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Chapter 13: Methods for CpG methylation array profiling via bisulfite conversion

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Abstract

DNA methylation is a key factor in epigenetic regulation, and contributes to the pathogenesis of many diseases, including various forms of cancers, and epigenetic events such X inactivation, cellular differentiation and proliferation, and embryonic development. The most conserved epigenetic modification in plants, animals, and fungi is 5-methylcytosine (5mC), which has been well characterized across a diverse range of species. Many technologies have been developed to measure modifications in methylation with respect to biological processes, and the most common method, long considered a gold standard for identifying regions of methylation, is bisulfite conversion. In this technique, DNA is treated with bisulfite, which converts cytosine residues to uracil, but does not affect cytosine residues that have been methylated, such as 5-methylcytosines. Following bisulfite conversion, the only cytosine residues remaining in the DNA, therefore, are those that have been methylated. Subsequent sequencing can then distinguish between unmethylated cytosines, which are displayed as thymines in the resulting amplified sequence of the sense strand, and 5-methylcytosines, which are displayed as cytosines in the resulting amplified sequence of the sense strand, at the single nucleotide level. In this chapter, we describe an array-based protocol for identifying methylated DNA regions. We discuss protocols for DNA quantification, bisulfite conversion, library preparation, and chip assembly, and present an overview of current methods for the analysis of methylation data.

Keywords

DNA; methylation; CpG; epigenetics; bisulfite conversion

1. Introduction

In 1975, Riggs proposed a model that DNA methylation of cytosine residues in the context of CpG dinucleotides should affect binding of regulatory proteins [1]. Since then, pioneering work has shown that DNA methylation directly silences genes, oftentimes in heritable patterns and with varying levels of regulation [1, 2]. With the advancement of technology

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and a better understanding of methylation processes, numerous subsequent studies have implicated methylation in the molecular etiologies of many diseases and genetic events, including neurodegenerative and cardiovascular diseases, diabetes and insulin secretion, various forms of cancers, X-inactivation, cellular differentiation and proliferation, and embryonic development [3–8].

Epigenetic regulation occurs through a highly conserved enzymatic mechanism that incorporates histone deacetylases, methyl-binding proteins, and DNA methyltransferases (DNMTs) [9]. The 5-methylcytosine (5mC) residue within a CpG sequence context is a highly conserved epigenetic marker in plants, fungi, and animals [10]. Approximately 60–80% of the 28 million CpG sites in the human genome are methylated [11], and given the role of DNA methylation in a diverse array of biological roles and gene regulation, the investigation and identification of CpG sites is important for a better understanding of disease pathogenesis and progression.

The identification and characterization of CpG sites is a growing area of research and there are many platforms available for assessment of DNA methylation, each with advantages and disadvantages in specific applications [12, 13]. Bisulfite sequencing technology is considered the gold standard for detection of methylated DNA due to the qualitative and quantitative ability to identify 5mC at a single base pair resolution. Initially developed by Frommer et al [13], this method converts unmethylated cytosine residues into uracil residues, which are then displayed as thymines on the resulting amplified sense strand in single-stranded DNA sequencing. In contrast, 5mCs do not respond to bisulfite treatment and will be displayed as cytosine residues on the amplified sense strand, therefore, allowing them to be distinguished from unmethylated cytosine residues [14].

Although new approaches for assessing methylation status are continuously emerging, a popular bisulfite conversion method is methyl-DNA immunoprecipitation (MeDIP), which involves denaturation and precipitation of cleaved DNA using a 5mC antibody, followed by sequencing [15]. Another commonly used technology, shotgun sequencing, has the ability to achieve single-base resolution of bisulfite sequencing, although it cannot distinguish between 5-hydroxymethylcytosine and 5mC [16]. The HumanMethylation450 BeadChip array (Illumina; San Diego, CA) allows the analysis of 19,755 unique CpG islands and 3091 probes at non-CpG sites. The Infinium HumanMethylation450 BeadChip array (Illumina) can interrogate the methylation status of more than 450,000 CpGs throughout the genome, 19,755 unique CpG islands with additional coverage in shore regions and miRNA promoters, as well as 3091 probes at non-CpG sites [17]. Although not as comprehensive as sequencing-based approaches, the 450k array provides simpler analysis and interpretation. Some currently popular methods for 450k array data analysis include *methylni*, *minfi*, *wateRmelon*, *RnBeads*, *ChAMP* and *COHCAP*, which are discussed in greater detail below.

The purpose of this chapter is to provide a detail protocol for performing array-based interrogation of CpG methylation using human genomic DNA. We start with a description of DNA quantification using Quant-iT PicoGreen, and follow with a protocol for bisulfite conversion that includes techniques for library preparation, chip assembly, and chip analysis

on the Illumina platform (Figure 1). We also present an approach for sample analysis and provide an overview of current packages for the analysis of methylation data.

