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(54) METHOD FOR DETERMINATION OF DNA METHYLATION

(57) The present invention relates to a method of measuring the content of methylated DNA in a DNA region of interest in a genomic DNA contained in a biological specimen, and so on.

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Description

TECHNICAL FIELD

[0001] The present invention relates to a method of measuring the content of methylated DNA in a DNA region of interest in a genomic DNA contained in a biological specimen, and so on.

BACKGROUND ART

[0002] As a method for evaluating the methylation state of DNA in an objective DNA region in a genomic DNA contained in a biological specimen, for example, there is known a method of measuring the content of methylated DNA in an objective DNA region in a genomic DNA (see, for example, Nucleic Acids Res., 1994, Aug 11; 22(15): 2990-7, and Proc. Natl. Acad. Sci. U.S.A., 1997, Mar 18; 94(6): 2284-9 for reference). In such a measuring method, first, it is necessary to extract DNA containing the objective DNA region from a DNA sample derived from a genomic DNA, and the extracting operation is complicated.

[0003] As a method of measuring the content of methylated DNA in an objective region of extracted DNA, for example, (1) a method of amplifying an objective region by subjecting the DNA to a chain reaction for DNA synthesis by DNA polymerase after modification of the DNA with a sulfite or the like (Polymerase Chain Reaction; hereinafter also referred to as PCR), and (2) a method of amplifying an objective region by subjecting the DNA to PCR after digestion of the DNA using a methylation sensitive restriction enzyme are known. Both of these methods require time and labor for DNA modification for detection of methylation, subsequent purification of the product, preparation of a reaction system for PCR, and checking of DNA amplification.

DISCLOSURE OF THE INVENTION

[0004] It is an object of the present invention to provide a method of measuring the content of methylated DNA in an objective DNA region in a genomic DNA contained in a biological specimen in a simple and convenient manner.

[0005] That is, the present include the following inventions.

[Invention 1]

[0006] A method of measuring the content of methylated DNA in a objective DNA region in a genomic DNA contained in a biological specimen, comprising:

(1) First step of subjecting a DNA sample derived from the genomic DNA contained in the biological specimen to a digestion treatment with a methylation-sensitive restriction enzyme;
(2) Second step of obtaining methylated single-stranded DNA from the DNA sample that has been subjected to the digestion treatment and obtained in First step, and binding the single-stranded DNA to an immobilized methylated DNA antibody, thereby selecting the single-stranded DNA; and
(3) Third step comprising, as a pre step of each of the following regular steps:

a step (First pre step) of separating the single-stranded DNA selected in Second step from the immobilized immobilized methylated DNA antibody to provide DNA in a single-stranded state (plus strand);
a step (Second pre step) of extensionally-forming a double-stranded DNA from a single-stranded DNA (plus strand) containing the objective DNA region by a single extension of an extension primer, using the genomic DNA (plus strand) provided in a single-stranded state in First pre step and the extension primer, wherein the extension primer (forward primer) comprises the nucleotide sequence (minus strand) complementary to a partial nucleotide sequence (plus strand) of the nucleotide sequence of the DNA in a single-stranded state (plus strand), the partial nucleotide sequence (plus strand) being located on further 3'-end side than the 3'-end of the nucleotide sequence (plus strand) of the objective DNA region; and
a step (Third pre step) of temporarily separating the double-stranded DNA extensionally formed in Second pre step into a single-stranded DNA (plus strand) containing the objective DNA region and a single-stranded DNA (minus strand) containing the nucleotide sequence complementary to the objective DNA region;
and as regular steps:

(a) Step A (regular step) of extensionally forming double-stranded DNA from the single-stranded DNA containing the objective DNA region, by a single extension of the extension primer, using as a template the generated single-stranded DNA (plus strand) containing the objective DNA region, and the forward primer

as the extension primer; and
(b) Step B (regular step) of extensionally forming double-stranded DNA from the single-stranded DNA containing the objective DNA region, by a single extension of an extension primer, using as a template the generated single-stranded DNA (minus strand) containing the nucleotide sequence complementary to the objective DNA region, and using as the extension primer an extension primer (reverse primer) comprising the nucleotide sequence (plus strand) complementary to a partial nucleotide sequence (minus strand) of the nucleotide sequence of the single-stranded DNA (minus strand) containing the nucleotide sequence complementary to the objective DNA region, the partial nucleotide sequence (minus strand) being located on further 3'-end side than the 3'-end of the nucleotide sequence (minus strand) complementary to the nucleotide sequence (plus strand)of the objective DNA region; and wherein

[0007] Third step further comprises:
amplifying the methylated DNA in the objective DNA region to a detectable level by repeating each regular step of Third step after temporarily separating the extensionally formed double-stranded DNA obtained in each of the regular steps into a single-stranded state; and quantifying the amount of the amplified DNA.

[Invention 2]

[0008] The method of Invention 1, wherein the immobilized immobilized methylated DNA antibody is a methylcytosine antibody.

[Invention 3]

[0009] The method of Invention 1 or 2, wherein the biological specimen is blood, a bodily fluid, serum, plasma, a cell lysate, or a tissue lysate from a mammal.

[Invention 4]

[0010] The method of any one of Inventions 1 to 3, wherein the DNA sample derived from the genomic DNA contained in the biological specimen is a DNA sample digested in advance with a restriction enzyme recognition cleavage site for which is not present in the objective DNA region of the genomic DNA, or a DNA sample purified in advance.

[Invention 5]

[0011] The method of any one of Inventions 1 to 4, wherein First step comprises:

First (A) step of mixing a single-stranded DNA (plus strand) containing the objective DNA region and a masking oligonucleotide comprising a nucleotide sequence complementary to a nucleotide sequence of a recognition site for a methylation-sensitive restriction enzyme, thereby selecting single-stranded DNA in which the recognition site for the methylation-sensitive restriction enzyme is protected; and
First (B) step of digesting the single-stranded DNA selected in First (A) step with the methylation-sensitive restriction enzyme.

[Invention 6]

[0012] The method of any one of Inventions 1 to 5, wherein the methylation-sensitive restriction enzyme is a restriction enzyme the restriction site for which is included in the objective DNA region in the genomic DNA contained in the biological specimen, or the methylation-sensitive restriction enzyme is HhaI.

[Invention 7]

[0013] The method of any one of Inventions 1 to 6, wherein Second step is performed without digestion treatment with the methylation-sensitive restriction enzyme in First step.

[Invention 8]

[0014] The method of any one of Inventions 1 to 7, wherein Second step comprises:

Second (A) step of separating into methylated single-stranded DNA the methylated double-stranded DNA contained in the DNA sample that has been subjected to the digestion treatment and obtained in First step; and
Second (B) step of binding the methylated single-stranded DNA obtained in Second (A) step to an immobilized methylated DNA antibody; and wherein

a counter oligonucleotide is added when separating the methylated double-stranded DNA into the methylated single-stranded DNA in Second (A) step.

BRIEF DESCRIPTION OF THE DRAWINGS

[0015]

Fig. 1 shows results of 2% agarose gel electrophoresis of amplification products obtained by amplifying methylated DNA in the region comprising the nucleotide sequence of SEQ ID NO: 23 by PCR from a prepared sample in Example 1.
From the leftmost lane in the drawing, results in a DNA marker "MK", a sample "M" of a solution of a partially methylated oligonucleotide GPR7-2079-2176/98 mer-M(7) in which the recognition sequence of HpaII is methylated, subjected to an "A" treatment, a sample "H" of a solution of a partially methylated oligonucleotide GPR7-2079-2176/98 mer-HM(5) in which part of the recognition sequence of HpaII is not methylated, subjected to an "A" treatment, a sample "U" of a solution of an unmethylated oligonucleotide GPR7-2079-2176/98 mer-UM, subjected to an "A" treatment, a sample "M" of a solution of a partially methylated oligonucleotide GPR7-2079-2176/98 mer-M(7) in which the recognition sequence of HpaII is methylated, subjected to a "B" treatment, a sample "H" of a solution of a partially methylated oligonucleotide GPR7-2079-2176/98 mer-HM(5) in which part of the recognition sequence of HpaII is not methylated, subjected to a "B" treatment, a sample "U" of a solution of an unmethylated oligonucleotide GPR7-2079-2176/98 mer-UM, subjected to a "B" treatment, a sample "M" of a solution of a partially methylated oligonucleotide GPR7-2079-2176/98 mer-M(7) in which the recognition sequence of HpaII is methylated, subjected to a "C" treatment, a sample "H" of a solution of a partially methylated oligonucleotide GPR7-2079-2176/98 mer-HM(5) in which part of the recognition sequence of HpaII is not methylated, subjected to a "C" treatment, and a sample "U" of a solution of an unmethylated oligonucleotide GPR7-2079-2176/98 mer-UM, subjected to a "C" treatment are shown.
Fig. 2 shows results of 1.5% agarose gel electrophoresis of amplification products obtained by amplifying methylated DNA in the target DNA region comprising the nucleotide sequence of SEQ ID NO: 28 by PCR from a prepared sample in Example 2. From the leftmost lane in the drawing, results in a DNA marker "MK", a solution "MD" of a methylated DNA fragment MX (negative control), a solution "D" of an unmethylated DNA fragment X (negative control), a solution "MC" of a methylated DNA fragment MX, a solution "C" of an unmethylated DNA fragment X, a solution "MB" of a methylated DNA fragment MX, a solution "B" of an unmethylated DNA fragment X, a solution "MA" of a methylated DNA fragment MX, and a solution "A" of an unmethylated DNA fragment X are shown.
Fig. 3 shows results of 1.5% agarose gel electrophoresis of amplification products obtained by amplifying methylated DNA in the target DNA region comprising the nucleotide sequence of SEQ ID NO: 45 by PCR from a prepared sample in Example 3. From the leftmost lane in the drawing, results in a DNA marker "MK", a solution "MD" of a methylated DNA fragment MY (negative control), a solution "D" of an unmethylated DNA fragment Y (negative control), a solution "MC" of a methylated DNA fragment MY, a solution "C" of an unmethylated DNA fragment X, a solution "MB" of a methylated DNA fragment MY, a solution "B" of an unmethylated DNA fragment Y, a solution "MA" of a methylated DNA fragment MA, and a solution "A" of an unmethylated DNA fragment Y are shown.
Fig. 4 shows results of 1.5% agarose gel electrophoresis of amplification products obtained by amplifying methylated DNA in the target DNA region comprising the nucleotide sequence of SEQ ID NO: 53 by PCR from a prepared sample in Example 4. From the leftmost lane in the drawing, results in a DNA marker "MK", a solution "MD" of a methylated DNA fragment MT (negative control), a solution "D" of an unmethylated DNA fragment T (negative control), a solution "MC" of a methylated DNA fragment MT, a solution "C" of an unmethylated DNA fragment T, a solution "MB" of a methylated DNA fragment MT, a solution "B" of an unmethylated DNA fragment T, a solution "MA" of a methylated DNA fragment MT, and a solution "A" of an unmethylated DNA fragment T are shown.
Fig. 5 shows results of 1.5% agarose gel electrophoresis of amplification products obtained by amplifying methylated DNA in the target DNA region comprising the nucleotide sequence of SEQ ID NO: 53 by PCR from a prepared sample in Example 5. From the leftmost lane in the drawing, results in a DNA marker "MK", a solution "MD" of a methylated yeast genomic DNA (negative control), a solution "D" of an unmethylated yeast genomic DNA (negative control), a solution "MC" of a methylated yeast genomic DNA, a solution "C" of an unmethylated yeast genomic DNA, a solution "MB" of a methylated yeast genomic DNA, a solution "B" of an unmethylated yeast genomic DNA, a solution "MA" of a methylated yeast genomic DNA, and a solution "A" of an unmethylated yeast genomic DNA

are shown.

MODE FOR CARRYING OUT THE INVENTION

[0016] As the "biological specimen" in the present invention, for example, a cell lysate, a tissue lysate (here the term "tissue" is used in a broad sense including blood, lymph node and so on) or biological samples including bodily sections such as plasma, serum and lymph, bodily secretions (urine, milk and so on) and the like and a genomic DNA obtained by extracting these biological samples, in mammals can be recited. As a biological specimen, for example, samples derived from microorganisms, viruses and the like can be recited, and in such a case, "a genomic DNA" in the present measuring method also means genomic DNA of microorganisms, viruses and the like.

[0017] When the specimen derived from a mammal is blood, use of the present measuring method in a regular health check or a simple examination is expected.

[0018] For obtaining a genomic DNA from a specimen derived from a mammal, for example, DNA may be extracted using a commercially available DNA extraction kit.

[0019] When blood is used as a specimen, plasma or serum is prepared from blood in accordance with a commonly used method, and using the prepared plasma or serum as a specimen, free DNA (including DNA derived from cancer cells such as gastric cancer cells) contained in the specimen is analyzed. This enables analysis of DNA derived from cancer cells such as gastric cancer cells while avoiding DNA derived from hemocytes, and improves the sensitivity of detection of cancer cells such as gastric cancer cells and a tissue containing the same.

[0020] The DNA sample derived from genomic DNA may be a DNA sample digested in advance with a restriction enzyme recognition cleavage site for which is not present in the objective DNA region of the genomic DNA, or a DNA sample purified in advance by a prescribed method.

[0021] Usually, a gene (a genomic DNA) consists of four kinds of bases. In these bases, such a phenomenon is known that only cytosine is methylated, and such methylation modification of DNA is limited to cytosine in a nucleotide sequence represented by 5'-CG-3' (C represents cytosine, and G represents guanine. Hereinafter, the nucleotide sequence is also referred to as "CpG"). The site to be methylated in cytosine is its position 5. In DNA replication prior to cell division, only cytosine in "CpG" of a template chain is methylated immediately after replication, however, cytosine in "CpG" of a newly-generated strand is immediately methylated by the action of methyltransferase. Therefore, the methylation state of DNA will be passed to new two sets of DNA even after DNA replication. The term "methylated DNA" in the present invention means DNA occurring by such methylation modification.

[0022] The term "CpG pair" in the present invention means double-stranded oligonucleotide in which a nucleotide sequence represented by CpG and a CpG that is complement with this are base-paired.

[0023] The term "objective DNA region" (hereinafter, also referred to as an "objective region") used in the present invention means a DNA region for which presence or absence of methylation of cytosine included in the region is to be examined, and has a recognition site of at least one kind of methylation sensitive restriction enzyme. A DNA region containing at least one cytosine in a nucleotide sequence represented by CpG which is present in a nucleotide sequence of a promoter region, an untranslated region, or a translated region (coding region) of a useful protein gene 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, Fibrillin2, Neurofilament3, disintegrin and metalloproteinase domain 23, G protein-coupled receptor 7, G-protein coupled somatostatin and angiotensin-like peptide receptor, Solute carrier family 6 neurotransmitter transporter noradrenalin member 2 and so on can be recited.

[0024] 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. Cytosine in the nucleotide sequence represented by CpG which is present in the nucleotide sequence of SEQ ID NO: 1, in particular, cytosine in CpG which is present in a region where CpGs are densely present in the nucleotide sequence of SEQ ID NO: 1 exhibits high methylation frequency (namely, a high methylation state (hypermethylation)) in, for example, cancer cells such as gastric cancer cells. More concretely, as cytosine exhibiting high methylation frequency in gastric cancer cells, for example, cytosines represented by base Nos. 1539, 1560, 1574, 1600, 1623, 1635, 1644, 1654, 1661, 1682, 1686, 1696, 1717, 1767, 1774, 1783, 1785, 1787, 1795 and so on in the nucleotide sequence of SEQ ID NO: 1 can be recited.

[0025] 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. Cytosine in the nucleotide sequence represented by CpG which is present in the nucleotide sequence of SEQ ID NO: 2, in particular, cytosine in CpG which is present in a region where CpGs are densely present in the nucleotide sequence of SEQ ID NO: 2 exhibits high methylation frequency (namely, a high methylation state (hypermethylation)) in, for example, cancer cells such as gastric cancer cells. More concretely, as cytosine exhibiting high methylation frequency in gastric cancer cells, for example, cytosines represented by base Nos. 1316, 1341, 1357, 1359, 1362, 1374, 1390, 1399, 1405, 1409, 1414, 1416, 1422, 1428, 1434, 1449, 1451, 1454, 1463, 1469, 1477, 1479, 1483, 1488, 1492, 1494, 1496, 1498, 1504, 1510, 1513, 1518, 1520 and so on in the nucleotide sequence of SEQ ID NO: 2 can be recited.

