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Methylation Profiling Using Methylated DNA Immunoprecipitation and Tiling Array Hybridization

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Abstract

DNA methylation is an important epigenetic modification that regulates development and plays a role in the pathophysiology of many diseases. It is dynamically changed during germline development. Methylated DNA immunoprecipitation (MeDIP) is an efficient, cost-effective method for locus-specific and genome-wide analysis. Methylated DNA fragments are enriched by a 5-methylcytidine-recognizing antibody, therefore allowing the analysis of both CpG and non-CpG methylation. The enriched DNA fragments can be amplified and hybridized to tiling arrays covering CpG islands, promoters, or the entire genome. Comparison of different methylomes permits the discovery of differentially methylated regions that might be important in disease- or tissue-specific expression. Here, we describe an established MeDIP protocol and tiling array hybridization method for profiling methylation of testicular germ cells.

Keywords

MeDIP; DNA methylation; Tiling arrays

1. Introduction

DNA methylation plays a fundamental role in epigenetic regulation of gene expression and genomic imprinting. In germ cells, methylation is under tight control both to temporally and spatially regulating the expression of genes important for development. X chromosome inactivation and imprinting marks in the germ line are mediated by methylation. Various methods have been developed for analysis of DNA methylation, including bisulfite-based PCR or sequencing, restriction enzyme-based analysis of methylated sites, and immunoprecipitation of methylated fragments. Methylated DNA immunoprecipitation (MeDIP) was first described by Weber et al. (1) and subsequently used for genome-wide analysis of plant and mammalian methylomes (2, 3). MeDIP products can be used for PCR analysis of an individual locus or hybridized to tiling arrays (MeDIP-chip) for genome-wide analysis. The MeDIP-chip provides cost-effective and high-resolution profiling of the methylome. With the advance of high-throughput next-gen sequencing, sequencing of MeDIP product (MeDIP-seq) or direct sequencing of bisulfite-converted DNA has been applied to resolve methylome in single-base resolution (4, 5). However, MeDIP-chip is still attractive for genomic research because of its rapid and lower cost.

For MeDIP analysis, 1–5 µg of genomic DNA is required. If the downstream analysis is whole-genome array hybridization, 5 µg of DNA is necessary. Enrichment of methylated DNA by anti-5-methylcytidine antibody is effective and sensitive; the degree of enrichment is reflective of the degree of methylation. The antibody can bind to both CpG- or non-CpG-methylated sites. Alternatively, recombinant proteins of the methylated DNA-binding

domain (MBD) are used to enrich methylated CpG fragments (6). Both antibody and MBD are sensitive and specific; however, a nonspecific IgG control or input DNA should be included to assess background noise during hybridization.

In this chapter, antibody-based MeDIP is described. We provide procedures for amplification, fragmentation, and labeling of immunoprecipitated DNA for hybridization of tiling arrays using the Affymetrix platform.

2. Materials

2.1. Extraction and Sonication of Genomic DNA

1. Any commercial kit for the extraction of high-quality genomic DNA can be utilized. The procedures described here utilize Gentra Puregene Cell Kit (Qiagen) for purification of RNA-free genomic DNA from cultured cells (see Note 1).
2. Sonics® Ultrasonic Processor Model GE505 (Sonics).
3. Sterile 1× PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂ HPO₄, 1.47 mM KH₂ PO₄, pH 7.4).
4. TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0).

2.2. MeDIP

1. Antibody: Mouse monoclonal anti-5-methylcytidine (5-mC), Clone 33D3 (Eurogentec).
2. Sheep anti-mouse IgG conjugated Dynabeads (Invitrogen).
3. Proteinase K (Roche).
4. Digestion buffer: 50 mM Tris–HCl (pH 8.0), 10 mM EDTA, 0.5% SDS.
5. 1× IP buffer: 10 mM sodium phosphate (pH 7.0), 140 mM NaCl, 0.05% Triton X-100.
6. Phenol:chloroform:isoamyl alcohol (25:24:1, v/v).
7. Magnetic rack (Invitrogen).

