UnMethylCollector™

(version A)

Catalog No. 55004

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Overview

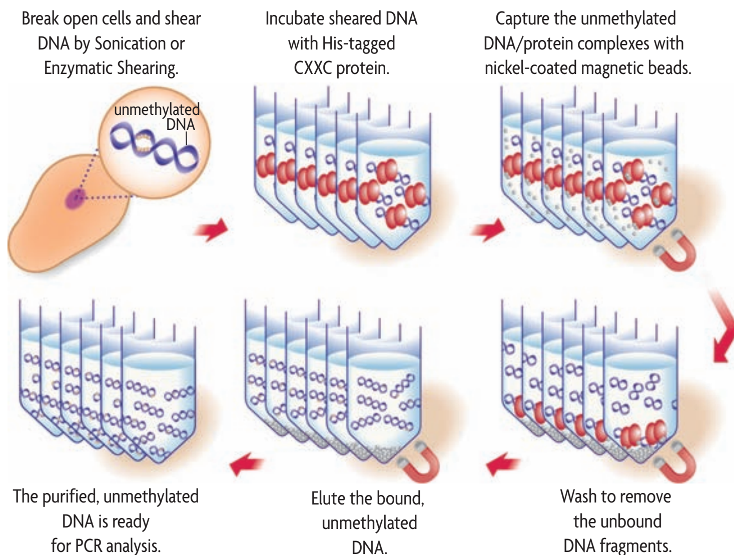
Active Motif's UnMethylCollector™ Kit provides an efficient method for isolating unmethylated CpG dinucleotides from limited amounts of cell or tissue DNA. Most CpG island enrichment methods require clusters of methylated CpGs. Since CpG islands are normally unmethylated[1, 2], isolation and evaluation of many human promoters contained within CpG islands has been difficult. Active Motif's UnMethylCollector Kit resolves this issue by utilizing a His-tagged recombinant CXXC protein domain from mouse Mbd1 in order to specifically bind unmethylated CpGs. This novel technology allows for positive identification of hypomethylated promoters or can be used to confirm the negative results obtained by methyl-CpG capture methods, such as Active Motif's MethylCollector™ product line (Cat. Nos. 55002 & 55005). This simple and sensitive technique is suitable for use in many downstream applications, such as real time or endpoint PCR analysis of the methylation status of particular promoters in normal and diseased samples, rapid screening of the methylation status of multiple loci, sequencing, or amplification and labeling for microarray analysis. It can also be used to detect changes in DNA methylation in other situations, including normal cellular differentiation and aging.

In the UnMethylCollector method*, His-tagged recombinant CXXC protein specifically binds unmethylated DNA fragments that have been prepared by enzymatic digestion or sonication. The His-CXXC is added to the DNA fragments, and these protein-DNA complexes are captured with nickel-coated magnetic beads. Subsequent wash steps are performed using an optimized buffer to separate the unmethylated fragments from the rest of the genomic DNA. The unmethylated DNA is then eluted from the beads in the presence of high salt. Due to the high efficiency of UnMethylCollector 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 1600 cells (~10 ng DNA).

product	format	catalog no.
UnMethylCollector™	30 rxns**	55004

*Patent Pending

**UnMethylCollector provides sufficient reagents to perform 30 reactions with excess reagents for 5 control reactions.



Flow chart of the UnMethylCollector process.

In UnMethylCollector, genomic DNA of interest is sheared by either enzymatic digestion or sonication. The sheared DNA is then incubated with His-tagged recombinant CXXC protein, which has a high affinity for unmethylated DNA. These protein-DNA complexes are captured with nickel-coated magnetic beads and washes are then performed to remove the rest of the genomic DNA. The unmethylated DNA is then eluted from the beads and 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[3-6]. Cellular 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 unmethylated[7]. Aberrant hypermethylation of CpG islands and subsequent transcriptional repression is one of the earliest and most common somatic genome alterations in multiple human cancers[8, 9]. 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 identified[10]. Aberrant methylation of CpG islands thus seems to be a tumor type-specific event[9, 11] and current efforts have concentrated on finding ways to exploit the diagnostic and therapeutic implications of these abnormalities[12, 13].

