Chapter 8
Application of Microarrays for DNA Methylation Profiling

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Abstract Comprehensive analyses of the human epigenome may be of critical importance in understanding the molecular mechanisms of complex diseases, development, aging, tissue specificity, parental origin effects, and sex differences, among other systemic aspects of human biology. However, traditional DNA methylation methods allowed for screening of only relatively short DNA fragments. The advent of microarrays has provided new possibilities in DNA methylation analysis, because this technology is able to interrogate a very large number of loci in a highly parallel fashion. There are several permutations of the microarray application in DNA methylation profiling, and such include microarray analysis of bisulfite modified DNA and also the enriched unmethylated or hypermethylated DNA fractions using methylation-sensitive restriction enzymes or antibodies against methylated cytosines. The method described in detail here is based on the analysis of the enriched unmethylated DNA fraction, using a series of treatments with methylation-sensitive restriction enzymes, adaptor ligation, PCR amplification, and quantitative mapping of unmethylated DNA sequences using microarrays. The key advantages of this approach are the ability to investigate DNA methylation patterns using very small DNA amounts and relatively high informativeness in comparison to the other restriction-enzyme- based strategies for DNA methylation profiling [1].

Keywords DNA methylation, microarrays, epigenetic profiling, epigenetic biomarkers, whole genome approach, epigenetics

1 Introduction

The key principles and some technical details of this method are described in our recent article [1] and the strategy for enrichment of unmethylated portions of the genome is presented in Fig. 8.1. Briefly, genomic DNA (gDNA) is

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digested with methylation-sensitive restriction enzymes, such as *Hpa*II and *Hin6*I. Whereas methylated restriction sites remain unaltered, the sites containing unmethylated CpGs are cleaved by the enzymes, and DNA fragments with 5′-CpG protruding ends are generated. In the next step, the double-stranded adapter CG-1 is ligated to the CpG overhangs. At this point, it is expected that most of the relatively short (<1.5 kb) and amplifiable DNA fragments derive from the unmethylated DNA regions. Some ligation fragments, however, still may contain methylated cytosines. A large proportion of these fragments are eliminated by treatment with McrBC, thereby increasing the specificity of the enrichment of the unmethylated DNA fraction. McrBC cleaves DNA containing methylcytosine on one or both strands, recognizing two half sites of the form (G/A)^{m}C; these half sites can be separated by up to 3 kb, but the optimal separation is 55–103 base pairs. The remaining pool of unmethylated DNA fragments then is enriched by aminoallyl-PCR amplification that uses primers complementary to the adapter CG-1. An important advantage of using protruding ends in the adapter-ligation step is that degraded gDNA fragments will not be ligated and amplified and, therefore, will not interfere with DNA methylation analysis (which is especially useful when analyzing tissues with relatively long postmortem interval or paraffin-embedded samples). The enriched unmethylated DNA fractions then are labeled with fluorescent dyes and hybridized to microarrays. Several different types of microarrays can be used for epigenetic analysis; for example, oligonucleotide arrays of individual genes or microarrays containing relatively larger DNA fragments of gene regulatory regions, such as CpG islands [1]. Yet, it is evident that epigenetic profiling should be performed in a systematic, unbiased fashion and not limited to the traditionally preferable regions, such as CpG islands. Numerous other genomic loci exist that may be sites for important epigenetic modification, including enhancers, imprinting control elements, and the regions that encode regulatory RNA elements. It therefore is beneficial to use high-density tiling arrays that can cover entire chromosomes and even entire genomes represented by millions of oligonucleotides on glass chips. Whole genome tiling arrays are already available for several species and will soon be available for the entire human genome [1–3].

2 Materials

2.1 Adapter Design

1. Adapter storage buffer ST (100 mL): 10 m*M* Tris-HCl, pH 8.5, 50 m*M* NaCl.
2. Primer for preparation of universal adapter CG-1:

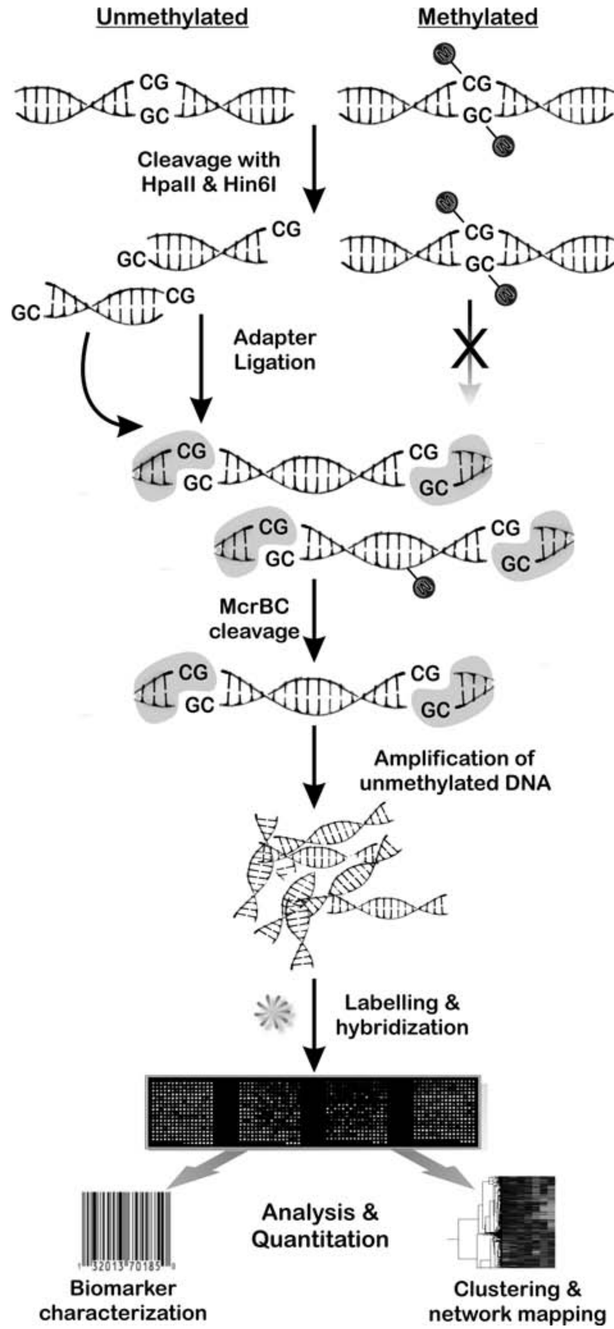

Fig. 8.1 Schematic outline of the microarray-based method for identification of DNA methylation differences in genomic DNA. Samples are cleaved by methylation-sensitive restriction endonucleases, such as *Hpa*II and *Hin6*I, ligated to the CpG-overhang specific adapter, then cut by McrBC to eliminate residual methylated DNA fragments. The resulting unmethylated DNA fragments are selectively enriched by adapter-specific aminoallyl-PCR, labeled, and hybridized to microarrays

primer CG-1a: 5′-CGTGGAGACTGACTACCAGAT-3′
primer CG-1b: 5′-AGTTACATCTGGTAGTCAGTCTCCA-3′.

