MethylCollector™

(version B2)

Catalog No. 55002

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Overview

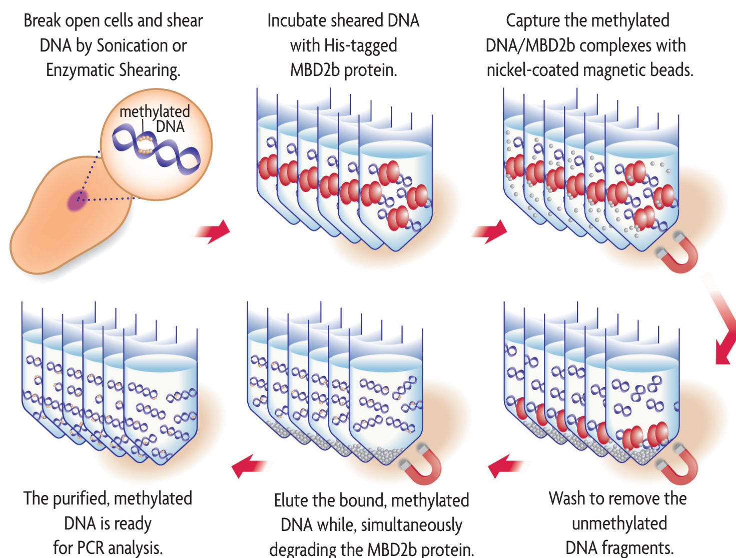
Active Motif's MethylCollector Kit provides an efficient method for isolating CpG-methylated DNA[1] from limited amounts of cell or tissue samples. MethylCollector has many powerful applications including enabling researchers to rapidly screen the methylation status of multiple loci and may be particularly 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 method*, 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 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 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 800 cells (~5 ng DNA).

product	format	catalog no.
MethylCollector™	25 rxns**	55002

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

**MethylCollector provides sufficient reagents to perform 25 reactions with excess reagents for 5 positive and 5 negative control reactions.



Flow chart of the MethylCollector process.

In MethylCollector, genomic DNA of interest is sheared by either enzymatic digestion or sonication. The sheared DNA is then incubated with His-tagged recombinant MBD2b protein, which has a high affinity 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 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[2-5]. 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[6]. Aberrant hypermethylation of CpG islands and subsequent transcriptional repression is one of the earliest and most common somatic genome alterations in multiple human cancers[7, 8]. 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[9]. Aberrant methylation of CpG islands thus seems to be a tumor type-specific event8, 10 and current efforts have concentrated on finding ways to exploit the diagnostic and therapeutic implications of these abnormalities[11, 12].

Methyl-CpG binding proteins appear to be central players in the process of DNA methylation-dependent gene silencing[13]. 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[14]. After the recognition of the MBD, four additional genes were found to contain this domain, namely MBD1, MBD2, MBD3 and MBD4[15]. In general, all MBD proteins, except MBD4, have been reported to be associated with histone deacetylase subunits as part of large multi-subunit complexes[16, 17]. A few studies support the notion of selectivity in the association of a particular MBD with particular promoters[18, 19], but some results rather indicate that the CpG distribution along the sequence may influence the interaction of each MBD protein with DNA[20]. The MBD2b protein has been found to possess one of the highest affinities for methylated DNA among MBD proteins[20].

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[21]. 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[22] 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[23]. 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 Kit is for research use only. Not for use in diagnostic procedures.

Sensitivity: MethylCollector can be performed on 5 ng - 1 µg of genomic DNA.

Nature of the MethylCollector Assay: MethylCollector is primarily designed as a rapid and efficient method to compare levels of methylation (at user-specified genomic loci) in various DNA samples. In order to do this accurately, the DNAs to be tested should be prepared identically (*e.g.*, purified and fragmented by the same methods) and an equal amount of each sample should be used in MethylCollector. In the below example, the assay was performed on equal amounts of identically prepared HeLa and A431 genomic DNA. The results demonstrate A431 DNA is much more heavily methylated than HeLa DNA at the MCJ locus.

