#95176 Store at RT, 4°C & -20°C

SimpleDIP™ Hydroxymethylated DNA IP (hMeDIP) Kit

1 Kit (10 immunoprecipitations)

Cell Signaling TECHNOLOGY®

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rev. 02/08/16

For Research Use Only. Not For Use In Diagnostic Procedures.

Components Ship As: 45838S	Item #	Kit Quantity	Storage Temp
SimpleDIP™ Cell Lysis Buffer	31482	6 ml	4°C
SimpleDIP™ DNA IP Buffer (10X)	49291	5 ml	4°C
ChIP Elution Buffer (2X)	7009	1.5 ml	4°C
TE Buffer	74252	10 ml	4°C
3M Sodium Acetate, pH 5.2	89173	1.2 ml	4°C
ChIP-Grade Protein G Magnetic Beads	9006	200 µl	4°C
DNA Binding Buffer	10007	12 ml	RT
DNA Wash Buffer (add 4x volume ethanol before use)	10008	2.5 ml	RT
DNA Elution Buffer	10009	1.0 ml	RT
DNA Purification Columns	10010	15 columns	RT
Components Ship As: 27188S	**Item #**	**Kit Quantity**	**Storage Temp**
Proteinase K	10012	20 µl	-20°C
RNase A	7013	50 µl	-20°C
5-Hydroxymethylcytosine (5-hmC) (HMC31) Mouse mAb	51660	100 µl	-20°C
Mouse (G3A1) mAb IgG1 Isotype Control (DIP Formulated)	98528	100 µl	-20°C
SimpleDIP™ Hydroxymethyl Control Spike-In DNA	86179	20 µl	-20°C
SimpleDIP™ Hydroxymethyl Control Primers	20906	150 µl	-20°C

Storage: Please store components at the temperatures indicated on the individual tube labels.

Reagents Not Supplied:

1. Magnetic Separation Rack #7017/#14654
2. Phenol/Choloroform/Isoamyl Alcohol (25:24:1) Saturated with 10 mM Tris, pH 8.0, 1 mM EDTA
3. Chloroform:Isoamyl Alcohol (24:1)
4. Ethanol (96-100%)
5. 1X PBS
6. Trypsin
7. Nuclease-free water #12931
8. Taq DNA polymerase
9. dNTP mix
10. Real-Time PCR SYBR™ Green Reaction Mix

Description: The SimpleDIP™ 5-Hydroxymethylcytosine DNA IP (hMeDIP) Kit provides enough reagents to perform up to 10 genomic DNA preparations and 10 IPs from mammalian cells and is optimized for 1 µg of genomic DNA per IP. The kit components are stable for 1 year from date of shipment when stored as directed.

Specificity/Sensitivity: The SimpleDIP™ Hydroxymethylated DNA IP (hMeDIP) Kit can be utilized to detect endogenous levels of 5-hydroxymethylcytosine modifications in mammalian cells (see Figure 1). The 5-Hydroxymethylcytosine (5-hmC) (HMC31) Mouse mAb has been validated for specificity using ELISA, dot blot, and hMeDIP assays and shows high specificity for its target DNA modification (see Figures 2-4). A positive control IP spike-in DNA fragment containing 5-hydroxymethylcytosine and positive control primer set for amplification of this fragment are included in the kit. This spike-in DNA and primer set can be used as a positive control for IP with any mammalian cell type.

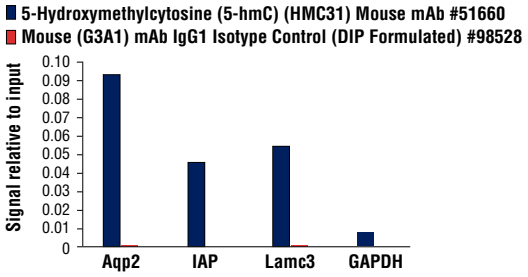

FIGURE 1. DNA immunoprecipitations were performed with 1 µg of genomic DNA from mES cells and either 10 µl of 5-Hydroxymethylcytosine (5-hmc) (HMC31) Mouse mAb #51660 or 10 µl of Mouse (G3A1) mAb IgG1 Isotype Control (DIP Formulated) using SimpleDIP™ Hydroxymethylated DNA IP (hMeDIP) Kit #95176. The enriched DNA was quantified by real-time PCR using mouse Aqp2 exon 1 primers, SimpleDIP™ Mouse Intracisternal-A Particle (IAP) LTR Primers, mouse Lamc3 exon 1 primers, and SimpleChIP® Mouse GAPDH Intron 2 Primers #8986. The amount of immunoprecipitated DNA in each sample is represented as signal relative to the total amount of input DNA, which is equivalent to one.