2.3. Amplification of MeDIP DNA

1. Sequenase Version 2.0 DNA Polymerase (13 U/μl) (USB).
2. 5× sequenase reaction buffer: 200 mM Tris–HCl (pH 7.5), 100 mM MgCl₂, 250 mM NaCl.
3. Sequenase dilution buffer: 10 mM Tris–HCl (pH 7.5), 5 mM DTT, 0.1 mM EDTA.
4. Primer A (200 μM): GTTTCACGTCACGGTC(N)₉, HPLC purified.
5. Primer B (100 μM) GTTTCACGTCACGGTC, HPLC purified.
6. DMSO, BSA, DTT, dNTP, MgCl₂ (molecular grade, various sources).
7. 10× PCR buffer: 200 mM Tris–HCl (pH 8.4), 500 mM KCl.
8. dNTP/dUTP mix: 10 mM of dATP, 10 mM of dCTP, 10 mM of dGTP, 8 mM of dTTP, and 2 mM of dUTP.

¹In selection of kits, you should pay attention to the appropriateness of the product for your cell types (e.g., culture cells, yeast, blood cells, or fixed tissues).

9. Platinum Taq (5 U/μl) (Invitrogen).
10. Thermocycler (Applied Biosystems).
11. GeneChip Sample Cleanup Module (Affymetrix) or MinElute Reaction Cleanup Kit (Qiagen).

2.4. Assessment of Amplified Product by qPCR

1. SYBR Green Master Mix (Applied Biosystems).
2. Control primer pairs for amplifying known methylated and unmethylated loci.
3. Realtime PCR machine (Applied Biosystems).

2.5. Tiling Array Hybridization

1. GeneChip WT Double-Stranded DNA Terminal Labeling Kit (Affymetrix).
2. GeneChip Hybridization, Wash and Stain Kit (Affymetrix).
3. GeneChip Human or Mouse Tiling Array 2.0R (Affymetrix).
4. Control Oligonucleotide B2, 3 nM (Affymetrix).
5. GeneChip® Hybridization Oven 640 (Affymetrix).
6. GeneChip Fluidics Station 450 or 400 (Affymetrix).
7. GeneChip Scanner 3000 7G (Affymetrix).

3. Methods

3.1. Extraction of Genomic DNA

1. Trypsinize cells and resuspend cells in PBS. Count cells.
2. Make an aliquot of ~1 million of cells into a 1.5-ml microcentrifuge tube. Centrifuge at $13,000\text{--}16,000 \times g$ for 5 s to collect cell pellet.
3. Discard PBS. Lyse cells immediately or freeze at -80°C .
4. Add 300 μl of cell lysis solution. Lyse cells by pipetting up and down ~50–60 times or vigorous vortexing until the solution becomes aqueous.
5. (Optional) Add 1.5 μl of RNase A solution. Mix and incubate at 37°C for 30 min.
6. Add 100 μl protein precipitation solution. Vortex for 20 s. Centrifuge at maximum speed for 1 min.
7. Add 300 μl of isopropanol to a clean 1.5-ml microcentrifuge tube. Transfer the supernatant and mix by inverting 50 times.
8. Centrifuge at maximum speed for 2 min.
9. Discard supernatant. Add 300 μl of 70% ethanol and invert several times to wash the DNA pellet.
10. Centrifuge at maximum speed for 1 min.
11. Discard the supernatant and remove any residual ethanol. Air dry the DNA for ~10–15 min.
12. Add ~50–100 μl of DNA hydration solution. Hydrate the DNA pellet by pipetting up and down.

13. Incubate at 65°C for 1 h.
14. Take 1 µl for determining the concentration at 260, 280, and 320 nm, respectively.
15. DNA concentration (µg/µl) = $(A_{260} - A_{320}) \times 0.05 \times \text{dilution factor}$.
16. Adjust DNA to ~0.1 µg/µl with TE. Proceed to sonication or store the DNA at -20°C.

