hMeDIP

(version B1)

Catalog No. 55010

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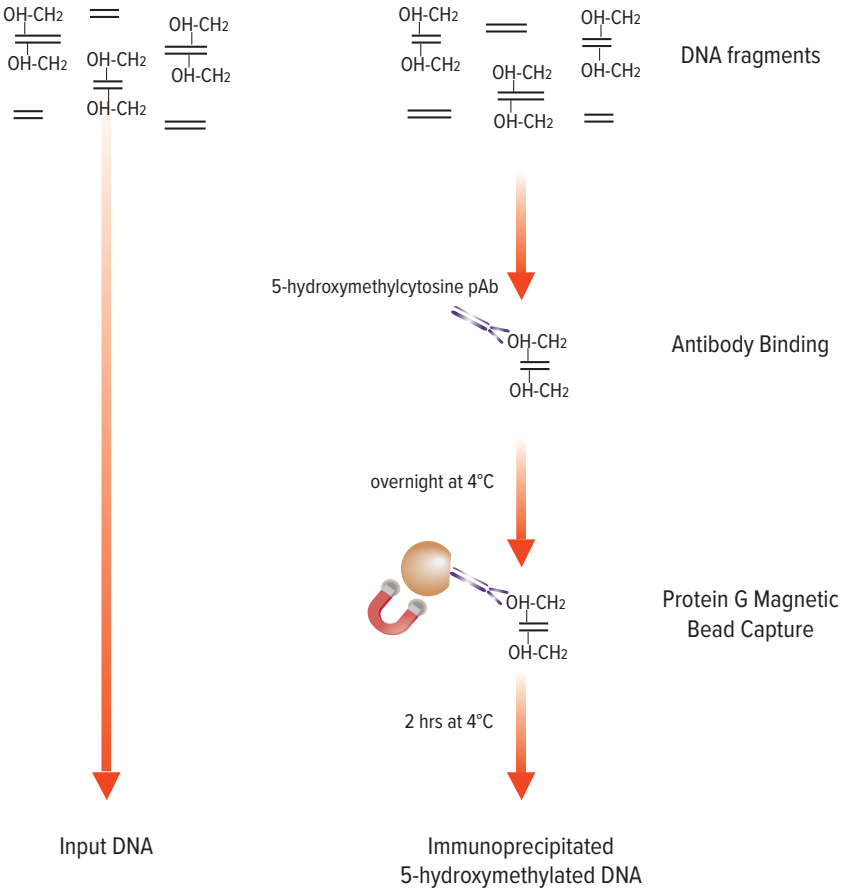
Overview

The hMeDIP Kit is designed to immunoprecipitate and enrich for DNA fragments containing 5-hydroxymethylcytosine (5-hmC). The hMeDIP Kit contains a highly specific purified 5-hydroxymethylcytosine 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 unmethylated, 5-methylcytosine and 5-hydroxymethylcytosine DNA controls and 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 hydroxymethylated DNA.

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

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

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.

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 technique 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, or in combination with microarrays for genome-wide methylation analysis. MeDIP can also be used to prepare samples for use in high-throughput sequencing (HTS) 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. Recently, 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.

To better understand the functions of 5-hmC, Active Motif has developed the hMeDIP Kit. The hMeDIP assay uses a purified 5-hydroxymethylcytosine antibody (5-hmC) to selectively enrich for DNA fragments containing 5-hmC. 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-hmC and 5-mC. Another advantage of the hMeDIP kit is that the 5-hmC antibody works efficiently to immunoprecipitate either double-stranded or single-stranded DNA.

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[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 recognized by the restriction enzymes used, but do not provide information about other methylation sites. Methylation-sensitive restriction enzymes are also unable to directly differentiate between 5-mC and 5-hmC methylation.
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

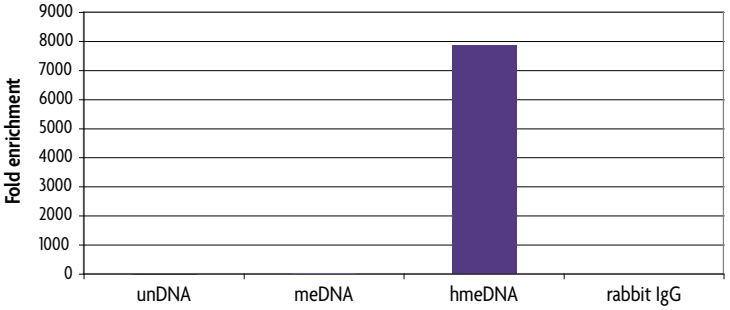
The hMeDIP kit is designed to enrich for 5-hydroxymethylcytosine (5-hmC) containing DNA.