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Applications: **W**—Western **IP**—Immunoprecipitation **IHC**—Immunohistochemistry **ChIP**—Chromatin Immunoprecipitation **IF**—Immunofluorescence **F**—Flow cytometry **E-P**—ELISA-Peptide **Species Cross-Reactivity:** **H**—human **M**—mouse **R**—rat **Hm**—hamster **Mk**—monkey **Mi**—mink **C**—chicken **Dm**—D. melanogaster **X**—Xenopus **Z**—zebrafish **B**—bovine **Dg**—dog **Pg**—pig **Sc**—S. cerevisiae **Ce**—C. elegans **Hr**—Horse **All**—all species expected Species enclosed in parentheses are predicted to react based on 100% homology.

Introduction: Methylation of DNA at cytosine residues in mammalian cells is a heritable, epigenetic modification that is critical for proper regulation of gene expression, genomic imprinting and development (1,2). DNA methylation is a repressive epigenetic mark established *de novo* by two enzymes, DNMT3a and DNMT3b, and it is maintained by DNMT1 (3,4). Methylation of DNA was originally thought to be passively depleted during DNA replication. However, subsequent studies showed that Ten-Eleven Translocation (TET) family members can cause the hydroxylation of methylated cytosine, supporting the notion of active cytosine demethylation (5). Additionally, hydroxymethylated cytosine (5-hmC) can be oxidized to form carboxylcytosine and formylcytosine by TET proteins, which in turn can be excised by thymine-DNA glycosylase (TDG), effectively linking cytosine oxidation to the base excision repair pathway (6,7). This highlights a mechanism whereby a methylated cytosine can be reverted back to an unmethylated state without relying on passive replacement during DNA replication. Moreover, genome-wide studies on the various cytosine modifications revealed that there is indeed large-scale, active demethylation in the mammalian genome (8).

DNA methylation normally occurs in a bi-modal fashion, such that CpG dinucleotides are largely methylated across the genome except in specific short stretches of CpG rich sequences associated with gene promoters, known as CpG islands where methylation is virtually absent (9). Cancer cell genomes often undergo global hypomethylation, while CpG-islands become hypermethylated, causing their associated promoters to become repressed (10). There is evidence that a number of aberrantly hypermethylated CpG-islands found in carcinomas occur at tumor suppressor genes such as RB1, MLH1, and BRCA1 (11).

TET protein-mediated cytosine hydroxymethylation was initially demonstrated in mouse brain and embryonic stem cells (5,12). Since then this modification has been discovered in many tissues, with the highest levels found in the brain (13). Initially thought to be an intermediate species, there is mounting evidence showing that 5-hmC is a distinct epigenetic mark with various unique functions (14-16). The modified base itself is stable in vivo and interacts with various readers including MeCP2 (16,17). The global level of 5-hmC increases during brain development and 5-hmC is enriched at genic regions and poised enhancers. Furthermore, there is an inverse correlation between levels of 5-hmC and histone H3K9 and H3K27 trimethylation, suggesting a role for hmC in gene activation (17). Lower amounts of 5-hmC have been reported in various cancers including myeloid leukemia and melanoma (18-19).

DNA immunoprecipitation (DIP) is a technique that uses antibodies to immunoenrich for regions of the genome containing modified cytosine residues. This assay was first used with a 5-methylcytosine antibody to identify differentially methylated sites within normal and transformed cells (20). Investigators can use the DIP assay to look at specific genomic loci or look across the entire genome by utilizing next-generation sequencing (NGS) (21).

