MeDIP

(version C2)

Catalog No. 55009

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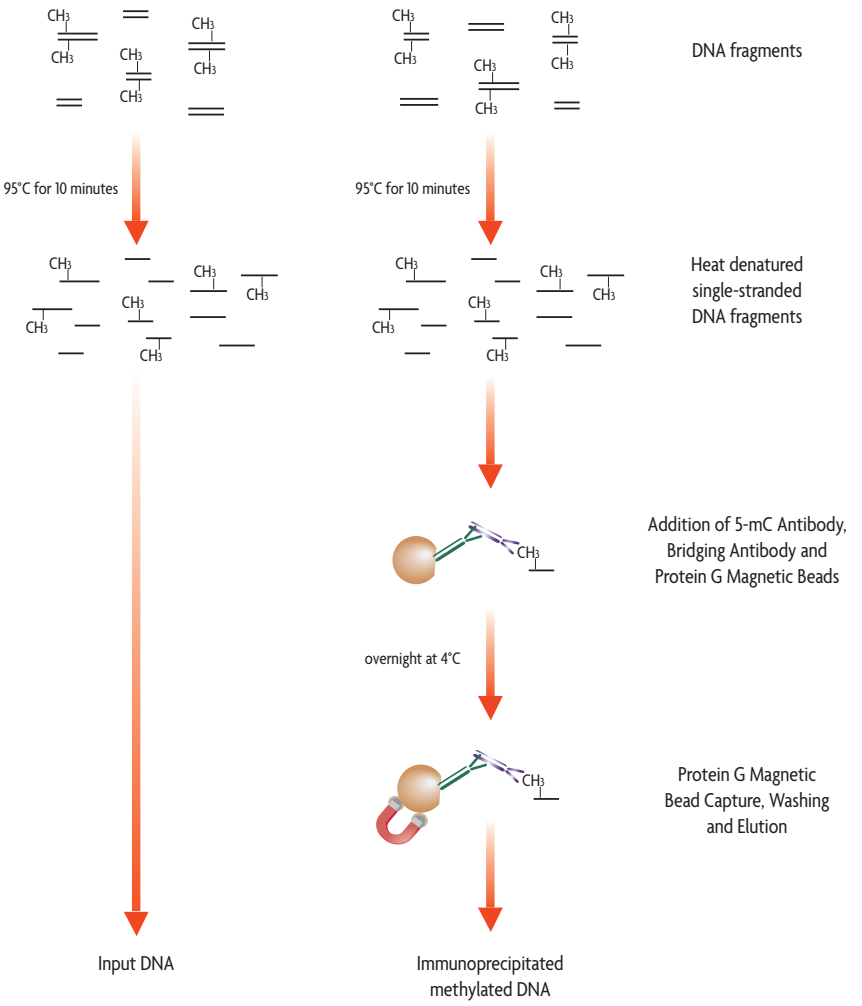
Overview

The MeDIP Kit is designed to immunoprecipitate and enrich for DNA fragments containing 5-methylcytosine (5-mC). The MeDIP Kit contains a highly specific monoclonal 5-methylcytosine antibody and the necessary buffers to perform methylated DNA immunoprecipitation (MeDIP). Active Motif's fast, magnetic protocol has been streamlined to minimize the number of wash and incubation steps, saving you valuable time. The kit also includes control human genomic DNA and positive control PCR primers that can be used to verify the efficiency of the enrichment. Additionally, the kit contains a bar magnet for easy separation and elution of the enriched methylated DNA.

Active Motif also offers a hMeDIP kit to study 5-hydroxymethylcytosine (5-hmC) methylation. This kit can be purchased separately; please see the ordering table below.

product	format	catalog no.
MeDIP	10 rxns	55009
hMeDIP	10 rxns	55010

Flow Chart of Process



Introduction

Methylated DNA Immunoprecipitation (MeDIP)

Over the last decade, the study of DNA methylation and its role in epigenetic cell signaling has grown rapidly[1-4]. Methylation of CpG dinucleotides, which occurs at the fifth position of the cytosine pyrimidine ring, is of particular interest. Methylation which occurs outside of CpG dinucleotides (CpA or CpT) is also of great interest, as it has been reported to account for 15-20% of total cytosine methylation in embryonic stem cells[20].