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

Cross-reactivity: The hMeDIP Kit includes a highly specific antibody for 5-hydroxymethylcytosine immunoprecipitation. The antibody has reactivity with a wide range of species as it detects any DNA containing 5-hydroxymethylcytosine. The unbound genomic DNA can be sequentially immunoprecipitated with the 5-methylcytosine antibody in the MeDIP Kit (Catalog No. 55009) to recover both 5-methylcytosine containing and 5-hydroxymethylcytosine containing DNA from the same sample.

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

hMeDIP Assay



Example of hMeDIP results using the 5-hydroxymethylcytosine antibody and "spiked" control DNA.

MseI digested human genomic DNA (500 ng) was "spiked" with 25 pg of either methylated APC DNA, hydroxymethylated APC DNA or unmethylated APC DNA. These samples were then processed using the hMeDIP kit with the 5-hydroxymethylcytosine pAb. Eluted DNA was purified and tested using real time PCR with the included APC PCR primer mix. The 5-hydroxymethylcytosine specifically enriched the IP sample containing the hydroxymethylated APC DNA, but did not enrich for the methylated or unmethylated DNA. The APC locus analyzed in this experiment is not methylated in human genomic DNA and therefore should not amplify. This experiment was performed to detect the presence of the spiked control DNA only. The fold enrichment represents the amount of IP DNA recovered from each spike normalized against the negative control rabbit IgG IP reaction. The hMeDIP Kit is specific for 5-hmC enrichment.

hMeDIP Kit Components and Storage

hMeDIP 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-hydroxymethylcytosine pAb (1 µg/µl)	50 µg	-20°C
Rabbit IgG (1 µg/µl)	100 µl	-20°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
Hydroxymethylated APC DNA (50 ng/µl)	10 µl	-20°C
Methylated APC DNA (50 ng/µl)	10 µl	-20°C
Unmethylated APC DNA (50 ng/µl)	10 µl	-20°C
APC PCR Primer Mix (2.5 µM)	400 µl	-20°C
Protein G magnetic beads*	250 µl	4°C
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
- DNA purification kit (e.g. QIAGEN MinElute PCR Purification Kit, Catalog no. 28004)
- (Optional, alternative to DNA purification kit) Phenol/Chloroform purification reagents. 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

hMeDIP 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 hMeDIP assay.

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 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 Appendix. Store the Input DNAs at -20°C until ready to use.

Control DNAs

A 338 base pair fragment containing 122 cytosine residues from the APC (adenomatosis polyposis coli) gene promoter was amplified by PCR. The unmethylated, 5-methylcytosine and 5-hydroxymethylcytosine containing versions were created by the inclusion of either unmethylated dCTP, 5-methyl dCTP or 5-hydroxymethyl dCTP in the PCR reaction mix.

Separate control reactions can be set up to verify the immunoprecipitation efficiency. For details on setting up control reactions, please refer to Appendix. Each control DNA should be "spiked" into sample DNA according to the protocol and the IP reactions performed as indicated. We recommend maintaining a control DNA spike to fragmented DNA ratio of 1:20,000 (*e.g.* 25 pg spike DNA standards in 500 ng fragmented genomic DNA = 1:20,000 ratio). Each control DNA is provided at a concentration of 50 ng/µl.

The Methylated DNA Standards are also available separately as Catalog No. 55008.

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 if working with single-stranded DNA in order to prevent re-annealing.

Assay Protocol

Read the entire protocol before use.

NOTES BEFORE STARTING

Sample Preparation: Fragmentation of Genomic DNA

Prior to starting the hMeDIP 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 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 "Appendix: Troubleshooting" 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 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.

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)	_ µl
10X NEB Buffer 4	10 µl
100X BSA	1 µl
Mse I (10 U/µl)	1 µl
dH_2O	_ µl
Total volume	**100 µl**

Note 1: The DNA volume depends 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. See "Appendix. Troubleshooting" 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) 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.

hMeDIP Protocol

The 5-hydroxymethylcytosine antibody included in the hMeDIP kit efficiently immunoprecipitates double-stranded DNA, therefore it is not necessary to denature the DNA prior to immunoprecipitation. However, if it is desired to work with single-stranded DNA, please follow the recommendations for denaturing DNA in the Appendix.