When performing the DIP assay, cells are lysed and the nucleic acids are recovered using phenol-chloroform extraction and ethanol precipitation. RNA is then removed by RNase A digestion, and genomic DNA is isolated by a second round of phenol-chloroform extraction and ethanol precipitation. The resulting genomic DNA is then fragmented by either restriction enzyme digestion or sonication and subjected to immunoprecipitation (IP) using antibodies specific to the modified cytosine residue. Any sequences containing the modified cytosines will be enriched by the immunoselection process. After IP, the DNA is purified and analyzed by various methods.

Standard PCR methods can be employed if an investigator is analyzing cytosine modifications at a particular region of the genome. PCR is used to measure the relative abundance of a particular DNA sequence enriched by the modified cytosine IP versus an IP with a non-specific antibody control. PCR products are run on an agarose or acrylamide gel to facilitate quantification, and the level of enrichment of the DNA sequence is determined relative to the total amount of input DNA (percent of input). The level of enrichment can also be expressed as fold enrichment above background (enrichment relative to that of the non-specific antibody control). Real-time PCR provides a more accurate, gel-free system for the quantification of DNA enrichment. Alternatively, the DIP assay can be combined with next-generation sequencing, which allows for genome-wide analysis of modified cytosines (10).

The SimpleDIP™ Hydroxymethylated DNA IP (hMeDIP) Kit #95176 contains buffers and reagents needed to perform the DIP assay with mammalian cells (see Figure 1). After cell lysis and genomic DNA extraction, the DNA is fragmented into small fragments (200-500 bp). DNA IPs are performed using 5-Hydroxymethylcytosine (5-hmC) (HMC31) Mouse mAb #51660 and ChIP-Grade Protein G Magnetic Beads #9006. After elution from the beads, the DNA is purified using DNA purification spin columns provided in the kit. After DNA purification, the enrichment of particular DNA sequences can be analyzed by a variety of methods including standard PCR, quantitative real-time PCR, or next-generation sequencing.

In addition to providing buffers and reagents required to perform the DIP assay, the SimpleDIP™ Kit provides highly validated monoclonal antibodies to ensure specific and robust signal. These antibodies have been rigorously validated using ELISA, dot blot, and MeDIP assays and show high specificity for their target DNA modifications (see Figures 2-4). The kit also includes DNA that contains exclusively hydroxymethyl cytosine, which can be spiked-in to the IPs as a control. Thus, spiked-in DNA will be immunoprecipitated with 5-Hydroxymethylcytosine (5-hmC) (HMC1) Mouse mAb #51660, but not with the Mouse (G3A1) mAb IgG1 Isotype Control (DIP Formulated) #98528. The relative enrichment can then be quantified using the SimpleDIP™ Hydroxymethyl Control Primers #20906.

The SimpleDIP™ Kit provides enough reagents to perform up to 10 genomic DNA preparations and 10 IPs and is optimized for 1 µg of genomic DNA per IP. The SimpleDIP™ protocol can be performed in as little as two days and can easily be scaled up or down for use with more or less cells.

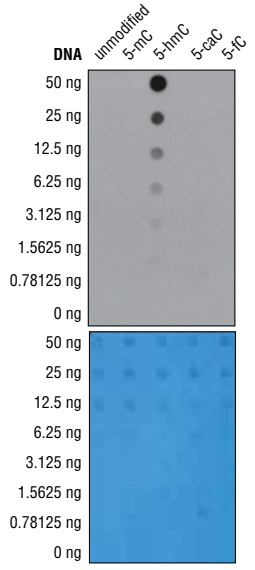

Figure 2. 5-Hydroxymethylcytosine (5-hmC) (HMC31) Mouse mAb #51660 specificity was determined by dot blot. The same sequence of a 387 base pair DNA fragment was generated by PCR using exclusively unmodified cytosine, 5-methylcytosine (5-mC), 5-hydroxymethylcytosine (5-hmC), 5-carboxylcytosine (5-caC), or 5-formylcytosine (5-fC). The respective DNA fragments were blotted onto a nylon membrane, UV cross-linked, and probed with 5-Hydroxymethylcytosine (5-hmC) (HMC31) Mouse mAb. The top panel shows the antibody only binding to the DNA fragment containing 5-hmC, while the bottom panel shows the membrane stained with methylene blue.

