Chapter 13

Genome-Wide DNA Methylation Profiling: The mDIP-Chip Technology

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Summary

Aberrant DNA methylation is one of the major characteristics of tumor cells in addition to genetic and other epigenetic alterations. Evidence shows that both regional hypermethylation and global hypomethylation can occur in cancer cells. Increased DNA methylation can be found at select tumor-suppressor gene promoters, causing the silencing of these genes in tumorigenic cells. At the same time, a global decrease in DNA methylation is frequently observed in cancer cells, which may contribute to genome instability. Unlike genetic mutations, hypermethylation at tumor-suppressor gene promoters can be reversed with epigenetic therapy by using DNA demethylating agents.

To better understand the mechanisms of cancer initiation and progression, and to better assess the effects of epigenetic therapy, a reliable high-throughput method for genome-wide DNA methylation analysis is needed. Recently, the process of coupling methylated DNA immunoprecipitation (mDIP) with microarray hybridization has been proven to be a successful strategy to map genome-wide DNA methylation patterns in different cell types.

Key words: DNA methylation, CpG island, Immunoprecipitation, Microarray, Cancer

1. Introduction

DNA methylation refers to the addition of a methyl group to the 5th carbon of the cytosine pyrimidine ring. DNA methylation is universal in vertebrates and is part of the epigenetic code, which refers to heritable change in gene function without a change in DNA sequence. DNA methylation typically occurs at CpG dinucleotides and is catalyzed by a family of DNA methyltransferases (DNMTs) that include de novo DNA methyltransferases (DNMT3A and DNMT3B) and the maintenance DNA

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methyltransferase (DNMT1) *(1, 2)*. Approximately 70% of CpGs in the mammalian genome are methylated, most of which are in heterochromatic regions containing various repetitive elements. However, CpG islands, which are defined as stretches of DNA containing a high concentration of CpG dinucleotides and frequently locating in the gene promoter region, are mostly unmethylated in normal cells and tissues.

It is well established that cancer cells exhibit abnormal DNA methylation patterns compared to normal cells. Using classic methylation assays such as Southern blot analysis and bisulfite genomic sequencing, we know that the global level of DNA methylation, especially in repetitive regions, is reduced in cancer cells. Concurrently with global hypomethylation, silencing of tumor-suppressor genes by DNA hypermethylation in the promoter CpG islands is frequently observed in tumor cells *(3–5)*. In fact, recent data suggest that genetic mutations in tumor-suppressor genes may only count for a small fraction of cancer cases and a majority of cancers could be attributed to epigenetic alterations *(6, 7)*. These epigenetic alterations include DNA methylation changes, chromatin alterations, and loss of imprinting *(8)*.

While determining the methylation pattern of a particular locus is well established with the bisulfite sequencing method, the tools for mapping genome-wide DNA methylation are just emerging *(9–14)*. We describe here a powerful method that can profile levels of DNA methylation at the genome-wide scale in gene promoters, CpG islands, introns and exons, and intergenic regions. This method first enriches methylated DNA fragments through immunoprecipitation of the methylated DNA with a monoclonal antibody against 5-methyl-cytosine *(11, 15)*. By coupling methylated DNA immunoprecipitation (mDIP) with DNA chip technology (mDIP-Chip), we can obtain a picture of genome-wide DNA methylation patterns. This method has been successfully used to describe promoter DNA methylation patterns in human normal and cancer cell lines, as well as the entire genome methylation in plant *Arabidopsis thaliana (11, 13, 14, 16)*.

2. Materials

2.1. DNA Extraction from Cells

1. *Trypsin–EDTA*. 0.25% Trypsin, 2.21 mM EDTA in HBSS (Fisher).
2. *Cell lysis buffer*. 100 mM Tris–HCl, 200 mM NaCl, 5 mM EDTA of pH 8.0, and 0.2% SDS (w/v) (*see* **Note 1**).

