MethylCollector™ MBD Capture Kit

(version A1)

Catalog No. 55026

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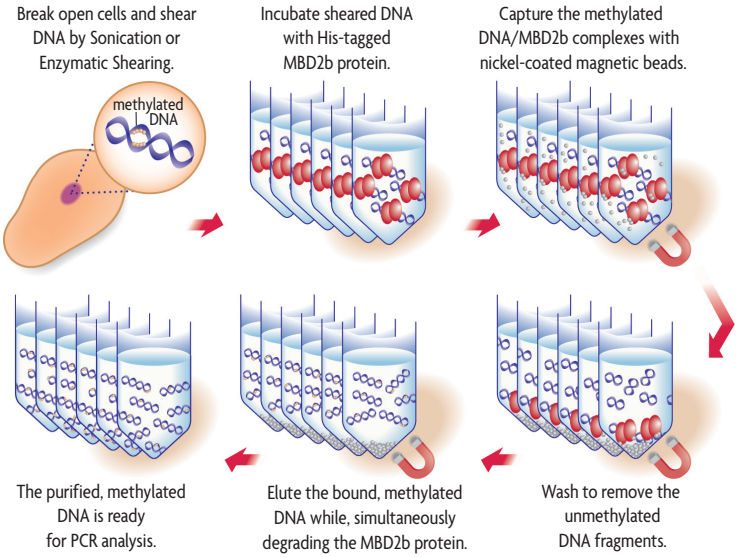
Overview

Active Motif's MethylCollector MBD Capture Kit provides an efficient method for isolating CpG-methylated DNA from limited amounts of cell or tissue samples. MethylCollector has many powerful applications including enabling researchers to rapidly screen the methylation status of multiple loci and may be particularly useful for analyzing the methylation levels of candidate genes in tumor tissue or cells. It can also be used to detect changes in DNA methylation in other situations, including normal cellular differentiation and aging.

In the MethylCollector MBD Capture Kit, His-tagged recombinant MBD2b protein specifically binds CpG-methylated DNA fragments that have been prepared by enzymatic digestion or sonication. These protein-DNA complexes are captured with nickel-coated magnetic beads and subsequent wash steps are performed 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 the MethylCollector MBD Capture protocol 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 cells (~1 ng DNA).

product	format	catalog no.
MethylCollector™ MBD Capture Kit	30 rxns**	55026

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



Flow chart of the MethylCollector MBD Capture process.

In MethylCollector MBD Capture, genomic DNA of interest is sheared by either enzymatic digestion or sonication. The sheared DNA is then incubated with a His-tagged recombinant MBD2b protein, which specifically binds CpG-methylated DNA fragments. These protein-DNA complexes are captured with nickel-coated magnetic beads and washes are then performed to remove fragments with little or no methylation. The methylated DNA is then eluted from the beads. Real time or endpoint PCR can be performed on the resulting supernatant using specific primers to amplify the locus of interest. Alternatively library prep and DNA sequencing can be performed in order to analyze whole-genome methylation profiles.

Kit Performance and Benefits

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

Sensitivity: MethylCollector MBD Capture can be performed on 1 ng - 1 µg of fragmented genomic DNA.

Nature of the MethylCollector MBD Capture Assay: MethylCollector MBD Capture is an MBD2b based assay 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.

GAPDH Glyceraldehyde-3-phosphate dehydrogenase is important for metabolism. Because this gene is often constitutively expressed, it is considered to be an actively transcribed housekeeping gene containing an unmethylated promoter in healthy tissues. The region amplified by this primer pair is 69 base pairs and contains 7 CpGs.

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

NBR2 Neighbor of BRCA1 gene 2 is located near the breast cancer gene BRCA1. Evidence indicates that NBR2 and BRCA1 share a bi-directional promoter. This region of the NBR2 gene is methylated in healthy tissues. The region amplified by this primer pair is 103 base pairs and contains 7 CpGs.

With high salt binding conditions, the *Mse* I digested control human, male genomic DNA provided in the kit should have at least a 10-fold enrichment of methylated DNA bound and eluted from the protein as detected with the NBR2 PCR Primer Mix. This means that of the total DNA recovered, there is ten times as much methylated DNA recovered in the eluted fraction as compared to the unbound fraction for the same locus.

