Chapter

Library Preparation for Whole Genome Bisulfite Sequencing of Plant Genomes

Kendall R. Corbin and Carlos M. Rodriguez Lopez

Abstract

Epigenetic mechanisms are a key interface between the environment and the genotype. These mechanisms regulate gene expression in response to plant development and environmental stimuli, which ultimately affects the plant's phenotype. DNA methylation, in particular cytosine methylation, is probably the best studied epigenetic modification in eukaryotes. It has been associated to the regulation of gene expression in response to cell/tissue differentiation, organism development and adaptation to changing environments. Whole genome bisulfite sequencing (WGBS) is considered the gold standard to study DNA methylation at a genome level. Here we present a protocol for the preparation of whole genome bisulfite sequencing libraries from plant samples (grapevine leaves) which includes detailed instructions for sample collection and DNA extraction, sequencing library preparation and bisulfite treatment.

Keywords: whole genome bisulfite sequencing, methylome analysis, DNA methylation, epigenetic modifications, *Vitis vinifera*

1. Introduction

Plants being sessile have developed strategies to adapt to their environment, specifically via epigenetic modification of their genome [1, 2]. Epigenetic mechanisms, both heritable and reversible, allow an organism to respond to its environment through changes in gene expression, without changing the underlying genome [3–6]. One of the most widely studied epigenetic mechanisms is cytosine methylation (5mC), which is the result of a methyl group replacing a hydrogen in the cyclic carbon-5 of cytosines. In plants, methylation of cytosine bases can occur in three contexts (DNA base sequences) CG, CHG or CHH, where H is any nucleotide other than G [7]. Plant nuclear genomes are known to contain more extensive and expansive DNA methylation than that found in animals [8]. DNA methylation has been identified in a range of plants and plays a role in a wide variety of biological processes from plant development and organ differentiation to response to stress [9–20].

Due to the functional importance of DNA methylation in many species, a plethora of DNA methylation analysis approaches has been developed in recent years. These can be mainly grouped into three functional types that (1) indicate the methylation status of a specific sequence; (2) reveal the degree and patterning of DNA methylation across partly characterized genomes; or (3) facilitate the discovery and sequencing of new epialleles [7]. From a technical point of view, such methodologies can be grouped into those using global estimation of all nucleic base species (e.g. HPLC and LC-MS/MS),

methylation-sensitive restriction enzymes [18, 21, 22], high-resolution melting analysis [10, 23, 24], methylcytosine-specific antibodies and methylated DNA-binding domains [25, 26], bisulfite conversion of DNA, and third-generation DNA sequencing technologies, including single molecule real-time (SMRT) sequencing and nanopore sequencing (for extensive reviews in these methodologies, see [27–29]).

Of all these techniques currently available, only bisulfite conversion of DNA and third-generation DNA sequencing provide a single-base resolution view of methylated cytosines across the selected target sequence. This approach is not limited by genome size and may be applied to a relatively small fraction of a genome or a whole genome. More recently developed techniques are capable of reading 5mC, and other DNA modifications, without the need for any chemical alteration of the target DNA molecule. However, their throughput, accuracy and affordability are still not sufficient for routine use. Bisulfite conversion of DNA, in turn, is based on the selective chemical modification of unmethylated cytosines (C) into uracils (U) (which are read as thymines (T) by DNA polymerases during PCR amplification) (**Figure 1**), while leaving unchanged 5mC (**Figure 2**). Due to its high throughput, reliability and low cost, bisulfite conversion is considered the "gold standard" DNA methylation analysis. Next-generation sequencing (NGS) allows the rapid sequencing of whole genomes. Combined with bisulfite conversion of the target DNA, it also permits the identification of methylated cytosines at a single-base resolution of whole genomes (i.e. whole genome bisulfite sequencing (WGBS)).