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[5]. Aberrant hypermethylation of CpG islands and subsequent transcriptional repression is one of the earliest and most common somatic genome alterations in multiple human cancers[6, 7]. 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[8]. Aberrant methylation of CpG islands thus seems to be a tumor type-specific event[7, 9] and current efforts have concentrated on finding ways to exploit the diagnostic and therapeutic implications of these abnormalities[10, 11].

Methylated DNA Immunoprecipitation is a an immunocapture method in which an antibody specific for methylated cytosines is used to immunoprecipitate methylated genomic DNA fragments[12]. The affinity of the antibody used in MeDIP enables the detection of methylated cytosines regardless of their context. This means that MeDIP can be used for the detection of any methylated cytosine and is not restricted to analysis of CpG methylation. The enriched DNA can be used for individual analysis of the methylation status of a particular gene by PCR, in combination with microarrays for genome-wide methylation analysis, or to prepare samples for use in Next-Gen sequencing techniques.

Traditional MeDIP uses a monoclonal antibody against 5-methylcytosine (5-mC) for immunoprecipitation of single-stranded DNA, as the 5-methylcytosine antibody has a higher affinity for single stranded DNA. The discovery of 5-hydroxymethylcytosine (5-hmC) as a modification within genomic DNA has lead to additional research to analyze the function of this modification[13, 14]. The 5-hydroxymethylcytosine modification results from the enzymatic conversion of 5-methylcytosine into 5-hydroxymethylcytosine by the TET family of cytosine oxygenases. While the precise function of 5-hmC has yet to be determined, it has been postulated that it could represent a pathway to demethylated DNA, as 5-hydroxymethylcytosine is repaired as mismatched DNA and replaced with unmethylated cytosine.

To better understand the functions of 5-mC and 5-hmC, Active Motif has developed the MeDIP and hMeDIP Kits. The MeDIP assay uses a monoclonal 5-methylcytosine antibody (5-mC) to selectively enrich for DNA fragments containing 5-mC. This selectivity is important as most common approaches to analyze DNA methylation, such as enzymatic approaches and bisulfite conversion, are unable to distinguish between 5-mC and 5-hmC. The MeDIP and hMeDIP Kits can be run in parallel to analyze differences in DNA methylation patterns.

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 affinities for 5-methylcytosine can be used to determine the methylation status of specific CpG dinucleotides[15]. Methylation-sensitive restriction enzymes have several limitations, such as the fact that the methylation-sensitive restriction merely informs on the methylation status of the cytosine residues which are within the restriction site sequence, but do not provide information about other methylation sites.
2. **Bisulfite conversion:** Bisulfite conversion 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[16]. Bisulfite-based techniques can be cumbersome, involving time- and labor-intensive chemical treatments that damage DNA and limit throughput. Additionally, bisulfite conversion does not differentiate between 5-mC and 5-hmC methylation.
3. **Methyl-CpG Binding proteins:** 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 MBD2b protein has been found to possess one of the highest affinities for methylated DNA among MBD proteins and has the greatest capacity to differentiate between methylated and unmethylated DNA[17]. Methyl-CpG binding proteins are limited to the evaluation of methylated DNA in a CpG context as the proteins do not recognize methylated cytosines that exist outside of a CpG dinucleotide. The MBD proteins are only capable of binding to 5-mC methylation; they cannot be used to enrich for 5-hmC methylation.

Kit Performance and Benefits

The MeDIP kit is designed to enrich for single-stranded DNA containing 5-methylcytosines (5-mC).

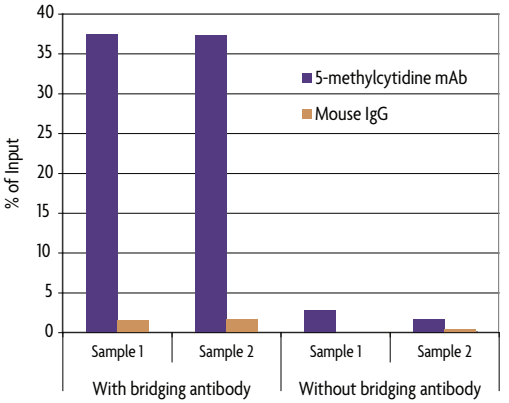
Range of detection: We recommend using between 100 ng and 1 µg fragmented genomic DNA per IP reaction.

