[0335] Twenty (20) μL of each obtained solution, 0.5 μL of SssI methylase (available from NEB Inc.), 5 μL of 10xNEBuffer2 (available from NEB Inc.), and 0.5 μL of 3.2 mM S-adenosyl methionine (available from NEB Inc.) were mixed, and added with sterilized ultrapure water to prepare a reaction liquid having a liquid amount of 50 μL. The reaction liquid was incubated at 37°C for 30 minutes.

[0336] Prepared were 0.02 μM TE buffer solutions of 5'-end biotin-labeled oligonucleotide B4 having the nucleotide sequence of SEQ ID NO: 39 capable of binding by complementation with the DNA fragment S of SEQ ID NO: 38 and 5'-end biotin-labeled oligonucleotide B5 having the nucleotide sequence of SEQ ID NO: 44 capable of binding by complementation with the DNA fragment T of SEQ ID NO: 43. Also prepared was a TE buffer solution (0.02 μM for each) of 5'-end biotin-labeled oligonucleotide mixing equivalent amounts of 5'-end biotin-labeled oligonucleotide B4 having the nucleotide sequence of SEQ ID NO: 39 and 5'-end biotin-labeled oligonucleotide B5 having the nucleotide sequence of SEQ ID NO: 44.

[0337] For each reaction liquid of the DNA fragment Solutions MA to MD, the following treatment was conducted.

[0338] In a PCR tube, 40 μL of the reaction liquid prepared in the above, 10 μL of the 5'-end biotin-labeled oligonu-

cleotide solution, 10 μL of a buffer (330 mM Tris-Acetate pH 7.9, 660 mM KOAc, 100 mM $MgOAc_2$, 5 mM Dithiothreitol), 10 μL of a 100 mM $MgCl_2$ solution and 10 μL of a 1 mg/mL BSA solution were added, and further the mixture was added with sterilized ultrapure water to make the liquid amount 100 μL, and mixed. Then the PCR tube was heated at 95°C for 10 minutes, cooled rapidly to 70°C, and retained for 10 minutes at this temperature. Then the PCR tube was cooled to 50°C and retained for 10 minutes, and further retained for 10 minutes at 37°C, and then the PCR tube was returned to room temperature, to promote formation of a conjugate of the 5'-end biotin-labeled oligonucleotide and the DNA fragment.

[0339] The entire obtained mixture was transferred to a well coated with streptavidin, and left still at room temperature for about 30 minutes, to immobilize the conjugate of the 5'-end biotin-labeled oligonucleotide and the DNA fragment to the well. Thereafter, the solution was removed by pipetting, and each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4)].

[0340] Synthesized were masking oligonucleotide M4 having the nucleotide sequence of SEQ ID NO: 40 capable of binding by complementation with 5'-end biotin-labeled oligonucleotide B4 having the nucleotide sequence of SEQ ID NO: 39 and masking oligonucleotide M5 having the nucleotide sequence of SEQ ID NO: 45 capable of binding by complementation with 5'-end biotin-labeled oligonucleotide B5 having the nucleotide sequence of SEQ ID NO: 44, and respective 0.1 μM TE buffer solutions were prepared. Prepared was a TE buffer solution (each 0.1 μM) mixing equivalent amounts of masking oligonucleotide M4 having the nucleotide sequence of SEQ ID NO: 40 and masking oligonucleotide M5 having the nucleotide sequence of SEQ ID NO: 45.

[0341] Each well was added with 100 μL of a methylcytosine antibody [available from Aviva Systems Biology, 0.5μg/mL 0.1% BSA-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4) solution], and added with 1 μL of the masking oligonucleotide solution, and left still for 1 hour at room temperature. Then the solution was removed by pipetting, and each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4)].

[0342] Then, each well was added with 100 μL of an Eu-N1-labeled mouse IgG antibody [available from Perkin Elmer, 0.05μg/mL 0.1% BSA-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4) solution], and then left still at room temperature for 1 hour. After leaving still, each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4)].

[0343] Each well was added with 150 μL of Enhancement Solution (available from Perkin Elmer), stirred at room temperature for 5 minutes, and left still for 15 minutes at room temperature. Then fluorescence was measured at excitation 340 nm/fluorescence 612 nm, and an average value of duplicate was calculated for the obtained measurements.

[0344] The results are shown in Figs. 11 to 13. It was revealed that the DNA fragment is selected by forming a complex with immobilized 5'-end biotin-labeled oligonucleotide B4 (Fig. 11), immobilized 5'-end biotin-labeled oligonucleotide B5 (Fig. 12), the 5'-end biotin-labeled oligonucleotides which are mixture of the foregoing two kinds (Fig. 13), and the methylcytosine antibody, and is quantified and detected with excellent sensitivity. It was revealed that when two kinds of the 5'-end biotin-labeled oligonucleotides are mixed (Fig. 13), in particular, quantification and detection with better sensitivity can be achieved compared with the case of detection by a single oligonucleotide (Fig. 11 and Fig. 12).

[0345] In the present experiment, it was revealed that a DNA fragment can be quantified and detected by forming and selecting a complex of a methylcytosine antibody, a methylated DNA fragment, and an immobilized 5'-end biotin-labeled oligonucleotide, and quantifying and detecting the methylcytosine antibody in the complex according to its identification function. It was also revealed that by using a plurality of immobilized 5'-end biotin-labeled oligonucleotides, more sensitive quantification and detection are realized than the case where one kind of immobilized 5'-end biotin-labeled oligonucleotide is used (that is, not only using one target DNA region but also using a plurality of target DNA regions at the same time).

Example 10

[0346] Yeast strain X2180-1A of baker's yeast was cultured in a YPD medium (1% Yeast extract, 2% Peptone, 2% Glucose, pH 5.6 to 6.0) to a turbidity of OD_{600} 0.6 to 1.0, and centrifuged at 10,000 g for 10 minutes, to prepare 1×10^7 of yeast cells. From the prepared yeast cells, a yeast genome was acquired using a generally used preparation method of a yeast genome as described in Methods in Yeast Genetics (Cold Spring Harbor Laboratory).

[0347] The prepared yeast cells were suspended in Buffer A (1 M sorbitol, 0.1 M EDTA, pH 7.4), added with 2-mercaptoethanol (final concentration 14 mM) and 100 U zymolase (10 mg/ml), and incubated under stirring at 30°C for an hour until the solution became clear. After collecting a protoplast by centrifugation at 550 g for 10 minutes, it was suspended in Buffer B (50 mM Tris-HCl, pH 7.4, 20 mM EDTA), added with sodium dodecyl sulfate in 1% (w/v), and then incubated at 65°C for 30 minutes. Sequentially, 5 M CH_3COOK was added and mingled in a volume ratio of 2/5, and the mixture was cooled on ice for 30 minutes, and then centrifuged at 15,000 g for 30 minutes to collect the supernatant. The collected supernatant was added with 3 M CH_3COONa in a volume ratio of 1/10 and an equal amount

of isopropanol and mingled well, and the precipitate obtained by centrifugation at 15,000 g at 4°C for 30 minutes was rinsed with 70% ethanol and collected. After drying, the precipitate was dissolved in 1 mL of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), and added with RNase A (available from Sigma) in a concentration of 40 μg/ml, incubated at 37°C for an hour, and then the mixture was added with proteinase K (available from Sigma) and sodium dodecyl sulfate in a concentrations of 500 μg/mL and 1% (w/v), respectively, and shaken at 55°C for about 16 hours. After end of the shaking, the mixture was extracted with phenol [saturated with 1 M Tris-HCl (pH 8.0)]·chloroform. An aqueous layer was collected, added with NaCl in a concentration of 0.5 N, and allowed to precipitate from ethanol, and the generated precipitate was collected. The collected precipitate was rinsed with 70% ethanol, to obtain genomic DNA.

[0348] For the obtained yeast genomic DNA, the following solutions were prepared.

Solution A: Yeast genomic DNA 100 ng/20 μL TE buffer solution

Solution B: Yeast genomic DNA 10 ng/20 μL TE buffer solution

Solution C: Yeast genomic DNA 1 ng/20 μL TE buffer solution

Solution D: TE buffer solution (negative control solution)

[0349] Twenty (20) μL of each obtained solution, 10 U of restriction enzyme XspI, and 5 μL of 10x buffer optimum for XspI (200 mM Tris-HCl pH 8.5, 100 mM $MgCl_2$, 10 mM Dithiothreitol, 1000 mM KCl) were mixed, and added with sterilized ultrapure water to prepare a reaction liquid having a liquid amount of 50 μL. The reaction liquid was incubated at 37°C for 1 hour.

[0350] Twenty (20) μL of each obtained solution, 0.5 μL of SssI methylase (available from NEB Inc.), 5 μL of 10xNEBuffer2 (available from NEB Inc.), and 0.5 μL of 3.2 mM S-adenosyl methionine (available from NEB Inc.) were mixed, and added with sterilized ultrapure water to prepare a reaction liquid having a liquid amount of 50 μL. The reaction liquid was incubated at 37°C for 30 minutes.

[0351] Prepared was a 0.02 μM TE buffer solution of 5'-end biotin-labeled oligonucleotide B4 having the nucleotide sequence of SEQ ID NO: 39 capable of binding by complementation with a plus strand of DNA fragment S' comprising the target DNA region of SEQ ID NO: 46.

<DNA fragment>

[0352]

S':5'-

TAGGTGAGCTACGTGTGTTTGGGCGTCGTGCACTGGCTCACTTGTACGCGCAGAAATGGCAG

CTTGTACGATTGGTGACCCGCCTTTTCGACACTGGACCGCTATGGACGTGGCGGCGGTGTGG

CGGCGGCTCAATGACCTGTGGCGCCCGTTTGTGGCGTGCGATAGTCGAGCCGCCTGTCACGT

GCGCGGCCGCCCTGCTCCGTTTGACGCGATGCATAGCATGCGACCACCCAGTAATCATACTG

CTGACGCTATTGGTCACGTGGTTATGGCAGCTGCTGTTGACTGCGGTGGCGTCCCGTTTCCA

CACCGTACGTGAGCACATGTCTGGATTGC -3' (SEQ ID NO: 46)

<5'-end biotin-labeled oligonucleotide>

[0353]

B4:5'- AGACATGTGCTCACGTACGGT-3' (SEQ ID NO: 39)

[0354] For respective reaction liquids of yeast genomic DNA Solutions A to D, the following treatment was conducted.

[0355] In a PCR tube, 40 μL of the reaction liquid prepared in the above, 10 μL of the 5'-end biotin-labeled oligonucleotide solution, 10 μL of a buffer (330 mM Tris-Acetate pH 7.9, 660 mM KOAc, 100 mM $MgOAc_2$, 5 mM Dithiothreitol),

10 μL of a 100 mM $MgCl_2$ solution and 10 μL of a 1 mg/mL BSA solution were added, and further the mixture was added with sterilized ultrapure water to make the liquid amount 100 μL, and mixed. Then the PCR tube was heated at 95°C for 10 minutes, cooled rapidly to 70°C, and retained for 10 minutes at this temperature. Then the PCR tube was cooled to 50°C and retained for 10 minutes, and further retained for 10 minutes at 37°C, and then the PCR tube was returned to room temperature, to promote formation of a conjugate of the 5'-end biotin-labeled oligonucleotide and the DNA fragment.

[0356] The entire obtained mixture was transferred to a well coated with streptavidin, and left still at room temperature for about 30 minutes, to immobilize the conjugate of the 5'-end biotin-labeled oligonucleotide and the DNA fragment to the well. Thereafter, the solution was removed by pipetting, and each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4)].

[0357] Synthesized was masking oligonucleotide M4 having the nucleotide sequence of SEQ ID NO: 40 capable of binding by complementation with 5'-end biotin-labeled oligonucleotide B4 having the nucleotide sequence of SEQ ID NO: 39, and a 0.1 μM TE buffer solution was prepared.

<5'-end biotin-labeled oligonucleotide>

[0358]

B4:5'- AGACATGTGCTCACGTACGGT -3' (SEQ ID NO: 39)

<Masking oligonucleotide>

[0359]

M4:5'- ACGGCGCACCACGAGACTAT -3' (SEQ ID NO: 40)

[0360] Each well was added with 100 μL of a methylcytosine antibody [available from Aviva Systems Biology, 0.5 μg/mL 0.1% BSA-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4) solution], and added with 1 μL of the masking oligonucleotide solution, and left still for 1 hour at room temperature. Then the solution was removed by pipetting, and each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4)].

[0361] Then, each well was added with 100 μL of an Eu-N1-labeled mouse IgG antibody [available from Perkin Elmer, 0.05 μg/mL 0.1% BSA-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4) solution], and then left still at room temperature for 1 hour. After leaving still, each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4)].

[0362] Each well was added with 150 μL of Enhancement Solution (available from Perkin Elmer), stirred at room temperature for 5 minutes, and left still for 15 minutes at room temperature. Then fluorescence was measured at excitation 340 nm/fluorescence 612 nm, and an average value of duplicate was calculated for the obtained measurements.

[0363] The result is shown in Fig. 14, it was revealed that in the yeast genomic DNA solution, the DNA fragment is selected by forming a complex with immobilized 5'-end biotin-labeled oligonucleotide B4 and the methylcytosine antibody, and thus quantified and detected with excellent sensitivity.

[0364] In the present experiment, it was revealed that genomic DNA can be quantified and detected by forming and selecting a complex of a methylcytosine antibody, a methylated DNA fragment, and an immobilized 5'-end biotin-labeled oligonucleotide, and quantifying and detecting the methylcytosine antibody in the complex according to its identification function.

Example 11

[0365] Yeast strain X2180-1A of baker's yeast was cultured in a YPD medium (1% Yeast extract, 2% Peptone, 2% Glucose, pH 5.6 to 6.0) to a turbidity of OD_{600} 0.6 to 1.0, and centrifuged at 10,000 g for 10 minutes, to prepare 1×10^7 of yeast cells. From the prepared yeast cells, a yeast genome was acquired using a generally used preparation method of a yeast genome as described in Methods in Yeast Genetics (Cold Spring Harbor Laboratory).

[0366] The prepared yeast cells were suspended in Buffer A (1 M sorbitol, 0.1 M EDTA, pH 7.4), added with 2-mercaptoethanol (final concentration 14 mM) and 100 U zymolase (10 mg/ml), and incubated under stirring at 30°C for an hour until the solution became clear. After collecting a protoplast by centrifugation at 550 g for 10 minutes, it was suspended in Buffer B (50 mM Tris-HCl, pH 7.4, 20 mM EDTA), added with sodium dodecyl sulfate in 1% (w/v), and

then incubated at 65°C for 30 minutes. Sequentially, 5 M CH_3COOK was added and mingled in a volume ratio of 2/5, and the mixture was cooled on ice for 30 minutes, and then centrifuged at 15,000 g for 30 minutes to collect the supernatant. The collected supernatant was added with 3 M CH_3COONa in a volume ratio of 1/10 and an equal amount of isopropanol and mingled well, and the precipitate obtained by centrifugation at 15,000 g at 4°C for 30 minutes was rinsed with 70% ethanol and collected. After drying, the precipitate was dissolved in 1 mL of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), and added with RNase A (available from Sigma) in a concentration of 40 μg/ml, incubated at 37°C for an hour, and then the mixture was added with proteinase K (available from Sigma) and sodium dodecyl sulfate in a concentrations of 500 μg/mL and 1% (w/v), respectively, and shaken at 55°C for about 16 hours. After end of the shaking, the mixture was extracted with phenol [saturated with 1 M Tris-HCl (pH 8.0)]·chloroform. An aqueous layer was collected, added with NaCl in a concentration of 0.5 N, and allowed to precipitate from ethanol, and the generated precipitate was collected. The collected precipitate was rinsed with 70% ethanol, to obtain genomic DNA.

[0367] For the obtained yeast genomic DNA, the following solutions were prepared.

Solution A: Yeast genomic DNA 100 ng/20 μL TE buffer solution
Solution B: Yeast genomic DNA 10 ng/20 μL TE buffer solution
Solution C: Yeast genomic DNA 1 ng/20 μL TE buffer solution
Solution D: TE buffer solution (negative control solution)

[0368] Twenty (20) μL of each obtained solution, 10 U of restriction enzyme XspI, and 5 μL of 10x buffer optimum for XspI (200 mM Tris-HCl pH 8.5, 100 mM $MgCl_2$, 10 mM Dithiothreitol, 1000 mM KCl) were mixed, and added with sterilized ultrapure water to prepare a reaction liquid having a liquid amount of 50 μL. The reaction liquid was incubated at 37°C for 1 hour.

[0369] Twenty (20) μL of each obtained solution, 0.5 μL of SssI methylase (available from NEB Inc.), 5 μL of 10xNEBuffer2 (available from NEB Inc.), and 0.5 μL of 3.2 mM S-adenosyl methionine (available from NEB Inc.) were mixed, and added with sterilized ultrapure water to prepare a reaction liquid having a liquid amount of 50 μL. The reaction liquid was incubated at 37°C for 30 minutes.

[0370] Prepared was a 0.02 μM TE buffer solution of 5'-end biotin-labeled oligonucleotide B5 having the nucleotide sequence of SEQ ID NO: 44 capable of binding by complementation with a plus strand of DNA fragment T' comprising the target DNA region of SEQ ID NO: 47.

<DNA fragment>

[0371]

T':5'-
TAGGAAATACATTCCGAGGGCGCCCGCACAAGGCCTATTATTAGAGGGACCTGTGTTTGACG
GGTATAACACTAAGTTGCGCAATTTGCTGTATTGCGAAATCCGCCCGGACGATATCACTCTT
GAGCGCATGTGCCGTTTCCGAGAACGCCAGATCTGTACTGCGATCGCACACGAGGAGACACA
GCGTCACGTGTTTTGCCATTTTGTACGACAAATGAACCGCCTGGCCACGCCTCTAATC -3'
(SEQ ID NO: 47)

<5'-end biotin-labeled oligonucleotide>

[0372]

B5:5'- AGTACAGATCTGGCGTTCTCG-3' (SEQ ID NO: 44)

[0373] For respective reaction liquids of yeast genomic DNA Solutions A to D, the following treatment was conducted.

[0374] In a PCR tube, 40 μL of the reaction liquid prepared in the above, 10 μL of the 5'-end biotin-labeled oligonucleotide solution, 10 μL of a buffer (330 mM Tris-Acetate pH 7.9, 660 mM KOAc, 100 mM $MgOAc_2$, 5 mM Dithiothreitol), 10 μL of a 100 mM $MgCl_2$ solution and 10 μL of a 1 mg/mL BSA solution were added, and further the mixture was added with sterilized ultrapure water to make the liquid amount 100 μL, and mixed. Then the PCR tube was heated at 95°C

for 10 minutes, cooled rapidly to 70°C, and retained for 10 minutes at this temperature. Then the PCR tube was cooled to 50°C and retained for 10 minutes, and further retained for 10 minutes at 37°C, and then the PCR tube was returned to room temperature, to promote formation of a conjugate of the 5'-end biotin-labeled oligonucleotide and the DNA fragment.

[0375] The entire obtained mixture was transferred to a well coated with streptavidin, and left still at room temperature for about 30 minutes, to immobilize the conjugate of the 5'-end biotin-labeled oligonucleotide and the DNA fragment to the well. Thereafter, the solution was removed by pipetting, and each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4)].

[0376] Synthesized was masking oligonucleotide M5 having the nucleotide sequence of SEQ ID NO: 45 capable of binding by complementation with 5'-end biotin-labeled oligonucleotide B5 having the nucleotide sequence of SEQ ID NO: 44, and a 0.1 μM TE buffer solution was prepared.

[0377] Each well was added with 100 μL of a methylcytosine antibody [available from Aviva Systems Biology, 0.5 μg/mL 0.1% BSA-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4) solution], and added with 1 μL of the masking oligonucleotide solution, and left still for 1 hour at room temperature. Then the solution was removed by pipetting, and each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4)].

[0378] Then, each well was added with 100 μL of an Eu-N1-labeled mouse IgG antibody [available from Perkin Elmer, 0.05 μg/mL 0.1% BSA-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4) solution], and then left still at room temperature for 1 hour. After leaving still, each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4)].

[0379] Each well was added with 150 μL of Enhancement Solution (available from Perkin Elmer), stirred at room temperature for 5 minutes, and left still for 15 minutes at room temperature. Then fluorescence was measured at excitation 340 nm/fluorescence 612 nm, and an average value of duplicate was calculated for the obtained measurements.

[0380] The result is shown in Fig. 15, it was revealed that in the yeast genomic DNA solution, the DNA fragment is selected by forming a complex with immobilized 5'-end biotin-labeled oligonucleotide B5 and the methylcytosine antibody, and thus quantified and detected with excellent sensitivity.

[0381] In the present experiment, it was revealed that genomic DNA can be quantified and detected by forming and selecting a complex of a methylcytosine antibody, a methylated DNA fragment, and an immobilized 5'-end biotin-labeled oligonucleotide, and quantifying and detecting the methylcytosine antibody in the complex according to its identification function.

Example 12

[0382] Yeast strain X2180-1A of baker's yeast was cultured in a YPD medium (1% Yeast extract, 2% Peptone, 2% Glucose, pH 5.6 to 6.0) to a turbidity of OD_{600} 0.6 to 1.0, and centrifuged at 10,000 g for 10 minutes, to prepare 1×10^7 of yeast cells. From the prepared yeast cells, a yeast genome was acquired using a generally used preparation method of a yeast genome as described in Methods in Yeast Genetics (Cold Spring Harbor Laboratory).

[0383] The prepared yeast cells were suspended in Buffer A (1 M sorbitol, 0.1 M EDTA, pH 7.4), added with 2-mercaptoethanol (final concentration 14 mM) and 100 U zymolase (10 mg/ml), and incubated under stirring at 30°C for an hour until the solution became clear. After collecting a protoplast by centrifugation at 550 g for 10 minutes, it was suspended in Buffer B (50 mM Tris-HCl, pH 7.4, 20 mM EDTA), added with sodium dodecyl sulfate in 1% (w/v), and then incubated at 65°C for 30 minutes. Sequentially, 5 M CH_3COOK was added and mingled in a volume ratio of 2/5, and the mixture was cooled on ice for 30 minutes, and then centrifuged at 15,000 g for 30 minutes to collect the supernatant. The collected supernatant was added with 3 M CH_3COONa in a volume ratio of 1/10 and an equal amount of isopropanol and mingled well, and the precipitate obtained by centrifugation at 15,000 g at 4°C for 30 minutes was rinsed with 70% ethanol and collected. After drying, the precipitate was dissolved in 1 mL of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), and added with RNase A (available from Sigma) in a concentration of 40 μg/ml, incubated at 37°C for an hour, and then the mixture was added with proteinase K (available from Sigma) and sodium dodecyl sulfate in a concentrations of 500 μg/mL and 1% (w/v), respectively, and shaken at 55°C for about 16 hours. After end of the shaking, the mixture was extracted with phenol [saturated with 1 M Tris-HCl (pH 8.0)]·chloroform. An aqueous layer was collected, added with NaCl in a concentration of 0.5 N, and allowed to precipitate from ethanol, and the generated precipitate was collected. The collected precipitate was rinsed with 70% ethanol, to obtain genomic DNA.