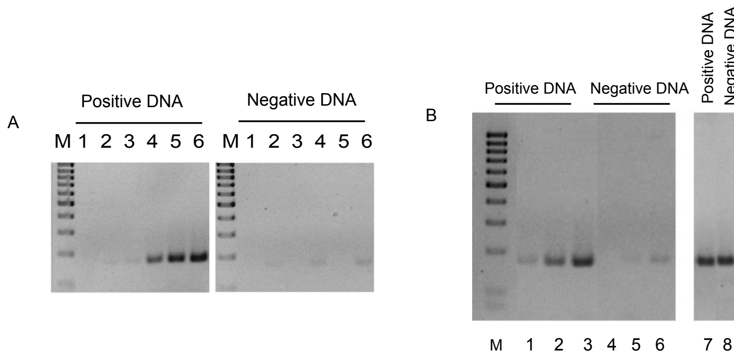


Figure 1: Agarose gel analysis of PCR products generated with MethylCollector.

A431 (positive control) and HeLa (negative control) genomic DNAs were sheared, either by enzymatic digestion with *Mse* I (A) or by sonication (B). Increasing amounts of fragmented DNA were then incubated for 1 hour with 1 µg of the His-MBD2b in the presence of magnetic beads. After washing, CpG-methylated complexes were eluted. The isolated DNA was then analyzed by 36 cycles of PCR using the control primers provided in the kit. PCR performed on the MethylCollector-enriched positive control DNA generates robust signals, proportional to the amount of DNA starting material (A, left panel, lanes 4 to 6; B, lanes 1 to 3). Very limited signal is observed with negative control DNA (A, right panel, lanes 4 to 6; B, lanes 4 to 6), which is unmethylated in this particular locus. No signal is observed in samples where His-MBD2b protein was omitted from the binding reaction (A, left and right panels, lanes 1 to 3). Finally, the PCR products from 10 ng of each input DNA are of equal intensity (B, compare lanes 7 and 8), confirming that identical amounts of A431 and HeLa genomic DNAs were initially used in the reactions. Taken together, these results indicate that MethylCollector specifically enriches for methylated DNA fragments and that this enrichment is due to the His-MBD2b protein.

A.

Lane 1: 5 ng DNA, 0 µg MBD2b protein
Lane 2: 40 ng DNA, 0 µg MBD2b protein
Lane 3: 100 ng DNA, 0 µg MBD2b protein
Lane 4: 5 ng DNA, 1 µg MBD2b protein
Lane 5: 40 ng DNA, 1 µg MBD2b protein
Lane 6: 100 ng DNA, 1 µg MBD2b protein

B.

Lane 1: 20 ng DNA, 1 µg MBD2b protein
Lane 2: 100 ng DNA, 1 µg MBD2b protein
Lane 3: 500 ng DNA, 1 µg MBD2b protein
Lane 4: 20 ng DNA, 1 µg MBD2b protein
Lane 5: 100 ng DNA, 1 µg MBD2b protein
Lane 6: 500 ng DNA, 1 µg MBD2b protein
Lane 7: 10 ng A431 DNA used in input PCR
Lane 8: 10 ng HeLa DNA used in Input PCR

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 protein (0.1 µg/µl)	350 µl	-80°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	21 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
Positive Control DNA (A431)	60 µl	-20°C for 6 months
Negative Control DNA (HeLa)	60 µl	-20°C for 6 months
MCJ PCR Primer Mix	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
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

- DEPC and sterile water
- Filter pipette tips
- *Taq* polymerase (5 U/µl) (Example: New England Biolabs M0267L or GeneSpin STS-T1000)
- Microcentrifuge tubes and microcentrifuge
- dNTP mixture (5 mM each)
- PCR cycler