Methyl-CpG binding proteins appear to be central players in the process of DNA methylation-dependent gene silencing[14]. 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 MeCP2[15]. After the recognition of the MBD, four additional genes were found to contain this domain, namely MBD1, MBD2, MBD3 and MBD4[16]. In general, all MBD proteins, except MBD4, have been reported to be associated with histone deacetylase subunits as part of large multi-subunit complexes[17, 18]. A few studies support the notion of selectivity in the association of a particular MBD with particular promoters[19, 20], but other results indicate that the CpG distribution along the sequence may influence the interaction of each MBD protein with DNA[21].

MBD1 is unique in that it contains three zinc-coordinating CXXC domains[22-24] Other chromatin-associated proteins, such as DNA methyltransferase 1 (DNMT1), mixed lineage leukemia (MLL) and CpG binding protein (CGBP) also contain CXXC domains. Both MLL and CGBP have been shown to bind nonmethylated CpG sites *in vitro*[22, 25, 26]. The specificity of the CXXC domain is able to enrich for DNA fragments containing only a single unmethylated CpG.

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-dinucleotides[27]. 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 conversion[28] 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 sonication[29]. 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 UnMethylCollector Kit is for research use only. Not for use in diagnostic procedures.

Sensitivity: UnMethylCollector can be performed on 10 ng - 1 µg of genomic DNA.

Nature of the UnMethylCollector Assay: The UnMethylCollector is designed as a simple, efficient technique to enrich for unmethylated 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.

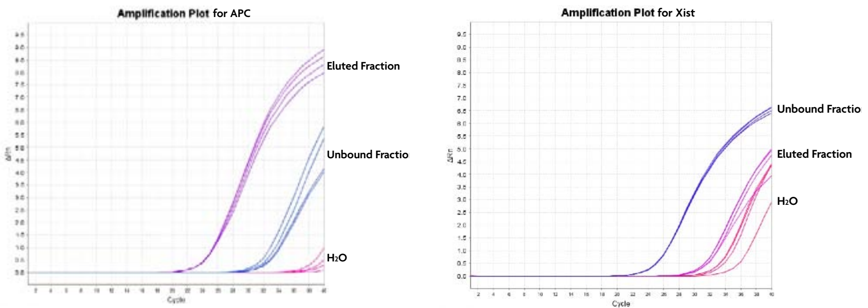


Figure 1: Real time PCR analysis of the control male genomic DNA with methylated and unmethylated promoters. 200 ng of human, male genomic DNA was digested with *Mse* I and tested in the UnMethylCollector Kit. Eluted DNA was analyzed using PCR primers for both methylated and unmethylated promoters. (Left) APC, adenomatosis polyposis coli, is an unmethylated promoter in healthy tissues. The early amplification of the DNA in the eluted fraction verifies APC as an unmethylated promoter. (Right) Xist is a methylated promoter in males and the methylated DNA fragments of this promoter will not bind to the His-CXXC protein. This is verified with the early amplification of the DNA in the unbound fraction. The APC promoter was verified as unmethylated using bisulfite conversion and sequencing.

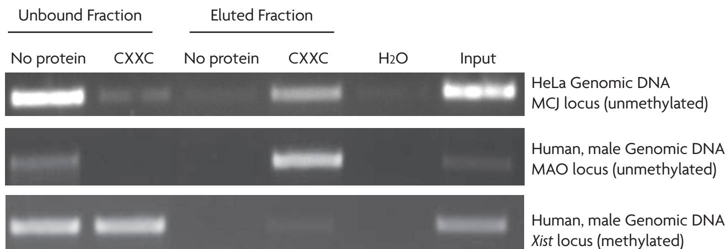


Figure 2: Specific isolation of unmethylated DNA using UnMethylCollector Kit. 100 ng of *Mse* I digested genomic DNA was tested in the presence and absence of His-tagged CXXC protein in the UnMethylCollector Kit. Both unbound and eluted fractions were collected and analyzed in PCR for 36 cycles. Both the MCJ locus, which is unmethylated in HeLa, and the MAO locus, which is unmethylated in males, were efficiently captured and eluted. The Xist locus, which is methylated in males, did not bind to the His-tagged CXXC protein and therefore can only be detected in the unbound fraction.