2.2 Methylation-Sensitive Cleavage of DNA

1. *Hpa*II 10 U/µL (Fermentas).
2. *Hin6*I 10 U/µL (Fermentas, *see* **Note 1**).
3. Spike DNA (optional, see Sect. 3, Methods).

2.3 Ligation

1. ATP stock-solution (NEB). Make 10 µ*M* stock solution, and store as aliquots at −20 °C.
2. T4 DNA Ligase (Fermentas).

2.4 McrBC Digestion

1. McrBC enzyme (NEB).
2. Guanosine triphosphate (GTP, supplied with the enzyme): Make 10X aliquots, since GTP is highly unstable.
3. Agarose (Peqlab).
4. Ethidium bromide (10 mg/ml solution, Bio-Rad).
5. Gel electrophoresis apparatus.

2.5 CpG-Specific Adapter Amplification

1. 100 m*M* nucleotide stock solutions (Fermentas).
2. 50 m*M* aminoallyl-dUTP (Ambion).
3. Labeling nucleotides (20 m*M*): Thaw one vial aminoallyl-dUTP. Add 16.5 µL of H_2O to this stock tube (contains 50 µL aa-dUTP). To this, add 16.6 µL dTTP, 41.6 µL dGTP, 41.6 µL dATP, and 41.6 µL dCTP (from 100 m*M* stock nucleotide tube). Mix and store at −20 °C.
4. Taq polymerase, 5 U/µL (NEB).
5. 200 pmol/µL CG-1b-primer (see Sect. 2.1).

2.6 Purification of Amplification Products

1. Microcon YM-50 columns (Millipore).
2. Spectrophotometer.

2.7 Labeling

1. Na_2CO_3 (Sigma). Prepare 0.1 *M* solution in water.
2. FluoroLink monofunctional dyes, Cy3 and Cy5 (Amersham Biosciences).
3. DMSO (Sigma).
4. Hydroxylamine (Sigma) (10 mL): 4 *M* hydroxylamine. Make aliquots and store at −20 °C.
5. Sodium bicarbonate buffer (0.1 *M*, pH 9.0): Add 0.42 g of sodium bicarbonate to a flask. Add 50 mL of water, and mix with a magnetic stirrer until it dissolves completely. The pH should be around 8.5. Adjust the pH to 9.0 by adding ~2.5–3.0 mL of the 0.1 *M* Na_2CO_3 solution. Monitor the pH change carefully, and do not exceed pH 9.3. Filter sterilize the buffer (prepare as fresh as possible). Aliquot and store at −20 °C. Do not refreeze the buffer. Use each aliquot only once.
6. Prior to coupling, prepare the dyes. The Cy dyes come in packages, which contain five vials. Dissolve one vial of dye in 72 µL DMSO. Aliquot 4.8 µL in 15 light-protected amber reaction tubes, dry immediately in a speedvac, and store at −20 °C protected from light (*see* **Note 2**).
7. MinElute PCR-purification kit (Qiagen).
8. 3 *M* Sodium acetate solution (pH 5.2).

2.8 Hybridization

1. SlideHyb glass array hybridization buffer 2 (Ambion).
2. Yeast tRNA (Sigma).
3. Bovine serum albumin (Sigma).
4. Cot-1 DNA (Roche Diagnostics).
5. Oligonucleotides poly(dA)-poly(dT).
6. Isopropanol (>99.8%, Sigma).
7. Wash solution I: 2X SSC, 0.5 % SDS. Filter-sterilize the buffer (*see* **Note 3**).
8. Wash solution II: 0.5X SSC, 0.5 % SDS. Filter-sterilize the buffer.
9. Coverslips: Hybri-Slips (Sigma).

3 Methods

Here, we present a complete protocol for DNA methylation profiling using the unmethylated fraction of the genome. This protocol works with most microarray types, as exemplified here with CpG island and oligonucleotide microarrays. A detailed description of how specific "epigenetic" microarrays are designed and how the slides are processed can be found elsewhere [1]. DNA samples can be either interrogated as pairs (tester and control) on one array, as described in this protocol, or hybridized independently, with only one fluorescent dye (e.g., for Affymetrix tiling

arrays). This protocol provides two examples: (1) a comparison of DNA derived from human control fibroblast cells, with the same cells treated with either staurosporine (STS) or retinoic acid (RA), and (2) lymphocyte DNA from Prader-Willi syndrome (PWS) patients compared with healthy controls and Angelman syndrome (AS) patients.

3.1 Adapter Design

In the CpG-specific adapter design, the following aspects must be taken into account:

1. It must contain a CpG overhang, which fits to the restriction site of the enzymes used (Fig. 8.2a).
2. The two nucleotides next to the CpG overhang have to be different from the nucleotides within the recognition sequence of all the enzymes used in the restriction

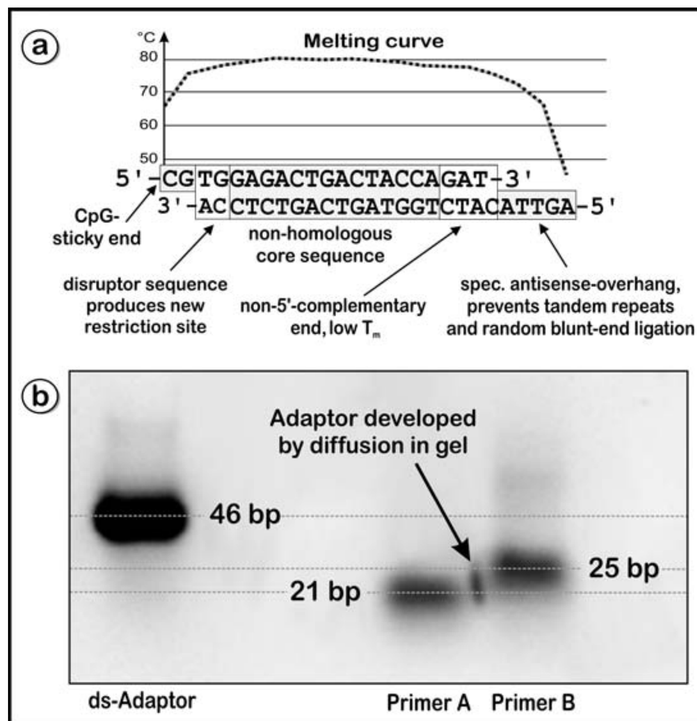

Fig. 8.2 Preparation of the CpG-specific adapter. (a) CG1-adapter components. (b) Annealing of the CG primers produces a double-stranded adapter that can be identified by electrophoresis as a strong band (200 pmol loaded; 2.5 % agarose gel)

digests. This ensures that, after ligation of the adapters, the old restriction site is disrupted and the sequence cannot be recut by the enzymes.

