The present invention provides methods for detecting biomarkers based on Abscription®, abortive transcription technology. Particularly, the present invention provides bisulfate free methods for detecting methylation of CpG islands from small samples of DNA. The methods are suitable for multiplexing and can be used to analyze multiple CpG islands from a single sample in a short time.
ABSCRIPTION BASED MOLECULAR DETECTION

RELATED APPLICATION

[0001] This application claims the benefit of priority under 35 USC §119 of U.S. Provisional Applications Serial No. 61/160,335 filed March 15, 2009, the entire disclosure of which is incorporated herein by reference.

BACKGROUND OF THE INVENTION

[0002] Cancer is actively avoided through the expression of numerous tumor-suppressor genes that regulate the cell division cycle and mediate interactions among cells. Studies of benign and malignant tumors have shown that cancer develops through a multi-step process where randomly accumulated changes either enhance the expression of proto-oncogenes or reduce the expression or function of tumor-suppressor and DNA repair genes. Somatic mutations account for some of these changes in tumor-suppressor and DNA repair genes. However, it has recently become apparent that epigenetic changes, such as DNA hypermethylation and hypomethylation, also play a large role in the development of cancer through inactivation or enhancement of tumor suppressors or proto-oncogenes. Hypermethylation of CpG promoter islands occurs at an early stage of cancer development and is found in virtually all tumors, making it potentially very useful as a diagnostic marker, allowing cancer to be noninvasively detected in the early stages when treatment is most effective. For example, hypermethylation of the promoter region of genes such as DAP kinase, p16, and MGMT has reportedly been detected in the sputum of smokers up to 3 years prior to the diagnosis of squamous cell lung carcinoma (Belinsky et al. (2006). Cancer Res. 66:3338-44, Palmisano et al. 2000. Cancer Res. 60:5954-8). Similarly, hypermethylation of a small panel of genes may be a valuable early detection indicator for non-small cell lung cancer. Hypermethylation of a small panel of genes was detected in the early stages of breast cancer but was not detected in normal or benign breast tissue Krassenstein et al. (2004). Clin Cancer Res. 10:28-32.

[0003] Methylation of cytosines in CpG islands is an early event in most cancers that leads to reduced expression of many genes, including tumor suppressor genes. Surveys of CpG island methylation in tumor cell DNA suggest that this epigenetic change is common enough to rival the impact of mutation in tumor progression. Early detection of abnormal methylation can lead to regular screening and early diagnosis when treatment is most effective. Early epigenetic changes are often detectable in blood serum or other bodily fluids (urine, sputum, saliva), not just in the tumor tissue itself, which means that epigenetic diagnostic testing can be done noninvasively using these fluids. Diagnostic tests based on CpG island methylation may also be utilized for drug development, identification of patient populations that will respond to these drugs and post treatment monitoring.

[0004] Recent improvements in the sensitivity of methylation detection and elucidation of methylation signatures for specific cancers have made it feasible to assay for tumors by sampling DNA from bodily fluids. Tumor cells release DNA into blood from a relatively early stage of the disease. From 3% to over 90% of the DNA in the blood of cancer patients has been found to be of tumor origin
The development of PCR-based methylation assays has made it possible to detect the presence of tumors noninvasively from blood, sputum, and urine. In one study methylation detection was more sensitive than urine cytology in detecting aberrant premalignant cells. Clinical sensitivities (the proportion of confirmed cases detected) are typically low in surveys of blood samples using a single methylation marker. However, clinical specificity (the proportion of normal controls that test negative) from blood samples approaches 100%. The clinical sensitivities should increase with methylation tests that assess more than a single CpG island.

The appearance of abnormally methylated DNA in bodily fluids by itself does not help to pinpoint which organ is affected by a tumor. Surprisingly little information is needed to establish the tissue origin of a tumor. Numerous methylation screening studies have established methylation signatures; collections of methylated CpG islands that are strongly associated with cancers from particular organs. In one survey, aberrant methylation of 3 to 4 candidate CpG islands was sufficient to identify from 70-90% of 15 cancer types (Esteller et al. (2001) Cancer Res. 61:3225-9). Methylation profiles developed for primary tumors could be applied to tumor cell lines to accurately identify the tissue origin of their parent tumors (Graziano et al. (2004) Clin. Cancer Res. 10:2784). This result suggests that methylation signatures of different tumor types are not greatly affected by the selective pressures associated with growth in culture. The availability of methylation profiles should greatly enhance the ability to detect cancer in inaccessible organs through a simple blood test.

Detection of methylation in clinical samples would enable early detection of cancer. The development of simple and sensitive multiplex detection assays will allow small clinical samples to be profiled for the status of multiple CpG islands. This kind of information will be valuable in diagnosis and treatment.

**Methods for Detecting DNA Methylation**

A number of methods have been used to detect methylated-CpG (mCpG) in target DNA. The three primary methods in current use are detailed below.

**Bisulfite Methods.** The most commonly used methylation detection methods are based on bisulfite modification of DNA, resulting in deamination of cytosine residues to uracil while leaving the methylated cytosines unchanged. Upon PCR amplification, the methylated cytosine is copied to cytosine and uracil is copied to thymine. As a result, the retention of cytosine at a specific position indicates methylation. The modified DNA is then analyzed, e.g. by sequence analysis, methylation-specific PCR (MSP) (Herman et al. (1996) Proc. Natl. Acad. Sci. USA 93:9821-26), or hybridization (e.g. to a microarray or blot). In MSP, a pair of methylation-specific oligonucleotide primers is added to the bisulfite-treated DNA and PCR is performed in order to amplify the target DNA. Fluorescence-based quantitative real-time PCR can also be performed on bisulfite-modified DNA (Eads et al. (2000) Nucl. Acids Res. 28:E32; Zeschnigk et al. (2004) Nucl. Acids Res. 32:e125).
Calibrated, fluorescence-based variants of MSP exploit real-time PCR to provide quantification of the amount of methylated DNA in a sample. An important underlying assumption of these PCR-based methods is that the few CpG sites that are recognized by the primers/probes reflect the overall status of the target CpG island. While this is usually true for heavily methylated or completely unmethylated islands, partially methylated targets are probably not readily scored in methylated- or unmethylated-specific reactions.

An advantage of bisulfite modification is that it differentially marks methylated versus unmethylated sites allowing sequencing methods to detect methylation patterns. Sequencing of cloned bisulfite-treated DNA is the most commonly used method for methylation detection. It provides information on the success of the bisulfite treatment in addition to sampling a greater number of CpG sites than the MSP-based methods. Due to its complexity and expense, however, bisulfite sequencing is better suited for marker discovery than clinical diagnostics. Bisulfite treatment destroys a large percentage of the input DNA, resulting in limited sensitivity and a requirement for large amounts of DNA. Quality control assessments of bisulfite treated DNA are necessary before performing a detection assay to avoid misleading results. There is a potential of false-positive results for MSP-based assays due to incomplete cytosine deamination during bisulfite treatment. Amplification of bisulfite-treated DNA is affected by PCR bias favoring unmethylated DNA. While this problem can usually be corrected by optimizing primer annealing conditions, it may complicate primer design and testing. Template biases can be eliminated with the use of digital bisulfite-PCR. Dilution of the DNA sample to an average of less than one copy per reaction eliminates competition among templates. Individual molecules can be sequenced without biases introduced by cloning.

Commercial kits, reagents and systems employing bisulfite treatment for analyzing mCpG are available. Epigenetics (Berlin) offers two variants of the MethyLight assay, adaptations of quantitative real-time PCR, called Quantitative MethyLight (QM) and Heavy Methyl (HM). QM utilizes Taqman® probes to generate a fluorescent signal. During the course of amplification, the fluor is cleaved from the Taqman® probe resulting in fluorescence that can be detected in real-time (Wojdacz & Dobrovic (2007) Nucl. Acids Res. 35:e41). HM is an adaptation of QM in which blocker oligonucleotides are added to the reaction. These blocker oligonucleotides prevent amplification of unmethylated DNA, resulting in increased assay sensitivity (Cottrell et al. (2004) Nucl. Acids Res. 32:e10). Pyrosequencing® is also utilized for methylation quantification from bisulfite-modified DNA, as exemplified by the Pyro Q-CpG™ system from Biotage (Uppsala, Sweden; Tost et al. (2003) Biotechniques 35:152-56).

Although bisulfite modification is a widely used, the extensive DNA degradation it causes can introduce sampling errors when few molecules are long enough to be amplified (Ehrich et al. (2007) Nucl. Acids Res. 35:e29). Furthermore, the assays are time-consuming, require a harsh base denaturation step, and have a high-probability of false-positive results due to incomplete cytosine deamination during bisulfite treatment.
Methylation-Sensitive Restriction Enzyme Digestion Methods. A second type of method for detecting mCpG in DNA relies on differential cleavage by restriction endonucleases. DNA is treated with either a MSRE (methylation-sensitive restriction endonuclease) or a MDRE (methylation dependent restriction endonuclease), amplified and then analyzed by microarray or gel electrophoresis. MSREs such as HpaII and Acil cut a DNA sequence only if it is unmethylated. MDREs are restriction endonuclease that require methylation of a DNA sequence for cleavage. By treating a sample of DNA with either of these enzymes and subsequent comparison to a control sample, the methylation state of the DNA sample can be determined. If digestion of a specific DNA sample occurs after treatment with a MDRE, then the DNA can be assumed to be methylated. Conversely, if the DNA is uncut when treated with a MSRE, then the sample can be assumed to be methylated. By comparing the amount of cut versus uncut DNA, the level of methylation can be estimated. A common read-out for this type of methylation analysis is the subsequent amplification and fluorescent labeling of the digested DNA. The fragments can then be hybridized to a library microarray and analyzed or simply resolved by electrophoresis.


An advantage of MSRE/MDRE digestion is that no pre-treatment of the DNA is necessary, although it is often performed in conjunction with bisulfite treatment of DNA in a procedure called COBRA (Xiong & Laird (1997) Nucl. Acids Res. 25:2532-34). Some disadvantages with this procedure are that it is lengthy and is dependent on the presence of MSRE/MDRE recognition sequences within a target DNA. Furthermore, this approach is relatively inefficient, which can reduce the reliability of the results. The only CpG sites that are assessed are those within a small number of restriction enzyme recognition sites and status of those sites may not reflect the status of the entire CpG island in which the site reside. Incomplete digestion leads to frequent false positives, especially when cleavage reactions are subjected to a subsequent amplification step. Restriction endonuclease cleavage assays have poor sensitivity compared to bisulfite methods, such as MSP, allowing detection of not less than 10% methylated DNA in a sample (Singer-Sam et al. Nucleic Acids Res. 1990. 18:687; Yegnasubramanian et al. Nucleic Acids Res. (2006) 34:e19).

