

MethylCollector™ Ultra

(version A)

Catalog No. 55005

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TABLE OF CONTENTS	Page
Overview	1
Flow Chart of Process	2
Introduction	2
Traditional Methods to Study DNA Methylation	3
Kit Performance and Benefits	4
Kit Components and Storage	5
Additional Materials Required	5
Protocols	
Fragmentation of Genomic DNA	7
Example Fragmentation Protocols	8
Data Analysis and Use of Inputs	9
PCR Primer Design	9
MethylCollector™ Ultra Protocol	10
Real Time PCR Analysis	13
Endpoint PCR Analysis	14
References	15
Appendix	
Section A. Use of Magnetic Beads and Included Bar Magnet	16
Section B. Troubleshooting Guide	18
Section C. Related Products	18
Technical Services	20

Overview

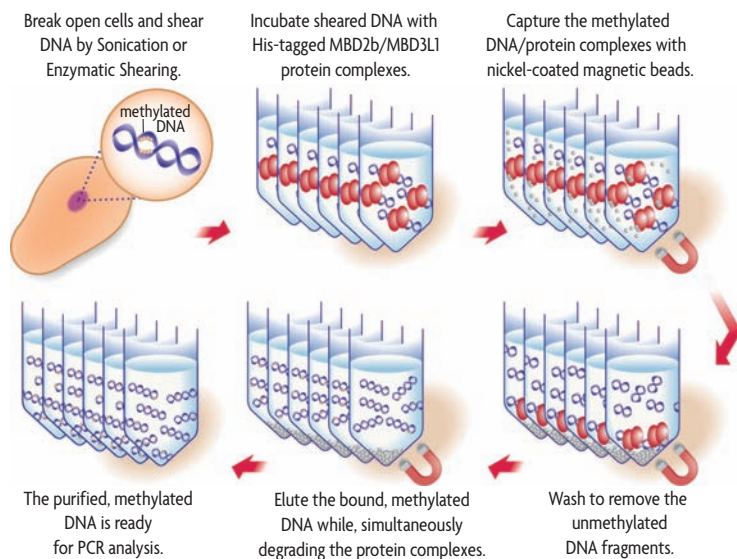
Active Motif's MethylCollector™ Ultra Kit is an improved alternative for the enrichment of CpG-methylated DNA¹ from limited amounts of cell or tissue samples. The method is based on the Methylated CpG Island Recovery Assay (MIRA), which utilizes the high affinity of the MBD2b/MBD3L1 complex for methylated DNA². MethylCollector Ultra is suitable for use in many downstream applications, such as real time or endpoint PCR analysis of the methylation status of particular loci in normal and diseased samples, rapid screening of the methylation status of multiple loci, bisulfite conversion followed by cloning and sequencing, or amplification and labeling for microarray analysis³. It can also be used to detect changes in DNA methylation during normal cellular differentiation and aging.

In the MethylCollector Ultra method*, His-tagged recombinant MBD2b and its binding partner MBD3L1 are combined together in order to increase the affinity of MBD2b for CpG-methylated DNA. The kit works using DNA fragments that have been prepared by enzymatic digestion or sonication from cell or tissue samples. The MBD2b/MBD3L1 protein complex is added to the DNA fragments, and it specifically binds to CpG-methylated DNA. These protein-DNA complexes are then captured with nickel-coated magnetic beads and subsequent wash steps are performed with a stringent high-salt buffer to remove fragments with little or no methylation. The methylated DNA is then eluted from the beads in the presence of Proteinase K. Due to the high efficiency of MethylCollector Ultra and the enormous amplification capability and specificity of PCR, analysis of the methylation status of a specific genomic DNA locus can be performed on DNA isolated from less than 170 cells (~1 ng DNA).

product	format	catalog no.
MethylCollector™ Ultra	30 rxns**	55005

*Technology covered under U.S. Patent No. 7,425,415.

**MethylCollector™ Ultra provides sufficient reagents to perform 30 reactions with excess reagents for 5 control reactions.



Flow chart of the MethylCollector Ultra process.

In MethylCollector Ultra, genomic DNA of interest is sheared by either enzymatic digestion or sonication. The sheared DNA is then incubated with a His-tagged recombinant MBD2b/MBD3L1 protein complex. The interaction of MBD2b with its binding partner MBD3L1 increases the affinity of MBD2b for CpG-methylated DNA. These protein-DNA complexes are captured with nickel-coated magnetic beads and stringent washes are then performed to remove fragments with little or no methylation. The methylated DNA is then eluted from the beads and real time or endpoint PCR is performed on the resulting supernatant using specific primers to amplify the locus of interest.

Introduction

Over the last decade, the study of DNA methylation and its role in epigenetic cell signaling has grown rapidly^{4,7}. Methylation of CpG-dinucleotides, which occurs at the fifth position of the cytosine pyrimidine ring, is of particular interest.

Although CpG dinucleotides are generally methylated throughout the genome of normal somatic cells, CpG islands (clusters of CpG dinucleotides in gene regulatory regions) are usually unmethylated⁸. Aberrant hypermethylation of CpG islands and subsequent transcriptional repression is one of the earliest and most common somatic genome alterations in multiple human cancers^{9,10}. Somewhat paradoxically, a decrease in the total amount of cytosine methylation is observed in many neoplastic tissues, but the genome context of this hypomethylation has not been identified¹¹. Aberrant methylation of CpG islands thus seems to be a tumor type-specific event^{10,12} and current efforts have concentrated on finding ways to exploit the diagnostic and therapeutic implications of these abnormalities^{13,14}.

