Chapter 9

Methylated DNA Immunoprecipitation (MeDIP) from Low Amounts of Cells

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Abstract

Methylated DNA immunoprecipitation (MeDIP) is an immunocapturing approach for unbiased enrichment of DNA that is methylated on cytosines. The principle is that genomic DNA is randomly sheared by sonication and immunoprecipitated with an antibody that specifically recognizes 5-methylcytidine (5mC), which can be combined with PCR or high-throughput analysis (microarrays, deep sequencing). The MeDIP technique has been originally used to generate DNA methylation profiles on a genome scale in mammals and plants. Here we provide an optimized version of the MeDIP protocol suitable for low amounts of DNA, which can be used to study DNA methylation in cellular populations available in small quantities.

Key words: DNA methylation, MeDIP, Cytosine, CpG, Profiling, Epigenomics, Microarrays, Deep sequencing

1. Introduction

DNA methylation occurs on the carbon 5 of cytosines and plays essential roles in genome regulation in a variety of organisms and in both normal and disease contexts (1). To better understand the role of this epigenetic mark, several strategies have been developed to assess the distribution of cytosine methylation at a genome-wide scale (2). Some of these technologies use methylation-sensitive (e.g., *Hpa*II) or methylation-specific (e.g., *Mcr*BC) restriction enzymes, with the caveat that they are biased towards specific restriction motifs. Other methods combine sodium bisulfite conversion and deep sequencing, which offers a powerful readout at a single-nucleotide resolution but requires large sequencing efforts when applied genome-wide (3). Alternative strategies use affinity purification of methylated DNA that can be coupled to microarray hybridization or deep sequencing. These are based on the use of

Nora Engel (ed.), *Genomic Imprinting: Methods and Protocols*, Methods in Molecular Biology, vol. 925,
DOI 10.1007/978-1-62703-011-3_9,

methyl-binding protein domains (MBD) that recognize methylated DNA, or in the case of MeDIP on the use of antibodies that specifically recognizes 5-methylcytidine (5mC). These affinity methods provide valuable tools for a rapid and unbiased profiling of DNA methylation at more limited costs.

The principle of MeDIP is that genomic DNA is randomly sonicated and immunoprecipitated with a monoclonal antibody directed against 5mC (4). The methylated fraction of the genome can be analyzed at a single-gene resolution by conventional PCR and real-time PCR, or on a genome-wide scale by microarray hybridization or deep sequencing. It is however important to keep in mind that enrichment-based methods also have certain limitations. First, they offer an incomplete resolution (defined by the size of sonicated fragments), and for this reason bisulfite sequencing still remains the method of choice when detailed methylation information at single nucleotide resolution is required. Second, there is a confounding effect of the DNA sequence because the methylation enrichment also depends on the local CpG concentration. Indeed, low MeDIP enrichments can indicate either an unmethylated state or the absence of sufficient CpG targets in very CpG-poor regions of the genome. This effect can be corrected by applying bioinformatics normalization to obtain absolute cytosine methylation levels with a relatively good accuracy (5–7). As a consequence, it also appears that the accuracy of MeDIP measurements decreases in regions that are very CpG-poor. The classical MeDIP protocol was originally designed to work with relatively large amounts of DNA (at least 2 μg) (8). Here we describe an optimized protocol that can be used to immunoprecipitate methylated DNA from as low as 20,000 cells (9) (Fig. 1).

2. Materials

2.1. Isolation of Genomic DNA

1. Eppendorf LoBind® and standard 1.5 ml microtubes.
2. Lysis buffer: 20 mM Tris pH 8.0, 4 mM EDTA, 20 mM NaCl, 2% SDS.
3. Proteinase K, 10 mg/ml stock. Store at −20 °C.
4. Dry heating block for 1.5 ml microtubes.
5. PCI (phenol–chloroform–isoamyl alcohol 25:24:1).
6. Linear polyacrylamide (LPA), 5 mg/ml stock, used as a coprecipitant.
7. Refrigerated microcentrifuge.
8. Qubit® Fluorometer (Invitrogen) for quantification of low amounts of nucleic acids.