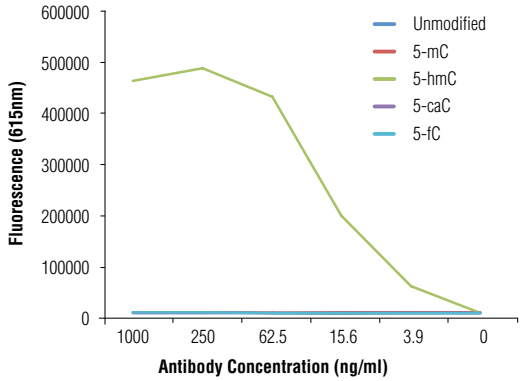

Figure 3. The specificity of 5-Hydroxymethylcytosine (5-hmC) (HMC31) Rabbit mAb #51660 was determined by an ELISA. The antibody was titrated against a single-stranded DNA oligo containing either unmodified cytosine or differentially modified cytosine (5-mC, 5-hmC, 5-caC, or 5-fC). As shown in the graph, the antibody only binds to the oligo containing 5-hmC.

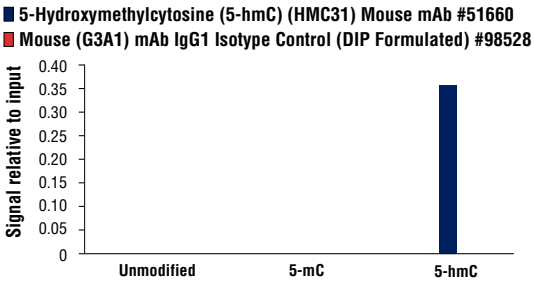

Figure 4. The specificity of 5-Hydroxymethylcytosine (5-hmC) (HMC31) Rabbit mAb #51660 was determined by MeDIP. DNA IPs were performed with genomic DNA prepared from mouse embryonic stem cells, spiked with DNA containing either unmodified cytosine, 5-methylcytosine (5-mC), or 5-hydroxymethylcytosine (5-hmC). IPs were performed using SimpleDIP™ Hydroxymethylated DNA IP (hMeDIP) Kit #95176. The enriched DNA was quantified by real-time PCR using primers specific to the spiked-in control DNA sequence. The amount of immunoprecipitated DNA in each sample is represented as signal relative to the total amount of input DNA, which is equivalent to one.

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#95176 DNA Immunoprecipitation Protocol

Section I. Genomic DNA Extraction

Before Starting:

- Stimulate or treat 5 million cells for each experiment. This number of cells will generate approximately 30 µg of DNA (30 IPs).
- Remove and warm SimpleDIP™ Cell Lysis Buffer in a 37°C water bath and ensure SDS is in solution.

Number of Cells Used	Approximate Yield
1 million	6 µg
5 million	30 µg
10 million	60 µg

1a. For suspension cells, count cells using a hemocytometer.

1b. For adherent cells, remove media and wash cells with 10 ml ice-cold 1X PBS, completely removing wash from culture dish. Add 2 ml of trypsin to remove the cells from the plate. Add 8 ml of media with serum to neutralize the trypsin after cells are completely detached and mix thoroughly. Count cells using a hemocytometer.

2. Transfer 5 million cells to a 15 ml conical tube, centrifuge at 250 x g in a bench top centrifuge for 5 min at 4°C. Wash pellet twice with 10 ml ice-cold 1x PBS. Repeat centrifugation after each wash.

3. Resuspend the cell pellet from step 2 in 500 µl of SimpleDIP™ Cell Lysis Buffer.

4. Transfer cells and buffer into a 1.5 ml microcentrifuge tube. Add 2 µl of Proteinase K to the tube and incubate overnight (12-18 hr) with shaking at 55°C.

5. Add 500 µl of phenol/chloroform/isoamyl alcohol (25:24:1) and mix thoroughly by vortexing for 30 sec.

6. Separate layers by centrifugation at 10,000 x g for 5 min in a microcentrifuge. Carefully transfer the top aqueous layer to a new tube.

7. Add 500 µl of chloroform/isoamyl alcohol (24:1) to the material and mix thoroughly by vortexing for 30 sec.

8. Separate layers by centrifugation at 10,000 x g for 5 min in a microcentrifuge. Carefully transfer the top aqueous layer to a new tube.