Active Motif's Methylated DNA Standards (Catalog No. 55008) are not recommended for use as spike controls in the MeDIP Kit. The standards contain a concentrated amount of cytosine residues that are difficult to denature into single-stranded DNA for use in the MeDIP Kit. However, the Methylated DNA Standards can be used in the hMeDIP Kit (Catalog No. 55010) as that assay uses double-stranded DNA.

Cross-reactivity: The MeDIP Kit includes a highly specific monoclonal antibody for 5-methylcytosine immunoprecipitation. The antibody has reactivity with a wide range of species as it detects any DNA containing 5-methylcytosine. The unbound genomic DNA can be sequentially immunoprecipitated with the 5-hydroxymethylcytosine antibody in the hMeDIP Kit (Catalog No. 55010) to recover both 5-methylcytosine containing and 5-hydroxymethylcytosine containing DNA from the same sample.

Assay time: MeDIP includes an overnight incubation with the 5-methylcytosine antibody and approximately 2 hours of hands-on time.

MeDIP Assay



Example of MeDIP results using the 5-methylcytosine antibody and the control human genomic DNA. *MseI* digested human genomic DNA (500 ng) was processed in duplicate using the MeDIP Kit and either 5-methylcytosine monoclonal antibody or the negative control mouse IgG in the presence or absence of bridging antibody. Eluted DNA was purified and tested using real time PCR with the included ZC3H13 PCR primer mix. The methylated ZC3H13 locus is specifically enriched in the IP reactions using the 5-methylcytosine antibody and bridging antibody, while reactions containing the negative control mouse IgG or lacking bridging antibody were not enriched.

MeDIP Kit Components and Storage

MeDIP Kits are for research use only. Not for use in diagnostic procedures. All components are guaranteed stable for 6 months from date of receipt when stored properly.

Reagents	Quantity	Storage
5-methylcytosine mAb (1 µg/µl)	20 µg	-20°C
Bridging Antibody (1 µg/µl)	500 µl	-20°C
Mouse IgG (1 µg/µl)	100 µl	4°C
Protease Inhibitor Cocktail (PIC)	100 µl	-20°C
Buffer C	10 ml	-20°C
Buffer D	10 ml	-20°C
Elution Buffer AM2	1.6 ml	-20°C
Neutralization Buffer	1.6 ml	-20°C
Human genomic DNA, *Mse*I digested (20 ng/µl)	250 µl	-20°C
ZC3H13 PCR Primer Mix (2.5 µM)	400 µl	-20°C
Protein G magnetic beads*	250 µl	4°C
Sterile water	1 ml	RT
0.2 ml PCR tubes	1 pack	RT
Bar magnet and glue dots	1 ea	RT

*Protein G magnetic beads are shipped on dry ice and will arrive frozen. DO NOT refreeze the magnetic beads after their first use. Once thawed, the Protein G magnetic beads should be stored at 4°C.

Additional materials required

- Sample DNA that has been fragmented between 200-600 bp in size
- End-to-end rotator (e.g. Labquake from Barnstead/Thermolyne)
- DNase-free water
- (Optional) DNA Purification Kit (*e.g.* QIAGEN MinElute PCR Purification Kit, Catalog no. 28004)
- (Optional) Phenol/Chloroform purification reagents (Alternative to DNA purification kit) Phenol:Chloroform:Isoamyl Alcohol (25:24:1, v/v/v, pH 8.0), glycogen (20 mg/ml), 5 M ammonium acetate, 100% ethanol

Buffer Preparation and Recommendations

Samples

MeDIP reactions can be performed using 100 ng - 1 µg of fragmented genomic DNA. Fragments should range in size from 200-600 bp. Use the recommended protocols for preparing fragmented DNA by restriction enzyme digestion or sonication prior to starting the MeDIP assay.

Input

Set aside a quantity of fragmented genomic DNA equivalent to 10% of the DNA being used in the IP reaction for use as Input DNA (*e.g.* for 500 ng DNA in IP reaction, set aside an additional 50 ng DNA for Input). Separate Input DNA should be saved for each DNA source tested. The Input DNA can be used in real time PCR to quantify the amount of enriched DNA recovered from the immunoprecipitation reaction. For details on real time PCR analysis and the use of Input DNA, see the Appendix. Store the Input DNAs at -20°C until ready to use.

Control DNAs

Control human genomic *Mse*I digested DNA is included as an optional validation of immunoprecipitation efficiency. The control DNA can be run in parallel to sample DNA and analyzed with real time PCR using the provided ZC3H13 PCR primer mix. The ZC3H13 locus should be methylated in the control DNA and is expected to be enriched in IP samples using the 5-methylcytosine monoclonal antibody. The control DNA is provided at a concentration of 20 ng/µl. We recommend using 500 ng per control IP reaction.

