

Sample Preparation Protocol for DNA Methylation Microarrays

Outline

This protocol describes the process for preparing samples for NimbleChip™ DNA methylation microarrays using the *Mse* I restriction enzyme for genomic DNA fragmentation followed by enrichment of methylated DNA using an anti 5-methyl cytidine antibody. CpG dinucleotides are left intact when *Mse* I (5'-T▼TAA) is used to digest DNA.

Protocol Information & Safety

- Wear gloves and take precautions to avoid sample contamination.
- Store all samples at -20°C or on ice.

Required Equipment & Labware*

* See last page for component and supplier list.

- Freezer capable of temperatures to -80°C
- Microcentrifuge with 12,000 x g capability
- Thermocycler
- Refrigerated clinical centrifuge with tube adaptors compatible with 15ml and 50ml conical (Falcon) tubes and 1.5ml microcentrifuge tubes.
- Cold box or refrigerator (4°C) equipped with rotating platform
- Rotating platform at 55°C
- Heat block(s) capable of temperatures to 98°C
- Spectrophotometer
- SpeedVac
- Liquid nitrogen

Sample Preparation Timeline

Listed below is a recommended timeline for completing the required steps for sample preparation.

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Step 1. Extract DNA

Extract genomic DNA using the Qiagen DNeasy Kit, following the manufacturer's protocol **except:**

After step 2 in the manufacturer's protocol, add 4µl RNaseA (100mg/ml) to the reaction, mix thoroughly, and incubate at 37°C for 30min before continuing onto step 3 of the manufacturer's protocol.

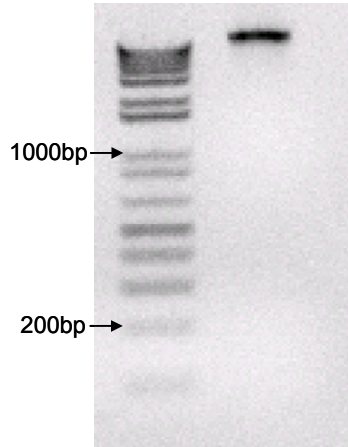


Figure 1. Example of Agarose Gel Electrophoresis for Nondegraded Genomic DNA

Step 2. Perform *Mse* I Digestion

1. Digest 6µg of high-quality genomic DNA (from Step 1) with 24U of *Mse* I (5'-T▼TAA) and supplement the reaction with 100ng/µl BSA overnight (12 - 15h) at 37°C:

	Amount
Sample	6µg
10X NEB2 Buffer	4µl
BSA (1µg/µl)	4µl
<i>Mse</i> I (10,000U/ml)	2.4µl
Water (to 40µl)	To volume
Total	40µl

2. Stop the reaction by heating the samples for 20min at 65°C.
3. Purify samples using the Qiagen Quick PCR Purification Kit.
4. Quantify DNA with a spectrophotometer.
5. Run 300ng of *Mse* I digested DNA on a 2% agarose gel to verify a fragment size of 200 - 1,000bp.

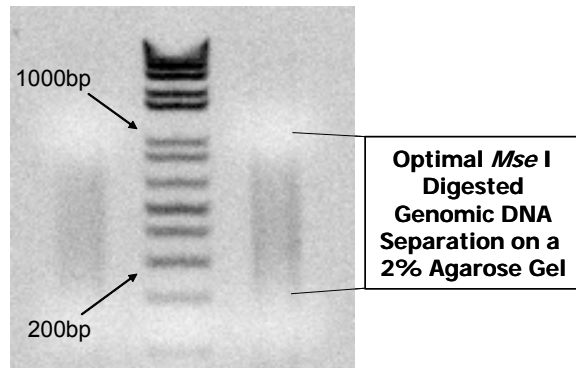


Figure 2. Example of Agarose Gel Electrophoresis for *Mse I* Digested Genomic DNA

Step 3. Add Antibody to DNA

This procedure uses a 1:1 ratio of antibody to DNA:

1. Dilute 1.25µg *Mse I* digested DNA (from Step 2) to a final volume of 300µl in TE buffer (10mM Tris HCl, pH7.5; 1mM EDTA).
2. Heat-denature for 10min at 95°C, and immediately cool on ice for 5min.
3. Remove 60µl (250ng) and store at -20°C for use as control (input) DNA.
4. To the remaining 240µl of DNA, add 60µl of 5X IP buffer:

5X IP Buffer (0.2µm filter sterilize; store at 4°C)	Volume
100mM Na-Phosphate pH7.0	50ml
5M NaCl	14ml
10% Triton X-100	2.5ml
Water	33.5ml
Total	100ml

5. Add 1µg of antibody (monoclonal mouse anti 5-methyl cytidine).
6. Incubate the DNA-antibody mixture overnight on a rotating platform at 4°C. Rotate at a low enough speed to prevent significant foaming.

Step 4. Bind Beads to DNA:Antibody Mixture

1. Pre-wash 24 μ l of Protein A agarose beads.
 - a. Resuspend the bottle of agarose beads by shaking.
 - b. Transfer 48 μ l to a 1.5ml centrifuge tube and centrifuge at 6,000rpm for 2min at 4°C. Remove the supernatant.
 - c. Add 600 μ l PBS-BSA 0.1% (1ml PBS + 2 μ l 50mg/ml BSA).
 - d. Incubate 5min on a rotating platform at 4°C.
 - e. Centrifuge at 6,000rpm for 2min at 4°C. Carefully remove the supernatant, while not disturbing the pellet.
 - f. Repeat steps c - e.
2. Resuspend beads in 24 μ l 1X IP buffer (dilute 5X IP buffer; see recipe above) to yield a 50% slurry.
3. Add 48 μ l of beads (50% slurry) to the 300 μ l of DNA-antibody mixture.
4. Incubate on a rotating platform for 2h at 4°C.