Chromatin Immunoprecipitation Methods. A third method that is commonly employed for detecting mCpG is chromatin immunoprecipitation (ChIP). Typically, cells are fixed, and then methylated DNA is immunoprecipitated by the use of antibodies specific for methyl binding proteins. The resulting DNA is amplified, labeled and analyzed by hybridization in a microarray assay. The advantages of this method are that the assay can be performed from live cells with little or no DNA purification required. The assay also has increased sensitivity, as unwanted and contaminant DNA are removed prior to analysis. However, the ChIP procedure is very time-consuming, involves several steps and requires expensive reagents. Some assays may take as long as five days to complete.
Methods using Methyl Binding Proteins. An alternative and more sensitive approach to separating methylated from unmethylated DNAs involves the use of methyl-CpG binding domain (MBD) proteins or antibodies against 5-methyl-C. MBD proteins have high affinity for methylated CpG sites and very low affinity for unmethylated DNA (Fraga et al. Nucleic Acids Res. (2003) 31:1765-74). Samples are incubated with immobilized MBD protein in a variety of formats (magnetic beads, columns, the walls of PCR tubes). Methylated DNA capture is usually followed by amplification of the captured DNA. MBD-based DNA detection has the major advantage that all of the methylated sites can contribute to binding, thereby allowing an entire island to be sampled for methyl-CpGs. This characteristic makes the binding assay less vulnerable to false negatives that affect MSP and restriction endonuclease-based assays when unmethylated sites in a partially methylated island correspond to priming/probe sites (Yegnasubramanian et al. Nucleic Acids Res. (2006) 34:e19). This situation is likely to be common in clinical samples containing early stage tumor cells that contain partially methylated CpG islands. MBD based binding assays are very sensitive, allowing detection of as little as 160 pg of methylated DNA (equivalent to ~25 cells) or 1 methylated molecule in 500 unmethylated molecules (Gebhard et al. Nucleic Acids Res. (2006) 34:e8256). This is close to the sensitivity of MSP (1 methylated molecule/1,000 unmethylated molecules). The COMPARE MBD assay can be as sensitive as real-time MSP (1 methylated molecule/10,000 unmethylated molecules) by including digestion with HpaII (an MSRE) before the binding step. Cleavage of unmethylated DNAs at a location between PCR priming sites gives high sensitivity with DNA mixtures that contain artificially methylated DNAs that are fully methylated (Yegnasubramanian et al. Nucleic Acids Res. (2006) 34:e19). However this strategy could suffer the disadvantage associated with the use of restriction endonucleases in that some partially methylated islands will be scored as unmethylated in clinical samples (Yegnasubramanian, et al. supra).

Given the importance of CpG methylation in cancer development and progression, a rapid, reliable, and sensitive test for methylated CpG DNA would provide an important and useful tool for cancer screening.

SUMMARY OF THE INVENTION

The present invention provides methods for detecting target polynucleotides in a sample. The methods generally involve contacting a sample containing a target polynucleotide with a primer pair that specifically hybridizes to and amplifies a target sequence of the polynucleotide. For this step, the primer pair includes a first primer with a 3’ sequence complementary to a first sequence flanking the target polynucleotide sequence, and a 5’ capture tag. The second primer of the pair has a 3’ sequence complementary to a second sequence flanking the polynucleotide target sequence on the opposite strand, and a 5’ sequence that provides a means for directing Abscription. Following amplification (e.g. PCR) using this primer pair, the the amplified target sequence is contacted with an immobilized molecule that binds the 5’ capture tag, to capture the amplified target sequence. At least one Abscript is then transcribed from the means for directing Abscription and the Abscript detected as an indication of the presence of the target polynucleotide.
Capture will typically be via an affinity reagent or binding pair bound or capable of being bound to a solid support. For example, the 5’ capture tag can be biotin, which can be readily incorporated into oligonucleotide primers, and the molecule that binds to the 5’ capture tag can be streptavidin immobilized on a solid support. A wide variety of solid supports are suitable for use in the methods of the present invention, such as beads, tubes, and microtiter plates. Conveniently, streptavidin and other binding pair molecules can be bound to magnetic beads which permit rapid separation of the solid phase from unbound reagents in solution. In certain embodiments of the invention, unbound reagents, primers, and polynucleotides can be washed from immobilized and captured polynucleotides prior to the subsequent steps in the procedure, which may increase the efficiency of the method. However, this is not necessary as the entire method can be performed in a single pot or tube without separation steps.

PCR is typically used for the amplification step, using for example, a thermostable DNA polymerase or a thermostable RNA polymerase. However, a variety of target amplification methods known in the art may be suitable for use in the methods of the present invention.

A variety of methods are available for detecting Abscripts as described herein, including, but not limited to mass spectrometry, capillary electrophoresis or thin layer chromatography. In certain aspects, a detectably labeled nucleotide or other label can be incorporated into Abscript signals generated by the methods of the invention to increase the sensitivity or expand the detection techniques that may be used. For example, the detectably labeled nucleotide can be a fluorescent nucleotide.

Abscripts generated by the present invention will generally be short, e.g. 3-20 nucleotides in length. Abscripts as small as 3 nucleotides in length are typically used in the methods described herein.

The second primer of the pair used during amplification has a 3’ sequence complementary to a second sequence flanking the target sequence of the polynucleotide on the opposite strand, and a 5’ sequence that provides a means for directing Abscript.

In certain embodiments, the means is provided by an α-TAP (Target Attachment Probe) sequence that is used to tag or identify the target. The α-TAP is designed to be complementary to a TAP sequence and permits the attachment of an APC (Abortive Promoter Cassette). To maintain the α-TAP as a single-strand that is thereby available for hybridization to the a TAP sequence, a non-natural nucleotide can be included between the 5’ α-TAP sequence and the 3’ sequence complementary to the sequence flanking the target in the primer. Non-natural nucleotides, such as etheno-deoxyadenosine, are not recognized by polymerases during amplification. Thus, sequences downstream from the non-natural nucleotide in a primer are not replicated and those sequences remain single-stranded.

Once an APC is bound to the amplified target through the TAP-α-TAP hybrid that is formed, Abscripts are transcribed from the APC as an indication or signal for detecting the presence of the target. The APC that is bound is either a double-strand region or is made double stranded by hybridization of a probe.

In certain embodiments of the invention, a fully duplex APC can be generated during the amplification reaction from a primer sequence that includes one strand of the APC. Conveniently,
Abscription can be performed during the amplification by including a thermostable RNA polymerase and nucleotides in the reaction. Thus, in these embodiments, the second primer for the amplification reaction includes an APC sequence. As duplex APCs are generated (e.g. by PCR), Abscripts are transcribed from the APC and can be detected as they are produced (e.g. in real-time), or analyzed at a later time by Abscript detection methods described herein.

The invention provides rapid, sensitive, and specific methods for detecting a variety of target polynucleotides of interest, including DNA and RNA targets, with an expanded repertoire of detection techniques as compared to PCR. Unlike PCR, the methods are also suitable for detecting polynucleotide targets that are modified, such as methylated DNA targets. According to such methods, methylated genomic DNA fragments are first isolated by cleaving a genomic DNA sample containing a methylated target polynucleotide (such as a CpG island), with a restriction enzyme that does not cleave the target polynucleotide, or generates suitably representative fragments of the target for during cleavage. The cleaved genomic DNA is then contacted with an immobilized methyl binding domain, such as the GST-MBD2 fusion protein described herein. In this way, methylated genomic DNA fragments are immobilized and therefore isolated from the non-methylated DNA fragments in the sample. Optionally, the methylated genomic DNA fragments can be eluted from the immobilized MBD and recovered prior to analysis. For example, where the GST-MBD2 fusion protein is used for immobilizing methylated CpG island targets, the GST portion of the fusion protein can be bound to a glutathione resin (before or after interaction with DNA), and the bound methylated DNA fragments can be eluted with glutathione.

The methods of the invention are also suitable for multiplexing. According to certain embodiments of the invention, the a plurality of different target polynucleotides, are processed simultaneously by including a plurality of first and second primer pairs in the reactions, each primer pair being designed to specifically hybridize to a different target polynucleotide. By designing different, unique APCs for each target that are attached to the target through the primed amplification (either as part of the APC-containing primer or via the TAP-\(\alpha\)-TAP hybrid, as described herein), the presence of each of the plurality of target can be identified through the APC signal that is generated. For example, each APC can be designed to be distinguishable on the basis of molecular weight or nucleotide sequence. According to these embodiments of the invention, at least 5, 10, 20, 50, 100 or more targets can be detected in a single assay.

**BRIEF DESCRIPTION OF THE DRAWINGS**

FIG. 1 illustrates the process of abortive transcription, which is exploited by the Abscription\textsuperscript{®} methods of the invention. Abortive transcription occurs on most promoters when RNA polymerase (RNAP) is trapped at the promoter repeatedly making short abortive transcripts (typically 2 to 12 nt long). During abortive transcription, the RNAP does not translocate or leave the promoter. During normal transcription, RNAP eventually undergoes a conformational change to a stable, processive elongation complex, a process called promoter escape, and then continues transcription until a termination signal is
Artificial promoters, or Abortive Promoter Cassettes (APCs) have been developed that trap RNAP in an abortive complex, reiteratively synthesizing thousands of identical short oligonucleotides per minute. Each APC is designed to make a different Abscript of specific length and sequence which can be separated and quantified.

FIG. 2 illustrates detection of protein, RNA and DNA targets using Abscription®. APCs are attached to a Target Attachment Probe (TAP), which specifically binds to the molecular target (FIG. 2A). For detection of DNA and RNA, TAPs include oligonucleotides that specifically hybridize to or proteins that bind specifically to the nucleic acid target (FIG. 2B). For protein detection, APCs can be attached to any molecule that binds to the protein target, such as an antibody or a ligand. FIG. 2C illustrates an embodiment of the invention where the APC is attached to an antibody. The target protein can be "sandwiched" between an APC-antibody complex and a second antibody immobilized on a solid support, for capture and detection of the protein targets similar to strategies used in enzyme linked immunosorbent assay (ELISA).

FIG. 3 illustrates dinucleotide initiation of Abscription® and termination after the addition of one NTP. Abscript length can be limited by inclusion of chain terminating NTPs (3′-0-Me-NTPs at R3) as depicted, or by omitting one or more NTPs from the reaction. R1 = Affinity tag, Fluorescent Tag; R2 = OH, OMe, H; R3 = OH, OMe, H; R4 = OH, OMe, H.

FIGs. 4A and 4B illustrate detection of Abscripts by mass spectrometry. An Abscription® reaction that included the initiator GpA and GTP was fractionated by reverse-phase HPLC. The output of the column was introduced into a mass spectrometer. FIG. 4A shows the column profile in terms of total ion count as a function of retention time. FIG. 4B shows the ion spectrum for the trinucleotide Abscript GAG peak (retention time 5.4 min). Singly- and doubly-charged GAG species have m/z values of 956, 1 and 978.2, respectively. The sodium adduct of the double charged species has a m/z of 978.2.

FIG. 5 is an illustration of the structure of a GST-MBD Protein used in methylation detection methods of the invention. FIG. 5A shows the linkage of a MBD domain to the carboxyl end of a GST domain. The amino acid sequence linking the domains (SEQ ID NO: 1) contains a thrombin cleavage site indicated by the arrow. FIG. 5B shows the amino acid sequence for the DNA binding domain of mouse MBD2b (SEQ ID NO:2). The underlined amino acids correspond to conserved residues among the DNA binding domains of MBD proteins (Ohki et al. (1999) EMBO J. 18:6653-61). FIG. 5C shows the results of methylated DNA fractionation using immobilized GST-MBD. The S (supernatant) fraction contains amplified unmethylated SNRPN CpG island DNA. E1 contains amplified methylated SNRPN CpG island DNA that was eluted from the immobilized protein. Fractions E2 and E3 are two additional serial elutions from the same immobilized protein. FIG. 5D shows the fractionation of PTGS2 DNA, which is unmethylated, in HeLa cells. All of the PTGS2 DNA is recovered in the unbound supernatant fraction S.