Methyl-CpG binding proteins appear to be central players in the process of DNA methylation-dependent gene silencing¹⁵. This family of proteins takes its definition from the methyl-CpG binding domain (MBD), the minimum portion with specific affinity for a single, symmetrically methylated CpG pair. The MBD was characterized by deletion studies of MeCP2¹⁶. After the recognition of the MBD, four additional genes were found to contain this domain, namely MBD1, MBD2, MBD3 and MBD4¹⁷. In general, all MBD proteins, except MBD4, have been reported to be associated with histone deacetylase subunits as part of large multi-subunit complexes^{18,19}. A few studies support the notion of selectivity in the association of a particular MBD with particular promoters^{20,21}, but other results indicate that the CpG distribution along the sequence may influence the interaction of each MBD protein with DNA²².

The MBD2b protein has been found to possess one of the highest affinities for methylated DNA among MBD proteins and has the greatest capacity to differentiate between methylated and unmethylated DNA²². The combination of MBD2b with its binding partner MBD3L1, methyl-CpG-binding protein 3-like-1, generates a higher affinity for methylated DNA than MBD2b protein alone^{2,23}. The specificity of the MBD2b/MBD3L1 complex is able to enrich for methylated DNA fragments containing as few as 5 methylated CpGs, and is more efficient at methylated DNA enrichment than antibody-based immunoprecipitation methods.

Traditional Methods to Study DNA Methylation

To date, there are several methods used for methylation analysis:

1. **Methylation-sensitive restriction enzyme analysis:** Isoschizomers of bacterial restriction endonucleases with different sensitivities for 5-methylcytosine can be used to determine the methylation status of specific CpG-dinucleotides²⁴. Methylation-sensitive restriction enzymes have several limitations including that methylation-sensitive restriction merely informs on the methylation status of the cytosine residues which are recognized by the restriction enzymes used.
2. **Bisulfite conversion:** Bisulfite conversion²⁵ consists of the treatment of double-stranded genomic DNA with sodium bisulfite, leading to deamination of unmethylated cytosines into uracil. PCR is then performed with primers that differentiate between methylated and unmethylated sequences. Bisulfite-based techniques can be cumbersome, involving time- and labor-intensive chemical treatments that damage DNA and limit throughput. Additionally, PCR primer design becomes difficult due to reduction in genome complexity after bisulfite treatment, leading to an inability to elucidate the methylation pattern at CpG dinucleotides in a genomic locus of interest.
3. **Methylated DNA Immunoprecipitation (MeDIP):** In this assay, an antibody specific for methylated cytosines (anti-5-methylcytosine antibody) is used to immunoprecipitate methylated DNA from genomic DNA fragmented by enzymatic digestion or sonication²⁶. The resulting enrichment is usually analyzed by PCR based methods; thus MeDIP can be combined with DNA microarrays for genome-wide analysis of CpG methylation. However, this technique is relatively time-consuming, requires a large amount of fragmented DNA starting material and only works with denatured DNA.

Kit Performance and Benefits

The MethylCollector Ultra Kit is for research use only. Not for use in diagnostic procedures.

Range of detection: MethylCollector Ultra can be performed on 1 ng - 1 µg of fragmented genomic DNA.

Sensitivity: Enriches methylated DNA fragments with as few as 5 methylated CpGs.

Nature of the MethylCollector Ultra Assay: MethylCollector Ultra is an improved technique for the enrichment of methylated CpG islands. The methylation status of specific promoters contained within CpG islands can be analyzed using either endpoint or real time PCR analysis of the locus of interest with customer designed PCR primers. Control human, male genomic DNA that was digested with *Mse* I is included in the kit along with PCR primers specific for both unmethylated and methylated promoters. The *Mse* I digested control human, male genomic DNA provided in the kit should have at least a 2-fold enrichment of methylated DNA bound and eluted from the protein complex as detected with the *Xist* PCR primer mix. There should be less than 5% of methylated DNA detected from the eluted fraction with the *APC* PCR primer mix.

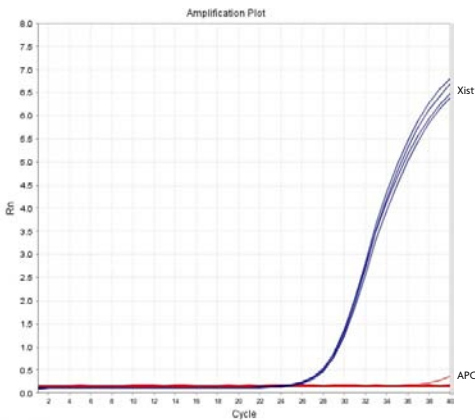


Figure 1: Real time PCR analysis of unmethylated (*APC*) and methylated (*Xist*) promoters with genomic DNA.

100 ng of *Mse* I digested human, male genomic DNA was tested in the MethylCollector Ultra Kit. Eluted DNA was cleaned and analyzed using real time PCR for both unmethylated and methylated promoters. *APC* is an unmethylated promoter in healthy tissues, therefore it is not captured by the His-MBD2b/MBD3L1 protein complex as verified by the lack of amplification with the *APC* primers. *Xist* is a methylated promoter in males and is thereby enriched in the MethylCollector Ultra Kit as verified by the amplification of the eluted, methylated DNA.