2. Materials

2.1. PicoGreen Assay

1. DNA extracted from samples of interest
2. Quant-iT™ PicoGreen dsDNA Reagent Kit (Thermo Fisher Scientific; Waltham, MA)
3. Black plate with clear flat bottom
4. 1x Tris-EDTA (TE) buffer
5. Spectrofluorometer

2.2. Bisulfite Conversion

1. 500 ng of extracted DNA
2. EZ DNA Methylation™ Kit (Zymo Research; Irvine, CA)
3. DNA/RNase/PCR inhibitor-free plates and tubes
4. Thermocycler

2.3. Methylation Array

1. Infinium HumanMethylation450 BeadChip Kit (Illumina; San Diego, CA)
2. 96-well 0.2 ml skirted microplate
3. 960 well 0.8 ml microplate (MIDI)
4. 150-mL reservoirs
5. Heat sealing foil sheets
6. 0.1 N NaOH
7. 100% Ethanol
8. 100% 2-propanol
9. 95% formamide/1 mM EDTA
10. Wash rack
11. Infinium standard glass back plates and spacer (Illumina; San Diego, CA)
12. Illumina Hybridization Oven (Illumina; San Diego, CA)
13. Hyb chamber, gasket and insert (Illumina; San Diego, CA)
14. Tecan
15. iScan or HiScan (Illumina; San Diego, CA)

3. Methods

3.1. PicoGreen Assay

1. Prepare a 2 µg/mL working solution of the lambda DNA standard, by diluting the 100 µg/mL sample provided in the Quant-iT™ PicoGreen dsDNA Reagent Kit in TE buffer. For a five-point standard curve ranging from 1 ng/mL to 1 µg/mL, proceed to Step 3. For a low-range standard curve ranging from 25 pg/mL to 25 ng/mL, prepare a 40-fold dilution of the 2 µg/mL DNA solution to yield a 50 ng/mL DNA stock solution and proceed to Step 6.
2. Prepare an aqueous working solution of the Quant-iT PicoGreen reagent by making a 200-fold dilution of the concentrated DMSO solution in TE (Note 1).
3. For the high-range standard curve, dilute the 2 µg/mL DNA stock solution with TE buffer as shown in Table 1 and add 1.0 mL of aqueous solution of Quant-iT PicoGreen reagent to each cuvette. Mix well and incubate for 2–5 minutes at room temperature, protected from light.
4. Measure the sample fluorescence using a spectrofluorometer or fluorescence microplate reader and standard fluorescein wavelengths (excitation ~480 nm, emission ~520 nm) (Note 2).
5. Subtract the fluorescence value of the reagent blank from readings for all of the samples.
6. For the low-range standard curve, dilute the 50 ng/mL DNA stock solution with TE buffer in disposable cuvettes, as shown in Table 2. Add 1.0 mL of the aqueous working solution of the Quant-iT PicoGreen reagent to each cuvette. Mix well and incubate for 2–5 minutes at room temperature, protected from light. Continue with Steps 2 and 3. Adjust the fluorometer gain to accommodate the lower fluorescence signals.

Sample analysis

1. Dilute the experimental DNA solution in TE to a final volume of 1.0 mL in disposable cuvettes or test tubes (Note 3).
2. Add 1.0 mL of the aqueous working solution of the Quant-iT PicoGreen reagent to each sample. Incubate for 2–5 minutes at room temperature, protected from light.
3. Measure the fluorescence of the sample using the instrument parameters that correspond to those used when generating your standard curve (Note 4).
4. Subtract the fluorescence value of the reagent blank from those of all the samples. Determine the DNA concentration of the sample from the standard curve generated in DNA standard curve.

3.2. Bisulfite Conversion using the EZ DNA Methylation™ Kit

1. Prepare the CT Conversion Reagent by combining 750 µl water and 210 µl M-Dilution Buffer to a tube of CT Conversion Reagent. Mix at room temperature with frequent vortexing or shaking for 10 minutes (Note 5).
2. Prepare the M-Wash Buffer by adding 24 ml of 100% ethanol to the 6 ml M-Wash Buffer concentrate (D5001) or 96 ml of 100% ethanol to the 24 ml M-Wash Buffer concentrate (D5002).
3. Add 5 µl of M-Dilution Buffer to the DNA sample and adjust the total volume to 50 µl with water. Mix by flicking or pipetting up and down.
4. Incubate the sample at 37°C for 15 minutes.
5. Add 100 µl of the CT Conversion Reagent to each sample and mix.
6. Incubate in the dark at 50°C for 12–16 hours.
7. Incubate at 0–4°C for 10 minutes.
8. Add 400 µl of M-Binding Buffer to a Zymo-Spin IC™ column and place the column into the collection tube provided in the kit.
9. Load the Sample from Step 7 into the Zymo-Spin IC Column containing the M-Binding Buffer. Close the cap and mix by inverting the column several times.
10. Centrifuge at full speed (10,000 x g) for 30 seconds. Discard flow-through.
11. Add 100 µl of M-Wash Buffer to the column. Centrifuge at full speed for 30 seconds.
12. Add 200 µl of M-Desulphonation Buffer to the column and let stand at room temperature (20–30°C) for 15–20 minutes. Following incubation, centrifuge at full speed for 30 seconds.
13. Add 200 µl of M-Wash Buffer to the column. Centrifuge at full speed for 30 seconds. Repeat this step.
14. Place the columns in a 1.5 ml microcentrifuge tube. Add 10 µl of M-Elution Buffer directly to the column matrix. Centrifuge at full speed to elute the DNA.