Buffer C and Buffer D

We recommend to use ice-cold Buffer C and Buffer D for the wash steps. Please chill both buffers on ice before use. This is especially important when working with single-stranded DNA in order to prevent re-annealing.

Read the entire protocol before use.

NOTES BEFORE STARTING

Sample Preparation: Fragmentation of Genomic DNA

Prior to starting the MeDIP assay, genomic DNA should be fragmented using either mechanical fragmentation (*e.g.* sonication) or restriction digestion with a methylation-insensitive restriction enzyme to yield fragments ranging in size from 200 to 600 base pairs.

Methylated DNA immunoprecipitation will enrich for methylated cytosines regardless of their sequence context. If trying to evaluate methylation of CpG dinucleotides, such as in CpG islands, please follow the recommendations below for fragmentation of genomic DNA.

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 "Troubleshooting" in the Appendix for further discussion).

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 single DNA sample will be used for simultaneous analysis of many CpG islands (*e.g.*, when the isolated DNA will be analyzed by microarray or MeDIP-Seq 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

Example fragmentation protocols are provided for both restriction digest and mechanical fragmentation. We suggest using 4 µg of purified genomic DNA when performing restriction digestion and 20 µg of purified genomic DNA for sonication. MeDIP and hMeDIP reactions can be performed on 100 ng - 1 µg of fragmented genomic DNA. Reactions may be scaled based on sample size.

Restriction digest

This protocol can be modified depending on the amount of isolated genomic DNA, the restriction enzyme being used, or supplier. 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	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.

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.

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) 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.

MeDIP Protocol

The 5-methylcytosine antibody included in the MeDIP kit requires the use of single-stranded DNA for efficient immunoprecipitation. It is critical to use ice-cold buffers and keep your samples on ice following the initial denaturation step in order to prevent the DNA from re-annealing.

Note: For Next-Gen sequencing analysis please see "Library Preparation Protocol for MeDIP-Seq" in the Appendix for instructions on how to prepare your genomic DNA. Because MeDIP requires single-stranded fragments, library prep protocols which are designed for double-stranded DNA, will not work. If you wish to perform NGS analysis following MeDIP we suggest using the xGen ssDNA & Low-Input DNA Library Preparation Kit from IDT (Catalog # 10009859). Alternatively, if a standard double-stranded NGS DNA library prep protocol is desired, adapter ligation can be done prior to Step 1: IP Reaction.

Step 1: IP Reaction

1. Set up a 200 µl PCR tube for each IP reaction to be performed. If desired, control reactions should be set up for the mouse IgG as well as the control DNA. We recommend using 500 ng of the control DNA per IP reaction.
2. Calculate the amount of reagent needed for each IP reaction.

   Fragmented DNA: Recommended range between 100 ng and 1 µg.

   Input DNA: Set aside an additional quantity of fragmented genomic DNA equivalent to 10% of the DNA being used in the IP reaction for use as Input DNA. Separate Input DNA should be saved for each DNA source tested. Input DNA should be heat denatured and cooled as in instructions #4-5 below, then stored at -20°C.

   Sterile water: Determine the amount of sterile water needed to bring each reaction to a final volume of 95 µl per IP.
3. Add reagents in the order listed below to each PCR tube.

Reagents	Sample DNA	Mouse IgG Negative Control	(Optional) Control DNA
Sterile water	_____ µl	_____ µl	60 µl
Buffer C	10 µl	10 µl	10 µl
Fragmented DNA	_____ µl	_____ µl	25 µl
Total Volume	**95 µl**	**95 µl**	**95 µl**

4. Incubate the samples in a PCR machine at 95°C for 10 minutes to denature the DNA.
5. Immediately transfer the samples to a 4°C ice bath and incubate for 10 minutes. It is critical

to keep DNA on ice at all times to maintain the DNA in it's single-stranded form. Quick spin tubes at 4°C.

6. Add the remaining reagents in the order listed in the table below to each PCR tube. The final reaction volume will be 100 µl.