With low salt binding conditions, the *Mse* I digested control human, male genomic DNA provided in the kit should have at least a 10-fold enrichment of methylated DNA bound and eluted from the protein as detected with the Xist PCR Primer Mix. This means that of the total DNA recovered, there is ten times 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 in the eluted fraction using the GAPDH PCR Primer Mix for either low or high salt binding conditions.

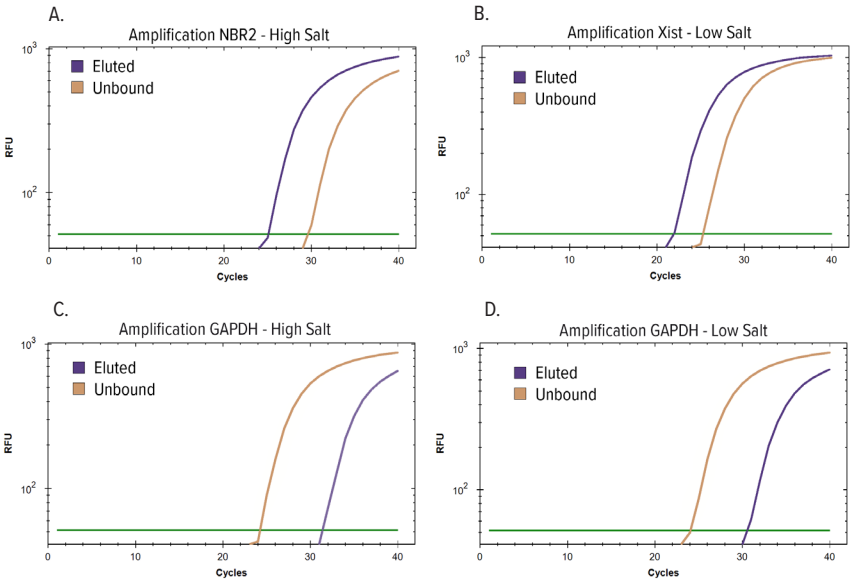


Figure 1: Real time PCR analysis of control PCR primer sets.

MethylCollector MBD Capture was performed using 100 ng of *Mse* I digested human, male genomic DNA under both low and high salt conditions. Eluted DNA was purified and analyzed using real time PCR for both methylated and unmethylated promoters. **A)** Amplification plot using the provided NBR2 PCR primer mix with the unbound and eluted fractions under high salt binding conditions. NBR2 is methylated in the control DNA and shows early amplification of the eluted fractions. **B)** Amplification plot using the provided Xist PCR primer mix with the unbound and eluted fractions under low salt binding conditions. Xist is methylated in the control DNA and shows early amplification in the eluted fractions. **C)** Amplification plot using the provided GAPDH PCR primer mix with the unbound and eluted fractions under high salt binding conditions. GAPDH is unmethylated in the control DNA and shows late amplification of the eluted fractions. **D)** Amplification plot using the provided GAPDH PCR primer mix with the unbound and eluted fractions under low salt binding conditions. GAPDH is unmethylated in the control DNA and shows late amplification of the eluted fractions.