At this point, the DNA can be analyzed or stored at temperatures -20°C. For long-term storage (e.g. over a year), store DNA at temperatures -70°C (Note 6).

3.3. Methylation Array

The following steps describe the transfer of bisulfite-converted DNA to a MSA4 plate, the denaturation and neutralization of samples, and the preparation of samples for amplification.

1. Preheat the Illumina Hybridization Oven to 37°C in a post-PCR area.
2. Thaw the MA1, RPM, and MSM reagents to room temperature.
3. Apply a MSA4 barcode label to a new 96-well 0.8 ml microplate (MIDI) (Note 7).

4. Select “MSA4 Tasks” at the robot PC and select the WG#-BCD plate type (midi or TCY), making sure that the “Use Barcodes” checkbox is cleared, and enter the number of DNA samples (48 or 96) in the “Basic Parameters” field.
5. Remove caps from the MA1, RPM, and MSM tubes, and then place them in the robot standoff tube rack according to the bed map.
6. Vortex the sealed plate at 1600 rpm for 1 minute, and centrifuge at $280 \times g$ for 1 minute.
7. Add 15 ml 0.1 N NaOH to the quarter reservoir, and place the reservoir on the robot bed according to the bed map.
8. Place the WG#-BCD and MSA4 plates on the robot bed according to the bed map, and click “Run” on the robot PC. Then, select the batch you want to run, and click “OK”. Click “OK” again to confirm the required DNAs.
9. After the robot adds the 0.1 N NaOH to the DNA in the MSA4 plate, follow the instructions at the prompt.
10. Seal the plate with a cap mat, vortex at 1600 rpm for 1 minute, and centrifuge at $280 \times g$ at 22°C for 1 minute (Note 8).
11. Place the MSA4 plate back to the robot bed, and click “OK”.
12. When the process is done, seal the MSA4 plate with a cap mat, invert it at least 10 times to mix contents, and centrifuge at $280 \times g$ for 1 minute.
13. Select “Infinium HD Methylation | Incubate MSA4” in the Illumina LIMS left pane.
14. Scan the barcode of the MSA4 plate, click “Verify”, and then “Save”.
15. Incubate in the Illumina Hybridization Oven for 20–24 hours at 37°C .

The following steps describe the enzymatic fragmentation of the amplified DNA samples.

1. Preheat the heat block with the midi plate insert to 37°C .
2. Thaw FMS tubes to room temperature and gently invert at least 10 times to mix contents.
3. If you plan to resuspend the MSA4 plate, remove the RA1 reagent from the freezer to thaw.
4. Pulse-centrifuge the MSA4 plate at $280 \times g$, remove the cap, and place it on the robot bed according to the bed map. At the robot PC, select “MSA4 Tasks | Fragment MSA4”. Make sure that the “Use Barcodes” checkbox is cleared, and in the “Basic Parameters” pane, change the value for number of MSA4 plates and number of DNA samples per plate to incubate to correspond with the number of samples (Note 9).
5. Place the MSA4 plate on the robot bed according to the bed map.

6. Place FMS tubes in the according bed map, remove the cap, and click “Run”. Click “OK” when finished, remove the plate, and seal it with a cap mat.
7. Vortex at 1600 rpm for 1 minute, pulse-centrifuge at 280 x *g*, and place on the 37°C heat block for 1 hour.

At this point the plate can be left in the 37°C heat block until ready to proceed, or sealed and stored at –25°C to –15°C for more than 24 hours.

PM1 and 2-propanol are added to the MSA4 plate to precipitate the DNA samples.

1. Preheat the block to 37°C, thaw the MSA4 plate to room temperature, and then pulse-centrifuge at 280 x *g*.
2. Thaw the PM1 reagent to room temperature and gently invert to mix at least 10 times.
3. Select “MSA4 Tasks | Precip MSA4” at the robot PC, and make sure the “Use Barcodes” checkbox is cleared.
4. Place the plate in the appropriate robot bed.
5. Place a half reservoir in the reservoir frame, and add 1 tube PM1 for 48 samples, or 2 tubes for 96 samples. Also, place a full reservoir in the reservoir frame, and add 20 ml 2-propanol for 48 samples, or 40 ml for 96 samples, and then click “Run”. Make sure the “Use Barcodes” checkbox is selected.
6. When prompted, remove the plate from the robot bed, seal it with the same cap mat that was removed earlier, vortex at 1600 rpm for 1 minute, incubate at 37°C for 5 minutes and pulse-centrifuge at 280 x *g* (Note 10).
7. Remove and discard the cap mat, place the plate back in the robot bed, and click “OK”.
8. When prompted, seal the plate with a new, dry cap mat. Invert the plate 10 times to mix, incubate at 4°C for 30 minutes, centrifuge at 3000 x *g* at 4°C for 20 minutes, and immediately remove the MSA4 plate from the centrifuge. Remove and discard the cap mat.
9. Quickly invert the MSA4 plate and drain the liquid onto an absorbent pad, then smack the plate down on a dry area of the pad, avoiding the liquid that was drained onto the pad.
10. Leave the plate uncovered and inverted on the tube rack for 1 hour at room temperature to dry the pellet.

