CpGenome™ Universal DNA Modification Kit

Catalog No. S7820

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Not for use in diagnostic procedures.

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Introduction

Methylation of cytosines located 5' to guanosine is known to have a profound effect on the expression of many eukaryotic genes (1). In normal cells methylation occurs predominantly in CG-poor regions, while CG-rich areas, called CpG-islands remain unmethylated. The exceptions are the extensive methylation of CpG islands associated with transcriptional inactivation of regulatory regions of imprinted genes (2, 3) and genes on the inactive X-chromosome of females (4, 5). Aberrant methylation of normally unmethylated CpG islands has been documented as a relatively frequent event in immortalized and transformed cells (6) and has been associated with transcriptional inactivation of defined tumor suppresser genes in human cancers (7, 8). Hundreds of CpG islands are now known to exhibit the characteristic of hypermethylation in tumors (9).

Several methods have been developed to determine the methylation status of cytosine. These include digestion with methylation sensitive restriction enzymes as in restriction landmark genomic scanning, oligonucleotide arrays, genomic DNA sequencing and methylation specific PCR (MSP). Some techniques are more useful for discovery while others are better used for monitoring of known methylated cytosines. Genomic DNA sequencing, although time consuming and labor intensive, offers a more universal detection method (10, 11). MSP is now an established technology for the monitoring of abnormal gene methylation in selected gene sequences (12). Utilizing small amounts of DNA, this procedure offers sensitive and specific detection of 5-methylcytosine in promoters. It is being exploited to define tumor suppresser gene function, and to provide a new strategy for early tumor detection.

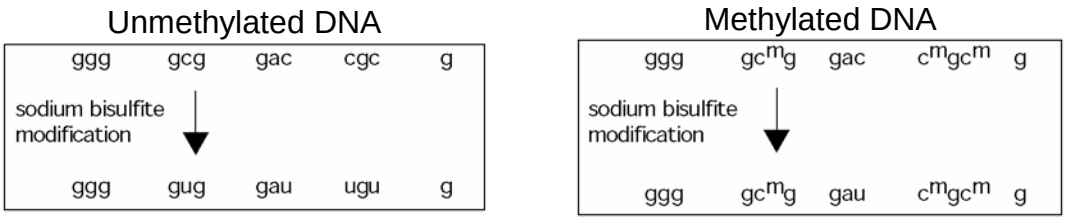
The initial step of both genomic sequencing and MSP is to perform a bisulfite modification of the DNA sample. MSP then involves PCR amplification with specific primers designed to distinguish methylated from unmethylated DNA. The CpGenome™ DNA Modification Kit contains the reagents for the initial bisulfite modification of the DNA required for both methodologies.

Principles of the Technique

Bisulfite Modification

The CpGenome™ DNA Modification Kit contains reagents required to perform a bisulfite modification on a DNA sample. As shown in Figure 1, in the bisulfite reaction, all unmethylated cytosines are deaminated and sulfonated, converting them to uracils, while 5-methylcytosines remain unaltered. Thus, the sequence of the treated DNA will differ depending on whether the DNA is originally methylated or unmethylated. Also, the initially complementary DNA strands will no longer be complementary after cytosine conversion. Primers for use in MSP can be designed to specifically amplify either a bisulfite-sensitive, unmethylated strand or a bisulfite-resistant, methylated strand, based upon these chemically-induced differences.

Figure 1. DNA treatment with Sodium Bisulfite



MSP

The PCR primers are designed to specifically amplify the promoter regions of the gene of interest. If the sample DNA was originally unmethylated, an MSP reaction product will be detectable when using the primer set (labeled as 'U') designed to be complementary to the unmethylated DNA sequence. No product will be generated using a primer set (labeled as 'M') designed to be complementary to the derivative methylated DNA sequence. Conversely, an MSP product will be generated only using the M primer set if the sample was originally methylated, and the U primers will not amplify such a template.