3. The melting temperature (T_m) should be about equal throughout the adapter and decreasing at the 3′ end opposite to the CpG overhang.
4. The core sequence of the adapter should be nonhomologous to any sequence in the gDNA. Additionally, it is advisable to avoid palindromic sequences, which can lead to mispriming.
5. If you want to cut the adapter from the target fragments, include a very specific, new recognition site within the adapters core sequence (close to CpG overhang), which does not cut frequently in the genome you analyze. Use a rare eight-mer (e.g., *Sda*I (5′-CC↑TGCA↓GG) for sticky ends or *Mss*I (GTTT↓AAAC) for blunt ends.
6. Use a specific, long antisense overhang, which prevents the forming of tandem repeats and random blunt-end ligation to the genome (e.g., through degraded DNA).
7. To avoid improper annealing between the two primers, choose a non-5′-complementary end (see Fig. 8.2a).

The adapter preparation involves the following program:

1. Dissolve the nonphosphorylated ssDNA oligonucleotides in ST buffer with a concentration of approximately 800 pmol. The presence of some salt is necessary for the oligos to hybridize into a double-stranded (ds) adapter. Keep some of the CG-1b oligo, as it will be later used as a primer to amplify the unmethylated DNA fraction.
2. Measure the concentration in a spectrophotometer and adjust the concentration to 400 pmol (*see* **Note 4**).
3. Mix together equal molar amounts of each complementary oligonucleotide to a final concentration of 200 pmol, and heat for 5 min in a thermalcycler at 80 °C. Cool down at 1 °C/min until the mixture reaches room temperature (RT). The longer the cooling period, the lower is the risk of hairpin structures.
4. Check a small amount of the ds adapter on a 2.5% agarose-gel (see Fig. 8.2b), which should generate a strong band. Run the single-stranded primers next to the adapter as a control.
5. Store the adapters at −20 °C.

3.2 Methylation-Sensitive Cleavage of DNA

Over 260 methylation-sensitive restriction enzymes (MSREs, including isoschizomers) are available; however, not all enzymes are useful and informative for DNA methylation profiling. Informative MSREs are defined by the number of cleavage fragments that can be ligated to adapters, efficiently amplified, and are not lost during column-purification steps [3]. Some enzymes, although they cut frequently in the genome, produce fewer informative fragments than enzymes that do not cut as

frequently. For example, the nonpalindromic *Aci*I (5′-CCGC-3′) recognizes more than twice as many CpG sites in CpG island regions than *Hpa*II but, on the other hand, produces fewer fragments in the size range that can be detected by PCR or amplified fragment-length polymorphism (AFLP) methods (Table 8.1). Different requirements for the enzyme reduces the list of potentially useful and informative MSREs to about 17, which would cover up to 85% of all CpG island CpG dinucleotides, but less than 50% of all CpG dinucleotides in other genomic regions [3]. Here, we demonstrate the technology with a double digestion using *Hpa*II and *Hin6*I enzymes (*see* **Note 5**). Before the test DNA can be processed, it is advantageous to add "spiking" DNA to the gDNA samples. The spiking DNA consists of "alien" (exogenous) DNA, which allows monitoring of each step of the experiment (*see* **Note 6**); as with any microarray experiment, controls are crucial for monitoring experimental variability. Suitable spiking DNA consists of sequences that are frequently spotted on commercially available microarrays, such as *Arabidopsis*, RNA spikes, or artificial sequences.

1. 400 ng of genomic DNA is digested with 10 U *Hpa*II plus 10 U *Hin6*I for 6 h at 37 °C. The DNA should be highly concentrated to keep the total volume of the reaction below 20 µL.
2. The samples are then incubated for 10 min at 65 °C to inactivate the enzymes. Let the sample cool down at 1 °C/min. This step ensures reannealing of small DNA fragments that can denature during heat inactivation.
3. Immediately proceed with the ligation reaction. It is important that the digested DNA is processed as soon as possible to avoid degradation of the CpG overhangs.

3.3 Ligation

1. Take 200 ng of the digested DNA for a standard ligation. If digested DNA is stored too long before ligation, ligation efficiency decreases.
2. Adjust the buffer concentration to 1X, taking into account that the unpurified restriction product still contains salts from the previous DNA cleavage reaction. That means that the buffer has to be supplemented for only the extra volume added to the DNA.
3. Add ATP to a final concentration of 1 m*M*.
4. Add 120 pmol of the double-stranded CG adapter and mix (*see* **Note 7**).
5. Adjust the volume of the reaction with water to 18 µL, mix, then heat the sample for 10 min at 45 °C in a water bath or thermal cycler (*see* **Note 8**).
6. Take the sample out of the cycler and chill on ice immediately.
7. Add quickly 2 µL (10 U) of the ligase to a final volume of 20 µL, mix well without introducing bubbles and place the tubes back into the cycler. Incubate for 16 h, followed by a short, 5 min, inactivation step at 65 °C. To favor reannealing of short denatured DNA fragments, let the sample cool down at 1 °C/min until room temperature is reached (*see* **Note 9**).
8. Store the ligation mixture at –20 °C (4 °C if the reaction is processed at the same day).