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 rabbit IgG as well as the included control DNAs. For notes on using the included control DNAs, please see Appendix.
2. Calculate the amount of reagent needed for each IP reaction.

   Fragmented DNA: Recommended range between 100 ng and 1 µg. 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 and store at -20°C. Separate Input DNA should be saved for each DNA source.

   Sterile water: Determine the amount of sterile water needed to bring the reaction to a final volume of 96 µl per IP.
3. Add reagents in the order listed below to each PCR tube.

Reagents	Sample	Rabbit IgG Negative Control
Sterile water	_____ µl	_____ µl
Buffer C	10 µl	10 µl
Fragmented DNA	_____ µl	_____ µl
PIC	1 µl	1 µl
Total Volume	96 µl	96 µl

4. Add 4 µl 5-hydroxymethylcytosine pAb to the sample IP reactions. Add 4 µl rabbit IgG to the negative control reaction. The total reaction volume is now 100 µl.
5. Cap the PCR tubes tightly. Incubate overnight with end-to-end rotation at 4°C.

Step 2: DNA Capture

1. Quickly spin the PCR tubes to collect the contents at the bottom.
2. 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.

3. Using a P-200 pipet and an uncut tip, add 25 µl Protein G magnetic beads to each tube.
4. Incubate the antibody-bound DNA with the magnetic beads for 2 hours at 4°C with end-to-end rotation.

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-methylcytosine 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 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 room temperature with end-to-end rotation to keep the beads in suspension.
6. Briefly centrifuge the tubes to collect liquid from the cap.
7. Add 50 µl Neutralization Buffer and pipette up and down to mix.
8. Place tubes in magnetic stand and allow beads to pellet onto tube sides.
9. Transfer the supernatant, which contains the enriched DNA, to a fresh tube.
10. For downstream applications like real-time PCR and Next-Generation Sequencing, DNA should be further purified. See Appendix, Section D for purification recommendations. The hMeDIP DNA fragments are double-stranded and will work with commerical DNA library prep kits designed for sub-nanogram levels of DNA. Please contact technical support for guidance.

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Appendix

Section A. Denaturing DNA (Optional)

Denaturing the DNA is not required for hMeDIP. However, if it is desired to work with single-stranded DNA, please follow the recommendations for denaturing DNA below.

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 rabbit IgG as well as the included control DNAs. For notes on using the included control DNAs, please see Appendix, Section B.
2. Calculate the amount of reagent needed for each IP reaction.

   Fragmented DNA: Recommended range between 100 ng and 1 µg. 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. 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 the 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	Rabbit IgG Negative Control
Sterile water	_____ µl	_____ µl
Buffer C	10 µl	10 µl
Fragmented DNA	_____ µl	_____ µl
Total Volume	95 µl	95 µl

4. Incubate the samples in a PCR machine at 95°C for 10 minutes to denature the DNA.
5. Quickly 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 1 µl PIC to each PCR tube.
7. Add 4 µl 5-hydroxymethylcytosine pAb to the sample IP reactions. Add 4 µl rabbit IgG to the negative control reaction. The total reaction volume is now 100 µl.
8. Cap the PCR tubes tightly. Incubate overnight with end-to-end rotation at 4°C.
9. Proceed with Step 2: DNA Capture on page 10.

Section B. Use of Included Control DNAs

Control DNAs are included as an optional validation of immunoprecipitation efficiency. When control DNA is "spiked" into the sample genomic DNA and immunoprecipitated, it can be analyzed in real time PCR using the provided APC PCR primer mix to verify that the immunoprecipitation reactions selectively enriched for 5-hydroxymethylcytosine DNA as compared to 5-methylcytosine or unmethylated DNA. The APC locus in not methylated in normal, human genomic DNA, therefore if the source of the genomic DNA used is normal human, the only enrichment you should expect is from the "spiked" hmeDNA. For sources of genomic DNA other than normal human, you will need to run a sample IP reaction without any control DNA alongside the spike reactions to determine the amount of enrichment from naturally occurring APC methylation in your genomic DNA sample versus the amount of enrichment due to the control DNA spike. There should be an increase in enrichment in spiked samples as compared to genomic DNA alone.

1. Set up a 200 µl PCR tube for each IP reaction to be performed.
2. Calculate the amount of reagent needed for each IP reaction.

   Fragmented DNA: Recommended range between 100 ng and 1 µg. 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 and store at -20°C. Separate Input DNA should be saved for each DNA source.