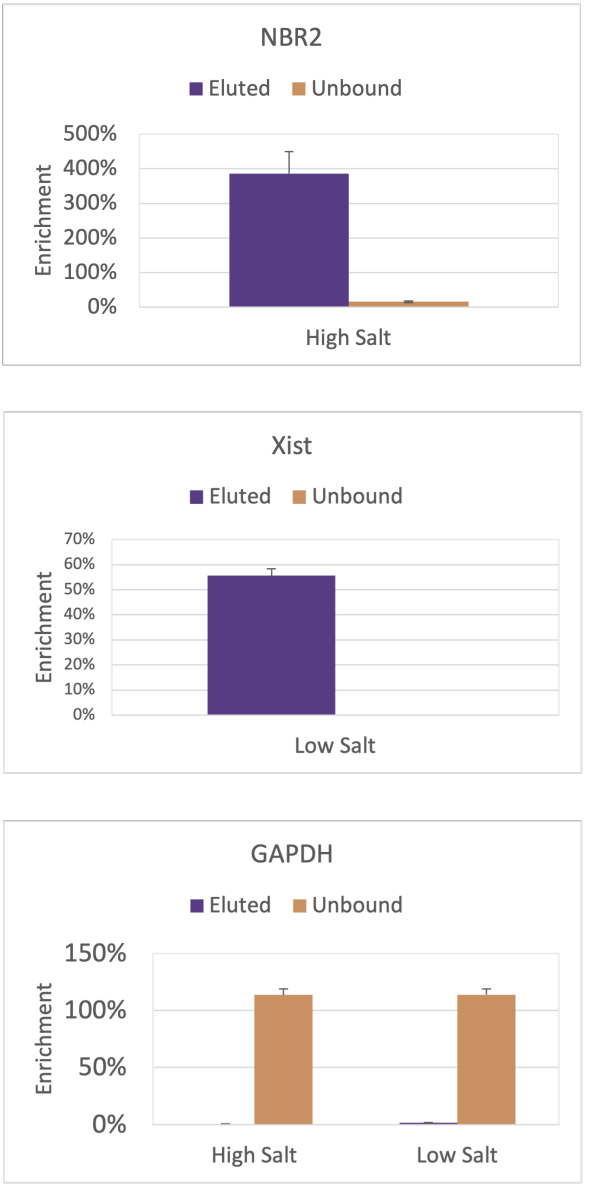


Figure 2: Percent enrichment of MethylCollector MBD Capture binding reactions.

MethylCollector MBD Capture was performed with 100 ng of Mse I digested human, male genomic DNA using both high and low salt binding conditions. Unbound and eluted DNA was cleaned and analyzed in real time PCR. The amount of DNA recovered in the eluted fraction was divided by the amount of input DNA used in the binding reaction to produce a percent enrichment. The methylated promoters NBR2 and Xist had greater than 10-fold enrichment of eluted DNA as compared to unbound DNA for the same locus, while the unmethylated GAPDH promoter was found almost exclusively 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 can be stored frozen upon arrival, however, we recommend long-term storage at 4°C. **Once the magnetic beads have been thawed, do not re-freeze the beads.**

Reagents	Quantity	Storage / Stability
His-MBD2b protein	350 µl	-80°C for 6 months
High Salt Binding Buffer	35 ml	-20°C for 6 months
Low Salt Binding Buffer	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 Mse I digested (20 ng/µl)	250 µl	-20°C for 6 months
GAPDH PCR Primer Mix (2.5 pmol/µl)	400 µl	-20°C for 6 months
Xist PCR Primer Mix (2.5 pmol/µl)	400 µl	-20°C for 6 months
NBR2 PCR Primer Mix (2.5 pmol/µl)	400 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 (20 mg/ml)	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

- Fragmented DNA sample
- Sample PCR primer sets
- 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 C) or use commercially available stands
- Rotisserie shaker
- Phenol/chloroform or DNA clean-up kit for purification of DNA such as Active Motif's Chromatin IP DNA Purification Kit (Catalog No. 58002).
- 5 M Ammonium acetate (see Troubleshooting Guide, Appendix - Section D, 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/µl) (Example: New England Biolabs M0267L or GeneSpin STS-T1000)
- SYBR Green mix for real time PCR analysis
- PCR cycler
- (Optional) 5M Betaine stock solution for use with endpoint PCR

NOTES BEFORE STARTING

Fragmentation of Genomic DNA

The provided His-MBD2b protein 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. 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(s) 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 DNA sample will be used for simultaneous analysis of many CpG islands. When the isolated DNA will be analyzed by NGS sequencing or by microarray methods, or when a CpG region of interest is not flanked by suitable restriction sites, sonication is recommended. 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 are provided for both restriction digest and mechanical fragmentation on page 9. We suggest using 4 µg of purified genomic DNA when performing restriction digestion and 20 µg of purified genomic DNA for sonication. MethylCollector MBD Capture reactions can be performed on 1ng - 1 µg of fragmented genomic DNA.

Example Framentation 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 4	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: MethylCollector MBD Capture 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 using a DNA purification Kit. See “Appendix D. Troubleshooting” on page 25 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)

Restriction enzymes are not always ideal to fragment and isolate the DNA sequences of interest. In this case 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) Using a tip probe sonicator, sonicate on ice with 15 pulses of 20 seconds (30% amplitude if using Active Motif’s EpiShear™ Sonicator, Catalog Nos. 53051 & 53052), 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.