Table 8.1 Methylation-sensitive restriction enzymes

MSRE	Cut site 5′–3′	Approx. % of CpGs in CpG islands*	Approx. % of CpGs in non-CpG islands*	Fragments/kb in CpG islands*	Fragments/kb in non-CpG islands*
*Aci*I (*Ssi*I)	C**CG**C, G**CG**G	30.60	17.36	3.23	1.79
*Hin6*I** (*HinP1*I)	G**CG**C	14.40	5.05	3.98	0.61
*Hpa*II (BsiSI)	C**CG**G	11.70	9.33	3.98	1.18
*Hin1*I (BsaHI)	GR**CG**YC	2.6	0.9	1.92	0.11
*Hpy*CH4IV (TaiI)	A**CG**T	1.66	6.73	1.24	0.97
*Bsp*119I (*Asu*II)	TT**CG**AA	0.11	0.13	0.11	<0.02
*Bsu*15I (*Cla*II)	AT**CG**AT	<0.05	0.39	<0.02	0.02

Note: Methylation-sensitive restriction enzymes. All the MSREs produce sticky ends that can be ligated to the CG-1 adapter for high throughput microarray-based DNA methylation profiling. Other MSREs that fit to the CG-1 adapter are Psp1406I, *Xmi*I, *Bst*BI or *Nar*I; however, these enzymes have very few restriction sites in the human genome or are too expensive to be used in the required amount.

* Indicates the number of 75 bp, 2 kb long, that is, “informative,” fragments, derived from CpG-island and non-CpG-island sequences in the human genome [3]. R = A/G; Y = C/T.

***See* **Note 1**.

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3.4 McrBC Digestion

1. Perform the reaction with NEB buffer 2, 1X BSA (10X), and 2X GTP (2 m*M*).
2. Add buffer only for the extra volume of the reaction. The ligation already took place in a restriction enzyme buffer.
3. ATP inhibits the reaction; therefore, make sure that the DNA sample was either purified before McrBC cleavage or the present ATP was degraded by heating the sample for some time during the "cool-down" phase of the ligation procedure.
4. Digest the DNA with 10 U/µg of McrBC for 8 h at 37 °C (*see* **Note 10**).
5. Heat inactivate the enzyme for 20 min at 65 °C. Store the ligation mixture at −20 °C (4 °C if the reaction is processed at the same day).

3.5 CpG-Specific Adapter Amplification

The protocol is based on aminoallyl (aa) nucleotide incorporation followed by coupling to N-hydroxysuccinimide (NHS) functionalized dyes. This indirect labeling method is advantageous compared to direct incorporation of dye labeled nucleotides in gDNA. The aminoallyl-nucleotide is better tolerated by the Taq polymerase than other fluorescent nucleotides analogues (*see* **Note 11**). While direct incorporation of dye labeled nucleotide protocols typically have incorporation efficiencies in the range of 2–5 dye molecules per 1,000 nucleotides, the aminoallyl protocol can achieve frequencies of incorporation in the range of 10–20 dye molecules per 1,000 nucleotides. Furthermore, the reagent costs for the aminoallyl-NHS protocols are lower. Aminoallyl-dUTP contains a reactive amino group on a two-carbon spacer attached to the methyl group on the base portion of dUTP. During the coupling reaction, after DNA synthesis, this amino group reacts with the NHS ester of the monoreactive Cy3 and Cy5 dyes.

1. For each 200 ng DNA, prepare the PCR-mixture as follows (final volume 100 µL):

Volume	Component	Final concentration
x µL	dd-H_2O	
10 µL	10X buffer	1X
14 µL	$MgCl_2$(25 m*M*)	3.5 m*M*
1.25 µL	Allyl-dNTP mix (20 m*M*)	250 µ*M*
1 µL	CG1b primer (200 pmol)	~1 pmol for each ng DNA
x µL	Adapter ligation	200 ng
3 µL	Taq polymerase (5 U/µL)	15 U (depending on amount of starting DNA)

2. Depending on the thermalcycler, the PCR reaction may have to be split into several tubes. Run the following program:

Step	Time	Temp	Cycles	Notes
1	5 min	72 °C	1	Fills in 3′ recessed ends*
2	1 min	95 °C	1	Starting denaturation
3	30 s	93 °C	24	Denaturation
4	2 min + 3 sec/cycle	68 °C		Annealing and extension
5	5 min	72 °C	1	Final extension
6	hold	4 °C		Storage

*This amplicon-PCR does not work with a hot-start procedure.

3. Check the size of the amplified DNA by separating 8 µL product on a 1% agarose gel (the size of DNA should range from approximately 150 to 2000 bp; see Fig. 8.3).

3.6 Purification of Amplification Product

The overall yield of the amplicon PCR is relatively high (20–100 µg). However, most commercially available column-based PCR cleanup kits cannot handle this large amount of DNA without clogging. Therefore, our laboratory uses YM-50 Microcon columns for cleanup, but other systems may work as well (*see* **Note 12**).

1. Add 500 µL of nuclease-free water to the empty columns and spin ~6 min at ~9,000 *g* until the column is dry (the columns contain small amounts of Tris on their membrane that can inhibit the subsequent labeling reaction. Therefore, the columns have to be washed prior to adding the allyl-PCR product).
2. Insert the Microcon sample reservoir into a 1.5 mL vial. Pipette the whole PCR product into the reservoir. Spin as per guidelines (approximately 1 min at ~9,000 *g*).

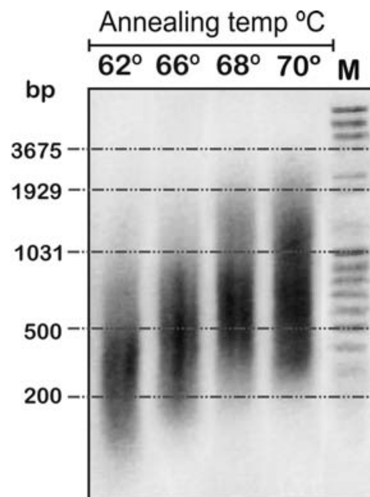

Fig. 8.3 The adapter-amplification results in a typical smear in the size range of 150–2,000 bp. To some extent, the lengths of the amplified fragments depend on the primer annealing temperature of the PCR reaction. Usually, an increased annealing or elongation temperature produces larger DNA fragments

The exact spin time depends on temperature and rotor and may have to be elucidated empirically. Be sure to align the strap of the collection tube cap toward the center of the rotor. Following the spin, the membrane still should be slightly wet.
3. Add 500 µL of dd-H_2O and spin again for 6 min at ~9,000 *g*. The membrane should look wet but not contain a visible amount of liquid. If there is liquid, spin it down for 1 min.
4. Add additional 90 µL of nuclease-free dd-H_2O (*see* **Note 13**).
5. Place the vial with the column into the Eppendorf shaker and shake at 400 rpm for 1 min (this helps to elute the entire DNA, which may be bound to the membrane). If you have no Eppendorf shaker, vortex the column slightly for several seconds.
6. Uncap the Microcon unit. Separate the sample reservoir from the filtrate cup, and place the sample reservoir upside down into a new vial. Spin for 3 min at ~1,500 *g* in invert spin mode to elute DNA.
7. Remove the column. Cap the vial with the eluate (~100 µL) for storage at –20 °C.
8. Use 5 µL of the eluate to measure the DNA concentration in a spectrophotometer.