   Sterile water: Determine the amount of sterile water needed to bring the IP reaction to a final volume of 96 µl per IP.

   Control DNAs: The amount of spiked control DNA (meDNA, hmeDNA and unDNA) should be determined based on the amount of DNA used in the IP reaction. We recommend maintaining a spiked control DNA to fragmented DNA ratio of 1:20,000.

   For each control DNA prepare a 10% excess of spiked control DNA in fragmented genomic DNA. Mix well by vortexing. Remove 10% of the spiked genomic DNA reaction and set aside for use as Input DNA for quantification during real time PCR. Set aside a separate Input DNA for each spiked control (**i.e.** meDNA, hmeDNA and unDNA). Input DNA can be stored at -20°C until ready to use. Some example calculations are shown below for reference.

Control spike DNA	Fragmented Genomic DNA	Quantity removed for Input DNA	Final control spike DNA per IP rxn	Final genomic DNA per IP rxn	Ratio of spike to genomic
5.5 pg	110 ng	10% volume	5 pg	100 ng	1:20,000
27.5 pg	550 ng	10% volume	25 pg	500 ng	1:20,000
55 pg	1.1 µg	10% volume	50 pg	1 µg	1:20,000

Control DNAs are provided at a concentration of 50 ng/µl. Please follow the recommendations in the table below for genomic DNA concentration. We recommend performing the first dilution of control DNA in sterile water (Tube 1), followed by subsequent dilutions into genomic DNA at the appropriate stock concentration. The dilutions are performed in genomic DNA in order to avoid over-diluting the genomic DNA concentration used in the final IP reactions. Follow the same dilution steps for each of the control DNAs (meDNA, hmeDNA and unDNA). Mix each dilution step by vortexing.

Genomic DNA concentration	**4 ng/µl**	**20 ng/µl**	**40 ng/µl**
Spike + genomic DNA quantity	**5 pg in 100 ng**	**25 pg in 500 ng**	**50 pg in 1 µg**
Tube 1	5 µl control DNA into 5 ml H_20 = 50 pg/µl	5 µl control DNA into 995 µl H_20 = 0.25 ng/µl	5 µl control DNA into 495 µl H_20 = 0.5 ng/µl
Tube 2	2 µl Tube 1 into 18 µl genomic DNA = 5 pg/µl	2 µl Tube 1 into 18 µl genomic DNA = 25 pg/µl	2 µl Tube 1 into 18 µl genomic DNA = 50 pg/µl
Tube 3	2 µl Tube 2 into 48 µl genomic DNA = 0.2 pg/µl	2 µl Tube 2 into 48 µl genomic DNA = 1 pg/µl	2 µl Tube 2 into 48 µl genomic DNA = 2 pg/µl
Input DNA volume	2.5 µl Tube 3	2.5 µl Tube 3	2.5 µl Tube 3
IP reaction volume	25 µl Tube 3	25 µl Tube 3	25 µl Tube 3

3. Set up IP reactions by adding reagents in the order listed to each PCR tube.

Reagents	**Sample DNA**	**Rabbit IgG Control**	**meDNA spike**	**hmeDNA spike**	**unDNA spike**
Sterile water	_____ µl	_____ µl	_____ µl	_____ µl	_____ µl
Buffer C	10 µl	10 µl	10 µl	10 µl	10 µl
Fragmented DNA	_____ µl	_____ µl	None	None	None
Fragmented DNA + meDNA control	None	None	_____ µl	None	None
Fragmented DNA + hmeDNA control	None	None	None	_____ µl	None
Fragmented DNA + unDNA control	None	None	None	None	_____ µl
PIC	1 µl	1 µl	1 µl	1 µl	1 µl
Total Volume	**96 µl**	**96 µl**	**96 µl**	**96 µl**	**96 µl**

4. Add 4 µl 5-hydroxymethylcytosine pAb to the sample IP reactions and the meDNA, hmeDNA and unDNA control IP reactions. Add 4 µl rabbit IgG to the negative control reaction. The total reaction volume is now 100 µl.
5. Cap the PCR tubes tightly. Incubate overnight with end-to-end rotation at 4°C.
6. Proceed with Step 2: DNA Capture on page 10

Notes about Input DNA

- **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-hydroxymethylcytosine DNA present in the final elution. This Input can be used to quantify DNA in both the Sample IP reactions and the negative control rabbit IgG reactions.
- **meDNA spike Input:** To be used with the provided APC PCR primer set. This standard curve will be used to determine the amount of meDNA captured in the final elution of the meDNA control reaction.
- **hmeDNA spike Input:** To be used with the provided APC PCR primer set. This standard curve will be used to determine the amount of hmeDNA captured in the final elution of the hmeDNA control reaction.
- **unDNA spike Input:** To be used with the provided APC PCR primer set. This standard curve will be used to determine the amount of unDNA captured in the final elution of the unDNA control reaction.