FIG. 6 is a flow diagram illustrating the strategy for α-TAP Abscription®-based CpG methylation detection including the binding of a TAP-APC to an amplified fragment of a target CpG island. Steps in the process are indicated by numerals. FIG. 6A illustrates the initial capture of
methylated CpG containing DNA. **Step 1:** Methylated DNA fragments are separated from unmethylated DNA fragments with an immobilized GST-MBD protein. **Step 2:** Methylated DNA fragments are released by heat treatment or exposure to protease or glutathione. **FIG. 6B** shows amplification tagging and capture of tagged DNA fragments. **Step 3:** A target CpG island is tagged with an affinity label such (as biotin (B), as shown) and a single-strand extension during PCR, through the incorporation of a 3' biotinylated primer and a primer containing a non-coding nucleotide between the primer sequence and an anti-TAP sequence (α-TAP). **Step 4:** The biotinylated amplicon is bound to streptavidin-magnetic beads. **Step 5:** The APC is bound to the amplicon by hybridization between the TAP sequence and the α-TAP sequence. Abscription® is performed by contacting an RNA polymerase (RNAP) with the bead-immobilized complexes containing APCs.

**[0036]** **FIGS. 7A-7C** illustrate the interactions between exemplary target- specific amplification primers and anti-TAP (α-TAP) primer/probes. **FIG.7A** illustrates the PCR primers used in CpG island amplification and their relative locations in the amplicon. **FIGs. 7B and 7C** illustrate unfavorable assay outcomes by poorly designed α-TAP primer/probes.

**[0037]** **FIG. 8.** shows the relative sensitivities of DNA detection by TaqMan® PCR versus the Abscription®/PCR method depicted in **FIG. 6.** A fragment of the GSTPI CpG island from unfractionated methylated HeLa DNA was amplified from starting copy numbers/PCR of 9000 to 30. **FIG. 8A** shows the TaqMan® PCR results. TaqMan® PCR primers were SEQ ID NO: 3 and SEQ ID NO: 4. Detection of 9000 copies required 28 PCR cycles. **FIG. 8 B** shows the results of the Abscription®/PCR detection following 29 PCR cycles. Abscription/PCR primers were SEQ ID NO: 12 and SEQ ID NO: 13. The TAP-APC was made by annealing SEQ ID NO: 28 and SEQ ID NO: 32. The APC encoded the Abscript GAG. Abscripts were detected using thin layer chromatography (TLC) and UV shadowing. Area refers to the area of the chromatographic peak containing the Abscript.

**[0038]** **FIG. 9** shows detection of CY5™ labeled Abscripts using TLC after PCR amplification of the indicated amounts of input DNA followed by 2 hr and 8 hr of Abscription®.

**[0039]** **FIG.10** is a flow diagram showing the strategy for methylated DNA detection using direct incorporation of an APC into amplicons with the use of an APC-primer. Numerals indicate steps in the strategy. **Steps 1 and 2** depict the fractionation of methylated DNA using immobilized GST-MBD protein, as illustrated in **FIG. 6A.** **Step 3** shows the relationship between the targeted sequence and the primers that are used to attach an APC to the amplicon. The leftward primer is a conventional PCR primer. The rightward primer has a 3' priming sequence and a single-stranded APC at the 5' end. **Steps 4 and 5** represent PCR amplification of the target. **Step 6** represents the Abscription® step following PCR. The PCR reaction is supplemented with RNA polymerase, initiator and one or more NTPs.

**[0040]** **FIG. 11** illustrates the validation of an APC promoter pair for the GAPDH CpG island. **FIG. 11 A** shows the evaluation of background signal due to self-priming by the APC primer C443 and background due to the formation of primer-dimers between C443 (SEQ ID NO:33) and the reverse primer C446(SEQ ID NO:34). PCR reactions lacking DNA containing C443 alone or a combination of C443
and C446 were performed at a range of annealing temperatures from 56.4°C to 68.5°C followed by 1 hr of Abscription®. The production of Abscripts was assayed by TLC-UV shadowing. Only the positive control containing HeLa DNA produced the Abscript GAG. FIG. 11 B shows the results of LC-MS detection of Abscripts from the same sample sets.

DETAILED DESCRIPTION

Definitions

[0041] It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention claimed. As used herein, the use of the singular includes the plural unless specifically stated otherwise. As used herein, "or" means "and/or" unless stated otherwise. As used herein, the terms "comprises," "comprising," "includes," and "including," or any other variations thereof, are intended to cover a non-exclusive inclusion, such that a process, method, composition, reaction mixture, kit, or apparatus that comprises a list of elements does not include only those elements but may include other elements not expressly listed or inherent to such process, method, composition, reaction mixture, kit, or apparatus. The section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described.

[0042] Unless specific definitions are provided, the nomenclatures utilized in connection with, and the laboratory procedures and techniques of molecular biology, biochemistry, and organic chemistry described herein are those known in the art. Standard chemical and biological symbols and abbreviations are used interchangeably with the full names represented by such symbols and abbreviations. Thus, for example, the terms "deoxyribonucleic acid" and "DNA" are understood to have identical meaning. Standard techniques may be used e.g., for chemical syntheses, chemical analyses, recombinant DNA methodology, and oligonucleotide synthesis. Reactions and purification techniques may be performed e.g., using kits according to manufacturer's specifications, as commonly accomplished in the art or as described herein. The foregoing techniques and procedures may be generally performed according to conventional methods well known in the art and as described in various general or more specific references that are cited and discussed throughout the present specification. See e.g., Sambrook et al. Molecular Cloning: A Laboratory Manual (2d ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989)); Ausubel et al. Current Protocols in Molecular Biology (John Wiley & Sons Inc., N.Y. (2003)), the contents of which are incorporated by reference herein in their entirety for any purpose.

[0043] "About" as used herein means that a number referred to as "about" comprises the recited number plus or minus 1-10% of that recited number. For example, "about" 50 nucleotides can mean 45-55 nucleotides or as few as 49-51 nucleotides depending on the situation. Whenever it appears herein, a numerical range, such as "45-55", refers to each integer in the given range; e.g., "45-55 nucleotides" means that the nucleic acid can contain 45 nucleotides, 46 nucleotides, etc., up to and including 55 nucleotides.

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"Transcription" as used herein, refers to the enzymatic synthesis of an RNA copy of one strand of DNA (i.e., template) catalyzed by an RNA polymerase (e.g., a DNA-dependent RNA polymerase).

"Abortive transcription" is an RNA polymerase-mediated process that reiteratively synthesizes and terminates the synthesis of oligonucleotides that correspond to at least one portion of a complementary nucleic acid template sequence. Abortive oligonucleotides synthesized in vivo vary in length of nucleotides, and are complementary to a sequence at or near the transcription initiation site.

"Abscription™" is a form of abortive transcription optimized for in vitro analytical use to reiteratively produce short, uniform RNA transcripts or "abscripts" from synthetic or naturally occurring promoter sequences at high frequency in vitro. The term "Abscripts" (capitalized), is used herein to distinguish optimized, synthetic transcripts produced in an Abscription® reaction or assay, from the more general term "abscripts," which also encompasses short abortive transcripts that are produced during the normal course of transcription as it occurs in nature.

"Reiterative" refers to the repetitive synthesis of multiple identical or substantially identical copies of a sequence of interest.

"Terminator" or "transcription terminator" as used herein, refers to an RNA chain terminating compound, complex or process. A terminator of the invention can, for example, be a nucleotide analog, which can be incorporated into an RNA chain during RNA synthesis to prevent the addition of additional nucleotides to the RNA chain.

"Amplification" as used herein, refers to the process of making identical copies of a polynucleotide, such as a DNA fragment or region. Amplification is generally accomplished by polymerase chain reaction (PCR), but other methods known in the art may be suitable to amplify DNA fragments of the invention.

A "target DNA sequence" or "target DNA" is a DNA sequence of interest for which detection, characterization or quantification is desired. The actual nucleotide sequence of the target DNA may be known or not known. Target DNAs are typically DNAs for which the CpG methylation status is interrogated. A "target DNA fragment" is a segment of DNA containing the target DNA sequence. Target DNA fragments can be produced by any method including e.g., shearing or sonication, but most typically are generated by digestion with one or more restriction endonucleases.

As used herein, a "template" is a polynucleotide from which a complementary oligo- or polynucleotide copy is synthesized.

"Synthesis" generally refers to the process of producing a nucleic acid, via chemical or enzymatic means. Chemical synthesis is typically used for producing single strands of a nucleic acid that can be used and primers and probes. Enzyme mediated "synthesis" encompasses both transcription and replication from a template. Synthesis includes making a single copy or multiple copies of the target. "Multiple copies" means at least 2 copies. A "copy" does not necessarily mean perfect sequence complementarity or identity with the template sequence. For example, copies can include nucleotide
analogs, intentional sequence alterations (such as sequence alterations introduced through a primer comprising a sequence that is hybridizable, but not complementary, to the template), and/or sequence errors that occur during synthesis.

[0053] The terms "polynucleotide" and "nucleic acid (molecule)" are used interchangeably to refer to polymeric forms of nucleotides of any length. The polynucleotides may contain deoxyribonucleotides, ribonucleotides and/or their analogs. Nucleotides may be modified or unmodified and have any three-dimensional structure, and may perform any function, known or unknown. The term "polynucleotide" includes single-stranded, double-stranded and triple helical molecules. The following are non-limiting embodiments of polynucleotides: a gene or gene fragment, exons, introns, mRNA, tRNA, rRNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes and primers.

[0054] "Oligonucleotide" refers to polynucleotides of between 2 and about 100 nucleotides of single- or double-stranded nucleic acid, typically DNA. Oligonucleotides are also known as oligomers or oligos and may be isolated from genes and other biological materials or chemically synthesized by methods known in the art. A "primer" refers to an oligonucleotide containing at least 6 nucleotides, usually single-stranded, that provides a 3'-hydroxyl end for the initiation of enzyme-mediated nucleic acid synthesis. A "polynucleotide probe" or "probe" is a polynucleotide that specifically hybridizes to a complementary polynucleotide sequence. As used herein, "specifically binds" or "specifically hybridizes" refers to the binding, duplexing, or hybridizing of a molecule to another molecule under the given conditions. Thus, a probe or primer "specifically hybridizes" only to its intended target polynucleotide under the given binding conditions, and an antibody "specifically binds" only to its intended target antigen under the given binding conditions. The given conditions are those indicated for binging or hybridization, and include buffer, ionic strength, temperature and other factors that are well within the knowledge of the skilled artisan. The skilled artisan will also be knowledgeable about conditions under which specific binding can be disrupted or dissociated, thus eluting or melting e.g. antibody-antigen, receptor-ligand and primer-target polynucleotide combinations.

[0055] "Nucleic acid sequence" refers to the sequence of nucleotide bases in an oligonucleotide or polynucleotide, such as DNA or RNA. For double-strand molecules, a single-strand may be used to represent both strands, the complementary stand being inferred by Watson-Crick base pairing.

[0056] The terms "complementary" or "complementarity" are used in reference to a first polynucleotide (which may be an oligonucleotide) which is in "antiparallel association" with a second polynucleotide (which also may be an oligonucleotide). As used herein, the term "antiparallel association" refers to the alignment of two polynucleotides such that individual nucleotides or bases of the two associated polynucleotides are paired substantially in accordance with Watson-Crick base-pairing rules. Complementarity may be "partial," in which only some of the polynucleotides' bases are matched according to the base pairing rules. Or, there may be "complete" or "total" complementarity between the polynucleotides. Those skilled in the art of nucleic acid technology can determine duplex stability.
empirically by considering a number of variables, including, for example, the length of the first polynucleotide, which may be an oligonucleotide, the base composition and sequence of the first polynucleotide, and the ionic strength and incidence of mismatched base pairs.