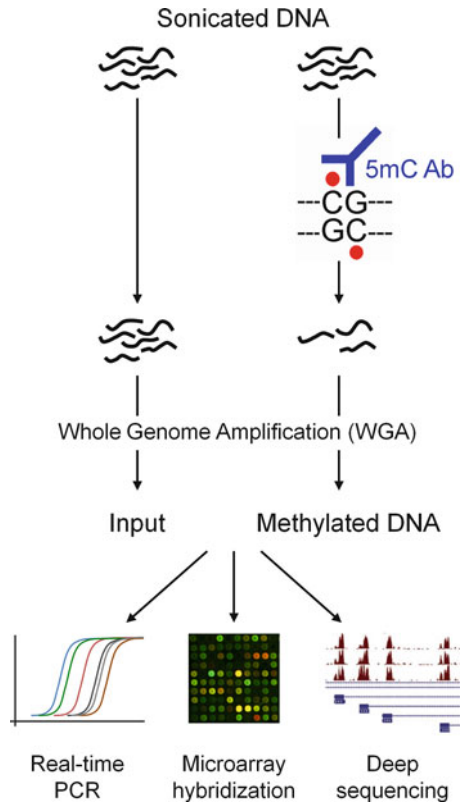

Fig. 1. Principle of MeDIP (methylated DNA immunoprecipitation). Genomic DNA is randomly sheared by sonication and immunoprecipitated with an antibody that recognizes 5-methylcytidine (5mC Ab). A portion of the sonicated DNA is left untreated and serves as input control. When MeDIP is performed on low amounts of starting DNA, a whole genome amplification (WGA) step is performed on the input and methylated DNA. Enrichments in the methylated fraction can be measured at a single gene resolution by real-time PCR, or on a global scale by microarray hybridization and deep sequencing. The deep sequencing image capture is reprinted by permission from Macmillan Publishers Ltd: *Nature Biotechnology* 28:1097-105, © 2010 -12).

2.2. Sonication

1. Eppendorf LoBind® 1.5 ml microtubes.
2. Diagenode Bioruptor® sonicator (standard model), with an automated cooling system that allows for continuous cooling of the water bath.
3. Equipment for small size agarose gel electrophoresis.

2.3. Immunoprecipitation of Methylated DNA

1. Eppendorf LoBind® 2 and 1.5 ml microtubes, and standard 1.5 ml microtubes.
2. Magnetic rack for microtubes, used for recovering the magnetic beads.
3. Dry heating block for 2 ml microtubes, with shaking.
4. IP buffer 10×: 100 mM Na-phosphate pH 7.0, 1.4 M NaCl, 0.5% Triton X-100. Store at room temperature.

5. 1 M Na-phosphate pH 7.0 buffer: Mix 39 ml 2 M monobasic sodium phosphate (NaH_2PO_4) (276 g/l), 61 ml 2 M dibasic sodium phosphate (Na_2HPO_4) (284 g/l), and 100 ml H_2O.
6. IP buffer 1×: Dilute 1 ml IP buffer 10× in 9 ml H_2O. Store at room temperature.
7. Mouse anti 5-methylcytidine monoclonal antibody, clone 33D3, available at a 1 mg/ml concentration from various suppliers such as Eurogentec or AbD Serotec. Other mouse monoclonal antibodies such as the ones developed by Diagenode work equally well. Store the antibody as 5 µl aliquots at −20 °C.
8. Vortex Genie 2 shaker with a platform for microtubes, placed at room temperature.
9. Overhead rotator for microtubes, placed in a 4 °C cold room.
10. Magnetic beads: Dynabeads M-280 Sheep anti-mouse IgG (Invitrogen).
11. PBS-BSA 0.05%: Mix 9.5 ml PBS with 0.5 ml BSA at 10 mg/ml concentration.
12. Proteinase K digestion buffer: 50 mM Tris pH 8.0, 10 mM EDTA, 0.5% SDS.
13. Proteinase K, 10 mg/ml stock. Store at −20 °C.
14. PCI (phenol–chloroform–isoamyl alcohol 25:24:1).
15. Linear polyacrylamide (LPA), 5 mg/ml stock, used as a coprecipitant.
16. Refrigerated microcentrifuge.