Methylation specific PCR permits sensitive detection of altered DNA. Because it is a PCR-based assay, it is extremely sensitive, facilitating the detection of low numbers of methylated alleles and the evaluation of DNA from small samples, including paraffin-embedded materials. MSP also allows examination of all CpG sites, not just those within sequences recognized by methylation sensitive restriction enzymes. Increasing the number of such sites that can be assessed allows rapid, fine mapping of methylation patterns throughout CpG regions. In addition, the bisulfite modification is ideally suited for analysis of CpG islands since it converts the majority of cytosines to uracils, making a region of the genome which is CG rich more easily amplified by PCR.

Important Note

Please read the entire manual prior to using the CpGenome™ DNA Modification Kit. Useful information on primer design and other considerations for methylation-specific PCR (MSP) can be found in the Appendix. Should additional questions arise, please contact Technical Support Services at www.millipore.com/techservice.

Kit Components

Sufficient reagents are provided in the CpGenome™ DNA Modification Kit to perform 100 bisulfite reactions.

Table 1: DNA Modification Kit Components

Part No.	Description	Quantity	Storage Conditions
90412	DNA Modification Reagent I	23 g	-15 to -25°C Protect from light.
90413	DNA Modification Reagent II	135 g	-15 to -25°C
90414	DNA Modification Reagent III	700 µL	-15 to -25°C (long term) 2 to 8°C (short term)
90415	DNA Modification Reagent IV	200 µL	-15 to -25°C

Materials Required But Not Supplied

Equipment and Supplies

a. Water bath incubator or heat block at 37°C and 50°C.

b. Microcentrifuge (to 12,000 X g)

c. Screw-cap microcentrifuge tubes, 1.5-2.0 mL

d. Narrow range pH indicator paper (pH 0-6, three different indicators per strip, and/or pH 4-7, one indicator per strip) or a dedicated pH electrode.

Reagents

a. NaOH pellets

b. 70%, 90% and 100% EtOH

c. β-mercaptoethanol

d. TE Buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5)

Storage

Store at -15 to -25°C as indicated in Table 1. The DNA Modification Reagent III (90414) can be stored at 2 to 8°C upon receipt of the kit.