[0384] From the obtained genomic DNA, a DNA fragment to be used as a test sample (S, SEQ ID NO: 38, the region corresponding to the nucleotide number 271743-272083 of yeast chromosome VII shown in Genbank Accession No. NC_001139) was amplified by conducting PCR using oligonucleotide primers (PF3 and PR3) designed for PCR of SEQ ID NO: 36 and SEQ ID NO: 37 and the following reaction condition.

[0385] As a reaction liquid of PCR, 10 ng of genomic DNA as a template, each 3 μL of 5 μM of the above primer solutions, 5 μL of each 2 mM dNTP, and 5 μL of 10×buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% Gelatin) were mixed with 0.25 μL of 5U/μL thermostable DNA polymerase (AmpliTaq Gold), and added with sterilized ultrapure water to prepare a reaction liquid having a liquid amount of 50 μL. After retaining the reaction liquid at 95°C for 10 minutes, PCR was conducted by 40 cycles each consisting of 20 seconds at 95°C, 30 seconds at 58°C and 30 seconds at 72°C.

[0386] After conducting PCR, amplification was checked by 2% agarose gel electrophoresis, and the DNA fragment S was purified by Wizard SV Gel/PCR Kit (PROMEGA Corporation).

[0387] For the DNA fragment S, the following solutions to which genomic DNA derived from human blood purchased from Clontech has been added were prepared respectively in duplicate.

Solution A: DNA fragment S 10ng/20 μL TE buffer solution (containing 5ng/μL genomic DNA derived from human blood)
Solution B: DNA fragment S 1ng/20 μL TE buffer solution (containing 5ng/μL genomic DNA derived from human blood)
Solution C: DNA fragment S 0.1ng/20 μL TE buffer solution (containing 5ng/μL genomic DNA derived from human blood)
Solution D: TE buffer solution (negative control solution)(containing 5ng/μL genomic DNA derived from human blood)

[0388] Twenty (20) μL of each obtained solution, 0.5 μL of SssI methylase (available from NEB Inc.), 5 μL of 10xNEBuffer2 (available from NEB Inc.), and 0.5 μL of 3.2 mM S-adenosyl methionine (available from NEB Inc.) were mixed, and added with sterilized ultrapure water to prepare a reaction liquid having a liquid amount of 50 μL. The reaction liquid was incubated at 37°C for 30 minutes.

[0389] Prepared was 0.02 μM TE buffer solution of 5'-end biotin-labeled oligonucleotide B4 having the nucleotide sequence of SEQ ID NO: 39 capable of binding by complementation with a plus strand of the DNA fragment S comprising the target DNA region of SEQ ID NO: 38.

[0390] Each obtained reaction liquid was subjected to the following treatments.

[0391] In a PCR tube, 40 μL of the reaction liquid prepared in the above, 10 μL of the 5'-end biotin-labeled oligonucleotide solution, 10 μL of a buffer (330 mM Tris-Acetate pH 7.9, 660 mM KOAc, 100 mM $MgOAc_2$, 5 mM Dithiothreitol), 10 μL of a 100 mM $MgCl_2$ solution and 10 μL of a 1 mg/mL BSA solution were added, and further the mixture was added with sterilized ultrapure water to make the liquid amount 100 μL, and mixed. Then the PCR tube was heated at 95°C for 10 minutes, cooled rapidly to 70°C, and retained for 10 minutes at this temperature. Then the PCR tube was cooled to 50°C and retained for 10 minutes, and further retained for 10 minutes at 37°C, and then the PCR tube was returned to room temperature, to promote formation of a conjugate of the 5'-end biotin-labeled oligonucleotide and the DNA fragment.

[0392] The entire obtained mixture was transferred to a well coated with streptavidin, and left still at room temperature for about 30 minutes, to immobilize the conjugate of the 5'-end biotin-labeled oligonucleotide and the DNA fragment to the well. Thereafter, the solution was removed by pipetting, and each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4)].

[0393] Synthesized was a masking oligonucleotide M4 having the nucleotide sequence of SEQ ID NO: 40 capable of binding by complementation with 5'-end biotin-labeled oligonucleotide B4 having the nucleotide sequence of SEQ ID NO: 39, and 0.1 μM TE buffer solution was prepared.

[0394] Each well was added with 100 μL of a methylcytosine antibody [available from Aviva Systems Biology, 0.5 μg/mL 0.1% BSA-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4) solution], and added with 1 μL of the masking oligonucleotide solution, and left still for 1 hour at room temperature. Then the solution was removed by pipetting, and each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4)].

[0395] Then, each well was added with 100 μL of an Eu-N1-labeled mouse IgG antibody [available from Perkin Elmer, 0.05μg/mL 0.1% BSA-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4) solution], and then left still at room temperature for 1 hour. After leaving still, the solution was removed by pipetting, and each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4)].

[0396] Each well was added with 150 μL of Enhancement Solution (available from Perkin Elmer), stirred at room temperature for 5 minutes, and left still for 15 minutes at room temperature. Then fluorescence was measured at excitation 340 nm/fluorescence 612 nm, and an average value of duplicate was calculated for the obtained measurements.

[0397] The result is shown in Fig. 16. It was revealed that a DNA fragment is selected accurately by the immobilized 5'-end biotin-labeled oligonucleotide, and quantified and detected with excellent sensitivity.

[0398] In the present experiment, it was revealed that an yeast-derived DNA fragment in human genomic DNA can be quantified and detected by forming and selecting a complex of a methylcytosine antibody, a methylated DNA fragment, and an immobilized 5'-end biotin-labeled oligonucleotide, and quantifying and detecting the methylcytosine antibody in the complex according to its identification function.

Example 13

[0399] Yeast strain X2180-1A of baker's yeast was cultured in a YPD medium (1% Yeast extract, 2% Peptone, 2% Glucose, pH 5.6 to 6.0) to a turbidity of OD_{600} 0.6 to 1.0, and centrifuged at 10,000 g for 10 minutes, to prepare 1×10^7 of yeast cells. From the prepared yeast cells, a yeast genome was acquired using a generally used preparation method of a yeast genome as described in Methods in Yeast Genetics (Cold Spring Harbor Laboratory).

[0400] The prepared yeast cells were suspended in Buffer A (1 M sorbitol, 0.1 M EDTA, pH 7.4), added with 2-mercaptoethanol (final concentration 14 mM) and 100 U zymolase (10 mg/ml), and incubated under stirring at 30°C for an hour until the solution became clear. After collecting a protoplast by centrifugation at 550 g for 10 minutes, it was suspended in Buffer B (50 mM Tris-HCl, pH 7.4, 20 mM EDTA), added with sodium dodecyl sulfate in 1% (w/v), and then incubated at 65°C for 30 minutes. Sequentially, 5 M CH_3COOK was added and mingled in a volume ratio of 2/5, and the mixture was cooled on ice for 30 minutes, and then centrifuged at 15,000 g for 30 minutes to collect the supernatant. The collected supernatant was added with 3 M CH_3COONa in a volume ratio of 1/10 and an equal amount of isopropanol and mingled well, and the precipitate obtained by centrifugation at 15,000 g at 4°C for 30 minutes was rinsed with 70% ethanol and collected. After drying, the precipitate was dissolved in 1 mL of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), and added with RNase A (available from Sigma) in a concentration of 40 μg/ml, incubated at 37°C for an hour, and then the mixture was added with proteinase K (available from Sigma) and sodium dodecyl sulfate in a concentrations of 500 μg/mL and 1% (w/v), respectively, and shaken at 55°C for about 16 hours. After end of the shaking, the mixture was extracted with phenol [saturated with 1 M Tris-HCl (pH 8.0)]·chloroform. An aqueous layer was collected, added with NaCl in a concentration of 0.5 N, and allowed to precipitate from ethanol, and the generated precipitate was collected. The collected precipitate was rinsed with 70% ethanol, to obtain genomic DNA.

[0401] From the obtained genomic DNA, a DNA fragment to be used as a test sample (T, SEQ ID NO: 43, the region corresponding to the nucleotide number 384523-384766 of yeast chromosome VII shown in Genbank Accession No. NC_001139) was amplified by conducting PCR using oligonucleotide primers (PF4 and PR4) designed for PCR of SEQ ID NO: 41 and SEQ ID NO: 42 and the following reaction condition.

[0402] As a reaction liquid of PCR, 10 ng of genomic DNA as a template, each 3 μL of 5 μM of the above primer solutions, 5 μL of each 2 mM dNTP, and 5 μL of 10×buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% Gelatin) were mixed with 0.25 μL of 5U/μL thermostable DNA polymerase (AmpliTaq Gold), and added with sterilized ultrapure water to prepare a reaction liquid having a liquid amount of 50 μL. After retaining the reaction liquid at 95°C for 10 minutes, PCR was conducted by 40 cycles each consisting of 20 seconds at 95°C, 30 seconds at 58°C and 30 seconds at 72°C.

[0403] After conducting PCR, amplification was checked by 2% agarose gel electrophoresis, and the DNA fragment T was purified by Wizard SV Gel/PCR Kit (PROMEGA Corporation).

[0404] For the DNA fragment T, the following solutions to which genomic DNA derived from human blood purchased from Clontech has been added were prepared respectively in duplicate.

Solution A: DNA fragment T 10ng/20 μL TE buffer solution (containing 5ng/μL genomic DNA derived from human blood)
Solution B: DNA fragment T 1ng/20 μL TE buffer solution (containing 5ng/μL genomic DNA derived from human blood)
Solution C: DNA fragment T 0.1ng/20 μL TE buffer solution (containing 5ng/μL genomic DNA derived from human blood)
Solution D: TE buffer solution (negative control solution)(containing 5ng/μL genomic DNA derived from human blood)

[0405] Twenty (20) μL of each obtained solution, 0.5 μL of SssI methylase (available from NEB Inc.), 5 μL of 10xNEBuffer2 (available from NEB Inc.), and 0.5 μL of 3.2 mM S-adenosyl methionine (available from NEB Inc.) were mixed, and added with sterilized ultrapure water to prepare a reaction liquid having a liquid amount of 50 μL. The reaction liquid was incubated at 37°C for 30 minutes.

[0406] Prepared was 0.02 μM TE buffer solution of 5'-end biotin-labeled oligonucleotide B5 having the nucleotide sequence of SEQ ID NO: 44 capable of binding by complementation with a plus strand of the DNA fragment T comprising the target DNA region of SEQ ID NO: 43.

<5'-end biotin-labeled oligonucleotide>

[0407]

B5:5'- AGTACAGATCTGGCGTTCTCG -3' (SEQ ID NO: 44)

[0408] Each obtained reaction liquid was subjected to the following treatments.

[0409] In a PCR tube, 40 μL of the reaction liquid prepared in the above, 10 μL of the 5'-end biotin-labeled oligonucleotide solution, 10 μL of a buffer (330 mM Tris-Acetate pH 7.9, 660 mM KOAc, 100 mM $MgOAc_2$, 5 mM Dithiothreitol), 10 μL of a 100 mM $MgCl_2$ solution and 10 μL of a 1 mg/mL BSA solution were added, and further the mixture was added with sterilized ultrapure water to make the liquid amount 100 μL, and mixed. Then the PCR tube was heated at 95°C for 10 minutes, cooled rapidly to 70°C, and retained for 10 minutes at this temperature. Then the PCR tube was cooled to 50°C and retained for 10 minutes, and further retained for 10 minutes at 37°C, and then the PCR tube was returned to room temperature, to promote formation of a conjugate of the 5'-end biotin-labeled oligonucleotide and the DNA fragment.

[0410] The entire obtained mixture was transferred to a well coated with streptavidin, and left still at room temperature for about 30 minutes, to immobilize the conjugate of the 5'-end biotin-labeled oligonucleotide and the DNA fragment to the well. Thereafter, the solution was removed by pipetting, and each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4)].

[0411] Synthesized was a masking oligonucleotide M5 having the nucleotide sequence of SEQ ID NO: 45 capable of binding by complementation with 5'-end biotin-labeled oligonucleotide B5 having the nucleotide sequence of SEQ ID NO: 44, and 0.1 μM TE buffer solution was prepared.

[0412] Each well was added with 100 μL of a methylcytosine antibody [available from Aviva Systems Biology, 0.5 μg/mL 0.1% BSA-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4) solution], and added with 1 μL of the masking oligonucleotide solution, and left still for 1 hour at room temperature. Then the solution was removed by pipetting, and each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4)].

[0413] Then, each well was added with 100 μL of an Eu-N1-labeled mouse IgG antibody [available from Perkin Elmer, 0.05μg/mL 0.1% BSA-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4) solution], and then left still at room temperature for 1 hour. After leaving still, the solution was removed by pipetting, and each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4)].

[0414] Each well was added with 150 μL of Enhancement Solution (available from Perkin Elmer), stirred at room temperature for 5 minutes, and left still for 15 minutes at room temperature. Then fluorescence was measured at excitation 340 nm/fluorescence 612 nm, and an average value of duplicate was calculated for the obtained measurements.

[0415] The result is shown in Fig. 17. It was revealed that a DNA fragment is selected accurately by the immobilized 5'-end biotin-labeled oligonucleotide, and quantified and detected with excellent sensitivity.

[0416] In the present experiment, it was revealed that an yeast-derived DNA fragment in human genomic DNA can be quantified and detected by forming and selecting a complex of a methylcytosine antibody, a methylated DNA fragment, and an immobilized 5'-end biotin-labeled oligonucleotide, and quantifying and detecting the methylcytosine antibody in the complex according to its identification function.

Example 14

[0417] Yeast strain X2180-1A of baker's yeast was cultured in a YPD medium (1% Yeast extract, 2% Peptone, 2% Glucose, pH 5.6 to 6.0) to a turbidity of OD_{600} 0.6 to 1.0, and centrifuged at 10,000 g for 10 minutes, to prepare 1×10^7 of yeast cells. From the prepared yeast cells, a yeast genome was acquired using a generally used preparation method of a yeast genome as described in Methods in Yeast Genetics (Cold Spring Harbor Laboratory).

[0418] The prepared yeast cells were suspended in Buffer A (1 M sorbitol, 0.1 M EDTA, pH 7.4), added with 2-mercaptoethanol (final concentration 14 mM) and 100 U zymolase (10 mg/ml), and incubated under stirring at 30°C for an hour until the solution became clear. After collecting a protoplast by centrifugation at 550 g for 10 minutes, it was suspended in Buffer B (50 mM Tris-HCl, pH 7.4, 20 mM EDTA), added with sodium dodecyl sulfate in 1% (w/v), and then incubated at 65°C for 30 minutes. Sequentially, 5 M CH_3COOK was added and mingled in a volume ratio of 2/5, and the mixture was cooled on ice for 30 minutes, and then centrifuged at 15,000 g for 30 minutes to collect the supernatant. The collected supernatant was added with 3 M CH_3COONa in a volume ratio of 1/10 and an equal amount of isopropanol and mingled well, and the precipitate obtained by centrifugation at 15,000 g at 4°C for 30 minutes was rinsed with 70% ethanol and collected. After drying, the precipitate was dissolved in 1 mL of TE buffer (10 mM Tris-HCl,

pH 8.0, 1 mM EDTA), and added with RNase A (available from Sigma) in a concentration of 40 μg/ml, incubated at 37°C for an hour, and then the mixture was added with proteinase K (available from Sigma) and sodium dodecyl sulfate in a concentrations of 500 μg/mL and 1% (w/v), respectively, and shaken at 55°C for about 16 hours. After end of the shaking, the mixture was extracted with phenol [saturated with 1 M Tris-HCl (pH 8.0)]·chloroform. An aqueous layer was collected, added with NaCl in a concentration of 0.5 N, and allowed to precipitate from ethanol, and the generated precipitate was collected. The collected precipitate was rinsed with 70% ethanol, to obtain genomic DNA.

[0419] From the obtained genomic DNA, a DNA fragment to be used as a test sample (S, SEQ ID NO: 38, the region corresponding to the nucleotide number 271743-272083 of yeast chromosome VII shown in Genbank Accession No. NC_001139) was amplified by conducting PCR using oligonucleotide primers (PF3 and PR3) designed for PCR of SEQ ID NO: 36 and SEQ ID NO: 37 and the following reaction condition.

[0420] As a reaction liquid of PCR, 10 ng of genomic DNA as a template, each 3 μL of 5 μM of the above primer solutions, 5 μL of each 2 mM dNTP, and 5 μL of 10×buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% Gelatin) were mixed with 0.25 μL of 5U/μL thermostable DNA polymerase (AmpliTaq Gold), and added with sterilized ultrapure water to prepare a reaction liquid having a liquid amount of 50 μL. After retaining the reaction liquid at 95°C for 10 minutes, PCR was conducted by 40 cycles each consisting of 20 seconds at 95°C, 30 seconds at 58°C and 30 seconds at 72°C.

[0421] After conducting PCR, amplification was checked by 2% agarose gel electrophoresis, and the DNA fragment S was purified by Wizard SV Gel/PCR Kit (PROMEGA Corporation).

[0422] From the obtained genomic DNA, a DNA fragment to be used as a test sample (T, SEQ ID NO: 43, the region corresponding to the nucleotide number 384523-384766 of yeast chromosome VII shown in Genbank Accession No. NC_001139) was amplified by conducting PCR using oligonucleotide primers (PF4 and PR4) designed for PCR of SEQ ID NO: 41 and SEQ ID NO: 42 and the following reaction condition.

[0423] As a reaction liquid of PCR, 10 ng of genomic DNA as a template, each 3 μL of 5 μM of the above primer solutions, 5 μL of each 2 mM dNTP, and 5 μL of 10×buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% Gelatin) were mixed with 0.25 μL of 5U/μL thermostable DNA polymerase (AmpliTaq Gold), and added with sterilized ultrapure water to prepare a reaction liquid having a liquid amount of 50 μL. After retaining the reaction liquid at 95°C for 10 minutes, PCR was conducted by 40 cycles each consisting of 20 seconds at 95°C, 30 seconds at 58°C and 30 seconds at 72°C.

[0424] After conducting PCR, amplification was checked by 2% agarose gel electrophoresis, and the DNA fragment T was purified by Wizard SV Gel/PCR Kit (PROMEGA Corporation).

[0425] For each of the DNA fragment S and the DNA fragment T, the following solutions to which genomic DNA derived from human blood purchased from Clontech has been added were prepared.

Solution A: DNA fragment S or DNA fragment T 10 ng/10 μL TE buffer solution (containing 5ng/μL genomic DNA derived from human blood)
Solution B: DNA fragment S or DNA fragment T 1 ng/10 μL TE buffer solution (containing 5ng/μL genomic DNA derived from human blood)
Solution C: DNA fragment S or DNA fragment T 0.1 ng/10 μL TE buffer solution (containing 5ng/μL genomic DNA derived from human blood)
Solution D: TE buffer solution (negative control solution) (containing 5ng/μL genomic DNA derived from human blood)

[0426] Equivalent amounts of Solution A of the DNA fragment S and Solution A of the DNA fragment T were mixed, to prepare DNA fragment-mixed Solution MA, equivalent amounts of Solution B of the DNA fragment S and Solution B of the DNA fragment T were mixed, to prepare DNA fragment-mixed Solution MB, equivalent amounts of Solution C of the DNA fragment S and Solution C of the DNA fragment T were mixed, to prepare DNA fragment-mixed Solution MC, and equivalent amounts of Solution D of the DNA fragment S and Solution D of the DNA fragment T were mixed, to prepare DNA fragment-mixed Solution MD. Three sets in duplicate were prepared respectively, for each of DNA fragment-mixed Solutions MA , MB, MC and MD.

[0427] Twenty (20) μL of each obtained solution, 0.5 μL of SssI methylase (available from NEB Inc.), 5 μL of 10xNEBuffer2 (available from NEB Inc.), and 0.5 μL of 3.2 mM S-adenosyl methionine (available from NEB Inc.) were mixed, and added with sterilized ultrapure water to prepare a reaction liquid having a liquid amount of 50 μL. The reaction liquid was incubated at 37°C for 30 minutes.

[0428] Prepared were 0.02 μM TE buffer solutions of 5'-end biotin-labeled oligonucleotide B4 having the nucleotide sequence of SEQ ID NO: 39 capable of binding by complementation with a plus strand of the DNA fragment S comprising the target DNA region of SEQ ID NO: 38 and 5'-end biotin-labeled oligonucleotide B5 having the nucleotide sequence of SEQ ID NO: 44 capable of binding by complementation with a plus strand of the DNA fragment T comprising the target DNA region of SEQ ID NO: 43. Also prepared was a TE buffer solution (0.02 μM for each) mixing equivalent amounts of 5'-end biotin-labeled oligonucleotide B4 having the nucleotide sequence of SEQ ID NO: 39 and 5'-end biotin-

labeled oligonucleotide B5 having the nucleotide sequence of SEQ ID NO: 44.

[0429] For each reaction liquid of the DNA fragment Solutions MA to MD, the following treatment was conducted.

[0430] In a PCR tube, 40 μL of the reaction liquid prepared in the above, 10 μL of the 5'-end biotin-labeled oligonucleotide solution, 10 μL of a buffer (330 mM Tris-Acetate pH 7.9, 660 mM KOAc, 100 mM $MgOAc_2$, 5 mM Dithiothreitol), 10 μL of a 100 mM $MgCl_2$ solution and 10 μL of a 1 mg/mL BSA solution were added, and further the mixture was added with sterilized ultrapure water to make the liquid amount 100 μL, and mixed. Then the PCR tube was heated at 95°C for 10 minutes, cooled rapidly to 70°C, and retained for 10 minutes at this temperature. Then the PCR tube was cooled to 50°C and retained for 10 minutes, and further retained for 10 minutes at 37°C, and then the PCR tube was returned to room temperature, to promote formation of a conjugate of the 5'-end biotin-labeled oligonucleotide and the DNA fragment.

[0431] The entire obtained mixture was transferred to a well coated with streptavidin, and left still at room temperature for about 30 minutes, to immobilize the conjugate of the 5'-end biotin-labeled oligonucleotide and the DNA fragment to the well. Thereafter, the solution was removed by pipetting, and each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4)].

[0432] Synthesized were masking oligonucleotide M4 having the nucleotide sequence of SEQ ID NO: 40 capable of binding by complementation with 5'-end biotin-labeled oligonucleotide B4 having the nucleotide sequence of SEQ ID NO: 39 and masking oligonucleotide M5 having the nucleotide sequence of SEQ ID NO: 45 capable of binding by complementation with 5'-end biotin-labeled oligonucleotide B5 having the nucleotide sequence of SEQ ID NO: 44, and respective 0.1 μM TE buffer solutions were prepared. Prepared was a TE buffer solution (each 0.1 μM) mixing equivalent amounts of masking oligonucleotide M4 having the nucleotide sequence of SEQ ID NO: 40 and masking oligonucleotide M5 having the nucleotide sequence of SEQ ID NO: 45.

[0433] Each well was added with 100 μL of a methylcytosine antibody [available from Aviva Systems Biology, 0.5μg/mL 0.1% BSA-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$ 154 mM NaCl pH7.4) solution], and added with 1 μL of the masking oligonucleotide solution, and left still for 1 hour at room temperature. Then the solution was removed by pipetting, and each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2PO\ 7H_2O$, 154 mM NaCl pH7.4)].

[0434] Then, each well was added with 100 μL of an Eu-N1-labeled mouse IgG antibody [available from Perkin Elmer, 0.05μg/mL 0.1% BSA-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4) solution], and then left still at room temperature for 1 hour. After leaving still, each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4)].