9. Add 50 µl of 3M Sodium Acetate, pH 5.2, then 1.0 ml of 100% ethanol chilled at -20°C. Incubate at -20°C overnight or -80°C for 1 hr to precipitate DNA.

10. Pellet DNA by centrifugation at 10,000 x g for 5 min in a microcentrifuge.

11. Carefully remove supernatant and wash pellet with 70% ethanol chilled at -20°C. Decant supernatant and air dry or vacuum dry pellet.

12. Resuspend pellet in 500 µl of TE Buffer and add 2 µl of RNase A. Incubate for 30 min at 37°C.

13. Repeat steps 5-11 and then resuspend pellet in 200 µl TE Buffer.

Section II. Genomic DNA Shearing and Quantification

1. Sonicate genomic DNA (from Section I, Step 13) for 5 pulses for 15 sec each at medium setting, keeping tube on ice for 30 sec in between each pulse.
- Genomic DNA from mouse embryonic stem cells was fragmented to sub 500 bp with 5 sets of 15 sec pulses using a VirTis VIRSONIC 100 Ultrasonic Homogenizer/Sonicator (The VirTis Company, Gardiner, NY) at setting 6 with a 1/8-inch probe.
- Please see Appendix A for further optimization of sonication conditions.

2. Remove 5 µl of the genomic DNA from Step 1 and determine DNA fragment size by electrophoresis on a 1% agarose gel with a 100 bp DNA marker. DNA should be sheared to a length of approximately 100-500 bp.

3. To determine DNA concentration, transfer 2 µl of genomic DNA from Step 1 to 98 µl TE Buffer to give a 50-fold dilution and read the OD_{260}. The concentration of DNA in µg/ml is OD_{260} x 2,500. DNA concentration should ideally be between 50 and 150 µg/ml.

Section III. DNA Immunoprecipitation

Note: The 5-Hydroxymethylcytosine (5-hmC) (HMC31) Mouse mAb binds hydroxymethylated genomic DNA only in the context of single-stranded DNA. However, next-generation sequencing library prep kits require double-stranded DNA for the adaptor ligation step and won't work efficiently with the heat-denatured DNA from the hMeDIP protocol. Therefore, before setting up the DNA immunoprecipitation, the user must perform the adaptor ligation step as recommended by the manufacturer's DNA library preparation protocol. The user should then use 1 µg of adaptor-ligated DNA for the DNA immunoprecipitation.

Before Starting:

- Remove and warm 10X SimpleDIP™ IP Buffer in a 37°C water bath and ensure there is no precipitate.

1. In one tube, prepare enough IP mix for the desired number of IPs (see table below). When determining the number of IPs, the user should include the negative control Mouse (G3A1) mAb IgG1 Isotype Control (DIP Formulated) #98528 sample and 1 additional IP to account for the 10% input. Place mix on ice.

Reagent	Amount per IP/Input
10x SimpleDIP™ DNA IP Buffer	50 µl
Sonicated genomic DNA	1 µg
SimpleDIP™ Hydroxymethyl Control Spike-In DNA (optional)	1 µl
dH_2O	Up to 500 µl final volume

2. Remove a 50 µl sample of the diluted DNA and transfer to a microfuge tube. This is the 10% input sample.

3. For each IP, transfer 500 µl of IP mix to a 1.5 ml microcentrifuge tube and heat each tube for 10 min at 95°C to denature DNA. Be sure to also heat the 10% input. Quickly put samples on an ice water bath for 5 min.
- From this point forward, it is important to keep all buffers cold and keep samples on ice to maintain single stranded DNA. The input can now be stored at -20°C until further use.

4. Add 10 µl of 5-Hydroxymethylcytosine (5-hmC) (HMC31) Mouse mAb #51660 or 10 µl of Mouse (G3A1) mAb IgG1 Isotype Control (DIP Formulated) #98528 to the appropriate IP samples. Incubate samples overnight at 4°C with rotation.

5. Resuspend ChIP-Grade Protein G Magnetic Beads #9006 by gently vortexing. Immediately, add 20 µl of beads to each sample and incubate for 2 hours at 4°C with rotation. Proceed to Section IV.