At this point the DNA can be resuspended as described below sealed and stored at –25°C to –15°C for no more than 24 hours.

The following steps describe the resuspension of precipitated DNA samples.

1. Before proceeding, preheat the Illumina Hybridization Oven to 48°C and the heat sealer for 20 minutes.

2. Thaw the RA1 reagent in a 20°C to 25°C water bath. Gently mix to dissolve any crystals that might be present.
3. At the robot PC, select “MSA4 | Resuspend MSA4”, and in the Basic Run Parameters change the value of number of MSA4 plates and number of DNA samples per plate to correspond with the number of samples being processed.
4. After placing the MSA4 plate on the robot bed, place a quarter reservoir in the reservoir frame, and add 4.5 ml of RA1 for 48 samples, or 9 ml for 96 samples.
5. Make sure the “Use Barcodes” checkbox is selected and then click “Run”.
6. Click “OK” in the message box, remove the MSA4 plate, and apply a foil seal by firmly holding the heat sealer block down for 3 seconds.
7. Immediately remove the plate from the heat sealer and forcefully roll the rubber plate sealer over the plate until 96 indentations become visible.
8. Place the sealed MSA4 plate in the Illumina Hybridization Oven to incubate for 1 hour at 48°C.
9. Vortex the plate at 1800 rpm for 1 minute and pulse centrifuge at 280 x *g*.

At this point, the samples can be hybridized to the BeadChip, where it is safe to leave the RA1 at room temperature, or stored as the MSA4 plate at –25°C to –15°C for no more than 24 hours. The plate can also be stored at –80°C for longer than 24 hours.

The following steps describe the dispensation of the fragmented, resuspended DNA samples onto BeadChips and incubation of BeadChips in the Illumina Hybridization Oven for sample hybridization.

1. Preheat the heat block to 95°C and the Illumina Hybridization Oven to 48°C. Set the rocker speed to 5.
2. Make sure you have the correct Robot Tip Alignment Guide for the Infinium assay you are running. The barcode says Guide-B for the 12x1 HD BeadChip or Guide-E for the 8x1 HD Beadchip. Also, wash and dry the entire one-piece Robot Tip Alignment Guide.
3. Denature the samples in the MSA4 plate on the heat block at 95°C for 20 minutes.
4. Remove the BeadChips from 2°C-8°C storage, but leaving them in the original packaging until ready to use. During the 20-minute incubation, prepare the Hyb Chambers.
5. Place the BeadChip Hyb Chamber gaskets into the BeadChip Hyb Chambers.
6. Dispense 400 µl PB2 reagent into the humidifying buffer reservoirs in the Hyb Chambers, and then immediately place the lid on the Hyb Chamber to prevent evaporation. At this point, the lid does not need to be locked.

7. Leave the closed Hyb Chambers on the bench at room temperature until the BeadChips are loaded with DNA sample. Load BeadChips into the Hyb Chamber within 1 hour (Note 11).
8. After the 20-minute incubation, remove the MSA4 plate from the heat block and let sit at room temperature for 30 minutes.
9. Remove all BeadChips from packaging (Note 12).
10. Place BeadChips into the Robot BeadChip Alignment Fixtures with the barcode end aligned to the ridges on the fixture.
11. Select “MSA4 Tasks | Hyb-Multi BC2” at the robot PC.
12. In the Basic Run Parameters pane, change the value for number of MSA4 plates and number of DNA samples per plate to correspond with the number of samples being processed.
13. Place the Robot BeadChip Alignment Fixtures onto the robot bed according to the bed map.
14. Pulse-centrifuge the plate to 280 x *g* and place it onto the robot bed. Remove the foil seal, make sure that the “Use Barcodes” checkbox is checked, and click “Run”.
15. Place the Robot Tip Alignment Guide on top of the Robot BeadChip Alignment Fixture with the Guide-B barcode upside down and facing away. Push both the Robot Tip Alignment Guide and Robot BeadChip Alignment Fixture to the upper left corner in its section of the robot bed.
16. At the robot PC, click “OK” to confirm that the Robot Tip Alignment Guide has been placed on top of the Robot BeadChip alignment fixture. The robot scans the barcode on the Robot Tip Alignment Guide to confirm that the correct tip guide is being used. The robot dispenses sample to the BeadChips.
17. Click “OK” in the message box.
18. Carefully remove the Robot BeadChip alignment fixtures from the robot bed and visually inspect all sections of the BeadChips. Make sure that DNA sample covers all the sections of each bead stripe. Record any sections that are not completely covered.
19. Make sure that the Illumina Hybridization Oven is set to 48°C.
20. Carefully remove each BeadChip from the Robot BeadChip alignment fixtures when the robot finishes (Note 13).
21. Carefully place each BeadChip in a Hyb Chamber insert, orienting the barcode end so that it matches the barcode symbol on the insert.
22. Load the Hyb Chamber inserts containing loaded BeadChips inside the Illumina Hyb Chamber. Position the barcode over the ridges indicated on the Hyb Chamber.