Protocol

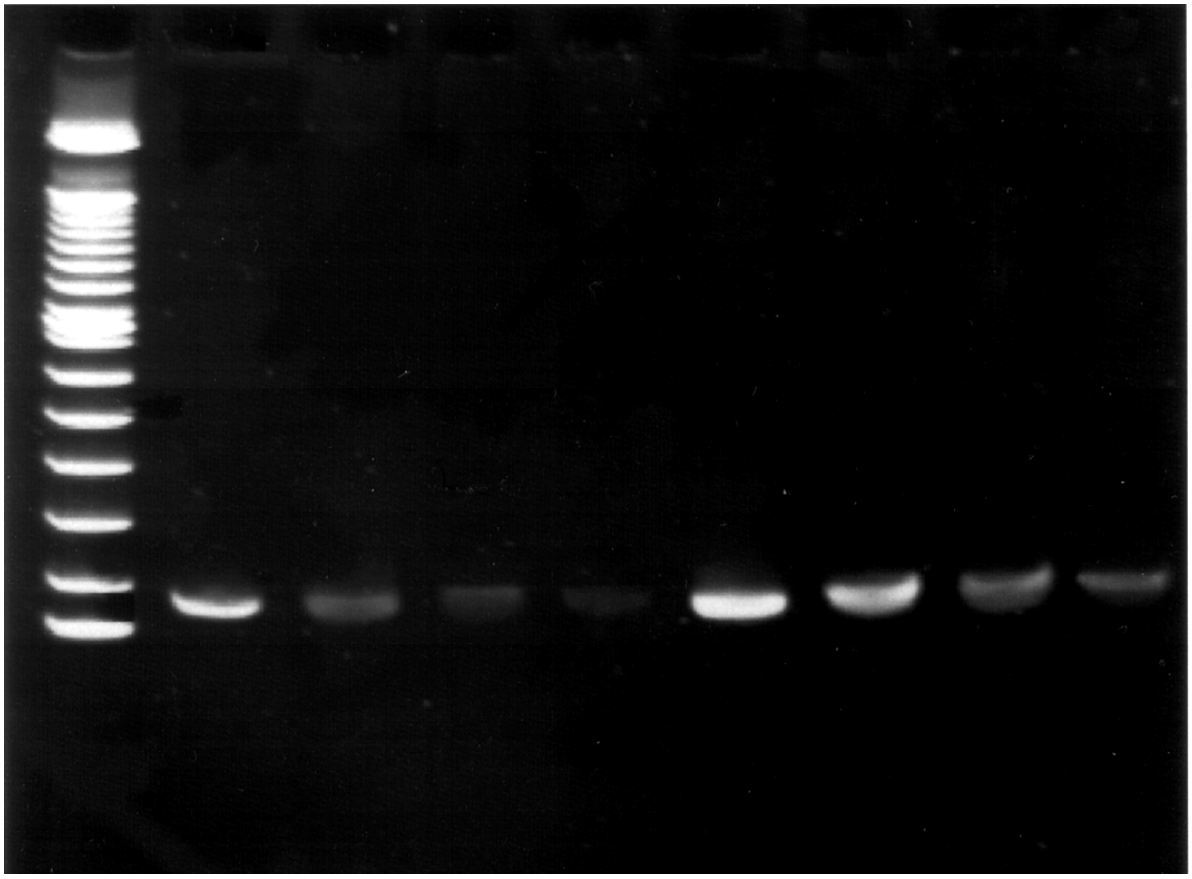
A. DNA Purification

Genomic DNA can be purified from experimental samples (e.g. cells or frozen tissue) using any of a number of standard DNA purification protocols (13). For paraffin-embedded specimens, we recommends using the EX-WAX™ DNA Extraction Kit (S4530). For tissues and cells, we recommend using the Non-Organic DNA Extraction Kit (S4520). For a reliable chemical modification, the DNA should be intact, undegraded and as pure as possible.

B. Recovery of Starting Material

Beginning the modification procedure with 1 µg of DNA when the sample is abundant is recommended. However, it is not required with the CpGenome™ DNA Modification Kit. Sufficient DNA can be recovered to perform MSP when the amount of starting material is as little as 0.001 µg of DNA, as shown in Figure 2.

Figure 2: Sensitivity of CpGenome™ DNA Modification Kit



Chemical Modification of the DNA was performed using the CpGenome™ DNA Modification Kit. PCR of the modified DNA was performed using the U primer set and Unmethylated (U) Control DNA from the CpG WIZ™ p16 Amplification Kit (Catalog No. S7800). 100 base pair ladder (Lane 1). 1.0 µg DNA, 0.1 µg DNA, 0.01 µg DNA, and 0.001 µg DNA without the addition of DNA Modification Reagent IV (Lanes 2-5). 1.0 µg DNA, 0.1 µg DNA, 0.01 µg, and 0.001 µg DNA with the addition of DNA Modification Reagent IV (Lanes 6-9).

C. Modification Protocol

The process for conversion of cytosine to uracil using the CpGenome™ kit is as follows. Bases are first exposed by denaturing the DNA to its single stranded form using mild heat at an alkaline pH. Reagent I, containing a sodium salt of bisulfite ion (HSO_3^-), causes unmethylated cytosine to be sulfonated and hydrolytically deaminated, yielding a uracil sulfonate intermediate. DNA is then bound to a micro-particulate carrier (Reagent III) in the presence of another salt (Reagent II), and is desalted by repeated centrifugation and resuspension in 70% EtOH. The conversion to uracil is completed by alkaline desulfonation and desalting is repeated in 90% EtOH. The DNA is finally eluted from the carrier by heating in TE Buffer.

To prevent PCR contamination read Sec. V. *Appendix, Laboratory Setup and Precautions*, before beginning.

STEP 1. Reagent Preparation

3 M NaOH stock **(freshly prepared prior to each use)**

Dissolve 1 g of dry NaOH pellets in 8.3 mL of water. Exercise appropriate caution and lab practices when using this caustic base.