[0026] 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. Cytosine in the nucleotide sequence represented by CpG which is present in the nucleotide sequence of SEQ ID NO: 3, in particular, cytosine in CpG which is present in a region where CpGs are densely present in the nucleotide sequence of SEQ ID NO: 3 exhibits high methylation frequency (namely, a high methylation state (hypermethylation)) in, for example, cancer cells such as gastric cancer cells. More concretely, as cytosine exhibiting high methylation frequency in gastric cancer cells, for example, cytosines represented by base Nos. 329, 335, 337, 351, 363, 373, 405, 424, 427, 446, 465, 472, 486 and so on in the nucleotide sequence of SEQ ID NO: 3 can be recited.

[0027] 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. Cytosine in the nucleotide sequence represented by CpG which is present in the nucleotide sequence of SEQ ID NO: 4, in particular, cytosine in CpG which is present in a region where CpGs are densely present in the nucleotide sequence of SEQ ID NO: 4 exhibits high methylation frequency (namely, a high methylation state (hypermethylation)) in, for example, cancer cells such as gastric cancer cells. More concretely, as cytosine exhibiting high methylation frequency in gastric cancer cells, for example, cytosines represented by base Nos. 329, 333, 337, 350, 353, 360, 363, 370, 379, 382, 384, 409, 414, 419, 426, 432, 434, 445, 449, 459, 472, 474, 486, 490, 503, 505 and so on in the nucleotide sequence of SEQ ID NO: 4 can be recited.

[0028] 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. Cytosine in the nucleotide sequence represented by CpG which is present in the nucleotide sequence of SEQ ID NO: 5, in particular, cytosine in CpG which is present in a region where CpGs are densely present in the nucleotide sequence of SEQ ID NO: 5 exhibits high methylation frequency (namely, a high methylation state (hypermethylation)) in, for example, cancer cells such as gastric cancer cells. More concretely, as cytosine exhibiting high methylation frequency in gastric cancer cells, for example, cytosines represented by base Nos. 1153, 1160, 1178, 1187, 1193, 1218, 1232, 1266, 1272, 1292, 1305, 1307, 1316, 1356, 1377, 1399, 1401, 1422, 1434 and so on in the nucleotide sequence of SEQ ID NO: 5 can be recited.

[0029] 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. Cytosine in the nucleotide sequence represented by CpG which is present in the nucleotide sequence of SEQ ID NO: 6, in particular, cytosine in CpG which is present in a region where CpGs are densely present in the nucleotide sequence of SEQ ID NO: 6 exhibits high methylation frequency (namely, a high methylation state (hypermethylation)) in, for example, cancer cells such as gastric cancer cells. More concretely, as cytosine exhibiting high methylation frequency in gastric cancer cells, for example, cytosines represented by base Nos. 1172, 1175, 1180, 1183, 1189, 1204, 1209, 1267, 1271, 1278, 1281, 1313, 1319, 1332, 1334, 1338, 1346, 1352, 1358, 1366, 1378, 1392, 1402, 1433, 1436, 1438 and so on in the nucleotide sequence of SEQ ID NO: 6 can be recited.

[0030] 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. Cytosine in the nucleotide sequence represented by CpG which is present in the nucleotide sequence of SEQ ID NO: 7, in particular, cytosine in CpG which is present in a region where CpGs are densely present in the nucleotide sequence of SEQ ID NO: 7 exhibits high methylation frequency (namely, a high methylation state (hypermethylation)) in, for example, cancer cells such as gastric cancer cells. More concretely, as cytosine exhibiting high methylation frequency in gastric cancer cells, for example, cytosines represented by base Nos. 1714, 1716, 1749, 1753, 1762, 1795, 1814, 1894, 1911, 1915, 1925, 1940, 1955, 1968 and so on in the nucleotide sequence of SEQ ID NO: 7 can be recited.

[0031] 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. Cytosine in the nucleotide sequence represented by CpG which is present in the nucleotide sequence of SEQ ID NO: 8, in particular, cytosine in CpG which is present in a region where CpGs are densely present in the nucleotide sequence of SEQ ID NO: 8 exhibits high methylation frequency (namely, a high methylation state (hypermethylation)) in, for example, cancer cells such as gastric cancer cells. More concretely, as cytosine exhibiting high methylation frequency in gastric cancer cells, for example, cytosines represented by base Nos. 35, 43, 51, 54, 75, 85, 107, 127, 129, 143, 184, 194, 223, 227, 236, 251, 258 and so on in the nucleotide sequence of SEQ ID NO: 8 can be recited.

[0032] 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. Cytosine in the nucleotide sequence represented by CpG which is present in the nucleotide sequence of SEQ ID NO: 9, in particular, cytosine in CpG which is present in a region where CpGs are densely present in the nucleotide sequence of SEQ ID NO: 9 exhibits high methylation frequency (namely, a high methylation state (hypermethylation)) in, for example, cancer cells such as gastric cancer cells. More concretely, as cytosine exhibiting high methylation frequency in gastric cancer cells, for example, cytosines represented by base Nos. 1539, 1551, 1571, 1579, 1581, 1585, 1595, 1598, 1601, 1621, 1632, 1638, 1645, 1648, 1665, 1667, 1680, 1698, 1710,

1724, 1726, 1756 and so on in the nucleotide sequence of SEQ ID NO: 9 can be recited.

[0033] 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. Cytosine in the nucleotide sequence represented by CpG which is present in the nucleotide sequence of SEQ ID NO: 10 exhibits high methylation frequency (namely, a high methylation state (hypermethylation)) in, for example, cancer cells such as pancreas cancer cells. More concretely, as cytosine exhibiting high methylation frequency in pancreas cancer cells, for example, cytosines represented by base Nos. 1282, 1284, 1301, 1308, 1315, 1319, 1349, 1351, 1357, 1361, 1365, 1378, 1383 and so on in the nucleotide sequence of SEQ ID NO: 10 can be recited.

[0034] 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. Cytosine in the nucleotide sequence represented by CpG which is present in the nucleotide sequence of SEQ ID NO: 11 exhibits high methylation frequency (namely, a high methylation state (hypermethylation)) in, for example, cancer cells such as pancreas cancer cells. More concretely, as cytosine exhibiting high methylation frequency in pancreas cancer cells, for example, cytosines represented by base Nos. 679, 687, 690, 699, 746, 773, 777, 783, 795, 799, 812, 823, 830, 834, 843 and so on in the nucleotide sequence of SEQ ID NO: 11 can be recited.

[0035] 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. Cytosine in the nucleotide sequence represented by CpG which is present in the nucleotide sequence of SEQ ID NO: 12 exhibits high methylation frequency (namely, a high methylation state (hypermethylation)) in, for example, cancer cells such as pancreas cancer cells. More concretely, as cytosine exhibiting high methylation frequency in pancreas cancer cells, for example, cytosines represented by base Nos. 428, 432, 443, 451, 471, 475, 482, 491, 499, 503, 506, 514, 519, 532, 541, 544, 546, 563, 566, 572, 580 and so on in the nucleotide sequence of SEQ ID NO: 12 can be recited.

[0036] 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. Cytosine in the nucleotide sequence represented by CpG which is present in the nucleotide sequence of SEQ ID NO: 13 exhibits high methylation frequency (namely, a high methylation state (hypermethylation)) in, for example, cancer cells such as pancreas cancer cells. More concretely, as cytosine exhibiting high methylation frequency in pancreas cancer cells, for example, cytosines represented by base Nos. 998, 1003, 1007, 1011, 1016, 1018, 1020, 1026, 1028, 1031, 1035, 1041, 1043, 1045, 1051, 1053, 1056, 1060, 1066, 1068, 1070, 1073, 1093, 1096, 1106, 1112, 1120, 1124, 1126 and so on in the nucleotide sequence of SEQ ID NO: 13 can be recited.

[0037] 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. Cytosine in the nucleotide sequence represented by CpG which is present in the nucleotide sequence of SEQ ID NO: 14 exhibits high methylation frequency (namely, a high methylation state (hypermethylation)) in, for example, cancer cells such as pancreas cancer cells. More concretely, as cytosine exhibiting high methylation frequency in pancreas cancer cells, for example, cytosines represented by base Nos. 1480, 1482, 1485, 1496, 1513, 1526, 1542, 1560, 1564, 1568, 1570, 1580, 1590, 1603, 1613, 1620 and so on in the nucleotide sequence of SEQ ID NO: 14 can be recited.

[0038] 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. Cytosine in the nucleotide sequence represented by CpG which is present in the nucleotide sequence of SEQ ID NO: 15 exhibits high methylation frequency (namely, a high methylation state (hypermethylation)) in, for example, cancer cells such as pancreas cancer cells. More concretely, as cytosine exhibiting high methylation frequency in pancreas cancer cells, for example, cytosines represented by base Nos. 470, 472, 490, 497, 504, 506, 509, 514, 522, 540, 543, 552, 566, 582, 597, 610, 612 and so on in the nucleotide sequence of SEQ ID NO: 15 can be recited.

[0039] 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. Cytosine in the nucleotide sequence represented by CpG which is present in the nucleotide sequence of SEQ ID NO: 16 exhibits high methylation frequency (namely, a high methylation state (hypermethylation)) in, for example, cancer cells such as pancreas cancer cells. More concretely, as cytosine exhibiting high methylation frequency in pancreas cancer cells, for example, cytosines represented by base Nos. 1002, 1010, 1019, 1021, 1051, 1056, 1061, 1063, 1080, 1099, 1110, 1139, 1141, 1164, 1169, 1184 and so on in the nucleotide sequence of SEQ ID NO: 16 can be recited.

[0040] The term "methylated DNA antibody" means an antibody that binds to a methylated base in DNA as its antigen. Concretely, it may be a methylcytosine antibody, and an antibody having a property of recognizing and binding to cytosine methylated at position 5 in single-stranded DNA can be recited. Also a commercially available methylated DNA antibody may be applicable as far as it specifically recognizes and specifically binds to DNA in a methylated state according to the present invention.

[0041] A methylated DNA antibody can be prepared by a conventional immunological technique from a methylated base, methylated DNA or the like as an antigen. Concretely, a methylcytosine antibody can be obtained by selecting from antibodies prepared against an antigen such as 5-methylcytidine, 5-methylcytosine or DNA containing 5-methylcytosine according to specific binding to methylcytosine in DNA as an index.

[0042] As an antibody obtainable by immunizing an animal against an antigen, after immunizing with a purified antigen, an antibody of an IgG fraction (polyclonal antibody), and an antibody produced by a single clone (monoclonal antibody) can be used. In the present invention, since an antibody capable of specifically recognizing methylated DNA or methylcytosine is desired, it is preferable to use a monoclonal antibody.

[0043] 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 pancreatic cell (B cell) derived from an immunized mouse and a myeloma cell, and an antibody produced by the hybridoma is selected, and thus a methylcytosine antibody (monoclonal antibody) is prepared. 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-methylcytosine or DNA or the like containing 5-methylcytosine may be administered as an antigen to an animal used for immunization. As an administration method, 5-methyl cytidine, 5-methylcytosine or DNA or the like containing 5-methylcytosine

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.

[0044] 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 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.

[0045] 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.

[0046] 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.

[0047] Single-stranded DNA is able to bind with an anti methylation antibody as far as at least one position of a CpG existing therein is methylated. The term "methylated single-stranded DNA" in the present invention means single-stranded DNA in which at least one potision of a CpG existing in single-stranded DNA is methylated, rather than meaning exclusively single-stranded DNA in which every CpG existing in single-stranded DNA is methylated.

[0048] The expression "an amount of amplified DNA obtained by (amplifying methylated DNA in a target DNA region to a detectable level"" means an amount itself after amplification of methylated DNA in a target region comprised by genomic DNA contained in a biological specimen, namely, an amount determined in Third step of the present invention as described below. For example, when the biological specimen is 1 mL of serum, it means an amount of DNA amplified based on the methylated DNA contained in 1 mL of serum.

[0049] In First step, a DNA sample derived from genomic DNA contained in a biological specimen is subjected to a digestion treatment with a methylation-sensitive restriction enzyme.

[0050] The "methylation-sensitive restriction enzyme" in the present invention, for example, a restriction enzyme or the like that does not digest a recognition sequence containing methylated cytosine, but digests only a recognition sequence containing unmethylated cytosine. In other words, in the case of DNA wherein cytosine contained in a recognition sequence inherently recognizable by the methylation sensitive restriction enzyme is methylated, the DNA will not be cleaved even when the methylation sensitive restriction enzyme is caused to act on the DNA. On the other hand, in the case of DNA wherein cytosine contained in a recognition sequence inherently recognizable by the methylation sensitive restriction enzyme is not methylated, the DNA will be cleaved when the methylation sensitive restriction enzyme is caused to act on the DNA. Concrete examples of such methylation sensitive restriction enzymes include HpaII, BstUI, NarI, SacII, and HhaI which are restriction enzymes recognition cleavage site for which is present in the objective DNA region of the genomic DNA contained in a biological specimen. The aforementioned methylation sensitive restriction enzymes have already been revealed by Gruenbaum et al. (Nucleic Acid Research, 9, 2509-2515).

[0051] As a method of examining whether or not digestion by the methylation sensitive restriction enzyme occurs, concretely, for example, a method of conducting PCR using a pair of primers capable of amplifying DNA containing cytosine which is a target of analysis in a recognition sequence while using the DNA as a template, and examining whether or not the DNA is amplified (amplified product) can be recited. When the cytosine which is a target of analysis is methylated, an amplified product is obtained. On the other hand, when the cytosine which is a target of analysis is not methylated, an amplified product is not obtained. In this manner, by comparing the amounts of amplified DNA, it is possible to measure the methylated rate of the cytosine which is a target of analysis. In brief, when genomic DNA contained in the biological specimen is methylated, it is possible to distinguish whether or not cytosine in CpG pair existing in the recognition site of the methylation sensitive restriction enzyme in genomic DNA contained in the biological specimen is methylated by utilizing the characteristic that the methylation sensitive restriction enzyme fails to cleave methylated DNA. In other words, when cytosine in at least one CpG pair existing in the recognition site of the methylation sensitive restriction enzyme in genomic DNA contained in the biological specimen is not methylated, the DNA having such a recognition site will be cleaved by the methylation sensitive restriction enzyme when it is subjected to a digestion treatment with the methylation sensitive restriction enzyme. Further, when cytosine in every CpG pair existing in the recognition site of the methylation sensitive restriction enzyme in genomic DNA contained in the biological specimen is

methylated, the DNA having such a recognition site will not be cleaved by the methylation sensitive restriction enzyme. Therefore, by conducting PCR using a pair of primers capable of amplifying the objective DNA region as will be described later after executing the digestion treatment, an amplified product by PCR will not be obtained when cytosine in at least one CpG pair existing in the recognition site of the methylation sensitive restriction enzyme in genomic DNA contained in the biological specimen is not methylated, and on the other hand, an amplified product by PCR will be obtained when cytosine in every CpG pair existing in the recognition site of the methylation sensitive restriction enzyme in genomic DNA contained in the biological specimen is methylated.

[0052] Concretely, First step may be executed, for example, in the following manner when genomic DNA contained in the biological specimen is genomic DNA from mammals. Genomic DNA from mammals is added with 3 μL of an optimum 10 x buffer (330 mM Tris-Acetate pH 7.9, 660 mM KOAc, 100 mM $MgOAc_2$, 5 mM Dithiothreitol), 3 μL of 1 mg/mL BSA aqueous solution, each 1.5 μL of a methylation sensitive restriction enzyme such as HpaII or HhaI (10 U/μL), and the resultant mixture is added with sterilized ultrapure water to make the liquid amount 30 μL, and incubated at 37°C for one to three hours.

[0053] Preferred embodiments of the treatment with a methylation-sensitive restriction enzyme in First step include addition of a masking oligonucleotide. In particular, the treatment may comprise First (A) step of mixing a single-stranded DNA (plus strand) containing the objective DNA region and a masking oligonucleotide comprising a nucleotide sequence complementary to a nucleotide sequence of a recognition site for a methylation-sensitive restriction enzyme, thereby selecting single-stranded DNA in which the recognition site for the methylation-sensitive restriction enzyme is protected; and First (B) step of digesting the single-stranded DNA selected in First (A) step with the methylation-sensitive restriction enzyme. In particular, for example, a masking oligonucleotide may be added to a solution for the treatment with a methylation-sensitive restriction enzyme.

[0054] By undergoing First (A) and First (B) steps, any DNA sample derived from a genomic DNA contained in a biological specimen may be digested with a methylation-sensitive restriction enzyme with double-stranded DNA exclusively as a substrate, even if the DNA sample is single-stranded DNA. First (A) and First (B) steps may be performed either simultaneously or sequentially.

