METHOD FOR MULTIPLEX DETECTION OF METHYLATED DNA

TECHNICAL FIELD

The present disclosure relates to a method of detecting methylation of target DNA in a multiplex manner and a composition for detecting methylation of target DNA, and more particularly to a method for detecting methylation of target DNA, comprising: constructing an oligonucleotide, which comprises a target-specific sequence capable of binding complementarily to the target DNA and an artificial primer not complementary to the target DNA; linearly amplifying the target DNA for linear target enrichment (hereinafter referred to as LTE) by using the oligonucleotide as a primary primer; amplifying the linearly amplified target DNA using an oligonucleotide separate from the oligonucleotide used as a primary primer, which is capable of binding complementarily to the linearly amplified target DNA, and a universal primer not complementary to the linearly amplified target DNA; and detecting the presence or absence of the amplification product by a probe.

BACKGROUND ART

In the genomic DNA of mammalian cells, there is the fifth base in addition to A, C, G and T, namely, 5-methylcytosine, in which a methyl group is attached to the fifth carbon of

the cytosine ring (5-mC). 5-mC is always attached only to the C of a CG dinucleotide (5'-mCG-3'), which is frequently marked CpG. The C of CpG is mostly methylated by attachment with a methyl group. The methylation of this CpG inhibits a repetitive sequence in genomes, such as Alu or transposon, from being expressed. In addition, this CpG is a site where an epigenetic change in mammalian cells appears most often. The 5-mC of this CpG is naturally deaminated to T, and thus, the CpG in mammal genomes shows only 1% of frequency, which is much lower than a normal frequency (1/4 x 1/4=6.25%).

Regions in which CpGs are exceptionally integrated are known as CpG islands. The term "CpG islands" refer to sites which are 0.2-3 kb in length, and have a C+G content of more than 50% and a CpG ratio of more than 3.75%. There are about 45,000 CpG islands in the human genome, and they are mostly found in promoter regions regulating the expression of genes. Actually, the CpG islands occur in the promoters of housekeeping genes accounting for about 50% of human genes. It is known that aberrant DNA methylation occurs mainly in the 5′ regulatory region of the gene to reduce the expression of the gene.

Herein, the 5′ regulatory region of the gene includes a promoter region, an enhancer region and a 5′ untranslated region. Recently, an attempt to examine the promoter methylation of tumor-related genes in blood, sputum, saliva,

feces or urine and to use the examined results for the diagnosis and treatment of various cancers, has been actively conducted.

It is well known that DNAs are released from abnormal cells in the cancer tissue of cancer patients into blood by processes including apoptosis and necrosis, and thus exist as cell-free tumor DNA in the serum or plasma of the blood, and methylated DNA fragments are also present in the cell-free tumor DNA. The presence of this aberrant DNA methylation has been used as a marker for diagnosing cancer.

Meanwhile, generally, methods for analyzing gene methylation are performed by detecting a control gene, which is not involved in methylation, by PCR in order to confirm the suitability of the PCR and the presence or absence of input DNA, and performing PCR for detecting methylation of target DNA in parallel thereto.

In particular, as methods of analyzing methylation by real-time PCR, the following methods can be taken into consideration: i) a method that uses primers independent of methylation of target DNA as primers for real-time PCR and uses a methylation-specific detection primer, which is capable of hybridizing to the methylated target DNA, for detection of the PCR amplification product; ii) a method that uses target DNA methylation-specific primers as primers for real-time PCR and uses a detection probe capable of

hybridizing to a methylation-independent sequence contained in the PCR amplification product; and iii) a method that uses target DNA methylation-specific primers as primers for real-time PCR and uses a methylation-specific detection probe, which is capable of hybridizing to methylated target DNA, for detection of the PCR amplification product.

However, these methods all use non-enriched DNA directly as a template and necessarily use two primers (forward and reverse) for amplifying target DNA, at the same time. Accordingly, there is a problem that two primers are further required at a time whenever the target to be amplified in one tube (single reactor) increases.

Primers for multiplex PCR are designed such that different primers in a single tube can have similar hybridization properties. The annealing temperature and primer concentration can be calculated to some extent or may also be used empirically. As non-specific hybridization increases each time a primer pair is added, reaction conditions should be modified whenever each primer pair is added. In addition, artifacts may occur due to depletion of the primer pair or the like. The use of 5'-tagged oligonucleotides in PCR reaction has been reported. However, a key feature of this amplification method is that it comprises a step of annealing of each primer and isolating primer extension reactions, and thus is not suitable for the

actual multiplex PCR concept. Thus, creating perfect conditions for multiplex PCR is a very difficult and costly process. Therefore, it is necessary to develop a multiplex PCR method capable of simultaneously amplifying several targets to the same extent under the same conditions regardless of various characteristics of different primers.

Under this technical background, the inventors of this application have made extensive efforts to solve the above-described problems and develop a method for detecting methylated target DNA, which has high detection limit and accuracy, and as a result, have found that when target DNA is enriched by asymmetric linear amplification using a universal primer-linked oligonucleotide and is detected using this enriched target DNA as a template and a universal primer is linked to a target-specific oligonucleotide, methylated DNA can be detected with desired high sensitivity and accuracy, even if only one primer is added for multiplex detection of the target DNA, unlike a conventional art, thereby completing the present disclosure.

DISCLOSURE OF INVENTION

TECHNICAL PROBLEM

It is an object of the present disclosure to provide a method of detecting methylated DNA in a multiplex manner using a universal primer.

Another object of the present disclosure is to provide a composition for multiplex detection of methylated DNA, which comprises a universal primer.

Still another object of the present disclosure is to provide a kit for multiplex detection of methylated DNA, which comprises the composition.

TECHNICAL SOLUTION

To achieve the above object, the present disclosure provides a method for detecting methylated DNA, comprising the steps of: method for detecting methylated DNA, comprising: (a) treating a target DNA-containing sample with at least one reagent which modifies a non-methylated DNA site to be distinguished from a methylated DNA site; (b) constructing an oligonucleotide, which comprises a target-specific sequence designed to be capable of binding complementarily to the target DNA treated with the reagent, and a universal primer that does not bind complementarily to the target DNA; (c) performing one direction asymmetric linear amplification using, as a template, the target DNA treated with the reagent in step (a), and using, as a primer, the oligonucleotide constructed in step (b); (d) amplifying the target DNA using an oligonucleotide, which is capable of binding complementarily to the DNA linearly amplified in step (c), and a universal primer; and (e) detecting methylation of

the target DNA by a probe capable of hybridizing complementarily to the target DNA sequence amplified in step (d).

The present disclosure also provides a composition for detecting methylated DNA, comprising: at least one reagent treated to a target DNA-containing sample, which modify a non-methylated DNA to be distinguished from a methylated DNA; an oligonucleotide, which comprises a target-specific sequence capable of binding complementarily to a target DNA sequence treated with the reagent, and a universal primer that does not bind complementarily to the target DNA; an oligonucleotide, which are capable of binding complementarily to a linearly amplified target DNA, and a universal primer; and a probe capable of hybridizing complementarily to the linearly amplified target DNA sequence.