Reagents	Sample	Mouse IgG Negative Control	(Optional) Control DNA
PIC	1 µl	1 µl	1 µl
5-methylcytosine antibody	2 µl	–	2 µl
Bridging antibody	2 µl	2 µl	2 µl
Mouse IgG	–	2 µl	–
Total Volume	**100 µl**	**100 µl**	**100 µl**

7. Quickly spin the Protein G magnetic beads vial to remove beads from the cap. Cut off the end of a P-1000 pipet tip. Set the pipet to a volume of 200 µl. Resuspend the beads by gently pipetting up and down. Ensure that the beads are fully resuspended before use.
8. Using a P-200 pipet and an uncut tip, add 25 µl Protein G magnetic beads to each tube.
9. Cap the PCR tubes tightly. Incubate overnight with end-to-end rotation at 4°C.

Step 2: Washing and Elution

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 immunoprecipitation with a 5-hydroxymethylcytosine antibody, place supernatant in a microcentrifuge tube and store at -20°C. Otherwise, carefully remove and discard the supernatant. To use the magnet provided in the kit, please see the Appendix.
2. Wash beads three times with 200 µl ice-cold Buffer C. 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 200 µl ice-cold Buffer C 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.

3. Wash beads two times with 200 µl ice-cold Buffer D. 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.
4. Resuspend the washed beads with 50 µl Elution Buffer AM2 by pipetting 2-3 times.
5. Incubate for 15 minutes at 4°C with end-to-end rotation to keep the beads in suspension.
6. Briefly centrifuge the tubes to collect liquid from the cap.
7. Place tubes in magnetic stand and allow beads to pellet onto tube sides.
8. Transfer the supernatant, which contains the enriched DNA, to a fresh tube.
9. Add 50 µl Neutralization Buffer and pipette up and down to mix.
10. For downstream applications like real-time PCR and Next-Generation Sequencing, DNA should be further purified. See Section C of the Appendix for purification recommendations.

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Appendix

Section A. Protocol for Library Preparation for MeDIP-Seq

Because MeDIP requires single-stranded fragments, library prep protocols which are designed for double-stranded DNA, will not work. There are two options recommended for library preparation for MeDIP-Seq when using this kit. Our recommendation is to use a library prep kit designed for single-stranded DNA like the xGen™ ssDNA & Low-Input DNA Library Prep Kit (IDT Catalog no. 10009859) ***after*** the immunoprecipitation (MeDIP) protocol. Alternatively, adapters can be ligated to the fragmented double-stranded DNA ***prior to*** performing the immunoprecipitation (MeDIP) protocol and then library amplification can be performed after MeDIP.

Option 1: The easiest option is to use a DNA library prep kit designed for single-stranded DNA after completing the immunoprecipitation (MeDIP) protocol. After completing the MeDIP protocol, purify the DNA using a purification kit like the MinElute PCR Purification Kit (QIAGEN Catalog no. 28004), eluting in 15 µl. The entire eluted volume of DNA can then be used directly in a library prep kit designed for ssDNA. The Active Motif MeDIP protocol has been validated using the xGen™ ssDNA & Low-Input DNA Library Prep Kit (IDT Catalog no. 10009859). Confirm successful library amplification by checking DNA yield, size range, and concentration using standard methods, such as Nanodrop, gel electrophoresis and/or Bioanalyzer analysis. Minimal yield should be 1-2 µg of DNA with a concentration range of 50-100 ng/µl. For more detailed instructions please contact Active Motif Technical Support.

Option 2: Alternatively, end repair and adapter ligation can be done while the DNA is double-stranded, after fragmentation, and before denaturing the DNA in the immunoprecipitation (MeDIP) protocol. After adapter ligation and before MeDIP, the DNA should be purified using a DNA clean-up system like the QIAGEN MinElute PCR Purification Kit (QIAGEN Catalog no. 28004), eluting in 30-50 µl elution buffer (EB). Use the adapter ligated DNA as the fragmented DNA in Step 1 of the MeDIP protocol. Check the concentration on a Nanodrop before proceeding with MeDIP. After MeDIP, DNA should be purified before library amplification. Confirm successful library amplification by checking DNA yield, size range, and concentration using standard methods, such as nanodrop, gel electrophoresis and/or Bioanalyzer analysis. Minimal yield should be 1-2 µg of DNA with a concentration range of 50-100 ng/µl. For more detailed instructions please contact Active Motif Technical Support.

Section B. 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.