[0055] The term "masking oligonucleotide" means oligonucleotide having a nucleotide sequence complementary to the nucleotide sequence of the recognition site of the methylation sensitive restriction enzyme, and is oligonucleotide that forms double strand by complementary base-pairing at least one site (even every site is possible) of several recognition sites of the methylation sensitive restriction enzyme contained in the objective DNA region in the single-stranded DNA (that is, the site is made into double-stranded state), thereby enabling the methylation sensitive restriction enzyme that uses only double-stranded DNA as a substrate to digest the site, and improving digestion efficiency at the site for the methylation sensitive restriction enzyme capable of digesting single-stranded DNA (methylation sensitive restriction enzyme capable of digesting single-stranded DNA also digests double-stranded DNA, and digestion efficiency thereof is higher with respect to double-stranded DNA than with respect to single-stranded DNA), and means oligonucleotide not inhibiting formation of double strand between single-stranded DNA containing the objective DNA region and single-stranded immobilized oligonucleotide. Further, when a sample is a single-stranded DNA, the masking oligonucleotide should be oligonucleotide that is unavailable in a reaction for extending an extension primer by using a later-described reverse primer (plus strand) as the extension primer and the masking oligonucleotide (minus strand) as a template. As a nucleotide length, 8 to 200 bases long is preferred.

[0056] The masking oligonucleotide to be mixed with a DNA sample derived from genomic DNA may be one kind or plural kinds. When plural kinds are used, many of recognition sites of the methylation sensitive restriction enzyme in the single-stranded DNA containing the objective DNA region become double-strand state, and "DNA remaining undigested" as will be described later by the methylation sensitive restriction enzyme can be minimized. For example, it is particularly useful to use the masking oligonucleotide designed in accordance with a site intended not to be digested when it is methylated and intended to be digested when it is not methylated among several recognition sequences of the methylation sensitive restriction enzyme contained in the objective DNA region (for example, the site that is methylated at 100% in a diseased patient specimen, but is not methylated at 100% in a healthy specimen).

[0057] As a concern in a digestion treatment in First step, a fear that a recognition sequence containing non-methylated cytosine cannot be completely digested (so called "DNA remaining undigested") can be recited. When such a fear is problematic, since the "DNA remaining undigested" can be minimized if recognition sites of the methylation sensitive restriction enzyme abundantly exist, it is considered that as the objective DNA region, the one having one or more recognition sites of the methylation sensitive restriction enzyme is preferred and the more the better.

[0058] One preferable embodiment is that "a DNA sample derived from a genomic DNA contained in a biological specimen" is a DNA sample digested in advance with a restriction enzyme recognition cleavage site for which in not present in the objective DNA region possessed by the genomic DNA. Here, when a digested substance of a genomic DNA contained in a biological specimen is selected with the use of present immobilized oligonucleotide, shorter template DNA is more likely to be selected, and when the objective region is amplified by PCR, shorter template DNA is more preferred. Therefore, a digestion treatment may be executed while using a restriction enzyme whose recognition cleavage

site excludes the objective DNA region directly on the DNA sample derived from a genomic DNA contained in a biological specimen. As a method of digesting with a restriction enzyme recognition cleavage site for which is not present in the objective DNA region, a commonly used restriction enzyme treatment method may be used. These embodiments are preferred because the methylation amount can be determined accurately by digesting the biological specimen itself in advance with a restriction enzyme as described above. Such a method is useful for avoiding the "DNA remaining undigested" as described above.

[0059] As a method of digesting a sample derived from a genomic DNA contained in a biological specimen with the methylation sensitive restriction enzyme, when the biological specimen is a genomic DNA itself, the method similar to that described above is preferred, and when the biological specimen is a tissue lysate, a cell lysate or the like, a digestion treatment may be executed using a large excess of methylation sensitive restriction enzyme, for example, a methylation sensitive restriction enzyme in an amount of 500 times (10 U) or more with respect to 25 ng of the DNA amount, according to a similar method as described above.

[0060] Basically, genomic DNA exists as double-stranded DNA. Therefore, in the present operation, not only a methylation sensitive restriction enzyme (for example, HhaI) capable of digesting single-stranded DNA, but also a methylation sensitive restriction enzyme capable of digesting double-stranded DNA (for example, HpaII, BstUI, NarI, SacII, HhaI and the like) may be used.

[0061] As another embodiment of First step, executing Second step without executing a digestion treatment with a methylation sensitive restriction enzyme capable of digesting single-stranded DNA can be recited. When there is no nucleotide sequence that is cleaved by a methylation sensitive restriction enzyme capable of digesting single-stranded DNA in the objective DNA region, Second step may be executed without executing First step.

[0062] In Second step of the present measuring method, methylated single-stranded DNA is obtained from the DNA sample that has been subjected to the digestion treatment and obtained in First step, and the single-stranded DNA is bound to an immobilized methylated DNA antibody, thereby selecting the single-stranded DNA. Second step may comprise Second (A) step of separating into methylated single-stranded DNA the methylated double-stranded DNA contained in the DNA sample that has been subjected to the digestion treatment and obtained in First step; and Second (B) step of binding the methylated single-stranded DNA obtained in Second (A) step to an immobilized methylated DNA antibody.

[0063] In Second (A) step of the present measuring method, in "separating into methylated single-stranded DNA the methylated double-stranded DNA contained in the DNA sample that has been subjected to the digestion treatment and obtained in First step", a commonly used operation for making double-stranded DNA into single-stranded DNA may be conducted. Concretely, a DNA sample derived from genomic DNA contained in a biological specimen may be dissolved in an appropriate amount of ultrapure water, heated at 95°C for 10 minutes, and rapidly cooled on ice.

[0064] In Second (B) step, the methylated single-stranded DNA obtained in Second (A) step is bound to an immobilized methylated DNA antibody, thereby selecting the single-stranded DNA. The immobilized methylated DNA antibody is used for selecting methylated single-stranded DNA from a DNA sample derived from genomic DNA contained in a biological specimen. The immobilized methylated DNA antibody may be one immobilizable to a support, and the expression "one immobilizable to a support" means that a immobilized methylated DNA antibody can be immobilized to a support directly or indirectly. For achieving such immobilization, a immobilized methylated DNA antibody may be immobilized to a support according to a commonly used genetic engineering operation method or a commercially available kit, apparatus or the like (binding to a solid phase). Concretely, a method of immobilizing a biotinylated immobilized methylated DNA antibody obtained by biotinylating an immobilized methylated DNA antibody to a support coated with streptavidin (for example, a PCR tube coated with streptavidin, magnetic beads coated with streptavidin and so on) can be recited.

[0065] Also there is a method of letting a molecule having an active functional group such as an amino group, a thiol group, or an aldehyde group covalently bind to an immobilized methylated DNA antibody, and letting the resultant bound body covalently bind to a support made of glass, a polysaccharide derivative, silica gel, the synthetic resin or thermostable plastic whose surface is activated with a silane coupling agent or the like. Covalent bonding may be achieved, for example, using a spacer formed by serially connecting five triglycerides, a cross linker or the like.

[0066] An immobilized methylated DNA antibody may be directly immobilized to a support, or an antibody against an immobilized methylated DNA antibody (secondary antibody) may be immobilized to a support, and a methylated antibody may be bound to the secondary antibody to achieve immobilization to a support.

[0067] It suffices that the present immobilized immobilized methylated DNA antibody is immobilized to a support when single-stranded DNA (plus strand) containing the objective DNA region is selected, and (1) immobilization may be achieved by binding between the present immobilized immobilized methylated DNA antibody and a support before binding between the single-stranded DNA (plus strand) and the present immobilized immobilized methylated DNA antibody, or (2) immobilization may be achieved by binding between the present immobilized methylated DNA antibody and a support after binding between the single-stranded DNA (plus strand) and the present immobilized immobilized methylated DNA antibody.

[0068] In Second (B) step, when "the single-stranded DNA is selected by binding between methylated single-stranded

DNA and an immobilized immobilized methylated DNA antibody," it may be concretely executed in the following manner, for example, using a "biotin-labeled biotinylated methylated cytosine antibody" as an immobilized immobilized methylated DNA antibody.

(a) An avidin-coated PCR tube is added with an appropriate amount (for example, 0.1 μg/50 μL) of a biotinylated methylated cytosine antibody, left still at room temperature for about an hour, to promote immobilization between the biotinylated methylated cytosine antibody and streptavidin. Then the remaining solution is removed and washing is performed. 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, pH 7.4)] is added in a proportion of 100 μL/tube, and the solution is removed. This washing operation is repeated several times, to leave the biotinylated methylated cytosine antibody immobilized to a support inside the PCR tube.

(b) Double-stranded DNA derived from genomic DNA contained in a biological specimen is mixed with a buffer (for example, 33 mM Tris-Acetate pH 7.9, 66 mM KOAc, 10 mM $MgOAc_2$, 0.5 mM Dithiothreitol) and heated at 95°C for several minutes. Then the reaction is rapidly cooled to about 0 to 4°C, and kept for several minutes at this temperature to cause formation of single-stranded DNA. Then the reaction is returned to room temperature.

(c) The formed single-stranded DNA is added to an avidin-coated PCR tube to which a biotinylated methylated cytosine antibody is immobilized, and then left still at room temperature for about an hour, to promote binding between the biotinylated methylated cytosine antibody and methylated single-stranded DNA among the single-stranded DNA (formation of a bound body)(in this stage, at least single-stranded DNA containing an unmethylated DNA region does not form a bound body). Thereafter, the remaining solution is removed and washing is performed. A washing buffer [for example, a 0.05% Tween 20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO{\cdot}7H_2O$, 154 mM NaCl, pH 7.4)] is added in a proportion of 100 μL/tube, and the solution is removed. This washing operation is repeated several times, to leave the bound body inside the PCR tube (selection of a bound body).

[0069] The buffer used in (b) is not limited to the above buffer and may be any buffer that is suited for separating double-stranded DNA derived from genomic DNA from a biological sample into single-stranded DNA.

[0070] The washing operation in (a) and (c) is important for removing an unimmobilized immobilized methylated DNA antibody suspended in the solution, unmethylated single-stranded DNA that does not bind with a immobilized methylated DNA antibody and hence is suspended in the solution, and DNA suspended in a solution digested by a restriction enzyme as will be described later, from the reaction solution. The washing buffer is not limited to the foregoing washing buffer, and any buffer suited for removing the free immobilized methylated DNA antibody, single-stranded DNA and so on suspended in the solution and the like is applicable, and a DELFIA buffer (available from Perkin Elmer, Tris-HCl pH 7.8 with Tween 80), a TE buffer and the like may be used.

[0071] In Second (A) step, as a preferred embodiment in separating methylated single-stranded DNA, addition of a counter oligonucleotide and the like can be recited. A counter oligonucleotide means a short oligonucleotide comprising a part of the same nucleotide sequence as that of the objective DNA region. It may be designed to have a length of usually 10 to 100 bases, and more preferably 20 to 50 bases. Here, a counter oligonucleotide is not designed on the nucleotide sequence where a forward primer or a reverse primer complementarily binds with the target DNA region. A counter oligonucleotide is added in excess relative to genomic DNA, and is added so as to prevent a complementary strand of a target DNA region (minus strand) and a single strand of a target DNA region (plus strand) from re-binding by complementation when binding with an immobilized methylated DNA antibody is caused after making a target DNA region into a single strand (plus strand). This is because in measuring a methylation frequency of DNA or an index value having correlation therewith while a methylated DNA antibody is bound to the target DNA region, the target region is more likely to bind with the methylated DNA antibody when it is a single strand. Preferably, a counter oligonucleotide is added in an amount of at least 10 times, usually 100 times or more relative to the target DNA region.

[0072] "Adding a counter oligonucleotide in separating methylated single-stranded DNA" may be concretely achieved by mixing a DNA sample derived from genomic DNA contained in a biological specimen with a counter oligonucleotide to form a double strand between the complementary strand of the target DNA region and the counter oligonucleotide so as to select methylated single-stranded DNA from a DNA sample derived from genomic DNA contained in a biological specimen. For example, the DNA sample and the counter oligonucleotide are added to 5 μL of a buffer (330 mM Tris-Acetate pH 7.9, 660 mM KOAc, 100 mM $MgOAc_2$, 5 mM Dithiothreitol), 5 μL of a 100 mM $MgCl_2$ solution, and 5 μL of a 1 mg/mL of BSA solution, and the resultant mixture is added with sterile ultrapure water to a liquid volume of 50 μL, and mixed, heated at 95°C for 10 minutes, rapidly cooled to 70°C, kept at this temperature for 10 minutes, then cooled to 50°C, kept at this temperature for 10 minutes, and then kept at 37°C for 10 minutes and returned to room temperature.

[0073] Third step comprises, as a pre step of each of the following regular steps:

a step (First pre step) of separating the single-stranded DNA selected in Second step from the immobilized immobilized methylated DNA antibody to provide DNA in a single-stranded state (plus strand);

a step (Second pre step) of extensionally-forming a double-stranded DNA from a single-stranded DNA (plus strand) containing the objective DNA region by a single extension of an extension primer, using the genomic DNA (plus strand) provided in a single-stranded state in First pre step and the extension primer, wherein the extension primer (forward primer) comprises the nucleotide sequence (minus strand) complementary to a partial nucleotide sequence (plus strand) of the nucleotide sequence of the DNA in a single-stranded state (plus strand), the partial nucleotide sequence (plus strand) being located on further 3'-end side than the 3'-end of the nucleotide sequence (plus strand) of the objective DNA region; and
a step (Third pre step) of temporarily separating the double-stranded DNA extensionally formed in Second pre step into a single-stranded DNA (plus strand) containing the objective DNA region and a single-stranded DNA (minus strand) containing the nucleotide sequence complementary to the objective DNA region;
and as regular steps:

(a) Step A (regular step) of extensionally forming double-stranded DNA from the single-stranded DNA containing the objective DNA region, by a single extension of the extension primer, using as a template the generated single-stranded DNA (plus strand) containing the objective DNA region, and the forward primer as the extension primer; and
(b) Step B (regular step) of extensionally forming double-stranded DNA from the single-stranded DNA containing the objective DNA region, by a single extension of an extension primer, using as a template the generated single-stranded DNA (minus strand) containing the nucleotide sequence complementary to the objective DNA region, and using as the extension primer an extension primer (reverse primer) comprising the nucleotide sequence (plus strand) complementary to a partial nucleotide sequence (minus strand) of the nucleotide sequence of the single-stranded DNA (minus strand) containing the nucleotide sequence complementary to the objective DNA region, the partial nucleotide sequence (minus strand) being located on further 3'-end side than the 3'-end of the nucleotide sequence (minus strand) complementary to the nucleotide sequence (plus strand) of the objective DNA region; and wherein Third step further comprises amplifying the methylated DNA in the objective DNA region to a detectable level by repeating each regular step of Third step after temporarily separating the extensionally formed double-stranded DNA obtained in each of the regular steps into a single-stranded state; and quantifying the amount of the amplified DNA.

[0074] In Third step, first, as First pre step step among the respective pre steps of the following regular steps, single-stranded DNA selected in Second step is temporarily separated from the immobilized immobilized methylated DNA antibody into DNA in a single-stranded state. Concretely, for example, by adding an annealing buffer to single-stranded DNA selected in Second step, a mixture is obtained. Then the resultant mixture is heated at 95°C for several minutes, to obtain DNA in a single-stranded state (plus strand). Thereafter, in Second pre step, concretely, for example, DNA in a single-stranded state (plus strand) obtained in First pre step and a forward primer are mixed in a solution that is prepared by adding 17.85 μL of sterile ultrapure water, 3 μL of an optimum buffer (for example, 100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$), 3 μL of 2 mM dNTP, and 6 μL of 5 N betaine, and adding the resultant mixture with 0.15 μL of AmpliTaq (a kind of DNA polymerase: 5 U/μL) to a liquid volume of 30 μL, and incubated at 37°C for about two hours, to extensionally form double-stranded DNA from the single-stranded DNA (plus strand) containing an objective DNA region. In Third pre C step, concretely, for example, the double-stranded DNA extensionally formed in Second pre step is added with an annealing buffer to obtain a mixture, and the DNA is temporarily separated into single-stranded DNA containing the target DNA region by heating the mixture at 95°C for several minutes.

[0075] Thereafter, the following regular steps are conducted.

(i) The reaction is rapidly cooled to a temperature lower than Tm of the forward primer by about 0 to 20°C, and kept at this temperature for several minutes for annealing the forward primer to the generated single-stranded DNA (plus strand) containing the target DNA region.
(ii) Thereafter, the reaction is returned to room temperature.
(iii) Double-stranded DNA is extensionally formed from single-stranded DNA comprising the nucleotide sequence complementary to the objective DNA region by one extension of an extension primer by using the DNA in a single-stranded state annealed in the above (i) as a template, and the forward primer as an extension primer (namely, Step A). Concretely, it may be executed, for example, according to the later-described explanation, or the operation method in an extension reaction in Second pre step of the present invention as described above.
(iv) Single-stranded DNA is made into extensionally formed double-stranded DNA by one extension of an extension primer by using the generated single-stranded DNA (minus strand) comprising the nucleotide sequence complementary to the target DNA region as a template, and an extension primer (reverse primer) comprising a nucleotide sequence (plus strand) which is complementary to a partial nucleotide sequence (minus strand) of the nucleotide sequence comprised by the single-stranded DNA (minus strand) containing the target DNA region, the partial nu-

cleotide sequence (minus strand) located on further 3'-end side than 3'-end of the nucleotide sequence (minus strand) complementary to the nucleotide sequence (plus strand) of the target DNA region as the extension primer (namely, Step B). Concretely, it may be executed, for example, according to the operation method in an extension reaction in Second pre step similarly to Step A of the above (iii).