3.7 Labeling

3.7.1 Coupling

The amount of unmethylated DNA fraction to be hybridized to an array depends on the surface area of the microarray. In our experience, 2 µg of the eluate is enough for a standard slide.

1. For all steps, shield all samples thath contain fluorescent dyes from light!
2. Dry the DNA sample (2 µg) in a speedvac.
3. Resuspend the DNA pellet in 9 µL of the 0.1 *M* sodium bicarbonate buffer. Add 3 µL DMSO. Let sample sit for 10–15 min.
4. Denature the sample for 2 min at 100 °C. In our experience, single-stranded DNA incorporates the Cy dyes better than native DNA.
5. Quickly spin down and add the sample to a dry aliquot of monofunctional Cy3 or Cy5 dye. Resuspend the dye pellets by pipetting carefully; try not to introduce air bubbles (if it happens, centrifuge the sample for a few seconds, this will get rid of the bubbles).
6. Incubate for 2 h at 30 °C in the dark.

3.7.2 Quenching

After the coupling step, reactive groups on the dyes must be quenched to prevent cross reactivity or exchange of dye molecules between active aminoallyl nucleotides. This protocol uses hydroxylamine to quench.

1. Add 4.5 µL of 4 *M* hydroxylamine. Mix well by pipetting up and down.
2. Incubate for 15 min at room temperature with slight agitation (e.g., on an Eppendorf shaker, ~400 rpm) in the dark.
3. Combine the Cy3 and C5 samples.

3.7.3 Cleanup of Product

To remove unincorporated dyes, columns have to be applied that efficiently purify fragments in the size range of ~50–2,000 bp. For this protocol, we are using the Qiagen MinElute PCR-Purification Kit.

1. Add 25 µL of dd-H_2O to the sample.
2. Add 3 µL of 3 *M* sodium acetate (pH 5.2) to ensure a low pH of the mixture. It is important to keep in mind the DNA binding curve for silica, on which this kit is based, is favorable at low pH but falls off precipitously around pH 8.0. Therefore, it is essential that the pH of the reaction be below pH 7.5.
3. Add 275 µL (5X vol.) of buffer PB and mix. Apply to column and centrifuge at 10,000 *g* for 1 min.
4. Discard the flow-through and wash the DNA five times with 750 µL buffer PE at 10,000 *g* for 1 min. A critical step for minimizing the background is to effectively wash away all unbound dye molecules during the final cleanup; small amounts of unincorporated dye can have great absorbance and thus give misleading results. If the color from the dyes still is visible in the wash, continue running wash buffer through the column until the eluate is clear.
5. Centrifuge the column for an additional 1 min at 10,000 *g*.
6. Place the column in a new, clean 1.5 mL microcentrifuge tube and elute by adding 25 µL prewarmed (~50 °C) elution buffer (EB) to the center of the membrane. Let it stand for 1 min at room temperature and spin at 10,000 *g* for 1 min.
7. Repeat the elution step with an additional 25 µL (final volume 50 µL).

3.7.4 Determination of Incorporated Dyes

1. Place the entire 50 µL sample in a clean microcuvette. Measure the absorbance of the Cy3/Cy5 sample in a spectrophotometer at 260 nm (DNA), 550 nm (Cy3 fluorescence), and 650 nm (Cy5 fluorescence). Be very careful about contamination in the cuvette as you will be recovering the sample for hybridization. Wash carefully after each measurement.
2. Calculate the amount of recovered DNA: $A_{260} \times 50 \times$ total volume of sample (µL) = ng of target.
3. Calculate the frequency of incorporation (FOI). For Cy3 incorporation, $86.5 \times (A_{550}/A_{260})$; and for Cy5 incorporation, $51.9 \times (A_{650}/A_{260})$. The results are expressed as the number of Cy-dCTP incorporated per 1,000 nucleotides of DNA (*see* **Note 14**). Optimal frequencies of incorporation are 15–20 and higher, although anything higher than 10 gives satisfactory results. Using targets with an FOI less than 6 may give high background or very weak signals.

3.8 Hybridization

Before starting to hybridize, it is recommended that the coverslips already are cut, the hybridization chambers ready, and all solutions prepared and adjusted to the correct temperature. To prepare the prehybridization buffer, combine 2 mL of SlideHyb glass array hybridization buffer 2, 80 µL of 20 µg/µL yeast tRNA, and 200 µg bovine serum albumin. As the hybridization buffer, use SlideHyb buffer 2 (*see* **Note 15**). Add 50 µL of 20 µg/µl yeast tRNA and 200 µL COT-1 DNA (1 µg/µL). The COT fraction of human placental gDNA consists mainly of repetitive DNA elements. COT DNA is expensive and may be omitted if the microarray used does not contain larger amounts of repetitive sequences. If using cDNA arrays, 10–20 µg poly(dA)-poly(dT), which blocks hybridization to polyA tails of cDNA, may be added. Also, scan one array prior to hybridization to determine basal level of background on the array (*see* **Note 16**).

3.8.1 Prehybridization

1. Briefly heat the prehybridization solution in an amber reaction tube to 72 °C and let it cool down to room temperature.
2. To locate the area on the microarray that has to be hybridized, place the dry microarray on a hybridization template that shows the dimensions of the slide and the area where the grids are printed.
3. Add water into the hybridization chamber to prevent drying of the samples.
4. Pipette the prehybridization solution to the designated area of the slide. Avoid surface contact with objects such as pipette tips. If any bubbles appear, try to remove these with a syringe needle.
5. When bubble free, apply the coverslip carefully (use coverslips larger than the grid area). Use tweezers.
6. Put the slide into a hybridization chamber and prehybridize the array for 1 h at 45 °C.
7. After incubation, move the slides to a 45 °C jar containing dd-water and remove the coverslip carefully, without using force.
8. Transfer the array into a new jar containing 45 °C dd-water and leave them there for 2 min, moving the slide up and down several times. Repeat this step two more times.
9. Transfer the slide into a new jar containing room-temperature isopropanol and repeat the washing by moving the slide up and down several times (~1 min).
10. Immediately blow dry the slide with pressurized air. The prehybridized arrays can now be hybridized or stored in a dark place for later usage.