[0435] Each well was added with 150 μL of Enhancement Solution (available from Perkin Elmer), stirred at room temperature for 5 minutes, and left still for 15 minutes at room temperature. Then fluorescence was measured at excitation 340 nm/fluorescence 612 nm, and an average value of duplicate was calculated for the obtained measurements.

[0436] The results are shown in Figs. 18 to 20. It was revealed that the DNA fragment is selected by forming a complex with immobilized 5'-end biotin-labeled oligonucleotide B4 (Fig. 18), immobilized 5'-end biotin-labeled oligonucleotide B5 (Fig. 19), the 5'-end biotin-labeled oligonucleotides which are mixture of the foregoing two kinds (Fig. 20), and the methylcytosine antibody, and is quantified and detected with excellent sensitivity. It was revealed that when two kinds of the 5'-end biotin-labeled oligonucleotides are mixed (Fig. 20), in particular, quantification and detection with better sensitivity can be achieved compared with the case of detection by a single oligonucleotide (Fig. 18 and Fig. 19).

[0437] In the present experiment, it was revealed that an yeast-derived DNA fragment in human genomic DNA can be quantified and detected by forming and selecting a complex of a methylcytosine antibody, a methylated DNA fragment, and an immobilized 5'-end biotin-labeled oligonucleotide, and quantifying and detecting the methylcytosine antibody in the complex according to its identification function. It was also revealed that by using a plurality of immobilized 5'-end biotin-labeled oligonucleotides, more sensitive quantification and detection are realized than the case where one kind of immobilized 5'-end biotin-labeled oligonucleotide is used (that is, not only using one target DNA region but also using a plurality of target DNA regions at the same time).

Example 15

[0438] Yeast strain X2180-1A of baker's yeast was cultured in a YPD medium (1% Yeast extract, 2% Peptone, 2% Glucose, pH 5.6 to 6.0) to a turbidity of OD_{600} 0.6 to 1.0, and centrifuged at 10,000 g for 10 minutes, to prepare 1×10^7 of yeast cells. From the prepared yeast cells, a yeast genome was acquired using a generally used preparation method of a yeast genome as described in Methods in Yeast Genetics (Cold Spring Harbor Laboratory).

[0439] The prepared yeast cells were suspended in Buffer A (1 M sorbitol, 0.1 M EDTA, pH 7.4), added with 2-mercaptoethanol (final concentration 14 mM) and 100 U zymolase (10 mg/ml), and incubated under stirring at 30°C for an hour until the solution became clear. After collecting a protoplast by centrifugation at 550 g for 10 minutes, it was

suspended in Buffer B (50 mM Tris-HCl, pH 7.4, 20 mM EDTA), added with sodium dodecyl sulfate in 1% (w/v), and then incubated at 65°C for 30 minutes. Sequentially, 5 M CH_3COOK was added and mingled in a volume ratio of 2/5, and the mixture was cooled on ice for 30 minutes, and then centrifuged at 15,000 g for 30 minutes to collect the supernatant. The collected supernatant was added with 3 M CH_3COONa in a volume ratio of 1/10 and an equal amount of isopropanol and mingled well, and the precipitate obtained by centrifugation at 15,000 g at 4°C for 30 minutes was rinsed with 70% ethanol and collected. After drying, the precipitate was dissolved in 1 mL of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), and added with RNase A (available from Sigma) in a concentration of 40 μg/ml, incubated at 37°C for an hour, and then the mixture was added with proteinase K (available from Sigma) and sodium dodecyl sulfate in a concentrations of 500 μg/mL and 1% (w/v), respectively, and shaken at 55°C for about 16 hours. After end of the shaking, the mixture was extracted with phenol [saturated with 1 M Tris-HCl (pH 8.0)]·chloroform. An aqueous layer was collected, added with NaCl in a concentration of 0.5 N, and allowed to precipitate from ethanol, and the generated precipitate was collected. The collected precipitate was rinsed with 70% ethanol, to obtain genomic DNA.

[0440] From the obtained genomic DNA, a DNA fragment to be used as a test sample (S, SEQ ID NO: 38, the region corresponding to the nucleotide number 271743-272083 of yeast chromosome VII shown in Genbank Accession No. NC_001139) was amplified by conducting PCR using oligonucleotide primers (PF3 and PR3) designed for PCR of SEQ ID NO: 36 and SEQ ID NO: 37 and the following reaction condition.

[0441] As a reaction liquid of PCR, 10 ng of genomic DNA as a template, each 3 μL of 5 μM of the above primer solutions, 5 μL of each 2 mM dNTP, and 5 μL of 10×buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% Gelatin) were mixed with 0.25 μL of 5U/μL thermostable DNA polymerase (AmpliTaq Gold), and added with sterilized ultrapure water to prepare a reaction liquid having a liquid amount of 50 μL. After retaining the reaction liquid at 95°C for 10 minutes, PCR was conducted by 40 cycles each consisting of 20 seconds at 95°C, 30 seconds at 58°C and 30 seconds at 72°C.

[0442] After conducting PCR, amplification was checked by 2% agarose gel electrophoresis, and the DNA fragment S was purified by Wizard SV Gel/PCR Kit (PROMEGA Corporation).

[0443] For the DNA fragment S, the following solutions were prepared respectively in duplicate.

Solution A: DNA fragment S 10ng/20 μL TE buffer solution
Solution B: DNA fragment S 1ng/20 μL TE buffer solution
Solution C: DNA fragment S 0.1ng/20 μL TE buffer solution
Solution D: TE buffer solution (negative control solution)

[0444] Twenty (20) μL of each obtained solution, 0.5 μL of SssI methylase (available from NEB Inc.), 5 μL of 10xNEBuffer2 (available from NEB Inc.), and 0.5 μL of 3.2 mM S-adenosyl methionine (available from NEB Inc.) were mixed, and added with sterilized ultrapure water to prepare a reaction liquid having a liquid amount of 50 μL. The reaction liquid was incubated at 37°C for 30 minutes.

[0445] Prepared was 0.02 μM TE buffer solution of 5'-end biotin-labeled oligonucleotide B4 having the nucleotide sequence of SEQ ID NO: 39 capable of binding by complementation with a plus strand of the DNA fragment S comprising the target DNA region of SEQ ID NO: 38.

[0446] Synthesized were counter oligonucleotides C6, C7, C8, C9, C10, C11, C12, C13 and C14 having the nucleotide sequences of SEQ ID NO: 48, SEQ ID NO: 49, SEQ ID NO: 50, SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 53, SEQ ID NO: 54, SEQ ID NO: 55 and SEQ ID NO: 56, respectively capable of binding by complementation with a minus strand of DNA fragment S comprising the target DNA region of SEQ ID NO: 38, and respective 0.01 μM TE buffer solutions were prepared.

<Counter oligonucleotides>

[0447]

C6:5'- GCGTCGTGCACTGGCTCACTTGTACGCGCA -3' (SEQ ID NO: 48)
C7:5'- CTTGTACGATTGGTGACCCGCCTTTTCGAC -3' (SEQ ID NO: 49)
C8:5'- ACTGGACCGCTATGGACGTGGCGGCGGTGT -3' (SEQ ID NO: 50)
C9:5'- GGCGGCGGCTCAATGACCTGTGGCGCCCGT -3' (SEQ ID NO: 51)
C10:5'- TTGTGGCGTGCGATAGTCGAGCCGCCTGTC -3' (SEQ ID NO: 52)
C11:5'- ACGTGCGCGGCCGCCCTGCTCCGTT -3' (SEQ ID NO: 53)
C12:5'- TGACGCGATGCATAGCATGCGACCACCCAG -3' (SEQ ID NO: 54)
C13:5'- ACTGCTGACGCTATTGGTCACGTGGTTATG -3' (SEQ ID NO: 55)
C14:5'- CTGCTGTTGACTGCGGTGGCGTCCCGTTTC -3' (SEQ ID NO: 56)

[0448] Each obtained reaction liquid was subjected to the following treatments.

[0449] In a PCR tube, 40 μL of the reaction liquid prepared in the above, 10 μL of the 5'-end biotin-labeled oligonucleotide solution, 10 μL of the counter oligonucleotide solution, 10 μL of a buffer (330 mM Tris-Acetate pH 7.9, 660 mM KOAc, 100 mM $MgOAc_2$, 5 mM Dithiothreitol), 10 μL of a 100 mM $MgCl_2$ solution and 10 μL of a 1 mg/mL BSA solution were added, and further the mixture was added with sterilized ultrapure water to make the liquid amount 100 μL, and mixed. Then the PCR tube was heated at 95°C for 10 minutes, cooled rapidly to 70°C, and retained for 10 minutes at this temperature. Then the PCR tube was cooled to 50°C and retained for 10 minutes, and further retained for 10 minutes at 37°C, and then the PCR tube was returned to room temperature, to promote formation of a conjugate of the 5'-end biotin-labeled oligonucleotide and the DNA fragment.

[0450] The entire obtained mixture was transferred to a well coated with streptavidin, and left still at room temperature for about 30 minutes, to immobilize the conjugate of the 5'-end biotin-labeled oligonucleotide and the DNA fragment to the well. Thereafter, the solution was removed by pipetting, and each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$ 154 mM NaCl pH7.4)].

[0451] Synthesized was a masking oligonucleotide M4 having the nucleotide sequence of SEQ ID NO: 40 capable of binding by complementation with 5'-end biotin-labeled oligonucleotide B4 having the nucleotide sequence of SEQ ID NO: 39, and 0.1 μM TE buffer solution was prepared.

[0452] Each well was added with 100 μL of a methylcytosine antibody [available from Aviva Systems Biology, 0.5 μg/mL 0.1% BSA-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4) solution], and added with 1 μL of the masking oligonucleotide solution, and left still for 1 hour at room temperature. Then the solution was removed by pipetting, and each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2SO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4)].

[0453] Then, each well was added with 100 μL of an Eu-N1-labeled mouse IgG antibody [available from Perkin Elmer, 0.05μg/mL 0.1% BSA-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$ 154 mM NaCl pH7.4) solution], and then left still at room temperature for 1 hour. After leaving still, the solution was removed by pipetting, and each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4)].

[0454] Each well was added with 150 μL of Enhancement Solution (available from Perkin Elmer), stirred at room temperature for 5 minutes, and left still for 15 minutes at room temperature. Then fluorescence was measured at excitation 340 nm/fluorescence 612 nm, and an average value of duplicate was calculated for the obtained measurements.

[0455] The result is shown in Fig. 21. It was revealed that a DNA fragment is selected accurately by the immobilized 5'-end biotin-labeled oligonucleotide, and quantified and detected with excellent sensitivity.

[0456] In the present experiment, it was revealed that a DNA fragment can be quantified and detected by forming and selecting a complex of a methylcytosine antibody, a methylated DNA fragment, and an immobilized 5'-end biotin-labeled oligonucleotide, and quantifying and detecting the methylcytosine antibody in the complex according to its identification function.

Example 16

[0457] Yeast strain X2180-1A of baker's yeast was cultured in a YPD medium (1% Yeast extract, 2% Peptone, 2% Glucose, pH 5.6 to 6.0) to a turbidity of OD_{600} 0.6 to 1.0, and centrifuged at 10,000 g for 10 minutes, to prepare 1×10^7 of yeast cells. From the prepared yeast cells, a yeast genome was acquired using a generally used preparation method of a yeast genome as described in Methods in Yeast Genetics (Cold Spring Harbor Laboratory).

[0458] The prepared yeast cells were suspended in Buffer A (1 M sorbitol, 0.1 M EDTA, pH 7.4), added with 2-mercaptoethanol (final concentration 14 mM) and 100 U zymolase (10 mg/ml), and incubated under stirring at 30°C for an hour until the solution became clear. After collecting a protoplast by centrifugation at 550 g for 10 minutes, it was suspended in Buffer B (50 mM Tris-HCl, pH 7.4, 20 mM EDTA), added with sodium dodecyl sulfate in 1% (w/v), and then incubated at 65°C for 30 minutes. Sequentially, 5 M CH_3COOK was added and mingled in a volume ratio of 2/5, and the mixture was cooled on ice for 30 minutes, and then centrifuged at 15,000 g for 30 minutes to collect the supernatant. The collected supernatant was added with 3 M CH_3COONa in a volume ratio of 1/10 and an equal amount of isopropanol and mingled well, and the precipitate obtained by centrifugation at 15,000 g at 4°C for 30 minutes was rinsed with 70% ethanol and collected. After drying, the precipitate was dissolved in 1 mL of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), and added with RNase A (available from Sigma) in a concentration of 40 μg/ml, incubated at 37°C for an hour, and then the mixture was added with proteinase K (available from Sigma) and sodium dodecyl sulfate in a concentrations of 500 μg/mL and 1% (w/v), respectively, and shaken at 55°C for about 16 hours. After end of the shaking, the mixture was extracted with phenol [saturated with 1 M Tris-HCl (pH 8.0)]·chloroform. An aqueous layer was collected, added with NaCl in a concentration of 0.5 N, and allowed to precipitate from ethanol, and the generated precipitate was collected. The collected precipitate was rinsed with 70% ethanol, to obtain genomic DNA.

[0459] From the obtained genomic DNA, a DNA fragment to be used as a test sample (T, SEQ ID NO: 43, the region corresponding to the nucleotide number 384523-384766 of yeast chromosome VII shown in Genbank Accession No. NC_001139) was amplified by conducting PCR using oligonucleotide primers (PF4 and PR4) designed for PCR of SEQ ID NO: 41 and SEQ ID NO: 42 and the following reaction condition.

[0460] As a reaction liquid of PCR, 10 ng of genomic DNA as a template, each 3 μL of 5 μM of the above primer solutions, 5 μL of each 2 mM dNTP, and 5 μL of 10xbuffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% Gelatin) were mixed with 0.25 μL of 5U/μL thermostable DNA polymerase (AmpliTaq Gold), and added with sterilized ultrapure water to prepare a reaction liquid having a liquid amount of 50 μL. After retaining the reaction liquid at 95°C for 10 minutes, PCR was conducted by 40 cycles each consisting of 20 seconds at 95°C, 30 seconds at 58°C and 30 seconds at 72°C.

[0461] After conducting PCR, amplification was checked by 2% agarose gel electrophoresis, and the DNA fragment T was purified by Wizard SV Gel/PCR Kit (PROMEGA Corporation).

[0462] For the DNA fragment T, the following solutions were prepared respectively in duplicate.

Solution A: DNA fragment T 10ng/20 μL TE buffer solution
Solution B: DNA fragment T 1ng/20 μL TE buffer solution
Solution C: DNA fragment T 0.1ng/20 μL TE buffer solution
Solution D: TE buffer solution (negative control solution)

[0463] Twenty (20) μL of each obtained solution, 0.5 μL of SssI methylase (available from NEB Inc.), 5 μL of 10xNEBuffer2 (available from NEB Inc.), and 0.5 μL of 3.2 mM S-adenosyl methionine (available from NEB Inc.) were mixed, and added with sterilized ultrapure water to prepare a reaction liquid having a liquid amount of 50 μL. The reaction liquid was incubated at 37°C for 30 minutes.

[0464] Prepared was 0.02 μM TE buffer solution of 5'-end biotin-labeled oligonucleotide B5 having the nucleotide sequence of SEQ ID NO: 44 capable of binding by complementation with a plus strand of the DNA fragment T comprising the target DNA region of SEQ ID NO: 43.

[0465] Synthesized were counter oligonucleotides C15, C16, C17 and C18 having the nucleotide sequences of SEQ ID NO: 57, SEQ ID NO: 58, SEQ ID NO: 59 and SEQ ID NO: 60, respectively capable of binding by complementation with a minus strand of DNA fragment T comprising the target DNA region of SEQ ID NO: 43, and respective 0.01 μM TE buffer solutions were prepared.

<Counter oligonucleotides>

[0466]

C15:5'- GGACCTGTGTTTGACGGGTAT -3' (SEQ ID NO: 57)
C16:5'- AACACTAAGTTGCGCAATTTGCTGT -3' (SEQ ID NO: 58)
C17:5'- ATTGCGAAATCCGCCCGGACGATAT -3' (SEQ ID NO: 59)
C18:5'- CACTCTTGAGCGCATGTGCCGTTTC -3' (SEQ ID NO: 60)

[0467] Each obtained reaction liquid was subjected to the following treatments.

[0468] In a PCR tube, 40 μL of the reaction liquid prepared in the above, 10 μL of the 5'-end biotin-labeled oligonucleotide solution, 10 μL of the counter oligonucleotide solution, 10 μL of a buffer (330 mM Tris-Acetate pH 7.9, 660 mM KOAc, 100 mM $MgOAc_2$, 5 mM Dithiothreitol), 10 μL of a 100 mM $MgCl_2$ solution and 10 μL of a 1 mg/mL BSA solution were added, and further the mixture was added with sterilized ultrapure water to make the liquid amount 100 μL, and mixed. Then the PCR tube was heated at 95°C for 10 minutes, cooled rapidly to 70°C, and retained for 10 minutes at this temperature. Then the PCR tube was cooled to 50°C and retained for 10 minutes, and further retained for 10 minutes at 37°C, and then the PCR tube was returned to room temperature, to promote formation of a conjugate of the 5'-end biotin-labeled oligonucleotide and the DNA fragment.

[0469] The entire obtained mixture was transferred to a well coated with streptavidin, and left still at room temperature for about 30 minutes, to immobilize the conjugate of the 5'-end biotin-labeled oligonucleotide and the DNA fragment to the well. Thereafter, the solution was removed by pipetting, and each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2 PO_4, 3 mM Na_2HPO $7H_2O$ 154 mM NaCl pH7.4)].

[0470] Synthesized was a masking oligonucleotide M5 having the nucleotide sequence of SEQ ID NO: 45 capable of binding by complementation with 5'-end biotin-labeled oligonucleotide B5 having the nucleotide sequence of SEQ ID NO: 44, and 0.1 μM TE buffer solution was prepared.

[0471] Each well was added with 100 μL of a methylcytosine antibody [available from Aviva Systems Biology, 0.5

μg/mL 0.1% BSA-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4) solution], and added with 1 μL of the masking oligonucleotide solution, and left still for 1 hour at room temperature. Then the solution was removed by pipetting, and each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4)].

[0472] Then, each well was added with 100 μL of an Eu-N1-labeled mouse IgG antibody [available from Perkin Elmer, 0.05μg/mL 0.1% BSA-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4) solution], and then left still at room temperature for 1 hour. After leaving still, the solution was removed by pipetting, and each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4)].

[0473] Each well was added with 150 μL of Enhancement Solution (available from Perkin Elmer), stirred at room temperature for 5 minutes, and left still for 15 minutes at room temperature. Then fluorescence was measured at excitation 340 nm/fluorescence 612 nm, and an average value of duplicate was calculated for the obtained measurements.

[0474] The result is shown in Fig. 22. It was revealed that a DNA fragment is selected accurately by the immobilized 5'-end biotin-labeled oligonucleotide, and quantified and detected with excellent sensitivity.

[0475] In the present experiment, it was revealed that a DNA fragment can be quantified and detected by forming and selecting a complex of a methylcytosine antibody, a methylated DNA fragment, and an immobilized 5'-end biotin-labeled oligonucleotide, and quantifying and detecting the methylcytosine antibody in the complex according to its identification function.

Example 17

[0476] Yeast strain X2180-1A of baker's yeast was cultured in a YPD medium (1% Yeast extract, 2% Peptone, 2% Glucose, pH 5.6 to 6.0) to a turbidity of OD_{600} 0.6 to 1.0, and centrifuged at 10,000 g for 10 minutes, to prepare 1×10^7 of yeast cells. From the prepared yeast cells, a yeast genome was acquired using a generally used preparation method of a yeast genome as described in Methods in Yeast Genetics (Cold Spring Harbor Laboratory).

[0477] The prepared yeast cells were suspended in Buffer A (1 M sorbitol, 0.1 M EDTA, pH 7.4), added with 2-mercaptoethanol (final concentration 14 mM) and 100 U zymolase (10 mg/ml), and incubated under stirring at 30°C for an hour until the solution became clear. After collecting a protoplast by centrifugation at 550 g for 10 minutes, it was suspended in Buffer B (50 mM Tris-HCl, pH 7.4, 20 mM EDTA), added with sodium dodecyl sulfate in 1% (w/v), and then incubated at 65°C for 30 minutes. Sequentially, 5 M CH_3COOK was added and mingled in a volume ratio of 2/5, and the mixture was cooled on ice for 30 minutes, and then centrifuged at 15,000 g for 30 minutes to collect the supernatant. The collected supernatant was added with 3 M CH_3COONa in a volume ratio of 1/10 and an equal amount of isopropanol and mingled well, and the precipitate obtained by centrifugation at 15,000 g at 4°C for 30 minutes was rinsed with 70% ethanol and collected. After drying, the precipitate was dissolved in 1 mL of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), and added with RNase A (available from Sigma) in a concentration of 40 μg/ml, incubated at 37°C for an hour, and then the mixture was added with proteinase K (available from Sigma) and sodium dodecyl sulfate in a concentrations of 500 μg/mL and 1% (w/v), respectively, and shaken at 55°C for about 16 hours. After end of the shaking, the mixture was extracted with phenol [saturated with 1 M Tris-HCl (pH 8.0)]·chloroform. An aqueous layer was collected, added with NaCl in a concentration of 0.5 N, and allowed to precipitate from ethanol, and the generated precipitate was collected. The collected precipitate was rinsed with 70% ethanol, to obtain genomic DNA.

[0478] From the obtained genomic DNA, a DNA fragment to be used as a test sample (S, SEQ ID NO: 38, the region corresponding to the nucleotide number 271743-272083 of yeast chromosome VII shown in Genbank Accession No. NC_001139) was amplified by conducting PCR using oligonucleotide primers (PF3 and PR3) designed for PCR of SEQ ID NO: 36 and SEQ ID NO: 37 and the following reaction condition.

[0479] As a reaction liquid of PCR, 10 ng of genomic DNA as a template, each 3 μL of 5 μM of the above primer solutions, 5 μL of each 2 mM dNTP, and 5 μL of 10xbuffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% Gelatin) were mixed with 0.25 μL of 5U/μL thermostable DNA polymerase (AmpliTaq Gold), and added with sterilized ultrapure water to prepare a reaction liquid having a liquid amount of 50 μL. After retaining the reaction liquid at 95°C for 10 minutes, PCR was conducted by 40 cycles each consisting of 20 seconds at 95°C, 30 seconds at 58°C and 30 seconds at 72°C.

[0480] After conducting PCR, amplification was checked by 2% agarose gel electrophoresis, and the DNA fragment S was purified by Wizard SV Gel/PCR Kit (PROMEGA Corporation).

[0481] For the DNA fragment S, the following solutions to which genomic DNA derived from human blood purchased from Clontech has been added were prepared respectively in duplicate.

Solution A: DNA fragment S 10ng/20 μL TE buffer solution (containing 5ng/μL genomic DNA derived from human blood)

Solution B: DNA fragment S 1ng/20 μL TE buffer solution (containing 5ng/μL genomic DNA derived from human

blood)
Solution C: DNA fragment S 0.1ng/20 μL TE buffer solution (containing 5ng/μL genomic DNA derived from human blood)
Solution D: TE buffer solution (negative control solution)(containing 5ng/μL genomic DNA derived from human blood)

[0482] Twenty (20) μL of each obtained solution, 0.5 μL of SssI methylase (available from NEB Inc.), 5 μL of 10xNEBuffer2 (available from NEB Inc.), and 0.5 μL of 3.2 mM S-adenosyl methionine (available from NEB Inc.) were mixed, and added with sterilized ultrapure water to prepare a reaction liquid having a liquid amount of 50 μL. The reaction liquid was incubated at 37°C for 30 minutes.