(v) By repeating the regular steps of Third step after temporarily separating the extensionally formed double-stranded DNA obtained in each of the regular steps into a single-stranded state (for example, Step A and Step B), the methylated DNA in the objective DNA region is amplified to a detectable level and a content of the amplified DNA is quantified.

[0076] In Third step, concretely the reaction starting from First pre step and up to regular steps may be executed as a single PCR reaction. Also, from First pre step step to Third pre step, each reaction may be independently executed, and only regular steps may be executed as a PCR reaction.

[0077] As a method of amplifying an objective DNA region (namely, an objective region) contained in the selected single-stranded DNA, for example, PCR may be used. Using a primer preliminarily labeled with fluorescence or the like and utilizing the label as an index in amplifying the target region make it possible to evaluate presence or absence of an amplification product without executing a burdensome operation such as electrophoresis. As a PCR reaction solution, for example, a reaction solution obtained by mixing DNA obtained in Second step of the present measuring method with 0.15 μL of a 50 μM primer solution, 2.5 μL of 2 mM dNTP, 2.5 μL of a 10 x buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 20 mM $MgCl_2$, 0.01% Gelatin), and 0.2 μL of AmpliTaq Gold (one kind of thermostable DNA polymerase: 5 U/μL), and adding sterilized ultrapure water to make the liquid volume 25 μL can be recited.

[0078] Since an objective DNA region (namely, an objective region) often has a GC rich nucleotide sequence, the reaction may be occasionally executed with addition of an appropriate amount of betaine, DMSO or the like. In one exemplary reaction condition, the reaction solution as described above is kept at 95°C for 10 minutes, and then a cycle including incubation of 30 seconds at 95°C, 30 seconds at 55 to 65°C, and 30 seconds at 72°C is repeated 30 to 40 times. After conducting such PCR, the obtained amplification product is detected. For example, when a preliminarily labeled primer is used, an amplification amount by a PCR reaction can be evaluated by measuring an amount of a fluorescent label after executing washing and purification operations similar to those as described above. When PCR is conducted using a normal primer that is not labeled, a probe or the like that is labeled with a gold colloid particle, fluorescence or the like is caused to anneal, and detection may be achieved by measuring an amount of the probe bound to the target region. Also, for determining an amount of an amplification product more accurately, for example, a real time PCR method may be used. Real time PCR is a method in which PCR is monitored in real time, and the obtained monitor result is analyzed kinetically, and is known as a high-accuracy quantitative PCR method capable of detecting a very small difference of as small as twice in a gene amount. As such a real time PCR method, for example, a method using a probe such as a template-dependent nucleic acid polymerase probe, a method using an intercalator such as SYBR-Green and the like can be recited. As an apparatus and a kit for the real time PCR method, those commercially available may be used. As described above, detection may be executed by any method conventionally well-known without any particular limitation. These methods make it possible to conduct the operations up to detection without requiring change of the reaction container.

[0079] The present invention may be used in the following situations.

[0080] It is known that DNA methylation abnormality occurs in various diseases (for example, cancer), and it is believed that the degree of various diseases can be measured by detecting this DNA methylation abnormality.

[0081] For example, when there is a DNA region where methylation occurs at 100% in genomic DNA contained in a diseased biological specimen, and the present invention is executed for the DNA region, the amount of methylated DNA will increase. For example, when there is a DNA region where methylation does not occur at 100% in genomic DNA contained in a diseased biological specimen, and the present measuring method is executed for the DNA region, the amount of methylated DNA will be approximately 0. For example, when there is a DNA region which is in hypomethylation in genomic DNA contained in a specimen derived from a healthy subject, and in hypermethylation in genomic DNA contained in a specimen derived from a disease subject, and the present measuring method is executed for the DNA region, the amount of methylated DNA would be approximately 0 for the healthy subject, and a significantly higher value than that of the healthy subject will be exhibited by the disease patient, so that the "degree of disease" can be determined based on this difference in value. The "degree of disease" used herein has the same meaning commonly used in this field of art, and concretely means, for example, malignancy when the biological specimen is a cell, and means, for example, abundance of disease cells in the tissue when the biological specimen is a tissue. Further, when the biological specimen is plasma or serum, it means the probability that the individual has the disease. Therefore, the present measuring method makes it possible to diagnose various diseases by examining methylation abnormality.

[0082] Restriction enzymes, primers or probes that can be used in various methods for measuring a methylated DNA amount in a target region in the present measuring method are useful as reagents of a detection kit. The present invention also provides a detection kit containing these restriction enzymes, primers or probes as reagents, and a detection chip

in which these primers, probes and so on are immobilized on a support, and a scope of the present measuring method or the present methylation rate measuring method of course embraces use in the form of a detection kit or a detection chip as described above utilizing the substantial principle of the method.

Examples

[0083] In the following, the present invention will be explained in detail by way of examples, however, the present invention will not be limited to these examples.

Example 1

[0084] A commercially available methylated cytosine antibody (available from Aviva Systems Biology) was labeled with biotin using a commercially available biotinylating kit (Biotin Labeling Kit-NH_2, available from DOJINDO Laboratories) according to the method described in the catalogue. The obtained biotin-labeled methylated cytosine antibody was refrigerated as a solution [about 0.1 μg/100 μL solution of an antibody in a 0.1% BSA-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO \cdot 7H_2O$, 154 mM NaCl, pH 7.4)].

[0085] To each PCR tube coated with streptavidin (a total of 9 tubes), 50 μL of the synthetically obtained biotin-labeled methylcytosine antibody solution was added and immobilized to the PCR tube by leaving it still for about an hour at room temperature. Then, after removing the solution by pipetting, 100 μL of a washing buffer [0.05% Tween 20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO \cdot 7H_2O$, 154 mM NaCl, pH 7.4)] was added, and then the buffer was removed by pipetting. This operation was repeated another two times (these correspond to preparation of an immobilized methylated DNA antibody used in the present measuring method).

[0086] A partially methylated oligonucleotide GPR7-2079-2176/98 mer-M(7) in which a recognition site of HpaII comprising the nucleotide sequence of SEQ ID NO: 17 is methylated; a partially methylated oligonucleotide GPR7-2079-2176/98 mer-HM(5) in which part of a recognition site of HpaII having the nucleotide sequence of SEQ ID NO: 18 is not methylated; and an unmethylated oligonucleotide GPR7-2079-2176/98 mer-UM having the nucleotide sequence of SEQ ID NO: 19 were synthesized, and a 0.001 pmol/10 μL solution in a TE buffer was prepared for each oligonucleotide.

<Partially methylated oligonucleotide in which recognition sequence of HpaII is methylated>

[0087] N denotes methylated cytosine.

GPR7-2079-2176/98mer-M(7):

5′-

GTTGGCCACTGCGGAGTCGNGCNGGGTGGCNGGCCGCACCTACAGNGCCGNGNGNGCGGTGA

GCCTGGCCGTGTGGGGGATCGTCACACTCGTCGTGC-3′ (SEQ ID NO: 17)

<Partially methylated oligonucleotide in which recognition sequence of HpaII is not methylated>

[0088] N denotes methylated cytosine.

GPR7-2079-2176/98mer-HM(5):

5′-

GTTGGCCACTGCGGAGTCGCGCCGGGTGGCNGGCCGCACCTACAGNGCCGNGNGNGCGGTGA

GCCTGGCCGTGTGGGGGATCGTCACACTCGTCGTGC-3′ (SEQ ID NO: 18)

<Unmethylated oligonucleotide>

[0089]

GPR7-2079-2176/98mer-UM:

5'-

GTTGGCCACTGCGGAGTCGCGCCGGGTGGCCGGCCGCACCTACAGCGCCGCGCGCGCGGTGA

GCCTGGCCGTGTGGGGGATCGTCACACTCGTCGTGC-3' (SEQ ID NO: 19)

[0090] Each of the obtained solutions (each solution was prepared in triplicate) was subjected to the following A treatment, B treatment or C treatment (each prepared singly).

[0091] A treatment group (no treatment group): The sample prepared above was added with 5 μL of a buffer (330 mM Tris-Acetate pH 7.9, 660 mM KOAc, 100 mM $MgOAc_2$, 5 mM Dithiothreitol), and 5 μL of BSA (Bovine serum albumin 1 mg/mL), and the resultant mixture was added with sterile ultrapure water to a liquid volume of 50 μL.

[0092] B treatment group (HpaII treatment group): The sample prepared above was added with 5 μL of a buffer (330 mM Tris-Acetate pH 7.9, 660 mM KOAc, 100 mM $MgOAc_2$, 5 mM Dithiothreitol), 5 μL of BSA (Bovine serum albumin 1 mg/mL), and 10 U of HpaII, and the resultant mixture was added with sterile ultrapure water to a liquid volume of 50 μL.

[0093] C treatment group (addition of masking oligonucleotide + HpaII treatment group): The sample prepared above was added with 5 μL of a buffer (330 mM Tris-Acetate pH 7.9, 660 mM KOAc, 100 mM $MgOAc_2$, 5 mM Dithiothreitol), 5 μL of BSA (Bovine serum albumin 1 mg/mL), 10 U of HpaII, and 5 pmol of the oligonucleotide MA comprising the nucleotide sequence of SEQ ID NO: 20 as masking oligonucleotide, and the resultant mixture was added with sterile ultrapure water to a liquid volume of 50 μL.

<Masking oligonucleotide>

[0094] MA: 5'-GCCACCCGGCGCGA-3' (SEQ ID NO: 20)

[0095] Each reaction mixture was incubated overnight at 37°C (these correspond to First step of the present invention).

[0096] The PCR tube coated with streptavidin to which a biotin-labeled methylcytosine antibody was immobilized was added with 50 μL of a reaction solution of an oligonucleotide prepared as described above, and left still for an hour at room temperature. Then the solution was removed by pipetting, and 100 μL of a washing buffer [0.05% Tween 20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO \cdot 7H_2O$, 154 mM NaCl, pH 7.4)] was added, and then the buffer was removed by pipetting. This operation was repeated another two times (these correspond to Second step of the present invention).

[0097] Next, the above PCR tube was subjected to PCR using each solutions of a primer comprising the nucleotide sequences of SEQ ID NO: 21 and a primer comprising the nucleotide sequences of SEQ ID NO: 22 (PF1 and PR1), and the following reaction condition, to amplify methylated DNA in a target DNA region (GPR7-2079-2176, SEQ ID NO: 23, methylated cytosine is also denoted by C).

<Primers>

[0098]

PF1: 5'-GTTGGCCACTGCGGAGTCG-3' (SEQ ID NO: 21)
PR1: 5'-GCACGACGAGTGTGACGATC-3' (SEQ ID NO: 22)

<Target DNA region>

[0099]

GPR7-2079-2176: 5'-

GTTGGCCACTGCGGAGTCGCGCCGGGTGGCCGGCCGCACCTACAGCGCCGCGCGCGCGGTGA

GCCTGGCCGTGTGGGGGATCGTCACACTCGTCGTGC-3' (SEQ ID NO: 23)

[0100] A reaction solution of PCR was prepared by mixing each 5 μL of a solution of primer comprising the nucleotide sequence of SEQ ID NO: 21 and a solution of primer comprising the nucleotide sequence of SEQ ID NO: 22 prepared to 3 μM, each 5 μL of 2 mM dNTPs, 5 μL of a buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% Gelatin), 0.25 μL of 5 U/μL thermostable DNA polymerase (AmpliTaq Gold), and 10 μL of a 5 N betaine aqueous solution to DNA which is a template, and adding sterile ultrapure water to a liquid volume of 50 μL. The reaction solution was kept at 95°C for 10 minutes, and then subjected to PCR conducting 25 cycles of incubation each including 30 seconds at 95°C, 30 seconds at 59°C, and 45 seconds at 72°C.

[0101] After conducting PCR, the amplification of DNA was checked by 2% agarose gel electrophoresis (these correspond to Third step of the present invention). The result is shown in Fig. 1.

[0102] In the case of A treatment group (no treatment group), in the partially methylated oligonucleotide GPR7-2079-2176/98 mer-M(7) in which the recognition sequence of HpaII is methylated, and the partially methylated oligonucleotide GPR7-2079-2176/98 mer-HM(5) in which part of the recognition sequence of HpaII is not methylated, amplification of DNA was observed, and an amplification product thereof (target DNA region: GPR7-2079-2176) was obtained. In the unmethylated oligonucleotide GPR7-2079-2176/98 mer-UM, amplification of DNA was not observed, and an amplification product thereof was not obtained. Also in the case of B treatment group (HpaII treatment group), the result was similar to that in A treatment group. In the case of C treatment group (addition of masking oligonucleotide + HpaII treatment group), in the partially methylated oligonucleotide GPR7-2079-2176/98 mer-M(7) in which the recognition sequence of HpaII is methylated, amplification of DNA was observed, and an amplification product thereof (target DNA region: GPR7-2079-2176) was obtained. Contrarily, in the cases of the partially methylated oligonucleotide GPR7-2079-2176/98 mer-HM(5) in which part of the recognition sequence of HpaII is not methylated, and in the unmethylated oligonucleotide GPR7-2079-2176/98 mer-UM, amplification of DNA was not observed, and an amplification product thereof was not obtained.

[0103] From the above, it was demonstrated that single-stranded DNA containing a methylated target DNA region can be selected by an immobilized methylated cytosine antibody, and a target DNA region in which methylation sensitive restriction enzyme recognition site is unmethylated and protected by a masking oligonucleotide can be digested by a treatment with a methylation sensitive restriction enzyme after addition and mixing of a masking oligonucleotide, and only methylated DNA can be amplified to a detectable level and an amount of amplified DNA can be quantified while unmethylated DNA in the target DNA region is not amplified.

Example 2

[0104] A commercially available methylated cytosine antibody (available from Aviva Systems Biology) was labeled with biotin using a commercially available biotinylating kit (Biotin Labeling Kit-NH_2, available from DOJINDO Laboratories) according to the method described in the catalogue. The obtained biotin-labeled methylated cytosine antibody was refrigerated as a solution [about 0.25 μg/μL solution of an antibody in a 0.1% BSA-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO \cdot 7H_2O$, 154 mM NaCl, pH 7.4)].

[0105] To each PCR tube coated with streptavidin (a total of 8 tubes), 50 μL of the synthetically obtained biotin-labeled methylcytosine antibody solution was added and immobilized to the PCR tube by leaving it still for about an hour at room temperature. Then, after removing the solution by pipetting, 100 μL of a washing buffer [0.05% Tween 20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO \cdot 7H_2O$, 154 mM NaCl, pH 7.4)] was added, and then the buffer was removed by pipetting. This operation was repeated another two times (these correspond to preparation of an immobilized methylated DNA antibody used in the present measuring method).

[0106] For genomic DNA derived from human blood purchased from Clontech, a DNA fragment (X, SEQ ID NO: 26, a region corresponding to the base numbers 25687390 to 25687775 shown in Genbank Accession No. NT_029419 and so on) to be used as a test sample was amplified by conducting PCR using an oligonucleotide primer of SEQ ID NO: 24 and an oligonucleotide primer of SEQ ID NO: 25 (PF2 and PR2) and the following reaction condition.

<Oligonucleotide primers designed for PCR>

[0107]

PF2: 5'-CTCAGCACCCAGGCGGCC-3' (SEQ ID NO: 24)
PR2: 5'-CTGGCCAAACTGGAGATCGC-3' (SEQ ID NO: 25)

<DNA fragment>

[0108]

X: 5'-

CTCAGCACCCAGGCGGCCGCGATCATGAGGCGCGAGCGGCGCGCGGGCTGTTGCAGAGTCTT

GAGCGGGTGGCACACCGCGATGTAGCGGTCGGCTGTCATGACTACCAGCATGTAGGCCGACG

CAAACATGCCGAACACCTGCAGGTGCTTCACCACGCGGCACAGCCAGTCGGGGCCGCGGAAG

CGGTAGGTGATGTCCCAGCACATTTGCGGCAGCACCTGGAAGAATGCCACGGCCAGGTCGGC

CAGGCTGAGGTGTCGGATGAAGAGGTGCATGCGGGACGTCTTGCGCGGCGTCCGGTGCAGAG

CCAGCAGTACGCTGCTGTTGCCCAGCACGGCCACCGCGAAAGTCACCGCCAGCACGGCGATC

TCCAGTTTGGCCAG-3' (SEQ ID NO: 26)

[0109] As a reaction solution of PCR, 5 ng of genomic DNA which is a template, mixed with each 3 μL of oligonucleotide primer solutions prepared to 5 μM, each 5 μL of 2 mM dNTPs, 5 μL of a 10 × buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% Gelatin), and 0.25 μL of 5 U/μL thermostable DNA polymerase (AmpliTaq Gold, available from ABI), and added with sterile ultrapure water to a liquid volume of 50 μL was used. The reaction solution was kept at 95°C for 10 minutes, and then subjected to PCR conducting 40 cycles of incubation each including 30 seconds at 95°C, 30 seconds at 61°C, and 45 seconds at 72°C.