As used herein, the term "hybridization" is used in reference to the base-pairing of complementary nucleic acids, including polynucleotides and oligonucleotides containing 6 or more nucleotides. Hybridization and the strength of hybridization (i.e., the strength of the association between the nucleic acids) is impacted by such factors as the degree of complementary between the nucleic acids, the stringency of the reaction conditions involved, the melting temperature (Tm) of the formed hybrid, and the G:C ratio within the duplex nucleic acid. Generally, "hybridization" methods involve annealing a complementary polynucleotide to a target nucleic acid (i.e., the sequence to be detected either by direct or indirect means). The ability of two polynucleotides and/or oligonucleotides containing complementary sequences to locate each other and anneal to one another through base pairing interactions is a well-recognized phenomenon.

A "complex" is an assembly of components. A complex may or may not be stable and may be directly or indirectly detected. For example, as described herein, given certain components of a reaction and the type of product(s) of the reaction, the existence of a complex can be inferred. For example, in the abortive transcription method described herein, a complex is generally an intermediate with respect to a final reiterative synthesis product, such as a final abortive transcription or replication product.

"Methylation" refers to the addition of a methyl group (-CH3) to a molecule, typically to a nucleotide base in DNA or RNA. "mCpG" refers to a 5'-CG-3' dinucleotide in which the C is methylated at position 5 (5-methylcytosine or 5-Me C). "CpG islands" are regions of genomic that contain a high frequency of the CpG dinucleotide. CpG Islands are in or near approximately 40% of promoters of mammalian genes and about 70% of human promoters have a high CpG content. See e.g. Fatemi et al. (2005) Nucleic Acids Res. 33:e176. doi:10.1093/nar/gni180. PMID 16314307.

"Promoter" as used herein, refers to a region of DNA that facilitates the transcription of an adjacent gene. Promoters are typically 5' and proximal to the start site of transcription initiation in a gene, and direct an RNA polymerase and associated transcription factors to the correct location for transcription of a the gene.

"Microarray" and "array," are used interchangeably to refer to an arrangement of a collection of compounds, samples, or molecules such as oligo- or polynucleotides. Arrays are typically "addressable" such that individual members of the collection have a unique, identifiable position within the arrangement. Arrays can be formed on a solid substrate, such as a glass slide, or on a semi-solid substrate, such as nitrocellulose membrane, or in vessels, such as tubes or microtiter plate wells. A typical arrangement for an array is an 8 row by 12 column configuration, such as with a microtiter plate, however, other arrangements suitable for use in the methods of the present invention will be well within the knowledge of the skilled artisan.
The term "solid support" refers to any solid phase that can be used to immobilize e.g., a capture probe or other oligo- or polynucleotide, a polypeptide, an antibody or other desired molecule or complex. Suitable solid supports will be well known in the art and include, but are not limited to, the walls of wells of a reaction tray, such as a microtiter plate, the walls of test tubes, polystyrene beads, paramagnetic or non-magnetic beads, glass slides, nitrocellulose membranes, nylon membranes, and microparticles such as latex particles. Typical materials for solid supports include, but are not limited to, polyvinyl chloride (PVC), polystyrene, cellulose, agarose, dextran, glass, nylon, latex and derivatives thereof. Further, the solid support may be coated, derivatized or otherwise modified to promote adhesion of the desired molecules and/or to deter non-specific binding or other undesired interactions. The choice of a specific "solid phase" is usually not critical and can be selected by one skilled in the art depending on the methods and assays employed. Conveniently, the solid support can be selected to accommodate various detection methods. For example, 96 or 384 well plates can be used for assays that will be automated, for example by robotic workstations, and/or those that will be detected using, for example, a plate reader. For methods of the present invention that may involve e.g., an autoradiographic detection step utilizing a film-based visualization, the solid support may be a thin membrane, such as a nitrocellulose or nylon membrane, a gel or a thin layer chromatography plate. Suitable methods for immobilizing molecules on solid phases include ionic, hydrophobic, covalent interactions and the like, and combinations thereof. However, the method of immobilization is not typically important, and may involve uncharacterized adsorbtion mechanisms. A "solid support" as used herein, may thus refer to any material which is insoluble, or can be made insoluble by a subsequent reaction. The solid support can be chosen for its intrinsic ability to attract and immobilize a capture reagent. Alternatively, the solid support can retain additional molecules which have the ability to attract and immobilize e.g., a "capture" reagent.

"Antibody" or "antibodies", as used herein, include naturally occurring species such as polyclonal and monoclonal antibodies as well as any antigen-binding portion, fragment or subunit of a naturally occurring molecule, such as for example Fab, Fab', and F(\(ab\))\(_2\) fragments of an antibody. Also contemplated for use in the methods of the invention are recombinant, truncated, single chain, chimeric, and hybrid antibodies, including, but not limited to, humanized and primatized antibodies, and other non-naturally occurring antibody forms.

The sequences of the promoter and the initially transcribed segment have significant effects on the lengths of the predominant Abscripts, as well as their rates of synthesis (Hsu et al. Biochemistry (2006) 45:8841-54.28). Multiple optimal highly abortive promoters, called Abortive Promoter Cassettes (APCs), have been developed and optimized to make Abscripts of different sequences and lengths (between 3 and 12 nt) at extremely high rates.

The generation of short Abscripts is very efficient because the RNAP does not dissociate from the promoter between rounds of truncated RNA synthesis, as it does after producing each full length transcript, and will continue to produce Abscripts at high turnover rates until substrates are depleted. This results in the very rapid production of thousands of Abscripts per APC each minute. Abscription® is a signal amplification, rather than a target amplification process.

The present invention provides simple and sensitive methods for the detection of CpG methylation in DNA via mCpG target site probes that include optimized methyl binding domain (MBD) polypeptides. mCpG target site probes can be coupled directly or indirectly to a signal generator, which produces a detectable signal that can be measured as an indicator of CpG methylation.

In certain embodiments of the invention, signal generation is based on an Abscription® process in which Abortive Promoter Cassettes (APCs) signal generators are bound to target mCpG sites via mCpG target specific probes. RNA polymerase produces uniform, short RNA molecules from synthetic or naturally occurring abortive promoters in APCs as signals (indicators) of the presence of methylated CpGs. In other embodiments of the invention, signal-generating cassettes can produce detectable RNA or DNA signals through PCR or other replication and/or amplification methods.

The methods of the invention offer significant advantages over current CpG methylation detection methods because bisulfite treatment is not required. Thus, the extensive DNA degradation and the reduction of sequence complexity associated with chemical treatment of target DNA can be avoided entirely in certain embodiments of the invention. The methods of the invention are rapid and can typically be performed in a single day. Furthermore, the invention can be adapted to multiplex and automated applications.

Certain Abscription®-based methylation detection assays of the present invention offer the unique capability of coupling a linear, robust signal amplification process (Abscription®) with a target amplification process (e.g. polymerase chain reaction or PCR). This provides for extremely high sensitivity and allows testing to use only small amounts of starting material. In addition, unlike other signal amplification methods, such as horseradish peroxidase or alkaline phosphatase, which generate the same signal molecule from each target in a sample, Abscription® based amplification can be formatted to generate a different signal from each target. These signals, in the form of short oligonucleotides, can then be detected by a variety of methods. Abscription®, based assays require fewer man-hours of labor than other DNA methylation detection assays, reagent cost is very competitive, and instrumentation cost is low. In addition, these assays, by including positive and negative control templates, result in highly specific detection and fewer false positives than other methods for target detection.
Abscription® Technology

[0070] Abscription® technology is based on the observation that prior to the initiation of full-length RNA transcription, a large number of short, abortive transcripts are synthesized by RNA polymerases before full-length RNA transcripts are made. As described below, abortive transcripts are a normal by-product of the transcription process, yet are distinguishable from full-length RNA transcripts (which are the functionally informative product of the transcription process), in both size and in the manner in which they are made.


[0073] **Abscription® Technology.** Abscription® technology exploits the natural phenomenon of abortive RNA synthesis to produce large numbers of detectable abortive transcripts (Abscripts). Abscription® is an isothermal, robust, linear signal generation system based on abortive transcription. In an Abscription® method, Abortive Promoter Cassettes (APCs) are bound to target molecules via Target Site Probes (TSPs). An RNA polymerase, such as *E. coli* RNA polymerase, then uses the APC as a template for generating large numbers of signals per target in the form of short, uniform RNA molecules or Abscripts (abortive transcripts).
Abscription® detection methods have three basic steps that can be adapted to detect a wide
variety of molecules of interest (i.e. targets). First, an APC is localized to a target molecule of interest
through a Target Site Probe (TSP). Second, Abscripts are synthesized from the localized APCs. Finally,
Abscripts are detected as a means of target detection and may be quantified as an indication of the amount
of a target molecule present. The process is very efficient because the RNAP does not move away or
dissociate from the promoter between rounds of abortive RNA synthesis, as it does after producing each
full-length transcript. Furthermore, only uniform, short RNA signals are synthesized, which can be
produced more quickly and with less effort than longer oligo- and polynucleotides.

Although the factors and conditions required for promoter escape (and hence the end of
abortive synthesis), are incompletely understood, sufficient knowledge is available to create a synthetic
environment that favors abortive transcript synthesis and precludes full-length RNA production. In one
embodiment, Abscription® is controlled at the synthesis stage to produce Abscripts that are initiated with
a defined dinucleotide initiator and then terminated after the addition of one or more NTPs as illustrated
in the nonlimiting example shown in FIG. 3. Abscript length can be limited to as short as 3 nucleotides
(nt) with the use of chain terminating NTPs (e.g., 3'-0-Me-NTPs) or by omitting one or more NTPs from
the reaction.

In other embodiments, Abscript length is controlled at the promoter/template stage, by
providing synthetic templates that have a discrete, limited number of nucleotides available for
transcription before a stop signal is reached. The uniformity of Abscript production from a single APC in
a single Abscription® reaction results in Abscript signals that are directly proportional to the amount of
target present. Thus Abscription® is both a qualitative and a quantitative system for measuring a target,
such as mCpG.

Abortive promoters can be incorporated into DNA targets through a target amplification
processes or formed on single-stranded DNA targets by hybridization of a second strand. More generally
however, Abscription® can be used to detect a wide variety of target molecules by binding APCs to those
targets (FIG. 2). For detection of protein, RNA or DNA, APCs are connected to a Target Attachment
Probe (TAP) which will bind to the molecular target (FIG. 2A). For detection of DNA and RNA, TAPs
include oligonucleotides or proteins that bind specifically to the nucleic acid target (FIG. 2B). For
protein detection, APCs can be attached to anything that binds to the protein target. Several assays have
been developed which employ two antibodies directed to the same target, similar to ELISA, one for
capture of the target and one for attachment of the APC (FIG. 2C).