Section IV. Washing and Elution of the Immunoprecipitated DNA

- Before starting prepare and chill on ice for each IP:
- 4 ml 1X SimpleDIP™ DNA-IP Buffer (400 µl 10X SimpleDIP™ IP Buffer + 3.6 ml water)
- Remove and warm 2X ChIP Elution Buffer in a 37°C water bath and ensure SDS is in solution.
- Prepare 150 µl 1X ChIP Elution Buffer (75 µl 2x ChIP Elution Buffer + 75 µl water) for each IP and 10% input sample.
- Set a water bath or thermomixer to 65°C.

1. Pellet protein G magnetic beads (from Section III, Step 5) by placing the tubes in a magnetic separation rack. Wait 1 to 2 min for solution to clear and then carefully remove the supernatant.

2. Add 1 ml of 1X SimpleDIP™ DNA IP buffer to the beads and incubate at 4°C for 5 min with rotation.

3. Pellet protein G magnetic beads by placing the tubes in a magnetic separation Rack. Wait 1 to 2 min for solution to clear and then carefully remove the supernatant.

4. Repeat Steps 2 and 3 three additional times, for a total of 4 washes.

5. Add 150 µl of the 1X ChIP Elution Buffer to each IP sample, including the 10% input sample tube. Set aside input sample at room temperature until Step 9.

6. Elute DNA from the antibody/protein G beads for 30 min at 65°C with gentle vortexing (1,200 rpm). A thermomixer works best for this step. Alternatively elutions can be performed at room temperature with rotation, but may not be as complete.

7. Pellet protein G magnetic beads by placing the tubes in a Magnetic Separation Rack and wait 1 to 2 min for solution to clear.

8. Carefully transfer eluted DNA to a new tube.

9. Immediately proceed to Section V. Alternatively, samples can be stored -20°C. However, to avoid formation of a precipitate, be sure to warm samples to room temperature before adding DNA Binding Buffer (Section V, Step 1).

#95176 DNA Immunoprecipitation Protocol

Section V. DNA Purification using Spin Columns

Before Starting:

- Add 10 ml of ethanol (96-100%) to DNA Wash Buffer before use. This step only has to be performed once prior to the first set of DNA purifications.
- Remove one DNA spin column/collection tube for each DNA sample from Section IV.

1. Add 750 µl DNA Binding Buffer to each DNA sample and vortex briefly.
   - 5 volumes of DNA Binding Buffer should be used for every 1 volume of sample.
2. Transfer 450 µl of each sample from Step 1 to a DNA Purification Column in a collection tube.
3. Centrifuge at 18,500 x g in a microcentrifuge for 30 sec.
4. Remove the spin column from the collection tube and discard the liquid. Replace spin column in the collection tube.
5. Transfer the remaining 450 µl of sample from Step 1 to the spin column in collection tube and repeat steps 3 and 4.
6. Add 750 µl of DNA Wash Buffer to the spin column in collection tube.
7. Centrifuge at 18,500 x g in a microcentrifuge for 30 sec.
8. Remove the DNA spin column from the collection tube and discard the liquid. Replace spin column in the collection tube.
9. Centrifuge at 18,500 x g in a microcentrifuge for 30 sec.
10. Discard collection tube and liquid. Retain spin column.
11. Add 50 µl of DNA Elution Buffer to each DNA spin column and place into a clean 1.5 ml microcentrifuge tube.
12. Centrifuge at 18,500 x g in a microcentrifuge for 30 sec to elute DNA.
13. Remove and discard DNA spin column. Eluate is now purified DNA. Samples can be stored at -20°C.

Section VI. Quantification of DNA by PCR

Recommendations:

- Use filter-tip pipette tips to minimize risk of contamination.
- The control primers included in the kit are specific for the SimpleDIP™ Hydroxymethyl Control Spike-In DNA #86179 and can be used for standard PCR or quantitative real-time PCR.
- A hot-start Taq polymerase is recommended to minimize the risk of non-specific PCR products.
- PCR primer selection is critical. Primers should be designed with close adherence to the following criteria:

Primer length	24 nucleotides
Optimum Tm	60°C
Optimum GC	50%
Amplicon size	150 to 200 bp (for standard PCR) 80 to 160 bp (for real-time quantitative PCR)

Standard PCR Method:

1. Label the appropriate number of 0.2 ml PCR tubes for the number of samples to be analyzed. These should include the 10% input sample and the negative control Mouse (G3A1) mAb IgG1 Isotype Control (DIP Formulated) #98528 and a tube with no DNA to control for DNA contamination.
2. Add 2 µl of the appropriate DNA sample to each tube.
3. Prepare a master reaction mix as described below, making sure to add enough reagent for two extra tubes to account for loss of volume. Add 18 µl of master mix to each reaction tube.