3. *Proteinase K (Fisher, molecular biology grade) solution (10 mg/mL).* Proteinase K is dissolved in H_2O to 20 mg/mL, and then an equal volume of 100% glycerol is added. $CaCl_2$ is added to 0.1 mM to stabilize the solution. The Proteinase K solution is aliquoted and stored at –20°C.
4. DNase-free RNase A (Sigma) is dissolved in H_2O at a concentration of 10 mg/mL, aliquoted, and stored at –20°C.
5. Ethyl Alcohol, 100%, molecular biology grade.
6. *TE buffer (pH 8.0).* 10 mM Tris–Cl (pH 8.0) and 1 mM EDTA (pH 8.0).

2.2. Immunoprecipitation

1. Mouse monoclonal antibody against 5-methylcytidine (Eurogentec, # MMS-900P-B) at a concentration of 1 µg/mL in 20 µL aliquots, stored at –20°C (*see* **Note 2**).
2. Dynabeads (M-280 sheep antimouse IgG, Invitrogen).
3. *1× PBS, 0.1% BSA.* Weigh 0.5 g of BSA and dissolve in 50 mL of 1× PBS, filter sterile, and store at 4°C.
4. *IP buffer.* For 10× stock, 100 mM Tris–Cl of pH 7.5, 500 mM NaCl, 10 mM EDTA. For making 1× IP buffer, 10× IP buffer is diluted with H_2O, store this buffer at 4°C.
5. *Low-salt wash solution.* Add 0.3 mL 5 M NaCl to 9.7 mL 1× IP buffer, store at 4°C.
6. *High-salt wash solution.* Add 0.6 mL 5 M NaCl to 9.4 mL 1× IP buffer, store at 4°C.
7. *Elute buffer with 1.5% SDS.* 10% SDS is added to 1× IP buffer so that the final concentration of SDS is 1.5%.
8. *Elute buffer with 0.5% SDS.* 10% SDS is added to 1× IP buffer so that the final concentration of SDS is 0.5%.
9. *Elute buffer with 0.1% SDS.* 10% SDS is added to 1× IP buffer so that the final concentration of SDS is 0.1%.
10. Phenol/Chloroform/Isoamyl Alcohol (25:24:1 mixture) (Fisher).

2.3. DNA Labeling and Hybridization to Microarray

1. BioPrime® Array CGH Genomic Labeling System (Invitrogen).
2. Cy3- dUTP and Cy5-dUTP (1 mM) (Perkin Elmer).
3. Nanodrop ND-1000 spectrophotometer (Nanodrop).
4. *Human Cot-1 DNA (Invitrogen).* 1 mg/mL store at –20°C.
5. *Yeast tRNA (Invitrogen).* Reconstitute in RNase-free H_2O to 5 mg/mL in 1 mL aliquots, store at –20°C.
6. Agilent 2× hybridization buffer (Agilent).
7. Agilent hybridization oven.

8. *20× SSC.* Dissolve 175.3 g NaCl, 88.2 g sodium citrate in 800 mL of H_2O. Adjust pH to 7.0 with a few drops of 14N HCl, and then bring the volume to 1 L with H_2O. Autoclave.
9. *Wash buffer 1.* Mix 25 mL of 20× SSC, 0.5 mL of 10% of Triton-X 102 and 475 mL of H_2O, filter sterile.
10. *Wash buffer 2.* Mix 5 mL of 20× SSC, 0.5 mL of 10% Triton-X 102 and 495 mL of H_2O, filter sterile.
11. Acetonitrile (Fisher).
12. Agilent Stabilization and Drying solution (Agilent).

2.4. Slide Scanning and Data Extraction

1. Agilent scanner (Carousel, 48-position).
2. Feature extraction software (Agilent).

2.5. Data Verification

1. *Bgl*II (New England Biolab).
2. Sodium Bisulfite (Sigma).
3. Hydroquinone (Sigma).
4. *6.3 M Sodium Hydroxide.* Dissolve 2.52 g of NaOH in 10 mL of H_2O.
5. Wizard® DNA Clean-up System (Promega).
6. 10 M Ammonium Acetate pH 7.3.
7. *Glycogen (Invitrogen).* 20 µg/µL store at –20°C.