Trinucleotide Synthesis

Trinucleotide Abscripts can be made exclusively with the inclusion of chain terminator NTPs
or by omitting one or more NTP. Abscripts can be labeled for detection or capture by incorporating
modified dinucleotides (Dissinger & Hanna, J. Biol. Chem (1990) 265:7662-8: Dissinger & Hanna, J.
In one embodiment, the assays described herein involve the production of different trinucleotide Abscripts that differ by molecular weight or mobility. Trinucleotides are made by RNAP at rates of 1000 to 2000 per minute on APCs by joining a dinucleotide initiator and a nucleoside triphosphate. Trinucleotide Abscripts can be detected without labeling by rapid TLC and UV shadowing or mass spectrometry. Alternatively, Abscripts can be detected through a label (e.g. a fluorescent moiety) incorporated into a dinucleotide initiator.

The present invention provides methods for detecting a polynucleotide in a sample by contacting a sample containing the polynucleotide with a primer pair that specifically hybridizes to and amplifies a target sequence of the polynucleotide. The primer pair includes a first primer that is complementary to the polynucleotide, flanks the target sequence, and contains a 5' capture tag. The second primer has three regions: a 3' sequence complementary to the polynucleotide that flanks the target sequence on the opposite side of the target sequence from the first primer; a 5' α-TAP sequence that is used to attach the APC following amplification; and a non-natural nucleotide between the 3' and 5' sequences, that is typically an etheno-deoxyadenosine.

The target sequence of the polynucleotide is then amplified (e.g. by polymerase chain reaction) using the first and second primers and the amplified target sequence is captured on a solid support containing a molecule that binds the 5' capture tag. Any available PCR technique or suitable nucleic acid amplification method can be employed for this step, such as PCR methods that use thermostable DNA polymerase and/or RNA polymerases. For example the capture tag can be biotin and the solid support can be streptavidin beads, such as magnetic beads. The captured amplicons can then be washed to remove unbound primers and, if desired, eluted from the solid support.

For detection of the target polynucleotide sequence, a probe is hybridized to the amplicon. This probe includes a 5' TAP sequence complementary to the α-TAP sequence. Due to the inclusion of the non-natural nucleotide in the second PCR primer, the α-TAP sequence is not copied during PCR and remains single-stranded during the amplification, thereby allowing the TAP sequence of the probe to hybridize without denaturing the amplified target. Etheno-deoxyadenosine can be used as the non-natural nucleotide, but the skilled artisan will be aware of additional suitable non-natural nucleotides (e.g. nucleotide analogs) that terminate replication and can thus be substituted for etheno-deoxyadenosine. The probe also includes an APC, which provides the template for synthesizing Abscripts using Abscript® methods as described above. Finally, the Abscripts are detected by any suitable method.
particularly the methods described herein for Abscript detection, such as mass spectrometry, capillary electrophoresis or thin layer chromatography. Typically, the Abscripts will have a length of from 3 to 20 nucleotides and may be labeled by incorporating a detectably-labeled (e.g. fluorescent) nucleotide during Abscription®.

[0083] In other embodiments of the invention, the TAP/α-TAP step can be eliminated by including an APC sequence at the 5' end of the second amplification primer and omitting the non-naturally occurring nucleotide. In such embodiments, a double-strand APC is generated during amplification adjacent to the amplified target sequence, which will direct Abscription® upon addition of RNAP and nucleotides. If these Abscription® reagents are present during amplification, Abscription® and amplification can be performed simultaneously in the same tube.

[0084] These methods of the invention can be adapted for multiplexing (i.e., detection of a plurality of polynucleotides simultaneously) by including primer pairs specific to each polynucleotide in the reaction. According to this embodiment of the invention, each primer pair is designed to specifically hybridize to and amplify a unique target sequence of a polynucleotide. The α-TAP sequence for each primer pair is also unique, thereby acting as an identifier for the target sequence. By hybridizing a complementary TAP sequence that includes a unique identifying APC following PCR amplification, the presence of each polynucleotide can be interrogated based on the Abscripts produced in the multiplex reaction. Thus a unique APC is attached to each amplified target polynucleotide and the distinguishable Abscript signal produced from the APC can be detected and measured as an indication of the presence of the target polynucleotide. For example, each APC can be designed to generate an Abscript distinguishable on the basis of molecule weight or nucleotide sequence. In a single reaction, 5, 10, 20 or more unique target sequences can be detected.

[0085] The present invention also provides methods for determining the methylation status of CpG islands without the use of deamination with bisulfite. Such method combines target amplification with a linear signal amplification process, Abscription®, making it extremely sensitive.

[0086] In certain embodiments of the invention, the target polynucleotide(s) is a methylated CpG island or a plurality of CpG islands (i.e. multiplexing). In these embodiments, genomic DNA containing methylated CpG islands is first cleaved using a predetermined restriction enzyme that does not cut the island or any of the islands in a multiplex assay. Methylated DNA fragments are then captured from the genomic DNA using an immobilized MBD reagent and the capture fragments interrogated for specific CpG sequences of interest. According to one method of the invention, the process begins by isolation of methylated DNA from fragmented genomic DNA using a methylated DNA enrichment process. In one aspect of the invention, the enrichment method uses a glutathione-S-transferase fusion protein which contains the methyl binding domain from mouse MBD2, which is highly specific for methylated DNA. Methylated DNA bound to MBD2-fusion protein is captured rapidly by glutathione magnetic beads and eluted directly into buffer for amplification by the polymerase chain reaction. CpG islands of interest are amplified using a pair of modified PCR primers. The first contains a biotin group for subsequent capture
of the targeted CpG island to streptavidin magnetic beads. The second primer contains an island-specific sequence that "marks" the amplicon for attachment of a specific APC. Once amplified, islands are captured to streptavidin beads; unique APCs are attached by hybridization; and Abscription® is initiated. Each CpG island thereby generates a different Abscript; therefore multiple CpG islands can be interrogated in each reaction.

[0087] Alternatively, an APC sequence can be incorporated into the second amplification primer, and an APC duplex generated during amplification. This approach allows amplification and Abscription® to be performed at the same time by including RNAP and nucleotides in the amplification reaction. Because this method couples target amplification with signal amplification, less starting DNA is required than with most methylated DNA detection methods, and less than 2 ng of genomic DNA is sufficient for the initial step of isolating methylated DNA. The entire assay is very rapid, requiring less operator time than most competing assays. The assay can be used as described with magnetic beads and can also be formatted for high throughput screening in a microtiter plate format.
EXAMPLES

EXAMPLE 1. Abscription ® Methods

Abscription ® has been previously described; see e.g. U.S. Pat. Application No. 09/984,664 (filed Oct 30, 2001) now U.S. Pat. No. 7,045,319; 10/425,037 (filed April 29, 2003); 10/600,581 (filed June 23, 2003); 10/602,045 (filed June 24, 2003); 10/607,136 (filed June 27, 2003), now U.S. Pat. No. 7,226,738; 10/686,713 (filed Oct. 17, 2003); 10/976,240 (filed Oct. 29, 2004); 10/790,766 (filed March 3, 2004); 10/488,971 (filed Oct. 18, 2004); and 10/551,775 (filed Sept. 14, 2006) the contents of each of which are incorporated by reference herein in their entirety.

EXAMPLE 2. Mass Spectrometry Detection of Abscripts

Trinucleotide Abscripts are detected by mass spectrometry following their fractionation from dinucleotide initiators by HPLC. The output of the fractionation is plotted as total ion count versus chromatographic retention time as illustrated in FIG. 4A. The chromatographic profile for any ion can be similarly plotted. The contributions of particular m/z species at a specific retention time can be summed to give the amount of Abscript as the area under the chromatographic peak. FIG. 4B shows the ion spectrum associated with the Abscript GAG (retention time of 5.4 min). The yield of GAG would be the sum of species with m/z values of 477.6, 956.1 and 978.2. These species account for doubly charged, singly charged and the sodium adduct respectively.

EXAMPLE 3. Preparation of GST-MBD Protein

A GST fusion protein that contains the methyl binding domain (MBD) from mouse MBD2 was constructed as illustrated in FIG. 5. The codons for the MBD domain were optimized for expression in E. coli. The construct contains a thrombin cleavage site between the GST and MBD domains. The GST protein also contains four surface cysteine residues that were used for attachment of APCs or Biotin. The details of the GST-MBD protein are provided in U.S. Patent Application No. 61/053,648, filed May 15, 2008 the contents of which are incorporated by reference herein, and in particular, Examples 2-11 describing the preparation and use of MBD fusion proteins.

The GST domain allows the fusion protein, or its complexes with methylated DNA, to be isolated on Glutathione resins or beads and eluted with glutathione, or to be captured or detected with antibodies that recognize GST.

MBD from the MBD2b protein was chosen for final constructs because MBD2b has the highest affinity among the known methyl CpG binding proteins for Me-CpG sites and the lowest cross reactivity with unmethylated CpGs. It has between a 25 to 100 fold higher affinity for Me-CpG sites than does MeCP2, and a 9.7 to 43 fold higher preference for methylated DNA than does MeCP2 (Fraga et al. (2003) Nucleic Acids Res., 31:1765-74). Additionally, there are no sequence context effects on MBD2
CpG recognition, as there are for MeCP2, which requires a run of 4 A-Ts near a CpG site. Therefore a greater number of mCpG sites are recognized by MDB2 than by MeCP2.

EXAMPLE 4. Immobilized GST-MBD2 Retains High Specificity for Methylated DNA Even With 2 ng or Less of Input DNA

The GST-MBD protein was attached to glutathione magnetic beads and used to isolate varying amounts of methylated DNA to determine the minimum starting DNA sample size that could be recovered with high specificity. HeLa genomic DNA or HeLa DNA artificially methylated with Sssl methylase was incubated with GST-MBD magnetic beads for 1 hour at 22°C with horizontal rotary mixing at 1000 rpm. Bound DNA was eluted from the beads after removal of the supernatant containing unbound DNA, by incubation at 80°C for 10 min with horizontal rotary mixing at 1000 rpm. Eluted DNA samples were tested for the presence of PTGS2 (GenBank GL34576917) DNA by qPCR using the primer pair 5'-ggtacgaaagccgaaaga-3' (SEQ ID NO:6) and 5'-tgtgggaaagctggaatatc-3' (SEQ ID NO:7) with SYBR® Green dye for detection. PTGS2 is unmethylated in HeLa and was expected to remain in the supernatant of the binding reaction. A recovery of 94% of a 2 ng genomic DNA input of artificially methylated DNA was observed in the eluted fraction while 100% of the unmodified PTGS2 DNA from HeLa was recovered in the supernatant fraction as expected. At an input of 1 ng of methylated HeLa DNA, 73% of PTGS2 DNA was bound and recovered with heat elution. No binding of the unmethylated version from unmodified HeLa was detected. The lowest DNA amount (2 ng) which could be visualized by agarose gel electrophoresis staining corresponds to approximately 300 cells. Even less DNA can be isolated and detected using Abscription® for amplicon detection.

EXAMPLE 5. Abscription® Based CpG Methylation Assay

FIG. 6 illustrates an overall protocol for determining the methylation status of multiple CpG islands. Briefly, fragmented methylated DNA from a genomic DNA sample is isolated, followed by amplification of specific CpG islands whose methylation status is under investigation. For the amplification, one primer contains an affinity tag, such as biotin, which allows retrieval and immobilization of the island. The second primer contains a sequence for attachment of the Abortive Promoter Cassette.