20 mM NaOH/90% EtOH **(freshly prepared prior to each use)**

To prepare 1 mL of this solution, combine 900 µL of 100% EtOH, 93.4 µL of H_2O and 6.6 µL of 3 M NaOH.

Dissolve Reagent I **(freshly prepared prior to each use)**

Warm bottle to room temperature before opening. For each sample to be modified, weigh 0.227 g of DNA Modification Reagent I and add to 0.571 mL water. Mix thoroughly by vortexing. Use appropriate caution when handling this reagent since it is irritating to the respiratory system and skin. Adjust the pH to 5.0 with approximately 20 µL of 3M NaOH, monitoring the pH with pH indicator paper (read out the pH immediately upon wetting with sample). Protect Reagent I solution form light. For best results this reagent should be used immediately following preparation.

Dissolve Reagent II

Warm bottle to room temperature before opening. Add 1 µL of ß-mercaptoethanol to 20 mL of deionized water. Add 750 µL of this solution to 1.35 g of DNA Modification II for each sample to be modified. Mix well to ensure complete dissolution. Excess reagent can be stored in a foil wrapped container at 2° to 8°C IN THE DARK for up to 6 weeks.

STEP 2. DNA Modification Procedure

1. In screwcap 1.5-2.0 mL microcentrifuge tubes: Add 7.0 µL 3M NaOH to 1.0 µg DNA in 100 µL of water (10 ng/µL) and mix .

   Note: *If the sample contains less than 1.0 µg of DNA, add 2 µL of DNA Modification reagent IV to the sample DNA and bring the total volume to 100 µL with water. Then add 7.0 µL 3M NaOH and mix.*

2. Incubate DNA 10 minutes at 50°C (heat block or water bath).
3. Add 550 µL of freshly prepared DNA Modification Reagent I and vortex.
4. Incubate at 50°C for 4-16 hours in a heat block or water bath protected from light.

STEP 3. Initial Desalting

1. Resuspend DNA Modification III by vortexing vigorously. Draw suspension into and out of a 1 ml plastic pipette tip 10x to disperse any remaining clumps.
2. Add 5 µL of well-suspended DNA Modification Reagent III to the DNA solutions in the tubes.
3 Add 750 µL of DNA Modification Reagent II and mix briefly.
4. Incubate at room temperature for 5-10 minutes.
5. Spin for 10 seconds at 5,000 X g to pellet the DNA Reagent III. (A small white pellet should be present.) Discard supernatant.
6. Add 1.0 mL of 70% EtOH, vortex, centrifuge for 10 seconds at 5,000 X g and discard supernatant. Perform this step for a total of 3 times.
7. After the supernatant from the third wash has been removed, centrifuge the tube at high speed for 2 minutes, and remove the remaining supernatant with a plastic pipette tip.

STEP 4. Completion of DNA Modification *(Desulfonation*), Second Desalting, and Elution

1. Add 50 µL of the 20 mM NaOH/90% EtOH solution to the appropriate samples.
2. Vortex briefly to resuspend the pellet, and incubate at room temperature for 5 minutes.
3. Spin for 10 seconds at 5,000 X g to move all contents to tip of tube. Add 1.0 mL of 90% EtOH and vortex to wash the pellet. Spin again and remove the supernatant. Repeat this step one additional time.
4. After the supernatant from the second wash has been removed, centrifuge the sample at high speed for 3 minutes.
5. Remove ALL of the remaining supernatant with a plastic pipette tip. Allow the tube to dry for 10-20 minutes at room temperature (alcohol odor should diminish).
6. Add TE Buffer.