[0483] Prepared was 0.02 μM TE buffer solution of 5'-end biotin-labeled oligonucleotide B4 having the nucleotide sequence of SEQ ID NO: 39 capable of binding by complementation with a plus strand of the DNA fragment S comprising the target DNA region of SEQ ID NO: 38.

[0484] Synthesized were counter oligonucleotides C6, C7, C8, C9, C10, C11, C12, C13, C14 and C19 having the nucleotide sequences of SEQ ID NO: 48, SEQ ID NO: 49, SEQ ID NO: 50, SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 53, SEQ ID NO: 54, SEQ ID NO: 55, SEQ ID NO: 56 and SEQ ID NO: 61, respectively capable of binding by complementation with a minus strand of DNA fragment S comprising the target DNA region of SEQ ID NO: 38, and respective 0.01 μM TE buffer solutions were prepared.

<Counter oligonucleotides>

[0485]

C6:5'- GCGTCGTGCACTGGCTCACTTGTACGCGCA -3' (SEQ ID NO: 48)
C7:5'- CTTGTACGATTGGTGACCCGCCTTTTCGAC -3' (SEQ ID NO: 49)
C8:5'- ACTGGATCGCTATGGACGTGGCGGCGGTGT -3' (SEQ ID NO: 50)
C9:5'- GGCGGCGGCTCAATGACCTGTGGCGCCCGT -3' (SEQ ID NO: 51)
C10:5'- TTGTGGCGTGCGATAGTCGAGCCGCCTGTC -3' (SEQ ID NO: 52)
C11:5'- ACGTGCGCGGCCGCCCTGCTCCGTT -3' (SEQ ID NO: 53)
C12:5'- TGACGCGATGCATAGCATGCGACCACCCAG -3' (SEQ ID NO: 54)
C13:5'- ACTGCTGACGCTATTGGTCACGTGGTTATG -3' (SEQ ID NO: 55)
C14:5'- CTGCTGTTGACTGCGGTGGCGTCCCGTTTC -3' (SEQ ID NO: 56)
C19:5'- AGGTGAGCTACGTGTGTTTGG -3' (SEQ ID NO: 61)

[0486] Each obtained reaction liquid was subjected to the following treatments.

[0487] In a PCR tube, 40 μL of the reaction liquid prepared in the above, 10 μL of the 5'-end biotin-labeled oligonucleotide solution, 10 μL of the counter oligonucleotide solution, 10 μL of a buffer (330 mM Tris-Acetate pH 7.9, 660 mM KOAc, 100 mM $MgOAc_2$, 5 mM Dithiothreitol), 10 μL of a 100 mM $MgCl_2$ solution and 10 μL of a 1 mg/mL BSA solution were added, and further the mixture was added with sterilized ultrapure water to make the liquid amount 100 μL, and mixed. Then the PCR tube was heated at 95°C for 10 minutes, cooled rapidly to 70°C, and retained for 10 minutes at this temperature. Then the PCR tube was cooled to 50°C and retained for 10 minutes, and further retained for 10 minutes at 37°C, and then the PCR tube was returned to room temperature, to promote formation of a conjugate of the 5'-end biotin-labeled oligonucleotide and the DNA fragment.

[0488] The entire obtained mixture was transferred to a well coated with streptavidin, and left still at room temperature for about 30 minutes, to immobilize the conjugate of the 5'-end biotin-labeled oligonucleotide and the DNA fragment to the well. Thereafter, the solution was removed by pipetting, and each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4)].

[0489] Synthesized was a masking oligonucleotide M4 having the nucleotide sequence of SEQ ID NO: 40 capable of binding by complementation with 5'-end biotin-labeled oligonucleotide B4 having the nucleotide sequence of SEQ ID NO: 39, and 0.1 μM TE buffer solution was prepared.

[0490] Each well was added with 100 μL of a methylcytosine antibody [available from Aviva Systems Biology, 0.5 μg/mL 0.1% BSA-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4) solution], and added with 1 μL of the masking oligonucleotide solution, and left still for 1 hour at room temperature. Then the solution was removed by pipetting, and each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4)].

[0491] Then, each well was added with 100 μL of an Eu-N1-labeled mouse IgG antibody [available from Perkin Elmer, 0.05μg/mL 0.1% BSA-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4) solution], and then left still at room temperature for 1 hour. After leaving still, the solution was removed by pipetting, and each well

was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4)].

[0492] Each well was added with 150 μL of Enhancement Solution (available from Perkin Elmer), stirred at room temperature for 5 minutes, and left still for 15 minutes at room temperature. Then fluorescence was measured at excitation 340 nm/fluorescence 612 nm, and an average value of duplicate was calculated for the obtained measurements.

[0493] The result is shown in Fig. 23. It was revealed that a DNA fragment is selected accurately by the immobilized 5'-end biotin-labeled oligonucleotide, and quantified and detected with excellent sensitivity.

[0494] In the present experiment, it was revealed that an yeast-derived DNA fragment in human genomic DNA can be quantified and detected by forming and selecting a complex of a methylcytosine antibody, a methylated DNA fragment, and an immobilized 5'-end biotin-labeled oligonucleotide, and quantifying and detecting the methylcytosine antibody in the complex according to its identification function.

Example 18

[0495] Yeast strain X2180-1A of baker's yeast was cultured in a YPD medium (1% Yeast extract, 2% Peptone, 2% Glucose, pH 5.6 to 6.0) to a turbidity of OD_{600} 0.6 to 1.0, and centrifuged at 10,000 g for 10 minutes, to prepare 1×10^7 of yeast cells. From the prepared yeast cells, a yeast genome was acquired using a generally used preparation method of a yeast genome as described in Methods in Yeast Genetics (Cold Spring Harbor Laboratory).

[0496] The prepared yeast cells were suspended in Buffer A (1 M sorbitol, 0.1 M EDTA, pH 7.4), added with 2-mercaptoethanol (final concentration 14 mM) and 100 U zymolase (10 mg/ml), and incubated under stirring at 30°C for an hour until the solution became clear. After collecting a protoplast by centrifugation at 550 g for 10 minutes, it was suspended in Buffer B (50 mM Tris-HCl, pH 7.4, 20 mM EDTA), added with sodium dodecyl sulfate in 1% (w/v), and then incubated at 65°C for 30 minutes. Sequentially, 5 M CH_3COOK was added and mingled in a volume ratio of 2/5, and the mixture was cooled on ice for 30 minutes, and then centrifuged at 15,000 g for 30 minutes to collect the supernatant. The collected supernatant was added with 3 M CH_3COONa in a volume ratio of 1/10 and an equal amount of isopropanol and mingled well, and the precipitate obtained by centrifugation at 15,000 g at 4°C for 30 minutes was rinsed with 70% ethanol and collected. After drying, the precipitate was dissolved in 1 mL of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), and added with RNase A (available from Sigma) in a concentration of 40 μg/ml, incubated at 37°C for an hour, and then the mixture was added with proteinase K (available from Sigma) and sodium dodecyl sulfate in a concentrations of 500 μg/mL and 1% (w/v), respectively, and shaken at 55°C for about 16 hours. After end of the shaking, the mixture was extracted with phenol [saturated with 1 M Tris-HCl (pH 8.0)]·chloroform. An aqueous layer was collected, added with NaCl in a concentration of 0.5 N, and allowed to precipitate from ethanol, and the generated precipitate was collected. The collected precipitate was rinsed with 70% ethanol, to obtain genomic DNA.

[0497] From the obtained genomic DNA, a DNA fragment to be used as a test sample (T, SEQ ID NO: 43, the region corresponding to the nucleotide number 384523-384766 of yeast chromosome VII shown in Genbank Accession No. NC_001139) was amplified by conducting PCR using oligonucleotide primers (PF4 and PR4) designed for PCR of SEQ ID NO: 41 and SEQ ID NO: 42 and the following reaction condition.

[0498] As a reaction liquid of PCR, 10 ng of genomic DNA as a template, each 3 μL of 5 μM of the above primer solutions, 5μ L of each 2 mM dNTP, and 5 μL of 10×buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% Gelatin) were mixed with 0.25 μL of 5U/μL thermostable DNA polymerase (AmpliTaq Gold), and added with sterilized ultrapure water to prepare a reaction liquid having a liquid amount of 50 μL. After retaining the reaction liquid at 95°C for 10 minutes, PCR was conducted by 40 cycles each consisting of 20 seconds at 95°C, 30 seconds at 58°C and 30 seconds at 72°C.

[0499] After conducting PCR, amplification was checked by 2% agarose gel electrophoresis, and the DNA fragment T was purified by Wizard SV Gel/PCR Kit (PROMEGA Corporation).

[0500] For the DNA fragment T, the following solutions to which genomic DNA derived from human blood purchased from Clontech has been added were prepared respectively in duplicate.

Solution A: DNA fragment T 10ng/20 μL TE buffer solution (containing 5ng/μL genomic DNA derived from human blood)

Solution B: DNA fragment T 1ng/20 μL TE buffer solution (containing 5ng/μL genomic DNA derived from human blood)

Solution C: DNA fragment T 0.1ng/20 μL TE buffer solution (containing 5ng/μL genomic DNA derived from human blood)

Solution D: TE buffer solution (negative control solution) (containing 5ng/μL genomic DNA derived from human blood)

[0501] Twenty (20) μL of each obtained solution, 0.5 μL of SssI methylase (available from NEB Inc.), 5 μL of 10xNEBuffer2 (available from NEB Inc.), and 0.5 μL of 3.2 mM S-adenosyl methionine (available from NEB Inc.) were

mixed, and added with sterilized ultrapure water to prepare a reaction liquid having a liquid amount of 50 μL. The reaction liquid was incubated at 37°C for 30 minutes.

[0502] Prepared was 0.02 μM TE buffer solution of 5'-end biotin-labeled oligonucleotide B5 having the nucleotide sequence of SEQ ID NO: 44 capable of binding by complementation with a plus strand of the DNA fragment T comprising the target DNA region of SEQ ID NO: 43.

[0503] Synthesized were counter oligonucleotides C15, C16, C17 and C18 having the nucleotide sequences of SEQ ID NO: 57, SEQ ID NO: 58, SEQ ID NO: 59 and SEQ ID NO: 60, respectively capable of binding by complementation with a minus strand of DNA fragment T comprising the target DNA region of SEQ ID NO: 43, and respective 0.01 μM TE buffer solutions were prepared.

<Counter oligonucleotides>

[0504]

C15:5'- GGACCTGTGTTTGACGGGTAT -3' (SEQ ID NO: 57)
C16:5'- AACACTAAGTTGCGCAATTTGCTGT -3' (SEQ ID NO: 58)
C17:5'- ATTGCGAAATCCGCCCGGACGATAT -3' (SEQ ID NO: 59)
C18:5'- CACTCTTGAGCGCATGTGCCGTTTC -3' (SEQ ID NO: 60)

[0505] Each obtained reaction liquid was subjected to the following treatments.

[0506] In a PCR tube, 40 μL of the reaction liquid prepared in the above, 10 μL of the 5'-end biotin-labeled oligonucleotide solution, 10 μL of the counter oligonucleotide solution, 10 μL of a buffer (330 mM Tris-Acetate pH 7.9, 660 mM KOAc, 100 mM $MgOAc_2$, 5 mM Dithiothreitol), 10 μL of a 100 mM $MgCl_2$ solution and 10 μL of a 1 mg/mL BSA solution were added, and further the mixture was added with sterilized ultrapure water to make the liquid amount 100 μL, and mixed. Then the PCR tube was heated at 95°C for 10 minutes, cooled rapidly to 70°C, and retained for 10 minutes at this temperature. Then the PCR tube was cooled to 50°C and retained for 10 minutes, and further retained for 10 minutes at 37°C, and then the PCR tube was returned to room temperature, to promote formation of a conjugate of the 5'-end biotin-labeled oligonucleotide and the DNA fragment.

[0507] The entire obtained mixture was transferred to a well coated with streptavidin, and left still at room temperature for about 30 minutes, to immobilize the conjugate of the 5'-end biotin-labeled oligonucleotide and the DNA fragment to the well. Thereafter, the solution was removed by pipetting, and each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4)].

[0508] Synthesized was a masking oligonucleotide M5 having the nucleotide sequence of SEQ ID NO: 45 capable of binding by complementation with 5'-end biotin-labeled oligonucleotide B5 having the nucleotide sequence of SEQ ID NO: 44, and 0.1 μM TE buffer solution was prepared.

<5'-end biotin-labeled oligonucleotide>

[0509]

B5:5'- AGTACAGATCTGGCGTTCTCG -3' (SEQ ID NO: 44)

<Masking oligonucleotide>

[0510]

M5:5'- CGAGAACGCCAGATCTGTACT -3' (SEQ ID NO: 45)

[0511] Each well was added with 100 μL of a methylcytosine antibody [available from Aviva Systems Biology, 0.5 μg/mL 0.1% BSA-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4) solution], and added with 1 μL of the masking oligonucleotide solution, and left still for 1 hour at room temperature. Then the solution was removed by pipetting, and each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4)].

[0512] Then, each well was added with 100 μL of an Eu-N1-labeled mouse IgG antibody [available from Perkin Elmer, 0.05μg/mL 0.1% BSA-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4) solution], and then left still at room temperature for 1 hour. After leaving still, the solution was removed by pipetting, and each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4,

3 mM Na_2HPO $7H_2O$, 154 mM NaCl pH7.4)].

[0513] Each well was added with 150 μL of Enhancement Solution (available from Perkin Elmer), stirred at room temperature for 5 minutes, and left still for 15 minutes at room temperature. Then fluorescence was measured at excitation 340 nm/fluorescence 612 nm, and an average value of duplicate was calculated for the obtained measurements.

[0514] The result is shown in Fig. 24. It was revealed that a DNA fragment is selected accurately by the immobilized 5'-end biotin-labeled oligonucleotide, and quantified and detected with excellent sensitivity.

[0515] In the present experiment, it was revealed that an yeast-derived DNA fragment in human genomic DNA can be quantified and detected by forming and selecting a complex of a methylcytosine antibody, a methylated DNA fragment, and an immobilized 5'-end biotin-labeled oligonucleotide, and quantifying and detecting the methylcytosine antibody in the complex according to its identification function.

Example 19

[0516] Yeast strain X2180-1A of baker's yeast was cultured in a YPD medium (1% Yeast extract, 2% Peptone, 2% Glucose, pH 5.6 to 6.0) to a turbidity of OD_{600} 0.6 to 1.0, and centrifuged at 10,000 g for 10 minutes, to prepare 1×10^7 of yeast cells. From the prepared yeast cells, a yeast genome was acquired using a generally used preparation method of a yeast genome as described in Methods in Yeast Genetics (Cold Spring Harbor Laboratory).

[0517] The prepared yeast cells were suspended in Buffer A (1 M sorbitol, 0.1 M EDTA, pH 7.4), added with 2-mercaptoethanol (final concentration 14 mM) and 100 U zymolase (10 mg/ml), and incubated under stirring at 30°C for an hour until the solution became clear. After collecting a protoplast by centrifugation at 550 g for 10 minutes, it was suspended in Buffer B (50 mM Tris-HCl, pH 7.4, 20 mM EDTA), added with sodium dodecyl sulfate in 1% (w/v), and then incubated at 65°C for 30 minutes. Sequentially, 5 M CH_3COOK was added and mingled in a volume ratio of 2/5, and the mixture was cooled on ice for 30 minutes, and then centrifuged at 15,000 g for 30 minutes to collect the supernatant. The collected supernatant was added with 3 M CH_3COONa in a volume ratio of 1/10 and an equal amount of isopropanol and mingled well, and the precipitate obtained by centrifugation at 15,000 g at 4°C for 30 minutes was rinsed with 70% ethanol and collected. After drying, the precipitate was dissolved in 1 mL of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), and added with RNase A (available from Sigma) in a concentration of 40 μg/ml, incubated at 37°C for an hour, and then the mixture was added with proteinase K (available from Sigma) and sodium dodecyl sulfate in a concentrations of 500 μg/mL and 1% (w/v), respectively, and shaken at 55°C for about 16 hours. After end of the shaking, the mixture was extracted with phenol [saturated with 1 M Tris-HCl (pH 8.0)]·chloroform. An aqueous layer was collected, added with NaCl in a concentration of 0.5 N, and allowed to precipitate from ethanol, and the generated precipitate was collected. The collected precipitate was rinsed with 70% ethanol, to obtain genomic DNA.

[0518] From the obtained genomic DNA, a DNA fragment to be used as a test sample (S, SEQ ID NO: 38, the region corresponding to the nucleotide number 271743-272083 of yeast chromosome VII shown in Genbank Accession No. NC_001139) was amplified by conducting PCR using oligonucleotide primers (PF3 and PR3) designed for PCR of SEQ ID NO: 36 and SEQ ID NO: 37 and the following reaction condition.

[0519] As a reaction liquid of PCR, 10 ng of genomic DNA as a template, each 3 μL of 5 μM of the above primer solutions, 5μ L of each 2 mM dNTP, and 5 μL of 10xbuffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% Gelatin) were mixed with 0.25 μL of 5U/μL thermostable DNA polymerase (AmpliTaq Gold), and added with sterilized ultrapure water to prepare a reaction liquid having a liquid amount of 50 μL. After retaining the reaction liquid at 95°C for 10 minutes, PCR was conducted by 40 cycles each consisting of 20 seconds at 95°C, 30 seconds at 58°C and 30 seconds at 72°C.

[0520] After conducting PCR, amplification was checked by 2% agarose gel electrophoresis, and the DNA fragment S was purified by Wizard SV Gel/PCR Kit (PROMEGA Corporation).

[0521] From the obtained genomic DNA, a DNA fragment to be used as a test sample (T, SEQ ID NO: 43, the region corresponding to the nucleotide number 384523-384766 of yeast chromosome VII shown in Genbank Accession No. NC_001139) was amplified by conducting PCR using oligonucleotide primers (PF4 and PR4) designed for PCR of SEQ ID NO: 41 and SEQ ID NO: 42 and the following reaction condition.

[0522] As a reaction liquid of PCR, 10 ng of genomic DNA as a template, each 3 μL of 5 μM of the above primer solutions, 5 μL of each 2 mM dNTP, and 5 μL of 10xbuffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% Gelatin) were mixed with 0.25 μL of 5U/μL thermostable DNA polymerase (AmpliTaq Gold), and added with sterilized ultrapure water to prepare a reaction liquid having a liquid amount of 50 μL. After retaining the reaction liquid at 95°C for 10 minutes, PCR was conducted by 40 cycles each consisting of 20 seconds at 95°C, 30 seconds at 58°C and 30 seconds at 72°C.

[0523] After conducting PCR, amplification was checked by 2% agarose gel electrophoresis, and the DNA fragment T was purified by Wizard SV Gel/PCR Kit (PROMEGA Corporation).

[0524] For each of the DNA fragment S and the DNA fragment T, the following solutions were prepared.

Solution A: DNA fragment S or DNA fragment T 10 ng/10 μL TE buffer solution
Solution B: DNA fragment S or DNA fragment T 1 ng/10 μL TE buffer solution
Solution C: DNA fragment S or DNA fragment T 0.1 ng/10 μL TE buffer solution
Solution D: TE buffer solution (negative control solution)

[0525] Equivalent amounts of Solution A of the DNA fragment S and Solution A of the DNA fragment T were mixed, to prepare DNA fragment-mixed Solution MA, equivalent amounts of Solution B of the DNA fragment S and Solution B of the DNA fragment T were mixed, to prepare DNA fragment-mixed Solution MB, equivalent amounts of Solution C of the DNA fragment S and Solution C of the DNA fragment T were mixed, to prepare DNA fragment-mixed Solution MC, and equivalent amounts of Solution D of the DNA fragment S and Solution D of the DNA fragment T were mixed, to prepare DNA fragment-mixed Solution MD. Three sets in duplicate were prepared respectively, for each of DNA fragment-mixed Solutions MA , MB, MC and MD.

[0526] Twenty (20) μL of each obtained solution, 0.5 μL of SssI methylase (available from NEB Inc.), 5 μL of 10xNEBuffer2 (available from NEB Inc.), and 0.5 μL of 3.2 mM S-adenosyl methionine (available from NEB Inc.) were mixed, and added with sterilized ultrapure water to prepare a reaction liquid having a liquid amount of 50 μL. The reaction liquid was incubated at 37°C for 30 minutes.

[0527] Prepared were 0.02 μM TE buffer solutions of 5'-end biotin-labeled oligonucleotide B4 having the nucleotide sequence of SEQ ID NO: 39 capable of binding by complementation with a plus strand of the DNA fragment S comprising the target DNA region of SEQ ID NO: 38 and 5'-end biotin-labeled oligonucleotide B5 having the nucleotide sequence of SEQ ID NO: 44 capable of binding by complementation with a plus strand of the DNA fragment T comprising the target DNA region of SEQ ID NO: 43. Also prepared was a TE buffer solution (0.02 μM for each) mixing equivalent amounts of 5'-end biotin-labeled oligonucleotide B4 having the nucleotide sequence of SEQ ID NO: 39 and 5'-end biotin-labeled oligonucleotide B5 having the nucleotide sequence of SEQ ID NO: 44.

[0528] Synthesized were counter oligonucleotides C6, C7, C8, C9, C10, C11, C12, C13, C14 and C19 having the nucleotide sequences of SEQ ID NO: 48, SEQ ID NO: 49, SEQ ID NO: 50, SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 53, SEQ ID NO: 54, SEQ ID NO: 55, SEQ ID NO: 56 and SEQ ID NO: 61, respectively capable of binding by complementation with a minus strand of DNA fragment S comprising the target DNA region of SEQ ID NO: 38, and respective 0.01 μM TE buffer solutions (counter oligonucleotide solution 1) were prepared. Also synthesized were counter oligonucleotides C15, C16, C17 and C18 having the nucleotide sequences of SEQ ID NO: 57, SEQ ID NO: 58, SEQ ID NO: 59 and SEQ ID NO: 60, respectively capable of binding by complementation with a minus strand of DNA fragment T comprising the target DNA region of SEQ ID NO: 43, and respective 0.01 μM TE buffer solutions (counter oligonucleotide solution 2) were prepared. Also prepared were respective 0.01 μM TE buffer solutions (counter oligonucleotide solution 3) of counter oligonucleotides C6, C7, C8, C9, C10, C11, C12, C13, C14, C15, C16, C17, C18 and C19 having the nucleotide sequences of SEQ ID NO: 48, SEQ ID NO: 49, SEQ ID NO: 50, SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 53, SEQ ID NO: 54, SEQ ID NO: 55, SEQ ID NO: 56, SEQ ID NO: 57, SEQ ID NO: 58, SEQ ID NO: 59, SEQ ID NO: 60 and SEQ ID NO: 61, respectively.