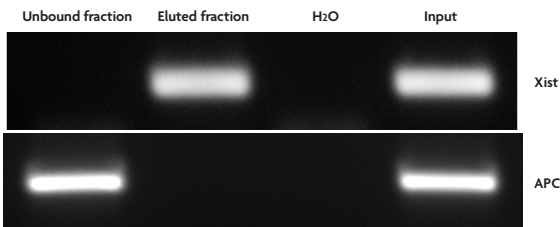


Figure 2: Specificity of the MBD2b/MBD3L1 protein complex for CpG methylated DNA.

MethylCollector Ultra was performed with 50 ng of *Mse* I digested human, male genomic DNA. Eluted DNA was cleaned and analyzed in endpoint PCR. The methylated *Xist* promoter is only detected in the eluted fraction while the unmethylated *APC* promoter is only detected in the unbound fraction, indicating the high specificity of MBD2b/MBD3L1.

Kit Components and Storage

Kit components arrive on dry ice. Upon receipt, we recommend storing each component at the temperatures listed in the table below. The magnetic beads may be frozen, however, we recommend long-term storage at 4°C. **Do not subject the magnetic beads to repeated freeze/thaws.**

Reagents	Quantity	Storage / Stability
His-MBD2b/MBD3L1 protein complex	350 µl	-20°C for 6 months
Binding Buffer AM7	35 ml	-20°C for 6 months
Elution Buffer AM1	3.5 ml	-20°C for 6 months
Protease Inhibitor Cocktail	100 µl	-20°C for 6 months
Proteinase K	70 µl	-20°C for 6 months
Proteinase K Stop Solution	70 µl	-20°C for 6 months
Human, male genomic DNA (<i>Mse</i> I digested) (20 ng/µl)	50 µl	-20°C for 6 months
APC PCR Primer Mix (2.5 pmol/µl)	50 µl	-20°C for 6 months
Xist PCR Primer Mix (2.5 pmol/µl)	50 µl	-20°C for 6 months
10X PCR Buffer	1.5 ml	-20°C for 6 months
10X PCR Loading Dye	1.5 ml	-20°C for 6 months
Magnetic Nickel Beads	350 µl	4°C for 6 months
Glycogen	35 µl	-20°C for 6 months
Bar Magnet	1	Room temperature
Mini Glue Dots	2 Dots	Room temperature
8-strip PCR tubes and caps	12 strips	Room temperature

Additional Materials Required

- Sterile DNase-free water
- Filter pipette tips
- Microcentrifuge tubes and microcentrifuge
- Magnetic stand. You can assemble a magnetic stand using the provided bar magnet and glue dots (see Appendix - Section A) or use commercially available stands
- Rolling shaker
- Phenol/chloroform
- 5 M Ammonium acetate (see Troubleshooting Guide, Appendix - Section B, for details regarding the use of 3 M sodium acetate, pH 5.2)

- 100% ethanol
- 70% ethanol
- dNTP mixture (5 mM each)
- *Taq* polymerase (5 U/ μ l) (Example: New England Biolabs M0267L or GeneSpin STS-T1000)
- PCR cycler

NOTES BEFORE STARTING

Fragmentation of Genomic DNA

The provided His-MBD2b/MBD3L1 protein complex has an enhanced affinity to bind methylated cytosines, particularly DNA fragments that contain five or more methylated cytosines. To enable clear interpretation of results, genomic DNA should be prepared such that DNA fragments containing a CpG region of interest do not contain methylated cytosines outside of this region (see “Appendix B. Troubleshooting” for further discussion). DNA can be fragmented by restriction digest or by mechanical means (*e.g.*, sonication).

Restriction digestion is especially useful for analysis of individual CpG islands. The genomic DNA is cut with a methylation-insensitive restriction enzyme (or enzymes) so that only CpGs of interest are contained within a particular restriction fragment. This fragment should be long enough (75 bp or longer) to allow for PCR analysis. Some useful methylation-insensitive restriction enzymes are shown in the below table. As might be expected, the enzymes whose recognition sites contain G and C bases cut more frequently in CpG islands than enzymes whose sites are composed only of A and T bases.

	Recognition Sequence	Number of fragments (per kb) in CpG islands	Number of fragments (per kb) in non-CpG islands
<i>Mse</i> I	TTAA	0.80	2.88
<i>Bfa</i> I	CTAG	1.56	1.55
<i>Tas</i> I	AATT	0.80	2.88
<i>Csp6</i> I	GTAC	2.23	1.41

Mechanical fragmentation is ideal when a single DNA sample will be used for simultaneous analysis of many CpG islands (*e.g.*, when the isolated DNA will be analyzed by microarray methods) or when a CpG region of interest is not flanked by suitable restriction sites. In general, the DNA should be sheared to an average fragment size of less than 500 bp to minimize the number of CpG islands on each fragment.

Example Fragmentation Protocols

Restriction digest

This protocol can be modified depending on the amount of isolated genomic DNA or the restriction enzyme being used. We recommend preparing high-quality genomic DNA using a commercially available kit or a standard established protocol. The quality of the genomic DNA can be assessed by agarose gel electrophoresis and DNA concentration can be determined by UV spectrophotometry.