[0110] After conducting PCR, amplification was checked by 1.5% agarose gel electrophoresis, and a DNA fragment X was purified with Wizard SV Gel/PCR Kit (PROMEGA).

[0111] For a part of the obtained DNA fragment solution, a reaction solution was prepared by mixing 1 μL of SssI methylase (available from NEB), 10 μL of a 10 × NEBuffer 2 (available from NEB), and 1 μL of S-adenosyl methionine (3.2 mM, available from NEB), and adding sterile ultrapure water to a liquid volume of 100 μL. The reaction solution was incubated at 37°C for 15 to 30 minutes, and further added with 1 μL of S-adenosyl methionine (3.2 mM, available from NEB) and incubated at 37°C for 15 to 30 minutes. This was then purified with Wizard SV Gel/PCR Kit (PROMEGA). These operations were repeated another 5 times, to obtain a methylated DNA fragment (MX, SEQ ID NO: 27).

<DNA fragment> (N denotes 5-methylcytosine.)

[0112]

MX: 5′-

CTCAGCACCCAGGNGGCNGNGATCATGAGGNGNGAGNGGNGNGNGGGCTGTTGCAGAGTCTT

GAGNGGGTGGCACACNGNGATGTAGNGGTNGGCTGTCATGACTACCAGCATGTAGGCNGANG

CAAACATGCNGAACACCTGCAGGTGCTTCACCANGNGGCACAGCCAGTNGGGGCNGNGGAAG

NGGTAGGTGATGTCCCAGCACATTTGNGGCAGCACCTGGAAGAATGCCANGGCCAGGTNGGC

CAGGCTGAGGTGTNGGATGAAGAGGTGCATGNGGGANGTCTTGNGNGGNGTCNGGTGCAGAG

CCAGCAGTANGCTGCTGTTGCCCAGCANGGCCACNGNGAAAGTCACNGCCAGCANGGNGATC

TCCAGTTTGGCCAG-3′ (SEQ ID NO: 27)

[0113] For each obtained DNA fragment X, the following solutions were prepared.

Solution A: 100 pg/5 μL solution in TE
Solution B: 10 pg/5 μL solution in TE
Solution C: 1 pg/5 μL solution in TE
Solution D: TE solution (negative control solution)

[0114] For each obtained DNA fragment MX, the following solutions were prepared.

Solution MA: 100 pg/5 μL solution in TE
Solution MB: 10 pg/5 μL solution in TE
Solution MC: 1 pg/5 μL solution in TE
Solution MD: TE solution (negative control solution)

[0115] For each of the solutions of the DNA fragment X and the solutions of the methylated DNA fragment MX, the following treatment was executed.

[0116] Five (5) μL of the DNA fragment solution prepared above was added with 2 μL of a buffer (330 mM Tris-Acetate pH 7.9, 660 mM KOAc, 100 mM $MgOAc_2$, 5 mM Dithiothreitol), 2 μL of BSA (Bovine serum albumin 1 mg/ml), 12 U of a methylation sensitive restriction enzyme HpaII, and the resultant mixture was further added with sterile ultrapure water to a liquid volume of 20 μL. Each of the mixture was incubated 37°C for 3 hours (these correspond to First step of the present measureing method).

[0117] Counter oligonucleotides C1 to C12 comprising the nucleotide sequences of SEQ ID NO: 29 to SEQ ID NO: 40 capable of complementarily base-pairing with a minus strand of the target DNA region X' comprising the nucleotide sequence of SEQ ID NO: 28 were synthesized, and each 0.01 μM solutions in TE buffer were prepared.

<Target DNA region>

[0118]

X': 5'-

CTCAGCACCCAGGCGGCCGCGATCATGAGGCGCGAGCGGCGCGCGGGCTGTTGCAGAGTCTT

GAGCGGGTGGCACACCGCGATGTAGCGGTCGGCTGTCATGACTACCAGCATGTAGGCCGACG

CAAACATGCCGAACACCTGCAGGTGCTTCACCACGCGGCACAGCCAGTCGGGGCCGCGGAAG

CGGTAGGTGATGTCCCAGCACATTTGCGGCAGCACCTGGAAGAATGCCACGGCCAGGTCGGC

CAGGCTGAGGTGTCGGATGAAGAGGTGCATGCGGGACGTCTTGCGCGGCGTCCGGTGCAGAG

CCAGCAGTACGCTGCTGTTGCCCAGCACGGCCACCGCGAAAGTCACCGCCAGCACGGCGATC

TCCAGTTTGGCCAG-3' (SEQ ID NO: 28)

<Counter oligonucleotides>

[0119]

C1:5' - GCCACCGCGAAAGTCACCGCCAGCACGGCG -3' (SEQ ID NO: 29)
C2:5' - GCCAGCAGTACGCTGCTGTTGCCCAGCACG -3' (SEQ ID NO: 30)
C3:5' - CGGGACGTCTTGCGCGGCGTCCGGTGCAGA -3' (SEQ ID NO: 31)
C4:5' - AGGCTGAGGTGTCGGATGAAGAGGTGCATG -3' (SEQ ID NO: 32)
C5:5' - ACCTGGAAGAATGCCACGGCCAGGTCGGCC -3' (SEQ ID NO: 33)
C6:5' - TAGGTGATGTCCCAGCACATTTGCGGCAGC -3' (SEQ ID NO: 34)
C7:5' - CGGCACAGCCAGTCGGGGCCGCGGAAGCGG -3' (SEQ ID NO: 35)
C8:5' - ATGCCGAACACCTGCAGGTGCTTCACCACG -3' (SEQ ID NO: 36)
C9:5' - ATGACTACCAGCATGTAGGCCGACGCAAAC -3' (SEQ ID NO: 37)
C10:5' - TGGCACACCGCGATGTAGCGGTCGGCTGTC -3' (SEQ ID NO: 38)
C11:5' - CGCGCGGGCTGTTGCAGAGTCTTGAGCGGG -3' (SEQ ID NO: 39)
C12:5' - CAGGCGGCCGCGATCATGAGGCGCGAGCGG -3' (SEQ ID NO: 40)

[0120] To the above reaction solution, 10 μL of a counter oligonucleotide solution prepared as described above, 5 μL of a buffer (330 mM Tris-Acetate pH 7.9, 660 mM KOAc, 100 mM $MgOAc_2$, 5 mM Dithiothreitol), 5 μL of a 100 mM $MgCl_2$ solution, and 5 μL of a 1 mg/mL BSA solution were added, and the resultant mixture was added with sterile ultrapure water to a liquid volume of 50 μL, and mixed. Thereafter, this PCR tube was heated at 95°C for 10 minutes, rapidly cooled to 70°C, and kept at this temperature for 10 minutes. Then the tube was cooled to 50°C and kept at this temperature for 10 minutes, and further kept at 37°C for 10 minutes, and returned to room temperature (these correspond to Second step of the present measuring method).

[0121] The PCR tube coated with streptavidin to which a biotin-labeled methylcytosine antibody was immobilized was added with 50 μL of a reaction solution of a DNA fragment prepared as described above, and left still for 30 minutes at room temperature. Then the solution was removed by pipetting, and 100 μL of a washing buffer [0.05% Tween 20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO \cdot 7H_2O$, 154 mM NaCl, pH 7.4)] was added, and then the buffer was removed by pipetting. This operation was repeated another two times (these correspond to Third step of the present measuring method).

[0122] Then by subjecting the above PCR tube to PCR using respective solutions of oligonucleotide primers PF2 and PR2 comprising the nucleotide sequences of SEQ ID NO: 24 and SEQ ID NO: 25, and the following reaction condition, methylated DNA in a target DNA region X' comprising the nucleotide sequence of SEQ ID NO: 28 was amplified.

<Oligonucleotide primers designed for PCR>

[0123]

PF2: 5'-CTCAGCACCCAGGCGGCC-3' (SEQ ID NO: 24)
PR2: 5'-CTGGCCAAACTGGAGATCGC-3' (SEQ ID NO: 25)

<Target DNA region>

[0124]

X': 5'-

CTCAGCACCCAGGCGGCCGCGATCATGAGGCGCGAGCGGCGCGCGGGCTGTTGCAGAGTCTT

GAGCGGGTGGCACACCGCGATGTAGCGGTCGGCTGTCATGACTACCAGCATGTAGGCCGACG

CAAACATGCCGAACACCTGCAGGTGCTTCACCACGCGGCACAGCCAGTCGGGGCCGCGGAAG

CGGTAGGTGATGTCCCAGCACATTTGCGGCAGCACCTGGAAGAATGCCACGGCCAGGTCGGC

CAGGCTGAGGTGTCGGATGAAGAGGTGCATGCGGGACGTCTTGCGCGGCGTCCGGTGCAGAG

CCAGCAGTACGCTGCTGTTGCCCAGCACGGCCACCGCGAAAGTCACCGCCAGCACGGCGATC

TCCAGTTTGGCCAG-3' (SEQ ID NO: 28)

[0125] As a reaction solution of PCR, DNA which is a template, mixed with each 3 μL of oligonucleotide primer solutions prepared to 5 μM, each 5 μL of 2 mM dNTPs, 5 μL of a buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% Gelatin), and 0.25 μL of 5 U/μL thermostable DNA polymerase (AmpliTaq Gold, available from ABI), and added with sterile ultrapure water to a liquid volume of 50 μL was used. The reaction solution was kept at 95°C for 10 minutes, and then subjected to PCR conducting 25 cycles of incubation each including 20 seconds at 95°C, 30 seconds at 61°C, and 30 seconds at 72°C.

[0126] After conducting PCR, amplification was checked by 1.5% agarose gel electrophoresis (these correspond to Fourth step of the present measuring method).

[0127] The result is shown in Fig. 2. In Solutions MA, MB and MC of the methylated DNA fragment MX, amplification was observed, and an amplification product thereof was obtained. In the negative control solution MD, amplification of DNA was not observed, and an amplification product was not obtained. In solutions A, B, C and D of the unmethylated DNA fragment X, amplification was not observed, and an amplification product thereof was not obtained.

[0128] From the above, it was demonstrated that DNA containing a methylated target DNA region can be selected by an immobilized methylcytosine antibody, and amplified DNA can be detected with higher sensitivity by amplifying methylated DNA to a detectable level.

Example 3

[0129] A commercially available methylated cytosine antibody (available from Aviva Systems Biology) was labeled with biotin using a commercially available biotinylating kit (Biotin Labeling Kit-NH_2, available from DOJINDO Laboratories) according to the method described in the catalogue. The obtained biotin-labeled methylated cytosine antibody was refrigerated as a solution [about 0.25 μg/μL solution of an antibody in a 0.1% BSA-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO \cdot 7H_2O$, 154 mM NaCl, pH 7.4)].

[0130] To each PCR tube coated with streptavidin (a total of 8 tubes), 50 μL of 0.1 μg/50 μL solution of the synthetically obtained biotin-labeled methylcytosine antibody was added and immobilized to the PCR tube by leaving it still for about an hour at room temperature. Then, after removing the solution by pipetting, 100 μL of a washing buffer [0.05% Tween 20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO \cdot 7H_2O$, 154 mM NaCl, pH 7.4)] was added, and then the buffer was removed by pipetting. This operation was repeated another two times (these correspond to preparation of an immobilized methylated DNA antibody used in the present measuring method).

[0131] For genomic DNA derived from human blood purchased from Clontech, a DNA fragment (Y, SEQ ID NO: 43, a region corresponding to the base numbers 76606 to 76726 shown in Genbank Accession No. ac009800 and so on) to be used as a test sample was amplified by conducting PCR using oligonucleotide primers (PF3 and PR3) of SEQ ID NO: 42 and SEQ ID NO: 43 and the following reaction condition.

<Oligonucleotide primers designed for PCR>

[0132]

PF3: 5'-TGAGCTCCGTAGGGCGTCC-3' (SEQ ID NO: 41)
PR3: 5'-GCGCCGGGTCCGGGCCC-3' (SEQ ID NO: 42)

<DNA fragment>

[0133]

Y: 5'-

GCGCCGGGTCCGGGCCCGATGCGTTGGCGGGCCAGGGCTCCGAGAACGAGGCGTTGTCCATC

TCAACGAGGGCAGAGGAGCCGGCGACCTGGCGTCCCCCAAGGACGCCCTACGGAGCTCA-3'

(SEQ ID NO: 43)

[0134] As a reaction solution of PCR, 5 ng of genomic DNA which is a template, mixed with each 3 μL of oligonucleotide primer solutions prepared to 5 μM, each 5 μL of 2 mM dNTPs, 5 μL of a 10 × buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% Gelatin), and 0.25 μL of 5 U/μL thermostable DNA polymerase (AmpliTaq Gold, available from ABI), and added with sterile ultrapure water to a liquid volume of 50 μL was used. The reaction solution was kept at 95°C for 10 minutes, and then subjected to PCR conducting 50 cycles of incubation each including 30 seconds at 95°C, 30 seconds at 60°C, and 45 seconds at 72°C.

[0135] After conducting PCR, amplification of DNA was checked by 1.5% agarose gel electrophoresis, and a DNA fragment Y was purified with Wizard SV Gel/PCR Kit (PROMEGA).

[0136] For a part of the obtained DNA fragment solution, a reaction solution was prepared by mixing 1 μL of SssI methylase (available from NEB), 10 μL of a 10 × NEBuffer 2 (available from NEB), and 1 μL of S-adenosyl methionine (3.2 mM, available from NEB), and adding sterile ultrapure water to a liquid volume of 100 μL. The reaction solution was incubated at 37°C for 15 to 30 minutes, and further added with 1 μL of S-adenosyl methionine (3.2 mM, available from NEB) and incubated at 37°C for 15 to 30 minutes. This was then purified with Wizard SV Gel/PCR Kit (PROMEGA). These operations were repeated another 5 times, to obtain a methylated DNA fragment (MY, SEQ ID NO: 44).

<DNA fragment> (N denotes 5-methylcytosine.)

[0137]

MY: 5'-

GNGCNGGGTCNGGGCCNGATGNGTTGGNGGGCCAGGGCTCNGAGAANGAGGNGTTGTCCATC

TCAANGAGGGCAGAGGAGCNGGNGACCTGGNGTCCCCCAAGGANGCCCTANGGAGCTCA-3'

(SEQ ID NO: 44)

[0138] For each obtained DNA fragment Y, the following solutions were prepared.

Solution A: 100 pg/5 μL solution in TE
Solution B: 10 pg/5 μL solution in TE
Solution C: 1 pg/5 μL solution in TE

Solution D: TE solution (negative control solution)

[0139] For each obtained DNA fragment MY, the following solutions were prepared.

Solution MA: 100 pg/5 μL solution in TE
Solution MB: 10 pg/5 μL solution in TE
Solution MC: 1 pg/5 μL solution in TE
Solution MD: TE solution (negative control solution)

[0140] For each of the solutions of the DNA fragment Y and the solutions of the methylated DNA fragment MY, the following treatment was executed.

[0141] Five (5) μL of the DNA fragment solution prepared above was added with 2 μL of a buffer (330 mM Tris-Acetate pH 7.9, 660 mM KOAc, 100 mM $MgOAc_2$, 5 mM Dithiothreitol), 2 μL of BSA (Bovine serum albumin 1 mg/ml), 12 U of a methylation sensitive restriction enzyme HpaII, and the resultant mixture was further added with sterile ultrapure water to a liquid volume of 20 μL. Each of the mixture was incubated 37°C for 3 hours (these correspond to First step of the present measureing method).

[0142] Counter oligonucleotides C13, C14, and C15 comprising the each nucleotide sequences of SEQ ID NO: 46, SEQ ID NO: 47, and SEQ ID NO: 48 capable of complementarily base-pairing with a minus strand of the target DNA region Y' comprising the nucleotide sequence of SEQ ID NO: 45 were synthesized, and each 0.01 μM solutions in a TE buffer were prepared.