2.4. Amplification and Analysis

1. Genomeplex® complete whole genome amplification kit WGA2 (Sigma-Aldrich).
2. Real-time PCR reagents and apparatus.

3. Methods

3.1. Isolation of Genomic DNA

This protocol is suitable for isolating genomic DNA from 20,000 to 200,000 mammalian cells. If extracting DNA from higher number of cells, please refer to the standard MeDIP protocol (8). The use of LoBind microtubes in the initial step allows to minimize the loss of DNA during the procedure.

1. Resuspend the cells in a LoBind 1.5 ml microtube in 300 µl lysis buffer containing 20 µl proteinase K (10 mg/ml stock) (see Note 1).
2. Incubate at 55 °C in the dry heating block for 3 h.

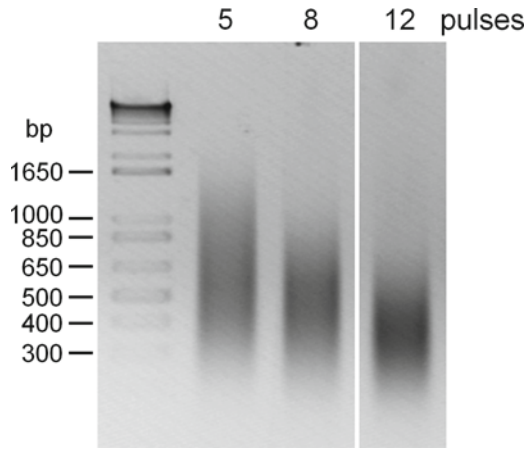

Fig. 2. Example of sonicated DNA migrating on a 1% agarose gel and stained with ethidium bromide. In this experiment, we sonicated 1 μg mouse genomic DNA in a volume of 150 μl H_2O and loaded 100 ng on the agarose gel. The numbers above the gel indicate the number of 30 s sonication pulses, which shows that 12 pulses leads to an optimal sonication under these conditions. Ideally, sheared DNA fragment should have an average size of 400 bp and be no longer than 1,000 bp.

3. Extract with 1 volume PCI. Transfer the upper phase in a new standard microtube (see Note 2).
4. Precipitate the DNA with 3 volumes (900 μl) ethanol containing 300 mM NaCl. Add 1 μl LPA if the amount of cells is <100,000. Store at −20 °C overnight.
5. Centrifuge for 40 min at full speed at 4 °C.
6. Wash the pellet with 500 μl ethanol 70% and centrifuge for 20 min at full speed at 4 °C.
7. Resuspend the pellet in 30 μl H_2O.
8. Quantify the amount of DNA with the Qubit fluorometer (see Note 3).

3.2. Sonication

Genomic DNA is randomly sheared by sonication to generate fragments between 200 and 1,000 bp. This step is crucial because the size of the DNA fragments will determine the resolution of the MeDIP assay.

1. Dilute 100–1,000 ng of genomic DNA in 150 μl H_2O in a 1.5 ml LoBind microtube.
2. Sonicate 12 times for 30 s (with 30 s intervals) with the Bioruptor (see Note 4).
3. If possible, verify the efficiency of the sonication by loading at least 50 ng on a 1% agarose gel. Ideally, the sheared fragments should have an average size of 400 bp and be no larger than 1,000 bp (Fig. 2) (see Note 5).