<Counter oligonucleotides>

[0529]

C6:5'- GCGTCGTGCACTGGCTCACTTGTACGCGCA -3' (SEQ ID NO: 48)
C7:5'- CTTGTACGATTGGTGACCCGCCTTTTCGAC -3' (SEQ ID NO: 49)
C8:5'- ACTGGACCGCTATGGACGTGGCGGCGGTGT -3' (SEQ ID NO: 50)
C9:5'- GGCGGCGGCTCAATGACCTGTGGCGCCCGT -3' (SEQ ID NO: 51)
C10:5'- TTGTGGCGTGCGATAGTCGAGCCGCCTGTC -3' (SEQ ID NO: 52)
C11:5'- ACGTGCGCGGCCGCCCTGCTCCGTT -3' (SEQ ID NO: 53)
C12:5'- TGACGCGATGCATAGCATGCGACCACCCAG -3' (SEQ ID NO: 54)
C13:5'- ACTGCTGACGCTATTGGTCACGTGGTTATG -3' (SEQ ID NO: 55)
C14:5'- CTGCTGTTGACTGCGGTGGCGTCCCGTTTC -3' (SEQ ID NO: 56)
C15:5'- GGACCTGTGTTTGACGGGTAT -3' (SEQ ID NO: 57)
C16:5'- AACACTAAGTTGCGCAATTTGCTGT -3' (SEQ ID NO: 58)
C17:5'- ATTGCGAAATCCGCCCGGACGATAT -3' (SEQ ID NO: 59)
C18:5'- CACTCTTGAGCGCATGTGCCGTTTC -3' (SEQ ID NO: 60)
C19:5'- AGGTGAGCTACGTGTGTTTGG -3' (SEQ ID NO: 61)

[0530] For each reaction liquid of the DNA fragment Solutions MA to MD, the following treatment was conducted using, as a combination of 5'-end biotin-labeled oligonucleotide solution and counter oligonucleotide solution, solution of 5'-

end biotin-labeled oligonucleotide B4 and counter oligonucleotide solution 1, solution of 5'-end biotin-labeled oligonucleotide B5 and counter oligonucleotide solution 2, or mixed solution of 5'-end biotin-labeled oligonucleotide B4 and 5'-end biotin-labeled oligonucleotide B5 and counter oligonucleotide solution 3.

[0531] In a PCR tube, 40 μL of the reaction liquid prepared in the above, 10 μL of the 5'-end biotin-labeled oligonucleotide solution, 10 μL of counter oligonucleotide solution, 10 μL of a buffer (330 mM Tris-Acetate pH 7.9, 660 mM KOAc, 100 mM $MgOAc_2$, 5 mM Dithiothreitol), 10 μL of a 100 mM $MgCl_2$ solution and 10 μL of a 1 mg/mL BSA solution were added, and further the mixture was added with sterilized ultrapure water to make the liquid amount 100 μL, and mixed. Then the PCR tube was heated at 95°C for 10 minutes, cooled rapidly to 70°C, and retained for 10 minutes at this temperature. Then the PCR tube was cooled to 50°C and retained for 10 minutes, and further retained for 10 minutes at 37°C, and then the PCR tube was returned to room temperature, to promote formation of a conjugate of the 5'-end biotin-labeled oligonucleotide and the DNA fragment.

[0532] The entire obtained mixture was transferred to a well coated with streptavidin, and left still at room temperature for about 30 minutes, to immobilize the conjugate of the 5'-end biotin-labeled oligonucleotide and the DNA fragment to the well. Thereafter, the solution was removed by pipetting, and each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4)].

[0533] Synthesized were a masking oligonucleotide M4 having the nucleotide sequence of SEQ ID NO: 40 capable of binding by complementation with 5'-end biotin-labeled oligonucleotide B4 having the nucleotide sequence of SEQ ID NO: 39 and a masking oligonucleotide M5 having the nucleotide sequence of SEQ ID NO: 45 capable of binding by complementation with 5'-end biotin-labeled oligonucleotide B5 having the nucleotide sequence of SEQ ID NO: 44, and respective 0.1 μM TE buffer solutions were prepared. Also prepared was a TE buffer solution (0.1 μM for each) mixing equivalent amounts of masking oligonucleotide M4 having the nucleotide sequence of SEQ ID NO: 40 and masking oligonucleotide M5 having the nucleotide sequence of SEQ ID NO: 45.

[0534] The following treatment was conducted using masking oligonucleotide M4 solution for each reaction liquid treated with 5'-end biotin-labeled oligonucleotide B4 solution, masking oligonucleotide M5 solution for each reaction liquid treated with 5'-end biotin-labeled oligonucleotide B5 solution, and mixed solution of masking oligonucleotide M4 and masking oligonucleotide M5 for each reaction liquid treated with mixed solution of 5'-end biotin-labeled oligonucleotide B4 and 5'-end biotin-labeled oligonucleotide B5.

[0535] Each well was added with 100 μL of a methylcytosine antibody [available from Aviva Systems Biology, 0.5μg/mL 0.1% BSA-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4) solution], and added with 1 μL of the masking oligonucleotide solution, and left still for 1 hour at room temperature. Then the solution was removed by pipetting, and each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4)].

[0536] Then, each well was added with 100 μL of an Eu-N1-labeled mouse IgG antibody [available from Perkin Elmer, 0.05μg/mL 0.1% BSA-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4) solution], and then left still at room temperature for 1 hour. After leaving still, each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO\ 7H_2O$, 154 mM NaCl pH7.4)].

[0537] Each well was added with 150 μL of Enhancement Solution (available from Perkin Elmer), stirred at room temperature for 5 minutes, and left still for 15 minutes at room temperature. Then fluorescence was measured at excitation 340 nm/fluorescence 612 nm, and an average value of duplicate was calculated for the obtained measurements.

[0538] The results are shown in Figs. 25 to 27. It was revealed that the methylated DNA fragment is selected by forming a complex with immobilized 5'-end biotin-labeled oligonucleotide and the methylcytosine antibody, and is quantified and detected with excellent sensitivity. It was revealed that when two kinds of the 5'-end biotin-labeled oligonucleotides are mixed (Fig. 27), in particular, quantification and detection with better sensitivity can be achieved compared with the case of detection by a single oligonucleotide (Fig. 25 and Fig. 26).

[0539] In the present experiment, it was revealed that a DNA fragment can be quantified and detected by forming and selecting a complex of a methylcytosine antibody, a methylated DNA fragment, and an immobilized 5'-end biotin-labeled oligonucleotide, and quantifying and detecting the methylcytosine antibody in the complex according to its identification function. It was also revealed that by using a plurality of immobilized 5'-end biotin-labeled oligonucleotides, more sensitive quantification and detection are realized than the case where one kind of immobilized 5'-end biotin-labeled oligonucleotide is used (that is, not only using one target DNA region but also using a plurality of target DNA regions at the same time).

Example 20

[0540] As a serum sample, mixed liquids of a TE buffer solution of genomic DNA derived from human blood DNA (Human Genomic DNA, #636401, Clontech) and serum collected from rat (Wistar Hannover) were prepared respectively in quadruplicate as follows.

Serum sample A: Genomic DNA derived from human blood 100 ng/10 μL TE buffer solution + rat serum 10 μL
Serum sample B: Genomic DNA derived from human blood 10 ng/10 μL TE buffer solution + rat serum 10 μL
Serum sample C: 0 ng/10 μL TE buffer solution + rat serum 10 μL (negative control)

[0541] For Serum samples A to C prepared in the above, Treatment 1 or Treatment 2 was conducted respectively in duplicate.

Treatment 1:

[0542] Twenty (20) μL of a serum sample and 4 μL of a buffer (500 mM Tris-HCl (pH 7.5), 100 mM $MgCl_2$, 10 mM DTT, 1000 mM NaCl) were mixed, and the mixture was added with sterilized ultrapure water to make a liquid amount 40 μL, and mixed. Then, the PCR tube was retained at 95°C for 10 minutes, retained at 4°C for 10 minutes, and then returned to room temperature. After centrifugation at 9100xg for 10 minutes, the supernatant was collected.

Treatment 2:

[0543] Twenty (20) μL of a serum sample and 4 μL of a buffer (330 mM Tris-Acetate (pH 7.9), 100 mM $Mg(OAc)_2$, 5 mM DTT, 660 mM KOAc) were mixed, and the mixture was added with sterilized ultrapure water to make a liquid amount 40 μL, and mixed. Then, the PCR tube was retained at 95°C for 10 minutes, retained at 4°C for 10 minutes, and then returned to room temperature. After centrifugation at 9100xg for 10 minutes, the supernatant was collected.

[0544] Thirty (30) μL of each solution prepared by Treatment 1 or Treatment 2, 2U of restriction enzyme MspI, and 5 μL of 10x buffer optimum for MspI (100 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 10 mM Dithiothreitol, 500 mM NaCl) were mixed, and added with sterilized ultrapure water to prepare a reaction liquid having a liquid amount of 50 μL. The reaction liquid was incubated at 37°C for 1 hour.

[0545] Fourty (40) μL of the solution obtained by the above enzyme treatment, 0.5 μL of SssI methylase (available from NEB Inc.), 5 μL of 10xNEBuffer2 (available from NEB Inc.), and 0.5 μL of 3.2 mM S-adenosyl methionine (available from NEB Inc.) were mixed, and added with sterilized ultrapure water to prepare a reaction liquid having a liquid amount of 50 μL. The reaction liquid was incubated at 37°C for 30 minutes.

[0546] As a specific oligonucleotide used for obtaining a target DNA region (W, SEQ ID NO: 66, region corresponding to the nucleotide number 178-262 shown in Genbank Accession No. AF458110) designed in Alu region known as human transposon and having the nucleotide sequence of SEQ ID NO: 66, was synthesized 5'-end biotin-labeled oligonucleotide B6 comprising the nucleotide sequence of SEQ ID NO: 67 that binds with a plus strand of the target DNA region W by complementation, and a 0.2 pmoL/10 μL TE buffer solution was prepared.

<Target DNA region>

[0547]

W:5'-

CGGGCGCGGTGGCTCACGCCTGTAATCCCAGCACTTTGGGAGGCCGAGGTGGGCGGATCACG

AGGTCAGGAGATCGAGACCATCC -3'(SEQ ID NO: 66)

<5'-end biotin-labeled oligonucleotide>

[0548]

B6:5'- GGATGGTCTCGATCTCCTGAC -3' (SEQ ID NO: 67)

[0549] Fifty (50) μL of the reaction liquid obtained in the above, 10 μL of the specific oligonucleotide solution, 10 μL of a buffer (330 mM Tris-Acetate pH 7.9, 660 mM KOAc, 100 mM $MgOAc_2$, 5 mM Dithiothreitol), 10 μL of a 100 mM $MgCl_2$ solution, 10 μL of a 1 mg/mL BSA solution were added, and the mixture was further added with sterilized ultrapure water to make a liquid amount 100 μL, and mixed. Then for forming a double strand between the target DNA region and

the specific oligonucleotide, the PCR tube was retained at 95°C for 10 minutes, rapidly cooled to 70°C, and retained at this temperature for 10 minutes. Then the PCR tube was cooled to 50°C and retained for 10 minutes, and further retained at 37°C for 10 minutes, and returned to room temperature.

[0550] One hundred (100) μL of the obtained reaction liquid was transferred to a 8-well strip coated with streptavidin (StreptaWell, #11645692001, Roche), and left still for about 30 minutes at room temperature, to immobilize the complex of the target DNA and the specific oligonucleotide to the 8-well strip through a biotin-streptavidin bond. Thereafter, the solution was removed by decantation, and each well was washed three times with 200 μL of a washing buffer [0.05%Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO_4 \cdot 7H_2O$, 154 mM NaCl pH 7.4)].

[0551] As a masking oligonucleotide used for masking an immobilized free specific oligonucleotide, was synthesized oligonucleotide M comprising the nucleotide sequence of SEQ ID NO: 68 that binds with the specific oligonucleotide by complementation, and a 0.1 pmoL/μL TE buffer solution was prepared.

<Masking oligonucleotide>

[0552]

M:5'- GTCAGGAGATCGAGACCATCC -3' (SEQ ID NO: 68)

[0553] Each well was added with 100 μL of a methylcytosine antibody [available from Aviva Systems Biology, 0.5 μg/mL 0.1% BSA-containing phosphate buffer (1 mM KH_2PO_4, 3 mM Na_2HPO $7H_2O$, 154 mM NaCl pH7.4) solution], and further added with 1 μL of a masking oligonucleotide solution, and left still for 1 hour at room temperature. Then the solution was removed by pipetting, and each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM Na_2HPO $7H_2O$, 154 mM NaCl pH 7.4)].

[0554] Then, each well was added with 100 μL of an Eu-N1-labeled mouse IgG antibody [available from Perkin Elmer, 0.05 μg/μL 0.1% BSA-containing phosphate buffer (1 mM KH_2PO_4, 3 mM Na_2HPO $7H_2O$, 154 mM NaCl pH7.4) solution] and left still for 1 hour at room temperature. After leaving still, each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM Na_2HPO $7H_2O$, 154 mM NaCl pH 7.4)].

[0555] Each well was added with 150 μL of Enhancement Solution (available from Perkin Elmer), stirred for 5 minutes at room temperature, and left still for 15 minutes at room temperature. Then fluorescence was measured at excitation 340 nm/fluorescence 612 nm, and an average value of duplicate was calculated for the obtained measurements.

[0556] The results are shown in Fig. 28 and Fig. 29. In both of Treatment 1 and Treatment 2, fluorescence intensity increased in a concentration dependent manner in Solution A (100 ng) and Solution B (10 ng) of genomic DNA derived from human blood, compared with Solution C (0 ng: control solution).

[0557] In this experiment, it was revealed that it is possible to detect and quantify human genomic DNA in serum with excellent sensitivity by forming and selecting a complex of a methylcytosine antibody, a methylated DNA fragment, and a 5'-end biotin-labeled oligonucleotide, and detecting the methylcytosine antibody in the complex according to its function. In Treatment 1, human genomic DNA in serum was detected with better sensitivity than in Treatment 2.

Example 21

[0558] As a serum sample, mixed liquids of a TE buffer solution of genomic DNA derived from human blood (Human Genomic DNA, #636401, Clontech) and a human serum purchased from Kohjin Bio Co., Ltd (individual human serum) were prepared respectively in quadruplicate as follows.

Serum sample A: Genomic DNA derived from human blood 50 ng/10 μL TE buffer solution + human serum 40 μL
Serum sample B: Genomic DNA derived from human blood 5 ng/10 μL TE buffer solution + human serum 40 μL
Serum sample C: 0 ng/10 μL TE buffer solution + human serum 40 μL (negative control solution)

[0559] For each of Serum samples A to C prepared in the above, following Treatment 1 or Treatment 2 was conducted respectively in duplicate.

Treatment 1:

[0560] Fifty (50) μL of a serum sample and 20 μL of a buffer (500 mM Tris-HCl (pH 7.5), 100 mM $MgCl_2$, 10 mM DTT, 1000 mM NaCl) were mixed, and the mixture was added with sterilized ultrapure water to make a liquid amount 100 μL, and mixed. Then, the PCR tube was retained at 95°C for 10 minutes, retained at 4°C for 10 minutes, and then returned to room temperature. After centrifugation at 9100xg for 10 minutes, the supernatant was collected.

Treatment 2:

[0561] Fifty (50) μL of a serum sample and 10 μL of a buffer (500 mM Tris-HCl (pH 7.5), 100 mM $MgCl_2$, 10 mM DTT, 1000 mM NaCl) were mixed, and the mixture was added with sterilized ultrapure water to make a liquid amount 100 μL, and mixed. Then, the PCR tube was retained at 95°C for 10 minutes, retained at 4°C for 10 minutes, and then returned to room temperature. After centrifugation at 9100xg for 10 minutes, the supernatant was collected.

[0562] Twenty (20) μL of the solution prepared in the above treatment, 2U of restriction enzyme MspI, and 5 μL of a 10x buffer optimum for MspI (100 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 10 mM Dithiothreitol, 500 mM NaCl) were mixed, and added with sterilized ultrapure water to prepare a reaction liquid having a liquid amount of 50 μL. The reaction liquid was incubated at 37°C for 1 hour.

[0563] Thirty (30) μL of the solution obtained by the above enzyme treatment, 0.5 μL of SssI methylase (available from NEB Inc.), 5 μL of 10xNEBuffer2 (available from NEB Inc.), and 0.5 μL of 3.2 mM S-adenosyl methionine (available from NEB Inc.) were mixed, and added with sterilized ultrapure water to prepare a reaction liquid having a liquid amount of 50 μL. The reaction liquid was incubated at 37°C for 30 minutes.

[0564] As a specific oligonucleotide used for obtaining a target DNA region (W, SEQ ID NO: 66, region corresponding to the nucleotide number 178-262 shown in Genbank Accession No. AF458110) designed in Alu region known as human transposon and having the nucleotide sequence of SEQ ID NO: 66, was synthesized 5'-end biotin-labeled oligonucleotide B6 comprising the nucleotide sequence of SEQ ID NO: 67 that binds with a plus strand of the target DNA region W by complementation, and a 0.2 pmoL/10 μL TE buffer solution was prepared.

[0565] Fifty (50) μL of the reaction liquid obtained in the above, 10 μL of the specific oligonucleotide solution, 10 μL of a buffer (330 mM Tris-Acetate pH 7.9, 660 mM KOAc, 100 mM $MgOAc_2$, 5 mM Dithiothreitol), 10 μL of a 100 mM $MgCl_2$ solution, 10 μL of a 1 mg/mL BSA solution were added, and the mixture was further added with sterilized ultrapure water to make a liquid amount 100 μL, and mixed. Then for forming a double strand between the target DNA region and the specific oligonucleotide, the PCR tube was retained at 95°C for 10 minutes, rapidly cooled to 70°C, and retained at this temperature for 10 minutes. Then the PCR tube was cooled to 50°C and retained for 10 minutes, and further retained at 37°C for 10 minutes, and returned to room temperature.

[0566] One hundred (100) μL of the obtained reaction liquid was transferred to a 8-well strip coated with streptavidin (StreptaWell, #11645692001, Roche), and left still for about 30 minutes at room temperature, to immobilize the complex of the target DNA and the specific oligonucleotide to the 8-well strip through a biotin-streptavidin bond. Thereafter, the solution was removed by decantation, and each well was washed three times with 200 μL of a washing buffer [0.05%Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO_4 \cdot 7H_2O$, 154 mM NaCl pH 7.4)].

[0567] As a masking oligonucleotide used for masking an immobilized free specific oligonucleotide, was synthesized oligonucleotide M comprising the nucleotide sequence of SEQ ID NO: 68 that binds with the specific oligonucleotide by complementation, and a 0.1 pmoL/μL TE buffer solution was prepared.

[0568] Each well was added with 100 μL of a methylcytosine antibody [available from Aviva Systems Biology, 0.5 μg/mL 0.1% BSA-containing phosphate buffer (1 mM KH_2PO_4, 3 mM Na_2HPO $7H_2O$, 154 mM NaCl pH7.4) solution], and further added with 1 μL of a masking oligonucleotide solution, and left still for 1 hour at room temperature. Then the solution was removed by pipetting, and each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM Na_2HPO $7H_2O$, 154 mM NaCl pH 7.4)].

[0569] Then, each well was added with 100 μL of an Eu-N1-labeled mouse IgG antibody [available from Perkin Elmer, 0.05 μg/μL 0.1% BSA-containing phosphate buffer (1 mM KH_2PO_4, 3 mM Na_2HPO $7H_2O$, 154 mM NaCl pH7.4) solution] and left still for 1 hour at room temperature. After leaving still, each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM Na_2HPO $7H_2O$, 154 mM NaCl pH 7.4)].

[0570] Each well was added with 150 μL of Enhancement Solution (available from Perkin Elmer), stirred for 5 minutes at room temperature, and left still for 15 minutes at room temperature. Then fluorescence was measured at excitation 340 nm/fluorescence 612 nm, and an average value of duplicate was calculated for the obtained measurements.

[0571] The results are shown in Fig. 30 and Fig. 31. In Treatment 1, fluorescence intensity increased in a concentration dependent manner in Solution A (50 ng) and Solution B (5 ng) of genomic DNA derived from human blood, compared with Solution C (0 ng: control solution) (Fig. 30). On the other hand, in Treatment 2, fluorescence intensity increased in a concentration dependent manner in Solution A (50 ng) of genomic DNA derived from human blood, compared with Solution C (0 ng: control solution), however, increase in fluorescence intensity was not found in Solution B (5 ng) (Fig. 31).

[0572] In this experiment, it was revealed that it is possible to detect and quantify human genomic DNA in serum with excellent sensitivity by forming and selecting a complex of a methylcytosine antibody, a methylated DNA fragment, and a 5'-end biotin-labeled oligonucleotide, and detecting the methylcytosine antibody in the complex according to its function. In Treatment 1, human genomic DNA in serum was detected with better sensitivity than in Treatment 2.

Example 22

[0573] As a serum sample, the following human serums were used. Human serums purchased from Kohjin Bio Co., Ltd (individual human serums)
Lot No.:

N51438 (healthy subject)
N51439 (healthy subject)
N51441 (healthy subject)

Human serums purchased from ProMedDx (individual human serums)
Lot No.:

11171268 (healthy subject, age 56, male)
11171292 (healthy subject, age 62, male)
11171297 (healthy subject, age 67, male)
11171314 (healthy subject, age 75, male)
11171327 (healthy subject, age 70, male)
11202510 (healthy subject, age 67, female)
11202522 (healthy subject, age 64, female)
11202527 (healthy subject, age 52, female)
11202615 (healthy subject, age 75, female)
11202618 (healthy subject, age 78, female)
10958886 (healthy subject, age 56, male)
10958979 (healthy subject, age 39, male)
10958980 (healthy subject, age 45, male)
10960268 (healthy subject, age 37, male)
10960272 (healthy subject, age 50, male)
10960276 (healthy subject, age 30, male)
10960285 (healthy subject, age 39, male)
11003457 (healthy subject, age 38, male)
11003479 (healthy subject, age 51, male)
11003480 (healthy subject, age 48, male)
11324997 (healthy subject, age 59, male)
11325001 (healthy subject, age 61, male)
10325022 (healthy subject, age 61, male)
11325032 (healthy subject, age 60, male)
11325062 (healthy subject, age 69, male)
10870623 (breast cancer patient, age 33, female)
10929521 (breast cancer patient, age 55, female)
10989644 (breast cancer patient, age 45, female)
11209430 (breast cancer patient, age 80, female)
10929514 (breast cancer patient, age 57, female)
10843055 (breast cancer patient, age 59, female)
10984680 (breast cancer patient, age 64, female)
11209428 (breast cancer patient, age 55, female)
10840414 (lung cancer patient, age 54, female)
10929506 (lung cancer patient, age 55, male)
11091955 (lung cancer patient, age 76, female)
11103346 (lung cancer patient, age 66, female)
11142322 (lung cancer patient, age 62, female)
11152564 (lung cancer patient, age 67, male)
11152571 (lung cancer patient, age 67, male)
11153198 (lung cancer patient, age 69, female)
11209435 (lung cancer patient, age 61, male)
11230621 (lung cancer patient, age 71, female)
11153192 (lung cancer patient, age 59, male)
10715942 (lung cancer patient, age 64, male)

10840422 (lung cancer patient, age 78, female)
10935547 (prostate cancer patient, age 83, male)
11000243 (prostate cancer patient, age 78, male)
11071226 (prostate cancer patient, age 84, male)

[0574] For each of the above serum samples, the following treatment was conducted respectively in duplicate.