3.2. Sonication of Genomic DNA

1. Dilute ~6 µg of genomic DNA in ~400 µl TE buffer in a 1.5-ml microcentrifuge tube.
2. Prechill the DNA by placing the tube on ice for at least 10 min.
3. Set up the sonicator with the following parameters:
  - a. Amplitude: 20%
  - b. Pulser: 15 s ON; 15 s OFF
  - c. Timer: 10 min
4. Put a microcentrifuge tube in a rack. Fix the rack tightly on an ice-water cup (see Note 2).
5. Dip the sonication probe into the microcentrifuge tube such that the probe is just above the bottom.
6. Start sonication.
7. After sonication, take 15 µl of the sonicated DNA and check the efficiency of fragmentation by electrophoresis on a 1.5% agarose gel. The size of the DNA should be ~100–500 bp and is shown in Fig. 1 (see Note 3).
8. Proceed to MeDIP or store the DNA at -20°C.

3.3. MeDIP

1. Determine the concentration of the sonicated DNA.
2. Use 5 µg of DNA for each MeDIP assay. Add TE to a final volume of 450 µl in a 1.5-ml microcentrifuge tube.
3. Incubate the DNA at 95°C for 10 min and cool immediately on ice for at least 2 min.
4. Add 51 µl of 10× IP buffer.
5. Add 10 µl of 5mC antibody (thaw on ice before use, see Note 4).
6. Incubate at 4°C for 2 h on an overhead shaker.
7. Vortex the Dynabeads and pipette 30 µl for each IP.
8. Precipitate the beads with a magnetic rack (1.5–2 min). Remove the storage buffer.
9. Wash the beads two times with 800 µl PBS–BSA 0.1%.

²It is critical to keep DNA on ice to avoid heat degradation.

³DNA fragmentation is critical for efficient pull-down of methylated DNA by antibody. Prolonged sonication at low energy input usually yields smaller fragments. An average size of DNA fragments of ~200–300 bp is ideal for MeDIP.

⁴Store aliquots of antibody at -20°C for long-term storage or at -4°C for up to 3 months.

10. Resuspend the beads in 30 μ l of 1 $\times$ IP buffer.
11. Add the beads to the IP mix. Incubate at 4°C for 2 h with overhead shaking.
12. Wash three times.
  - a. Precipitate beads with a magnetic rack. Remove supernatant.
  - b. Add 700 μ l of 1 $\times$ IP buffer.
  - c. Wash for 5 min by overhead shaking at room temperature.
13. After final wash, remove wash buffer and resuspend the beads in 250 μ l digestion buffer.
14. Add 3.5 μ l of proteinase K (23.4 mg/ml). Wrap the cap with parafilm.
15. Digest at 50°C for 3 h with shaking (800 rpm). Check regularly to ensure that the beads do not settle at the bottom.
16. Add 500 μ l of phenol/chloroform/isoamyl alcohol. Vortex for 30 s and centrifuge at 11,000 $\times g$ for 2 min. Transfer the supernatant to a new tube.
17. Repeat step 16.
18. Precipitate the DNA with 500 μ l of absolute ethanol, 20 μ l of 5 M NaCl, and 1 μ l of glycogen (20 μ g/ μ l).
19. Put in -20°C refrigerator overnight.
20. Centrifuge at 20,000 $\times g$ for 35 min.
21. Discard the supernatant. Wash the DNA pellet with 700 μ l of 70% ethanol. Centrifuge at 20,000 $\times g$ for 10 min.
22. Air dry the pellet. Resuspend in 10 μ l of nuclease-free water.
23. Take 2 μ l for qPCR. Keep the remaining 8 μ l for genome-wide amplification.