The present disclosure also provides a kit for detecting methylated DNA, which comprises the composition.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a conceptual view schematically showing a method for multiplex detection of methylated target DNA.

FIG. 2 shows the results of comparing the sensitivity of detection of methylated DNA between a method of the present disclosure and a conventional method.

FIG. 3 schematically shows a process of designing primers and a probe for target DNA, which are used in a method of the present disclosure.

FIGs. 4a and 4b show the results of comparing the sensitivity of detection of methylated DNA between a method of the present disclosure, which is performed using primers and a probe for target DNA, and a conventional method.

BEST MODE FOR CARRYING OUT THE INVENTION

Unless defined otherwise, all the technical and scientific terms used herein have the same meaning as those generally understood by one of ordinary skill in the art to which the invention pertains. Generally, the nomenclature used herein and the experiment methods, which will be described below, are those well known and commonly employed in the art.

In one aspect, the present disclosure is directed to a method for detecting methylated DNA, comprising the steps of:

(a) treating a target DNA-containing sample with at least one reagent which modifies a non-methylated DNA site to be distinguished from a methylated DNA site;

(b) constructing an oligonucleotide, which comprises a target-specific sequence designed to be capable of binding complementarily to the target DNA treated with the reagent,

and a universal primer that does not bind complementarily to the target DNA;

(c) performing one direction asymmetric linear amplification using, as a template, the target DNA treated with the reagent in step (a), and using, as a primer, the oligonucleotide constructed in step (b);

(d) amplifying the target DNA using an oligonucleotide, which is capable of binding complementarily to the DNA linearly amplified in step (c), and a universal primer that does not bind complementarily to the linearly amplified target DNA; and

(e) detecting methylation of the target DNA by a probe capable of hybridizing complementarily to the target DNA sequence amplified in step (d).

In the present disclosure, a method capable of early diagnosing disease by detecting methylated DNA with high sensitivity was developed, and the performance of the method was examined. In one example of the present disclosure, an internal control gene and a target DNA were first linearly amplified using a target-specific sequence capable of binding complementarily to the target DNA, and a universal primer that does not bind complementarily to the target DNA bound to the target-specific sequence, and then methylated DNA was analyzed by a detection method using an oligonucleotide capable of binding complementarily to a sequence

complementary to the target DNA, a probe capable of hybridizing complementarily to the target DNA sequence, and the universal primer that does not bind complementarily to the linearly amplified target DNA. As a result, it was confirmed that the method of the present disclosure could detect methylated DNA with higher sensitivity and accuracy than a conventional method.

Specifically, fecal DNA derived from normal person, and fecal DNA derived from patients with colorectal cancer at each stage, were treated with bisulfite, and non-methylated cytosines were all converted to uracil, and then the DNAs were linearly amplified using an oligonucleotide which is capable of binding specifically to each of methylated SDC2 (target DNA) and COL2A1 (internal control) and capable of binding complementarily to the target DNA fused with a universal primer, after which real-time PCR was performed using a specific probe capable of hybridizing to each target probe, an oligonucleotide capable of binding complementarily to a sequence complementary to the target DNA, and the universal primer (FIG. 1). As a result, it could be confirmed that the method of the present disclosure could detect methylated DNA with higher sensitivity and accuracy than a conventional qMSP (quantitative methylation specific PCR) performed using a primer that does not comprise the universal primer (FIGS. 2 and 4).

In the present disclosure, step (a) is a step of treating a target DNA-containing sample with at least one reagent which modifies a non-methylated DNA site to be distinguished from a methylated DNA site.

"Methylation" as used in the present disclosure means that a methyl group is attached to the 5th carbon atom of the cytosine base ring to form 5-methylcytosine (5-mC). 5-methylcytosine (5-mC) is always attached only to the C of a CG dinucleotide (5'-mCG-3'), which is frequently marked CpG. The C of CpG is mostly methylated by attachment with a methyl group. The methylation of this CpG inhibits a repetitive sequence in genomes, such as Alu or transposon, from being expressed. In addition, this CpG is a site where an epigenetic change in mammalian cells appears most often. The 5-mC of this CpG is naturally deaminated to T, and thus, the CpG in mammal genomes shows only 1% of frequency, which is much lower than a normal frequency (1/4 x 1/4=6.25%).

Regions in which CpGs are exceptionally integrated are known as CpG islands. The term "CpG islands" refer to sites which are 0.2-3 kb in length, and have a C+G content of more than 50% and a CpG ratio of more than 3.75%. There are about 45,000 CpG islands in the human genome, and they are mostly found in promoter regions regulating the expression of genes. Actually, the CpG islands occur in the promoters of housekeeping genes accounting for about 50% of human genes.

The presence of CpG methylation in the target DNA may be an indicator of disease. For example, CpG methylation of any one of the promoter, 5'-untranslated region and intron of the target DNA may be measured.

A CpG-containing gene is generally DNA. However, the method of the present disclosure may be applied to a sample containing DNA or a sample containing DNA and RNA, including mRNA, wherein the DNA or RNA may be single-stranded or double-stranded. Alternatively, the sample may also be a sample containing a DNA-RNA hybrid.

A nucleic acid mixture may also be used. As used herein, the term "multiplex" includes both the case in which there are a plurality of specific nucleic sequence regions to be detected in one kind of gene and the case in which a single tube (single reactor) includes a plurality of target DNAs. The specific nucleic acid sequence to be detected may be a large molecular fraction, and the specific sequence may be present from the beginning in the form of an isolated molecule constituting the entire nucleic acid sequence. The nucleic acid sequence need not be a nucleic acid present in a pure form, and the nucleic acid may be a small fraction in a complex mixture such as one containing the whole human DNA.

Specifically, the present disclosure is directed to a method for detecting a plurality of target DNA methylations in samples in a single reactor, wherein the sample may

contain a plurality of multiple target DNAs. The target DNA may be used without limitation as long as it is not only a control gene, but also any gene that affects the development or progression of cancer when it expression is inhibited by abnormal methylation.

The sample may be derived from the human body. For example, the sample may be derived from liver cancer, glioblastoma, ovarian cancer, colon cancer, head and neck cancer, bladder cancer, renal cell cancer, gastric cancer, breast cancer, metastatic cancer, prostate cancer, pancreatic cancer, or lung cancer patients. In the present disclosure, the sample may be any one selected from among solid or liquid tissue, cells, feces, urine, blood, serum and plasma.