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-CXXC protein (0.65 µg/µl)	350 µl	-20°C for 6 months
Binding Buffer AM8	50 ml	RT for 6 months
Binding Buffer AM9	5 ml	RT for 6 months
Elution Buffer AM3	5 ml	RT for 6 months
Protease Inhibitor Cocktail	100 µl	-20°C for 6 months
DTT (1 M)	100 µl	-20°C for 6 months
Human genomic DNA, *Mse* I digested	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 DNAase-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
- *Taq* polymerase (5 U/µl) (Example: New England Biolabs M0267L or GeneSpin STS-T1000)
- dNTP mixture (5 mM each)
- PCR cycler

NOTES BEFORE STARTING

Fragmentation of Genomic DNA

The provided His-CXXC protein binds specifically to unmethylated cytosines and the UnMethyl-Collector method enriches for DNA fragments that contain one or more unmethylated CpG dinucleotides. To enable clear interpretation of results, genomic DNA should be prepared such that DNA fragments contain unmethylated CpGs in the region of interest (see Troubleshooting Guide, Appendix B 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
***Mse* I**	TTAA	0.80	**2.88**
***Bfa* I**	CTAG	1.56	1.55
***Tas* I**	AATT	0.80	**2.88**
***Csp6* 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/µl)	10 µl
10X NEB Buffer 2	10 µl
100X BSA	1 µl
Mse I (10 U/µl)	1 µl
dH_2O	78 µl
Total volume	**100 µl**

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

Note 2: UnMethylCollector has been used with as little as 10 ng of restriction-digested genomic DNA. As a reference, a human cell contains about 6 picograms DNA; 10 ng of genomic DNA corresponds to 1,600 cells.

Note 3: For capturing large amounts of DNA (200 ng - 1 µg), it is recommended to use a more concentrated DNA stock such that the final volume of DNA per UnMethyl-Collector reaction is approximately 10 µl. If necessary, the DNA can be precipitated and concentrated following the digestion reaction.

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 Troubleshooting Guide, Appendix B 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 µg genomic DNA into a 1.5 ml microcentrifuge tube and adjust final volume to 300 µl 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

Unmethylated DNA isolated using UnMethylCollector 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 UnMethylCollector 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 5. No. 1 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 UnMethylCollector.

The *Mse* I digested control human, male genomic DNA provided in the kit should have a 10-fold enrichment of unmethylated DNA bound and eluted from the protein complex as detected with the APC PCR primer mix. There should be less than 5% of methylated DNA detected from the eluted fraction with the Xist PCR primer mix.

PCR Primer Design

The isolated DNA from the bound and unbound fractions obtained by UnMethylCollector 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 unmethylated 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.

UnMethylCollector Protocol

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING!

Step 1: Binding reaction

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

1. Thaw components from storage as needed for preparation. Keep frozen 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 AM8	99.25 µl	794 µl
1 M DTT	0.25 µl	2 µl
Protease Inhibitor Cocktail	0.5 µl	4 µl
Total Volume	**100 µl**	**800 µl**

Note: The provided Binding Buffer AM8 is optimal for efficient capture of DNA fragments that contain more than six unmethylated CpGs. For DNA fragments with very few CpGs (between 1 and 6 unmethylated CpGs), Binding Buffer AM9 is recommended, please see Troubleshooting on page 19 for more information.

3. Dilute the fragmented DNA in water if necessary. The UnMethylCollector protocol can be performed on a large range of input DNA amounts (10 ng to 1 µg). If using 200 ng - 1 µg of starting material, please see recommendations in the Troubleshooting section on page 19. We recommend 200 ng for the control *Mse* I digested human, male genomic DNA.

   Note 1: Input DNA for PCR analysis should be prepared just prior to starting the PCR. For preparation of Input DNA see Step 5 No. 1.

   Note 2: If the downstream application is high-throughput sequencing, please read the notes in Troubleshooting on page 19 before beginning the binding reaction.

4. Using the PCR tubes provided, fully resuspend magnetic beads by inverting and aliquot a 10 µl 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.)
5. In parallel prepare sample reactions and a positive control reaction. A negative control, no protein, reaction is also recommended. Add the remaining components in the order shown below to each PCR tube.