3. Methods

3.1. DNA Extraction and Preparation for mDIP

1. Cultured cells are harvested by Trypsin–EDTA digestion and spun down at 140 × *g* for 5 min. Supernatant is aspirated and the cell pellet (5×10^6 cells) is resuspended with 500 µL of DNA lysis buffer. 5 µL of proteinase K solution is added to a final concentration of 100 µg/mL. This step is also used for DNA extraction from dissected tissues (*see* **Note 3**).
2. The mixed cell lysis solution is incubated at 37°C or 55°C oven with agitation for overnight digestion.
3. For tissue samples, spin the cell lysis mixture at 16,100 × *g* for 15 min. Transfer the supernatant to a clean tube. This is the fraction you will use to extract DNA for the next step.
4. RNAse A solution is added to a final concentration of 20 µg/mL and the cell lysis is incubated for another 1–2 h at 37°C with agitation.
5. Add equal volume of Phenol/Chloroform/Isoamyl Alcohol (500 µL) to the mixed cell lysis solution. Shake the tube vigorously for 15 s (*see* **Note 4**).

6. Centrifuge the mixture at 16,100 × *g* for 10 min.
7. Transfer the supernatant with pipet to a clean Eppendorf tube (*see* **Note 5**).
8. Add 1/10 volume of 3 M NaOAc and 2 volumes of 100% EtOH. Mix well by inverting the tube several times (*see* **Note 6**).
9. Put the tube at −80°C for 30 min to help precipitate the DNA.
10. Spin down DNA at 16,100 × *g* at 4°C for 20 min.
11. Aspirate the supernatant and wash the DNA pallet with 70% of EtOH. Briefly vortex the tube so that the pellet is just lifted off the bottom of tube.
12. Centrifuge at 16,100 × *g* for 5 min and aspirate the supernatant.
13. Dissolve DNA in TE.
14. Check DNA concentration with a UV spectrometer. Also check the DNA quality by running 1 μL of DNA in a 1% agarose gel (*see* **Note 7**).

3.2. mDIP

1. Sonicate 5 μg (in 150 μL volume) genomic DNA to 300–400 bp for 20–25 s on ice with Branson sonifier at setting 3.
2. Run 5 μL on 2% agarose gel with a DNA ladder to check the size of sonication of DNA (*see* **Note 8**).
3. Measure the DNA concentration with UV spectrometer.
4. Mix 2 μg of sonicated DNA, 60 μL of 10× IP buffer, 20 μL of monoclonal 5′m-C antibody (−20°C freezer, 1 vial per sample), and H_2O to 600 μL.
5. Rotate the DNA and antibody mixture at 4°C overnight.
6. Pipet 100 μL of Dynabeads for each sample into an Eppendorf tube. The Dynabeads need to be washed to get rid of nonspecific binding. The washing procedure is facilitated by using a magnet Dynal Invitrogen.
7. Wash 100 μL of Dynabeads (M-280 sheep antimouse IgG, Invitrogen) twice with PBS, 0.1% BSA, and pipet off the supernatant.
8. Wash the beads with 1× IP twice.