MethylCollector MBD Capture Protocol

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING!

Step 1: Planning the experiment

1. Determine the number of reactions needed. For PCR or sequencing analysis, one reaction per sample is usually sufficient. For recommendations on downstream applications, such as whole genome amplification or Next-Gen sequencing, please refer to Appendix A.
2. Two different binding buffers are included in the kit. For high salt conditions (high stringency reactions) we recommend using High Salt Binding Buffer. For low salt conditions (lower stringency reactions) we recommend using Low Salt Binding Buffer. It may be necessary to perform experiments with both binding conditions to determine the optimal conditions for the target of interest.

Step 2: Binding reaction

1. Thaw components from storage as needed for preparation. Keep all components on ice when not in use.
2. Prepare Complete Binding Buffer for the desired binding conditions.

   For high salt binding conditions (high stringency): Prepare the appropriate amount of Complete Binding Buffer according to the table below. Store on ice.

Reagent	One rxn	8 rxns
High Salt Binding Buffer	100 µl	800 µl
Protease Inhibitor Cocktail	0.5 µl	4 µl
Total Volume	**100.5 µl**	**804 µl**

   For low salt binding conditions (lower stringency): Prepare the appropriate amount of Complete Binding Buffer according to the table below. Store on ice.

Reagent	One rxn	8 rxns
Low Salt Binding Buffer	100 µl	800 µl
Protease Inhibitor Cocktail	0.5 µl	4 µl
Total Volume	**100.5 µl**	**804 µl**

   Note: The provided High and Low Binding Buffers are 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 MBD Capture protocol can be performed on a large range of sample DNA amounts (1 ng to 1 µg). 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/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 of 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 (see page 21).

   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, then 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.)
6. **Binding Reaction:** Add the remaining components in the order shown below to each PCR tube. Pipet the His-MBD2b protein up and down several times to ensure homogeneity before use. Prepare a positive control reaction using the provided *Mse* I digested human, male genomic DNA.

Reagent	One rxn	Positive Control
Magnetic beads	10 µl	10 µl
Complete Binding Buffer	70 µl	75 µl
Fragmented genomic DNA	1 ng-1 µg (10 µl)	-
Control Human, male genomic DNA (*Mse* I digested)	-	5 µl
His-MBD2b protein	10 µl	10 µl
Total Volume	**100 µl**	**100 µl**

Note: It is recommended to aliquot the provided His-MBD2b protein into several small fractions for future use. Pipet the His-MBD2b protein up and down several times to ensure homogeneity before making aliquots. Store at -80°C.

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

Step 3: Wash beads

1. After the capture step is complete, spin the PCR tubes briefly and place tubes on a magnetic stand to pellet beads to the side of the tube. 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 5 of the protocol. Otherwise, remove and discard the supernatant. To use the magnet provided in the kit, please see Appendix C.
2. Wash beads four times with 200 µl Binding Buffer. Pipette 2-3 times gently to resuspend. Use the same stringency Binding Buffer for the wash steps as was used for the binding reactions.
    a. Place tubes on magnetic stand and allow beads to pellet on side of the tube.
    b. Carefully remove the supernatant and any residual bubbles.
    c. Add 200 µl 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 from the magnet and place in a separate rack to fully resuspend the beads.
    d. Repeat steps a-c one time.
3. In a microcentrifuge tube, prepare Complete Elution Buffer by adding 2 µl of Proteinase K to 98 µl of Elution Buffer AM1 for each reaction.
4. 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 4: Recovery of methylated DNA fragments

1. Resuspend washed beads with 100 µl Complete Elution Buffer by pipetting 2-3 times.
2. Incubate samples at 50°C for 30 minutes. Every 10 minutes, invert the tubes to ensure the beads are resuspended.
3. During this incubation, warm the Proteinase K Stop Solution at 37°C for 10 minutes.
4. Quick spin the tubes. Place tubes in magnetic stand and allow beads to pellet onto tube sides.
5. Carefully transfer the supernatant to a new microcentrifuge tube.
6. Add 2 µl of Proteinase K Stop Solution to each supernatant. Pipet 2-3 times to mix.
7. Proceed to Step 5, DNA clean up, or 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 5: DNA clean up

Prior to use in downstream applications it is necessary to clean up the DNA. DNA clean up can be performed using one of the methods listed below:

- **Purification columns** – use PCR clean up kits such as Active Motif's Chromatin IP DNA Purification Kit (Catalog No. 58002). Samples should be eluted in 50 µl volume, or other appropriate volume of water or buffer as needed for specific downstream applications.