This method permits the amplification and isolation of as many CpG islands in a sample as compatible PCR primers can be designed. Because the DNA is not deaminated with bisulfite treatment, which results in the conversion of "C"s to "dU", the problematic issues for high level multiplexing associated with the loss of sequence heterogeneity caused by deamination, are avoided. Since the primer sites are not rigidly limited to specific sequences in the target, there is sufficient flexibility in primer placement to allow the design of multiple compatible primer sets (Henegariu et al. Biotechniques (1997) 23:504-1 t; Onishi et al. J. Agric. Food Chem. (2005) 53:9713). A different APC is attached to each target CpG island, thereby generating a different Abscription signal for each. Thus, simultaneous detection of multiple CpG islands from a single sample can be achieved.
Briefly, native genomic DNA is fragmented and then bound to glutathione beads containing the Glutathione-S-Transferase (GST)-Methyl Binding Domain (MBD) fusion protein described above in EXAMPLE 3. Only methylated DNA binds (FIG. 6A, Step 1). After washing, the methylated DNA is eluted from the beads (FIG. 6A, Step 2) and islands to be interrogated are amplified by PCR (FIG. 6B, Step 3). One of the primers contains a capture tag (e.g. biotin) at the 5’ end (FIG. 7A). The second primer contains 2 regions. The first is a polynucleotide sequence complementary to the target DNA. The second region is an arbitrary sequence dissimilar to the target DNA or other islands (FIG. 7A). This second sequence is complementary to a Target Attachment Probe (TAP) sequence which is linked to an Abortive Promoter Cassette (APC). The complement to the TAP sequence is called an anti-TAP sequence (α-TAP). The α-TAP sequence remains single-stranded during the PCR amplification of the target DNA due to the inclusion of a non-natural nucleotide at the junction between the primer sequence that hybridizes to the target and the α-TAP sequence (the εA nucleotide in FIG. 7A). After amplification, the islands are immobilized, for example on streptavidin beads (FIG. 6B, Step 4), and remaining genomic DNA and primers are washed away. The TAP-APC polynucleotide is then contacted with the amplified target DNA and hybridizes to the single-stranded anti-TAP sequence (FIG. 6C, Step 5). Free TAP-APC is washed away, and Abscription® reagents are added to generate Abscript signals (FIG. 6C, Step 5). Further details of the method are given below.

Step 1: Cutting of genomic DNA

The initial digestion step was designed to generate fragments that contain CpG islands or large portions thereof. The restriction sites were chosen to fall outside of the region to be analyzed and are neither methylation dependent nor methylation sensitive. Restriction enzymes with 4 base recognition sequences were used. Table 1 lists the fragment sizes generated by Msel or DdeI for a sample of 6 CpG islands reported to be differentially methylated. Up to 3 μg of genomic DNA routinely were digested with 20 units of Msel (NEB, Beverly, MA) in the vendor's restriction buffer (NEB buffer 4). Cleavage reactions were incubated for at least 8 hr at 37°C. The extent of cleavage was measured by performing PCR on a sample of the digest using a primer that contains the Msel sequence. Positive controls are genomic DNA untreated with Msel and an amplification reaction of the Msel treated DNA using primers that are unaffected by Msel digestion.

The purpose of the digestion is to unlink the target islands from neighboring CpG sequences that might be normally methylated and thereby cause the transfer of an unmethylated island to the methylated DNA fraction. In most cases either Msel or DdeI produced a single fragment that accounts for the bulk of the island sequence without including many neighboring sequences. The exception was the excessive cleavage of MGMT with DdeI. In this case, the alternative enzyme produced satisfactory results. In cases where a CpG island is fragmented into several fragments, each segment can be analyzed with its own set of primers.
### Table 1. CpG Island Cleavage patterns

<table>
<thead>
<tr>
<th>CpG Island</th>
<th>Restriction Enzyme</th>
<th>Recognition Sequence</th>
<th>Fragment(s) generated (nt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC (GL224589817)</td>
<td>Msel</td>
<td>TTAA</td>
<td>520, 164</td>
</tr>
<tr>
<td></td>
<td>Ddel</td>
<td>CTNAG</td>
<td>820</td>
</tr>
<tr>
<td>CCNAI (GL224589804)</td>
<td>Msel</td>
<td>TTAA</td>
<td>1168</td>
</tr>
<tr>
<td></td>
<td>Ddel</td>
<td>CTNAG</td>
<td>523, 231, 177</td>
</tr>
<tr>
<td>GSTPI (GL34576917)</td>
<td>Msel</td>
<td>TTAA</td>
<td>1936</td>
</tr>
<tr>
<td></td>
<td>Ddel</td>
<td>CTNAG</td>
<td>261, 131</td>
</tr>
<tr>
<td>MGMT (GL34556)</td>
<td>Msel</td>
<td>TTAA</td>
<td>1967</td>
</tr>
<tr>
<td></td>
<td>Ddel</td>
<td>CTNAG</td>
<td>Excessively fragmented</td>
</tr>
<tr>
<td>RARB (GL35881)</td>
<td>Msel</td>
<td>TTAA</td>
<td>612, 163</td>
</tr>
<tr>
<td></td>
<td>Ddel</td>
<td>CTNAG</td>
<td>625</td>
</tr>
<tr>
<td>PTGS2 (GI:211904109)</td>
<td>Msel</td>
<td>TTAA</td>
<td>516, 417</td>
</tr>
<tr>
<td></td>
<td>Ddel</td>
<td>CTNAG</td>
<td>464, 229, 87, 37</td>
</tr>
</tbody>
</table>

**Step 2: Isolation and recovery of methylated DNA**

The methylated DNA capture step was formatted for magnetic beads bearing glutathione. The GST-MBD protein was preloaded onto the beads suspended in the DNA sample after removing excess GST-MBD protein. DNA binding was performed for 1 hr at room temperature (22-24°C) with mixing to maintain the beads in a suspended state (Eppendorf Thermomixer, 1000 rpm). DNA samples typically contained between 1 ng to 50 ng of genomic DNA in a 50 µl volume of binding buffer. At the completion of the binding step, the beads were pelleted to the side of the tube with a rare-earth magnet and the supernatant containing unmethylated DNA was removed. The beads were washed twice with 400 µl of a wash buffer containing the same NaCl concentration as the binding buffer (160 mM). Each wash was incubated for 5 min at room temperature with mixing (1000 rpm). A final wash was performed with TE buffer (10 mM Tris pH 8, 1 mM EDTA). The beads were suspended in 400 µl TE and immediately pelleted with the magnet. The TE wash buffer was discarded and the beads were suspended in 50 µl of elution buffer (10 mM Tris pH 8, 1 mM EDTA).

Methylated DNA can be eluted from the beads with several alternative methods. Beads in TE buffer can be incubated for 10 min at 80°C with mixing (1000 rpm). The beads are pelleted with the magnet and the eluted DNA is recovered. In an alternative method the beads are suspended in elution buffer containing 0.1% SDS. Complete elution of bound methylated DNA was achieved with a single 20 min incubation at 50°C. The eluted DNA is ready for PCR without further processing provided a nonionic detergent such as Tween-20 is included in the PCR buffer (see Goldenberger et al. PCR Methods Appl. (1995) 4:368-70). Elution can also be performed with exposure of the beads for 10 min to elution buffer containing a minimum of 20 mM reduced glutathione at pH 8. **FIG. 5C** shows the results.
of the fractionation of the SNRPN CpG island of HeLa DNA. Methylated DNA was released with heat treatment. SNRPN is an imprinted gene. One copy is fully methylated and the other copy is normally unmethylated. As expected half of the SNRPN copies were found in the supernatant (FIG. 5C fraction S, unmethylated fraction) and half of the copies were eluted from the beads in the first elution (FIG. 5C, fraction El, methylated fraction). All of the methylated DNA was extracted in the first elution. Two serial elutions after the first elution (E2 and E3) did not contain SNRPN DNA. The CpG island of the PTGS2 gene (unmethylated in HeLa) was used as a negative control (FIG. 5D). All of the PTGS2 DNA appeared in the supernatant fraction.

Step 3: PCR amplification with tagged primers

CpG island segments were amplified and labeled with a biotin affinity tag and a single-stranded oligonucleotide sequence that is used for attachment of an abortive promoter cassette (APC). PCR reactions contained 1x Hot Start Taq buffer (Fermentas) 0.8 mM dNTPs (0.2 mM each), 2 mM MgCl₂ and 5 % (v/v) DMSO. The biotinylated primer and the α-TAP primer were present at 1 µM each. Amplifications were performed with 2 units/20 µl reaction of TrueStart™ hot start Taq DNA polymerase (Fermentas). One unit equals the incorporation of 10 nmol of dNTPs in 30 min at 74°C. The cycling conditions were 95°C for 1 min, followed by up to 32 cycles of 95°C for 30 sec, 62°C for 30 sec, and 72°C for 30 sec. A final elongation step was at 72°C for 5 min. Completed reactions were held at 4°C. FIGS. 5C and 5D show detection of the fractionated, amplified DNAs by agarose gel electrophoresis.

α-TAP primer sequence design

Primers were optimized for high signal intensity by minimizing interactions among the three oligonucleotide primer/α-TAP sequences that are present in the PCR reaction. The biotinylated PCR primer and the 3’ end of the α-TAP were used to prime DNA synthesis during the amplification and were designed using primer software (Oligo Explorer 1.2) to minimize primer-primer interactions and the formation of primer hairpin loops. The α-TAP at the 5’ end of the second primer was designed to be incorporated into the amplicon and remain single-stranded due to the presence of a non-coding nucleotide, ethenoA (εA), which separates the primer sequence from the α-TAP. Most DNA polymerases including Taq polymerase cannot incorporate a dNTP opposite εA and terminate synthesis (FIG. 7) (see Patel et al. J. Biol. Chem. (2001) 276:5044-51). The nucleotide analog prevents the α-TAP sequence from being copied during PCR. Potential interactions between the α-TAP sequence and either primer sequence are minimized to avoid inhibition of PCR and false positive results from nonspecific immobilization of a fully single-stranded α-TAP.

Primer design went through 3 steps. First the priming sequences were optimized within the following criteria: 1) a primer length between 16 to 20 nt; 2) a Tₘ close to 60°C; 3) primer-primer interactions with base paired 3’ ends were eliminated; 4) other primer-primer interactions must have a Tₘ
of < 16°C; and 5) hairpin structures with Tm > 80°C were rejected. Primer pairs for 6 CpG islands were successfully developed using these criteria.

[0104] The second step in primer optimization was to eliminate hairpin structures in the α-TAP-primer oligonucleotide that might interfere with PCR or attachment of a TAP-APC. A collection of 6 α-TAP candidate sequences were designed based on RNA phage MS2, fr and Qβ sequences. Each candidate α-TAP was tested in silico and those that formed hairpins with a Tm > 26°C under our TAP-annealing conditions were modified to eliminate the hairpin and retested (Zuker, Nucleic Acids Res. (2003) 31:3406-15). The only limitation in modifying α-TAP sequences was that they do not acquire significant complementarity with the priming sequences. Potential hairpin interactions between an α-TAP and a linked primer sequence were tested in silico and if necessary the α-TAP sequence was changed to minimize hairpin stability and/or prevent primer extension from a hairpin. An exemplary primer-α-TAP oligonucleotide (SEQ ID NO:42) has a hairpin structure with a Tm of 41°C under PCR conditions used, but amplified CDKN2A DNA as efficiently as the primer lacking the α-TAP extension in combination with reverse primer SEQ ID NO:43. Finally, interactions between the α-TAP and the biotinylated primer were tested in primer design software using a CpG island sequence file with the α-TAP appended at the end of the file and choosing it as a primer along with the biotinylated primer sequence, α-TAP and reverse primer pairs were developed for CpG islands associated with the α-TAP appended at the end of the file and choosing it as a primer along with the biotinylated primer sequence, α-TAP and reverse primer pairs were developed for CpG islands as listed below in Table 2.