3.3. Immunoprecipitation of Methylated DNA

The sonicated DNA is then immunoprecipitated with a monoclonal antibody directed against 5-methylcytidine (5mC). Importantly, a portion of the sonicated DNA (at least 10 ng) should be left untreated to serve as input control. We describe here on optimized immunoprecipitation protocol for 75–100 ng sonicated DNA. For immunoprecipitation of larger amounts of DNA, you can refer to the standard MeDIP protocol (8).

1. Dilute 75–100 ng sonicated DNA in 135 µl H_2O in a 2 ml LoBind microtube (see Note 6).
2. Denature at 95 °C for 10 min in the dry heating block, and immediately cool on ice.
3. Add 15 µl IP buffer 10×.
4. Add 1/5 µl 5mC antibody (see Note 7).
5. Incubate for 2 h at 4 °C on the overhead rotator (see Note 8).
6. Prewash 2 µl magnetic beads with 500 µl PBS-BSA 0.05% in a standard 1.5 ml microtube. Incubate 5 min at room temperature with vortexing on the vortex Genie 2 (see Note 9).
7. Collect the magnetic beads on the magnetic rack and repeat the washing step with 500 µl PBS-BSA 0.05%.
8. Collect the magnetic beads on the magnetic rack and wash briefly with 500 µl IP buffer 1× to eliminate the traces of PBS-BSA.
9. Collect the magnetic beads on the magnetic rack and resuspend in 2 µl IP buffer 1× (see Note 9).
10. Transfer the magnetic beads to the sample.
11. Incubate for 2 h at 4 °C on the overhead rotator (see Note 10).
12. Collect the magnetic beads on the magnetic rack and wash with 700 µl IP buffer 1× by incubating 10 min at room temperature with vortexing on the vortex Genie 2.
13. Repeat the washing step with 700 µl IP buffer 1× twice.
14. Collect the magnetic beads on the magnetic rack and resuspend in 250 µl proteinase K digestion buffer.
15. Add 5 µl proteinase K and incubate 30 min at 50 °C in the dry heating block with shaking (see Note 11). Transfer the sample in a 1.5 ml LoBind microtube.
16. Extract with one volume (250 µl) PCI. Transfer the upper phase in a new standard 1.5 ml microtube (see Note 2).
17. Precipitate the DNA with 3 volumes (750 µl) ethanol containing 300 mM NaCl and 1 µl LPA. Store at −20 °C overnight.
18. Centrifuge for 40 min at full speed at 4 °C.
19. Wash the pellet with 500 µl ethanol 70% and centrifuge for 20 min at full speed at 4 °C.
20. Resuspend the pellet in 10 µl H_2O and store at −20 °C.

3.4. Amplification and Analysis

Enrichments in the MeDIP fraction can be measured by real-time PCR or microarray hybridization. If the MeDIP is performed with small amounts of starting material, it is necessary to perform a nonspecific amplification to increase the amount of DNA for downstream analyses, with the caveat that it might introduce amplification biases. We routinely use the Genomeplex® complete whole genome amplification kit WGA2 (Sigma-Aldrich), following the manufacturer's protocol on 10 ng of input DNA and the entire MeDIP product. This amplification step does not alter the MeDIP profiles at most targets, however we and others experienced that it can introduce amplification biases in the MeDIP enrichments at certain targets, especially the ones that are CpG-rich (9, 10). The MeDIP procedure can be validated after the whole genome amplification by performing real-time PCR in the input and MeDIP fraction on endogenous methylated and unmethylated controls. Real-time PCR can be performed on 10 ng of input and MeDIP DNA. Methylated controls are typically imprinting control regions or promoters of germline-specific genes that appear methylated in most somatic cells, whereas unmethylated negative controls are CpG island promoters that remain constitutively unmethylated in most cells or regions that contain very few CpGs. Table 1 gives a number of primers for real-time PCR in methylated and unmethylated controls that can be used to validate the MeDIP in human and mouse. Enrichments in the MeDIP fraction are calculated relative to one unmethylated negative control with the following formula: enrichment = $(IP_{target}/IN_{target})/(IP_{nc}/IN_{nc})$, with IP_{target} and IN_{target} representing the amount of the target sequence in the MeDIP and input fraction, and IP_{nc} and IN_{nc} representing the amount of the unmethylated negative control sequence in the MeDIP and input fraction. An example of typical enrichment profile obtained by MeDIP on small amounts of human or mouse genomic DNA is given in Fig. 3. Keep in mind to interpret the MeDIP results with caution because apparent low MeDIP enrichments can reflect in some cases an absence of sufficient CpGs in the target or a bias introduced during the whole genome amplification. For these reasons, we highly recommend to complement the MeDIP results with bisulfite sequencing at selected targets whenever possible. For genome-wide analyses, WGA-amplified input and MeDIP fractions can be differentially labeled with Cy3 and Cy5 and cohybridized to high-density oligonucleotides microarrays. It is also possible to couple the MeDIP procedure with deep sequencing; however, it is important to keep in mind that the MeDIP generates single-stranded DNA that cannot directly be used to generate libraries for deep sequencing. This can be circumvented by ligating the library adapter oligos between the sonication and the immunoprecipitation step (5, 7, 11, 12).