3.8.2 Hybridization

1. Place the complete 50 µL DNA sample in a speedvac (shield from light) until the solution is reduced to about 5 µL. Do not allow the labeled DNA to dry completely.

2. Preheat the hybridization solution prior to use for 5 min at 72 °C. Make sure the hybridization solution is thoroughly resuspended. Centrifuge for 1 min at 10,000 *g* to get rid of particulate material. Pipette the needed amount (~60 µL for a 12,200 feature CpG- island array) of the hybridization solution and keep it at room temperature for 10 min.
3. Add the DNA (~5 µL) to the hybridization mix, denature it for 5 min at 80 °C, and briefly spin down.
4. Add water into the hybridization chamber to prevent drying of the samples.
5. Pipette the hybridization solution to the designated area of the slide.
6. Apply the coverslip carefully to the bubble-free hybridzation solution. Again, avoid any contact with the array surface.
7. Place the slide quickly but carefully into a hybridization chamber to prevent renaturation of the probe and evaporation of the hybridization solution. Make certain that the array surface is level.
8. Protect the hybridization chambers from light, and hybridize for approximately 12–16 h at 50 °C (oligo arrays may require a lower temperature, whereas higher temperatures are needed for BAC/PAC arrays).

3.8.3 Washing

1. Fill three jars with Wash I and two jars with Wash II and heat them to 50 °C.
2. Remove the coverslip by rinsing the slide carefully in Wash I (jar 2). Take extreme care when handling the array. Allow coverslip to float off the surface. Do not attempt to force off the coverslip, as this will damage the array.
3. Place the array slide quickly into the second Wash I (jar 2) for 15 min (carry out all wash steps in the dark), moving the slide up and down from time to time.
4. Transfer the slides to a new jar (jar 3) and repeat the wash step for 15 min.
5. Place the array slide into Wash II (jar 4) for 15 min, moving the slide up and down from time to time.
6. Transfer the slides to a new Wash II (jar 5) and repeat the wash step (15 min).
7. Rinse the slide for 2 min within another jar in dd-water (jar 6) to remove all traces of SDS and SSC.
8. Transfer the slide into a new jar with isopropanol (jar 7) for 30 s, moving it up and down (*see* **Note 17**).
9. Blow dry the slide as quickly as possible using pressurized air. Any buffer left on the array will appear as haze on array and result in a strong green background.
10. Scan the array in the Cy3 and Cy5 channel (532 nm and 635 nm).

3.9 Examples

The preceding protocol, in combination with other epigenetic profiling methods, may help identify interindividual variation in genomewide methylation patterns as

well as epigenetic changes that arise during tissue differentiation or through the influence of various environmental factors. Figure 8.4 demonstrates how the technology can be used to detect abnormal DNA methylation patterns in genomic imprinting diseases, such as Prader-Willi syndrome. In approximately 25% of subjects affected with PWS, both copies of chromosome 15 are inherited from the mother (maternal disomy or UPD) [4]. However, the missing chromosome 15 from the father, which contains the paternally expressed genes, is required for normal development. The maternal genes are inactive due to methylation of the chromosomal

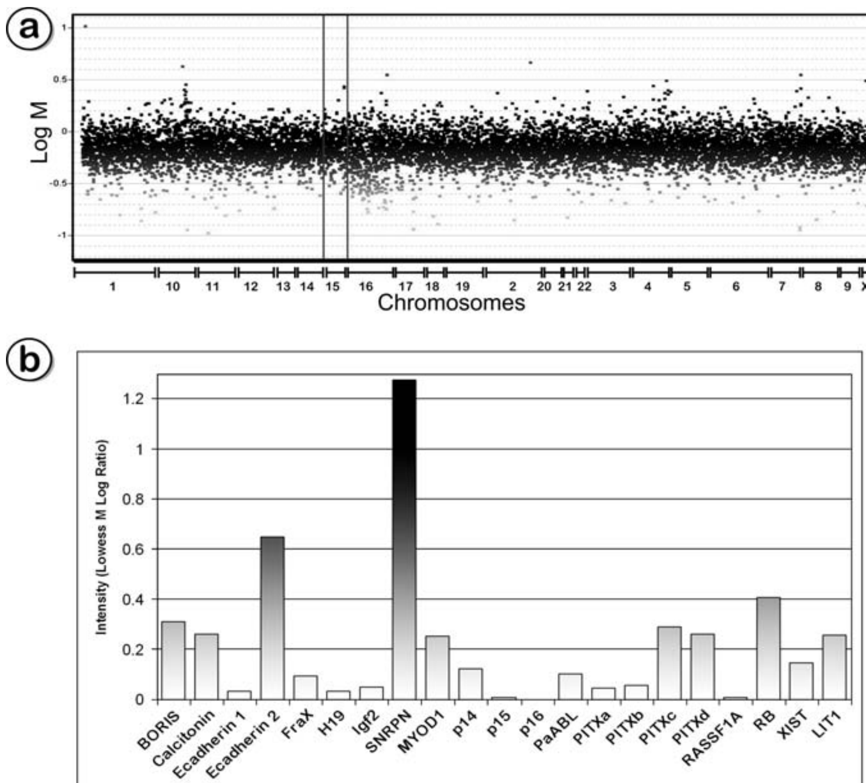

Fig. 8.4 Identification of imprinting patterns in PWS and AS patients. (a) Comparison of two PWS patients with healthy controls using a CpG-island array displays numerous interindividual methylation differences throughout the genome. Additionally, a proportion of the outliers may be caused by single nucleotide polymorphisms (SNPs; *see* **Note 18**). Chromosome 15 data points are highlighted. (b) Comparison of the unmethylated fraction of two PWS and two AS UPD patients (mean values) using an oligonucleotide-microarray. The AS patients have two paternal chromosomes; hence, they should exhibit no methylation of *SNRPN* (default methylated only on the maternal allele). The PWS patients have two maternal chromosomes, resulting in a fully methylated *SNRPN* gene (methylated only on the maternal allele). Of the tested genes, only *SNRPN* showed significant methylation differences (log ratio >1) between the PWS and AS cases