   Note: *The volume of TE to be added depends on the amount of starting DNA and on the required input concentration for the intended use. For example, if 25 µL of TE is added, the final concentration based on complete recovery of 1 µg DNA would be 40 ng/µL. Vortex rapidly and forcefully until the pellet is completely resuspended. Manually flick or tap the contents to tube tip (but do not centrifuge).*

7. Incubate the sample for 15 minutes at 50-60°C to elute the DNA.
8. Centrifuge at high speed for 2-3 minutes and transfer the sample (supernatant) to a new tube using a plastic pipette tip.
9. Proceed to MSP or sequencing, or store at –15°C to –25°C for up to 2 months, at –80°C for up to 6 months. Avoid repeated thawing and refreezing; store as aliquots. Do not store DNA in a self-defrosting freezer.
10. When removing thawed DNA from a tube for later use, avoid transfer of Reagent III to PCR reaction tubes. Always centrifuge the tube briefly first, to pellet any residual Reagent III solids.

Troubleshooting

? No products are visible in any lane.

1. Potential Problem: PCR amplification is not initiated.

Recommendations:

a. Confirm that all PCR components were added to the reaction tube.
b. Confirm that the time and temperature settings on the temperature cycler match those described in this manual.
c. If performing hot start PCR using AmpliTaq Gold®, verify the initial denaturation/activation time of 12 minutes at 95°C.
d. For all other hot start methods, confirm the proper use of the reagents.
e. Confirm that the PCR polymerase is still active.

2. Potential Problem: Experimental DNA samples were degraded prior to chemical modification.

Recommendation: Purify the genomic DNA again and repeat the chemical modification. Methylated DNA (unmodified) is available from Millipore (Catalog No. S7821) for use as a positive control.

3. Potential Problem: DNA samples were degraded after modification.

Recommendation: If chemically modified experimental DNA samples were stored at –20°C for more than two months prior to PCR, repeat the chemical modification on new genomic DNA samples. Do not store DNA in a self-defrosting freezer.

? The only lanes containing a PCR product are from those DNA samples amplified with the W primer set.

1. Potential Problem: Chemical modification of the experimental DNA samples did not work.

Recommendations:

a. DNA Modification Reagent I and 3 M NaOH stock must be freshly prepared prior to each use.
b. Sodium bisulfite modifies only single-stranded DNA. Double stranded DNA should be denatured in 200 mM NaOH prior to modification. Some highly G/C rich sequences may denature better if a restriction digestion step is inserted prior to denaturation. The cuts should be carefully targetted outside the intended template sequence. (CpG WIZ™ MSP primer kits do not require this step.)

2. Potential Problem: DNA was not recovered

Recommendations:

a. Be sure that a small white pellet of DNA carrier is seen when suspensions containing Reagent III are first centrifuged down. The pellet should be about 1 mm x 2 mm. If the pellet is inadequate, add more Reagent III, vortex, re-incubate for 10 minutes, and re-centrifuge.
b. Remove all alcohol before attempting to elute DNA from carrier.
c. Be sure that pellet is well resuspended in TE before elution.

3. Potential Problem: DNA was degraded during modification.

Recommendation: Incubation of DNA at pH 5 unavoidably causes some damage. Incubation in Reagent I at 50°C for over 16 hours could cause some methylated cytosines to be converted to thymidines.

4. Potential Problem: Some DNA sequences form a structure that is resistant to chemical modification. Some DNA samples be partially or randomly methylated, and after modification, these might not bind the MSP primers under the recommended PCR conditions.

Recommendation: These possibilities can be explored by cloning, amplifying and sequencing the modified DNA. The annealing temperature of the MSP reaction may be lowered by 3-6°C to increase primer binding, although this may promote artifacts due to mispriming.

5. Potential problem: Residual Reagent III suspension was transferred to PCR tubes with DNA (Reagent III may inhibit PCR).

Recommendation: Before removing an aliquot of modified DNA from a tube, always centrifuge briefly to pellet any residual solids, then carefully pipette only from the supernatant.

? W primer set produces an amplification product in some or all experimental samples, in addition to an amplification product from the U or M primer set.

1. Potential Problem: Chemical modification of the experimental DNA sample(s) is incomplete.

Recommendation: This will not jeopardize the validity of the assay as long as a product is also produced using the U or M primer set.