- a) Set up the following restriction digest (with *Mse* I as an example, New England Biolabs (NEB)):

Genomic DNA (400 ng/ μ l)	10 μ l
10X NEB Buffer 2	10 μ l
100X BSA	1 μ l
<i>Mse</i> I (10 U/ μ l)	1 μ l
dH ₂ O	78 μ l
Total volume	100 μl

Note 1: The DNA volume may vary depending on its initial concentration.

Note 2: MethylCollector Ultra has been used with as little as 1 ng of restriction-digested genomic DNA. As a reference, a human cell contains about 6 picograms DNA; 1 ng of genomic DNA corresponds to 170 cells.

- b) Mix well by pipetting and incubate at 37°C for 2 hours to overnight.
- c) Heat-inactivate *Mse* I by incubating the reaction mixture at 65°C for 20 minutes. If using an alternative restriction enzyme that cannot be heat-inactivated, the DNA can be purified by phenol/chloroform extraction and precipitation, or through use of a DNA purification column. See “Appendix B. Troubleshooting” on page 18 for comments about heat-inactivation.

Note 1: For greater accuracy, the digested DNA should be quantified.

Note 2: This digested DNA should be stored at -20°C until use.

Mechanical fragmentation (sonication)

Because *Mse* I or other restriction enzymes cannot always be used to fragment and isolate the DNA sequences of interest, sonication of the genomic DNA is an alternative method.

- a) Pipette 20 μ g genomic DNA into a 1.5 ml microcentrifuge tube and adjust final volume to 300 μ l by addition of 10 mM Tris-HCl pH 8.5.
- b) Sonicate on ice using 15 pulses of 20 seconds (30% amplitude if using a Sonics Vibracell VC 130 sonicator), with a 20-second pause on ice between each pulse. The sheared DNA can be visualized by ethidium staining after electrophoresis on a 3% agarose gel. The majority of the DNA fragments should be between 100 and 350 bp in length.

Data Analysis and Use of Input DNAs

Methylated DNA isolated using MethylCollector Ultra is usually analyzed by PCR amplification of the loci of interest. However, if the goal is to compare the methylation status of particular loci in different DNA samples, it is essential that MethylCollector Ultra be performed on the same amount of each DNA sample. Thus, DNA samples should be carefully quantified before use. In addition, Input DNA should be prepared for each of the different DNA samples (see Step 1. No. 4 in the Protocol) to clearly indicate the relative concentrations of the DNA samples. If possible, real time PCR is recommended for analysis of DNA isolated with MethylCollector Ultra.

The *Mse I* digested control human, male genomic DNA provided in the kit should have at least a 2-fold enrichment of methylated DNA bound and eluted from the protein complex as detected with the *Xist* PCR primer mix. This means that of the total DNA recovered, there is twice as much methylated DNA recovered in the eluted fraction as compared to the unbound fraction for the same locus. There should be less than 5% of methylated DNA detected from the eluted fraction with the APC PCR primer mix.

PCR Primer Design

The isolated CpG-methylated DNA fragments obtained by MethylCollector Ultra must be amplified by PCR for subsequent visualization by agarose gel electrophoresis.

Primer design considerations:

- i. Restriction-digested DNA: PCR primer pairs should amplify a restriction fragment (or portion of a restriction fragment) that contains a CpG-rich region of interest. Each amplicon must also be free of internal sites for the restriction enzyme.
- ii. Sonicated DNA: PCR primers should flank the CpG-containing region of interest and the amplicon should not contain any CpG-dinucleotides that are outside of this region. This will minimize amplification of fragments that are isolated as a result of methylated CpGs that are near, but not within, the CpG-rich region of interest.
- iii. PCR primers should be designed with the aid of a reliable primer design computer program (e.g., http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Due to the technical limitations of PCR, it is sometimes necessary to design more than one primer pair for a given fragment of interest.

The included control PCR primers are suitable for use in both endpoint and real time PCR analysis:

APC Adenomatosis polyposis coli is an unmethylated promoter in healthy tissues. Methylation of this promoter is associated with several types of cancers. The region amplified by this primer pair is 338 base pairs and contains 29 CpGs.

Xist X inactive specific transcript is a methylated promoter in human, male genomic DNA, but is non-methylated in females. The region amplified by this primer pair is 178 base pairs and contains 8 CpGs.

MethylCollector Ultra Protocol

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING!

Step 1: Binding reaction

In this section, the fragmented DNA is mixed with the recombinant His-MBD2b/MBD3L1 protein complex. The resulting protein-DNA complexes are captured by the magnetic beads.

1. Thaw components from storage as needed for preparation. Keep all components on ice when not in use.
2. Prepare the appropriate amount of Complete Binding Buffer according to the table below. Store on ice.

Reagent	One rxn	8 rxns
Binding Buffer AM7	100 μ l	800 μ l
Protease Inhibitor Cocktail	0.5 μ l	4 μ l
Total Volume	100.5 μl	804 μl

Note: The provided Binding Buffer is optimal for efficient capture of DNA fragments that contain five or more methylated CpGs.