23. In the Illumina LIMS left pane click “Infinium HD Methylation | Infinium Prepare Hyb Chamber”.
24. Scan the barcodes of the PB2 tubes and scan the BeadChip barcodes. Click “Verify” and “Save”.
25. Position the lid onto the Hyb Chamber by applying the backside of the lid first, and then slowly bringing down the front end to avoid dislodging the Hyb Chamber inserts.
26. Close the clamps on both sides of the Hyb Chamber so that the lid is secure and even on the base and there are no gaps (Note 14).
27. Place the Hyb Chamber in the 48°C Illumina Hybridization Oven with the clamps on the left and right sides of the oven and the Illumina logo facing front.
28. Incubate at 48°C for at least 16 hours, but no more than 24 hours.

Proceed to Wash BeadChips (Post-Amp) after the overnight incubation.

1. Add 330 ml 100% EtOH to the XC4 bottle, for a final volume of 350 ml. Each XC4 bottle has enough solution to process up to 24 BeadChips.
2. Shake the XC4 bottle vigorously to ensure complete resuspension. After it is resuspended, use XC4 at room temperature. The solution can be stored at 2°C-8°C for 2 weeks.
3. For optimal performance, wash and dry the Robot Tip Alignment Guides after every run.
4. Soak the tip guide inserts for 5 minutes in a 1% aqueous Alconox solution (1 part Alconox to 99 parts water) using a 400 ml Pyrex beaker (Note 15).
5. After the soak in the 1% Alconox solution, thoroughly rinse the tip guides with DiH₂O at least three times to remove any residual detergent.
6. Dry the Robot Tip Alignment Guide using a Kimwipe or lint-free paper towels. Use a laboratory air gun to dry. Be sure to inspect the tip guide channels, including the top and bottom. Tip guides must be dry and free of any residual contaminants before next use.

Prepare the BeadChips for the staining process.

1. Remove each Hyb Chamber from the Illumina Hybridization Oven and let cool for 30 minutes before opening.
2. Fill two wash dishes with PB1 (200 ml per dish) and fill the Multi-Sample BeadChip Alignment Fixture with 150 ml PB1.
3. Attach the wire handle to the rack and submerge the wash rack in the wash dish containing 200 ml PB1.

4. Remove the Hyb Chamber inserts from the Hyb Chambers, the BeadChip from the Hyb Chamber insert and finally, remove the cover seal from each BeadChip (Note 16).
5. Using powder-free gloved hands, hold the BeadChip securely by the edges in one hand. Avoid contact with the sample inlets. Make sure that the barcode is facing up and closest to you, and that the top side of the BeadChip is angled slightly away from you. Remove the entire seal in a single, continuous motion. Start with a corner on the barcode end and pull with a continuous upward motion away from you and towards the opposite corner on the topside of the BeadChip. Do not touch the exposed arrays.
6. Immediately and carefully slide each BeadChip into the wash rack, making sure that the BeadChip is submerged in the PB1.
7. Repeat until all BeadChips (a maximum of eight) are transferred to the submerged wash rack.
8. After all BeadChips are in the wash rack, move the wash rack up and down for 1 minute, breaking the surface of the PB1 with gentle, slow agitation.
9. Move the wash rack to the other wash dish containing clean PB1 and repeat Step 8.
10. When you remove the BeadChips from the wash rack, inspect them for remaining residue.
11. For each additional set of eight BeadChips, assemble the flow-through chambers for the first eight BeadChips and repeat the wash steps (Note 17).
12. If you plan to process more than four BeadChips, the 150 ml of PB1 used to fill the BeadChip alignment fixture can be reused for an additional set of four BeadChips (Note 18).
13. For each BeadChip to be processed, place a black frame into the BeadChip alignment fixture prefilled with PB1.
14. Place each BeadChip to be processed into a black frame, aligning its barcode with the ridges stamped onto the alignment fixture (Note 19).
15. Place a clear spacer onto the top of each BeadChip. Use the alignment fixture grooves to guide the spacers into proper position (Note 20).
16. Place the alignment bar onto the alignment fixture. The groove in the alignment bar fits over the tab on the alignment fixture.
17. Place a clean glass back plate on top of the clear spacer covering each BeadChip. The plate reservoir is at the barcode end of the BeadChip, facing inward to create a reservoir against the BeadChip surface.
18. Attach the metal clamps to the flow-through chambers as follows: gently push the glass back plate against the alignment bar with one finger. Place the first metal clamp around the flow-through chamber so that the clamp is

approximately 5 mm from the top edge. Place the second metal clamp around the flow-through chamber at the barcode end, approximately 5 mm from the reagent reservoir.