? U and M primer sets are both producing bands in some DNA samples.

Potential Problem: Sample heterogeneity.

Recommendations: The DNA sample may have been derived from heterogeneous original cell types, containing both methylated and unmethylated DNA.

? U or M primer sets are producing bands in all samples, including the "no DNA" controls.

Potential Problem: PCR reagents are contaminated with amplification products.

Recommendations: see Sec.V. *Appendix: Laboratory Setup and Precautions*

a. Use fresh aliquots of every PCR component (i.e. dNTPs, buffer, etc).
b. Use separate sets of pipettes for pre- vs. post-amplification liquid dispensing.
c. Devote a work area to pre- and post-amplification procedures.
d. Always use aerosol-resistant pipette tips.
e. Always use a clean labcoat and gloves.

A. Considerations for MSP

I. Primer Design

Methylation specific PCR consists of chemical modification followed by amplification. Chemical modification creates the sequence differences between the methylated and unmethylated DNA. Ideally, three sets of primers should be designed to anneal to the DNA, based upon these sequence differences. One primer set (U) will anneal to unmethylated DNA that has undergone a chemical modification. A second primer set (M) will anneal to methylated DNA that has undergone a chemical modification. A third primer set (W) will anneal to any DNA (unmethylated or methylated) that has NOT undergone chemical modification, hence, the "wild type", or W. This serves as a control for the efficiency of chemical modification.

The standard rules for primer design apply toward the creation of MSP primers. The primers should be approximately 20-21 bp in length and should possess similar dissociation temperatures. The product produced with each set of primers should be 100-200 bp in size. Internal secondary structure should be avoided. In order to minimize primer dimer formation, primers should not be complementary, especially at the 3' end. Since most CpG islands are located within the gene promoter, the optimal area for primer selection is the most G-C rich region closest to the transcription start site. Discrimination between methylated and unmethylated sequences by MSP seems to be greatest when the 3' ends of the primers are most different from one another.

Alternatively, the MethPrimer software package can be used to design primers for bisulfite-based PCR. Please visit http://www.urogene.org/methprimer for more information on how to access this tool.

II. Experimental Design

A thorough analysis of each modified DNA sample includes PCR amplifications using the U, M and W primer sets as described above. Unmodified DNA should be amplified with the W primer set, which serves as a positive control for PCR. In addition, a negative PCR control (i.e. no DNA) should be performed for each set of primers.

Depending on the DNA sample, a product may be produced using either the U or M primer sets or both sets may produce a product. Amplification using the W primer set with a modified DNA sample should not produce a product unless the modification was incomplete. However, PCR with the W primer set on unmodified DNA serves as a general positive PCR control product. Appearance of a product in the negative PCR control would indicate possible contamination.

III. "Hot Start" PCR

The three sets of primers used in MSP are derived from sequences closely related to each other, which introduces an opportunity for mispriming. In order to avoid this and other PCR-related artifacts, "hot start" PCR is recommended. "Hot start" PCR permits the Taq polymerase to begin the reaction only after the template and primers are in single stranded form.

There are several modifications of the standard PCR protocol which allow a "hot start" to occur. In one scenario, the PCR reaction mixture excluding the polymerase can be overlaid with mineral oil prior to heating to 95°C. At the end of the incubation, the enzyme is pipetted directly into the mixture under the mineral oil. A second method involves the physical separation of the polymerase and the rest of the PCR mix with a wax bead. The enzyme combines with the rest of the reaction mixture only

after the wax melts. In another variation, an anti-Taq antibody inhibits the polymerase during reaction set-up by forming a complex with the Taq enzyme. Taq polymerase becomes active when the complex is abolished due to antibody denaturation during the 95°C incubation.

Alternatively, AmpliTaq Gold® (Applied Biosystems) can be used. This enzyme is inactive until heated at 94-95°C for 9-12 minutes.