3. Dilute the fragmented DNA in water if necessary. The MethylCollector Ultra protocol can be performed on a large range of sample DNA amounts (1 ng to 1 μ g). We recommend 100 ng because it gives robust results without requiring a large amount of DNA.
4. In this step, the Input DNAs that will be used in the final PCR step are prepared.
 - a. **If performing real time PCR:** For the control genomic DNA provided in the kit, it is recommended that several Input DNA concentrations be run in triplicate. Input DNA should be tested at 0.01, 0.1, 1 and 10 ng/ μ l.
 - b. **If performing endpoint PCR:** For the control genomic DNA provided in the kit, PCR analysis is performed for 36 cycles on 25 ng of control DNA. The control DNA (provided at 20 ng/ μ l) should be diluted to 5 ng/ μ l for use in Input PCR. This can be done by diluting the DNA 1/4 in dH₂O (e.g., 5 μ l 20 ng/ μ l DNA + 15 μ l dH₂O to make 5 ng/ μ l DNA). 5 μ l of the 5 ng/ μ l DNA is used for Input PCR (see page 11).

Note: Customer sample Input DNA can be treated similarly. If your locus-specific PCR primers are efficient and PCR will be performed for 36 cycles, 25 ng of sample DNA can be used for the Input PCRs. However, PCR primer efficiency varies and you may want to try several amounts of Input DNA to be sure to obtain PCR products from reactions still in the linear phase of amplification.

5. Using the PCR tubes provided, fully resuspend magnetic beads by inverting and aliquot a 10 μ l slurry into each tube. If preparing more than 4 reactions, cap and re-invert the beads after every 4 aliquots. (**Note:** When working with magnetic beads, pipette gently.)

6. Add the remaining components in the order shown below to each PCR tube. Prepare a positive control reaction using the provided *Mse* I digested human, male genomic DNA.

Reagent	One rxn	Positive Control
Magnetic beads	10 μ l	10 μ l
Complete Binding Buffer	70 μ l	70 μ l
Fragmented genomic DNA (in a final volume of 10 μ l)	from 1 ng-1 μ g	-
Control Human, male genomic DNA (<i>Mse</i> I digested)	-	10 μ l
His-MBD2/MBD3L1 protein complex	10 μ l	10 μ l
Total Volume	100 μl	100 μl

Note: It is recommended to aliquot the provided His-MBD2b/MBD3L1 protein complex into several small fractions to avoid multiple freeze/thaw cycles. Store at -20°C .

8. Cap tubes and shake to mix thoroughly. Incubate on a rolling shaker for 1 hour at 4°C .

Step 2: Wash beads

- After the capture step is complete, spin the PCR tubes briefly and place tubes on a magnetic stand to pellet beads on the tube side. If further analysis of the unbound fraction will be performed, such as a comparison of the level of enrichment of methylated DNA, place supernatant in a microcentrifuge tube and set aside at 4°C for DNA clean up in Step 4 of the protocol. Otherwise, remove and discard the supernatant. To use the magnet provided in the kit, please see page 16 in the Appendix.
- Wash beads four times with 200 μ l Binding Buffer. Pipette 2-3 times gently to resuspend.
 - Place tubes on magnetic stand and allow beads to pellet on the side of the tube.
 - Carefully remove the supernatant and any residual bubbles.
 - Add 200 μ l Binding Buffer and resuspend the pellet completely by pipetting several times. Ensure that the beads do not stick to the pipette tips. Depending on the strength of the magnet being used, it may be necessary to remove the tubes to a separate rack before resuspending the beads.
 - Repeat steps a-c.
- After the final wash, place tubes on magnetic stand and allow beads to settle to the side. Remove and discard supernatant without disturbing the beads.

Step 3: Recovery of methylated DNA fragments

1. In a microcentrifuge tube, prepare Complete Elution Buffer by adding 2 μ l of Proteinase K to 98 μ l of Elution Buffer AM1 for each reaction.
2. Resuspend washed beads with 100 μ l Complete Elution Buffer by pipetting 2-3 times.
3. Incubate samples at 42°C for 30 minutes and quick spin to ensure all beads are collected.
4. During this incubation warm the Proteinase K Stop Solution at 37°C for 10 minutes.
5. Return tubes to room temperature and add 2 μ l of Proteinase K Stop Solution and pipet 2-3 times to mix.
6. Place tubes in magnetic stand and allow beads to pellet onto tube sides.
7. Carefully transfer the supernatant to a new microcentrifuge tube.
8. Proceed to Step 4, DNA clean up, or else DNA can be stored at -20°C. If the DNA is stored at -20°C, it is recommended to be reheated at 37°C for 10 minutes prior to use.

Step 4: DNA clean up

Prior to PCR amplification it is necessary to clean up the DNA. Use the following protocol to perform a phenol/chloroform extraction followed by ethanol precipitation. Alternatively, DNA can be purified using columns such as QIAquick PCR purification kit (Qiagen part no. 28104), or UltraClean PCR Clean-Up kit (Mo Bio Labs part no. 12500-50). Elute in 50 μ l volume (use other appropriate volume of water or buffer as needed for specific downstream applications).