At least one reagent that modifies a non-methylated DNA site to be distinguished from a methylated DNA site can be used without limitation as long as it can distinguish between a non-methylated cytosine base and a methylated cytosine base. For example, the reagent may be one or more selected from among bisulfite, hydrogen sulfite, disulfite, and combinations thereof, but is not limited thereto. Specifically, a cytosine base methylated by the reagent is not converted, but a cytosine base unmethylated by the reagent may be converted to uracil or to another base other than or cytosine.

In the present disclosure, step (b) is a step of constructing an oligonucleotide, which comprises a target-specific sequence designed to be capable of binding complementarily to the target DNA treated with the reagent, and a universal primer that does not bind complementarily to the target DNA.

For common multiplex PCR, pairs of forward and reverse primers, which correspond to the number of targets, should be constructed and used simultaneously. However, in the method of the present disclosure, only one target-specific sequence (reverse primer) capable of binding complementarily to a target DNA sequence by a universal primer may be used in a real-time PCR process for simultaneously detecting several multiple methylated DNAs, and only an oligonucleotide (forward primer) capable of binding complementarily to the linearly amplified DNA may be used in the same number as the targets to be detected. Accordingly, only a small number of primers may be used for simultaneous detection of methylation of several targets, and thus the complexity of PCR and variation of PCR efficiency are reduced.

The target-specific sequence is a sequence capable of binding complementarily to a target DNA, and as the target-specific sequence, a sequence capable of binding complementarily to a methylated site of the target DNA, as well as a sequence capable of binding complementarily to a

non-methylated site of the target DNA may also be used selectively.

The target-specific sequence may comprise, for example, one or more CpG dinucleotides. Specifically, the target-specific sequence may comprise a sequence having an identity of at least 50%, specifically, at least 55%, 60%, 70%, 80% or 90%, to one or more sequences selected from the group consisting of sequences represented by SEQ ID NOs: 2, 5, 9, 14, 17, 21 and 26.

The universal primer that can be used in the present disclosure is may be linked to either any one of a target-specific sequence (reverse primer) capable of binding complementarily to a target DNA sequence and an oligonucleotide (forward primer) capable of binding complementarily to the linearly amplified DNA, or both the reverse primer and the forward primer.

The universal primer can be used without limitation as long as it comprises a nucleotide sequence that does not bind complementarily to a target DNA amplifiable irrespective of the target DNA, but for example, may comprise a nucleotide sequence that is not present in the human genome. Specifically, the universal primer may comprise a sequence having an identity of at least 50%, specifically, at least 55%, 60%, 70%, 80% or 90%, to a nucleotide sequence represented by SEQ ID NO: 7. In addition, the universal

primer may comprise a sequence such as T7, SP6, M13 or the like, but is not limited thereto.

Further, the universal primer may be one or more sequences selected from the group consisting of sequences represented by SEQ ID NOs: 35 to 41. The universal primer may be, for example, a T7 sequence of 5′-TAATACGACTCACTATAGG-3′ (SEQ ID NO: 35), an SP6 sequence of 5′-TATTTAGGTGACACTATAG-3′ (SEQ ID NO: 36), or an M13 sequence of 5′-GTAAAACGACGGCCAG-3(SEQ ID NO: 37: -20F), 5′-GTTTTCCCAGTCACGAC-3′(SEQ ID NO: 38: -40F), 5′-CGCCAGGGTTTTCCCAGTCACGAC-3′ (SEQ ID NO: 39: -47F), 5′-GAAACAGCTATGACCATG-3′ (SEQ ID NO: 40: R) or 5′-AGCGGATAACAATTTCACACAGG-3′ (SEQ ID NO: 41: -48R).

The method of the present disclosure comprises a step (c) of performing asymmetric linear amplification using, as a template, the target DNA treated with the reagent in step (a), and using, as a primer, the oligonucleotide constructed in step (b).

A DNA treated with bisulfite in a real-time PCR process through the step (c) was not used immediately, but a linearly amplified DNA was used, and thus a detection rate and the sensitivity of detection are excellent (FIGS 2 and 4).

In the present disclosure, only a target DNA is asymmetrically amplified linearly in one direction, and thus this leads to enrichment of the target DNA. In one embodiment, linear amplification for enriching a methylated

target DNA may be performed by unidirectional PCR using as a primer an oligonucleotide comprising a universal primer bound to the 5′ end of a target-specific sequence.

The linear amplification means that an amplification product is produced linearly with respect to the number of amplification cycles, including double-strand denaturation, primer annealing and nucleic acid synthesis. The linear amplification is distinguished from a polymerase chain reaction (PCR) that produces an amplification product exponentially with respect to the number of amplification cycles.

Next, the method of the present disclosure comprises a step (d) of amplifying the target DNA using an oligonucleotide, which is capable of binding complementarily to the DNA linearly amplified in step (c), and the universal primer.

In some cases, step (d) may further comprise a step of detecting methylation of the target DNA by use of a self-reporting or energy transfer labeled primer.

As used herein, the term "self-reporting" is also named "energy transfer labeled", and may be used interchangeably with "energy transfer labeled". As used herein, "self-reporting universal primer" may be used interchangeably with the term "energy transfer labeled primer".

"Self-reporting" or "energy transfer labeled" means that the primer is capable of self-quenching or self-probing such that when amplification does not occur, fluorescence is not emitted due to self-quenching, but when amplification occurs, quenching is released and fluorescence is emitted. Self-reporting or energy transfer-labeled substances include, but are not limited to, TaqMan probes, fluorophores and molecular beacons.

In one example, the oligonucleotide (forward primer) capable of binding complementarily to the linearly amplified DNA may comprise a sequence capable of binding complementarily to the target DNA constructed in step (d)and amplifying the target DNA, for example, one or more CpG dinucleotides.

Specifically, the oligonucleotide may comprise a sequence having an identity of at least 50%, specifically, at least 55%, 60%, 70%, 80% or 90%, to one or more sequences selected from the group consisting of sequences represented by SEQ ID NOs: 1, 4, 8, 10 to 13, 15, 16, 18 to 20, 22 to 25, 27 and 28.

The description of the universal primer is applied in the same manner as in step (b) as mentioned above.

The method of the present disclosure comprises a step (e) of detecting methylation of the target DNA by a probe

capable of hybridizing complementarily to the target DNA sequence amplified in step (d).

In one example, the detection of methylation may be performed by any one method selected from the group consisting of PCR, methylation specific PCR, real-time methylation specific PCR, PCR Using Methylated DNA-specific binding protein, PCR Using Methylated DNA-specific binding antibody, quantitative PCR, DNA chip Assay, sequencing, Sequencing-by-synthesis, and Sequencing-by-ligation.