Table 1
Primer sequences for real-time PCR validation of MeDIP in somatic cells

Organism	Gene name	Comment	Sequence
Mouse	*Dpep3*	Methylated in somatic cells	Forward: GCAGGTTACCCACAGAGACG Reverse: GTGACCAAGACTGAGCACCA
Mouse	*Prss21*	Methylated in somatic cells	Forward: CAAGACGTTGGTGCCACTG Reverse: CACTGCCCCCAGTCTCAC
Mouse	*H19* ICR	Partially methylated in somatic cells	Forward: GCATGGTCCTCAAATTCTGCA Reverse: GCATCTGAACGCCCCAATTA
Mouse	IGd	Control sequence with very few CpGs	Forward: CCCTCTGGCCCTGAATTTAT Reverse: CACCCAGCAATGCTTCAGT
Mouse	*Tbx15*	Unmethylated CpG island	Forward: TCCCCCTTCTCTTGTGTCAG Reverse: CGGAAGCAAGTCTCAGATCC
Human	*TSH2B*	Methylated in somatic cells	Forward: CAGACATCTCCTCGCATCAA Reverse: GGAGGATGAAAGATGCGGTA
Human	*BRDT*	Methylated in somatic cells	Forward: CCCTTTGGCCTTACCAACTT Reverse: GCCCTCCCTTGAAGAAAAAC
Human	IG5	Control sequence with very few CpGs	Forward: GACCATGTCCAGGCAAAAGT Reverse: AGGCTCCTACAGACGTGGAA
Human	*UBE2B*	Unmethylated CpG island	Forward: CTCAGGGGTGGATTGTTGAC Reverse: TGTGGATTCAAAGACCACGA

4. Notes

1. Due to the viscosity of the solution after cell lysis, we recommend to add the proteinase K to the lysis buffer before mixing it with the cells.
2. We recommend not using a LoBind microtube for the ethanol precipitation step because we experienced that it hinders the recovery of the pellet.
3. Typically, up to 100 ng genomic DNA can be recovered from 20,000 diploid mammalian cells.
4. Because the sonication efficiency varies with DNA quality, quantity and sonicator settings, it is highly recommended to first verify the efficiency of the sonication on nonprecious DNA. For this, sonicate nonprecious DNA in the same conditions and verify the size of the sheared DNA by loading at least 50 ng sonicated DNA on a 1% agarose gel. If the sonication needs to be optimized for <50 ng, you can sonicate several tubes in parallel and then check the size of the pooled DNA.