Reagent	Volume for 1 PCR Reaction (18 µl)
Nuclease-free dH_2O	12.5 µl
10x PCR Buffer	2.0 µl
4 mM dNTP Mix	1.0 µl
5 µM Primers	2.0 µl
Taq DNA Polymerase	0.5 µl

4. Start the following PCR reaction program:

a.	Initial Denaturation	95°C	5 min
b.	Denature	95°C	30 sec
c.	Anneal	62°C	30 sec
d.	Extension	72°C	30 sec
e.	Repeat Steps b-d for a total of 34 cycles.		
f.	Final Extension	72°C	5 min

5. Remove 10 µl of each PCR product for analysis by 2% agarose gel or 10% polyacrylamide gel electrophoresis with a 100 bp DNA marker. The expected size of the PCR product for the postive control spike in #20906 is 120 bp.

Real-Time Quantitative PCR Method:

1. Label the appropriate number of PCR tubes or PCR plates compatible with the model of PCR machine to be used. PCR reactions should include the negative control Mouse (G3A1) mAb IgG1 Isotype Control (DIP Formulated) #98528, a tube or well with no DNA to control for contamination, and a serial dilution of the 10% input genomic DNA (undiluted, 1:5, 1:25, 1:125) to create a standard curve and determine the efficiency of amplification.
2. Add 2 µl of the appropriate DNA sample to each tube or well of the PCR plate.
3. Prepare a master reaction mix as described below. Add enough reagents for two extra reactions to account for loss of volume. Add 18 µl of reaction mix to each PCR reaction tube or well.

Reagent	Volume for 1 PCR Reaction (18 µl)
Nuclease-free H_2O	6 µl
5 µM primers	2 µl
SYBR™ Green Reaction Mix	10 µl

4. Start the following PCR Reaction program:

a.	Initial Denaturation	95°C	3 min
b.	Denature	95°C	15 sec
c.	Anneal	65°C	60 sec
d.	Repeat step b and c for a total of 40 cycles		

5. Analyze quantitative PCR results using the software provided with the real-time PCR machine. Alternatively, one can calculate the IP efficiency manually using the Percent Input Method and the equation shown below. With this method, signals obtained from each immunoprecipitation are expressed as a percent of the total input chromatin.

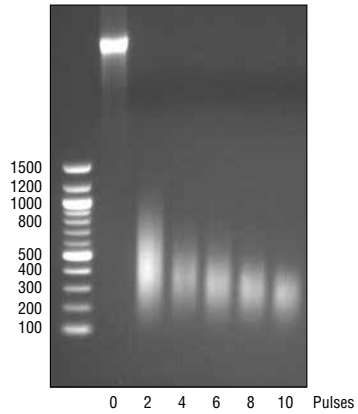
$$\text{Percent Input} = 10\% \times 2^{(C[T]\ 10\%\ \text{Input Sample} - C[T]\ \text{IP Sample})}$$

$C[T] = C_T$ = Threshold cycle of PCR reaction

Appendix A. Optimization of Sonication Conditions

Optimal conditions for shearing genomic DNA to 150-500 bp in length may depend on cell type and number of cells and the type of sonicator used. Below is a protocol to determine the optimal sonication conditions for a specific cell type and concentration of cells.

1. Prepare genomic DNA from 5 million cells as described in Section I, Steps 1-13
2. Sonicate on medium setting for 10 pulses for 15 sec each, keeping tube on ice for 30 sec in between each pulse. Take a 5 µl aliquot after every 2 pulses. Determine DNA fragment size by electrophoresis in a 1% agarose gel with a 100 bp DNA marker.
3. Observe which of the sonication conditions produces DNA in the desired range of 150-500 bp. These conditions can then be used in Section II, Step 1 in place of the suggested protocol step.