<Target DNA region>

[0143]

Y': 5'-

GCGCCGGGTCCGGGCCCGATGCGTTGGCGGGCCAGGGCTCCGAGAACGAGGCGTTGTCCATC

TCAACGAGGGCAGAGGAGCCGGCGACCTGGCGTCCCCCAAGGACGCCCTACGGAGCTCA-3'

(SEQ ID NO: 45)

<Counter oligonucleotides>

[0144]

C13: 5'-GCGTCCCCCAAGGACGCCCTACGGAGCTCA-3' (SEQ ID NO: 46)
C14: 5'-CTCAACGAGGGCAGAGGAGCCGGCGACCTG-3' (SEQ ID NO: 47)
C15: 5'-CGCCGGGTCCGGGCCCGATGCGTTGGCGGG-3' (SEQ ID NO: 48)

[0145] To the above reaction solution, 10 μL of a counter oligonucleotide solution prepared as described above, 5 μL of a buffer (330 mM Tris-Acetate pH 7.9, 660 mM KOAc, 100 mM $MgOAc_2$, 5 mM Dithiothreitol), 5 μL of a 100 mM $MgCl_2$ solution, and 5 μL of a 1 mg/mL BSA solution were added, and the resultant mixture was added with sterile ultrapure water to a liquid volume of 50 μL, and mixed. Thereafter, this PCR tube was heated at 95°C for 10 minutes, rapidly cooled to 70°C, and kept at this temperature for 10 minutes. Then the tube was cooled to 50°C and kept at this temperature for 10 minutes, and further kept at 37°C for 10 minutes, and returned to room temperature (these correspond to Second step of the present measuring method).

[0146] The PCR tube coated with streptavidin to which a biotin-labeled methylcytosine antibody was immobilized was added with 50 μL of a reaction solution of a DNA fragment prepared as described above, and left still for 30 minutes at room temperature. Then the solution was removed by pipetting, and 100 μL of a washing buffer [0.05% Tween 20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO \cdot 7H_2O$, 154 mM NaCl, pH 7.4)] was added, and then the buffer was removed by pipetting. This operation was repeated another two times (these correspond to Third step of the present measuring method).

[0147] Then by subjecting the above PCR tube to PCR using respective solutions of oligonucleotide primers PF3 and PR3 comprising the nucleotide sequences of SEQ ID NO: 41 and SEQ ID NO: 42, and the following reaction condition,

methylated DNA in a target DNA region Y' comprising the nucleotide sequence of SEQ ID NO: 45 was amplified.

<Oligonucleotide primers designed for PCR>

[0148]

PF3: 5'-TGAGCTCCGTAGGGCGTCC-3' (SEQ ID NO: 41)
PR3: 5'-GCGCCGGGTCCGGGCCC-3' (SEQ ID NO: 42)

<Target DNA region>

[0149]

Y': 5'-
GCGCCGGGTCCGGGCCCGATGCGTTGGCGGGCCAGGGCTCCGAGAACGAGGCGTTGTCCATC
TCAACGAGGGCAGAGGAGCCGGCGACCTGGCGTCCCCCAAGGACGCCCTACGGAGCTCA-3'
(SEQ ID NO: 45)

[0150] As a reaction solution of PCR, DNA which is a template, mixed with each 3 μL of oligonucleotide primer solutions prepared to 5 μM, each 5 μL of 2 mM dNTPs, 5 μL of a 10 × buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% Gelatin), and 0.25 μL of 5 U/μL thermostable DNA polymerase (AmpliTaq Gold, available from ABI), and added with sterile ultrapure water to a liquid volume of 50 μL was used. The reaction solution was kept at 95°C for 10 minutes, and then subjected to PCR conducting 25 cycles of incubation each including 20 seconds at 95°C, 30 seconds at 60°C, and 30 seconds at 72°C.

[0151] After conducting PCR, amplification of DNA was checked by 1.5% agarose gel electrophoresis (these correspond to Third step of the present measuring method).

[0152] The result is shown in Fig. 3. In Solutions MA, MB and MC of the methylated DNA fragment MY, amplification was observed. In the negative control solution MD, amplification of DNA was not observed. In solutions A, B, C and D of the unmethylated DNA fragment Y, amplification of DNA was not observed.

[0153] From the above, it was demonstrated that DNA containing a methylated target DNA region can be selected by an immobilized methylcytosine antibody, and amplified DNA can be detected with higher sensitivity by amplifying methylated DNA to a detectable level.

Example 4

[0154] A commercially available methylated cytosine antibody (available from Aviva Systems Biology) was labeled with biotin using a commercially available biotinylating kit (Biotin Labeling Kit-NH_2, available from DOJINDO Laboratories) according to the method described in the catalogue. The obtained biotin-labeled methylated cytosine antibody was refrigerated as a solution [about 0.25 μg/μL solution of an antibody in a 0.1% BSA-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO \cdot 7H_2O$, 154 mM NaCl, pH 7.4)].

[0155] To each PCR tube coated with streptavidin (a total of 8 tubes), 50 μL of 0.1 μg/50 μL solution of the synthetically obtained biotin-labeled methylcytosine antibody was added and immobilized to the PCR tube by leaving it still for about an hour at room temperature. Then, after removing the solution by pipetting, 100 μL of a washing buffer [0.05% Tween 20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO \cdot 7H_2O$, 154 mM NaCl, pH 7.4)] was added, and then the buffer was removed by pipetting. This operation was repeated another two times (these correspond to preparation of an immobilized methylated DNA antibody used in the present measuring method).

[0156] 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).

[0157] The prepared yeast cells were suspended in Buffer A (1 M sorbitol, 0.1 M EDTA, pH 7.4), added with 0.1% 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.

[0158] From the obtained genomic DNA, a DNA fragment to be used as a test sample (T, SEQ ID NO: 51, a region corresponding to the base numbers 384569 to 384685 of yeast chromosome VII shown in Genbank Accession No. NC_001139 and so on) was amplified by conducting PCR using oligonucleotide primers (PF4 and PR4) designed for PCR of SEQ ID NO: 49 and SEQ ID NO: 50 and the following reaction condition.

<Oligonucleotide primers designed for PCR>

[0159]

PF4: 5'-GGACCTGTGTTTGACGGGTAT-3' (SEQ ID NO: 49)
PR4: 5'-AGTACAGATCTGGCGTTCTCG-3' (SEQ ID NO: 50)

<DNA fragment>

[0160]

T: 5'-

GGACCTGTGTTTGACGGGTATAACACTAAGTTGCGCAATTTGCTGTATTGCGAAATCCGCCC

GGACGATATCACTCTTGAGCGCATGTGCCGTTTCCGAGAACGCCAGATCTGTACT-3'

(SEQ ID NO: 51)

[0161] As a reaction solution of PCR, 10 ng of genomic DNA which is a template, mixed with each 3 μL of oligonucleotide primer solutions prepared to 5 μM, each 5 μL of 2 mM dNTPs, 5 μL of a 10 × buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% Gelatin), and 0.25 μL of 5 U/μL thermostable DNA polymerase (AmpliTaq Gold, available from ABI), and added with sterile ultrapure water to a liquid volume of 50 μL was used. The reaction solution was kept at 95°C for 10 minutes, and then subjected to PCR conducting 40 cycles of incubation each including 20 seconds at 95°C, 30 seconds at 58°C, and 30 seconds at 72°C.

[0162] After conducting PCR, amplification was checked by 1.5% agarose gel electrophoresis, and a DNA fragment T was purified with Wizard SV Gel/PCR Kit (PROMEGA).

[0163] For a part of the obtained DNA fragment solution, a reaction solution was prepared by mixing 1 μL of SssI methylase (available from NEB), 10 μL of a 10 × NEBuffer 2 (available from NEB), and 1 μL of S-adenosyl methionine (3.2 mM, available from NEB), and adding sterile ultrapure water to a liquid volume of 100 μL. The reaction solution was incubated at 37°C for 15 to 30 minutes, and further added with 1 μL of S-adenosyl methionine (3.2 mM, available from NEB) and incubated at 37°C for 15 to 30 minutes. This was then purified with Wizard SV Gel/PCR Kit (PROMEGA). These operations were repeated another 3 times, to obtain a methylated DNA fragment (MT, SEQ ID NO: 52).

<DNA fragment> (N denotes 5-methylcytosine.)

[0164]

MT: 5′-

GGACCTGTGTTTGANGGGTATAACACTAAGTTGNGCAATTTGCTGTATTGNGAAATCNGCCN

GGANGATATCACTCTTGAGNGCATGTGCNGTTTCNGAGAANGCCAGATCTGTACT-3′

(SEQ ID NO: 52)

[0165] For each obtained DNA fragment T, the following solutions were prepared.

Solution A: 100 pg/5 μL solution in TE
Solution B: 10 pg/5 μL solution in TE
Solution C: 1 pg/5 μL solution in TE
Solution D: TE solution (negative control solution)

[0166] For each obtained DNA fragment MZ, the following solutions were prepared.

Solution MA: 100 pg/5 μL solution in TE
Solution MB: 10 pg/5 μL solution in TE
Solution MC: 1 pg/5 μL solution in TE
Solution MD: TE solution (negative control solution)

[0167] For each of the solutions of the DNA fragment T and the solutions of the methylated DNA fragment MT, the following treatment was executed.

[0168] Five (5) μL of the DNA fragment solution prepared above was added with 2 μL of a buffer (330 mM Tris-Acetate pH 7.9, 660 mM KOAc, 100 mM $MgOAc_2$, 5 mM Dithiothreitol), 2 μL of BSA (Bovine serum albumin 1 mg/ml), 12 U of a methylation sensitive restriction enzyme HpaII, and the resultant mixture was further added with sterile ultrapure water to a liquid volume of 20 μL. Each of the mixture was incubated 37°C for 3 hours (these correspond to First step of the present measureing method).

[0169] Counter oligonucleotides C16 to C19 each comprising the nucleotide sequences of SEQ ID NO: 54 to SEQ ID NO: 57 capable of complementarily base-pairing with a minus strand of the target DNA region T' comprising the nucleotide sequence of SEQ ID NO: 53 were synthesized, and each 0.01 μM solutions in a TE buffer were prepared.

<Target DNA region>

[0170]

T′: 5′-

GGACCTGTGTTTGACGGGTATAACACTAAGTTGCGCAATTTGCTGTATTGCGAAATCCGCCC

GGACGATATCACTCTTGAGCGCATGTGCCGTTTCCGAGAACGCCAGATCTGTACT-3′

(SEQ ID NO: 53)

<Counter oligonucleotides>

[0171]

C16: 5'-GGACCTGTGTTTGACGGGTAT-3' (SEQ ID NO: 54)
C17: 5'-AACACTAAGTTGCGCAATTTGCTGT-3' (SEQ ID NO: 55)
C18: 5'-ATTGCGAAATCCGCCCGGACGATAT-3' (SEQ ID NO: 56)
C19: 5'-CACTCTTGAGCGCATGTGCCGTTTC-3' (SEQ ID NO: 57)

[0172] To the above reaction solution, 10 μL of a counter oligonucleotide solution prepared as described above, 5 μL of a buffer (330 mM Tris-Acetate pH 7.9, 660 mM KOAc, 100 mM $MgOAc_2$, 5 mM Dithiothreitol), 5 μL of a 100 mM $MgCl_2$ solution, and 5 μL of a 1 mg/mL BSA solution were added, and the resultant mixture was added with sterile ultrapure water to a liquid volume of 50 μL, and mixed. Thereafter, this PCR tube was heated at 95°C for 10 minutes, rapidly cooled to 70°C, and kept at this temperature for 10 minutes. Then the tube was cooled to 50°C and kept at this temperature for 10 minutes, and further kept at 37°C for 10 minutes, and returned to room temperature (these correspond to Second step of the present measuring method).

[0173] The PCR tube coated with streptavidin to which a biotin-labeled methylcytosine antibody was immobilized was added with 50 μL of a reaction solution of a DNA fragment prepared as described above, and left still for 30 minutes at room temperature. Then the solution was removed by pipetting, and 100 μL of a washing buffer [0.05% Tween 20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO \cdot 7H_2O$, 154 mM NaCl, pH 7.4)] was added, and then the buffer was removed by pipetting. This operation was repeated another two times (these correspond to Third step of the present measuring method).

[0174] Then the above PCR tube was subjected to PCR using respective solutions of oligonucleotide primers PF4 and PR4 comprising the nucleotide sequences of SEQ ID NO: 49 and SEQ ID NO: 50, and the following reaction condition, methylated DNA in a target DNA region T' comprising the nucleotide sequence of SEQ ID NO: 53 was amplified.

<Oligonucleotide primers designed for PCR>

[0175]

PF4: 5'-GGACCTGTGTTTGACGGGTAT-3' (SEQ ID NO: 49)
PR4: 5'-AGTACAGATCTGGCGTTCTCG-3' (SEQ ID NO: 50)

<Target DNA region> (Also 5-methylcytosine is denoted by C.)

[0176]

T': 5'-

GGACCTGTGTTTGACGGGTATAACACTAAGTTGCGCAATTTGCTGTATTGCGAAATCCGCCC

GGACGATATCACTCTTGAGCGCATGTGCCGTTTCCGAGAACGCCAGATCTGTACT-3'

(SEQ ID NO: 53)

[0177] As a reaction solution of PCR, DNA which is a template, mixed with each 3 μL of oligonucleotide primer solutions prepared to 5 μM, each 5 μL of 2 mM dNTPs, 5 μL of a buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% Gelatin), and 0.25 μL of 5 U/μL thermostable DNA polymerase (AmpliTaq Gold, available from ABI), and added with sterile ultrapure water to a liquid volume of 50 μL was used. The reaction solution was kept at 95°C for 10 minutes, and then subjected to PCR conducting 28 cycles of incubation each including 20 seconds at 95°C, 30 seconds at 58°C, and 30 seconds at 72°C.

[0178] After conducting PCR, amplification of DNA was checked by 1.5% agarose gel electrophoresis (these correspond to Fourth step of the present measuring method).

[0179] The result is shown in Fig. 4. In Solutions MA, MB and MC of the methylated DNA fragment MT, amplification of DNA was observed, and an amplification product thereof was obtained. In the negative control solution MD, amplification of DNA was not observed, and an amplification product was not obtained. In solutions A, B, C and D of the unmethylated DNA fragment T, amplification of DNA was not observed, and an amplification product thereof was not obtained.

[0180] From the above, it was demonstrated that DNA containing a methylated target DNA region can be selected by an immobilized methylcytosine antibody, and amplified DNA can be detected with higher sensitivity by amplifying methylated DNA to a detectable level.

Example 5

[0181] A commercially available methylated cytosine antibody (available from Aviva Systems Biology) was labeled with biotin using a commercially available biotinylating kit (Biotin Labeling Kit-NH_2, available from DOJINDO Laboratories)

according to the method described in the catalogue. The obtained biotin-labeled methylated cytosine antibody was refrigerated as a solution [about 0.25 μg/μL solution of an antibody in a 0.1% BSA-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO \cdot 7H_2O$, 154 mM NaCl, pH 7.4)].

[0182] To each PCR tube coated with streptavidin (a total of 8 tubes), 50 μL of 0.1 μg/50 μL solution of the synthetically obtained biotin-labeled methylcytosine antibody was added and immobilized to the PCR tube by leaving it still for about an hour at room temperature. Then, after removing the solution by pipetting, 100 μL of a washing buffer [0.05% Tween 20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO \cdot 7H_2O$, 154 mM NaCl, pH 7.4)] was added, and then the buffer was removed by pipetting. This operation was repeated another two times (these correspond to preparation of an immobilized methylated DNA antibody used in the present measuring method).

[0183] 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).

[0184] The prepared yeast cells were suspended in Buffer A (1 M sorbitol, 0.1 M EDTA, pH 7.4), added with 0.1% 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.

[0185] Part of the obtained genomic DNA was mixed with 1 μL of SssI methylase (available from NEB), 10 μ*L of a 10 × NEBuffer 2 (available from NEB), and 1 μL of S-adenosyl methionine (3.2 mM, available from NEB), and added with sterile ultrapure water to a liquid volume of 100 μL. The reaction solution was incubated at 37°C for 15 to 30 minutes, added with 1 μL of S-adenosyl methionine (3.2 mM, available NEB) and incubated at 37°C for 15 to 30 minutes. This was then purified by Wizard SV Gel/PCR Kit (PROMEGA). This operation was repeated three times, and methylated genomic DNA was obtained.

[0186] For the obtained yeast genomic DNA, the following solutions were prepared.

Solution A: 10 ng/5 μL solution in TE
Solution B: 1 ng/5 μL solution in TE
Solution C: 0.1 ng/5 μL solution in TE
Solution D: TE solution (negative control solution)

[0187] For the obtained methylated yeast genomic DNA, the following solutions were prepared.