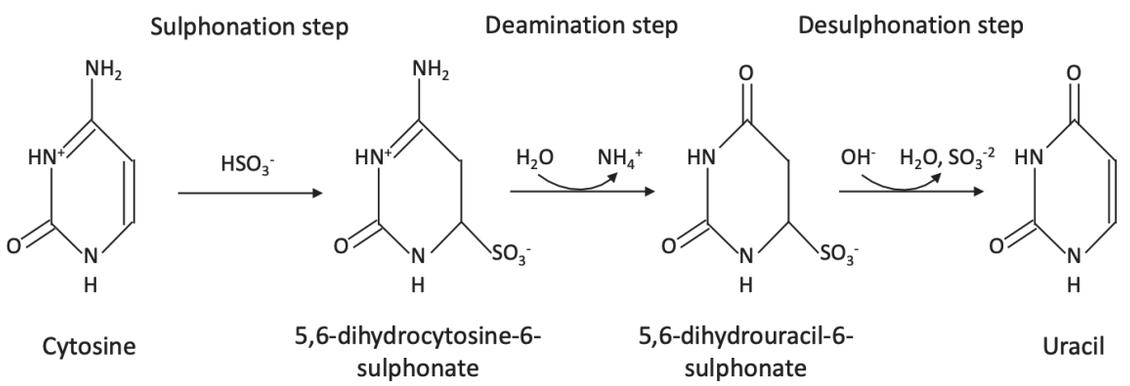

Figure 1.
Bisulfite conversion of unmethylated cytosines. Bisulfite conversion reaction starts with the addition of a sodium bisulfite group (sulphonation step) to the pyrimidine ring double bond between carbons 5 and 6 to form a 5,6-dihydrocytosine-6-sulphonate. Next, spontaneous and irreversible hydrolytic deamination results in a 5,6-dihydrouracil-6-sulphonate (deamination step). Finally, high pH conditions favor the loss of the sulphonate group (desulphonation step) to form uracil. Only unmethylated cytosines are susceptible to the bisulfite reaction. Methylated (5mC and 5-hmC) cytosines do not undergo conversion.

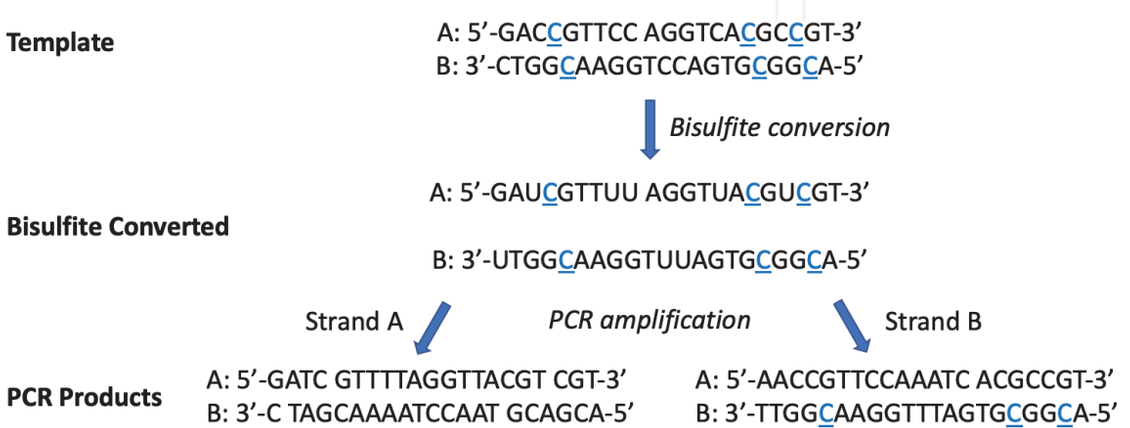

Figure 2.
Bisulfite conversion of a sample DNA sequence. Nucleotides highlighted in blue (methylated cytosines) are protected from bisulfite conversion and are maintained as cytosines. Unmethylated cytosines are converted to uracils. Loss of the original base pairing will yield two different PCR products from each DNA fragment.

2. Procedure

2.1 Equipment

i. Ultralow freezer (−80°C)

ii. Mortar and pestle—used to grind leaf samples prior to DNA extraction. Use a clean set for each sample to avoid cross-contamination. Wash both parts using warm water and soap, air-dry, wrap in aluminium foil and autoclave.