[0575] 40 μL of a serum sample and 20 μL of a buffer (500 mM Tris-HCl (pH 7.5), 100 mM $MgCl_2$, 10 mM DTT, 1000 mM NaCl) were mixed, and the mixture was added with sterilized ultrapure water to make a liquid amount 100 μL, and mixed. Then, the reaction was retained at 95°C for 10 minutes, retained at 4°C for 10 minutes, and then returned to room temperature. After centrifugation at 9100xg or 20400xg for 10 minutes, the supernatant was collected.

[0576] Twenty (20) μL of the solution prepared in the above treatment, 2U of restriction enzyme MspI, and 5 μL of a 10x buffer optimum for MspI (100 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 10 mM Dithiothreitol, 500 mM NaCl) were mixed, and added with sterilized ultrapure water to prepare a reaction liquid having a liquid amount of 50 μL. The reaction liquid was incubated at 37°C for 1 hour.

[0577] Thirty (30) μL of the solution obtained by the above enzyme treatment, 0.5 μL of SssI methylase (available from NEB Inc.), 5 μL of 10xNEBuffer2 (available from NEB Inc.), and 0.5 μL of 3.2 mM S-adenosyl methionine (available from NEB Inc.) were mixed, and added with sterilized ultrapure water to prepare a reaction liquid having a liquid amount of 50 μL. The reaction liquid was incubated at 37°C for 30 minutes.

[0578] As a specific oligonucleotide used for obtaining a target DNA region (W, SEQ ID NO: 66, region corresponding to the nucleotide number 178-262 shown in Genbank Accession No. AF458110) designed in Alu region known as human transposon and having the nucleotide sequence of SEQ ID NO: 66, was synthesized 5'-end biotin-labeled oligonucleotide B6 comprising the nucleotide sequence of SEQ ID NO: 67 that binds with a plus strand of the target DNA region W by complementation, and a 0.2 pmoL/10 μL TE buffer solution was prepared.

[0579] Fifty (50) μL of the reaction liquid obtained in the above, 10 μL of the specific oligonucleotide solution, 10 μL of a buffer (330 mM Tris-Acetate pH 7.9, 660 mM KOAc, 100 mM $MgOAc_2$, 5 mM Dithiothreitol), 10 μL of a 100 mM $MgCl_2$ solution, 10 μL of a 1 mg/mL BSA solution were added, and the mixture was further added with sterilized ultrapure water to make a liquid amount 100 μL, and mixed. Then for forming a double strand between the target DNA region and the specific oligonucleotide, the PCR tube was retained at 95°C for 10 minutes, rapidly cooled to 70°C, and retained at this temperature for 10 minutes. Then the PCR tube was cooled to 50°C and retained for 10 minutes, and further retained at 37°C for 10 minutes, and returned to room temperature.

[0580] One hundred (100) μL of the obtained reaction liquid was transferred to a 8-well strip coated with streptavidin (StreptaWell, #11645692001, Roche), and left still for about 30 minutes at room temperature, to immobilize the complex of the target DNA and the specific oligonucleotide to the 8-well strip through a biotin-streptavidin bond. Thereafter, the solution was removed by decantation, and each well was washed three times with 200 μL of a washing buffer [0.05%Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO_4 \cdot 7H_2O$, 154 mM NaCl pH 7.4)].

[0581] As a masking oligonucleotide used for masking an immobilized free specific oligonucleotide, was synthesized oligonucleotide M comprising the nucleotide sequence of SEQ ID NO: 68 that binds with the specific oligonucleotide by complementation, and a 0.1 pmoL/μL TE buffer solution was prepared.

[0582] Each well was added with 100 μL of a methylcytosine antibody [available from Aviva Systems Biology, 0.5 μg/mL 0.1% BSA-containing phosphate buffer (1 mM KH_2PO_4, 3 mM Na_2HPO $7H_2O$, 154 mM NaCl pH7.4) solution], and further added with 1 μL of a masking oligonucleotide solution, and left still for 1 hour at room temperature. Then the solution was removed by pipetting, and each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM Na_2HPO $7H_2O$, 154 mM NaCl pH 7.4)].

[0583] Then, each well was added with 100 μL of an Eu-N1-labeled mouse IgG antibody [available from Perkin Elmer, 0.05 μg/μL 0.1% BSA-containing phosphate buffer (1 mM KH_2PO_4, 3 mM Na_2HPO $7H_2O$, 154 mM NaCl pH7.4) solution] and left still for 1 hour at room temperature. After leaving still, each well was washed three times with 200 μL of a washing buffer [0.05% Tween20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM Na_2HPO $7H_2O$, 154 mM NaCl pH 7.4)].

[0584] Each well was added with 150 μL of Enhancement Solution (available from Perkin Elmer), stirred for 5 minutes at room temperature, and left still for 15 minutes at room temperature. Then fluorescence was measured at excitation 340 nm/fluorescence 612 nm.

[0585] DNA in the solution obtained by the above enzyme treatment (MspI treatment) was quantified by real-time PCR.

[0586] As a standard sample for measuring concentration, a MspI-treated human genomic DNA solution was prepared in the following manner. A 5 ng/μL TE buffer solution of genomic DNA derived from human blood (Human Genomic DNA, #636401, Clontech) was prepared, and 20 μL of the solution, 2 U of restriction enzyme MspI, and 5 μL of a 10xbuffer optimum for MspI (100 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 10 mM Dithiothreitol, 500 mM NaCl) were mixed, and added with sterilized ultrapure water to prepare a reaction liquid having a liquid amount of 50 μL. The reaction liquid was incubated at 37°C for 1 hour. For the obtained reaction liquid, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1, 10 ng/5 μL solutions were prepared by dilution with TE buffer.

[0587] For amplifying a target DNA region (W, SEQ ID NO: 66, region corresponding to the nucleotide number 178-262 shown in Genbank Accession No. AF458110) designed in Alu region known as human transposon and quantifying by real-time PCR, a forward primer (F1, SEQ ID NO: 69) and a reverse primer (R1, SEQ ID NO: 70) were designed.

<Forward primer>

[0588]

F1:5'- GGTGGCTCACGCCTGTAATC -3' (SEQ ID NO: 69)

<Reverse primer>

[0589]

R1:5'- GGATGGTCTCGATCTCCTGAC -3' (SEQ ID NO: 70)

[0590] A reaction liquid of PCR was prepared by mixing 5 μL of the MspI-treated human genomic DNA solution prepared in the above or the standard sample for measuring concentration prepared in the above serving as a template, each 1.5 μL of 5 μM solutions of forward primer F1 and reverse primer R1, O.lx amount of SYBR® Green I (Lonza), 2.5 μL of each 2 mM dNTP, 2.5 μL of a 10xPCR buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% Gelatin), and 0.125 μL of thermostable DNA polymerase (AmpliTaq Gold, 5 U/μL, ABI), and adding sterilized ultrapure water to make the liquid amount 25 μL. Real-time PCR was conducted using Mx3005P (Stratagene). After retaining the reaction liquid at 95°C for 10 minutes, PCR was conducted by 40 cycles each consisting of 30 seconds at 95°C, 30 seconds at 61°C and 45 seconds at 72°C, to amplify the target DNA region. According to a result of the real-time PCR, DNA in a serum sample was quantified.

[0591] The results are shown in Fig. 32 and Fig. 33. A measured value by the present method, and a value quantified by the real-time PCR were compared, to reveal that there is a correlation (coefficient of correlation: R = 0.74)(Fig. 32). Further, the results quantified for human serum samples aged 59 or younger were compared between cancer patients and healty subjects, to reveal that serum DNA concentration increases in cancer patients (Fig. 33).

[0592] In the present experiment, it was revealed that free DNA in human serum can be detected and quantified with excellent sensitivity by forming and selecting a complex of a methylcytosine antibody, a methylated DNA fragment, and a 5'-end biotin-labeled oligonucleotide, and quantifying and detecting the methylcytosine antibody in the complex according to its function.

INDUSTRIAL APPLICABILITY

[0593] According to the present invention, it becomes possible to provide a method for quantifying or detecting DNA having a target DNA region in a simple and convenient manner. Further, it becomes possible to provide a method for selecting a specimen derived from a cancer patient by using a specimen derived from a test subject (preferably serum) and comparing a result in the specimen and a result in a specimen derived from a healthy subject, and so on.

Free Text in Sequence Listing

SEQ ID NOs:17 to 70

Designed oligonucleotide

[0594]

SEQUENCE LISTING

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<213> Artificial Sequence

<220>
<223> Designed oligonucleotide

<400> 17

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<210> 18
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
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<400> 18
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<210> 19
<211> 386
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Claims

1. A method for quantifying or detecting DNA comprising a target DNA region contained in a specimen comprising:

   (1) First step of preparing from a specimen DNA for which the target DNA region is to be detected;
   (2) Second step of treating the DNA prepared in First step with a DNA methylation enzyme,
   (3) Third step of preparing single-stranded methylated DNA from the DNA treated in Second step,
   (4) Fourth step of forming a complex of a single-stranded methylated DNA comprising a methylated target DNA region, a methylated DNA antibody, and a specific oligonucleotide by mixing the single-stranded methylated DNA prepared in Third step, the methylated DNA antibody, and the specific oligonucleotide comprising a nucleotide sequence that does not inhibit binding between one or more methylated bases in the target DNA region in the single-stranded methylated DNA and the methylated DNA antibody, and that is capable of binding with the single-stranded DNA comprising the target DNA region by complementation, and
   (5) Fifth step of quantifying or detecting the DNA comprising the target DNA region in the single-stranded methylated DNA by quantifying or detecting the methylated DNA antibody contained in the complex formed in Fourth step by its identification function.

2. The method according to claim 1, wherein the complex is formed in a reaction system containing a divalent cation in Fourth step.

3. The method according to claim 2, wherein the divalent cation is a magnesium ion.

4. The method according to any one of claims 1 to 3, wherein the antibody contained in the complex formed in Fourth step has been bound to a support before starting of Fifth step.

5. The method according to any one of claims 1 to 3, wherein the specific oligonucleotide contained in the complex formed in Fourth step has been bound to a support before starting of Fifth step.

6. The method according to any one of claims 1 to 5, wherein the DNA methylation enzyme is a cytosine methylation enzyme.

7. The method according to any one of claims 1 to 6, wherein the DNA methylation enzyme is SssI methylase.

8. The method according to any one of claims 1 to 7, wherein the methylated DNA.antibody is a methylcytosine antibody.

9. The method according to any one of claims 1 to 8, wherein the specimen is any of the following specimen:

   (a) mammalian blood, body fluid, excreta, body secretion, cell lysate, or tissue lysate,
   (b) DNA extracted from one selected from the group consisting of mammalian blood, body fluid, excreta, body secretion, cell lysate, and tissue lysate,
   (c) DNA prepared by using as a template RNA extracted from one selected from the group consisting of mammalian tissue, cell, tissue lysate and cell lysate,
   (e) DNA extracted from cell, fungus or virus, or
   (f) DNA prepared by using as a template RNA extracted from cell, fungus or virus.

10. The method according to any one of claims 1 to 9, wherein DNA for which the target DNA region is to be detected

is any of the following DNAs (a) to (e):

(a) DNA digested in advance with a restriction enzyme recognition cleavage site for which is not present in the target DNA region,
(b) DNA purified in advance,
(c) free DNA in blood,
(d) DNA derived from microbial genome, or
(e) DNA generated from RNA by a reverse transcriptase.

11. The method according to any one of claims 1 to 10, wherein a counter oligonucleotide is added in forming the complex in Fourth step.

12. The method according to any one of claims 1 to 11, wherein concentration of a sodium salt in a solution used in a DNA extracting operation for preparing DNA from a specimen in First step is 100 mM or more and 1000 mM or less.

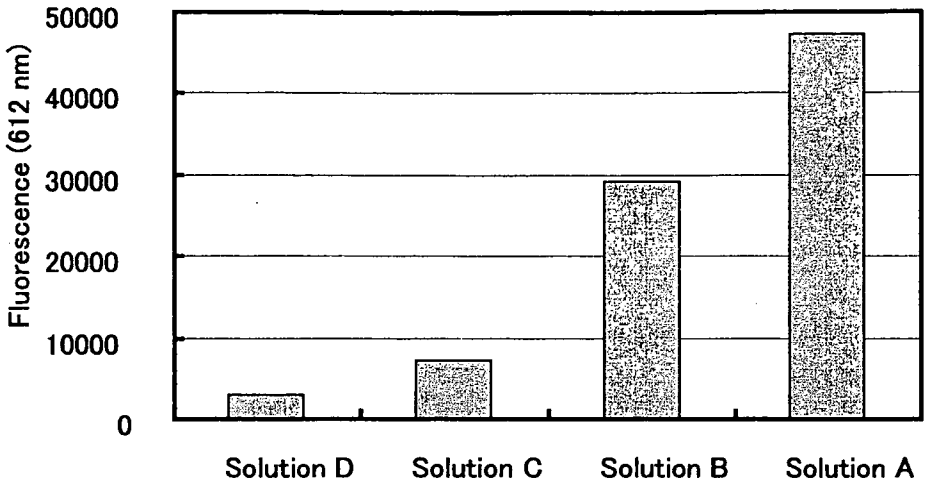
13. The method according to any one of claims 1 to 11, wherein concentration of a sodium salt in a solution used in a DNA extracting operation for preparing DNA from a specimen in First step is 100 mM or more and 200 mM or less.

14. A method for selecting a specimen from a cancer patient comprising the step of evaluating that a specimen from a test subject is a specimen from a cancer patient when there is significant difference between a quantification result or a detection result of DNA quantified or detected by using the specimen from the test subject according to the method of any one of Inventions 1 to 13 and a quantification result or a detection result of DNA quantified or detected by using a specimen from a healthy subject according to the same method, and identifying a specimen from a cancer patient based on a result of the evaluation.

15. The method according to claim 14, wherein the specimen is mammalian serum.

16. The method according to claim 14 or 15, wherein DNA. comprising a target DNA region is free DNA comprising the target DNA region in a mammalian serum.

Fig.1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Solution D
Solution C
Solution B
Solution A

Fig.2

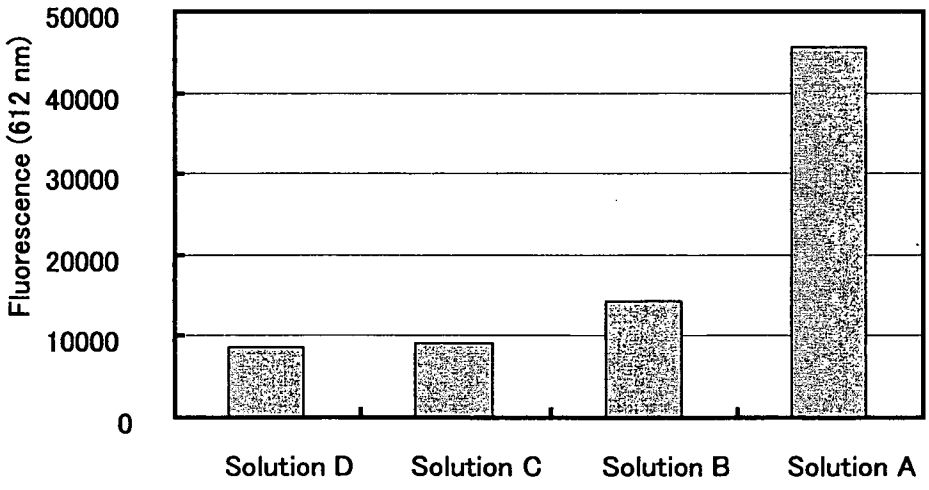
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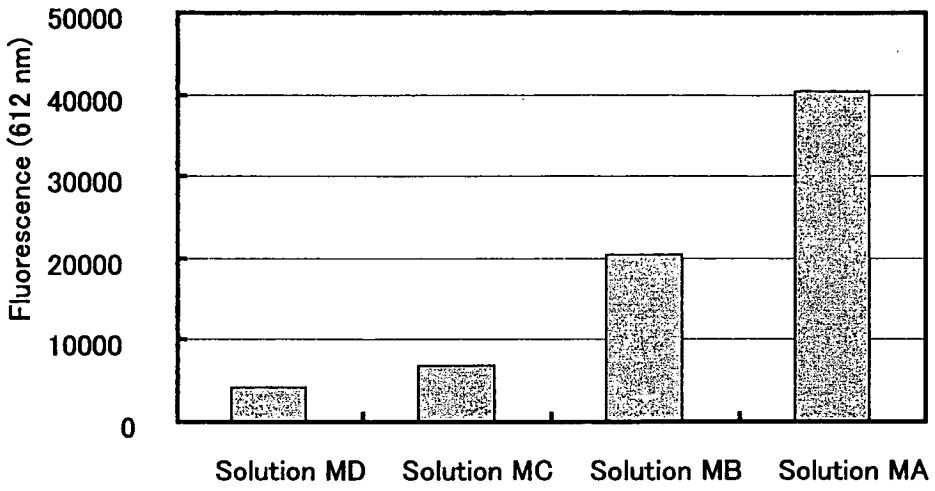
Fig.3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Solution MA

Fig.4

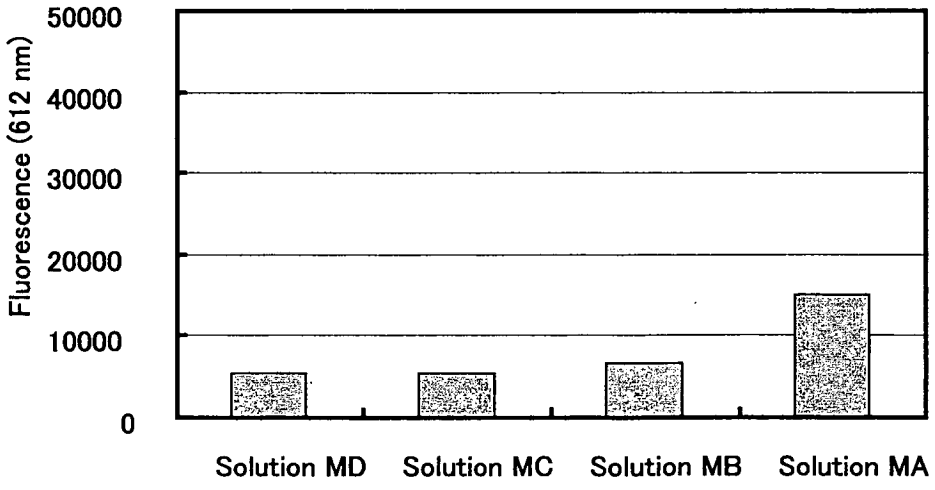
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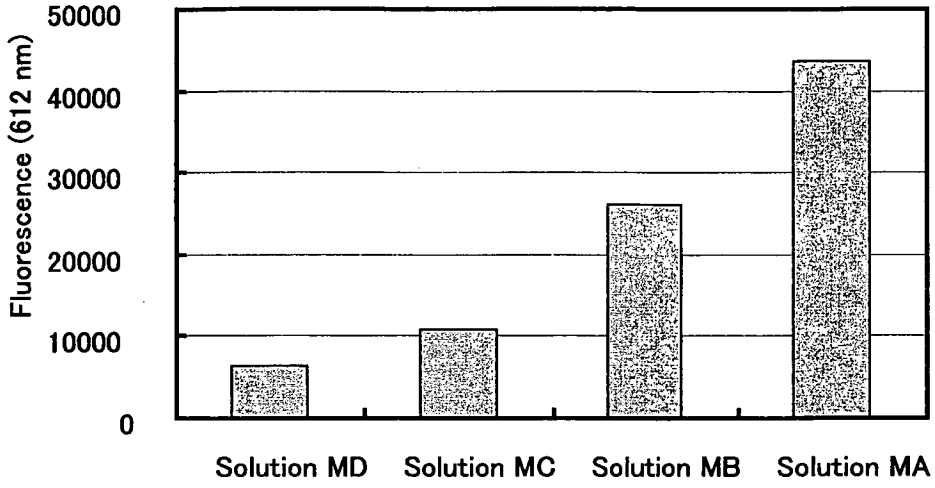
Fig.5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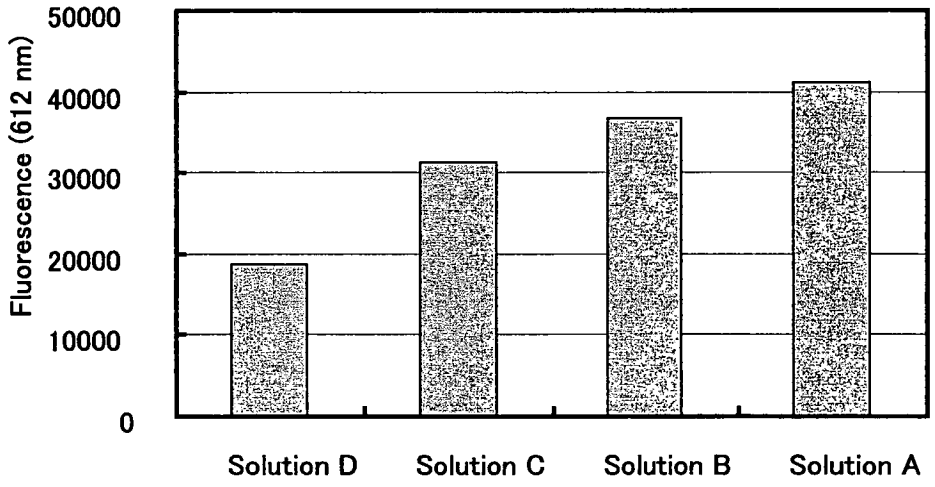
Solution MB
Solution MA

Fig.6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Solution D
Solution C
Solution B
Solution A

Fig.7

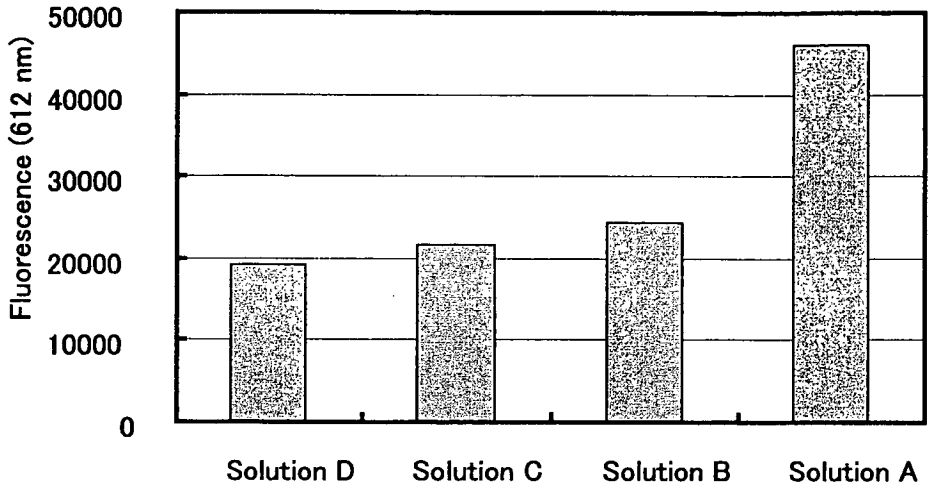
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Fig.8