Method for Detection of Methylation

(1) Methylation-specific PCR:

When genomic DNA is treated with bisulfite to detect methylation by the methylation-specific PCR, cytosine in the 5'-CpG'-3 region remains intact, if it was methylated, but the cytosine changes to uracil, if it was unmethylated. Accordingly, based on the base sequence converted after bisulfite treatment, PCR primer sets corresponding to a region having the 5'-CpG-3' base sequence are constructed. When genomic DNA is amplified by PCR, the PCR product is detected in the PCR mixture employing the primers corresponding to the methylated base sequence, if the genomic DNA was methylated, and this methylation can be quantitatively analyzed by agarose gel electrophoresis. Herein, the probe for detection of methylation may be a TaqMan probe,

a molecular beacon probe, or a self-reporting or energy transfer-labeled probe, but is not limited thereto.

(2) Real-time methylation specific PCR

Real-time methylation-specific PCR is a real-time measurement method modified from the methylation-specific PCR method and comprises treating genomic DNA with bisulfite, designing PCR primers corresponding to the methylated base sequence, and performing real-time PCR using the primers. Methods of detecting the methylation of the genomic DNA include two methods: a method of detection using a TanMan probe complementary to the amplified base sequence; and a method of detection using Sybergreen. Thus, the real-time methylation-specific PCR allows selective quantitative analysis of methylated DNA. Herein, a standard curve is plotted using an in vitro methylated DNA sample, and a gene containing no '-CpG-3' sequence in the base sequence is also amplified as a negative control group for standardization to quantitatively analyze the degree of methylation.

(3) PCR Using Methylated DNA-specific binding protein, quantitative PCR, And DNA Chip Assay

When a protein binding specifically only to methylated DNA is mixed with DNA, the protein binds specifically only to the methylated DNA. Thus, either PCR using a methylation-specific binding protein or a DNA chip assay allows selective isolation of only methylated DNA.

In addition, methylation of DNA can also be measured by a quantitative PCR method, and methylated DNA isolated with a methylated DNA-specific binding protein can be labeled with a fluorescent probe and hybridized to a DNA chip containing complementary probes, thereby measuring methylation of the DNA.

(4) Detection of Differential Methylation-Bisulfate Sequencing Method

Another method for detecting a methylated CpG-containing nucleic acid comprises the steps of: bringing a nucleic acid-containing sample into contact with an agent that modifies unmethylated cytosine; and amplifying the CpG-containing nucleic acid in the sample using CpG-specific oligonucleotide primers, wherein the oligonucleotide primers distinguish between modified methylated nucleic acid and non-methylated nucleic acid and detect the methylated nucleic acid. The amplification step is optional and desirable, but not essential. The method relies on the PCR reaction to distinguish between modified (e.g., chemically modified) methylated DNA and unmethylated DNA.

(5) Bisulfite Sequencing Method

Another method for detecting a methylated CpG-containing nucleic acid comprises the steps of: contacting a nucleic acid-containing sample with an agent that modifies a non-methylated cytosine; and amplifying the CpG-containing

nucleic acid in the sample by means of methylation-independent oligonucleotide primers. Herein, the oligonucleotide primers can amplify the nucleic acid without distinguishing modified methylated and non-methylated nucleic acids. The amplified product may be sequenced by the Sanger method using a sequencing primer or by a next-generation sequencing method linked with bisulfite sequencing for detection of methylated nucleic acid.

(6) Herein, the next-generation sequencing method may be performed by sequencing-by-synthesis and sequencing-by-ligation. This method is characterized in that a single DNA fragment is spatially separated in place of making a bacterial clone, and is amplified *in situ* (clonal amplification) and sequenced. Herein, it analyzes hundreds of thousands of fragments at the same time, and thus is called "massively parallel sequencing".

It is based on sequencing-by-synthesis, and relies on a method of obtaining a signal while sequentially attaching mono- or di-nucleotides. It includes pyrosequencing, ion torrent and Solexa methods.

NGS systems based on sequencing-by synthesis include a Roche 454 platform, an Illumina HiSeq platform, an Ion PGM platform (Life Technology), and a PacBio platform (Pacific BioSciences). The 454 and Ion PGM platforms use emersion PCR that is a clonal amplification method, and the HiSeq platform

uses Bridge amplification. The sequencing-by-synthesis method analyzes a sequence by detecting phosphate which is generated when synthesizing a DNA while sequentially attaching single nucleotides, hydrogen ion, or a pre-labeled fluorescence dye. To detect a sequence, the 454 platform uses a pyrosequencing method employing phosphate, and the Ion PGM platform uses hydrogen ion detection. The HiSeq and PacBio platforms analyze a sequence by detecting fluorescence.

Sequencing-by-ligation is a sequencing technique employing DNA ligase, and is performed by identifying nucleotides at specific positions in a DNA nucleotide sequence. Unlike most sequencing techniques employing polymerase, sequencing-by-ligation does not use a polymerase, and uses the characteristic in that DNA ligase does not ligate a mismatch sequence. It includes a SOLiD system. In this technique, two bases are read in each step, and the reading steps are independently repeated five times through the primer reset process. Thus, each base is read twice to increase accuracy.

In the case of sequencing-by-ligation, among dinucleotide primer sets made of 16 combinations, dinucleotide primers corresponding to the nucleotide sequence of interest are sequentially ligated, and a combination of the ligations is analyzed, thereby determining the nucleotide sequence of the DNA of interest.

Regarding the primers that are used in the present disclosure, when the target DNA is treated with the reagent (e.g., bisulfite) in step (a), cytosine in the 5'-CpG'-3 region remains intact, if it was methylated, but the cytosine changes to uracil, if it was not methylated. Accordingly, based on the base sequence converted after reagent (e.g., bisulfite) treatment, PCR primers corresponding to a region having the 5'-CpG-3' base sequence may be constructed.

The primers may be designed to be "substantially" complementary to each strand of the locus to be amplified of a target DNA. This means that the primers must be sufficiently complementary to hybridize with their respective strands under polymerization reaction conditions.

Methylation of the product amplified by an oligonucleotide, which is capable of binding complementarily to the DNA linearly amplified, and the universal primer in step (d) is detected by a probe capable of hybridizing to the target DNA, and the probe can be used without limitation as long as it can hybridizing to the target DNA to detect the methylation, but may comprise, for example, one or more CpG dinucleotides. In addition, there is a method in which detection is performed using a self-reporting fluorescent substance or an energy transfer labeling primer, in addition to the above-described oligonucleotide or universal primer.

Specifically, the probe may comprise a sequence having an identity of at least 50%, specifically, at least 55%, 60%, 70%, 80% or 90%, to one or more sequences selected from the group consisting of sequences represented by SEQ ID NOs: 3, 6, 29, 30, and 31 to 34.

In some embodiments, the probe may have a reporter or a quencher attached to both ends. The reporter may be one or more selected from the group consisting of FAM (6-carboxyfluorescein), Texas red, HEX (2', 4', 5', 7',-tetrachloro-6-carboxy-4,7-dichlorofluorescein), JOE, Cy3, and Cy5. The quencher may be one or more selected from the group consisting of TAMRA (6-carboxytetramethyl-rhodamine), BHQ1, BHQ2 and Dabcyl. The quencher may be one or more selected from the group consisting of TAMRA(6-carboxytetramethyl-rhodamine), BHQ1, BHQ2 and Dabcyl.