iii. NanoDrop™ 2000 Spectrophotometer—this UV-Vis spectrophotometer has the capability to quantify and assess the purity of small volumes of DNA (0.5 μL). The sample may be pipetted directly onto the optical measurement surface. Additional information regarding DNA quantification and quality assessment using NanoDrop can be found at https://www.thermofisher.com/us/en/home/industrial/spectroscopy-elemental-isotope-analysis/molecular-spectroscopy/ultraviolet-visible-visible-spectrophotometry-uv-vis-vis/uv-vis-vis-instruments/nanodrop-microvolume-spectrophotometers/nanodrop-nucleic-acid-quantification.html

iv. Thermocycler (PCR machine)—thermocyclers amplify segments of nucleic acid following a series of temperature-controlled enzymatic reactions.

v. Covaris M220 Focused-Ultrasonicator™ and MicroTUBE-50 (Covaris, catalog number: 520166) (or equivalent models and parts)—sonicators are used for shearing DNA to a desired size.

vi. Magnetic rack for 1.5 mL tubes—magnetic racks are used for separation and purification of nucleic acids in combination with paramagnetic beads (e.g. AMPure XP beads).

vii. Qubit Fluorometric Quantification and Qubit dsDNA HS (High Sensitivity) Assay Kit (Thermo Fisher Scientific, catalog number: Q32854). Qubit assays accurately quantify nucleic acids quickly and require small volumes of sample.

viii. Agilent Fragment Analyzer, Agilent Bioanalyzer (Agilent Technologies) or the Bio-Rad Experion (Bio-Rad Laboratories).

ix. High-speed centrifuge.

2.2 Consumables

i. Sterile microcentrifuge tubes 1.5 mL (Eppendorf® Safe-Lock™)

ii. 15 mL polypropylene centrifuge tubes (Laboratory Product Sales)

iii. Filtered pipette tips

iv. Wide-bore pipette tips

v. Sterile 200 μL PCR tubes

vi. Sterile 500 μL tubes

2.3 Chemicals and reagents

i. Molecular biology grade ethanol (MilliporeSigma or Fisher BioReagents)

ii. Molecular biology grade water (MilliporeSigma)

iii. Cetyltrimethylammonium bromide (CTAB)

iv. Ethylenediaminetetraacetic acid (EDTA)

v. Tris hydrochloride (Tris-HCl)

vi. Hydrochloric acid (HCl)

vii. Polyvinylpolypyrrolidone (PVP)

viii. Chloroform

ix. Octane

x. Sodium chloride (NaCl)

xi. RNAse A (Sigma-Aldrich, catalog number: R4642)

xii. Agencourt AMPure XP magnetic beads (Beckman Coulter, catalog number: A63880)

xiii. Q5® High-Fidelity 2× Master Mix (New England Biolabs, catalog number: M0492S)

xiv. 10× End Repair Buffer (New England Biolabs, catalog number: B6052S)

xv. End Repair Enzyme Mix (New England Biolabs, catalog number: E6051)

xvi. 10× dA-Tailing Reaction Buffer (New England Biolabs, catalog number: B6059S)

xvii. A-tailing Enzyme (e.g. Klenow Fragment (3′ → 5′ exo-) (New England Biolabs, catalog number: M0212S)

xviii. 10× T4 DNA Ligation Buffer and T4 DNA Ligase (New England Biolabs, catalog numbers: B0202S and M0202)

xix. TruSeq Sequencing adapters: adapters are ordered as lyophilized oligonucleotides with the specified modifications[1] from the provider of

[1] Order the oligonucleotides with standard desalting. Request that all cytosines are methylated. This will allow the sequence integrity of the adapters to be maintained after bisulfite treatment. Also, order the indexed adapter with a 5′ phosphate group and TruSeq Universal Adapter with phosphorothioate bond between the 3′ end C and T nucleotides.

your choice (sequences are provided below[2]). To prepare the adapters, resuspend both oligonucleotides with TE buffer to a final concentration of 200 μM. Then add 75 μL from each into a 200 μL sterile PCR tube. To allow annealing of the complementary sections of the oligos, heat the mixture using a thermocycler to 95°C for 1 min, and then slowly lower the temperature to 30°C at a rate of 1°C/min. This can be accomplished by programming your thermocycler with a single step PCR cycle at 95°C for 1 min followed by 65 cycles during which the temperature is reduced by 1°C each cycle. Store double-stranded adapters at −20°C.