3.4. Amplification of MeDIP DNA

3.4.1. Random Priming

1. Set up the random priming reaction in a PCR tube on ice:

MeDIP DNA	8 μ l
Nuclease-free water	1.15 μ l
5 $\times$ sequenase reaction buffer	4 μ l
200 μ M Primer A	4 μ l
Total	17.15 μ l

2. Start the random priming program of the thermocycler (see Note 5). Heat at 95°C for 4 min. Snap cool on ice and hold at 10°C.
3. Dilute 1 μ l of sequenase stock (13 U/ μ l) with 9 μ l of sequenase dilution buffer (enough for two reactions). Keep on ice.
4. Prepare the cocktail on ice (for one reaction):

10 mg/ml BSA	0.2 μ l
0.1 M DTT	1 μ l
10 mM dNTP mix	1.25 μ l
1.3 U/ μ l diluted sequenase	1 μ l
Total	3.45 μ l

5. Add 3.45 μ l of the cocktail. Mix by pipetting.
6. Put the PCR tube back to the thermocycler which is held at 10°C. Resume the program and keep the lid open.
7. Restart the program and heat again at 95°C for 4 min. Snap cool on ice and hold at 10°C.
8. Add 1 μ l of the diluted sequenase. Mix by pipetting.
9. Put the PCR tube back to the thermocycler. Resume the program and keep the lid open.
10. Repeat another two rounds of the random priming (steps 7 –9).
11. Add 26.4 μ l TE buffer to the reaction mix (total: 50 μ l).

3.4.2. PCR Amplification

1. Set up the PCR mix on ice (see Note 6):

Random primed DNA	30 μ l
10 $\times$ PCR buffer	20 μ l
dNTP/dUTP mix	7.5 μ l
100 μ M Primer B	8 μ l
DMSO	12 μ l
50 mM MgCl ₂	8 μ l
5 U/ μ l Platinum Taq	4 μ l
Nuclease-free water	110.5 μ l
Total	200 μ l

2. Place in a thermocycler and run the program:

⁵Set up a program for random priming:

95°C	4 min
10°C	5 min, 15 s
Ramp from 10 to 37°C by holding for 23 s for each increment of 1°C.	
37°C	8 min

⁶30 μ l of random-primed DNA is used for PCR amplification. If more DNA is required, scale up the PCR reaction and use all the 50 μ l of random-primed DNA.

92°C	2 min	
92°C	30 s	
40°C	30 s	
50°C	30 s	
72°C	1 min	Repeat 30 cycles
72°C	5 min	
4°C	∞	

3.5. Cleanup of Amplified DNA

1. Add 24 ml of absolute ethanol to the cDNA wash buffer for the first time use of the kit.
2. In a 1.5-ml microcentrifuge tube, mix 1 ml of cDNA binding buffer with the PCR product. Vortex for 3 s.
3. Spin down briefly and load equal volume of sample to two cDNA spin columns (see Note 7).
4. Centrifuge at $8,000 \times g$ for 1 min. Discard the flow-through.
5. Transfer the cDNA spin column to a new 2-ml collection tube. Add 750 μ l cDNA wash buffer to the column. Centrifuge $8,000 \times g$ for 1 min and discard the flow-through.
6. Open the cap and centrifuge at $20,000$ – $25,000 \times g$ for 5 min. Discard the flow-through and transfer the column to a new 1.5-ml collection tube.
7. Add 20 μ l of cDNA elution buffer to the membrane of the column. Incubate at room temperature for 1 min. Then, centrifuge at $20,000$ – $25,000 \times g$ for 1 min.
8. Repeat step 7 with another round of elution (see Note 8).
9. Pool the eluted DNA samples (from the same PCR product).
10. Take 1 μ l of the sample to determine concentration.
11. Calculate the concentration.

$$\text{DNA concentration } (\mu\text{g}/\mu\text{l}) = (A_{260} - A_{320}) \times 0.05 \times \text{dilution factor}$$

3.6. Assessment of Amplified Product by qPCR

This step is necessary before performing array hybridization as it ensures that your MeDIP product is 5mC enriched and maintained after amplification. Enrichment is shown in Fig. 2 (see Note 9).

1. The following samples are used in qPCR:
  - a. Sonicated genomic DNA (Input)
  - b. MeDIP DNA (IP)
  - c. MeDIP DNA with amplification (IPM)

⁷Each column has a binding capacity of 9–13 μ g of DNA. Increase the number of columns if more DNA is synthesized from PCR.