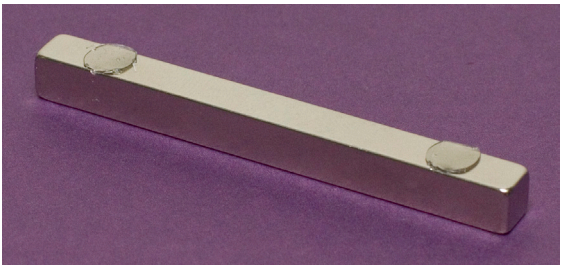
Assembly of Magnetic Stands

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.

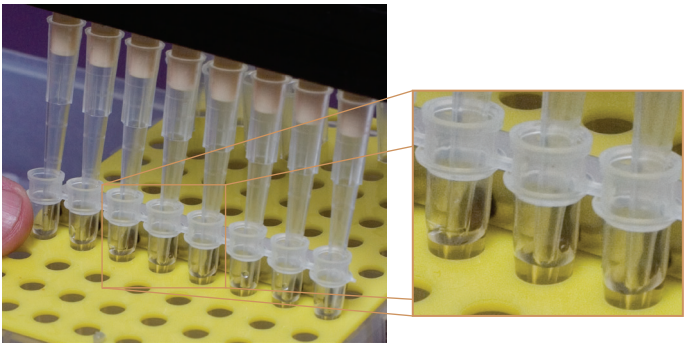


4. Remove the covering tape from the exposed side of the glue dots. Fix the magnet to the tip box so that it is against the PCR tubes. The magnetic stand is now ready for use.

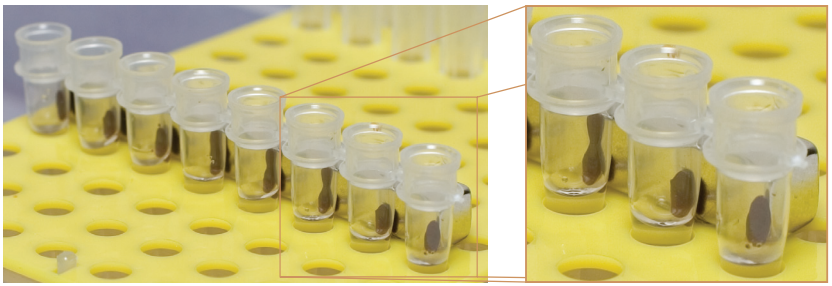
Note: Familiarize yourself with using the magnetic stand before performing with PCR tubes for the first time. Add 5 µl of magnetic beads to 100 µl Buffer C in one tube of an 8-well strip of PCR tubes. Use this tube with the assembled bar magnet stand to become familiar with use of the beads and magnet. 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 C: DNA Purification

DNA Purification

DNA should be purified prior to use in most downstream applications, such as real time PCR or library prep. DNA clean up can be performed using one of the methods listed below. Input DNAs should be removed from -20°C and processed in parallel with the sample IP reactions. For single-stranded DNA, the Input DNA should be thawed on ice.

- **Purification columns** – Use a DNA clean up kit, that has been validated to work with single-stranded DNA. We recommend the QIAGEN MinElute PCR Purification Kit (Qiagen part no. 28004). Elute in an appropriate volume of Buffer EB or sterile 10 mM Tris-HCl pH 7.5-8 for downstream application.
- **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.
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)

   1 sample volume of 5 M ammonium acetate

   2.5 sample volumes of ice-cold 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 ice-cold 70% ethanol. Do not disturb the pellet.
10. Centrifuge the tube for 10 minutes at 12,000 x *g*, 4°C.
11. Immediately 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 sterile 10 mM Tris-HCl pH 7.5-8. Use appropriate volume of buffer or water as needed for specific downstream application.
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 to ensure the DNA is not bound to the plastic of the tubes.

Section D. PCR Analysis

PCR Primer Design

The MeDIP Kit includes ZCH13 PCR primers for use with the provided human genomic DNA. Additional positive and negative control primer sets for MeDIP are available from Active Motif. 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 end point PCR or an amplicon of 100-150 bp for real time PCR.

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 the 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. IDT has a very good one. Due to the technical limitations of PCR, it is sometimes necessary to design more than one primer pair for a given fragment of interest. Primers that dimerize should be avoided as they compromise accurate quantitation..

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 20. Use the slope of the plotted standard curve in the primer efficiency equation above.

Real Time PCR Analysis

It is important to purify enriched DNA prior to use in downstream applications. The samples and Input DNAs should be subjected to a DNA clean-up step prior to real time PCR analysis (refer to the Appendix). Below is an example PCR reaction. Please follow the specific instructions for your real time PCR instrument.