  For more concentrated DNA, use the MinElute PCR Purification Kit (Qiagen part no. 28004) and elute in as low as 10 µl.

- **Phenol/chloroform extraction followed by ethanol precipitation** – follow the protocol listed below

Phenol/Chloroform Extraction & Ethanol Precipitation

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 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 mg/ml) (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. Do not disturb the pellet.
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 minutes (do not completely dry the pellet).
13. Resuspend the DNA pellet in 50 µl sterile DNase-free water., or use other appropriate volumes of buffer or water as needed for specific downstream applications.
14. This eluted DNA can be used immediately 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 downstream applications.

Appendix

Section A. Downstream Applications

The MBD2b protein used in the MethylCollector MBD Capture Kit can be used to selectively bind and enrich for methyl CpG dinucleotides that are often found in the promoter/regulatory regions of genes. The binding affinity of the MBD2b protein increases with the density of methyl CpG dinucleotides. The amount of DNA recovered from the MethylCollector MBD Capture Kit will depend on the global methylation status of the sample DNA. Usually only a small percentage of the starting material will be recovered, yet the recovered sample is specific for CpG-methylated DNA.

PCR Analysis

By amplifying a specific target site using PCR, it is possible to determine if there is an increase in methylation at that single site. Alternatively, the same site can be compared across different sample types, but it will be necessary to run MethylCollector MBD Capture on the same amount of each DNA sample and generate a separate standard curve for each sample.

Next-Gen Sequencing Analysis

To analyze whole-genome methylation profiles, researchers may chose to perform libray prep and Next-Gen sequencing following the enrichment of methylated DNA with MethylCollector MBD Capture. This process has been validated when DNA enriched with MethylCollector MBD Capture is end adapted for use in sequencing. We recommend using a kit like the Active Motif Next Gen DNA Library Kit, cat# 53216, to create libraries.

Section B. PCR Analysis

PCR Primer Design

MethylCollector MBD Capture includes PCR primers for use with the provided *Mse* I digested human, male genomic DNA. If possible, real time PCR is recommended for analysis of DNA isolated with MethylCollector MBD Capture. To design primers specific to the CpG region of interest in your sample, please follow the recommendations below.

Primer design considerations

i. Primers should flank the CpG region of interest and produce an amplicon between 100-350 bp in length for endpoint PCR or an amplicon of 100-150 bp for real time PCR. The CpG region needs to contain at least five methylated CpGs.

ii. Each primer should be approximately 18-22 nucleotides long, contain 50% GC content and have a Tm between 55°C-60°C.

iii. 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.

iv. 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.

v. PCR primers should be designed with the aid of a reliable primer design computer program. Due to the technical limitations of PCR, it is sometimes necessary to design more than one primer pair for a given fragment of interest.

vi. Potential primer pairs can be evaluated via computer simulation using a program such as UCSC Genome Browser to ensure the primers selected will produce a single amplicon in a CpG region of the species being amplified.

Determining Primer Efficiency

It is advised to determine the efficiency of the PCR primers being used. Primers with an efficiency less than 90% will have poor reproducibility. To calculate primer efficiency:

$$\text{Primer efficiency (\%)} = [10^{(-1/\text{slope})}-1] \times 100\%$$

To obtain the slope value, follow the instructions for generating and graphing a standard curve in the Data Analysis and Use of Input DNAs section on page 21. Use the slope of the plotted standard curve in the primer efficiency equation above.

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	11.8 µl
10X PCR Buffer	2.5 µl
10X PCR Loading Dye	2.5 µl
dNTP mixture (5 mM each dNTP)	1 µl
Forward Primer* (5 pmol/µl)	1 µl
Reverse Primer* (5 pmol/µl)	1 µl
Taq (5 U/µl)	0.2 µl
DNA sample (eluted or Input)	5 µl
(Optional) 1 M Betaine can be added to PCR reactions as an enhancer to improve PCR results	
Total Volume	**25 µl**

* The provided PCR Primer Mixes contain both Forward and Reverse primers for use with the provided control DNA. Use 2 µl of the PCR primer Mix in the PCR protocol described above. The Xist PCR primer mix has been optimized for use with low salt binding conditions, while NBR2 and GAPDH primer mixes can be used with either low or high salt binding conditions.