Table 2. α-TAP and Reverse Primer Pairs

<table>
<thead>
<tr>
<th>CpG Island</th>
<th>( \alpha )-TAP primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAPKI (SEQ ID NO:44)</td>
<td>5'-(EAT)[TTtcctacacgctgcccgggac]-3' (SEQ ID NO: 8)</td>
<td>5'-Biotin-gtcttcctacactccg-3' (SEQ ID NO: 9)</td>
</tr>
<tr>
<td>GAPDH (SEQ ID NO:45)</td>
<td>5'-(EAT)cctacacgctgcccgggac-3' (SEQ ID NO: 10)</td>
<td>5'-Biotin-gtcttcctacactccg-3' (SEQ ID NO: 11)</td>
</tr>
<tr>
<td>GSTP1 (SEQ ID NO:47)</td>
<td>5'-(EAT)cctacacgctgcccgggac-3' (SEQ ID NO: 12)</td>
<td>5'-Biotin-gtcttcctacactccg-3' (SEQ ID NO: 13)</td>
</tr>
<tr>
<td>MGMT (SEQ ID NO:46)</td>
<td>5'-(EAT)cctacacgctgcccgggac-3' (SEQ ID NO: 14)</td>
<td>5'-Biotin-gtcttcctacactccg-3' (SEQ ID NO: 15)</td>
</tr>
<tr>
<td>PTGS2 (SEQ ID NO:48)</td>
<td>5'-(EAT)cctacacgctgcccgggac-3' (SEQ ID NO: 16)</td>
<td>5'-Biotin-gtcttcctacactccg-3' (SEQ ID NO: 17)</td>
</tr>
<tr>
<td>SNRPN (SEQ ID NO:49)</td>
<td>5'-(EAT)cctacacgctgcccgggac-3' (SEQ ID NO: 18)</td>
<td>5'-Biotin-gtcttcctacactccg-3' (SEQ ID NO: 19).</td>
</tr>
</tbody>
</table>
TAP-APC Design.

[0105] TAP-APCs were made by hybridizing TAP-APC non-template strands to complementary APC template strands. The APC portions were double-stranded and the TAP segments were a single strands extending from the non-template strands. TAP-APCs were designed so that a collection of APCs either encoded the same abscript for use in single-plex reactions, or a collection of TAP-APCs each encoded a different abscript allowing for multiplex detection. Single-stranded single-plex TAP-APCs were designed for the α-TAPs as indicated below in Table 3.

Table 3. Single-Plex TAP APCs

<table>
<thead>
<tr>
<th>CpG Island</th>
<th>TAP APC</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAPKI</td>
<td>5′-catttttatgaccttttgacctgtggtgttgacacagaatataacgctcaatgtcaatgggagggag</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5′-ggagagattgcacgggtggttgacacagaataaacgctcaatgtcaatggttagttgaggt</td>
</tr>
<tr>
<td>GSTP1</td>
<td>5′-gtctgtggtgcctgggcttgacacagaataaacgctcaatgtcaatggttagttgaggt</td>
</tr>
<tr>
<td>MGMT</td>
<td>5′-taccttttggtggatggaggtggttgacacagaatataacgctcaatgtcaatggggttagttgaggt</td>
</tr>
<tr>
<td>PTGS2</td>
<td>5′-ctgtcttttggtctgctgctgctgttgacacagaataaacgctcaatgtcaatgggagggtagttgaggt</td>
</tr>
<tr>
<td>SNRPN</td>
<td>5′-gctactcgataggaggctggcttgacacagaataaacgctcaatgtcaatggggttagttgaggt</td>
</tr>
</tbody>
</table>

[0106] The single-stranded single-plex TAP-APCs were annealed to a common template strand encoding the same abscript (SEQ ID NO:32).

[0107] Multiplex-compatible TAP-APCs were each paired with their own template strand encoding a unique abscript. A collection of multiplex TAP-APCs (Table 4) could be used together to detect the CpG islands listed in Table 4.

Table 4. Multiplex-Compatible TSP-APCs

<table>
<thead>
<tr>
<th>CpG Island</th>
<th>TAP APC</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAPKI</td>
<td>5′-catttttatgaccttttgacctgtggtgttgacacagaatataacgctcaatgtcaatgggagggag</td>
</tr>
</tbody>
</table>
Step 4 and 5. Attachment of amplicons to streptavidin beads and binding of TAP-APCs

Biotinylated amplicons were bound to streptavidin beads to remove free probes and unbound APCs in succeeding steps of the assay.

In optimization experiments, complete binding of amplicons to the streptavidin magnetic beads in the presence of 10 pmol of biotinylated primer was observed with a total binding capacity of 40 pmol of biotinylated oligonucleotide. The binding time was optimized to minimize the time course of the assay using agarose gel electrophoresis to measure the removal of the amplicons from the buffer phase of the binding reaction. Quantitative binding of the biotinylated amplicons was observed within 5 min. of mixing the beads and the DNA samples.

Attachment of the TAP-APC to the amplicon was successfully performed either free in solution before the addition of streptavidin beads or after binding the amplicons to the beads followed by a wash step to remove free primer-αTAP oligonucleotides. The T_m for the TAPs range between 55.8-64°C under the annealing conditions used (150 mM Na+). TAP-APC was added at 0.5 μM and incubated at 51°C for a minimum of 15 min. High stringency was not required to achieve efficient binding. Analysis of the TAPs and α-TAP's indicated that hairpin formation was insignificant under these annealing conditions. The sequence complexity of the reaction was low even in preparations subjected to multiplex PCR. At most 3 pairs of α-TAPs and TAPs are present in a triplex reaction and these sequences can be arbitrarily changed to prevent cross-hybridization. This annealing step was optimized with respect to temperature in a gradient thermocycler and the shortest annealing time was determined using electrophoretic mobility shift of the amplicon as an endpoint.

The PCR reactions were diluted 1:1 in DNA binding buffer to give a final NaCl concentration of 150 mM. The appropriate TAP-APC was added to each DNA sample to a final concentration of 0.5 μM, followed by an incubation at 51°C for a minimum of 15 min.

Streptavidin magnetic beads were aliquoted to PCR tubes. The beads were washed with 100 μl of 50% (v/v) binding buffer. The washed beads were suspended in the DNA samples followed by incubation at 51°C for a minimum of 5 min.
The binding reaction was terminated by pelleting the beads with a magnet and removing the binding buffer. The beads were subjected to 2 washes in 180 µl of wash buffer containing the same NaCl concentration as the 50% (v/v) binding buffer (150 mM). Each wash step included a 5 min incubation at 51°C to replicate the stringency of the binding reaction and then the beads were rapidly pelleted with the magnet. A third wash was in 40 mM HEPES pH 7.5, 40 mM KCl. The beads could be stored refrigerated or could be immediately subjected to Abscription®.

**Step 6: Abscription®**

Beads containing bound amplicon:TAP-APCs were pelleted with a magnet to remove storage buffer and were suspended in 10 µl of Abscription® buffer containing 1 mM dinucleotide initiator (GpA), 1 mM NTP (GTP) and 0.4 units of RNA polymerase. One unit catalyzes the incorporation of 1 nmol of NTP in 60 min at 65°C. Abscription® reactions were incubated for 1 hr at 77°C.

Abscripts were detected by UV-shadowing by spotting 1.5 µl samples onto a silica gel TLC plate containing a fluor. TLCs were developed in an air-tight chamber containing 100 ml of solvent (Isopropanol:Ammonium hydroxide:Activator solution, 6:3:1). Abscripts were detected as dark spots under shortwave UV light as illustrated in FIG. 8B (UV-shadowing).

For LC-MS detection, 10 µl of Abscription® reaction was diluted into 20 µl of HPLC grade water in a 384 well plate. Ten microliters was processed and quantified by LC-MS as illustrated in FIG. 8C.

**Table 5. Sensitivities of TaqMan® and Abscription® assays**

<table>
<thead>
<tr>
<th>TaqMan®</th>
<th>Abscription® (1.5 hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TLC</td>
</tr>
<tr>
<td></td>
<td>Copies</td>
</tr>
<tr>
<td>9000</td>
<td>28</td>
</tr>
<tr>
<td>3000</td>
<td>30</td>
</tr>
<tr>
<td>1000</td>
<td>32</td>
</tr>
<tr>
<td>300</td>
<td>34</td>
</tr>
<tr>
<td>100</td>
<td>35</td>
</tr>
<tr>
<td>30</td>
<td>37</td>
</tr>
</tbody>
</table>

FIG. 8 shows the results of α-TAP Abscription®-based detection of titrated HeLa compared to TaqMan® PCR using the same priming sites for both methods. 5'-gcgggacctctcaga-3' (SEQ ID NO:3) and 5'-actcactgtgcggcagcagt-3' (SEQ ID NO:4) were used for the qPCR amplification. 5'-FAM-accacctttataagctggagggc-Iowa Black™ FQ quencher-3' (SEQ ID NO:5) was the fluorescent probe.
5'-actcactggt ggegaagact-3' (SEQ ID NO: 13) and 5'-cctccatccaaagtagcgggcggaccctccagaa -3' (SEQ ID NO: 12) were the α-TAP primer pair. **FIG. 8B** shows the results for TLC detection. Absorption/PCR primers were SEQ ID NO: 12 and SEQ ID NO: 13. The TAP-APC was made by annealing SEQ ID NO:28 and SEQ ID NO:32. The APC encoded the Abscript GAG. Abscripts were detected using thin layer chromatography (TLC) and UV shadowing. The limit of detection (LOD) at 29 cycles using UV-shadowing was 100 copies. **FIG. 8C** shows the LC-MS detection results for the same samples analyzed in **FIG. 8B**. DNA inputs for the PCR reactions varied from 30 to 9000 genomic copies. The LOD for LC-MS at 29 cycles was 30 copies. Area refers to the area of the chromatographic peak containing the Abscript (GAG).

[0118] TaqMan® PCR required 35 cycles for detection of 100 copies and 37 cycles for detection of 30 copies (**FIG. 8A** and **Table 5**). Absorption® based detection was more sensitive than TaqMan® even with TLC and UV-shadowing.

[0119] TLC based detection is more sensitive if UV-shadowing is replaced with detection of fluorescent Abscripts. Genomic DNA that was methylated in the CDKN2A CpG island was amplified with primers containing a biotin group (SEQ ID NO:43) and an anti-TAP sequence (SEQ ID NO:42). Amounts of starting genomic DNA were 10 ng, 2.5 ng, 640 pg, and 160 pg. This corresponded to 3000, 750, 188 or 47 copies of genomic DNA. After addition of the TAP-APC encoding AUC, Absorption® was carried out in the presence of the Cy5™ labeled dinucleotide ApU and CTP at 45C. Samples were withdrawn (1 μl) and analyzed by rapid TLC. Abscripts from 2.5 ng of starting DNA could be visualized after 2 hr of Absorption®, and 47 copies could be detected easily after 8 hours of Absorption® as shown in **FIG. 9**.