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.

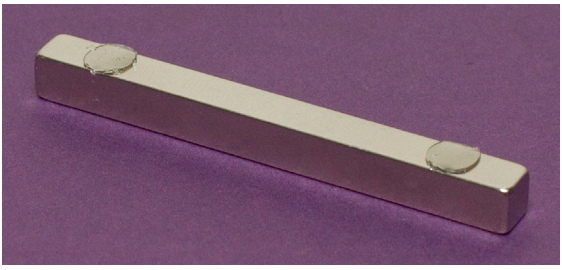
Assembly of Magnetic Stands

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.

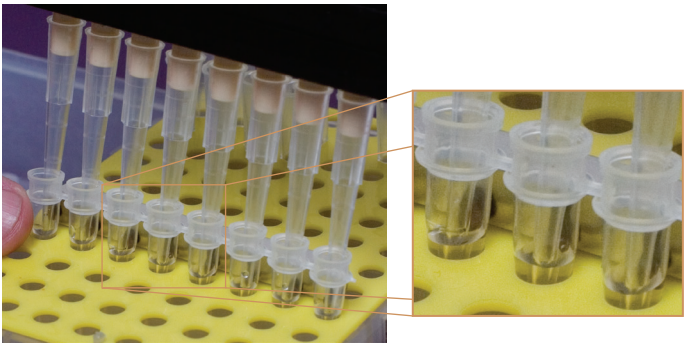


4. Remove the covering tape from the exposed side of the glue dots. Fix the magnet to the tip box so that it is against the PCR tubes. The magnetic stand is now ready for use.

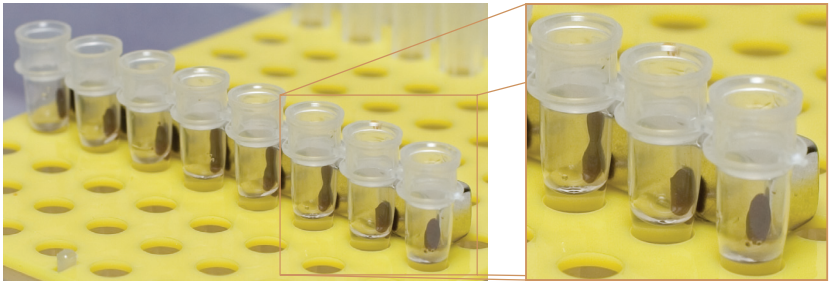
Note: Familiarize yourself with using the magnetic stand before performing with PCR tubes for the first time. Add 5 µl of magnetic beads to 100 µl Buffer C in one tube of an 8-well strip of PCR tubes. Use this tube with the assembled bar magnet stand to become familiar with use of the beads and magnet.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

DNA Purification

DNA should be purified prior to use in most downstream applications, such as real time PCR or library prep for next gen sequencing. 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. If the Denaturing DNA protocol was used as in Appendix, Section A, the Input DNA is single-stranded DNA and should be thawed on ice.

- **Purification columns** – Use a DNA clean up kit with a low elution volume such as 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 50 µl sterile DNase-free water or 10 mM Tris-HCl pH 7.5. Use other appropriate volumes of buffer or water as needed for specific downstream applications.
14. This eluted DNA can be used immediately in PCR or stored at -20°C. If the DNA is stored at -20°C, it will need to be reheated at 37°C for 10 minutes prior to use in PCR reactions to ensure the DNA is not bound to the plastic of the tubes.