In another aspect, the present disclosure is directed to a composition for detecting methylated DNA, comprising: at least one reagent treated to a target DNA-containing sample, which modify a non-methylated DNA to be distinguished from a methylated DNA;

an oligonucleotide, which comprises a target-specific sequence capable of binding complementarily to a target DNA sequence treated with the reagent, and a universal primer that does not bind complementarily to the target DNA;

an oligonucleotide, which are capable of binding complementarily to a linearly amplified target DNA, and a universal primer that does not bind complementarily to the linearly amplified target DNA; and

a probe capable of hybridizing complementarily to the linearly amplified target DNA sequence.

The composition according to the present disclosure overlaps with the above-described constitutions, including performing treatment with at least one reagent which modifies methylated DNA and non-methylated DNA so as to be distinguished between each other, performing linear amplification using an oligonucleotide which amplifies target DNA treated with the reagent and comprises a target-specific sequence capable of binding complementarily to a target DNA sequence and a universal sequence that does not hybridize to the target DNA, and detecting methylated DNA using an oligonucleotide, which is capable of binding complementarily to the linearly amplified DNA, a universal primer and a probe, and thus the detailed description thereof is omitted.

In still another aspect, the present disclosure is directed to a kit for detecting methylation of a target DNA, which comprises the composition.

In one example, the kit may comprise a carrier means compartmentalized to receive a sample therein, a container that receives a reagent therein, a container containing PCR

primers for amplification of a 5'-CpG-3' base sequence of a target DNA, and a container containing a probe for detecting an amplified PCR product.

Carrier means are suited for containing one or more containers such as vials, tubes, and the like, each of the containers comprising one of the separate elements to be used in the method. In view of the description provided herein of the inventive method, those of skill in the art can readily determine the apportionment of the necessary reagents among the containers.

EXAMPLES

Hereinafter, the present disclosure will be described in further detail with reference to examples. It will be obvious to a person having ordinary skill in the art that these examples are for illustrative purposes only and are not to be construed to limit the scope of the present disclosure.

Example 1: Determination of Detection Limit Using Methylated DNA Derived from Cell Line

HCT116, SW480 and HT-29 cell lines, which are human colorectal cancer cell lines, were purchased from the Korean Cell Line Bank (Seoul, Korea), and cultured in RPMI media (JBI, Seoul, South Korea) containing 10% fetal bovine serum

(JBI, Seoul, South Korea), penicillin and streptomycin in an incubator at 37°C under 5% carbon dioxide.

Genomic DNA was extracted using a QiaAmp DNA Mini kit (Qiagen, Hilden, Germany) and treated with sodium bisulfite by means of an EZ DNA Methylation-Gold kit (ZYMO Research, Irvine, USA). In brief, genomic DNA was treated with bisulfite at 65°C for 2.5 hours, and then desulfonated by leaving it at room temperature for 20 minutes. Next, it was bound to a Zymo-Spin IC column (Zymo Research, Irvine, USA), and then extracted with 10 μl of distilled water and stored at 20°C.

In order to determine the detection limit of the method of the present disclosure compare it with a meSDC2-qMSP that does not comprise the LTE process, methylated DNA derived from the HCT116 cell line was dispensed at a concentration of 100 to 10 pg, and then mixed with 20 ng of a human leukocyte genomic DNA (BioChain Insititute Inc., Hayward, CA) amplified using an Illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare, Cleveland, USA), followed by serial dilution.

Next, the DNA was amplified linearly using an oligonucleotide, which comprises the universal primer of SEQ ID NO: 7 linked with the SDC2-specific sequence of SEQ ID NO: 2, and an oligonucleotide which comprises the universal primer of SEQ ID NO: 7 linked with the COL2A1-specific sequence of SEQ ID NO: 5, under the following conditions:

95°C for 5 min, and then 35 cycles, each consisting of 95°C for 15 sec and 60°C for 1 min. Next, real-time PCR was performed using the probe of SEQ ID NO: 3, the oligonucleotide of SEQ ID NO: 1 that can bind complementarily to the linearly amplified DNA, the probe of SEQ ID NO: 6, the oligonucleotide of SEQ ID NO: 4 that can bind complementarily to the linearly amplified DNA, and the universal primer of SEQ ID NO: 7, under the following conditions: 95°C for 5 min, and then 40 cycles, each consisting of 95°C for 15 sec and 60°C for 1 min. The experiment was repeated 24 times (Table 1 and FIG. 1). Then, the Ct (cycle threshold) value was analyzed using the Rotor Gene Q software.

[Table 1] Primer and probe sequences

SEQ ID NO:	Description	Sequences
SEQ ID NO: 1	Oligonucleotide capable of binding complementarily to *SDC2*	5'-GTAGAAATTAATAAGTGAGAGGGC-3'
SEQ ID NO: 2	*SDC2*-specific sequence	5'- *AAAGATTCGGCGACCACCGA* ACGACTCAAACTCGAAAACTCG-3'
SEQ ID NO: 3	*SDC2* probe	5'-FAM-TTCGGGGCGTAGTTGCGGGCGG-3'
SEQ ID NO: 4	Oligonucleotide capable of binding complementarily	5'-GTAATGTTAGGAGTATTTTGTGGITA-3'

	to *COL2A1*	
SEQ ID NO: 5	*COL2A1*-specific sequence	5‘- *AAAGATTCGGCGACCACCGA* CTAICCCAAAAAAAC CCAATCCTA-3‘
SEQ ID NO: 6	*COL2A1* probe	5’-Cy5- AGAAGAAGGGAGGGGTGTTAGGAGAGG-3’
SEQ ID NO: 7	Universal primer	5‘-*AAAGATTCGGCGACCACCGA*-3’

Underlined: CpG dinucleotide; italic: universal primer; I: inosine nucleotide

* the sequences of SEQ ID NO: 2 and SEQ ID NO: 5 are *SDC2* and *COL2A1* target specific sequences, respectively, and correspond to sequences other than the universal primer sequence of SEQ ID NO: 7, and an oligonucleotide comprising a target-specific sequence and a universal primer that does not hybridize to the target DNA was used as a primer.

As a result, it was confirmed that the conventional method showed a detection rate of 100% in 200 pg of DNA, but showed a detection rate of 37.5% in 20 pg and a detection rate of 0% in 10 pg, whereas the method of the present disclosure showed a detection of 100% in 200 pg to 20 pg and showed a detection rate of 33.3% even in 10 pg (FIG. 2 and Table 2).