[0120] In this experiment, the Absorption® product was the Cy5™-labeled trimucleotide AUC. Cy5™ is actually a rather poor initiator compared to several other fluorescent dyes, reducing the turnover for dinucleotide synthesis to about 8% of that with unlabeled dinucleotides. For this reason, Cy5™ may not be the dye of choice for these assays, but can be replaced with other dyes, such as fluorescein or DyLight (Pierce), both of which give turnovers closer to 35% of that obtained with unlabeled initiators. By using dyes with approximately 4 fold higher efficiency, times may be reduced correspondingly, allowing detection of less than 50 copies of starting DNA in 2 hours. Fluorescein has the additional advantage of being detectable with a low cost, long wavelength UV light.

**EXAMPLE 6. Two-step CpG island methylation detection with APC-primers**

[0121] Using a two step detection method, methylated DNAs were isolated from a fragmented genomic DNA sample as in the three-step α-TAP method (**EXAMPLE 5**). Methylated DNA fragments were bound to immobilized GST-MBD protein (**FIG. 10**; Step 1) as described above. Methylated fragments were released by exposure to heat or glutathione after washes to remove unmethylated DNA (**FIG. 10**; Step 2).
Targeted CpG islands were amplified and tagged using a primer that contains an APC sequence at its 5’ end (FIG. 10; Step 3). The single-stranded form of the APC is inactive but becomes activated when it is converted into a double-stranded form during amplification of the target (FIG. 10; Step 4). Thus, Abscription® can be performed during the PCR reaction if initiator(s) NTP(s) and RNAP are included (FIG. 10; Step 6).

EXAMPLE 7. Design and validation of APC-primers.

APC-primers were designed to avoid self priming and the formation of primer dimers with the reverse primer. At least some of these events are likely to produce active duplex promoters that could create high levels of background Abscription®. Potential primer sequences were screened as described in EXAMPLE 5 for the potential to form primer dimers and to self prime. The APC portion of the APC primer is 44 nt long of this 33 nt can be changed without significantly affecting Abscription® activity. In most cases potential self priming or primer dimer interactions could be eliminated by changing the sequence of the APC segment of the APC-primer.

Primer pairs that were predicted to be free of potential interactions were tested by performing PCR reactions in the absence of DNA over a range of annealing temperatures to determine if the primers alone could produce background signal. First the APC-primer was tested to determine the level of self priming. Next, PCR reactions without DNA were performed with both the APC primer and the reverse primer to test for primer dimer effects. Completed PCR reactions were supplemented with 1 mM dinucleotide initiator, 1 mM NTP, 0.4 units of RNA polymerase and were analyzed by Abscription®.

FIG. 11 shows the Abscription® results for a well designed primer pair (SEQ ID NO:33 and SEQ ID NO:34) that targets the GAPDH CpG island. FIG. 1 A shows the TLC data for the APC primer alone (SEQ ID NO:34) and for the primer pair along with a positive control that included HeLa genomic DNA. The encoded Abscript GAG could only be detected in the positive control. FIG. 1 B shows LC-MS data for the same samples. Only the positive control produced the Abscript, while the reactions lacking DNA did not produce significant signal over a broad range of annealing temperatures. APC-primer /reverse primer pairs developed for CpG islands are given in Table 6 below.

Table 6. APC-Primer/Reverse Primer Pairs

<table>
<thead>
<tr>
<th>CpG Island</th>
<th>APC Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>5'-agagaatatttttctaaacattaatgtacaatgg_gaagcgag aacgtgtgccccagacc-3' (SEQ ID NO:33)</td>
<td>5'-ctgcctagggagagaga-3' (SEQ ID NO:34)</td>
</tr>
<tr>
<td>MGMT</td>
<td>5'-gtggttgcacattataacgctaatgtacaatggactcg aagctaggttcgcttgccg-3' (SEQ ID NO:35)</td>
<td>5'-ctggtgggagcagatgc-3' (SEQ ID NO:36)</td>
</tr>
<tr>
<td>PTGS2</td>
<td>5'-tacgacaacctgtataaattataatgtacaatgg_gaacg agaggtcagaaagcccagaga-3' (SEQ ID NO:48)</td>
<td>5'-gtgtgggaagcagttggaatc-3' (SEQ ID NO:38)</td>
</tr>
</tbody>
</table>
EXAMPLE 8. Comparison of Detection of methylated DNA from tumor cell lines with α-TAP and APC-primer methods

PCR reactions were performed with 0.4 units of Hot Start Taq (Fermentas) in the vendor’s IX buffer containing 2 mM MgC12, 0.8 mM dNTPs (0.2 mM each), and 5% (v/v) DMSO. One unit of Hot Start Taq incorporates 10 nmol of dNTPs in 30 min at 74°C. The APC-primer and the reverse primer were at 1 µM each. The cycling conditions were 95°C for 1 min, followed by up to 32 cycles of 95°C for 30 sec, 62°C for 30 sec, and 72°C for 30 sec. A final elongation step was at 72°C for 5 min. Completed reactions were held at 4°C. Completed PCR reactions (10 µl) were supplemented with 1 mM dinucleotide initiator, 1 mM NTP and 0.4 units of RNA polymerase. Absorption ® was then performed for up to 1 hr at 77°C.

Absorption ® could be performed during PCR if the dinucleotide initiator and the NTP were included in the PCR reaction at 1 mM each. A thermostable RNA polymerase was added at 0.4 units per 20 µl reaction. One unit catalyzes the incorporation of 1 nmol of NTP in 60 min at 65°C. Absorption ® reactions were incubated for 1 hr at 77°C. Abscripts could be detected as described in examples 2 and 5.

Table 7. Percent Methylation of Tumor Cell DNAs

<table>
<thead>
<tr>
<th>DNA sample Detection method</th>
<th>DAPKI</th>
<th>MGMT</th>
<th>GSTPI</th>
<th>PTGS2</th>
<th>GAPDH</th>
<th>SNRPN</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNCaP (Prostate) α-TAP detection</td>
<td>81</td>
<td>19</td>
<td>100</td>
<td>60</td>
<td>0.8 ± 0.3</td>
<td>49 ± 1.5</td>
</tr>
<tr>
<td>n=2</td>
<td>n=2</td>
<td>n=2</td>
<td></td>
<td></td>
<td>n=3</td>
<td>n=3</td>
</tr>
<tr>
<td>LNCaP (Prostate) APC-primer detection</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n=4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDA-PCA-2b (Prostate) α-TAP detection</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HeLa (Cervical) APC-primer</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n=5</td>
<td>n=2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Percent methylation ± SD
Table 7 shows the results of Abscription®-based detection of methylated DNA from tumor cell lines. Most replicate measurements were done with samples measured once from multiple fractionation experiments. The GAPDH CpG island was unmethylated which was expected if the tumors maintained the normal methylation status in this island. The SNRPN CpG island fit the prediction for an imprinted gene. The other islands were consistent with published results except for DAPK1 in LNCaP for which there are divergent conclusions on its methylation status (Yegnasubramanian et al. (2004) Cancer Res. 64:1975-86; Lin et al. (2001) Am J. Pathol. 159:1815-26; Paz et al. (2003) Cancer Res. 63:1114-21; Toyota et al. (2000) Cancer Res. 60:4044-48; Lodygin et al. (2005) Cancer Res. 65:4218-27).
WHAT I CLAIMED IS:

1. A method for detecting at least one target polynucleotide in a sample comprising:
   a) contacting a sample containing the at least one polynucleotide with a primer pair that specifically hybridizes to and amplifies a target sequence of the at least one polynucleotide, wherein the primer pair consists of:
      i) a first primer comprising
         (1) a 3’ sequence complementary to a first sequence flanking the target sequence of the polynucleotide, and
         (2) a 5’ capture tag;
      and
      ii) a second primer comprising:
         (1) a 3’ sequence complementary to a second sequence flanking the target sequence of the polynucleotide, and
         (2) a 5’ sequence that provides a means for directing Abscription;
   b) amplifying the target sequence from the first and second primers;
   c) contacting the amplified target sequence with an immobilized molecule that binds the 5’ capture tag, thereby capturing the amplified target sequence of the polynucleotide;
   d) transcribing at least one Abscript from the means for directing Abscription;
   and
   e) detecting at least one Abscript transcribed in step d).

2. The method of claim 1, wherein unbound reagents, primers and polynucleotides are washed from immobilized and captured polynucleotides prior to the following step.

3. The method of claim 1, wherein amplifying consists of performing a polymerase chain reaction.

4. The method of claim 3, wherein the polymerase chain reaction is performed with at least one of a thermostable DNA polymerase and a thermostable RNA polymerase.

5. The method of claim 1, wherein the 5’ capture tag is biotin and the molecule that binds to the 5’ capture tag is streptavidin.

6. The method of claim 1, wherein the molecule that binds to the 5’ capture tag is immobilized on a solid support selected from a magnetic bead and a microtiter plate.
7. The method of claim 1, wherein a detectably labeled nucleotide is incorporated into the at least one Abscript during step d).

8. The method of claim 7, wherein the detectably labeled nucleotide is a fluorescent nucleotide.

9. The method of claim 1, wherein detecting the at least one Abscript comprises mass spectrometry, capillary electrophoresis or thin layer chromatography.

10. The method of claim 1, wherein the at least one Abscript is 3-20 nucleotides in length.

11. The method of claim 10, wherein the at least one Abscript is 3 nucleotides in length.

12. The method of claim 1, wherein the means for directing Abscription comprises an APC.

13. The method of claim 1, wherein the means for directing Abscription comprises:
   i) A 5’ α-TAP sequence that identifies the unique CpG island; and
   ii) a non-natural nucleotide between the 5’ α-TAP sequence and the 3’ sequence complementary to the second sequence flanking the target sequence;

and step d) comprises:
   i) hybridizing a probe to the amplified target sequence, wherein the probe comprises a 5’ TAP sequence complementary to the α-TAP sequence and a 3’ APC; and
   ii) transcribing at least one Abscript from the APC.

14. The method of claim 13, wherein the non-natural nucleotide prevents replication of the α-TAP during amplification, and thereby the α-TAP sequence remains single-stranded during steps a) through c).

15. The method of claim 14, wherein the non-natural nucleotide is etheno-deoxyadenosine.

16. The method of claim 1, wherein the at least one target polynucleotide comprises a plurality of different target polynucleotides, and steps a) through e) are carried out simultaneously with a plurality of first and second primer pairs, each primer pair specifically hybridizing to a different target.

17. The method of claim 16, wherein the means for directing Abscription produces a unique Abscript for each of the plurality of targets.

18. The method of claim 17, wherein the unique APCs are distinguishable from each other on the basis of molecule weight or nucleotide sequence.
19. The method of claim 16, wherein the plurality of different target comprises at least 10 different targets.

20. The method of claim 1, wherein the at least one target polynucleotide is a methylated CpG island and the sample comprises isolated methylated genomic DNA fragments.

21. The method of claim 20, wherein the methylated genomic DNA fragments are isolated by:
   a) cleaving a genomic DNA sample containing at least one methylated target polynucleotide with a restriction enzyme that does not cleave the target polynucleotide CpG island;
   b) contacting the cleaved genomic DNA with an immobilized MBD, thereby immobilizing methylated genomic DNA from the sample; and
   c) optionally, recovering the methylated genomic DNA from the immobilized MBD, thereby isolating methylated genomic DNA fragments.

22. The method of claim 21, wherein the MBD is a GST-MBD2 fusion protein.

23. The method of claim 22, wherein the GST-MBD2 fusion protein is immobilized on a glutathione-containing solid support.
Figure 1

A

TAP

APC

B

Capture probe

APC

RNA or DNA target

RNA or DNA target

C

Protein

APC

Solid support

Antibody

Figure 2
Figure 5
Figure 8

Figure 9