I. TruSeq universal adapter: 5′-AATGATACGGCGACCACCGAGATCT ACACTCTTTCCCTACACGACGCTCTTCCGATC*T-3′

II. TruSeq INDEX adapter: 5′-P*GATCGGAAGAGCACACGTCTGAAC TCCAGTCAC[i7]ATCTCGTATGCCGTCTTCTGCTTG-3′

xx. Library amplification primers: primers are ordered as lyophilized oligonucleotides with standard desalting from the provider of your choice (sequences are provided below). To prepare the primers, resuspend both oligonucleotides with TE buffer to a final concentration of 100 μM. (This is your stock solution. Store at −20°C.) To prepare the Forward and Reverse Primer Mix, mix 10 μL from each in a new tube, and add 80 μL of molecular grade water to achieve a final concentration of 10 μM.

I. Forward primer: 5′-AATGATACGGCGACCACCGAGATCTACACTC TTTCCCTACACGA-3′

II. Reverse primer: 5′-CAAGCAGAAGACGGCATACGAGAT-3′

2.4 Additional items required

i. Insulated polystyrene box

ii. Pipettes

iii. Water bath

iv. Liquid nitrogen

v. Refrigerator (4°C) and freezer (−20°C)

3. Set-up

i. Label all tubes prior to starting any of the described protocols to reduce the likeliness of downstream errors.

[2] [i7] index sequences can be found at https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/experiment-design/illumina-adapter-sequences-1000000002694-09.pdf

ii. Use sterilized tools (scissors, knives, tweezers, etc.) for harvesting plant material, and clean utensils thoroughly between samples using 70% (v/v) ethanol.

iii. Gloves should be worn at all times while handling samples to minimize cross-contamination (change gloves as needed).

iv. DNA extractions, next-generation sequencing library preparations and bisulfite treatments should be carried out in a PCR cabinet or similar to minimize contamination.

v. General safety notes.

- Follow safe operating procedures when handling cryogenic products (dry ice and liquid nitrogen). Prior to usage (and transport) of cryogenic products, a risk assessment should be conducted to evaluate hazards and identify control measures that may be implemented to minimize the level of risk. Additional information about cryogenic materials precautions and safe handling procedures may be available from your local Office of Environmental Health and Safety.

- β-Mercaptoethanol (also known as 2-hydroxyethylmercaptan, BME or thioethylene glycol) is a toxic chemical that should be handled with extreme caution. Exposure to this product may cause respiratory issues, vomiting or skin irritation. Long-term exposure to this product can result in death. Personal protective equipment should be worn when handling this product and all experimental work conducted in a fume hood. Hazard control measures include wearing nitrile laboratory gloves (if gloves get splashed or tear, change immediately), safety glasses, closed toe shoes, a laboratory coat, and if spills are possible, a face shield. Safety documentation about this product, including information relevant to storage, transport and disposal, may be found on manufacturers Website.

3.1 Collection of plant material

i. Collect three individual leaves at bud burst (E-L 7 [30]) from the number of desired grapevines. The rationale for using immature vegetative tissue (leaves) is that cell number is fixed very early during development; thus the number of genome copies per gram of tissue is higher in younger leaves relative to older leaves. It is also advantageous to use younger plant material as some plant species accumulate secondary metabolites (such as alkaloids and flavonoids) as their tissues age. High levels of these metabolites can impede DNA extraction or PCR amplification [31].

Note: DNA methylation has been shown to change with the plant's circadian cycle [32] and during plant development [19]. Thus, when collecting samples for DNA methylation analysis from more than one plant, it is extremely important to harvest all plant tissue at approximately the same time of day and at the same developmental stage in order to minimize unwanted variability in DNA methylation.

ii. Immediately upon harvesting the leaves, put the material in a pre-labelled 1.5 mL centrifuge tube. Place the tubes in an insulated container (i.e. polystyrene box) and cover with dry ice (solid CO_2).

Note: By immediately snap-freezing the samples, changes in DNA methylation profiles induced during harvesting and cell death will be minimized.

iii. Store all samples at −80°C until required for DNA extraction.

Note: Storage of samples at ultralow temperatures will minimize DNA degradation. Avoid unnecessary freeze-thawing cycles, including during the period of material transport from the field to laboratory.