1. For notes on preparing standard curves with the Input DNA, please see page 20.
2. 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 ZC3H13 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.

Note: It is recommended to prepare triplicates of each sample and Input reaction.

3. Place tubes in a real time PCR instrument and program as below. The amplification conditions should be optimized for each target locus, master mix reagent and PCR instrument. A suggested starting point is:

   95°C for 2 minutes
   (95°C for 3 seconds, 60°C for 30 seconds) for 40 cycles

4. 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.

Data Analysis and Use of Input DNAs

Methylated DNA isolated using MeDIP and hMeDIP are 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 MeDIP and hMeDIP be performed on the same amount of each DNA sample. In addition, Input DNA should be prepared for each of the different DNA samples 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. For the MeDIP Assay, produce a standard curve at 3.3, 0.33, 0.033 and 0.0033 ng/µl in triplicate using the Input DNAs captured in Step 1 instruction #2. We recommend running a standard curve every time the PCR amplification is performed. An example standard curve linear regression plot is shown below.
2. Run each sample with the appropriate Input DNA standard (*i.e.* prepare a separate standard curve for each DNA source tested).
   - **Sample IP Input:** To be used with the PCR primers designed to analyze the sample DNA. This standard curve will be used to determine the amount of enriched 5-methylcytosine DNA present in the final elution. This Input can be used to quantify DNA in both the Sample IP reactions and the negative control mouse IgG reactions.
   - **(Optional) Control Human genomic DNA Input:** To be used with the provided ZC3H13 PCR primer set. This standard curve will be used to determine the amount of DNA captured in the final elution of the control reaction.
3. Every gene and/or primer set will generate a different amplification profile.
4. CT = Threshold Cycle and is the cycle number where the signal exceeds the background threshold level. CT values should be plotted for each gene to create a linear regression plot.
5. Plot CT versus log DNA concentration. The slope of the standard curve can be used to determine primer efficiency in the equation on page 18.

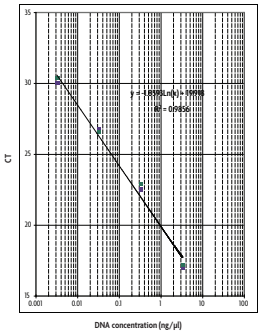


Figure 1: Example standard curve linear regression plot.

A standard curve for Input DNA is provided as a reference only. Ten-fold dilutions of Input DNA were tested in triplicate and plotted against the CT value. A new standard curve should be generated each time the assay is performed.

6. Using the CT value of the sample, extrapolate the DNA concentration of the sample DNA using the appropriate standard curve plot. To determine the total amount of enriched DNA in the sample, simply multiple the DNA concentration by the volume of enriched DNA.

7. Calculate the percent enrichment. Use the sample DNA quantity calculated above and compare it with the amount of DNA used in the initial immunoprecipitation reaction (Step 1).

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

$$\textbf{Fold enrichment} = \frac{\text{Amount of enriched Sample DNA material (ng)}}{\text{Amount of enriched rabbit IgG material (ng)}}$$

Section E: Troubleshooting Guide

Problem/question	Recommendation
Little or no enrichment of methylated DNA	The 5-methylcytosine antibody can be used only with single-stranded DNA. It is critical to place the DNA on ice immediately after heating to 95°C in order to prevent re-annealing.
	Fragmented DNA of less than 600 bp should be used in the IP reactions. The MeDIP and hMeDIP Kits are optimized for use with 100 ng - 1 µg of fragmented DNA per IP reaction. Using different DNA concentrations will alter the ratio of antibody:DNA and decrease the efficiency of the immunoprecipitation. A lack of antibody will decrease the recovery of methylated DNA, while an excess of antibody will decrease the specificity of the IP.
PCR amplification	Follow the recommendations for PCR primer design to prepare specific primers for your sample. The included ZC3H13 PCR primers are designed to work with the control human genomic DNA provided in the kit using real time PCR.
	Real time PCR results may vary depending on the SYBR Green master mix used.
Storage of DNA	Once DNA is enriched with MeDIP and hMeDIP, 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 MeDIP and hMeDIP protocols. 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.

Technical Services

If you need assistance at any time, please call or send an e-mail to Active Motif Technical Service at one of the locations listed below.

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