1. Add an equal volume of Phenol:Chloroform:Isoamyl Alcohol (25:24:1, v/v/v) to the eluted sample and, if desired, the unbound fraction from Step 2 No. 1.
2. Vortex the tube at maximum speed for 15 seconds.
3. Centrifuge the tube for 5 minutes at 12,000 $\times g$ at room temperature.
4. Carefully transfer the top aqueous phase to clean microcentrifuge tube without collecting any of the lower organic phase or precipitate that may occur between the phases.
5. To each sample add:
 - 1 μ l Glycogen (20 ng/ μ l) (included in the kit)
 - 1 sample volume of 5 M ammonium acetate
 - 2.5 sample volumes of 100% ethanol
6. Mix well and incubate at -80°C for at least 2 hours.
7. Centrifuge the tube for 20 minutes at 12,000 $\times g$, 4°C.
8. Carefully discard the supernatant without disturbing the pellet.
9. Add 500 μ l of cold 70% ethanol.
10. Centrifuge the tube for 10 minutes at 12,000 $\times g$, 4°C.
11. Carefully discard the supernatant without disturbing the pellet.
12. Air-dry the pellet for 5 minutes (do not completely dry the pellet).

13. Resuspend the DNA pellet in 50 µl sterile DNase-free water. Use other appropriate volumes of buffer or water as needed for specific downstream applications.
14. This eluted DNA can be used immediately in PCR or stored at -20°C. If the DNA is stored at -20°C, it will need to be reheated at 37°C for 10 minutes prior to use in PCR reactions.

Real Time PCR Analysis

This is an example PCR reaction. Please follow the specific instructions for your real time PCR instrument.

1. For one PCR Reaction:

Reagent	10 µl PCR reactions	20 µl PCR reactions
Fast SYBR Green master mix	5 µl	10 µl
Forward primer* (5 pmol/µl)	0.5 µl	1 µl
Reverse primer* (5 pmol/µl)	0.5 µl	1 µl
Sterile water	1 µl	3 µl
DNA sample (eluted or Input)	3 µl	5 µl
Total volume	10 µl	20 µl

* The provided PCR Primer Mixes contain Forward and Reverse primers for use with the provided control DNA. Use 1 µl of the PCR Primer Mix in the 10 µl reaction or 2 µl of the PCR primer mix in the 20 µl reaction.

Note: It is recommended to prepare triplicates of each sample and Input reaction. Input DNA should be tested at 0.01, 0.1, 1 and 10 ng/µl to obtain a standard curve.

2. Place tubes in a Real Time PCR instrument and program as below. The amplification conditions should be optimized for each target locus and PCR instrument. A suggested starting point is:
 - 95 °C for 2 minutes
 - (95 °C for 10 seconds, 60 °C for 30 seconds) for 40 cycles
3. Analyze the results. Data analysis varies depending on the instrument used. Obtain the standard curve from the Input samples. Use the standard curve to quantify the DNA in each sample.

APC Adenomatosis polyposis coli is an unmethylated promoter in healthy tissues. The control human, male genomic DNA should be unmethylated at this locus and will not amplify in the eluted fraction.

Xist X inactive specific transcript is a methylated promoter in human male genomic DNA, but is non-methylated in females. The control human, male genomic DNA should be methylated at this locus and will amplify early in the eluted fraction.

Endpoint PCR Analysis

A typical endpoint PCR protocol example follows below. This protocol was optimized for the control samples. For each new set of primers amplifying the promoter region of interest, the PCR conditions have to be optimized carefully (optimal T_m , number of cycles, etc.).

1. For one PCR reaction:

Reagent	One rxn
Sterile water	9.8 μ l
10X PCR Buffer	2.5 μ l
10X PCR compatible loading dye	2.5 μ l
dNTP mixture (5 mM each dNTP)	1 μ l
Forward Primer* (5 pmol/ μ l)	2 μ l
Reverse Primer* (5 pmol/ μ l)	2 μ l
<i>Taq</i> (5 U/ μ l)	0.2 μ l
DNA sample (eluted or Input)	5 μ l
Total Volume	25 μl

* The provided PCR Primer Mixes contain Forward and Reverse primers for use with the provided control DNA. Use 4 μ l of this mix in the typical PCR protocol described above.

2. Place tubes in a PCR thermocycler and program as below:

94°C for 3 minutes

(94°C for 20 seconds, 60°C for 30 seconds, 72°C for 30 seconds) for 36 cycles

Hold at 4°C

3. Endpoint PCR can be analyzed by agarose gel electrophoresis. Run reactions by loading 10 μ l from each of the PCRs on a thin 3% agarose gel at 125V for 50 minutes in parallel with an appropriate DNA ladder. Post-stain the gel with 1 μ g/ml ethidium bromide in 1X TAE buffer for 20 minutes. Observe gel under UV.

APC Adenomatosis polyposis coli is an unmethylated promoter in healthy tissues. The control human, male genomic DNA should be unmethylated at this locus and is not expected to produce a 338 base pair PCR product in the eluted DNA fraction.

Xist X inactive specific transcript is a methylated promoter in human male genomic DNA, but is non-methylated in females. The control human, male genomic DNA should be methylated at this locus and is expected to produce a PCR product for the 178 base pair region amplified by included PCR primer mix in the eluted fraction.

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Notes: The polymerase chain reaction (PCR) process for amplifying nucleic acid is covered by U.S. Patent Nos. 4,683,195 and 4,683,202 assigned to Hoffmann-La Roche. Patents pending in other countries.

Use of methylation-specific PCR (MSP) is protected by U.S. Patent Nos. 5,786,146, 6,017,704, 6,200,756 & 6,265,171 and International patent WO97/46705. No license under these patents to use the MSP process is conveyed to the purchaser by purchasing this product.

MethylCollector™ Ultra is covered under U.S. Patent No. 7,425,415.