19. Using scissors, trim the ends of the clear plastic spacers from the flow-through chamber assembly. Slip scissors up over the barcode to trim the other end.
20. Immediately wash the Hyb Chamber reservoirs with DiH_2O and scrub them with a small cleaning brush, ensuring that no PB2 remains in the Hyb Chamber reservoir.

If you are using Illumina LIMS, in the Illumina LIMS left pane, click “Infinium HD Methylation | Wash BeadChip”. Scan the reagent barcodes and the BeadChip barcodes, click “Verify”, and then click “Save”. Illumina LIMS records the data and queues the BeadChips for the next step (Note 21).

The following steps describe the washing of unhybridized and non-specifically hybridized DNA sample from the BeadChips, addition of nucleotides to extend the primers to the DNA, staining of the primers, disassembly of the Flow-Through Chambers, and coating of Beadchips for protection.

1. Thaw the RA1 reagent in a 20°C to 25°C water bath. Gently mix to dissolve any crystals that might be present.
2. Place all tubes (RA1, XC1, XC2, TEM, XC3, STM, ATM, PB1, XC4, Alconox Powder Detergent, 95% formamide/1mM EDTA) in a rack in the order in which they will be used. If frozen, allow them to thaw to room temperature, and then gently invert the reagent tubes at least 10 times to mix contents.
3. Ensure the water circulator is filled to the appropriate level. Turn on the water circulator and set it to 44°C using the Circulator Manager in the automation control software.
4. Remove bubbles trapped in the Chamber Rack.
5. Test several locations on the Chamber Rack, using the Illumina Temperature Probe. All locations should be at 44°C $\pm$ 0.5°C. If the temperature on the probe is not within $\pm$ 0.5°C, contact Illumina Technical Support.

The remaining steps must be performed without interruption.

1. Slide the chamber rack into column 36 on the robot bed. Make sure that it is seated properly.
2. Select “XStain Tasks | XStain HD BeadChip” at the robot PC. In the Basic Run Parameters pane, enter the number of BeadChips.
3. If you plan on imaging the BeadChips immediately after the staining process, turn on the iScan or HiScan now to allow the lasers to stabilize.

4. Place a quarter reservoir in the reservoir frame, according to the robot bed, and add 15 ml of 95% formamide/1mM EDTA to process 8 BeadChips, 17 ml to process 16 BeadChips, or 25 ml to process 24 BeadChips.
5. Place a half reservoir in the reservoir frame, according to the robot bed, and add 50 ml of CX3 to process 8 BeadChips, 100 ml to process 16 BeadChips, or 150 ml to process 24 BeadChips.
6. Place each reagent tube (XC1, XC2, TEM, STM, ATM) in the robot tube rack according to the bed map, and remove their caps.
7. When prompted, enter the stain temperature indicated on the STM tube. Do not load the BeadChips yet.
8. When the chamber rack reaches 44°C, quickly place each flow-through chamber assembly into the first row of the chamber rack. Refer to the robot bed map for the correct layout.
9. Click “OK” at the robot PC. When the robot finishes, immediately remove the flow-through chambers from the chamber rack. Place horizontally on the lab bench at room temperature.
10. Pour 310 ml PB1 per 8 BeadChips into a wash dish.
11. Place the staining rack inside the wash dish.
12. For each BeadChip, use the dismantling tool to remove the two metal clamps from the flow-through chamber. Remove the glass back plate, the spacer, and then the BeadChip. Immediately place each BeadChip into the staining rack that is in the wash dish with the barcode facing away from you. Make sure all BeadChips are submerged.
13. Slowly move the staining rack up and down 10 times, breaking the surface of the reagent.
14. Allow the BeadChips to soak for an additional five minutes.
15. Shake the XC4 bottle vigorously to ensure complete resuspension. If necessary, vortex until dissolved.
16. Pour 310 ml CX4 into a wash dish (Note 22).
17. Move the BeadChip staining rack into the XC4 dish.
18. Slowly move the staining rack up and down 10 times, breaking the surface of the reagent.
19. Allow the BeadChips to soak for an additional 5 minutes.
20. Lift the staining rack out of the solution and place it on a tube rack with the staining rack and BeadChips horizontal, barcodes facing up.
21. Remove the BeadChips from the staining rack with locking tweezers, working from top to bottom. Place each BeadChip on a tube rack to dry. Remove the rack handle if it facilitates removal of the BeadChips.

22. Dry the BeadChips in the vacuum desiccator for 50–55 minutes at 675 mm Hg (0.9 bar).
23. Make sure that the XC4 coating is dry before proceeding to the next step.
24. Clean the underside of each BeadChip with a ProStat EtOH wipe or Kimwipe soaked in EtOH (Note 23).
25. Clean and store the glass back plates and Hyb Chamber components.
26. If you are using Illumina LIMS, in the Illumina LIMS left pane click “Infinium HD Methylation | Coat BC2”. Scan the reagent barcodes and BeadChip barcodes, and then click “Save”. Illumina LIMS records the data and queues the BeadChips for the next step.