[Table 2] Comparison of detection rate between the conventional method (qMSP) and the method of the present disclosure (LTE-qMSP)

DNA concentration (pg)	Number of detections of methylated DNA by LTE-qMSP	Detection rate	Number of detections of methylated DNA by qMSP	Detection rate
10	8 out of 24	33.3	0 out of 24	N.D
20	24 out of 24	100	9 out of 24	37.5
50	24 out of 24	100	19 out of 24	79.2
100	24 out of 24	100	23 out of 24	95.8
200	24 out of 24	100	24 out of 24	100
Negative control	0 out of 24	N.D	0 out of 24	N.D

Example 2: Comparison between LTE-qMSP and qMSP

In order to evaluate the ability of the *SDC2* gene to diagnose colorectal cancer, 18 sets of methylation-specific detection primers and probes which can represent all CpG islands of the *SDC2* gene were designed (Table 3), and LTE-qMSP was performed. To this end, genomic DNA was isolated (QIAamp DNA Stool Mini kit, Qiagen) from the feces of each of 25 normal persons and 25 colorectal cancer patients, and

treated with bisulfite by an EZ DNA methylation-Gold kit. Then, the DNA was extracted with 10 μl of sterile distilled water and used in LTE-qMSP (Linear Target Enrichment-Methylation-specific real time PCR). Using the bisulfite-treated genomic DNA as a template, linear target enrichment (LTE) was performed using the designed target-specific sequence (R) shown in Table 3 below (FIG. 3). The LTE reaction was performed using a Rotor-Gene Q PCR system (Qiagen) and a total of 20 μl of a PCR reaction solution (template DNA, 10 μl; 5X AptaTaq DNA Master (Roche Diagnostics), 4 μl; *COL2A1* target-specific sequence, 1 μl (1 pmole); *SDC2* target-specific sequence, 1 μl (1 pmole); D.W. 4 μl) under the following PCR conditions: treatment at 95°C for 5 min, and then 35 cycles, each consisting of 95°C for 15 sec and 60°C for 1 min.

qMSP was performed using a Rotor-Gene Q PCR system (Qiagen). It was performed using a total of 40 μl of a PCR reaction solution (20 μl of a primary LTE product; 8 μl of 5X AptaTaq DNA Master (Roche Diagnostics); 1 μl (10 pmole) of a PCR primer capable of binding complementarily to DNA; 1 μl (10 pmole) of an oligonucleotide capable of binding complementarily to *SDC2*; DNA; 1 μl (5 pmole) of an oligonucleotide capable of binding complementarily to *COL2A1*; 1 μl (10 pmole) of a universal primer (SEQ ID NO: 7); 1 μl (5 pmole) of an *SDC2* TaqMan probe; 1 μl (2.5 pmole) of a *COL2A1*

TaqMan probe; 6 μl of D.W.) under the following PCR conditions: treatment at 95°C for 5 min, and then 40 cycles, each consisting of 95°C for 15 sec and a suitable annealing temperature (58°C to 61°C) for 1 min. Whether the PCR product would be amplified was determined by measuring the cycle threshold (Ct) value. Methylated and non-methylated control DNAs were tested along with sample DNAs using an EpiTect PCR control DNA set (Qiagen, cat. no. 59695). As an internal control gene, the *COL2A1* gene (Kristensen *et al.*, 2008) was used.

[Table 3] Primer and probe sequences for *SDC2* gene LTE-qMSP

Set	**Primers**	**Sequences (5'-->3')**	**Amplification product size (bp)**	**SEQ ID NO:**
1	F1	AAGAAAAGGATTGAGAAAAC	155	8
	R1	*AAAGATTCGGCGACCACCGA*CGAAAAA AATTCCTACAAAATTACACG		9
	Probe 1	CGTGTAATTTTGTAGGAATTTTTTTCG		29
2	F2	GGTTTGTCGGTGAGTAGAGTCGGC	124	10
	R1	*AAAGATTCGGCGACCACCGA*CGAAAAA AATTCCTACAAAATTACACG		9
	Probe 1	CGTGTAATTTTGTAGGAATTTTTTTCG		29
3	F3	GTTATAGCGCGGAGTCGCGGC	97	11
	R1	*AAAGATTCGGCGACCACCGA*CGAAAAA AATTCCTACAAAATTACACG		9
	Probe 1	CGTGTAATTTTGTAGGAATTTTTTTCG		29

4	F4	GGTTTTCGGAGTTGTTAATC	69	12
	R1	*AAAGATTCGGCGACCACCGA*CGAAAAAATTCCTACAAAATTACACG		9
	Probe 1	CGTGTAATTTTGTAGGAATTTTTTTCG		29
5	F5	TTATTTGGGAGTTATATTGTC	156	13
	R2	*AAAGATTCGGCGACCACCGA*CGCGCCGCGCCTCCCTCCCCG		14
	Probe 2	CGGGGAGGGAGGCGCGGCGCG		30
6	F6	TTTTAGTCGTTTAGGGGAGTTC	126	15
	R2	*AAAGATTCGGCGACCACCGA*CGCGCCGCGCCTCCCTCCCCG		14
	Probe2	CGGGGAGGGAGGCGCGGCGCG		30
7	F7	CGTAGTCGCGGAGTTAGTGGTTTC	152	16
	R3	*AAAGATTCGGCGACCACCGA*CGCTAACTTAAAAAAAAACTACG		17
	Probe 3	CGTAGTTTTTTTTTAAGTTAGCG		31
8	F8	CGCGTTGTTTTTTAGATATTTTC	121	18
	R3	*AAAGATTCGGCGACCACCGA*CGCTAACTTAAAAAAAAACTACG		17
	Probe 3	CGTAGTTTTTTTTTAAGTTAGCG		31
9	F9	CGCGCGGATCGCGCGTTTTCGTC	87	19
	R3	*AAAGATTCGGCGACCACCGA*CGCTAACTTAAAAAAAAACTACG		17
	Probe 3	CGTAGTTTTTTTTTAAGTTAGCG		31
10	F10	CGGTACGGGAAAGGAGTTCGCG	113	20
	R4	*AAAGATTCGGCGACCACCGA*CGACACGAAATTAATACTCCG		21

	Probe 4	CGGAGTATTAATTTCGTGTCG		32
11	F11	GTAGAAATTAATAAGTGAGAGGGC	144	1
	R5	*AAAGATTCGGCGACCACCGA*ACGACTC AAACTCGAAAACTCG		2
	Probe5	CGAGTTTTCGAGTTTGAGTCGT		33
12	F11	GTAGAAATTAATAAGTGAGAGGGC	144	1
	R5	*AAAGATTCGGCGACCACCGA*ACGACTC AAACTCGAAAACTCG		2
	Probe 5-1	TTCGGGGCGTAGTTGCGGGCGG		3
13	F12	TCGCGTTTTCGGGGCGTAGTTGC	119	22
	R5	*AAAGATTCGGCGACCACCGA*ACGACTC AAACTCGAAAACTCG		2
	Probe5	CGAGTTTTCGAGTTTGAGTCGT		33
14	F13	CGGCGGGAGTAGGCGTAGGAGGAGGAA GC	93	23
	R5	*AAAGATTCGGCGACCACCGA*ACGACTC AAACTCGAAAACTCG		2
	Probe 5	CGAGTTTTCGAGTTTGAGTCGT		33
15	F14	AGGAAGCGAGCGTTTTCGAGTTTC	71	24
	R5	*AAAGATTCGGCGACCACCGA*ACGACTC AAACTCGAAAACTCG		2
	Probe 5	CGAGTTTTCGAGTTTGAGTCGT		33
16	F15	AATCGTTGCGGTATTTTGTTTC	133	25
	R6	*AAAGATTCGGCGACCACCGA*CCAAAAA CCGACTACTCCCAACCG		26
	Probe 6	CGGTTGGGAGTAGTCGGTTTTTGG		34