Appendix

Section A. Use of Magnetic Beads and Included Bar Magnet

Caution: The included neodymium bar magnet is extremely powerful and is easily broken if handled incorrectly.

1. The magnet should be stored in the provided tube.
2. Be careful when working near metal objects or surfaces. A free magnet will jump great distances onto nearby metal surfaces with surprising speed. This can break the magnet.
3. If the magnet becomes attached to a flat metal surface, it should be removed by sliding it off the edge of surface. The magnet may be broken if you attempt to pull one end away from the metal.

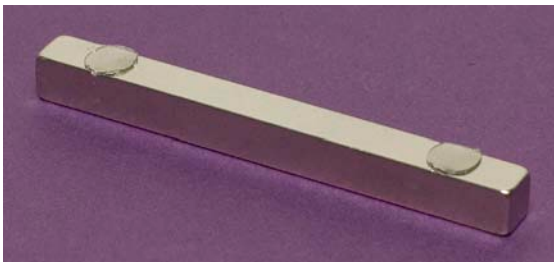
Assembly of Magnetic Stands

The provided Mini Glue Dots can be used to attach the bar magnet to an empty tip box to create an effective magnet stand.

Creating a magnetic stand for 8-well PCR strips:

Note: 8-well strip tubes for use with standard 96-well PCR cyclers are appropriate.

1. Remove the covering tape from one side of two glue dots.
2. Place a strip of PCR tubes in the wells of an empty tip box (200 μ l tips) and place the magnet directly against the tubes. This is the way the magnet will be positioned when the glue dots are used to affix it to the box.
3. Attach the glue dots on the bar magnet (the uncovered face of the dot is placed on the magnet) as shown below.



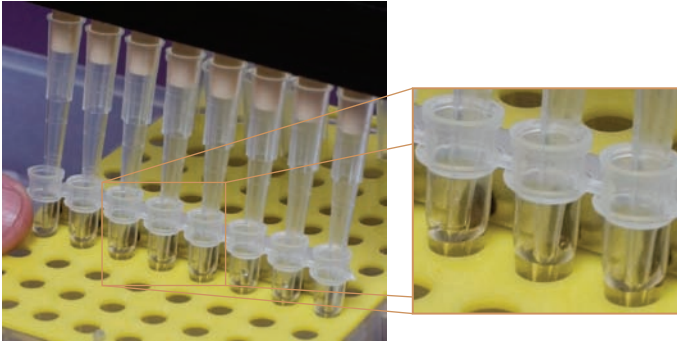
4. Remove the covering tape from the exposed side of the glue dots. Fix the magnet to the tip box so that it is against the PCR tubes. The magnetic stand is now ready for use.

Note: Familiarize yourself with using the magnetic stand before performing with PCR tubes for the first time. Add 5 μ l of magnetic beads to 100 μ l Binding Buffer AM7 in one tube of an 8-well strip of PCR tubes. Use this tube with the assembled bar

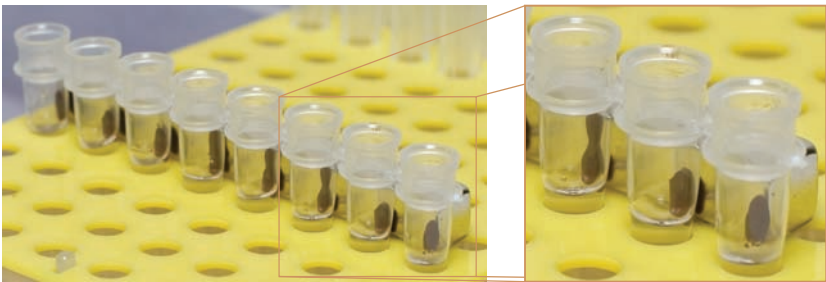
magnet stand to become familiar with use of the beads and magnet. It is difficult to re-suspend the beads if the tubes are directly adjacent to the magnet, so it is usually best to move the tubes away from the magnet for resuspension.

Washing should be performed as follows:

- a. Place the tubes in the rack against the magnet and allow the beads to be pinned to the side of the tube, as shown below.



- b. Remove supernatant with a 200 μ l pipette or a 200 μ l eight-channel pipette.



- c. Move the tube strip into a row that is not adjacent to the magnet.
- d. Add wash buffer and pipette up and down to fully resuspend the beads. Ensure that a minimal amount of beads cling to the tips when the resuspension is complete.
- e. Repeat steps a-d until desired washing steps are complete.

Centrifugation of 8-well PCR strip tubes:

When working with 8-well PCR strip tubes, it may be desirable to centrifuge the tubes to collect the liquid and beads from the inside of the caps. This is easily accomplished in a centrifuge fitted with adaptors for spinning microtiter plates. In this case, a standard 96-well plate can be placed in the adaptor to hold the tubes in place. Take care to ensure the rotor is balanced (*e.g.*, place a microtiter plate and tubes of appropriate mass in the rotor's opposing 96-well plate adaptor). Spin the plates briefly to let the rotor reach a speed of 1000 \times g before allowing the rotor to stop.