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

   94°C for 3 minutes
   (94°C for 20 seconds, 55°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 µl from each of the PCRs on a thin 3% agarose gel at 100 V for 60 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.

GAPDH Glyceraldehyde-3-phosphate dehydrogenase should be unmethylated in the control human, male genomic DNA and is not expected to produce a 69 base pair PCR product in the eluted DNA fraction.

Xist X inactive specific transcript is methylated in the control human, male ge-

nomic DNA and will produce a 178 base pair PCR product with the eluted fraction.

NBR2 Neighbor of BRCA1 gene 2 is methylated in the control human, male genomic DNA and is expected to produce a 103 base pair PCR product with the eluted fraction.

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 both 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 for the PCR protocol described above. The Xist PCR primer mix has been optimized for use with low salt binding conditions, while NBR2 and GAPDH primer mixes can be used with either low or high salt binding conditions.

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.

GAPDH Glyceraldehyde-3-phosphate dehydrogenase should be unmethylated in the control human, male genomic DNA and will not amplify in the eluted fraction.

Xist X inactive specific transcript is a methylated promoter in the control human, male genomic DNA and will amplify early in the eluted fraction.

NBR2 Neighbor of BRCA1 gene 2 is a methylated promoter in the control human, male genomic DNA and will amplify early in the eluted fraction.

Data Analysis and Use of Input DNAs

Methylated DNA isolated using MethylCollector MBD Capture 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 MBD Capture 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 2. No. 4 in the Protocol) to clearly indicate the relative concentrations of the DNA samples.

For real time PCR, generating a standard curve using the input DNA enables accurate determination of the enriched DNA concentration.

1. Produce a standard curve at 10, 1, 0.1 and 0.01 ng/µl in triplicate using the Input DNA from Step2, No. 4. We recommend running a standard curve every time MethylCollector MBD Capture is performed. However, if the primer efficiency has been determined to be greater than 90% according to the calculations on page 18, the standard curve can be generated once and stored for future use with the same DNA sample.
2. Run each sample with the appropriate DNA standard (*i.e.* prepare a separate standard curve for each cell line or species tested).
3. Every gene will generate a different amplification profile. CT = Threshold Cycle or cycle number where the signal exceeds the background threshold level. CT values should be plotted for each gene to create a linear regression plot.
4. Plot CT versus log DNA concentration. See Figure 3 below.

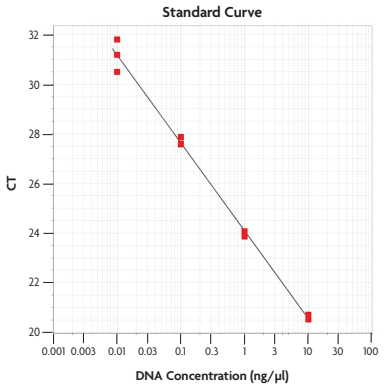


Figure 3: Example standard curve linear regression plot.

A standard curve for the human, male genomic DNA is provided as a reference only. Input DNA was tested in triplicate and plotted against the CT value. A new standard curve should be generated each time the assay is performed.

5. Using the CT value of the sample, extrapolate the DNA concentration of the sample DNA using the standard curve plot. To determine the amount of enriched DNA in the sample simply multiple the DNA concentration by the volume of enriched DNA.
6. Calculate the percent enrichment. Use the sample DNA quantity calculated above and compare it with the amount of DNA used in the initial binding reaction (Step 2, No. 6).

$$\textbf{Enrichment}\ (\%) = \frac{\text{Amount of enriched Sample DNA material}}{\text{Amount of fragmented DNA used in the binding reaction}} \times 100\%$$

7. *Optional:* Calculate the fold enrichment of methylated DNA. If the unbound material was collected in Step 3, No.1 and purified for analysis in PCR, the eluted samples can be compared to the unbound samples for the same locus in order to determine the fold enrichment.

$$\textbf{Fold enrichment} = \frac{\text{Sample DNA concentration in the eluted fraction}}{\text{Sample DNA concentration in the unbound fraction}}$$

Section C. 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.