17	F16	GATTCGTGTGCGCGGGTTGC	110	27
	R6	*AAAGATTCGGCGACCACCGA*CCAAAAA CCGACTACTCCCAACCG		26
	Probe 6	CGGTTGGGAGTAGTCGGTTTTTGG		34
18	F17	CGAGCGTTGGGTAGGAGGTTTC	88	28
	R6	*AAAGATTCGGCGACCACCGA*CCAAAAA CCGACTACTCCCAACCG		26
	Probe 6	CGGTTGGGAGTAGTCGGTTTTTGG		34

* Italic: universal sequence (SEQ ID NO: 7)

* Sequences of R1 to R6 are target-specific sequences which correspond to sequences other than the universal sequence of SEQ ID NO: 7.

* F1 to F17 correspond to oligonucleotides capable of binding complementarily to linearly amplified target DNA.

A. Results of comparison between LTE-qMSP and qMSP for set 6

According to the method described in Example 1, the sensitivity of detection by set 6 was compared between the LTE-qMSP method and the qMSP method. As a result, it was confirmed that the conventional method showed a detection rate of 100% in 200 pg of DNA, but showed a detection rate of 37.5% in 20 pg and a detection rate of 0% in 10 pg, whereas the method of the present disclosure showed a detection rate of 100% in 200 pg to 20 pg and a detection rate of 33.3% even in 10 pg (FIGS. 4a and Table 4).

[Table 4] Comparison of detection rate between the conventional method (qMSP) and the method of the present disclosure (LTE-qMSP)

DNA concentration (pg)	Number of detections of methylated DNA by LTE-qMSP	Detection rate	Number of detections of methylated DNA by qMSP	Detection rate
10	8 out of 24	33.3	0 out of 24	N.D
20	24 out of 24	100	9 out of 24	37.5
50	24 out of 24	100	19 out of 24	79.2
100	24 out of 24	100	23 out of 24	95.8
200	24 out of 24	100	24 out of 24	100
Negative control	0 out of 24	N.D	0 out of 24	N.D

B. Results of comparison between LTE-qMSP and qMSP for set 17

According to the method described in Example 1, the sensitivity of detection by set 17 was compared between the LTE-qMSP method and the qMSP method. As a result, it was

confirmed that the qMSP method showed a detection rate of 100% in 200 pg of DNA, but showed a detection rate of 45.0% in 20 pg and a detection rate of 0% in 10 pg, whereas the method of the present disclosure showed a detection rate of 100% in 200 pg to 20 pg and a detection rate of 91.7% in 20 pg and a detection rate of 29.2% even in 10 pg (FIGS. 4b and Table 5).

[Table 5] Comparison of detection rate between the conventional method (qMSP) and the method of the present disclosure (LTE-qMSP)

DNA concentration (pg)	Number of detections of methylated DNA by LTE-qMSP	Detection rate	Number of detections of methylated DNA by qMSP	Detection rate
10	7 out of 24	29.2	0 out of 24	N.D
20	22 out of 24	91.7	11 out of 24	45.0
50	23 out of 24	95.8	18 out of 24	75.0
100	24 out of 24	100	23 out of 24	95.8
200	24 out of 24	100	24 out of 24	100

Negative control	0 out of 24	N.D	0 out of 24	N.D

Example 3: Evaluation of the Ability of *SDC2* Gene to Diagnose Colorectal Cancer in Feces by LTE-qMSP

The degree of methylation in each of the samples shown in Table 3 of Example 2 was measured by the Ct value, and the sensitivity and specificity of each primer and probe set were calculated by ROC curve analysis (MedCalc program, Belgium) (Table 6).

Methylation of the *SDC2* gene was analyzed using fecal DNA derived from normal persons and colorectal cancer patients. As a result, it was confirmed that the sensitivity for diagnosis of colorectal cancer was as high as 76% (19/25) to 88.0% (22/25) and the specificity was as high as 88.0% (3/25) to 100% (0/25). This suggests that the use of methylation of the *SDC2* gene is highly useful for diagnosis of colorectal cancer.

[Table 6] Evaluation of the ability of *SDC2* gene to diagnose colorectal cancer

Primer and probe set	Cut-off (Ct)	P value	Sensitivity (%), n = 25	Specificity (%), n = 25
1	<32.1	< 0.001	76.0	92.0
2	<32.0	< 0.001	80.0	96.0
3	<32.3	< 0.001	76.0	88.0

4	<32.1	< 0.001	80.0	92.0
5	<32.0	< 0.001	84.0	96.0
6	<32.5	< 0.001	88.0	92.0
7	<32.5	< 0.001	76.0	96.0
8	<32.2	< 0.001	80.0	88.0
9	<32.3	< 0.001	88.0	100
10	<32.5	< 0.001	76.0	92.0
11	<32.0	< 0.001	80.0	100
12	<32.0	< 0.001	88.0	92.0
13	<32.1	< 0.001	88.0	88.0
14	<32.0	< 0.001	84.0	92.0
15	<32.2	< 0.001	80.0	96.0
16	<32.3	< 0.001	76.0	100
17	<32.5	< 0.001	84.0	100
18	<32.0	< 0.001	88.0	96.0

In order to further evaluate the ability of the LTE-qMSP method for multiplex detection of methylation, the ability of a combination of set 1 and set 2 to diagnose colorectal cancer was evaluated using fecal DNA in a single tube.

[Table 7] Evaluation of the ability of combination of set 1 and set 12 to diagnose colorectal cancer

Primer and probe set	Cut-off (Ct)	P value	Sensitivity (%), n = 25	Specificity (%), n = 25
1 + 12	<32.2	< 0.001	88.0	92.0

Clinical verification of a combination of set 1 and set 12 was performed, and as a result, it was confirmed that the sensitivity and the specificity were as extremely high as 88% and 92%, respectively. Thus, it was confirmed again that it is possible to detect methylation using the LTE-qMSP method in which several methylated targets are simultaneously amplified in a single tube.