3.2 Recipes for buffers, solutions and reagents

i. Ethanol (70, 80 and 95% v/v). Store at room temperature.

ii. CTAB DNA extraction buffer (per 100 mL): 20 mM sodium EDTA (1 mL of 0.5 M stock) and 100 mM Tris-HCl (10 mL of 1 M stock), adjust pH to 8.0 with HCl; add 1.4 M NaCl (8.2 g), 1% (w/v) PVP (1.0 g), and 2.0% (w/v) CTAB (2.0 g). Dissolve CTAB by heating to 60°C. Store at 37°C.

iii. Chloroform-octanol 24:1 (v/v). Store at room temperature.

iv. 5 M sodium chloride (NaCl)—dissolve 292 g of NaCl in 800 mL of water, and then adjust the volume to 1 L with water.

v. 1× Tris-EDTA buffer (TE buffer)—10 mM Tris-HCl and 1 mM EDTA, adjust pH to 8.0 and autoclave. Store at room temperature

4. Protocol

4.1 DNA extraction

DNA extraction is carried out following a modified CTAB protocol [33].

i. Pour liquid nitrogen on to a mortar and pestle.

Note: The mortar should be fully cooled in liquid nitrogen prior to and during usage. In addition, the sample must remain frozen during the grinding process. Accidental thawing may result in DNA degradation.

ii. Grind 500 mg of leaf material in a mortar and pestle. Continue to add liquid nitrogen to ensure the equipment remains cold.

Note: Over grinding of plant biomass will cause DNA shearing, which results in lower yields after bisulfite treatment due to degradation of small DNA fragments.

iii. Add 5 mL of CTAB extraction buffer to the ground leaves and mix with a sterile spatula.

iv. Transfer the slurry to a 15 mL polypropylene centrifuge tube. Rinse the mortar and pestle with 1 mL of extraction buffer, and add to the tube (added to original extract).

v. Add 50 mg polyvinylpolypyrrolidone (PVP), screw the cap on the tube tightly, and invert the tube several times to mix thoroughly.

Note: PVP is added at a concentration of 100 mg PVP/g leaf tissue used in step ii.

vi. Incubate the tube in a water bath set at 60°C for 25 min. Carefully remove the tube from the bath and cool to room temperature.

Note: Take care when removing the sample from the water bath, wear personal protective equipment (laboratory jacket, safety glasses and heat-resistant gloves).

vii. Centrifuge the homogenate for 5 min at 14,000 × *g* (room temperature), and transfer the supernatant to a clean 1.5 mL tube.

viii. Treat with 1 μL RNase A per 100 μL DNA solution and incubate at 37°C for 15 min.

Note: An RNAse treatment step is included to enzymatically digest RNA in the material, minimizing the amount of RNA extracted with the DNA. Contaminating RNA will result in the overestimation of DNA quantity.

ix. Add 6 mL of chloroform-octanol, and mix gently by inverting the tube 20–25 times to form an emulsion.

x. Spin at 14,000 × *g* for 15 min in a centrifuge (room temperature).

xi. Using a wide-bore pipette tip, transfer the top aqueous phase to a new 15 mL tube. A second chloroform-octanol extraction may be performed if the aqueous phase is cloudy due to the presence of PVP (repeat steps ix to xi).

xii. Add 3 mL of 5 M NaCl to the aqueous solution and mix well (invert gently by hand).

xiii. Add two volumes of cold (−20°C) 95% (v/v) ethanol and refrigerate (4–6°C) until DNA strands begin to appear.

Note: The solution should be left for at least 15 min but can stay refrigerated for longer if necessary.

xiv. Spin at 10,000 × *g* for 3 min (room temperature).

xv. Increase the speed of the centrifuge to 14,000 × *g*. Spin samples for an additional 3 min.

Note: Differential centrifugation steps aid in keeping the DNA at the bottom of the tube.

xvi. Carefully pour off supernatant and wash pellet with 1 mL of chilled (0–4°C) 70% (v/v) ethanol.

xvii. Remove ethanol by pipetting—do not disturb the DNA pellet. Air-dry the remaining ethanol by leaving the tubes uncovered at room temperature for 10 min.

xviii. Solubilize the DNA pellet in 200–300 μL TE buffer.

xix. Quantify isolated DNA using the NanoDrop™ 2000.

Note: TE buffer should be used as the reference blank.

xx. Normalize DNA concentrations to 20 ng/μL using molecular grade water.

xxi. Store DNA samples at −20°C (short-term) or −80°C (long-term).