⁸If a higher concentration of DNA is desired, apply the 20 μ l eluate to the same column and repeat elution.

⁹At least two pairs of primers are required for assessment of specificity of MeDIP. A positive-pair control is designed to amplify regions of known methylation, whereas a negative-pair control is for regions of no methylation.

2. Dilute Input DNA to 5 ng/μl, IP DNA (from step 23 of Subheading 3.3) to 40 μl, and IPM to 2 ng/μl with nuclease-free water.
3. Set up the real-time PCR master mix (one reaction):

SYBR Green Master Mix	12.5 μl
10 μM Control primer-F	1 μl
10 μM Control primer-R	1 μl
Nuclease-free water	6.5 μl
Total	21 μl

4. Make aliquots of 21 μl of master mix to each well of 96-well PCR plate.
5. Add 4 μl of Input, IP, and IPM to duplicated wells, respectively.
6. Run real-time PCR with the following condition:

95°C	10 min
95°C	15 s
60°C	1 min
95°C	1 min
Dissociation step	

7. Calculate the fold of enrichment:

Fold of enrichment = $2^{-(\Delta Ct_{PC} - \Delta Ct_{NC})}$, where $\Delta Ct_{PC} = Ct_{IP} - Ct_{Input}$ for positive control, $\Delta Ct_{NC} = Ct_{IP} - Ct_{Input}$ for negative control.

3.7. Tiling Array Hybridization

3.7.1. Fragmentation of Amplified DNA

1. Set up the fragmentation mix in a PCR tube:

Double-stranded DNA	9 μg
10× cDNA fragmentation buffer	4.8 μl
10 U/μl UDG	1.5 μl
100 U/μl APE1	2.25 μl
Nuclease-free water add up to 48 μl	
Total	48 μl

2. Flick mix and spin down the tube. Incubate at 37°C for 1 h, 93°C for 2 min, and 4°C for 2 min.
3. (Optional) Remove 1–2 μl of sample for checking efficiency of fragmentation using a Bioanalyzer.

3.7.2. Labeling of Fragmented DNA

1. Set up the labeling mix in a PCR tube:

Fragmented DNA	45 μ l
5 $\times$ TdT buffer	12 μ l
TdT	2 μ l
5 mM DNA-labeling reagents	1 μ l
Total	60 μ l

2. Flick mix and spin down the tube. Incubate at 37°C for 1 h, 70°C for 10 min, and 4°C for 2 min.
3. (Optional) Remove 1–2 μ l of sample for checking efficiency of labeling using gel-shift assay.

3.7.3. Hybridization of Labeled DNA on Tiling Arrays

1. Prepare the hybridization cocktail in a 1.5-ml microcentrifuge tube:

Fragmented and labeled DNA	60 μ l
Control oligonucleotide B2	4 μ l
2 $\times$ hybridization mix	120 μ l
DMSO	16.8 μ l
Nuclease-free water	39.2 μ l
Total	240 μ l

2. Vortex and spin down the cocktail.
3. Heat the hybridization cocktail at 99°C for 5 min. Cool to 45°C for 5 min. Centrifuge at maximum speed for 1 min to remove any precipitate.
4. Inject 200 μ l of the hybridization cocktail to the array through the lower left septum. Save the remaining cocktail at –20°C for future use.
5. Seal the septa with sticky labels to prevent leakage. Place the arrays in a 45°C hybridization oven at 60 rpm. Incubate for 16 h.
6. After hybridization, remove the hybridization cocktail and save for future use.

3.8. Array Washing, Staining, and Scanning

Refer to Affymetrix Chromatin Immunoprecipitation Assay Protocol for array washing, staining, and scanning.

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Fig. 1. Genomic DNA fragments after sonication. Mobilities of size markers are *indicated on the left*.

Fig. 2. Enrichment of methylated loci (RASSF1 and NPY) by MeDIP. ACTB is used as a negative-control locus.