13. Rotate at 4°C overnight.
14. Collect the supernatant as the unbound fraction.
15. Wash beads 4 times with 1× IP buffer and collect the supernatant as W1, W2, W3, and W4. Allow 3–5 min rotation at 4°C during each wash.
16. Wash beads once with IP containing 150 mM NaCl. Allow 3–5 min of rotation at 4°C. Collect the supernatant as low-salt washing fraction.
17. Wash beads once with IP containing 300 mM NaCl. Allow 3–5 min of rotation at 4°C. Collect the supernatant as high-salt washing fraction.
18. Freshly prepare elution buffer.
19. Elute in 200 μL IP with 1.5% SDS, vortex for 1 min, collect the buffer as elution fraction.
20. Elute in 200 μL IP with 0.5% SDS, vortex for 1 min, collect the buffer, and combine the elution from **step 19**.
21. Elute in 200 μL IP with 0.1% SDS, vortex for 1 min, collect the buffer, and combine the elution from **steps 19** and **20**.
22. Phenol/chloroform clean half of the unbound, W1, W2, W3, W4, low-salt, high-salt, and elution fractions. Resuspend the DNA from each fraction in 80 μL of H_2O.
23. Test the pull down efficiency of the mDIP procedure. Use 5 μL of DNA from each of the fraction for real-time PCR (*see* **Note 9**).
24. If each fraction of the DNA is proportional to what is expected, clean up the other half of the elute fraction and combine with the previous half elution fraction.
25. Check the Elute DNA concentration with the Nanodrop (*see* **Note 10**).

3.3. DNA Labeling, DNA Hybridization, and Feature Extraction

In our efforts to assay genome-wide DNA methylation patterns, we have successfully used Agilent oligo-microarrays that cover 27,800 CpG islands and 18,000 promoter regions. To perform DNA microarray hybridization, we have followed Agilent ChIP-on-chip protocol (http://www.Agilent.com) with BioPrime® array CGH genomic Labeling Kit from Invitrogen. Nevertheless, readers should be able to obtain satisfactory results with other commercially available DNA microarrays by following the specific instructions from oligo-microarray manufacturers. The following are our adopted procedures for Agilent oligo-microarrays.

1. In two separate Eppendorf tubes, mix 500 ng of input DNA or Elute DNA with 35 μL of 2.5× random primer solution and H_2O to 75 μL.
2. Mix the solution well by either pipeting or vortexing.

3. Place the tube on 95°C heat block and incubate for 5 min.
4. Immediately transfer the tubes on ice and cool for 5 min.
5. Add 8.2 μL of 10× dUTP, 1.5 μL of exo-Klenow, 1.5 μL of either Cy3- or Cy5-dUTP (1 mM), and 1.8 μL of H_2O to the tubes (*see* **Note 11**).
6. Mix the reaction by briefly vortexing and give the tube a quick spin down.
7. Incubate the reaction in a 37°C water bath for 4–5 h in dark.
8. Add 9 μL stop buffer to the reaction and mix. Samples are ready for clean up.
9. Clean the labeled probes using BioPrime® array CGH purification module.
10. Add 400 μL of Purification Buffer A to each reaction tube and mix them by vortexing for 30 s.
11. Transfer the samples to the purification columns in the 2-mL collection tubes.
12. Spin the column at 8,000 × *g* for 1 min at room temperature.
13. Discard flow through and add 600 μL of Purification Buffer B to the column and spin at 8,000 × *g* for 1 min at room temperature.
14. Discard the flow through and add 200 μL of Purification Buffer B to the column and spin at 8,000 × *g* for 1 min at room temperature.
15. Place the column in a new Eppendorf tube.
16. Add 50 μL of sterile water to the column and incubate 1 min at room temperature.
17. Spin the column at 8,000 × *g* for 1 min at room temperature.
18. Keep the elute on ice in dark.
19. Measure the quantity and quality of labeled DNA samples using Nanodrop (*see* **Note 12**).
20. Mix 5 μg of labeled Input DNA with 5 μg of labeled Elute DNA in an Eppendorf tube, and bring the volume to 130 μL with H_2O (*see* **Note 13**).
21. Add 50 μL of 10 × Agilent control target, 50 μL of human cot-1 DNA, and 20 μL of yeast tRNA to the tube (*see* **Note 14**).
22. Add 250 μL of 2× Agilent hybridization buffer, and mix well by pipeting.
23. Heat the mixture at 95°C for 3 min.
24. Immediately transfer the tube to a 37°C H_2O bath and incubate for 30 min.