INDUSTRIAL APPLICABILITY

As described above, the method for detecting methylated DNA according to the present disclosure uses a universal primer, and hence can efficiently amplify multiple target DNAs even when using only one additional primer, even if a sample contains various kinds of genes. Furthermore, the complexity of PCR (real-time PCR) which is used to detect methylation by linear amplification can decrease, and variation in the efficiency of PCR can decrease, indicating that the sensitivity of detection is significantly high and the method is useful. In addition, the method has an advantage in that it can enrich methylated target DNA with higher specificity in the step of linearly amplifying the target DNA (LTE step).

Although the present disclosure has been described in detail with reference to the specific features, it will be apparent to those skilled in the art that this description is

only for a preferred embodiment and does not limit the scope of the present disclosure. Thus, the substantial scope of the present disclosure will be defined by the appended claims and equivalents thereof.

CLAIMS

1. A method for detecting methylated DNA, comprising:

(a) treating a target DNA-containing sample with at least one reagent which modifies a non-methylated DNA site to be distinguished from a methylated DNA site;

(b) constructing a reverse primer, which comprises a target-specific sequence designed to be capable of binding complementarily to the target DNA treated with the reagent, and a universal primer that does not bind complementarily to the target DNA, and the universal primer is bound to the 5’ end of the target-specific sequence;

(c) performing one direction asymmetric linear amplification using, as a template, the target DNA treated with the reagent in step (a), and using, as a primer, the reverse primer constructed in step (b);

(d) amplifying the target DNA using a forward primer which is capable of binding complementarily to the DNA linearly amplified in step (c), and a universal primer that does not bind complementarily to the linearly amplified target DNA; and

(e) detecting methylation of the target DNA by a probe capable of hybridizing complementarily to the target DNA sequence amplified in step (d).

2. The method of claim 1, wherein the reagent is one or more selected from among bisulfite, hydrogen sulfite, disulfite, and combinations thereof.

3. The method of claim 1 or 2, wherein at least one cytosine base is converted to either uracil or another base that is dissimilar to cytosine, by the treatment with the reagent.

4. The method of any one of claims 1 to 3, wherein a plurality of target DNA methylations are detected in the sample in a single reaction.

5. The method of any one of claims 1 to 4, wherein the universal primer of the step (b) or (d) comprises one or more sequences selected from the group consisting of nucleotide sequences set forth in SEQ ID NOs: 7 and 35 to 41.

6. The method of any one of claims 1 to 5, wherein the target-specific sequence of step (b) binds complementarily to a methylated site and/or a non-methylated site of the target DNA.

7. The method of any one of claims 1 to 6, wherein the target-specific sequence of step (b) comprises one or more CpG dinucleotides.

8. The method of any one of claims 1 to 7, wherein the target-specific sequence of step (b) comprises one or more sequences selected from the group of sequences set forth in SEQ ID NOs: 2, 5, 9, 14, 17, 21 and 26.

9. The method of any one of claims 1 to 8, wherein step (e) comprises detecting methylation of the target DNA using one or more methods selected from the group consisting of PCR, methylation specific PCR, real-time methylation specific PCR, PCR using a methylated DNA-specific binding protein, PCR using a methylated DNA-specific binding antibody, quantitative PCR, DNA chip Assay, sequencing, sequencing-by-synthesis, and sequencing-by-ligation.

10. The method of any one of claims 1 to 9, wherein the forward primer of step (d) comprises one or more CpG dinucleotides.

11. The method of any one of claims 1 to 9, wherein the forward primer of step (d) comprises one or more sequences

selected from the group of sequences set forth in SEQ ID NOs: 1, 4, 8, 10 to 13, 15, 16, 18 to 20, 22 to 25, 27 and 28.

12. The method of any one of claims 1 to 11, wherein the probe of step (e) comprises one or more CpG dinucleotides.

13. The method of any one of claims 1 to 12, wherein the probe of step (e) comprises one or more sequences selected from the group of sequences set forth in SEQ ID NOs: 3, 6, 29, 30, and 31 to 34.

14. The method of any one of claims 1 to 13, wherein the probe of step (e) further comprises a reporter or quencher.

15. The method of any one of claims 1 to 14, wherein the forward primer of the step (d) and the universal primer of the step (d) have a self-reporting function or are energy transfer-labeled.

16. The method of claim 14, wherein the reporter is selected from the group consisting of FAM (6-carboxyfluorescein), Texas red, HEX (2', 4', 5', 7',-tetrachloro-6-carboxy-4,7-dichlorofluorescein), JOE, Cy3, and Cy5.

17. The method of claim 14, wherein the quencher is selected from the group consisting of TAMRA (6-carboxytetramethyl-rhodamine), BHQ1, BHQ2 and Dabcyl.

18. The method of any one of claims 1 to 17, wherein the target DNA is SDC2.

19. A composition for detecting methylated DNA, comprising:

a reverse primer which comprises a target-specific sequence capable of binding complementarily to a target DNA sequence treated with a reagent which modifies a non-methylated DNA to be distinguished from a methylated DNA, and a universal primer that does not bind complementarily to the target DNA, fused to the 5′ end of the target-specific sequence;

a forward primer capable of binding complementarily to a linearly amplified target DNA, and a universal primer not complementary to the linearly amplified target DNA; and

a probe capable of hybridizing complementarily to the linearly amplified target DNA sequence.

20. The composition of claim 19, wherein the reagent is selected from the group consisting of bisulfite, hydrogen sulfite, disulfite, and combinations thereof.

21. The composition of claim 19 or 20, wherein the composition is formulated to detect methylation of a plurality of target DNAs in a sample in a single reaction.

22. The composition of any one of claims 19 to 21, wherein the universal primer comprises one or more sequences selected from the group of nucleotide sequences set forth in SEQ ID NOs: 7 and 35 to 41.

23. The composition of any one of claims 19 to 22, wherein the target-specific sequence binds complementarily to a methylated site and/or a non-methylated site of the target DNA.

24. The composition of any one of claims 19 to 23, wherein the target-specific sequence comprises one or more CpG dinucleotides.

25. The composition of any one of claims 19 to 24, wherein the target-specific sequence comprises one or more sequences selected from the group of sequences set forth in SEQ ID NOs: 2, 5, 9, 14, 17, 21 and 26.

26. The composition of any one of claims 19 to 25, wherein the forward primer comprises one or more CpG dinucleotides.

27. The composition of any one of claims 19 to 26, wherein the forward primer comprises one or more sequences selected from the group of sequences set forth in SEQ ID NOs: 1, 4, 8, 10 to 13, 15, 16, 18 to 20, 22 to 25, 27 and 28.

28. The composition of any one of claims 19 to 27, wherein the probe comprises one or more CpG dinucleotides.

29. The composition of any one of claims 19 to 28, wherein the probe comprises one or more sequences selected from the group of sequences set forth in SEQ ID NOs: 3, 6, 29, 30, and 31 to 34.

30. A kit for detecting methylated DNA, which comprises the composition of any one of claims 19 to 29.