NOTES BEFORE STARTING

Fragmentation of Genomic DNA

The provided His-MBD2b protein binds to all methylated cytosines and the MethylCollector method enriches for DNA fragments that contain six 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" on page 15 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
MseI	TTAA	0.80	**2.88**
BfaI	CTAG	1.56	1.55
TasI	AATT	0.80	**2.88**
Csp6I	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: MethylCollector has been used with as little as 5 ng of restriction-digested genomic DNA. As a reference, a human cell contains about 6 picograms DNA; 5 ng of genomic DNA corresponds to 800 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 15 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

Methylated DNA isolated using MethylCollector is usually analyzed by PCR amplification of the loci of interest. If the method will be used to compare the methylation status of particular loci in different DNA samples, it is essential that MethylCollector be performed on the same amount of each DNA sample. Thus, DNA samples should be carefully quantified before use. In addition, each DNA sample should be treated as Input DNA (see Step 4 below) to clearly indicate the relative concentrations of the DNA samples. If possible, real-time PCR is recommended for analysis of DNA isolated with MethylCollector.

Primer design

The isolated CpG-methylated DNA fragments obtained by MethylCollector 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.

MethylCollector 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 protein. 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	100 µl	800 µl
Protease Inhibitor Cocktail	0.5 µl	4 µl
Total Volume	**100.5 µl**	**804 µl**

Note: The provided Binding Buffer is optimal for efficient capture of DNA fragments that contain more than six methylated CpGs. For DNA fragments with very little methylation (between 2 and 6 methylated-CpGs), a Low-Salt buffer is recommended, please see Troubleshooting on page 15 for more information.

3. Dilute the fragmented DNA in water if necessary. The MethylCollector protocol can be performed on a large range of input DNA amounts (5 ng to 1 µg). We recommend 100 ng because it gives robust results without requiring a large amount of DNA (please see Troubleshooting on page 15 for points regarding the amount of DNA starting material).
4. In this step, the Input DNAs that will be used in the final PCR step are prepared. When using the two control DNAs provided, PCR analysis is performed for 36 cycles and 0.25 ng of each control DNA is used in separate Input PCRs. The control DNAs (provided at 10 ng/µl) should be diluted to 0.05 ng/µl for use in Input PCR. This can be done by diluting each DNA 1/10 in dH_2O (*e.g.*, 1 µl 10 ng/µl DNA + 9 µl dH_2O to make 1 ng/µl DNA), followed by a second dilution of 1/20 (*e.g.*, 5 µl of 1 ng/µl DNA + 95 µl dH_2O to make the final concentration of 0.05 ng/µl). 5 µl of the 0.05 ng/µl 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, 0.25 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 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. Add the remaining components shown below to each PCR tube.

Reagent	One rxn
Magnetic beads	10 µl
Complete Binding Buffer	add to 100 µl
Fragmented genomic DNA	from 5 ng-1 µg
His-MBD2 (0.1 µg/µl)	10 µl
Total Volume	**100 µl**

7. In parallel, prepare two similar reactions with the provided positive and negative control DNAs.

Reagent	One rxn
Magnetic beads	10 µl
Complete Binding Buffer	70 µl
Control DNA	10 µl
His-MBD2 (0.1 µg/µl)	10 µl
Total Volume	**100 µl**

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

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 discard supernatant. To use the magnet provided in the kit, please see page 13 in the Appendix.
2. Wash beads four times with 200 µl Binding Buffer. Pipette 2-3 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 Binding Buffer 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 methylated DNA fragments

1. Prepare Complete Elution Buffer by adding 2 µl of Proteinase K to 98 µl of Elution Buffer for each reaction.
2. Resuspend washed beads with 100 µl 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 µl of Proteinase K Stop Solution.
6. Place tubes in magnetic stand and allow beads to pellet onto tube sides.