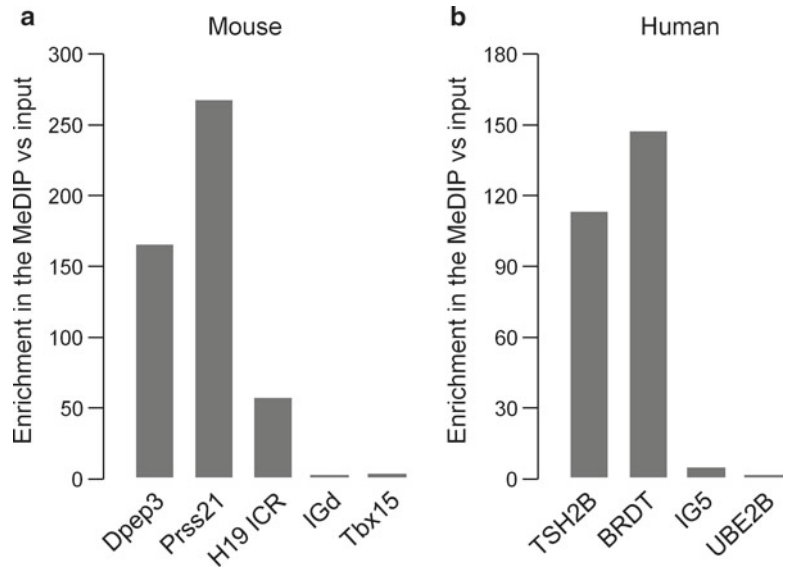

Fig. 3. Examples of MeDIP enrichment profiles measured by real-time PCR. MeDIP was performed with 200 ng sonicated DNA from mouse E9.5 embryos (**a**) or human primary fibroblasts (**b**), followed by whole genome amplification. The graphs show the enrichment in the MeDIP versus input fraction of methylated sequences over unmethylated negative controls. Values are normalized with the formula $(IP_{target}/IN_{target})/(IP_{nc}/IN_{nc})$ to the unmethylated negative controls (nc) IGd (mouse) or *UBE2B* (human), whose ratios are set to 1 (see Note 12).

To ensure a better consistency in the sonication, we also recommend always filling up the sonicator tube holder with empty microtubes containing 150 µl H_2O.

5. If sonicating small amounts of DNA, it is possible to monitor the efficiency of the sonication by sonicating in parallel an equal amount of nonprecious DNA and loading it on a 1% agarose gel.
6. We use 2 ml instead of 1.5 ml microtubes because it allows for a better mixing of small volumes.
7. For a better reproducibility, we suggest to first dilute 1 µl 5mC antibody in 4 µl IP buffer 1×, and then add 1 µl of the dilution to the sample. This amount of antibody has been optimized for somatic cells with a standard methylation level. It can be adjusted for cells with an unusual hyper- or hypomethylation state.
8. Verify that the sample is properly mixed in the microtube. Alternatively, you can also perform gentle horizontal shaking on a vortex Genie 2 with a platform for microtubes placed in a 4 °C cold room.
9. If performing several MeDIPs in parallel, you can wash all the beads together. For a better consistency, we also recommend to wash more beads than necessary for the experiment. For instance in the case of 5 MeDIPs, wash 15 µl of beads, resuspend in 15 µL IP buffer 1×, and use 2 µl per MeDIP reaction.

10. Verify that the sample is properly mixed in the microtube. Alternatively, you can also perform gentle horizontal shaking on a vortex Genie 2 with a platform for microtubes placed in a 4 °C cold room. In that case, the horizontal shaking should be strong enough to prevent the sedimentation of the magnetic beads.
11. The shaking speed must be sufficient to prevent the sedimentation of the magnetic beads. We routinely use 900 rpm.
12. Enrichment values of methylated over unmethylated controls in the MeDIP fraction can sometimes vary drastically from 100 to 10,000 between experiments, which reflects the stochastic nature of the MeDIP and WGA procedures.

Acknowledgments

This work was supported by the CEFIC Long Research Initiative (LRI-EMSG49-CNRS-08).

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