At this point, you can either proceed to Image BeadChip (Post-Amp), or store the BeadChips in the Illumina BeadChip Slide Storage Box inside a vacuum desiccator at room temperature. Be sure to image the BeadChips within 72 hours.

Image BeadChip (Post-Amp)

1. Follow the instructions in the iScan System User Guide or HiScan System User Guide to scan your BeadChips.
2. Use the Methylation NXT scan setting for your BeadChip.
3. Follow the instructions in the Decode File Client User Guide (11337856) available on the Illumina support website to download your DMAPs.
4. Use the GenomeStudio Methylation Module v1.8 Guide (11319130) to analyze your data. Use the Infinium >> Infinium HD setting to create a GenomeStudio project for your BeadChip. Analysis requires a sample sheet that describes the location of each sample.

3.4. Analysis of Illumina HumanMethylation450 BeadChip

3.4.1. Preprocessing—Raw IDAT files can be imported using the *minfi* package [18], followed by comprehensive evaluation of bisulfite conversion and subsequent array hybridization success rate. Filtering out problematic probes that failed to hybridize and are not represented by a minimum of three beads on the array is recommended before performing any downstream analysis. In practice, this step typically entails filtering out probes displaying a high detection p-value (e.g., >0.05). The number of non-specific (cross-reactive) Infinium HumanMethylation450 probes ranges between 8.6–25% depending on the criteria used [19, 20]. This is particularly problematic, since methylation measurements from a non-specific probe could represent signal from several genomic sites, alluding to false differential expression in further analyses. Therefore, it is of interest to remove probes that cross-hybridize to multiple genomic regions or are located on the sex chromosomes [21]. Finally, DNA methylation measurements can be confounded by the actual DNA sequence, particularly in independent case-control studies, and single nucleotide polymorphisms (SNPs) can be present within the remainder of the probe, although some studies suggest that DNA methylation measurements are not significantly affected by these [19].

3.4.2 Within-array Normalization—The comprehensive nature of the 450k array is due to the use of two different types of chemical assays (~30% Infinium I and 70% Infinium II). However, the two probe types display a different dynamic range potentially leading to type II bias during analysis [22]. Complete Infinium HumanMethylation450 within-array normalization thus encompasses Infinium I/II-type bias correction, dye bias adjustment, and background correction. The two-assay design has led to the development of a plethora of within-array normalization algorithms. The R package *wateRmelon* [23] takes advantage of known methylation patterns that have been previously associated with genomic imprinting, X chromosome inactivation, and 65 SNPs present on the array to test methods of correction and normalization, while also providing access to 15 different normalization methods. A non-comprehensive list of popular methods includes peak-based correction (PBC), Subset Quantile Normalization, Subset quantile for Within Array Normalization (SWAN) and Beta Mixture Quantile normalization (BMIQ) among others [22–26]. Color bias adjustment methods based on smooth quantile and shift-and-scaling normalization and Genome Studio are implemented in *lumi* and *methyllumi* R packages [27]. Finally, some of the freely available background correction methods include simple background subtraction, mixture models that model signal intensity and background noise separately (ENmix), and convolution models implemented in *methyllumi* (normal-exponential convolution using out-of-band probes) [28].

3.4.3 Between-array normalization and batch effects—Between-array normalization is essential to remove sources of non-biological variation related to external parameters, including reagent concentrations and temperature, unequal quantities of starting material, and detection efficiencies. Simple, yet effective options, derived from processing methods initially developed for gene expression arrays, are the different versions of the quantile normalization. Other between-array methods recently developed include shift and scaling normalization of the *lumi* package, local regression-based approaches [29], and unsupervised functional normalization [30]. Batch and side effects can generate artifacts on methylation measurements at the global level that could be partially alleviated due to between-array normalization. However, global between-array methods may be inefficient in settings where batch effects affect only a subset of probes. Popular methods for batch effect correction are the supervised methods *ComBat* [31], independent surrogate variable analysis (ISVA) [32], and surrogate variable analysis (SVA) [33] implemented in the *RnBeads* package.

3.4.4 Differential methylation analysis—After the preprocessing and normalization steps, the calculation of differentially methylated positions (DMPs) can be performed. In epigenome-wide association studies, one might want to consider correction for cell heterogeneity first [34]. The β -value is defined as the ratio of the methylated signal over the total signal used to express the degree of methylation obtained with Infinium array. The more heteroscedasticity resistant statistic, the M-value, is the log-transformed ratio of the methylated over the unmethylated signal. Statistical tests for simple, independent two-sample designs consisting of t-test or Mann-Whitney test perform best on M-values. In addition to single CpG analysis, one might also want to identify differentially methylated regions (DMRs). The intuition behind measurement of DMRs derives from the idea that

probes lying within the promoter of the same gene, or in a window of a given size, should exhibit the same methylation patterns. Depending on the genomic region, the *minfi* package implements two different functions for estimating DMRs [18]: *bumphunter* focuses on short range (1–2 kb) methylation changes (around gene promoters), while *cpgCollapse* covers long-range changes as represented by the 170,000 open sea probes on the 450k array. Another freely available DMR method includes *champ.lasso* function, implemented in Chip Analysis Methylation Pipeline (*ChAMP*), which uses a dynamic window based on genomic features to capture DMRs [35]. Other recently developed DMR calling methods include *DMRcate*, *FastDMA*, and *COHCAP* that identifies CpG islands showing consistent methylation pattern among CpG sites. The *COHCAP* package also allows for gene expression and DNA methylation data integration for identification of CpG islands that potentially regulate downstream gene expression [36].