Note: *Do not use a polymerase capable of 3-5' mismatch repair (i.e. proofreading).*

IV. Laboratory Setup and Precautions

One of the most important considerations when using the CpGenome™ DNA Modification Kit and performing PCR is the environment where the initial reaction mixtures are set up. The ideal environment is free of amplified DNA products, which can cause false-positive results. Some sources of PCR product contamination are contaminated pipettes and tips, gel box and buffer, tube racks, notebooks, lab coats and any other item exposed to amplified PCR products.

The following precautions should be followed in all steps of the assay protocol:

1 Always wear gloves.

2. Use sterile water for all solutions, aliquot the solutions in small amounts, and use fresh aliquots as working solutions. Discard working solutions after use.

3. Keep the assay solutions (10X PCR buffers, dNTPs, polymerase, etc.) separate from the amplified DNA.

4. Always use aerosol resistant pipette tips.

5. Separate micropipettors and work areas are recommended for the following three steps of the assay:
    - DNA modification and purification
    - Amplification set-up
    - Post-amplification analysis

6. Reagent II spills or aerosols can cause a yellow stain on cloth or paper upon drying out. Use extra care when vortexing, pipetting, centrifuging and opening tube lids. Optionally, work over absorbent paper and wear protective outer clothing.

7. Solids in Reagent III may dry out if the vial is subjected to prolonged storage between uses. Any dried solids on the plastic seal will interfere with a tight seal, allowing water to evaporate from the vial. Dried Reagent III may be reconstituted with several volumes of sterile deionized water.

Related Products

Description	Catalog No.
5-hmC Glucosyltransferase	14-1047
CpGenome Fast DNA Modification Kit	S7824
CpGenome™ Turbo Bisulfite Modification Kit	S7847
CpG MethylQuest™ DNA Isolation Kit	17-10035
CpG WIZ® BRCA1 Amplification Kit	S7830
CpG WIZ® Oct-4	S7840
CpG WIZ® RASSF1A Amplification Kit	S7813
CpG WIZ® ERa Amplification Kit	S7815
CpG WIZ® Fragile X Amplification Kit	S7807
CpG WIZ® hMLH1 Amplification Kit	S7811
CpG WIZ® GST-pi Amplification Kit	S7808
CpG WIZ® p16 Amplification Kit	S7800
CpG WIZ® p15 Amplification Kit	S7802
CpGenome™ 5mC and 5hmC DNA Standard Set	S8005
CpGenome™ Human Methylated & Non-Methylated DNA Set	S8001
CpGenome™ Human Methylated DNA Standard	S8001M
CpGenome™ Human Non-Methylated DNA Standard	S8001U
CpGenome™ Universal Methylated Mouse DNA Standard	S8000
CpG MethylQuest™ Protein	14-921
Anti-5-Hydroxymethylcytosine, clone AB3/63.3	MABE176
Anti-5-Methylcytosine, clone use mAb (162 33 D3)	NA81-50UG
Anti-5-Methylcytosine, clone 33D3	MABE146
Anti-DNA Methyltransferase 1	AB3429
Anti-DNA Methyltransferase 3a	AB3431
Anti-DNA Methyltransferase 3b	AB3433
Anti-DNMT3A2	07-2050
Anti-Kaiso, clone 6F	05-659
Anti-MBD1, C-terminus	07-2054
Anti-MBD2	07-198
Anti-MBD4	07-2057
Anti-MeCP2 (Rabbit Polyclonal)	07-013
Anti-Methylcytosine Dioxygenase TET1	09-872
Anti-Phospho-DNMT1(Ser714)	07-1594
DNA Methyltransferase Inhibitor	260920
DNA Methyltransferase Inhibitor II, SGI-1027	260921

For a complete list of DNA methylation products, visit www.millipore.com/epigenetics.

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Disclaimers

AmpliTaq Gold® is a trademark of Applied Biosystems.

TaKaRa is a registered trademark of TaKaRa Shuzo, Ltd.

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The CpGenome™ Methylation Products apply technologies exclusively licensed from The Johns Hopkins University School of Medicine. Methylation-specific PCR (MSP) technology is covered by U.S. Patent # 5,786,146.

Warranty

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