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region. In contrast, in UPD cases of Angelman syndrome, the imprinting is the other way around: Both copies of chromosome 15 are inherited from the father and unmethylated. As can be seen in Fig. 8.4a, many epigenetic differences between PWS patients and healthy controls could be observed. Most detected methylation differences are due to interindividual variation and are not disease specific. However, a comparison of the *SNRPN* gene on chromosome 15 with other imprinted genes on other chromosomes and nonimprinted control genes revealed that the different methylation levels at *SNRPN* could be identified (see Fig. 8.4b). Another example for the application of the presented protocol is shown in Fig. 8.5, where fibroblast cells were either treated with the neurogenic substance, staurosporine, or retinoic acid; several cell lines are known to undergo neuronal and partly cardiomyocyte

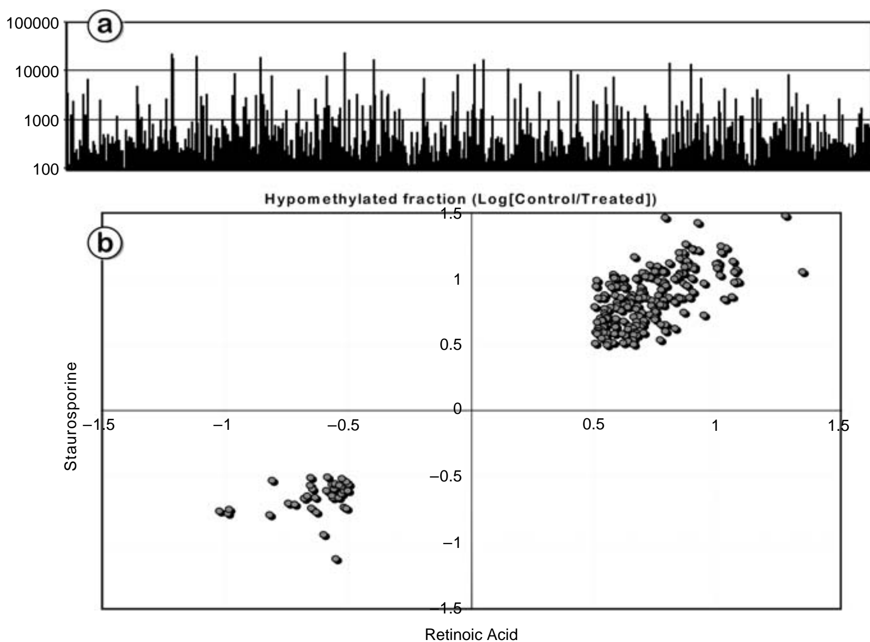

Fig. 8.5 Influence of STS- and RA-treatment on DNA methylation patterns in fibroblast cells. (a) Methylation differences detected with CpG-island microarrays after staurosporine treatment. Each bar indicates the absolute methylation difference at one CpG island reflected in intensity differences of fluorescent dyes on the array (x-axis absolute fluorescent differences between the Cy3 and Cy5 channels). (b) Cosegregation of methylation changes in STS- and RA-treated fibroblast cells. Shown are only the CpG-islands sequences that changed in both STS- and RA-treated cells (log[control/treated] >0.5) compared to untreated controls. Some of the genes that displayed a methylation difference after treatment of both substances, for example, were the postsynaptic density scaffolding protein (PDSP1), the roundabout homolog 2 and 3 precursor (receptor for SLIT2, and probably SLIT1, which are thought to act as molecular guidance cue in cellular migration, including axonal navigation and projection of axons to different regions during neuronal development), NT-3 growth factor receptor precursor, nexin 9, Galectin-1, and MAX interacting protein 1

differentiation in vitro after induction of these substances. Some genes change methylation after induction of staurosporine and retinoic acid, possibly due to common pathways [5].

4 Notes

1. *Hha*I is an isoschizomer of *Hin6*I; however, the *Hha*I restriction enzyme is not suitable for adapter-based methylation analyses, since this enzyme does not produce a CpG overhang that can be ligated to the adapter.
2. Alternatively, water also can be used to dissolve Cy dyes; however, the mono reactive ester is labile in water and not recommended. Do not use Dioxane, which was suggested elsewhere as an alternative to DMSO. It appears that Cy5 is not fully soluble in dioxane. DMSO on the other hand is hygroscopic and will absorb moisture from the air, which reacts with the NHS ester of the dye and significantly reduces the coupling reaction efficiency. Therefore, keep the DMSO supplied in an amber screw-capped vial at −20 °C, and let the vial warm to room temperature before opening to prevent condensation. While the dyes usually are good when first opened, they are sensitive to moisture, with a short half-life in aqueous environments. Therefore, take care to store aliquots of them desiccated, if possible under vacuum.
3. Wash buffer impurities can cause a grainy background; SDS will fluoresce in the Cy5 channel, while salt will fluoresce in the Cy3 channel. To eliminate this problem use high-quality SDS and SSC for preparation of wash buffers and filter sterilize. Insufficient washing also may contribute to a grainy background, therefore, increasing the wash times or slight agitation may help decrease the background.
4. Quantify in spectrophotometer. Remember the $OD_{260} = 1$ of single-stranded DNA is equivalent to 33 µg/mL not 50 µg/mL. This relationship, however, can be inaccurate for short fragments of DNA, such as oligonucleotides. Base composition and even linear sequence affect optical absorbance; hence, the precise value of the OD to mass relationship is unique for each oligo. For example, 1.0 OD_{260} of CCCCCCCCCCCC (homopolymeric deoxycytidine) equals 39 µg while 1.0 OD_{260} of AAAAAAAAAAAA (homopolymeric deoxyadenosine) equals only 20 µg. Do not believe the amount of primer as indicated by the manufacturer. Perform the appropriate calculation (800 pmol primer = 0.0008 × MW oligo).
5. To get the most out of restriction analyses, it is crucial to choose the right enzyme combination for the targets to be interrogated. For example, some MSREs cut relative frequently in CpG islands but rarely recognize a sequence outside of a CpG-island region, as is the case for *Hin6*I (5′-GCGC-3′) or *Bsp143*II (5′-PuGCGCPy-3′). In contrast, enzymes such as *Hpy*CH4IV (5′-ACGT-3′) cut predominantly outside of CpG-island sequences and are less useful in the interrogation of CpG islands, for instance, in CpG-island microarray-based studies [3]. Several other methods rely on the specific methylation-sensitive

cleavage of the rare cutter *Not*I (5′-GCGGCCGC-3′), for example, RLGS and AFLP methods and a couple of microarray approaches [6, 7]. However, *Not*I-sites are not well represented in the genome and provide only a very rough overview of methylation patterns. Hence, it is not advisable to include *Not*I in genomewide analyses of complex diseases.