Solution MA: 10 ng/5 μL solution in TE
Solution MB: 1 ng/5 μL solution in TE
Solution MC: 0.1 ng/5 μL solution in TE
Solution MD: TE solution (negative control solution)

[0188] For each of the above yeast genomic DNA solutions and methylated yeast genomic DNA solutions, the following treatment was executed.

[0189] A PCR tube was added with 5 μL of the genomic DNA solution prepared as described above, 5 U of a restriction enzyme XspI, and 1 μL of a 10 × buffer (200 mM Tris-HCl pH 8.5, 100 mM $MgCl_2$, 10 mM Dithiothreitol, 1000 mM KCl) suited for XspI, and added with sterile ultrapure water to a liquid volume of 10 μL. The reaction solution was incubated at 37°C for an hour.

[0190] The above solution was added with 2 μL of a buffer (330 mM Tris-Acetate pH 7.9, 660 mM KOAc, 100 mM $MgOAc_2$, 5 mM Dithiothreitol), 2 μL of BSA (Bovine serum albumin 1 mg/ml), 12 U of a methylation sensitive restriction enzyme HpaII, and the resultant mixture was further added with sterile ultrapure water to a liquid volume of 20 μL. Each of the mixture was incubated 37°C for 3 hours (these correspond to First step of the present measureing method).

[0191] Counter oligonucleotides C16 to C23 each comprising the nucleotide sequences of SEQ ID NO: 54 to SEQ ID NO: 57 and SEQ ID NO: 59 to SEQ ID NO: 62, capable of complementarily base-pairing with a minus strand of the target DNA region T'(a region corresponding to the base numbers 384523 to 384766 of yeast chromosome VII shown in Genbank Accession No. NC_001139 and so on) comprising the nucleotide sequence of SEQ ID NO: 58 were synthesized, and each 0.01 μM solutions in a TE buffer were prepared.

<Target DNA region>

[0192]

T': 5'-

TAGGAAATACATTCCGAGGGCGCCCGCACAAGGCCTATTATTAGAGGGACCTGTGTTTGACG

GGTATAACACTAAGTTGCGCAATTTGCTGTATTGCGAAATCCGCCCGGACGATATCACTCTT

GAGCGCATGTGCCGTTTCCGAGAACGCCAGATCTGTACTGCGATCGCACACGAGGAGACACA

GCGTCACGTGTTTTGCCATTTTGTACGACAAATGAACCGCCTGGCCACGCCTCTAATC-3'

(SEQ ID NO: 58)

<Counter oligonucleotides>

[0193]

C16: 5'-GGACCTGTGTTTGACGGGTAT-3' (SEQ ID NO: 54)
C17: 5'-AACACTAAGTTGCGCAATTTGCTGT-3' (SEQ ID NO: 55)
C18: 5'-ATTGCGAAATCCGCCCGGACGATAT-3' (SEQ ID NO: 56)
C19: 5'-CACTCTTGAGCGCATGTGCCGTTTC-3' (SEQ ID NO: 57)
C20: 5'-AATACATTCCGAGGGCGCCCGCACAAGGCC-3' (SEQ ID NO: 59)
C21: 5'-GCGATCGCACACGAGGAGACA-3' (SEQ ID NO: 60)
C22: 5'-AGCGTCACGTGTTTTGCCATTTTGTACGAC-3' (SEQ ID NO: 61)
C23: 5'-AAATGAACCGCCTGGCCACGCCTCTAATC-3' (SEQ ID NO: 62)

[0194] To the above reaction solution, 10 μL of a counter oligonucleotide solution prepared as described above, 5 μL of a buffer (330 mM Tris-Acetate pH 7.9, 660 mM KOAc, 100 mM $MgOAc_2$, 5 mM Dithiothreitol), 5 μL of a 100 mM $MgCl_2$ solution, and 5 μL of a 1 mg/mL BSA solution were added, and the resultant mixture was added with sterile ultrapure water to a liquid volume of 50 μL, and mixed. Thereafter, this PCR tube was heated at 95°C for 10 minutes, rapidly cooled to 70°C, and kept at this temperature for 10 minutes. Then the tube was cooled to 50°C and kept at this temperature for 10 minutes, and further kept at 37°C for 10 minutes, and returned to room temperature (these correspond to Second step of the present measuring method).

[0195] The PCR tube coated with streptavidin to which a biotin-labeled methylcytosine antibody was immobilized was added with 50 μL of a reaction solution of a DNA fragment prepared as described above, and left still for 30 minutes at room temperature. Then the solution was removed by pipetting, and 100 μL of a washing buffer [0.05% Tween 20-containing phosphate buffer (1 mM KH_2PO_4, 3 mM $Na_2HPO \cdot 7H_2O$, 154 mM NaCl, pH 7.4)] was added, and then the buffer was removed by pipetting. This operation was repeated another two times (these correspond to Third step of the present measuring method).

[0196] Then by subjecting the above PCR tube to PCR using respective solutions of oligonucleotide primers PF4 and PR4 comprising the nucleotide sequences of SEQ ID NO: 49 and SEQ ID NO: 50, and the following reaction condition, methylated DNA in a target DNA region T' comprising the nucleotide sequence of SEQ ID NO: 53 was amplified.

<Oligonucleotide primers designed for PCR>

[0197]

PF3: 5'-GGACCTGTGTTTGACGGGTAT-3' (SEQ ID NO: 49)
PR3: 5'-AGTACAGATCTGGCGTTCTCG-3' (SEQ ID NO: 50)

<Target DNA region>

[0198]

T': 5'-
GGACCTGTGTTTGACGGGTATAACACTAAGTTGCGCAATTTGCTGTATTGCGAAATCCGCCC
GGACGATATCACTCTTGAGCGCATGTGCCGTTTCCGAGAACGCCAGATCTGTACT-3'
(SEQ ID NO: 53)

[0199] As a reaction solution of PCR, DNA which is a template, mixed with each 3 μL of oligonucleotide primer solutions prepared to 5 μM, each 5 μL of 2 mM dNTPs, 5 μL of a buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% Gelatin), and 0.25 μL of 5 U/μL thermostable DNA polymerase (AmpliTaq Gold, available from ABI), and added with sterile ultrapure water to a liquid volume of 50 μL was used. The reaction solution was kept at 95°C for 10 minutes, and then subjected to PCR conducting 31 cycles of incubation each including 20 seconds at 95°C, 30 seconds at 58°C, and 30 seconds at 72°C.

[0200] After conducting PCR, amplification was checked by 1.5% agarose gel electrophoresis (these correspond to Fourth step of the present measuring method).

[0201] The result is shown in Fig. 5. In Solutions MA, MB and MC of methylated yeast genomic DNA, amplification of DNA was observed. In the negative control solution MD, amplification of DNA was not observed. In solutions A, B, C and D of unmethylated yeast genomic DNA, amplification of DNA was not observed.

[0202] From the above, it was demonstrated that DNA containing a methylated target DNA region can be selected by an immobilized methylcytosine antibody, and methylated DNA is amplified to a detectable level and the amplified DNA can be detected with higher sensitivity.

INDUSTRIAL APPLICABILITY

[0203] Based on the present invention, it becomes possible to provide a method of measuring the content of methylated DNA in an objective DNA region in a genomic DNA contained in a biological specimen in a simple and convenient manner, and so on.

Free Text in Sequence Listing

[0204]

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SEQ ID NO:21
Designed oligonucleotide primer for PCR
SEQ ID NO:22
Designed oligonucleotide primer for PCR

SEQ ID NO:23
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SEQ ID NO:24
Designed oligonucleotide primer for PCR
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ctcatcccag tgtctaaggc gctcccgggt ggtcttggga gttgcaagta gggaggaacg 1440
gccgggtaac cacctctttt ccctttatcc aagcagagcc tcggcgtgcc cccaggaccg 1500
gtaaagttcc tctcgccagc cgcatccatg cttctggcgc ggatgaaccc gcaggtgcag 1560
cccgagaaca acggggcgga cacgggtcca gagcagcccc ttcgggcgcg caaaactgcg 1620
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gacgcgcagc cccgggagac ctggggcaag aagatcgact tcctgctgtc cgtagtcggc 1740
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ggtgagcgtg gggtcgggct gggaatttga atctgggagg tccactgtct gcagcggtgg 1860
ctgggacagg agctggaata cacacggaag ggaggcgagg agacaggggc aaatctgggg 1920
cgcagaaaga actggacagg gctaacggga aaaaaaaaag attggagtcc tctggaaggt 1980

cattttccca ggctctttgc agagtacctc gagctcattc cagcggaagt gtcaggattg 2040
ggcaccctgg aagcaaaaca gcagaagagt gaaatcgagt catgacccta aagtcatggt 2100
aggggtatgg atggaaagga cagaatctgg ggtgccaggt tgggtggggg agcctgacct 2160
tttgatggtc tgctggaagg gaggtggaga ttccaagagc 2200

<210> 17
<211> 98
<212> DNA
<213> Artificial Sequence

<220>
<221> modified_base
<222> 20,23,31,46,51,53,55
<223> Designed oligonucleotide consisting of objective DNA domain (GPR7-2079-2176), n=m5c

<400> 17
gttggccact gcggagtcgn gcngggtggc nggccgcacc tacagngccg ngngngcggt 60
gagcctggcc gtgtgggggа tcgtcacact cgtcgtgc 98

<210> 18
<211> 98
<212> DNA
<213> Artificial Sequence

<220>
<221> modified_base
<222> 31,46,51,53,55
<223> Designed oligonucleotide consisting of objective DNA domain (GPR7-2079-2176),n=m5c

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gagcctggcc gtgtgggggа tcgtcacact cgtcgtgc 98

<210> 19
<211> 98
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<213> Artificial Sequence

<220>
<223> Designed oligonucleotide consisting of objective DNA domain (GPR7-2079-2176)

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<212> DNA
<213> Artificial Sequence

<220>
<223> Designed oligonucleotide for experiment

<400> 20
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<210> 21
<211> 19
<212> DNA
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<220>
<223> Designed oligonucleotide primer for PCR

<400> 21

gttggccact gcggagtcg 19

<210> 22
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Designed oligonucleotide primer for PCR

<400> 22
gcacgacgag tgtgacgatc 20

<210> 23
<211> 98
<212> DNA
<213> Artificial Sequence

<220>
<223> Designed oligonucleotide consisting of objective DNA domain (GPR7-2079-2176)

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gagcctggcc gtgtgggga tcgtcacact cgtcgtgc 98

<210> 24
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Designed oligonucleotide primer for PCR

<400> 24
ctcagcaccc aggcggcc 18

<210> 25
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Designed oligonucleotide primer for PCR

<400> 25
ctggccaaac tggagatcgc 20

<210> 26
<211> 386
<212> DNA
<213> Artificial Sequence

<220>
<223> Amplified oligonucleotide consisting of objective DNA domain (Genbank Accession No.NT_029419 25687390-25687775 Homo sapiens)

<400> 26
ctcagcaccc aggcggccgc gatcatgagg cgcgagcggc gcgcgggctg ttgcagagtc 60
ttgagcgggt ggcacaccgc gatgtagcgg tcggctgtca tgactaccag catgtaggcc 120
gacgcaaaca tgccgaacac ctgcaggtgc ttcaccacgc ggcacagcca gtcggggccg 180
cggaagcggt aggtgatgtc ccagcacatt tgcggcagca cctggaagaa tgccacggcc 240
aggtcggcca ggctgaggtg tcggatgaag aggtgcatgc gggacgtctt gcgcggcgtc 300
cggtgcagag ccagcagtac gctgctgttg cccagcacgg ccaccgcgaa agtcaccgcc 360
agcacggcga tctccagttt ggccag 386

<210> 27
<211> 386

<212> DNA
<213> Artificial Sequence

<220>
<221> modified_base
<222>
14,18,20,31,33,38,40,42,44,66,78,80,88,92,120,123,134,160,173,179,181,187,203,237,245,262,280,285,292,294,297,301,320,338,346,357,365,368
<223> Designed oligonucleotide consisting of objective DNA domain (Genbank Accession No.NT_029419 25687390-25687775 Homo sapiens), n=m5c

<400> 27
ctcagcaccc aggnggcngn gatcatgagg ngngagnggn gngngggctg ttgcagagtc 60
ttgagngggt ggcacacngn gatgtagngg tnggctgtca tgactaccag catgtaggcn 120
gangcaaaca tgcngaacac ctgcaggtgc ttcaccangn ggcacagcca gtnggggcng 180
nggaagnggt aggtgatgtc ccagcacatt tgnggcagca cctggaagaa tgccanggcc 240
aggtnggcca ggctgaggtg tnggatgaag aggtgcatgn gggangtctt gngnggngtc 300
nggtgcagag ccagcagtan gctgctgttg cccagcangg ccacngngaa agtcacngcc 360
agcanggnga tctccagttt ggccag 386

<210> 28
<211> 386
<212> DNA
<213> Artificial Sequence

<220>
<223> Designed oligonucleotide consisting of objective DNA domain (Genbank Accession No.NT_029419 25687390-25687775 Homo sapiens)

<400> 28
ctcagcaccc aggcggccgc gatcatgagg cgcgagcggc gcgcgggctg ttgcagagtc 60
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gacgcaaaca tgccgaacac ctgcaggtgc ttcaccacgc ggcacagcca gtcggggccg 180
cggaagcggt aggtgatgtc ccagcacatt tgcggcagca cctggaagaa tgccacggcc 240
aggtcggcca ggctgaggtg tcggatgaag aggtgcatgc gggacgtctt gcgcggcgtc 300
cggtgcagag ccagcagtac gctgctgttg cccagcacgg ccaccgcgaa agtcaccgcc 360
agcacggcga tctccagttt ggccag 386

<210> 29
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Designed counter oligonucleotide for making an objective DNA domain a single strand DNA

<400> 29
gccaccgcga aagtcaccgc cagcacggcg 30

<210> 30
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Designed counter oligonucleotide for making an objective DNA domain a single strand DNA

<400> 30
gccagcagta cgctgctgtt gcccagcacg 30

<210> 31
<211> 30
<212> DNA
<213> Artificial Sequence

<220>

<223> Designed counter oligonucleotide for making an objective DNA domain a single strand DNA

<400> 31
cgggacgtct tgcgcggcgt ccggtgcaga 30

<210> 32
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Designed counter oligonucleotide for making an objective DNA domain a single strand DNA

<400> 32
aggctgaggt gtcggatgaa gaggtgcatg 30

<210> 33
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Designed counter oligonucleotide for making an objective DNA domain a single strand DNA

<400> 33
acctggaaga atgccacggc caggtcggcc 30

<210> 34
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Designed counter oligonucleotide for making an objective DNA domain a single strand DNA

<400> 34
taggtgatgt cccagcacat ttgcggcagc 30

<210> 35
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Designed counter oligonucleotide for making an objective DNA domain a single strand DNA

<400> 35
cggcacagcc agtcggggcc gcggaagcgg 30

<210> 36
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Designed counter oligonucleotide for making an objective DNA domain a single strand DNA

<400> 36
atgccgaaca cctgcaggtg cttcaccacg 30

<210> 37
<211> 30

<212> DNA
<213> Artificial Sequence

<220>
<223> Designed counter oligonucleotide for making an objective DNA domain a single strand DNA

<400> 37
atgactacca gcatgtaggc cgacgcaaac 30

<210> 38
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Designed counter oligonucleotide for making an objective DNA domain a single strand DNA

<400> 38
tggcacaccg cgatgtagcg gtcggctgtc 30

<210> 39
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Designed counter oligonucleotide for making an objective DNA domain a single strand DNA

<400> 39
cgcgcgggct gttgcagagt cttgagcggg 30

<210> 40
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Designed counter oligonucleotide for making an objective DNA domain a single strand DNA

<400> 40
caggcggccg cgatcatgag gcgcgagcgg 30

<210> 41
<211> 19
<212> DNA
<213> Artificial Sequence

<220>
<223> Designed oligonucleotide primer for PCR

<400> 41
tgagctccgt agggcgtcc 19

<210> 42
<211> 17
<212> DNA
<213> Artificial Sequence

<220>
<223> Designed oligonucleotide primer for PCR

<400> 42
gcgccgggtc cgggccc 17

<210> 43
<211> 121
<212> DNA
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<220>
<223> Amplified oligonucleotide consisting of objective DNA domain (Genbank Accession No.AC009800 76606-76726 Homo sapiens)

<220>
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tctcaacgag ggcagaggag ccggcgacct ggcgtccccc aaggacgccc tacggagctc 120
a 121