4.2 DNA shearing

i. Aliquot 1 μg of genomic DNA (equivalent to 50 μL of DNA with a concentration of 20 ng/μL) into a Covaris MicroTUBE-50, and add 5 μL of molecular biology water. The final volume in the microtube is 55 μL.

ii. Shear DNA to 200 bp fragments using the Covaris M220 Focused-Ultrasonicator™, using the following specifications:

Duration, 90 s; peak power, 75 W; duty factor, 25%; cycles per burst, 1000

iii. Transfer 50 μL of the fragmented DNA to a clean, pre-labelled 200 μL PCR tube.

Note: Label the top and the side of the PCR tubes.

4.3 Sheared DNA end repair

i. Prepare End Repair Master Mix containing 8 μL molecular grade water, 7 μL of 10× end repair buffer and 5 μL end repair enzyme.

Note: When preparing Master Mixes, prepare 10% extra to account for pipetting errors, and allow enough reaction mix for all sample. For example, for 10 samples, prepare enough Master Mix for those samples plus one extra (11 in total): combine 88 μL molecular grade water, 77 μL of 10× end repair buffer and 55 μL end repair enzyme.

ii. Add 20 μL of End Repair Master Mix to each of the sheared samples.

iii. Incubate in a thermocycler at 20°C for 30 min.

Note: At this point remove AMPure XP beads from the refrigerator and allow the bottle to reach room temperature before use. Immediately before pipetting, resuspend the beads by vortexing vigorously. The AMPure purification system selectively binds DNA fragments to paramagnetic beads, allowing the removal of excess primers, nucleotides, salts and enzymes during a simple washing step. These clean-up steps result in a more purified PCR product. For further information about using AMPure XP for PCR purification, please refer to the manufacturer's manual.

iv. Capture DNA by adding 120 μL of AMPure XP beads, pipette up and down to achieve a homogenous mixture, and incubate at room temperature for 5 min.

v. Transfer the beads with captured DNA to a 1.5 mL tube.

vi. Place the tube on a magnetic rack for 2 min.

vii. Keep the tube on the magnetic rack and remove the supernatant using a pipette. Do not disturb the beads.

Note: The aim of this step is to remove the AMPure XP buffer. At this stage the DNA is captured by the beads which are kept in the tube by the magnet. The buffer can be discarded.

viii. Keep the tube on the magnetic rack and add 200 μL of 80% (v/v) ethanol.

Note: Due to the different evaporation rates of H_20 and ethanol, it is important to use freshly prepared ethanol.

ix. Incubate for 30 s on the magnetic rack and use a pipette to remove the ethanol.

x. Repeat steps viii and ix.

Note: After the second ethanol wash, remove as much ethanol as possible using a 10 μL pipette. These wash steps are important to remove any remains of the End Repair Master Mix. At this stage the DNA is captured by the AMPure beads which are kept in the tube by the magnet.

xi. Remove residual ethanol by leaving the tube open on the magnetic rack for 5 min (air-dry).

Note: Do not over dry the beads as it will lower DNA yields. Appearance of cracks on the bead pellet is indicative of over drying.

xii. Remove the tube from the magnetic rack, add 42 μL of molecular grade water, and pipette up and until beads are fully resuspended.

xiii. Leave the tube at room temperature for 5 min to allow the DNA to be released from the AMPure beads.

xiv. Place the tube in the magnetic rack and leave at room temperature for 2 min.

xv. Transfer 40 μL of the supernatant to a clean 200 μL PCR tube.

Note: At this stage the DNA is resuspended in the water. Beads can be safely discarded. Do not attempt to pipette the entire volume in the tube (42 μL) as some of the AMPure beads may be transferred which could affect later reactions. If beads are disturbed during pipetting, simply put the whole volume back in the tube and proceed from step xiv.

4.4 Fragmented DNA A-tailing

i. Prepare the A-tailing Master Mix containing 2 μL molecular grade water, 5 μL of 10× A-tailing buffer and 3 μL A-tailing enzyme.