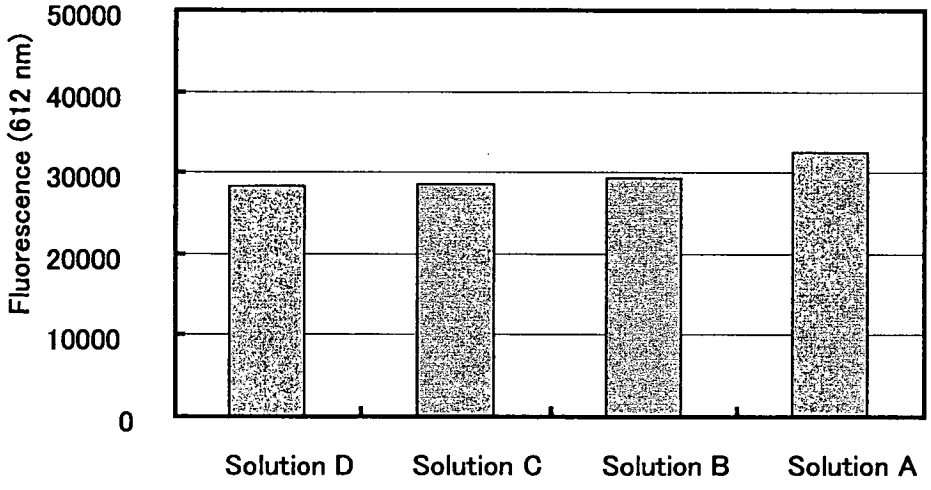
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Fig.9

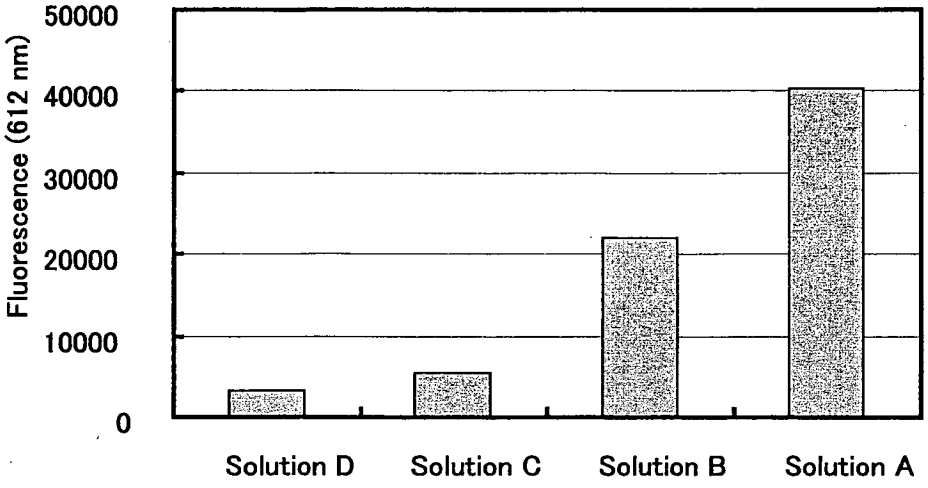
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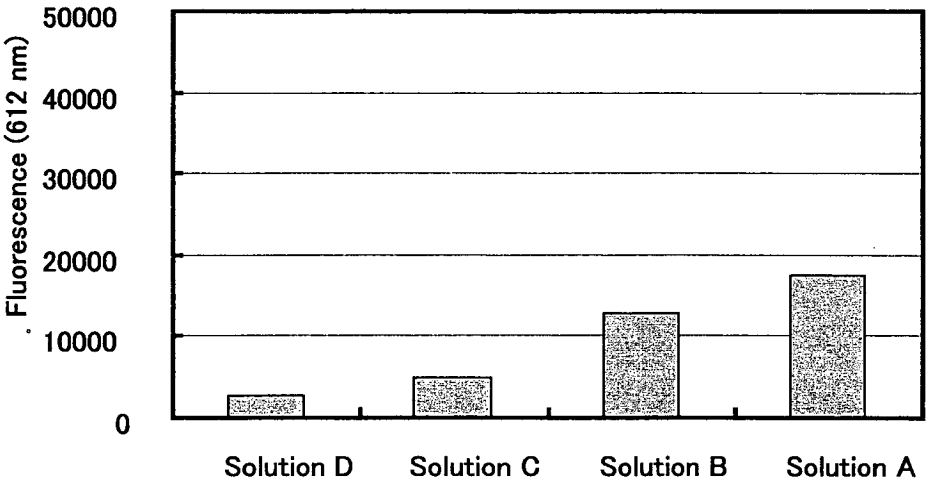
Fig.10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Solution C
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Fig.11

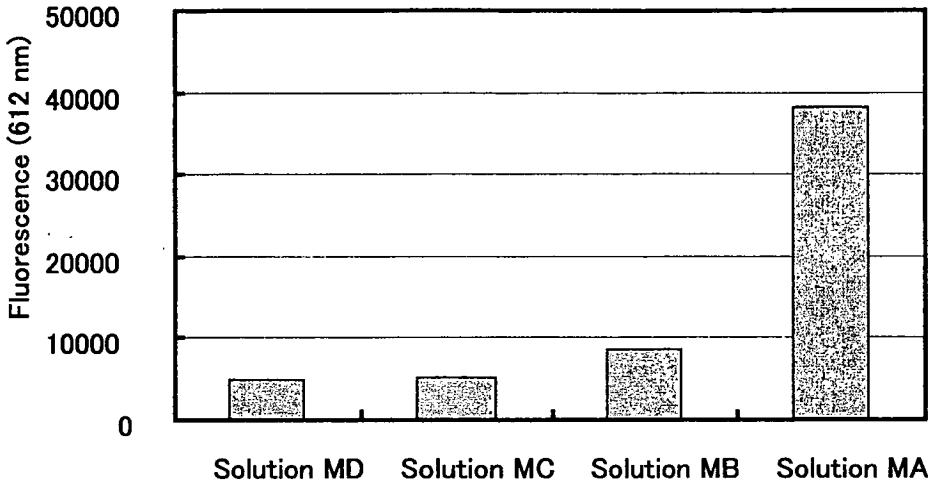
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Fig.12

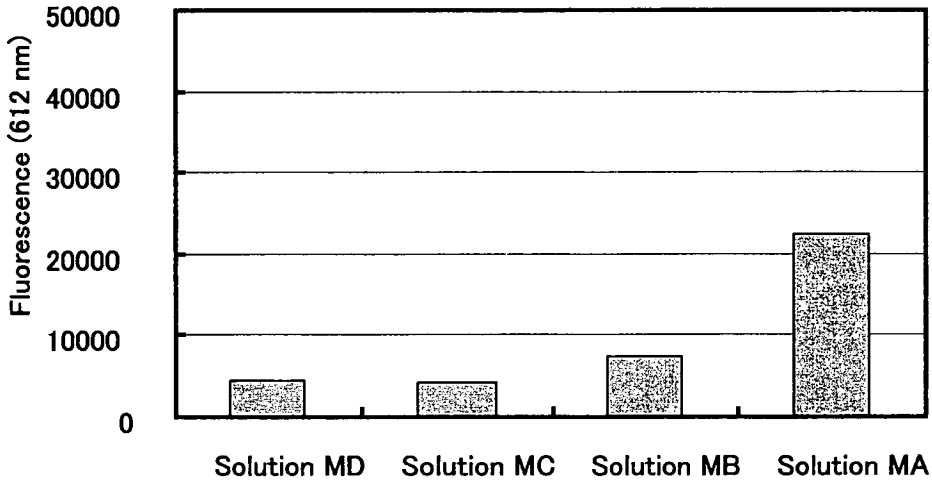
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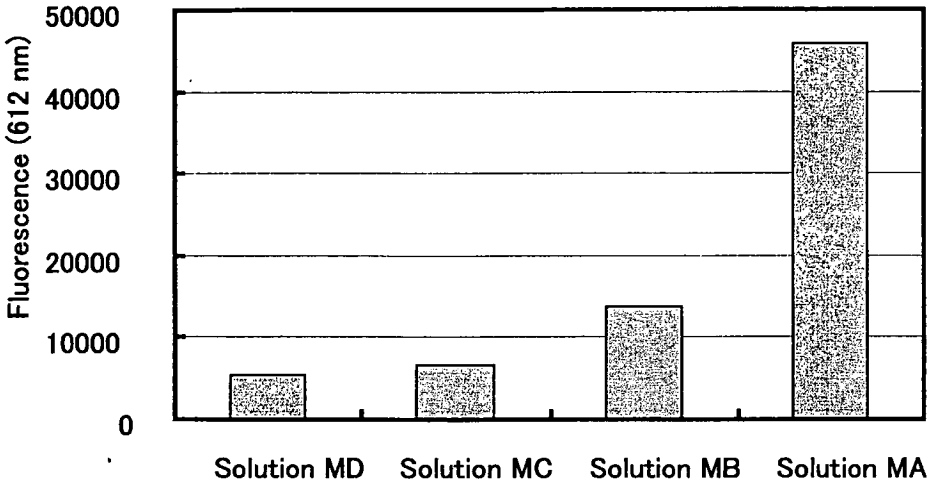
Fig.13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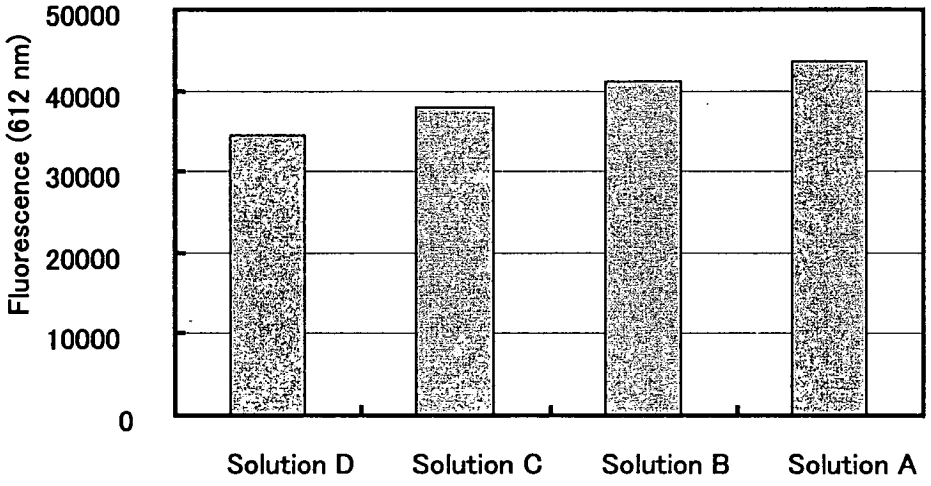
Fluorescence (612 nm)
Solution MD
Solution MC
Solution MB
Solution MA

Fig.14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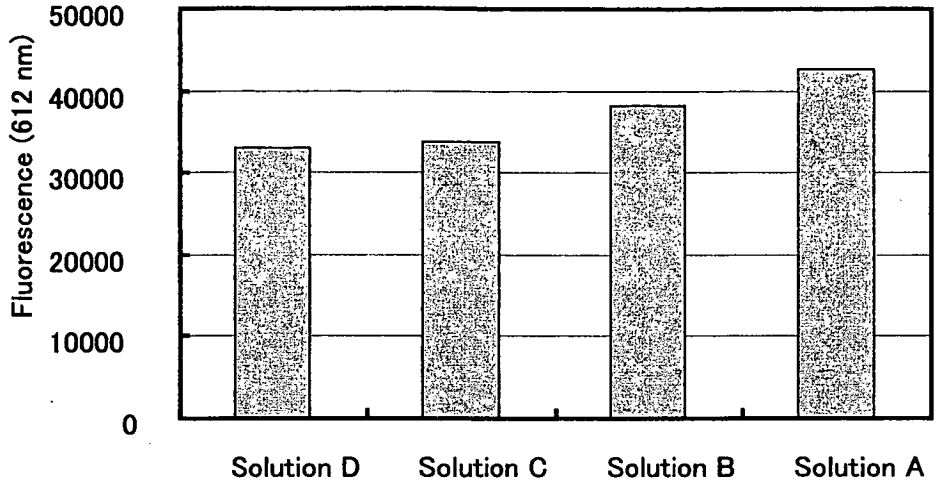
10000
0
Fluorescence (612 nm)
Solution D
Solution C
Solution B
Solution A

Fig.15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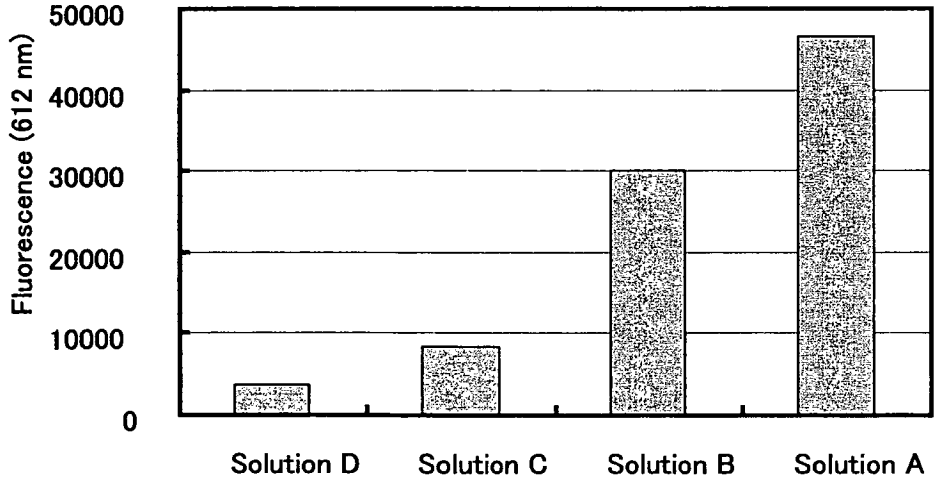
20000
10000
0
Fluorescence (612 nm)
Solution D
Solution C
Solution B
Solution A

Fig.16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20000
10000
0
Fluorescence (612 nm)
Solution D
Solution C
Solution B
Solution A

Fig.17

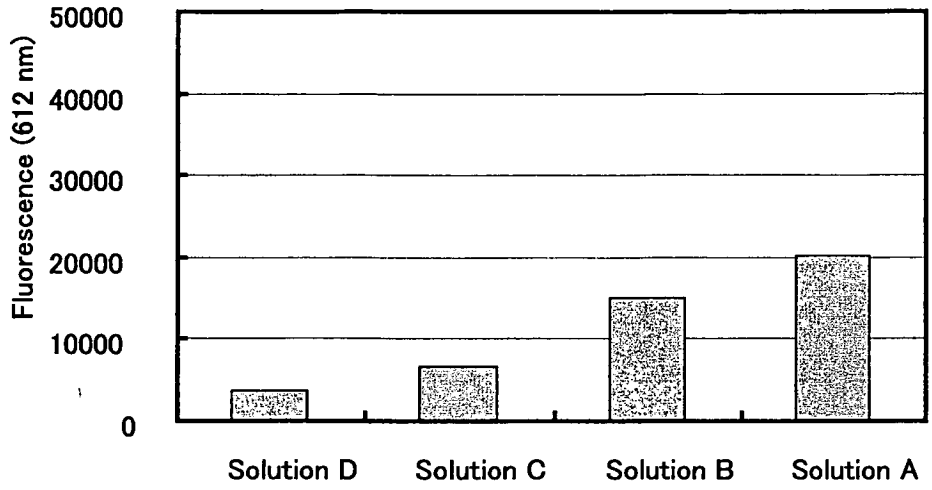

Fluorescence (612 nm)
50000
40000
30000
20000
10000
0
Solution D
Solution C
Solution B
Solution A

Fig.18

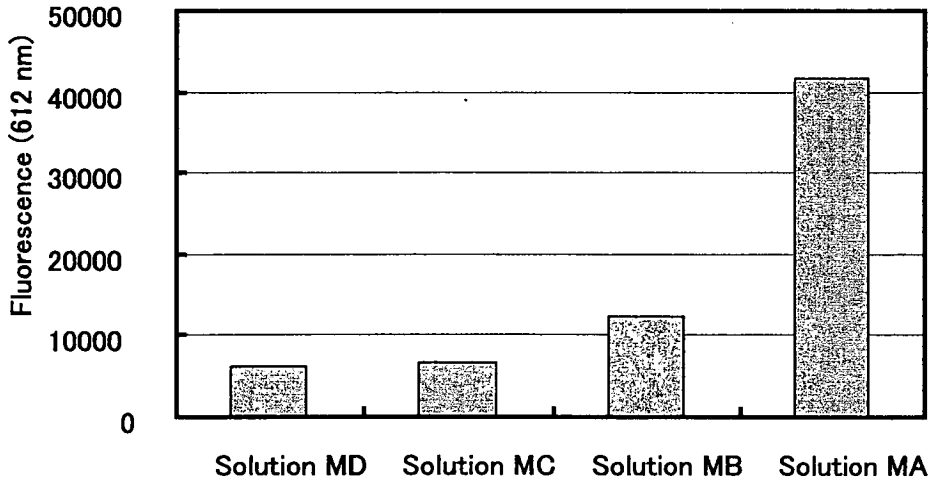
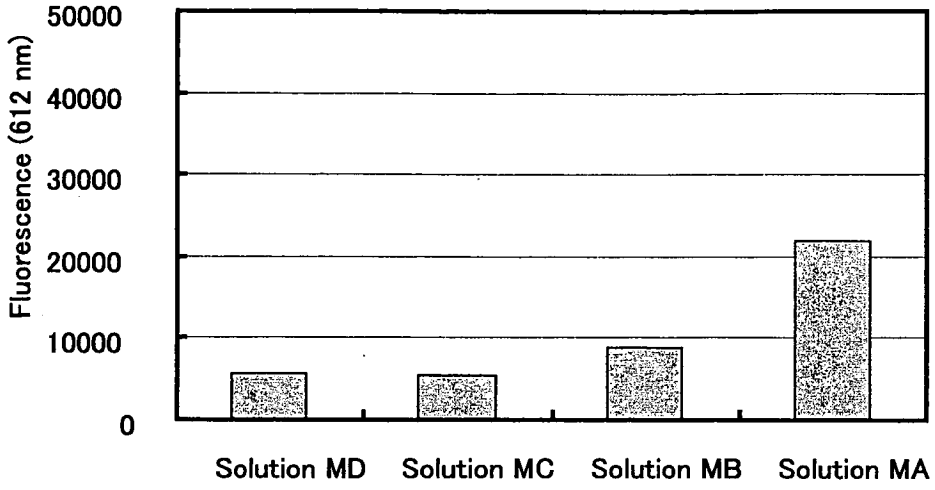

Fluorescence (612 nm)
50000
40000
30000
20000
10000
0
Solution MD
Solution MC
Solution MB
Solution MA

Fig.19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40000
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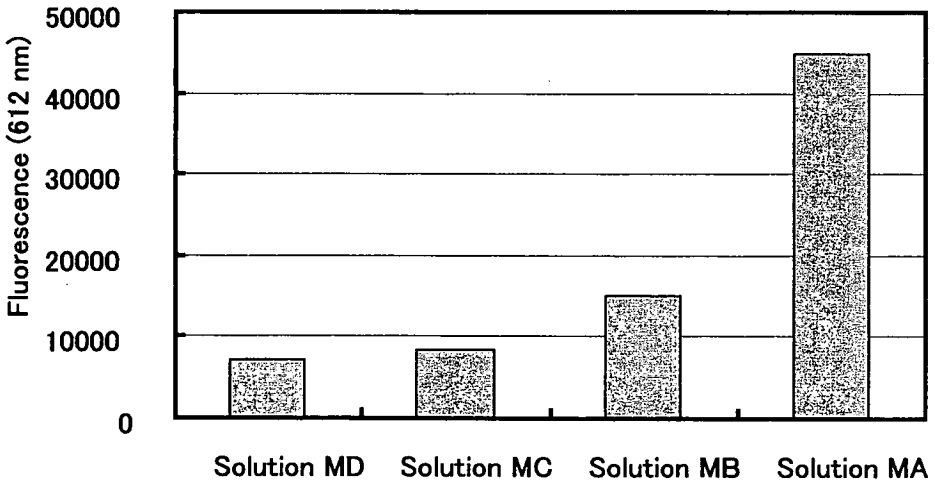
20000
10000
0
Fluorescence (612 nm)
Solution MD
Solution MC
Solution MB
Solution MA

Fig.20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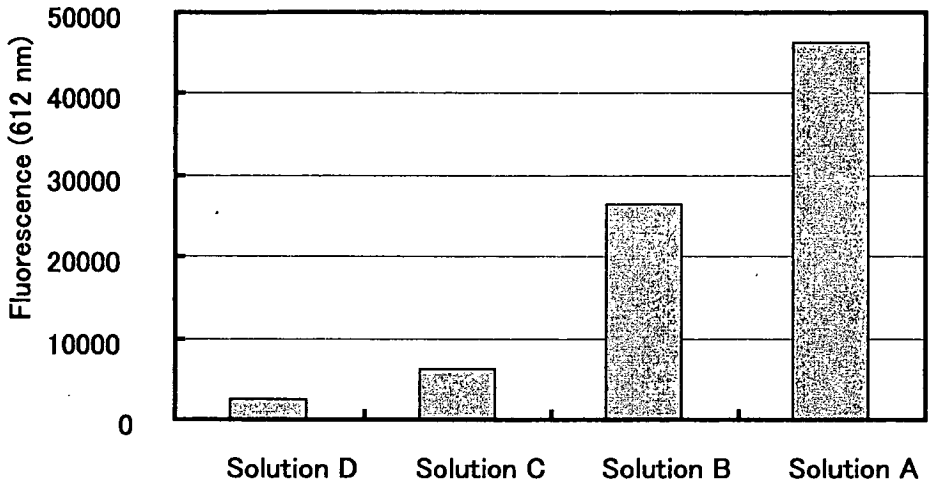
20000
10000
0
Fluorescence (612 nm)
Solution MD
Solution MC
Solution MB
Solution MA

Fig.21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40000
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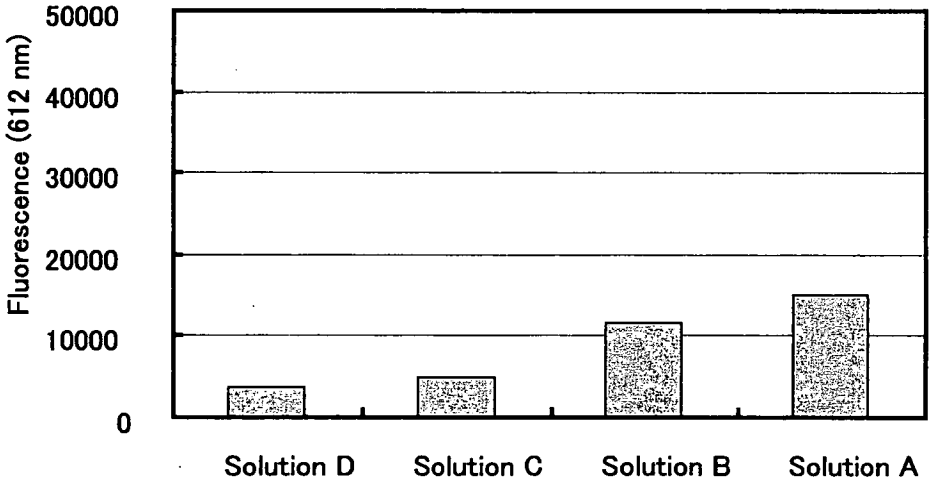
20000
10000
0
Fluorescence (612 nm)
Solution D
Solution C
Solution B
Solution A