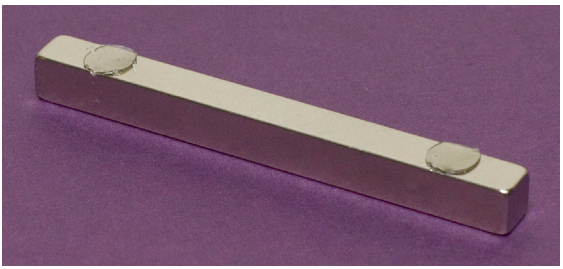
Assembly of Magnetic Stands

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.



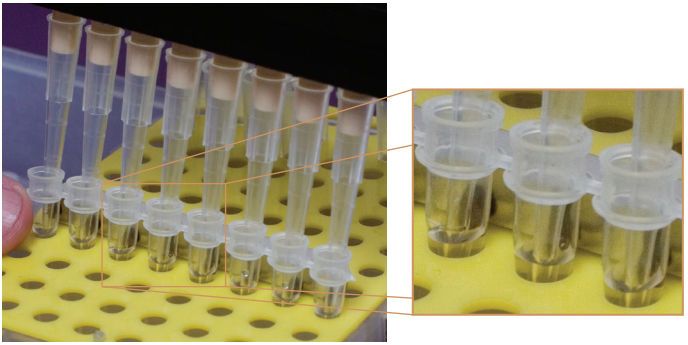
4. Remove the covering tape from the exposed side of the glue dots. Fix the magnet to the tip box so that it is against the PCR tubes. The magnetic stand is now ready for use.

Note: Familiarize yourself with using the magnetic stand before performing

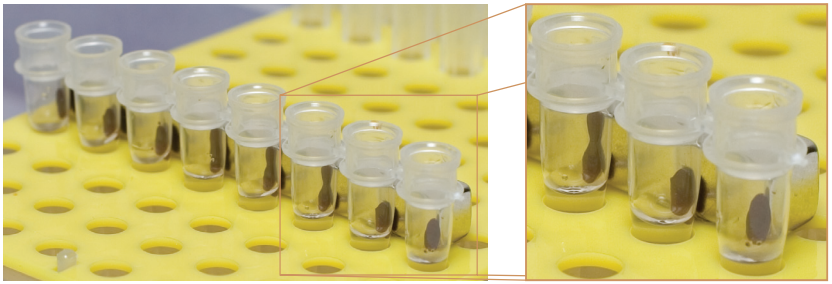
with PCR tubes for the first time. Add 5 µl of magnetic beads to 100 µl High Salt Binding Buffer in one tube of an 8-well strip of PCR tubes. Use this tube with the assembled bar magnet stand to become familiar with use of the beads and magnet. It is difficult to re-suspend the beads if the tubes are 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 D. Troubleshooting Guide

Problem/question	Recommendation
The target DNA fragment has less than 5 methylated CpGs.	The provided Binding Buffers are optimal for efficient capture of DNA fragments that have five or more methylated CpGs. MethylCollector MBD Capture is not recommended for isolation of DNA fragments containing less than 5 methylated CpGs.
Little or no enrichment of methylated DNA in the bound fraction	The binding conditions may be too stringent. Try using the Low Salt Binding Buffer to set up binding reactions for that particular target.
PCR amplification	It has been determined that using a hot-start polymerase (*i.e.* Phusion™ from NEB) instead of a classic *Taq* polymerase may increase the sensitivity of the assay.
	Selection of an appropriate qPCR master mix is important to achieve good amplification. Since all commercially available mixes have different compositions regarding chemical enhancers or inhibitors for non-specific amplification, results may vary depending on the master mix used.
	The addition of 1 M Betaine to endpoint PCR reactions can be used to enhance the PCR and improve the results. Betaine lowers the melting temperature of GC-rich regions making them comparable to AT regions.
Storage of DNA	Once DNA is prepared using MethylCollector MBD Capture, 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 precipitation 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 may also require use of different enzymes. In addition, the region of interest may not be flanked by suitable restriction sites and single-nucleotide polymorphisms (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 MethylCollector MBD Capture 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.

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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