6. Notes

1. We recommend that this solution be prepared in a plastic container, as the reagent may absorb to glass surfaces. The working solution should also be protected from light, as the Quant-iT PicoGreen reagent is photodegradable. For best results, prepare this solution within a few hours of using.
2. To ensure that sample readings remain in the detection range of the fluorometer, the instrument's gain should be set so that the sample containing the highest DNA concentration yields a fluorescence intensity near the instrument's maximum range.
3. A higher dilution of the experimental sample may diminish the interfering effect of certain contaminants, but extremely small sample volumes should be avoided because they are difficult to pipet accurately.
4. To minimize photobleaching effects, keep the time for fluorescence measurement constant for all samples.
5. Minimize the exposure to light of the CT Conversion Reagent, as it is light-sensitive. For best results, the CT Conversion Reagent should be used immediately following preparation. The prepared reagent can be stored overnight at room temperature, one week at 4°C, or up to one month at –20°C. Stored CT Conversion Reagent must be warmed to 37°C and vortexed prior to use.
6. 1–4 µl of eluted DNA is recommended for each PCR. The elution volume can be more than 10 µl, but small elution volumes are preferred for more concentrated DNA.
7. If you do not already have a WG#-BCD plate, add the bisulfite-converted DNA to either a Midi plate: 20 µl to each WG#-BCD plate well or to a TCY plate: 10 µl to each WG#-BCD plate well. Apply a barcode label to the new WG#-BCD plate.

8. When you remove a cap mat, set it aside, upside down, in a safe location for use later in the protocol. When you place the cap mat back on the plate, be sure to match it to its original plate and in the correct orientation.
9. If you are using Illumina LIMS, you cannot change the number of DNA samples on this screen. However, it will process the correct number of samples.
10. Set the centrifuge to 4°C in preparation for the next centrifuge step.
11. You can also prepare the Hyb Chambers later, during the 30-minute cool down.
12. When handling the BeadChip, avoid touching the beadstripe area and sample inlets.
13. For optimal performance, take care to keep the Hyb Chamber inserts containing BeadChips steady and level when lifting or moving. Avoid shaking and always keep parallel to the lab bench. Do not hold by the sides near the sample inlets.
14. Keep the Hyb Chamber steady and level when moving it or transferring it to the Illumina Hybridization Oven.
15. Do not use bleach or ethanol to clean the tip guide inserts.
16. Remove the cover seal over an absorbent cloth or paper towels, preferably in the hood to make sure that no solution splatters on you.
17. Confirm that you are using the correct Infinium standard glass back plates and spacers before assembling the flow-through chambers. Refer to Figure 2 for the correct flow-through chamber components.
18. Use 150 ml of PB1 for every additional set of eight BeadChips.
19. Inspect the surface of each BeadChip for residue left by the seal. Use a pipette tip to remove any residue under buffer and be careful not to scratch the bead area.
20. Be sure to use the clear plastic spacers, not the white ones.
21. Place all assembled flow-through chambers on the lab bench in a horizontal position while you perform the preparation steps for the XStain BeadChip. Do not place the flow-through chambers in the chamber rack until the preparation is complete.
22. Do not let the XC4 sit for longer than 10 minutes.
23. Do not touch the stripes with the wipe or allow EtOH to drip onto the stripes.

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Figure 1. Schematic representation of workflow to identify gene methylation in conjunction with Illumina Infinium HumanMethylation450 BeadChip Kit assay.

Figure 2. Assembly of Flow-Through Chambers.

This figure was adapted from the Illumina Infinium HumanMethylation450 BeadChip Kit protocol.

Table 1.

Protocol for preparing a high-range standard curve

TE (μl)	DNA stock (μl) *	Diluted PicoGreen Reagent (μl)	Final DNA Concentration
0	1000	1000	1 μg/mL
900	100	1000	100 ng/mL
990	10	1000	10 ng/mL
999	1	1000	1 ng/mL
1000	0	1000	blank

*
2 μg/mL

Table 2.

Protocol for preparing a low-range standard curve

TE (μl)	DNA stock (μl) *	Diluted PicoGreen Reagent (μl)	Final DNA Concentration
0	1000	1000	25 ng/mL
900	100	1000	2.5 ng/mL
990	10	1000	250 pg/mL
999	1	1000	25 pg/mL
1000	0	1000	blank

*
50 ng/mL