25. Take out the tube and centrifuge at 16,100 × *g* for 1 min.
26. Load a gasket slide in the Agilent SureHyb Chamber base with the label facing up.
27. Pipet 490 µL and slowly dispense the hybridization mixture onto the gasket slide (*see* **Note 15**).
28. Place a microarray on the gasket slide so that the active side is facing down and in contact with the hybridization buffer.
29. Place the SureHyb Chamber cover onto the sandwiched slide and slide on the clamp assembly. Tighten the clamp by hand.
30. Rotate the assembled chamber so that the slide is wet and make sure there are no stationary bubbles.
31. Hybridize the array in the hybridization oven for 40 h at 65°C with rotation speed at 10 rpm (*see* **Note 16**).
32. Prewarm wash buffer 2 in a 31°C oven overnight.
33. Disassemble the array, and place the array/gasket slide sandwich into a slide wash container with wash buffer 1. Separate the gasket slide from the array slide with a pair of forceps.
34. Transfer the slide into a new container with wash buffer 1. Make sure there is a stir bar in the container and wash with the rotation speed set at medium low.
35. Repeat **steps 2** and **3** for the other slides. You can wash four slides at a time.
36. Wash the slides in wash buffer 1 at room temperature for 5 min.
37. Wash the slides in wash buffer 2 at 31°C for 5 min with stirring at medium low.
38. Slowly move the slides out of wash buffer 2. The slides should be dry.
39. Wash the slides in acetonitrile for 1 min.
40. Dip the slides one at a time into Agilent Stabilization and Drying solution, slowly pulling the slide out (*see* **Note 17**).
41. Warm up the Agilent array scanner by turning it on 20 min before the scanning.
42. Put the washed slide into slide holder so that the numeric barcode is visible.
43. Scan for two colors at 5 µM resolution (*see* **Note 18**).
44. Import the scanned image into Agilent Feature Extraction software.
45. Select the appropriate protocol and grid file and start the feature extraction.
46. Save the Feature Extraction data to the computer.

3.4. Data Analysis and Verification

The methods for analyzing the mDIP data are still evolving. The array you use determines the way you calculate which genes are enriched for methylation. Here we provide ways to analyze both Promoter and CpG island microarrays. The power of your array data will depend on the quality of the hybridizations.

1. Open the Feature Extraction.txt file in your favorite math program (Excel, R, MatLab). The file can be imported into Excel as a tab-delimited file.
2. To compare the enrichment of methylation at a particular locus, sort by Log ratio (*see* **Note 19**). Higher log ratios indicate more enrichment of methylated DNA.
3. If using the CpG island arrays, you can compare the log ratios over an entire CpG island (*see* **Note 20**).
4. When performing replicate arrays, you can compare the average Log Ratio for each probe or set of probes for a CpG island.
5. To further increase you confidence of methylated probes, you can compute a t-test probability for each probe or set of probes. To compute a t-test, use the rProcessedSignal and gProcessedSignal columns from the Feature Extraction .txt file.
6. Pick genes that contain statistically significant probes to confirm. Currently, there is no definitive log ratio cut off for methylated probes. This has to be determined experimentally for each sample. We suggest confirming genes at either end of the log ratio spectrum as well as genes in between to find the threshold of methylation in your array experiments.
7. To confirm a gene's methylation status, use bisulfite sequencing *(17)*.
8. Digest DNA overnight in a 50-µL reaction with a restriction enzyme (*see* **Note 21**).
9. Run 5 µL of digested DNA on a 1% gel and check for a small shift in the genomic DNA band.
10. Heat 20 µL of digested DNA at 97°C for 5 min, and chill on ice for 5 min.
11. Add 1 µL of freshly prepared 6.3 M NaOH to DNA; vortex and centrifuge.
12. If not already, transfer DNA to a 0.2-mL PCR tube.
13. Incubate DNA at 39°C for 30 min.
14. Prepare sodium bisulfite solution by adding 4.05 g $Na_2S_2O_5$ to 8 mL of H_2O. Cover the tube with foil and allowing it to dissolve by incubate the tube at 55°C for 30 min.