6. Additionally, spike DNA can be used to test the methylation-sensitive cleavage reaction, ligation efficiency, McrBC digestion, and PCR reaction. Spiking DNA is added to the reactions with concentrations equivalent to the template concentration (1 genome equivalent, GE, assuming 3×10^9 bp in the human genome). For example, 16.2 pg of λ plasmid is added to 1 µg of template DNA. Make 12X GE stock solutions in 1 m*M* EDTA; aliquot into small volumes and store at –20 °C. To prepare a McrBC digestion control, digest pUC57 with *Hpa*II, ligate the CG adapter, and premethylate with SssI methylase. McrBC controls are added to the McrBC cleavage step as premethylated plasmid. Other suitable controls are ΦX174 to test forPCR bias or pBR322 to test the ligation efficiency [1]. The ΦX174 DNA has to be digested separately with *Hpa*II/*Hin6*I then ligated to the CG adapter. Complementary spiking oligonucleotides have to be representative sequences of *Hpa*II and *Hin6*I cleavage fragments.
7. The amount of CG adapter depends directly on the amount of restriction enzymes used. For each enzyme add ~0.3 pmol of the double-stranded CG adapter per ng DNA. For instance, in a triple digest (*Hpa*II/*Hin6*I/*Aci*I) of 500 ng DNA, it is advised to add 500 × 0.3 pmol × 3 = 450 pmol of the adapter.
8. All ligation components initially are combined without the T4-ligase. Since the CpG overhang of the adapter is complementary to itself, it could form adapter dimers during the ligation. Therefore, a heating step is introduced before the ligase is added to the mixture. In this way, adapters are dissociated to bind specifically to the DNA overhangs produced by the restriction enzymes.
9. It is important to reduce the amount of ATP in the mixture, since ATP interferes with the McrBC digestion by competing with GTP for a binding site in McrBC. Since ATP cannot be utilized by the enzyme to perform the enzymatic reaction, McrBC binds to the DNA without cleavage. The best way to decrease the amount of ATP is heat degradation, since free ATP is highly unstable.
10. McrBC makes one cut between each pair of half sites, cutting close to one half site or the other, but cleavage positions are distributed over several base pairs approximately 30 base pairs from the methylated base. Therefore, the enzyme does not produce defined DNA ends on cleavage. Also, when multiple McrBC half sites are present in DNA (as is the case with cytosine-methylated genomic DNA), the flexible nature of the recognition sequence results in an overlap of sites, so a smeared rather than a sharp banding pattern is produced. GTP is more labile than most other nucleotides, so it is recommended to aliquot the 100 m*M* solution supplied. McrBC cuts the DNA (both strands) even if the methylated cytosines are on only one strand (hemi-methylated).
11. This protocol works only with standard Taq polymerases. Other, high-processing polymerases usually cannot incorporate modified nucleotides efficiently enough to produce PCR fragments of the desired length.

12. Centrifugation of Microcon YM-50 columns forces liquid through the low-binding cellulose membrane. Solutes larger than the nominal cutoff of the units are retained: YM-50 columns have a double-stranded nucleotide cutoff of 100 bp. This includes about 46 bp from the adapters on both sides of the amplification products. Therefore, all methylation-sensitive restriction products below ~54 bp and unincorporated adapters and primers should be removed during cleanup. Recovery of amplification products generally is above 90%. Millipore columns also are available as plates, which could be suitable for high-throughput analysis. Recently, we also used Qiagen columns for purification; the overall amount that can be loaded is lower, however, the cleanup still produces enough material for several hybridizations with high-quality DNA.
13. The following aminoallyl/N-hydroxysuccinimide reaction requires that no free amines be present in the coupling reaction buffer. This means that no Tris buffer can be used during any steps, including the DNA preparation.
14. The numbers 86.5 and 51.9 are conversion factors calculated by using the average molecular weight of dNTPs (324.5 g/mole), the absorption coefficient of Cy3 and Cy5 (150,000/M^*cm and 250,000/M^*cm), and the concentration of Cy-labeled allyl-PCR products that absorb 1 AU of 260-nm light (50 μg/mL). When measuring ss-DNA, the concentration of Cy-labeled ss-DNA that absorbs 1 AU of 260-nm light is 37 μg/mL. The conversion factors changes then to 117 for Cy3 and 70.2 for Cy5. Note that sample volume is not factored in; this is because volumes cancel out when DNA and cyanine absorbance are measured simultaneously. Frequently, the FOI for the different dyes may differ. In our experience, Cy5 is not as well incorporated as Cy3, however, it really depends on several factors (humidity, ozone concentration, etc.) and that is why it is recommended to do reciprocal labeling.
15. Other hybridization buffers may be used. The optimal buffer depends on several experimental variables, such as type of glass slide used, the length and GC content of the attached nucleic acid targets, and the manufacturing protocol employed.
16. As arrays age, some surface chemistries, particularly poly-L-lysine, show an increase in autofluorescence in the Cy3 channel. Aged microarrays also may show a higher affinity for salt, which will fluoresce in the Cy3 channel. To minimize these effects, use arrays as fresh as possible and store them properly (in the dark and under vacuum, if possible).
17. Salt from the final wash buffer may precipitate out of solution onto the array surface on submersion into isopropanol. This is likely a result of a higher than intended concentration of salt in the wash buffer; accidentally switching the wash buffers can cause this phenomenon. A brief rewash in water followed by another isopropanol wash likely will remove the salt from the array.
18. SNPs within the recognition sites for *Hpa*II and *Hin6*I may simulate epigenetic differences [8]. To exclude the impact of DNA sequence variation, check the available SNP databases and identify the DNA sequence variation within the restriction sites of the used enzymes. From CpG-island microarray studies, the estimate is that 10–30% of methylation variation detected in among individuals, in fact,

could be due to DNA sequence variation [1]. For comparison, in a study for the Human Epigenome Project (HEP), interrogation of 3,273 unique CpG sites on chromosome 6 revealed that 101 CpGs overlapped with known SNPs (3%) [9]. Another way to differentiate DNA sequence effects from genuine epigenetic differences consists of performing an identical microarray experiment on a DNA sample that has been stripped of all methylated cytosines [1]. To eliminate all methylation, the whole genome has to be amplified (thereby replacing all ^{met}C with C), for example, using the Phi29 DNA polymerase. Amplified DNA samples then are subjected to the same steps as depicted in Fig. 8.1 and cohybridized on the microarrays. In this experiment, all the outliers must be a result of DNA sequence variations within the restriction sites of the enzymes used and can be eliminated from further epigenetic analyses.

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