◀ *Genomic DNA from 5 million mouse ES cells was fragmented with 0, 2, 4, 6, 8, and 10 sets of 15 sec pulses using a VirTis Virsonic 100 Ultrasonic Homogenizer/Sonicator at setting 6 with a 1/8-inch probe. DNA samples were then separated by electrophoresis on a 1% agarose gel next to a 100 bp ladder and stained with ethidium bromide.*

Appendix B. TroubleShooting Guide

Protocol Step	Issue	Causes and Resolutions
Genomic DNA Extraction	Concentration of fragmented DNA is too low.	Not enough cells were added to the genomic DNA extraction. Count a separate plate of cells before performing the genomic DNA extraction to ensure an exact count. The genomic DNA extraction protocol can support up to 10 million cells per 500 ml of SimpleDIP™ Cell Lysis Buffer. Adding more than 10 million cells may inhibit cell lysis and also decrease DNA concentration.
Genomic DNA Shearing and Quantification	OD260/280 ratio is lower than 1.8 (impure DNA).	Phenol and/or salt carryover occurred during the phenol/chloroform extractions. During the extractions, leave a small amount of the top layer behind ensuring that no phenol or salt is accidentally transferred with the DNA-containing supernatant.
	DNA fragments are the incorrect size.	Sonication power or the number of pulses was not sufficient to shear the DNA properly. See Appendix A for a DNA shearing optimization protocol.
DNA Immunoprecipitation	Can I alter the amount of antibody or DNA used in the IP?	The kit was optimized for 1 µg of antibody and 1 µg of genomic DNA. Adding less antibody or DNA may decrease the recovery of hydroxymethylated DNA, while adding additional antibody or DNA may decrease the specificity of the IP and generate false positive enrichments.
	Can other antibodies be used in the kit?	The protocol has been validated and optimized for use with the antibody included in the kit. Other antibodies may not perform optimally with the protocol provided in the kit.
Quantification of DNA by PCR	Little or no enrichment of hydroxymethylated DNA	In each IP, 1 µg of antibody and 1 µg of genomic DNA should be used. Using less of either may result in decreased recovery of hydroxymethylated DNA and weaker signal.
		The antibody will only bind to single-stranded DNA, so ensure that all protocol steps after denaturation are performed on ice to prevent reannealing.
		Incomplete elution of DNA from the beads may decrease recovery of hydroxymethylated methylated DNA and result in weaker signal. Elution of DNA from protein G beads is optimal at 65°C with frequent mixing to keep beads suspended in solution.
	High background in the IgG control immunoprecipitation.	In each IP, 1 µg of antibody and 1 µg of genomic DNA should be used. Using additional antibody or DNA may generate higher background by increasing the amount of non-specific interactions. Adding less DNA could cause the signal in the IgG PCR reaction to appear higher relative to your input.
		If performing gel-based PCR, scale back on the number of cycles to be sure you are analyzing PCR products within the linear amplification phase of PCR. Otherwise the differences in quantitites of starting DNA cannot be accurately measured. Alternatively, quantify your immunoprecipitations by real-time PCR.
DIP-Sequencing	Can this kit be used in sequencing?	Yes. However, next-generation sequencing library prep kits require double-stranded DNA for the adaptor ligation step and won't work efficiently with enriched heat-denatured DNA from the MeDIP protocol. Therefore, before setting up the DNA immunoprecipitation in Section III, the user must perform the adaptor ligation step as recommended by the manufacturer's DNA library preparation protocol. The user should then use 1 µg of adaptor-ligated DNA for the DNA immunoprecipitation.
Storage	When can the protocol be stopped and the material stored until the protocol is ready to be finished?	Cell pellets can be flash frozen and stored at -80°C after Section I, Step 2.
		Genomic DNA can be stored at -20°C after Section I, Step 9 or Step 13.
		Sheared DNA can be stored at -20°C after Section II, Step 3.
		DNA IP's can be stored at -20°C overnight after Section IV, Step 9. However, to avoid formation of precipitate, be sure to warm samples to room temperature before adding DNA Binding Reagent A in Section V, Step1.
		IP'd genomic DNA can be stored at -20°C after Section V, Step 13. However, be sure to heat frozen material to 37°C for 10 minutes before use in PCR, as heat treatment releases any DNA bound to the tube during storage.