Section B. Troubleshooting Guide

Problem/question	Recommendation
The target DNA fragment has less than 5 methylated CpGs.	The provided Binding Buffer is optimal for efficient capture of DNA fragments that have five or more methylated CpGs. MethylCollector™ Ultra is not recommended for isolation of DNA fragments containing less than 5 methylated CpGs.
PCR amplification	It has been determined that using a hot-start polymerase (<i>i.e.</i> Phusion™ from NEB) instead of a classic <i>Taq</i> polymerase may also increase the sensitivity of the assay.
Storage of DNA	Once DNA is prepared using MethylCollector Ultra, samples may be stored at -20°C prior to PCR analysis. However, we recommend heating the frozen material to 37°C for 10 minutes before use in PCR, as heat-treatment releases any DNA bound to the tube during storage.
Can I use 3 M sodium acetate, pH 5.2 instead of 5 M ammonium acetate in the ethanol precipitation?	Yes, 3 M sodium acetate, pH 5.2 can be used at 1/10th sample volume along with 2 sample volumes of 100% ethanol during the precipitation step. However, we have noticed that the ammonium acetate had better yield of recovery than the sodium acetate in a direct comparison of several samples.
Should I use Restriction Digest or Sonication to fragment my DNA?	Restriction Digest is very precise and reproducible, however, the DNA must be well purified and analysis of several loci would also require use of different enzymes. In addition, the region of interest may not be flanked by suitable restriction sites and SNPs between different cell types may confound results. In contrast, Sonication is random, which enables analysis of many loci simultaneously (microarray), but it may not be possible to shear DNA small enough to isolate CpG islands of interest. In addition, results may vary from shearing to shearing depending on sonicator used. Also it is difficult to prepare DNA from a small number of cells.
Heat inactivation or removal of restriction enzyme used to fragment DNA	After restriction digest, we recommend that samples be treated for 20 minutes at 65°C. Some enzymes (such as <i>Mse I</i>) will be inactivated by this treatment, while those that are not will be forced off the DNA. In most cases (even when using enzymes that are not heat-inactivated), DNA treated in this fashion should be suitable for use in the MethylCollector Ultra protocol. In some situations (<i>e.g.</i> , when the DNA used in a digest is contaminated with cellular proteins or when a large amount of restriction enzyme is required for the digest) it may be desirable to purify the digested DNA by purification columns or through phenol extraction/ethanol precipitation.
10X PCR Loading Dye	If PCR is performed using the 10X PCR Loading Dye provided, it is not necessary to add additional loading dye to the samples before running samples on agarose gel.

Section C. Related Products

DNA Methylation	Format	Catalog No.
MethylDetector™	50 rxns	55001
MethylCollector™	25 rxns	55002
Fully Methylated Jurkat DNA	10 µg	55003
UnMethylCollector™	30 rxns	55004

Antibodies	Application	Format	Catalog No.
DNMT1 mouse mAb	ChIP, IHC, IP, WB	100 µg	39204
DNMT2 rabbit pAb	WB	100 µg	39205
DNMT3A mouse mAb	ChIP, IF, IHC, WB	100 µg	39206
DNMT3B mouse mAb	ChIP, IF, IP, WB	100 µg	39207
MBD1 mouse mAb	WB	100 µg	39215
MBD2 mouse mAb	WB	100 µg	40965

MBD3 mouse mAb	WB	100 µg	39216
MBD4 mouse mAb	WB	100 µg	39217
MeCP2 rabbit pAb	WB	100 µg	39218

Recombinant Methylated Histones	Format	Catalog No.
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Recombinant Histone H3 (C110A)	50 µg	31207
Recombinant Histone H3 monomethyl Lys4	50 µg	31208
Recombinant Histone H3 dimethyl Lys4	50 µg	31209
Recombinant Histone H3 trimethyl Lys4	50 µg	31210
Recombinant Histone H3 monomethyl Lys9	50 µg	31211
Recombinant Histone H3 dimethyl Lys9	50 µg	31212
Recombinant Histone H3 trimethyl Lys9	50 µg	31213
Recombinant Histone H3 monomethyl Lys27	50 µg	31214
Recombinant Histone H3 dimethyl Lys27	50 µg	31215
Recombinant Histone H3 trimethyl Lys27	50 µg	31216
Recombinant Histone H4	50 µg	31223
Recombinant Histone H4 monomethyl Lys20	50 µg	31224
Recombinant Histone H4 dimethyl Lys20	50 µg	31225
Recombinant Histone H4 trimethyl Lys20	50 µg	31226

Histone Purification	Format	Catalog No.
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Histone Purification Kit	10 rxns	40025
Histone Purification Mini Kit	20 rxns	40026

Chromatin Assembly	Format	Catalog No.
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Chromatin Assembly Kit	10 rxns	53500
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Histone Acetyltransferase and Deacetylase Activity	Format	Catalog No.
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HAT Assay Kit (Fluorescent)	1 x 96 rxns	56100
Recombinant p300 protein, catalytic domain	5 µg	31205
HDAC Assay Kit (Fluorescent)	1 x 96 rxns	56200
HDAC Assay Kit (Colorimetric)	1 x 96 rxns	56210

Co-Immunoprecipitation	Format	Catalog No.
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Nuclear Complex Co-IP Kit	50 rxns	54001
Universal Magnetic Co-IP Kit	25 rxns	54002

SUMOylation	Format	Catalog No.
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SUMOlink™ SUMO-1 Kit	20 rxns	40120
SUMOlink™ SUMO-2/3 Kit	20 rxns	40220

Technical Services

If you need assistance at any time, please call Active Motif Technical Service at one of the numbers listed below.

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