Reagent	Sample (One rxn)	Positive Control	Negative Control
Magnetic beads	10 µl	10 µl	10 µl
Complete Binding Buffer	70 µl	70 µl	80 µl
His-CXXC (0.65 µg/µl)	10 µl	10 µl	-
Total Volume	**90 µl**	**90 µl**	**90 µl**

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

6. Pipet up and down to mix the above components. Incubate at room temperature for 5 minutes.
7. Add fragmented genomic DNA. For samples and negative control, use 10 ng - 1 µg sample DNA in a final volume of 10 µl. For the positive control, use 10 µl of the provided *Mse* I digested human, male genomic DNA.

Reagent	Sample (One rxn)	Positive Control	Negative Control
Human genomic DNA control (20 ng/µl)	-	10 µl	-
Fragmented Genomic DNA (in a final volume of 10 µl)	from 10 ng - 1 µg	-	from 10 ng - 1 µg

8. Cap tubes and shake to mix thoroughly. Incubate on a rolling shaker for 30 minutes at room temperature.

Step 2: Wash beads

1. 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. Carefully remove and SAVE the supernatant (unbound fraction) in a DNase-free 1.5 ml microcentrifuge tube. Set the unbound fraction aside at 4°C for DNA clean up in Step 4. To use the magnet provided in the kit, please see page 17 in the Appendix.
2. Wash beads four times with 200 µl Binding Buffer AM8 (use Binding Buffer AM8 for wash steps regardless of binding buffer used to capture unmethylated CpGs). Pipette 4-5 times gently to resuspend.
   a. Place tubes on magnetic stand and allow beads to pellet on the side of the tube.
   b. Carefully remove the supernatant and any residual bubbles.
   c. Add 200 µl Binding Buffer AM8 and resuspend the pellet completely by pipetting several times. Ensure that the beads do not stick to the pipette tips. In most cases, the beads can be completely resuspended while the tubes are in the magnetic stand. However, depending on the strength of the magnet being used, it may be necessary to move the

tubes to a separate rack before resuspending.

d. Repeat steps a-c.

3. 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 unmethylated DNA fragments

1. Resuspend washed beads with 100 µl Elution Buffer AM3 by pipetting 5-6 times.
2. Place tubes in magnetic stand and allow beads to pellet onto tube sides.
3. Carefully transfer the supernatant to a fresh DNase-free 1.5 ml microcentrifuge tube.
4. 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 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 on both the eluted unmethylated fraction and the unbound fraction from Step 2 No. 1. 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 µl 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.
2. Vortex the tube at maximum speed for 15 seconds.
3. Centrifuge the tube for 5 minutes at 12,000 x *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 µl Glycogen (20 ng/µl) (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 x *g*, 4°C.
8. Carefully discard the supernatant without disturbing the pellet.
9. Add 500 µl of cold 70% ethanol.
10. Centrifuge the tube for 10 minutes at 12,000 x *g*, 4°C.
11. Carefully discard the supernatant without disturbing the pellet.
12. Air-dry the pellet for 5-10 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.

Step 5: PCR amplification of unmethylated DNA fragments

In this step, the cleaned DNAs from the unbound and eluted fractions of the sample and negative control reaction, as well as the Input DNA sample (prepared below) will be amplified by PCR using customer-designed primers. In parallel, the cleaned DNAs from the unbound and eluted fractions of the positive control DNA fragment, as well as the control Input DNA sample will be amplified by PCR using the provided PCR control primer mixes. The provided PCR primer mixes are suitable for use in either endpoint or real time PCR analysis.

1. In this step, the Input DNAs that will be used in the PCR analysis 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/µl.
   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/µl) should be diluted to 5 ng/µl for use in Input PCR. This can be done by diluting the DNA 1/4 in dH_2O (*e.g.*, 5 µl 20 ng/µl DNA + 15 µl dH_2O to make 5 ng/µl DNA). 5 µl of the 5 ng/µl DNA is used for Input PCR.

   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.

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 reaction	20 µl PCR reaction
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, unbound 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 20 seconds
   (95°C for 3 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 amplify early 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 unbound 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 µl
10X PCR Buffer	2.5 µl
10X PCR compatible loading dye	2.5 µl
dNTP mixture (5 mM each dNTP)	1 µl
Forward Primer* (5 pmol/µl)	2 µl
Reverse Primer* (5 pmol/µl)	2 µl
Taq (5 U/µl)	0.2 µl
DNA sample (eluted, unbound or Input)	5 µl
Total Volume	**25 µl**

* The provided PCR Primer Mixes contain Forward and Reverse primers for use with the provided control DNA. Use 4 µl 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 by analyzed by agarose gel electrophoresis. Run reactions by loading 10 µl 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 µg/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 expected to produce a 338 base pair PCR product in the eluted fraction. There should be a strong band in the eluted fraction and a faint/no band in the unbound 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 unbound fraction. There should be a strong band in the unbound fraction and a faint/no band 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.