7. Carefully transfer the supernatant to a fresh PCR tube. 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 4: PCR amplification of methylated DNA fragments

In this step, the purified methylated DNA fragments and the Input DNA samples (prepared in Step 1, No. 4 above) will be amplified by PCR using customer-designed primers. In parallel, the purified positive and negative control DNA fragments and their respective input fractions will be amplified by PCR using the provided PCR control primer mix. PCR products can then be analyzed by agarose gel electrophoresis.

A typical 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
DEPC 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
Eluted DNA (or Input DNA)	5 µl
Total Volume	**25 µl**

* The provided MCJ PCR Primer Mix contains Forward and Reverse primers for use with the provided control DNAs. 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, 55°C for 30 seconds, 72°C for 30 seconds) for 36 cycles
   Hold at 4°C

Step 5: Agarose Gel Analysis of PCR products

Run reactions by loading 10 µl from each of the PCRs on a thin 3% 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.

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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 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.

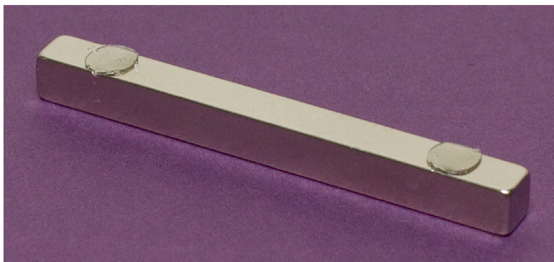
Assembly of Magnetic Stands

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 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.



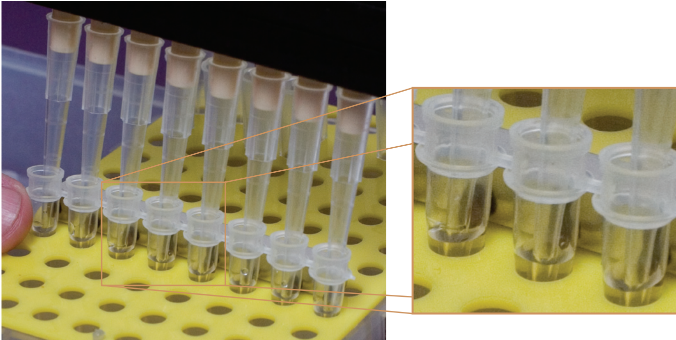
4. Remove the covering tape from the exposed side of the glue dots. Fix the magnet to the tip box so that it is against the PCR tubes. The magnetic stand is now ready for use.

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 ChIP Buffer 1 in one tube of an 8-well strip of PCR tubes. Use this tube with the assembled bar

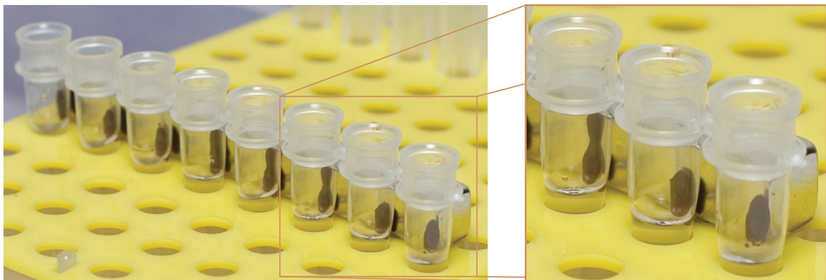
magnet stand to become familiar with use of the beads and magnet. It is difficult to re-suspend the beads if the tubes are directly adjacent to the magnet, so it is usually best to move the tubes away from the magnet for resuspension.

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 methylated CpGs.	The provided Binding Buffer is optimal for efficient capture of DNA fragments that have six or more methylated CpGs. For fragments with less than 6, we recommend preparing a Low-Salt Binding Buffer to use instead: 20 mM Tris-HCl (pH 7.5), 300 mM NaCl, 2 mM $MgCl_2$, 0.5 mM EDTA, 0.1% Igepal.
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 MethylCollector, 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.
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 MethylCollector 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.

Section C. Related Products

DNA Methylation	Format	Catalog No.
MethylDetector™	50 rxns	55001
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

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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