Fig.22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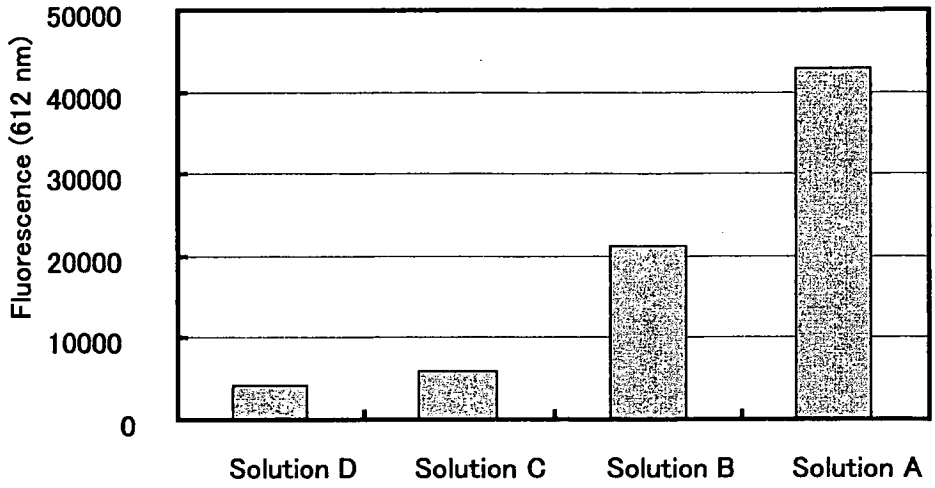
0
Fluorescence (612 nm)
Solution D
Solution C
Solution B
Solution A

Fig.23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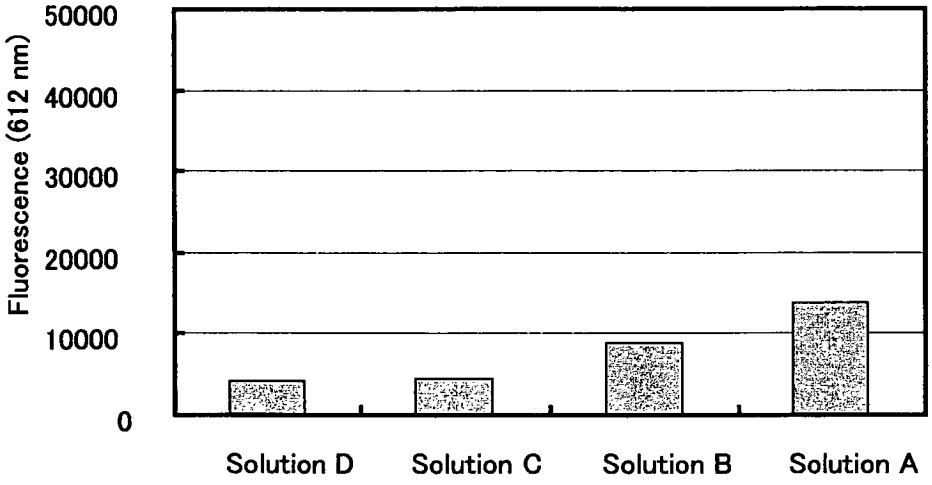
20000
10000
0
Fluorescence (612 nm)
Solution D
Solution C
Solution B
Solution A

Fig.24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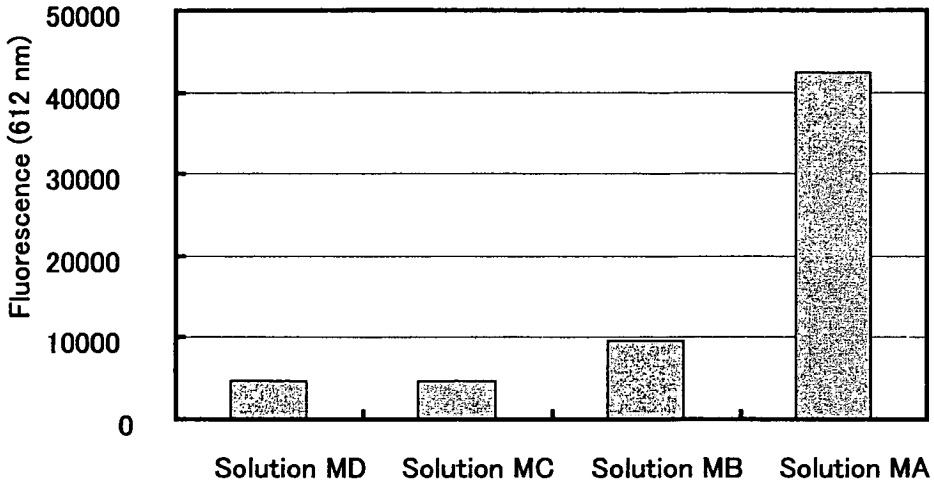
20000
10000
0
Fluorescence (612 nm)
Solution D
Solution C
Solution B
Solution A

Fig.25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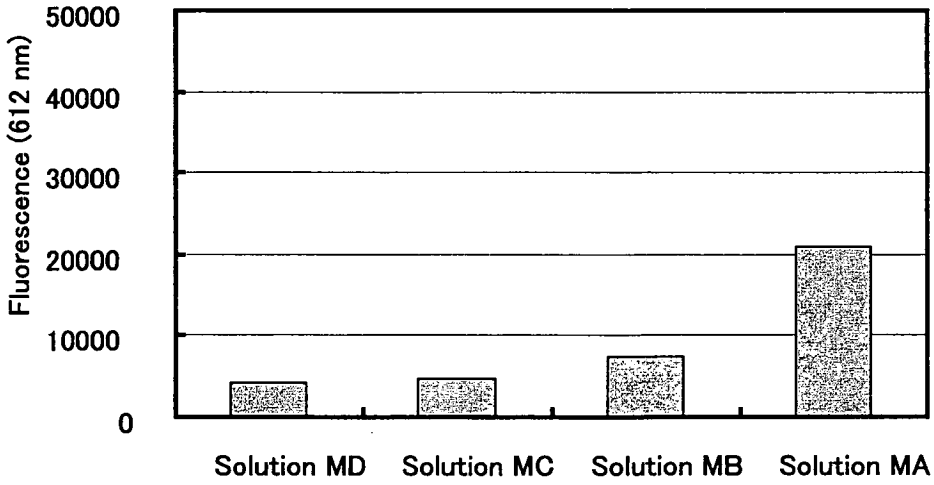
40000
30000
20000
10000
0
Fluorescence (612 nm)
Solution MD
Solution MC
Solution MB
Solution MA

Fig.26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20000
10000
0
Fluorescence (612 nm)
Solution MD
Solution MC
Solution MB
Solution MA

Fig.27

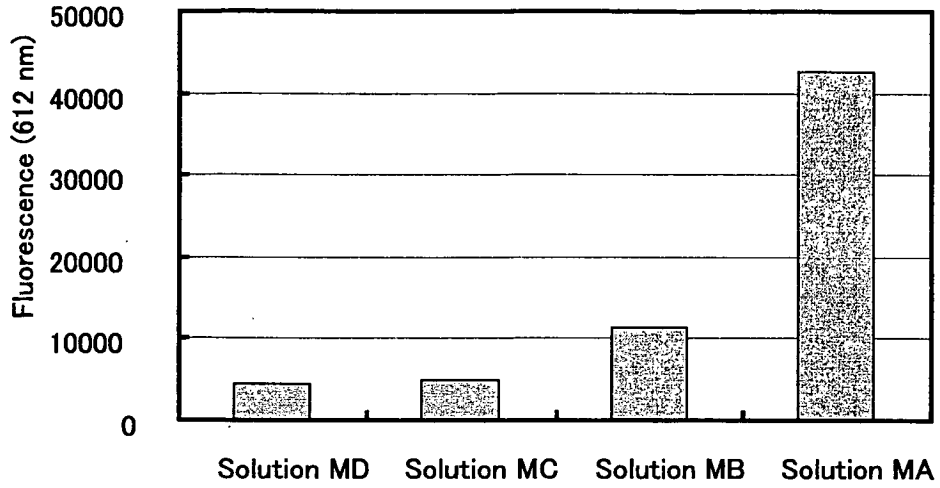

Fluorescence (612 nm)
50000
40000
30000
20000
10000
0
Solution MD
Solution MC
Solution MB
Solution MA

Fig.28

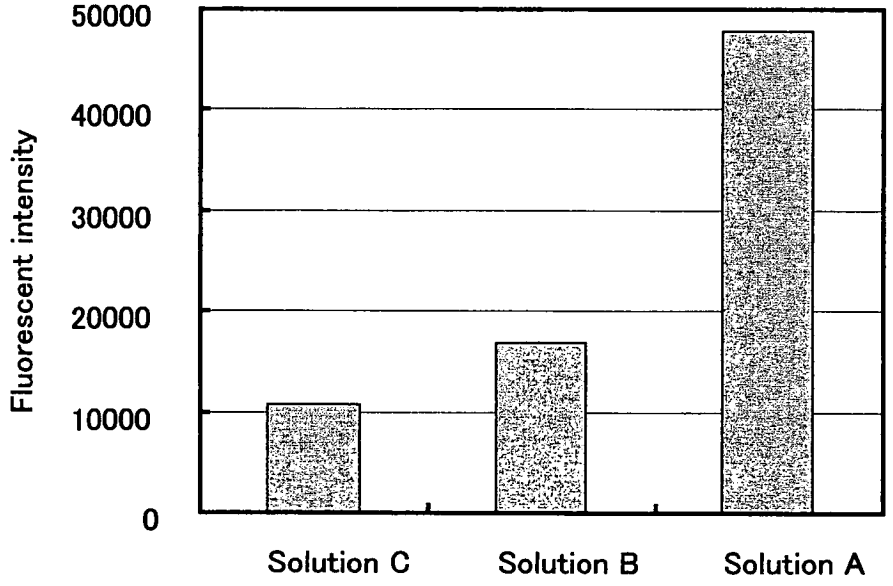
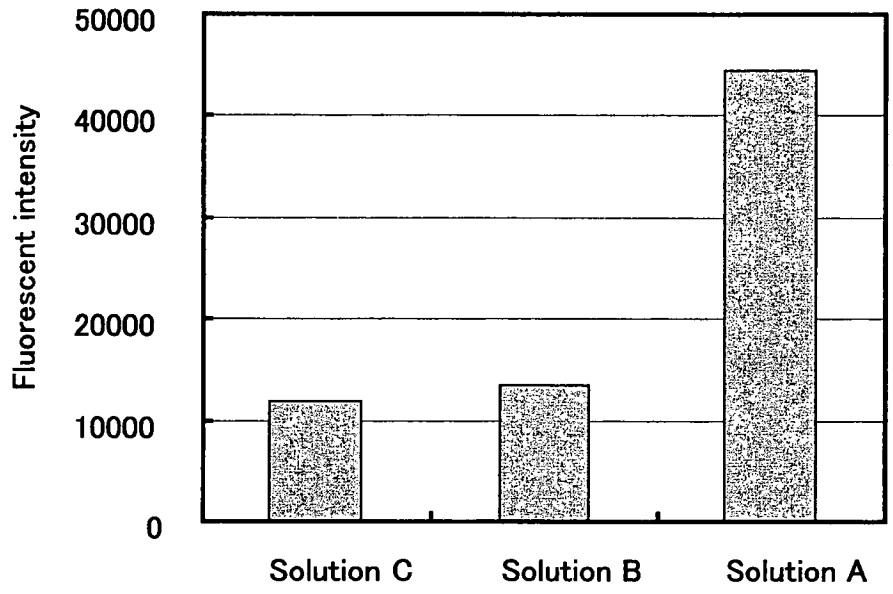

Fluorescent intensity
50000
40000
30000
20000
10000
0
Solution C
Solution B
Solution A

Fig.29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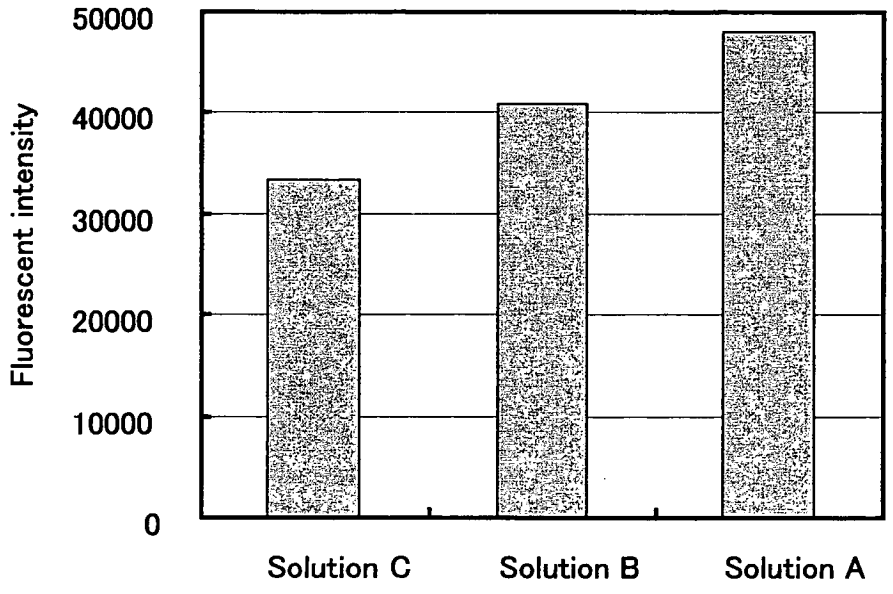
40000
30000
20000
10000
0
Fluorescent intensity
Solution C
Solution B
Solution A

Fig.30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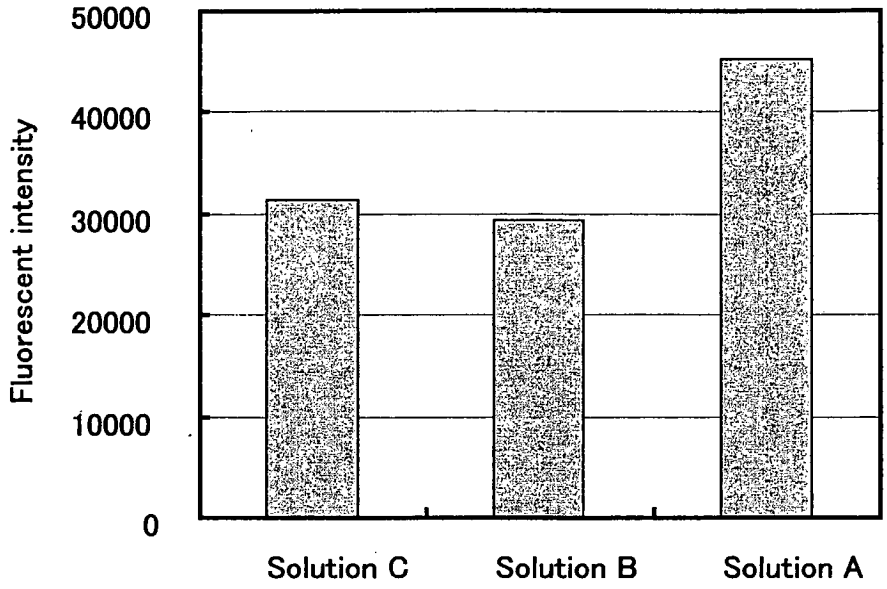
40000
30000
20000
10000
0
Fluorescent intensity
Solution C
Solution B
Solution A

Fig.31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40000
30000
20000
10000
0
Fluorescent intensity
Solution C
Solution B
Solution A

Fig.32

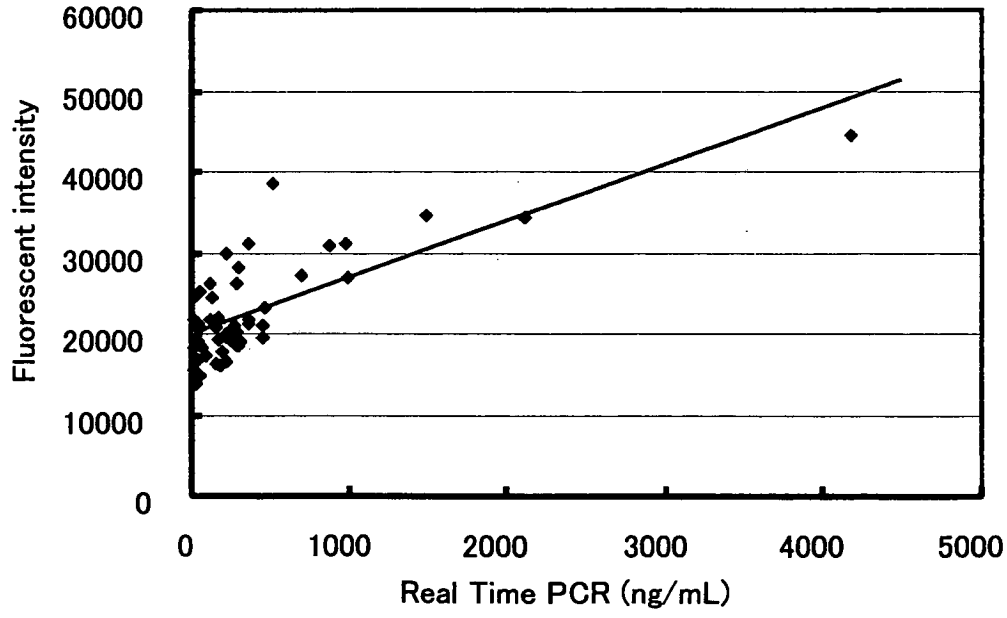
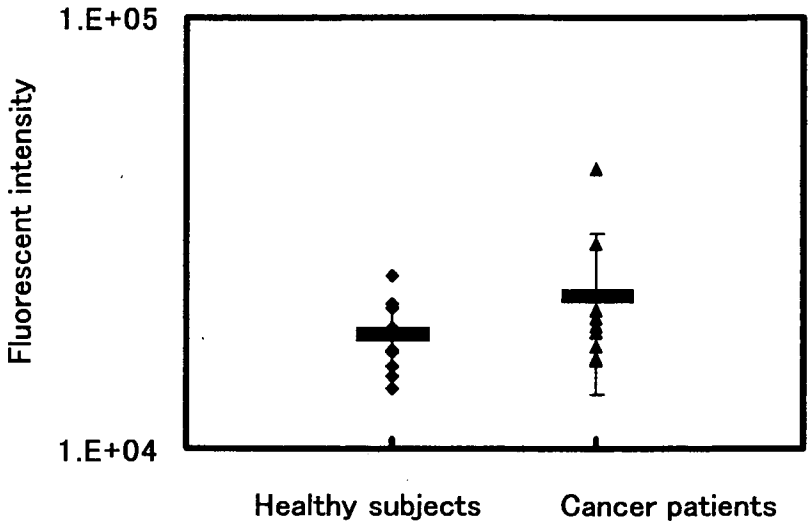

60000
50000
40000
30000
20000
10000
0
Fluorescent intensity
0
1000
2000
3000
4000
5000
Real Time PCR (ng/mL)

Fig.33


1.E+05
Fluorescent intensity
1.E+04
Healthy subjects
Cancer patients

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2009/061067

A. CLASSIFICATION OF SUBJECT MATTER
C12N15/09(2006.01)i, C12Q1/25(2006.01)i, C12Q1/68(2006.01)i, G01N33/53(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N15/09, C12Q1/25, C12Q1/68, G01N33/53

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Jitsuyo Shinan Koho 1922-1996 Jitsuyo Shinan Toroku Koho 1996-2009
Kokai Jitsuyo Shinan Koho 1971-2009 Toroku Jitsuyo Shinan Koho 1994-2009

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
CA/BIOSIS/MEDLINE/WPIDS(STN), JSTPlus/JMEDPlus/JST7580(JDreamII)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BAEK, T.J., et al., Development of a photodiode array biochip using a bipolar semiconductor and its application to detection of human papilloma virus. Anal. Bioanal. Chem., Mar 2008, Vol.390, No.5, p.1373-1378	1-16
A	JP 2004-536596 A (U.S. Genomics, Inc.), 09 December, 2004 (09.12.04), & US 2002/0197639 A1 & EP 1402071 A & WO 2002/101353 A2	1-16
A	JP 2004-347508 A (Japan Science and Technology Agency), 09 December, 2004 (09.12.04), & WO 2004/104582 A1	1-16

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 August, 2009 (19.08.09)	01 September, 2009 (01.09.09)
Name and mailing address of the ISA/ Japanese Patent Office Facsimile No.	Authorized officer Telephone No.

Form PCT/ISA/210 (second sheet) (April 2007)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2009/061067

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2005/080565 A1 (Japan Science and Technology Agency), 01 September, 2005 (01.09.05), & US 2008/0227652 A1 & EP 1717312 A1	1-16
A	GAO, L., et al., DNA microarray: a high throughput approach for methylation detection. Colloids Surf. B: Biointerfaces., 2005, Vol.40, No.3-4, p.127-131	1-16

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REFERENCES CITED IN THE DESCRIPTION

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Non-patent literature cited in the description

- *J. Cataract. Refract. Surg.,* 2007, vol. 33 (4), 635-641 **[0002]**
- *Environ. Mol. Mutagen.,* 1991, vol. 18 (4), 259-262 **[0002]**
- Methods in Yeast Genetics. Cold Spring Harbor Laboratory Press **[0071]**
- Molecular Cloning -A Laboratory Manual. Cold Spring Harbor Laboratory Press **[0071]**
- Methods in Yeast Genetics. Cold Spring Harbor Laboratory **[0304] [0325] [0346] [0365] [0382] [0399] [0417] [0457] [0476] [0495] [0516]**

专利名称(译)	检测或定量DNA的方法		
公开(公告)号	EP2305806A1	公开(公告)日	2011-04-06
申请号	EP2009762575	申请日	2009-06-11
[标]申请(专利权)人(译)	住友化学有限公司		
申请(专利权)人(译)	住友化学公司		
当前申请(专利权)人(译)	住友化学公司		
[标]发明人	TOMIGAHARA YOSHITAKA SATOH HIDEO TARUI HIROKAZU		
发明人	TOMIGAHARA, YOSHITAKA SATOH, HIDEO TARUI, HIROKAZU		
IPC分类号	C12N15/09 C12Q1/25 C12Q1/68 G01N33/53		
CPC分类号	G01N33/57484 C12Q1/6816 C12Q1/6827 C12Q1/6886 G01N33/5308		
优先权	2008152617 2008-06-11 JP		
其他公开文献	EP2305806A4		
外部链接	Espacenet		

摘要(译)

本发明涉及定量或检测具有靶DNA区域的DNA的方法，等等。

X:5′-

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GAGCGGGTGGCACACCGCGATGTAGCGGTCGGCTGTCATGACTACCAGCATGTAGGCCGACG

CAAACATGCCGAACACCTGCAGGTGCTTCACCACGCGGCACAGCCAGTCGGGGCCGCGGAAG

CGGTAGGTGATGTCCCAGCACATTTGCGGCAGCACCTGGAAGAATGCCACGGCCAGGTCGGC

CAGGCTGAGGTGTCGGATGAAGAGGTGCATGCGGGACGTCTTGCGCGGCGTCCGGTGCAGAG

CCAGCAGTACGCTGCTGTTGCCCAGCACGGCCACCGCGAAAGTCACCGCCAGCACGGCGATC

TCCAGTTTGGCCAG -3′(SEQ ID NO: 19)