The UnMethylCollector™ technology is Patent pending.

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.

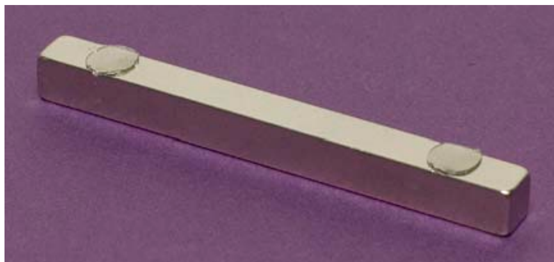
Assembly of Magnetic Stands

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 µl 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.



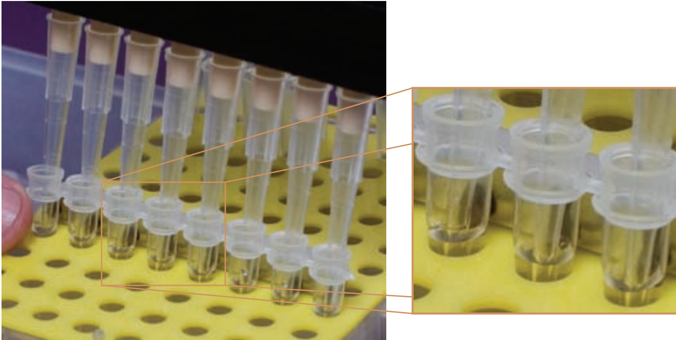
4. Remove the covering tape from the exposed side of the glue dots. Fix the magnet to the tip box so that it is against the PCR tubes. The magnetic stand is now ready for use.

   Note: Familiarize yourself with using the magnetic stand before performing with PCR tubes for the first time. Add 5 µl of magnetic beads to 100 µl Binding Buffer AM8 in one tube of an 8-well strip of PCR tubes. Use this tube with the assembled bar

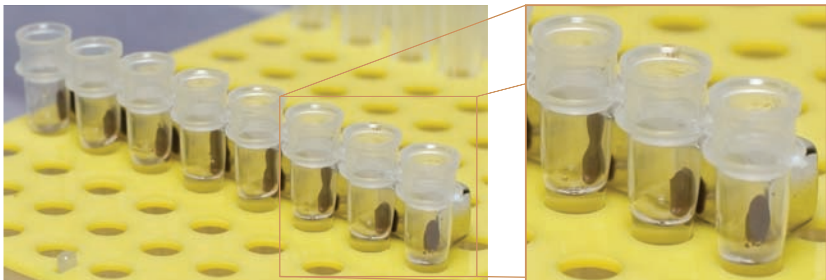
magnet stand to become familiar with use of the beads and magnet. It is difficult to re-suspend the beads if the tubes are directly adjacent to the magnet, so it is usually best to move the tubes away from the magnet for resuspension.

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 µl pipetteman or a 200 µl eight-channel pipetteman.



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 x *g* before allowing the rotor to stop.