Section E. PCR Analysis

PCR Primer Design

The hMeDIP Kit includes APC PCR primers for use with the provided methylated control DNAs. If possible, real time PCR is recommended for analysis of DNA isolated with MeDIP or hMeDIP. 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 (*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. 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 (http://genome.ucsc.edu/) 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 23. 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 Appendix D). 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 23.
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 APC 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 hMeDIP 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 calculation is provided on page 25.
2. Run each sample with the appropriate Input DNA standard (*i.e.* prepare a separate standard curve for each DNA source or spiked control tested. Sample IP, meDNA, hmeDNA and unDNA should each have their own standard curves).
   - **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-hydroxymethylcytosine DNA present in the final elution. This Input can be used to quantify DNA in both the Sample IP reactions and the negative control rabbit IgG reactions.
   - **(Optional) meDNA spike Input:** To be used with the provided APC PCR primer set. This standard curve will be used to determine the amount of meDNA captured in the final elution of the meDNA control reaction.
   - **(Optional) hmeDNA spike Input:** To be used with the provided APC PCR primer set. This standard curve will be used to determine the amount of hmeDNA captured in the final elution of the hmeDNA control reaction
   - **(Optional) unDNA spike Input:** To be used with the provided APC PCR primer set. This standard curve will be used to determine the amount of unDNA captured in the final elution of the unDNA 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. See Figure 1 on page 24. The slope of the standard curve can be used to determine primer efficiency in the equation on page 21.

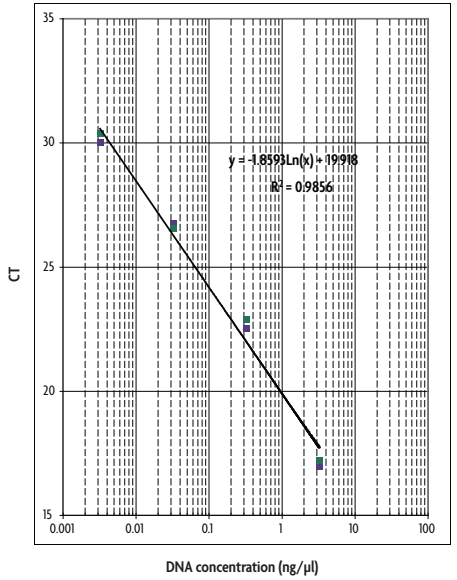


Figure 1: Example standard curve linear regression plot.

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).

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

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

Example Standard Curve Calculations

To illustrate how to set up a standard curve at the recommended concentrations of 3.3, 0.33, 0.033 and 0.0033 ng/µl, example calculations are shown below for the preparation of a standard curve using Input DNA from the optional spike reactions containing 25 pg spike DNA in 500 ng genomic DNA. It is critical to adjust DNA concentrations and DNA quantities based on the actual amount of spike DNA and genomic DNA used in the reactions in order to obtain accurate quantification of eluted material. Perform the following serial dilutions to generate the DNA standards listed in the table below.

Input: Contains 10% of initial IP reaction. (2.5 pg spike DNA in 50 ng genomic DNA)

Tube 1: Take the 2.5 µl Input DNA and add 12.5 µl sterile water. Vortex to mix.

Tube 2: Make a 1:10 dilution by transferring 5 µl DNA from Tube 1 into 45 µl sterile water. Vortex to mix.

Tube 3: Make a 1:10 dilution by transferring 5 µl DNA from Tube 2 into 45 µl sterile water. Vortex to mix.

Tube 4: Make a 1:10 dilution by transferring 5 µl DNA from Tube 3 into 45 µl sterile water. Vortex to mix.

Tube #	Genomic DNA conc.	Spike DNA conc.	Final volume	Volume per PCR	Genomic DNA qty.	Spike DNA qty.
Input	50 ng	2.5 pg	2.5 µl	N/A	N/A	N/A
1	3.3 ng/µl	0.166 pg/µl	15 µl	3 µl	10 ng/well	0.5 pg/well
2	0.33 ng/µl	0.0166 pg/µl	50 µl	3 µl	1 ng/well	0.05 pg/well
3	0.033 ng/µl	0.00166 pg/µl	50 µl	3 µl	0.1 ng/well	0.005 pg/well
4	0.0033 ng/µl	0.000166 pg/µl	50 µl	3 µl	0.01 ng/well	0.0005 pg/well

Use 3 µl of DNA from Tubes 1-4 to set up PCR reactions for the standard curve.

Plot the CT vs. log genomic DNA concentration as shown on page 24.

Section F: Troubleshooting Guide

Problem/question	Recommendation
Little or no enrichment of methylated DNA	The 5-hydroxymethylcytosine antibody can be used with either single or double-stranded DNA. If using the optional denaturation protocol for single-stranded DNA, it is important 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 on page 21 to prepare specific primers for your sample. The included APC PCR primers are designed to work with the methylated control DNAs provided in the kit.
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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