Step 5. Wash DNA:Antibody:Beads Mixture & Add Proteinase K Mix

1. Wash beads three times with 1X IP buffer:
 - a. Centrifuge the reaction from Step 3.4 at 6,000rpm for 2min at 4°C.
 - b. Add 1ml 1X IP buffer (dilute 5X IP buffer; see recipe on page 3).
 - c. Incubate 5min on a rotating platform at 4°C.
 - d. Centrifuge at 6,000rpm for 2min at 4°C. Carefully remove the supernatant, while not disturbing the pellet.
 - e. Repeat steps b - d twice more.
2. Resuspend beads in 250 μ l digestion buffer:

Digestion Buffer (0.2 μ m filter sterilize, store at 4°C)	Volume
1M Tris HCl, pH8.0	5ml
0.5M EDTA	2ml
10% SDS	5ml
Water	88ml
Total	100ml

3. Add 7 μ l Proteinase K Mix (10mg/ml) to resuspended beads.
4. Incubate overnight on a rotating platform at 55°C.

Step 6. Purify DNA

1. Add 250µl phenol. Vortex for 30sec and centrifuge at 14,000rpm for 5min at room temperature. The beads will form a layer between the phenol and the aqueous layer. Remove the aqueous supernatant and transfer to a sterile microcentrifuge tube.
2. Add 250µl chloroform:isoamyl alcohol. Vortex for 30sec and centrifuge at 14,000rpm for 5min at room temperature. Remove the aqueous supernatant and transfer to a sterile microcentrifuge tube.
3. Add 1µl glycogen (20mg/ml) and mix well.
4. Add 20µl 5M NaCl and then 500µl ethanol and mix well.
5. Precipitate in -80°C freezer for 30min.
6. Centrifuge at 14,000rpm for 15min at 4°C. Carefully remove the supernatant, while not disturbing the pellet.
7. Wash the pellet with 500µl cold 70% ethanol.
8. Centrifuge at 14,000rpm for 5min at 4°C. Carefully remove the supernatant, while not disturbing the pellet.
9. Completely dry samples in SpeedVac.
10. Resuspend in 30µl 10mM Tris HCl (pH8.5). Measure the DNA concentration. Expected yield is 300 - 500ng (10 - 15ng/µl).

Step 7. Amplify DNA

Roche NimbleGen's DNA methylation microarray services require 4µg of sample for Class I organisms and 8µg of sample for Class II or III organisms. Starting with 6µg genomic DNA requires the IP DNA and input DNA to be amplified before labeling and subsequent array hybridization. However, it is possible to scale up the IP experiment or pool multiple IP reactions to generate enough DNA for array use without further amplification (using the steps above), although much more genomic DNA and antibody will be needed.

1. Amplify IP and input DNA. Use 10ng IP DNA (from Step 6.10) and 10ng input DNA (from Step 3.3), respectively, for amplification with the Sigma WGA2 Kit. Follow the manufacturer's protocol. Be aware of the following:
 - The heat treatment step in the Fragmentation section of the manufacturer's protocol is optional, since the DNA is already sheared.
 - If the total DNA amount after IP is less than 10ng, consider one of the following:
 - Use the Sigma WGA3 Kit, following the manufacturer's protocol to perform a second round of amplification if the yield is not sufficient after using the WGA2 Kit.
 - Or, use the Sigma WGA4 Kit *instead of* the Sigma WGA2 Kit, following the manufacturer's protocol to produce amplicons from picogram amounts of total IP DNA.
2. Purify amplified DNA using the Qiagen Qiaquick PCR Purification Kit (catalog #28106) according to the manufacturer's protocol.
3. Store all samples at -20°C until ready for use.

Component and Supplier List

Component	Vendor(s)	Package Size	Item Number
Mse I (5'-T▼TAA)	New England Biolabs	500 units	R0525S
Beads: Protein A Agarose	Invitrogen	5ml	15918-014
Antibody: monoclonal mouse anti 5-methyl cytidine*	Diagenode	100µg	MAB-5MECYT-100
	Abcam	50µg	Ab10805
	Eurogentec	100µg	I-MECY-0100
DNeasy Kit	Qiagen		69506
WGA2 Kit	Sigma Aldrich	50 reactions	WGA2-50RXN
RnaseA	Promega	4mg/ml, 1µl	A7973
BSA	Invitrogen	150mg	15561-020
10X PBS	Invitrogen	500ml	70013-032
Glycogen	Roche Applied Science	20mg (1ml)	10901393001
0.5M EDTA	Sigma Aldrich	100ml	E-7889
Triton X-100	Sigma Aldrich		93426
Chloroform:Isoamyl Alcohol (24:1)	Sigma Aldrich		C0549
Absolute Ethanol	Sigma Aldrich	500ml	E702-3
Water: Reagent Grade, ACS, Nonsterile, Type 1	VWR	2.5gal	RC915025
Proteinase K, PCR Grade	Roche Applied Science	25mg	03115836001
Phenol: Equilibrated with TE, pH8.0	Sigma Aldrich		P-4557
1M Tris HCl, pH7.4	Sigma Aldrich	1 liter	T-2663
1M Tris HCl, pH8.0	Sigma Aldrich	1 liter	T-3038
1M Tris HCl, pH8.5	<i>Multiple Vendors</i>		
5M NaCl	Ambion	500ml	9759
10% SDS	Sigma Aldrich	100ml	L-4522
100mM Na Phosphate, pH7.0	Sigma Aldrich	100ml	82637

*Choose between the recommended vendors.