<210> 44
<211> 121
<212> DNA
<213> Artificial Sequence

<220>
<221> modified_base
<222> 2,5,11,17,22,28,41,47,52,67,82,85,93,106,113
<223> Designed oligonucleotide consisting of objective DNA domain (Genbank Accession No.AC009800 76606-76726 Homo sapiens) n=m5c

<400> 44
gngcngggtc ngggccngat gngttggngg gccagggctc ngagaangag gngttgtcca 60
tctcaangag ggcagaggag cnggngacct ggngtccccc aaggangccc tanggagctc 120
a 121

<210> 45
<211> 121
<212> DNA
<213> Artificial Sequence

<220>
<223> Designed oligonucleotide consisting of objective DNA domain (Genbank Accession No.AC009800 76606-76726 Homo sapiens)

<400> 45
gcgccgggtc cgggcccgat gcgttggcgg gccagggctc cgagaacgag gcgttgtcca 60
tctcaacgag ggcagaggag ccggcgacct ggcgtccccc aaggacgccc tacggagctc 120
a 121

<210> 46
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Designed counter oligonucleotide for making an objective DNA domain a single strand DNA

<400> 46
gcgtccccca aggacgccct acggagctca 30

<210> 47
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Designed counter oligonucleotide for making an objective DNA domain a single strand DNA

<400> 47
ctcaacgagg gcagaggagc cggcgacctg 30

<210> 48
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Designed counter oligonucleotide for making an objective DNA domain a single strand DNA

<400> 48
cgccgggtcc gggcccgatg cgttggcggg 30

<210> 49
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Designed oligonucleotide primer for PCR

<400> 49
ggacctgtgt ttgacgggta t 21

<210> 50
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Designed oligonucleotide primer for PCR

<400> 50
agtacagatc tggcgttctc g 21

<210> 51
<211> 117
<212> DNA
<213> Artificial Sequence

<220>
<223> Amplified oligonucleotide consisting of objective DNA domain (Genbank Accession No.NC001139 384569-384685 Saccharomyces cereviciae chromosome VII)

<400> 51
ggacctgtgt ttgacgggta taacactaag ttgcgcaatt tgctgtattg cgaaatccgc 60
ccggacgata tcactcttga gcgcatgtgc cgtttccgag aacgccagat ctgtact 117

<210> 52
<211> 117
<212> DNA
<213> Artificial Sequence

<220>
<221> modified_base
<222> 15,34,51,58,61,66,82,91,97,103
<223> Designed oligonucleotide consisting of objective DNA domain (Genbank Accession No.NC001139 384569-384685 Saccharomyces cereviciae chromosome VII) n=m5c

<400> 52
ggacctgtgt ttgangggta taacactaag ttgngcaatt tgctgtattg ngaaatcngc 60
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<210> 53
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<400> 53
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<210> 54
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<400> 54
ggacctgtgt ttgacgggta t 21

<210> 55
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Designed counter oligonucleotide for making an objective DNA domain a single strand DNA

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aacactaagt tgcgcaattt gctgt 25

<210> 56
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Designed counter oligonucleotide for making an objective DNA domain a single strand DNA

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attgcgaaat ccgcccggac gatat 25

<210> 57
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<213> Artificial Sequence

<220>
<223> Designed counter oligonucleotide for making an objective DNA domain a single strand DNA

<400> 57
cactcttgag cgcatgtgcc gtttc 25

<210> 58
<211> 244
<212> DNA
<213> Artificial Sequence

<220>
<223> Designed oligonucleotide primer for PCR

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taggaaatac attccgaggg cgcccgcaca aggcctatta ttagagggac ctgtgtttga 60
cgggtataac actaagttgc gcaatttgct gtattgcgaa atccgcccgg acgatatcac 120

tcttgagcgc atgtgccgtt tccgagaacg ccagatctgt actgcgatcg cacacgagga 180
gacacagcgt cacgtgtttt gccattttgt acgacaaatg aaccgcctgg ccacgcctct 240
aatc 244

<210> 59
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Designed counter oligonucleotide for making an objective DNA domain a single strand DNA

<400> 59
aatacattcc gagggcgccc gcacaaggcc 30

<210> 60
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Designed counter oligonucleotide for making an objective DNA domain a single strand DNA

<400> 60
gcgatcgcac acgaggagac a 21

<210> 61
<211> 30
<212> DNA
<213> Artificial Sequence

<220>
<223> Designed counter oligonucleotide for making an objective DNA domain a single strand DNA

<400> 61
agcgtcacgt gttttgccat tttgtacgac 30

<210> 62
<211> 29
<212> DNA
<213> Artificial Sequence

<220>
<223> Designed counter oligonucleotide for making an objective DNA domain a single strand DNA

<400> 62
aaatgaaccg cctggccacg cctctaatc 29

Claims

1. A method of measuring the content of methylated DNA in a objective DNA region in a genomic DNA contained in a biological specimen, comprising:

(1) First step of subjecting a DNA sample derived from the genomic DNA contained in the biological specimen to a digestion treatment with a methylation-sensitive restriction enzyme;
(2) Second step of obtaining methylated single-stranded DNA from the DNA sample that has been subjected to the digestion treatment and obtained in First step, and binding the single-stranded DNA to an immobilized

methylated DNA antibody, thereby selecting the single-stranded DNA; and
(3) Third step comprising, as a pre step of each of the following regular steps:

a step (First pre step) of separating the single-stranded DNA selected in Second step from the immobilized immobilized methylated DNA antibody to provide DNA in a single-stranded state (plus strand);
a step (Second pre step) of extensionally-forming a double-stranded DNA from a single-stranded DNA (plus strand) containing the objective DNA region by a single extension of an extension primer, using the genomic DNA (plus strand) provided in a single-stranded state in First pre step and the extension primer, wherein the extension primer (forward primer) comprises the nucleotide sequence (minus strand) complementary to a partial nucleotide sequence (plus strand) of the nucleotide sequence of the DNA in a single-stranded state (plus strand), the partial nucleotide sequence (plus strand) being located on further 3'-end side than the 3'-end of the nucleotide sequence (plus strand) of the objective DNA region; and
a step (Third pre step) of temporarily separating the double-stranded DNA extensionally formed in Second pre step into a single-stranded DNA (plus strand) containing the objective DNA region and a single-stranded DNA (minus strand) containing the nucleotide sequence complementary to the objective DNA region;
and as regular steps:

(a) Step A (regular step) of extensionally forming double-stranded DNA from the single-stranded DNA containing the objective DNA region, by a single extension of the extension primer, using as a template the generated single-stranded DNA (plus strand) containing the objective DNA region, and the forward primer as the extension primer; and
(b) Step B (regular step) of extensionally forming double-stranded DNA from the single-stranded DNA containing the objective DNA region, by a single extension of an extension primer, using as a template the generated single-stranded DNA (minus strand) containing the nucleotide sequence complementary to the objective DNA region, and using as the extension primer an extension primer (reverse primer) comprising the nucleotide sequence (plus strand) complementary to a partial nucleotide sequence (minus strand) of the nucleotide sequence of the single-stranded DNA (minus strand) containing the nucleotide sequence complementary to the objective DNA region, the partial nucleotide sequence (minus strand) being located on further 3'-end side than the 3'-end of the nucleotide sequence (minus strand) complementary to the nucleotide sequence (plus strand)of the objective DNA region; and wherein

Third step further comprises:

amplifying the methylated DNA in the objective DNA region to a detectable level by repeating each regular step of Third step after temporarily separating the extensionally formed double-stranded DNA obtained in each of the regular steps into a single-stranded state; and quantifying the amount of the amplified DNA.

2. The method of claim 1, wherein the immobilized immobilized methylated DNA antibody is a methylcytosine antibody.

3. The method of claim 1 or 2, wherein the biological specimen is blood, a bodily fluid, serum, plasma, a cell lysate, or a tissue lysate from a mammal.

4. The method of any one of claims 1 to 3, wherein the DNA sample derived from the genomic DNA contained in the biological specimen is a DNA sample digested in advance with a restriction enzyme recognition cleavage site for which is not present in the objective DNA region of the genomic DNA, or a DNA sample purified in advance.

5. The method of any one of claims 1 to 4, wherein First step comprises:

First (A) step of mixing a single-stranded DNA (plus strand) containing the objective DNA region and a masking oligonucleotide comprising a nucleotide sequence complementary to a nucleotide sequence of a recognition site for a methylation-sensitive restriction enzyme, thereby selecting single-stranded DNA in which the recognition site for the methylation-sensitive restriction enzyme is protected; and
First (B) step of digesting the single-stranded DNA selected in First (A) step with the methylation-sensitive restriction enzyme.

6. The method of any one of claims 1 to 5, wherein the methylation-sensitive restriction enzyme is a restriction enzyme

the restriction site for which is included in the objective DNA region in the genomic DNA contained in the biological specimen, or the methylation-sensitive restriction enzyme is HhaI.

7. The method of any one of claims 1 to 6, wherein Second step is performed without digestion treatment with the methylation-sensitive restriction enzyme in First step.

8. The method of any one of claims 1 to 7, wherein Second step comprises:

Second (A) step of separating into methylated single-stranded DNA the methylated double-stranded DNA contained in the DNA sample that has been subjected to the digestion treatment and obtained in First step; and Second (B) step of binding the methylated single-stranded DNA obtained in Second (A) step to an immobilized methylated DNA antibody; and wherein

a counter oligonucleotide is added when separating the methylated double-stranded DNA into the methylated single-stranded DNA in Second (A) step.

Fig.1

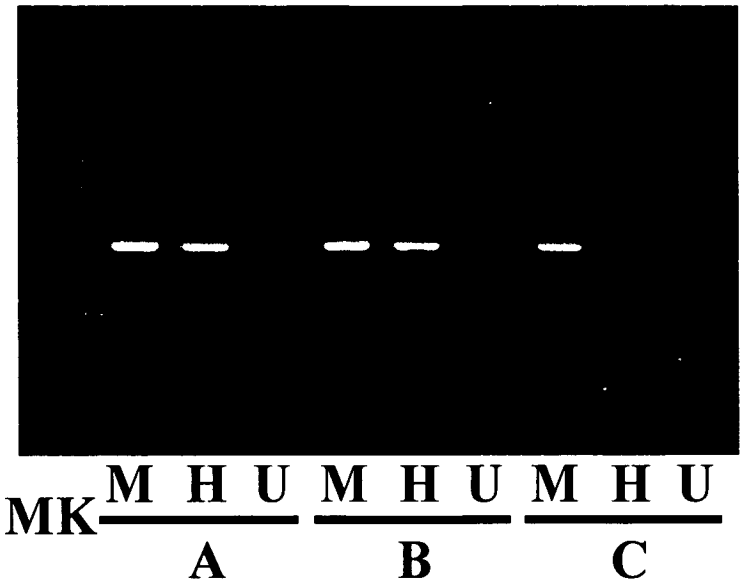

Fig.2

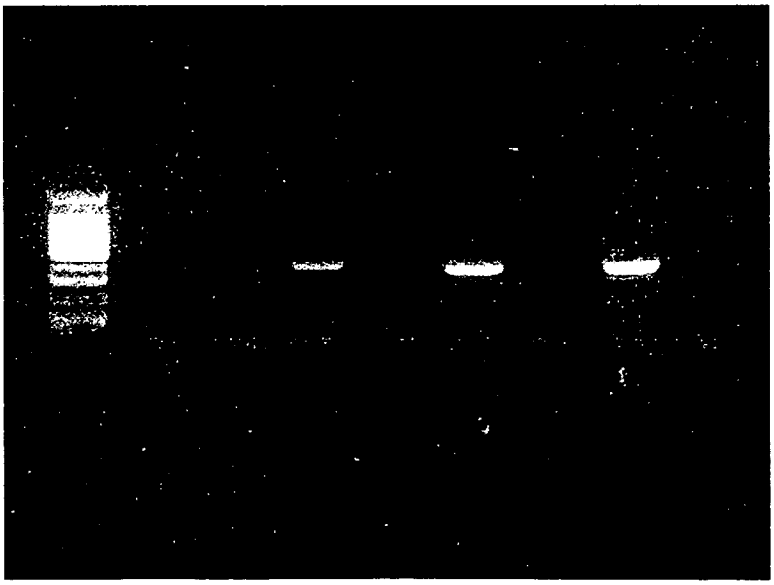

Fig.3

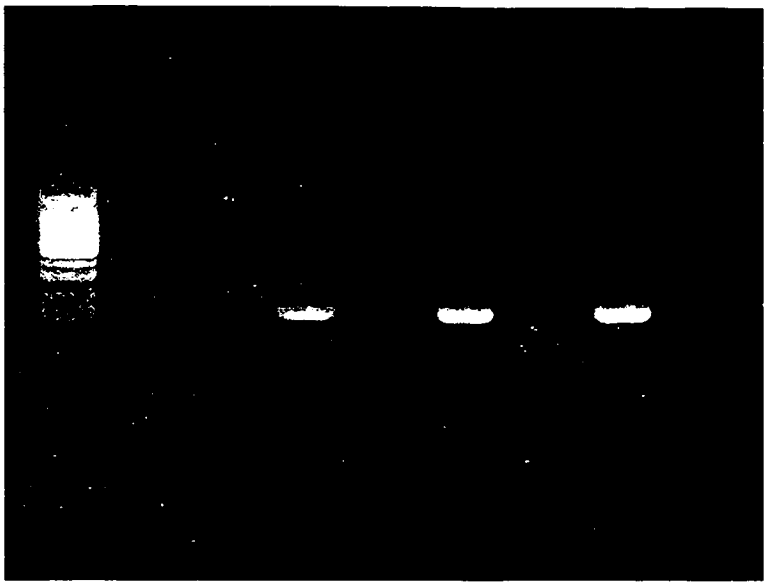

Fig.4

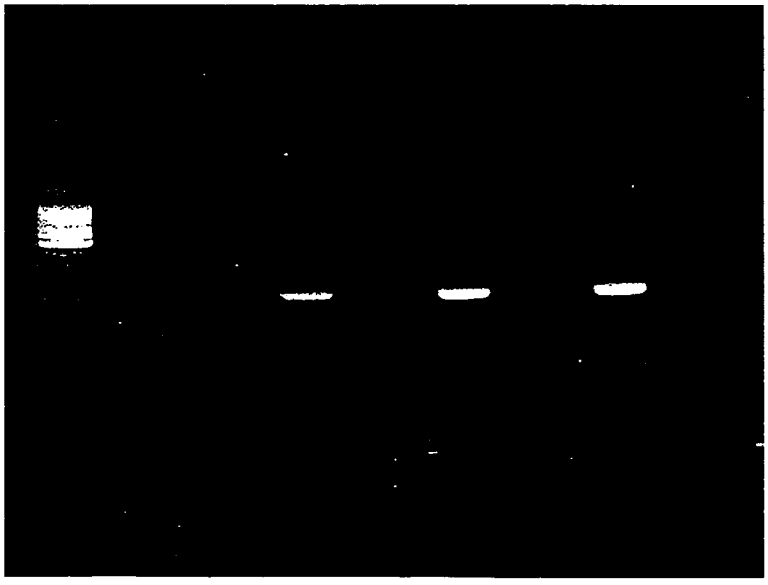

Fig.5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2009/056785

A. CLASSIFICATION OF SUBJECT MATTER
C12Q1/68(2006.01)i, *C12N15/09*(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)
C12Q1/68, C12N15/00-15/90

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	TONG, Yu K., et al., Detection of restriction enzyme-digested target DNA by PCR amplification using a stem-loop primer: application to the detection of hypomethylated fetal DNA in maternal plasma., Clinical chemistry, 2007, Vol.53, No.11, pages 1906-1914	1-8
Y	Edited by The Japanese Biochemical Society, Shin Seikagaku Jikken Koza 2 Kakusan I -Bunri Seisei-, Tokyo Kagaku Dojin, 1991, 1st edition, pages 169 to 172, 180	1-8
Y	WO 2007/119518 A1 (The University of Tokyo), 25 October, 2007 (25.10.07), Full text (Family: none)	1-8

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

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 May, 2009 (29.05.09)	09 June, 2009 (09.06.09)
Name and mailing address of the ISA/ Japanese Patent Office	Authorized officer
Facsimile No.	Telephone No.

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

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2009/056785

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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REFERENCES CITED IN THE DESCRIPTION

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Non-patent literature cited in the description

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专利名称(译)	测定DNA甲基化的方法		
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摘要(译)

本发明涉及测量生物样本中包含的基因组DNA中的目标DNA区域中甲基化DNA含量的方法，等等。

GPR7-2079-2176/98mer-M(7):

5′-

GTTGGCCACTGCGGAGTCGNGCNGGGTGGCNGGCCGCACCTACAGNGCCGNGNGNGCGGTGA

GCCTGGCCGTGTGGGGGATCGTCACACTCGTCGTGC-3′ (SEQ ID NO: 17)