15. Prepare hydroquinone solution by adding 0.11 g of hydroquinone to 5 mL of H_2O. Cover the tube with foil and allow it to dissolve by incubating the tube at 55°C for 30 min.
16. Once sodium bisulfite solution has dissolved, adjust pH with 333 µL of 6.3 M NaOH and then add 300 µL of the hydroquinone solution (*see* **Note 22**).
17. After 30 min at 39°C, add 208 µL of the sodium bisulfite solution to the DNA and mix by pipetting.
18. Incubate in a PCR machine with the following cycles
    (a) 55°C for 3 h
    (b) 95°C for 5 min
    (c) Goto **Step 1** 4 times
19. Add 1 mL of Promega's Wizard Clean-Up resin to sample. Desalt and elute according to the manufacturer's protocol.
20. Add 2.5 µL of 6.3 M NaOH to sample and incubate at 37° for 15 min.
21. Spin down briefly.
22. Add 22.5 µL of 10 M Ammonium Acetate of pH 7.3, 0.5 µL of glycogen, and 225 µL of 100% EtOH. Vortex to mix.
23. Place in –80°C for 30 min to help precipitate the DNA.
24. Spin down at 16,100 × *g* for 30 min at 4°C.
25. Resuspend the DNA in 30 µL of TE buffer and store at –20°C.
26. For Bisulfite Sequencing analysis, you can generate primers using MethPrimer (*see* **Note 23**).
27. Use 1/10th of your bisulfite treated DNA for each PCR.
28. After amplification, extract the PCR product by gel purification.
29. Sequence the PCR product using the reverse primer (*see* **Note 24**).

4. Notes

1. Unless stated otherwise, all water used to make the solutions is MilliQ water with a resistivity of 18.2 MΩ-cm.
2. Antibody should avoid repeated freeze and thaw cycles. Aliquot the antibody upon arrival so that one tube is good for one immunoprecipitation reaction for one sample.
3. For tissue samples, usually 500 µL of lysis buffer is enough for a tissue size less than 100 µL in volume.
4. This procedure should be done in a fume hood to prevent inhalation of phenol.

5. Phenol waste should be disposed of in a designated phenol waster container in a fume hood.
6. During DNA extraction procedure, vortexing should be prevented as this could cause shearing of genomic DNA.
7. The ratio of absorbance at 260/280 for DNA should be 1.8–2.0. For good quality of DNA, a high molecular weight band should be seen from the gel.
8. Usually, sonication should yield a DNA smear of 300–400 bp on the agarose gel. Make sure the sonication is complete so that there is no DNA larger than 500 bp.
9. Real-time PCR is performed on all the fractions of mDIP to test the efficacy of the procedure. Loci are picked either for methylated controls or for unmethylated controls. For instance some tumor-suppressor gene promoters can serve as positive control loci, while housekeeping gene promoters should be negative controls. Primers are designed within these loci. The Ct values were converted to a starting quantity and then all amounts were used to determine the total percentage of DNA in each fraction. Only mDIP procedures that resulted in high binding of methylated controls and low binding of unmethylated controls were used for microarray analysis. Quantitative PCR was done on a MyIQ Thermocycler (Biorad) using the Sybr Green Supermix (BioRad). A representative figure of real-time result is shown for an efficient mDIP procedure **(Fig. 1)**.
10. Typically, one IP procedure should yield 500–700 ng of DNA.
11. Typically Input DNA is labeled with Cy3-dUTP, while Eluted DNA is labeled with Cy5-dUTP.
12. Using the Microarray measure function of Nanodrop for this step. The total amount of label DNA should be >5 μg per reaction (>100 ng/μL). Cy3 label efficiency should be >3.5 pmol/μL and Cy5 labeling efficiency should be >2.5 pmol/μL. If the labeled samples do not reach these criteria, repeat the labeling procedure.
13. For Agilent 244K format CpG island array or promoter array, 5 μg of label probes for each channel is required to ensure good signal intensity.
14. Alternatively, Agilent 10× blocking reagent can be used to replace control targets and yeast tRNA. If used, bring volume in **Subheading 3.4, step 1** to 150 μL with H_2O.
15. Make sure that the hybridization mixture is added to the middle of the gasket slide.
16. Check the chambers after 20 min to ensure they are not leaking.