Section B. Troubleshooting Guide

Problem/question	Recommendation
The target DNA fragment has less than 6 unmethylated CpGs.	The provided Binding Buffer AM8 is optimal for efficient capture of DNA fragments that have six or more unmethylated CpGs. For fragments with less than 6, we recommend using Binding Buffer AM9 (included in kit).
DNA starting material	UnMethylCollector can be used with 10 ng - 1 µg of fragmented DNA. For capturing large amounts of DNA (200 ng - 1 µg) it is recommended to keep the DNA concentration high so that only 10 µl of DNA sample is used for each reaction. Following fragmentation, DNA can be precipitated and concentrated if necessary.
PCR amplification	It has been determined that using a hot-start polymerase (*i.e.* Phusion™ from NEB) instead of a classic *Taq* polymerase may also increase the sensitivity of the assay.
Storage of DNA	Once DNA is prepared using UnMethylCollector, 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 *Mse* 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 UnMethylCollector protocol. In some situations (*e.g.*, 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.
High-throughput sequencing analysis	The His-CXXC protein stock may contain some trace amounts of bacterial DNA. If the desired downstream application is high-throughput sequencing analysis, we recommend pre-treatment of the required amount of His-CXXC protein with DNase I for 2 hours at room temperature on a rolling shaker. Use 0.5 µl DNase I (1 U/µl) and 1 µl of 10X DNase I buffer for every 10 µl of His-CXXC protein. Following the DNase treatment, the protein is ready for use in the binding reaction. After the 5 minute binding reaction, place the tubes on the magnetic stand to pellet the beads (see Appendix Section A for instructions on using the magnetic stand). Wash the bead pellet twice with 200 µl Binding Buffer AM8 in order to remove the DNase I. Resuspend beads in 80 µl of either complete Binding Buffer AM8 or AM9 depending on the number of unmethylated CpGs per DNA fragment. Proceed with the protocol at Step 1 No. 7 for the addition of fragmented DNA.

Section C. Related Products

DNA Methylation	Format	Catalog No.
MethylDetector™	50 rxns	55001
MethylCollector™	25 rxns	55002
MethylCollector™ Ultra	30 rxns	55005
Fully Methylated Jurkat DNA	10 µg	55003

Antibodies	Application	Format	Catalog No.
CGBP rabbit pAb	WB	200 µl	39203
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

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

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

ChIP-IT™ Kits	Format	Catalog No.
ChIP-IT™ Express	25 rxns	53008
ChIP-IT™ Express Enzymatic	25 rxns	53009
ChIP-IT™ Express HT	96 rxns	53018
ChIP-IT™ Protein G Magnetic Beads	25 rxns	53014
Re-ChIP-IT™	25 rxns	53016
ChIP-IT™	25 rxns	53001
ChIP-IT™ w/o controls	25 rxns	53004
ChIP-IT™ Shearing Kit	10 rxns	53002
ChIP-IT™ Enzymatic	25 rxns	53006
ChIP-IT™ Enzymatic w/o controls	25 rxns	53007
Enzymatic Shearing Kit	10 rxns	53005
Salmon Sperm DNA/Protein G agarose	25 rxns	53003
ChIP-IT™ Control Kit – Human	5 rxns	53010
ChIP-IT™ Control Kit – Mouse	5 rxns	53011
ChIP-IT™ Control Kit – Rat	5 rxns	53012
Ready-to-ChIP HeLa Chromatin	10 rxns	53015
Ready-to-ChIP Hep G2 Chromatin	10 rxns	53019
Ready-to-ChIP K-562 Chromatin	10 rxns	53020
Ready-to-ChIP NIH/3T3 Chromatin	10 rxns	53021

Chromatin Assembly	Format	Catalog No.
Chromatin Assembly Kit	10 rxns	53500

Histone Acetyltransferase and Deacetylase Activity	Format	Catalog No.
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

Recombinant Methylated Histones	Format	Catalog No.
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

Control Acid Extracts	Format	Catalog No.
HeLa acid extract	100 µg	36200
HeLa acid extract (Paclitaxel treated)	100 µg	36201
HeLa acid extract (Sodium Butyrate treated)	100 µg	36202
HeLa acid extract (Etoposide treated)	100 µg	36203
HeLa acid extract (Anacardic acid treated)	100 µg	36204

Transcription Factor ELISAs	Format	Catalog No.
TransAM™ AP-1 Family	2 x 96-well plates	44296
TransAM™ AP-1 c-Jun	1 x 96-well plate	46096
TransAM™ GR	1 x 96-well plate	45496
TransAM™ HIF-1	1 x 96-well plate	47096
TransAM™ IRF-3 (Human)	1 x 96-well plate	48396
TransAM™ IRF-7	1 x 96-well plate	50196
TransAM™ NFATc1	1 x 96-well plate	40296
TransAM™ NFκB Family	2 x 96-well plates	43296
TransAM™ NFκB p50	1 x 96-well plate	41096
TransAM™ NFκB p52	1 x 96-well plate	48196
TransAM™ NFκB p65	1 x 96-well plate	40096
TransAM™ p53	1 x 96-well plate	41196
TransAM™ STAT Family	2 x 96-well plates	42296

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Technical Services

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