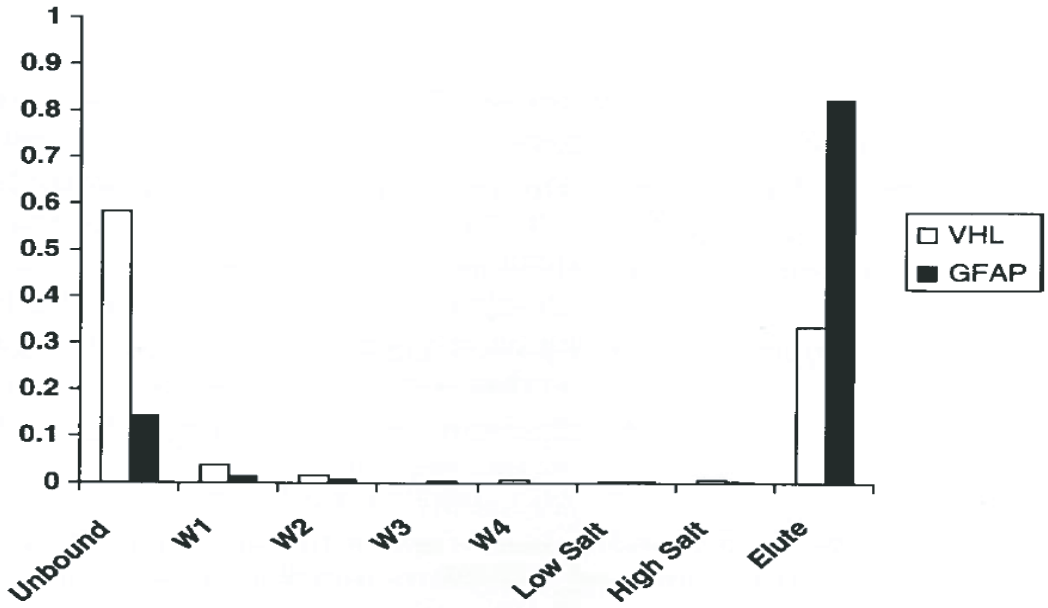

Fig. 1. Real-time PCR confirmation of a successful mDIP experiment using normal leukocytes' genomic DNA. As shown in figure, tumor-suppressor gene VHL promoter is hypermethylated in normal cells (*18*) and consistent with a low pull down efficiency. On the other hand, Gial specific gene GFAP is hypermethylated in normal leukocytes (our unpublished data) and showed a high efficiency of pull down. For more resolution of your sequence of interest, you can clone your PCR product into a Topo vector and generate sequences from at least ten clones.

17. Agilent Stabilization and Drying solution can easily form precipitation at room temperature. Incubate the solution overnight in a 37°C water bath to redissolve the precipitates before use. The acetonitrile and Stabilization and Drying solution should be set up in a fume hood.
18. For Agilent 244K format, we scan at 5 μm resolution. However, for other array format, the scanning resolution can vary.
19. Log Ratio in Feature Extraction is presented as Log10 ofrProcessedSignal over gProcessedSignal.
20. It is known that aberrant CpG island methylation spreads from its starting point throughout the whole CpG island *(19)*. Thus, one can gain great significance from comparing all the probes within one CpG island.
21. It is important to pick a Restriction Enzyme that will not digest the area of interest. A typical enzyme used for this procedure is *Bgl*II.
22. Make the sodium bisulfite and hydroquinone solution fresh each time.
23. The web address for MethPrimer is http://www.urogene.org/methprimer/index1.htmL.

24. In general, reverse primers gives better sequencing results than forward primers with PCR products from Bisulfite treated DNA samples. This is because the forward strand of PCR products from bisulfite converted DNA is T rich, while the reverse strand is A rich.

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