Note: When preparing Master Mixes, prepare 10% extra to account for pipetting errors and allow enough reaction mix for all samples.

ii. Add 10 μL of A-tailing Master Mix to each of the samples (200 μL PCR tube).

iii. Incubate in a thermocycler at 30°C for 30 min.

iv. Capture DNA by adding 90 μL of AMPure XP beads, pipette up and down to achieve a homogenous mix, and leave at room temperature for 5 min.

v. Transfer the beads with the capture DNA to a clean 1.5 mL tube.

vi. Place the tube on a magnetic rack for 2 min.

vii. Keep the tube on the magnetic rack and remove the supernatant without disturbing the beads using a pipette.

Note: The aim of this step is to remove the AMPure XP buffer. At this stage the DNA is captured by the beads which are kept in the tube by the magnet. The buffer can be safely discarded.

viii. Keep the tube on the magnetic rack and add 200 μL of 80% (v/v) ethanol.

ix. Incubate for 30 s on the magnetic rack and use a pipette to remove the ethanol.

x. Repeat steps viii and ix.

xi. Evaporate ethanol by leaving the tube open on the magnetic rack for 5 min.

xii. Remove the tube from the magnetic rack and resuspend the beads by adding 32 μL of molecular grade water and pipette up and down.

xiii. Leave the tube at room temperature for 5 min to allow the DNA to be released from the AMPure beads.

xiv. Place the tube in the magnetic rack and leave at room temperature for 2 min.

xv. Transfer 30 μL of the supernatant to a clean 200 μL PCR tube. Do not transfer beads.

4.5 Ligation of sequencing adapters

i. Prepare the Ligation Master Mix containing 5 μL of 10× Ligation Buffer, 2.5 μL T4 DNA Ligase and 7.5 μL molecular grade water.

ii. Add 5 μL of TruSeq Adapter to each of the samples in a 200 μL PCR tube.

Note: Add 5 μL of adapters (10 μM) for every 1 μg of starting DNA. If you are planning to multiplex more than one sample in each sequencing lane, use adapters with different index sequences.

iii. Add 15 μL of Ligation Master Mix to each of the samples in the 200 μL PCR tube and mix by pipetting up and down.

iv. Incubate in a thermocycler at 20°C for 15 min.

v. Capture DNA by adding 90 μL of AMPure XP beads, pipette up and down to achieve a homogenous mix. Leave at room temperature for 5 min.

vi. Transfer the beads with the captured DNA to a clean 1.5 mL tube.

vii. Place the tube on a magnetic rack for 2 min.

viii. Keep the tube on the magnetic rack, and remove the supernatant without disturbing the beads using a pipette.

Note: The aim of this step is to remove the AMPure XP buffer. At this stage the DNA is captured by the beads which are kept in the tube by the magnet. The buffer can be safely discarded.

ix. Keep the tube on the magnetic rack and add 200 μL of 80% (v/v) ethanol.

x. Incubate for 30 s on the magnetic rack and use a pipette to remove the ethanol.

xi. Repeat steps ix and x.

xii. Evaporate ethanol by leaving the tube open on the magnetic rack for 5 min.

xiii. Remove the tube from the magnetic rack, add 105 μL of molecular grade water, and pipette up and down until beads are resuspended.

xiv. Leave the tube at room temperature for 5 min to allow the DNA to be released from the AMPure beads.

xv. Place the tube in the magnetic rack and leave at room temperature for 2 min.

xvi. Transfer 100 μL of the supernatant to a clean 1.5 mL tube. Do not transfer beads.

4.6 Sequencing library fragment size selection

i. Add 60 μL of AMPure beads to capture DNA fragments >450 bp, pipette up and down to achieve a homogenous mix, and leave at room temperature for 5 min.

Note: Beads preferentially capture larger fragments of DNA. The size range that the beads capture is determined by the volume to volume ratio of AMPure XP buffer and DNA aqueous solution. In this case a ratio of 0.6 (60 μL AMPure XP buffer/100 μL DNA) will capture fragments above 450 bp.

ii. Place the tube on a magnetic rack for 2 min.

iii. With the tube on the magnetic rack, transfer 155 μL of supernatant to a new tube without disturbing the beads.

Note: Do not discard the supernatant in this case. The supernatant contains the fragment size range required for sequencing, while larger, unwanted fragments are still captured by the beads. At this stage the beads and the tube containing them can be discarded.

iv. Add 20 μL of beads to the 155 μL of supernatant collected in step iii, pipette up and down to achieve a homogenous mix, and leave at room temperature for 5 min.

v. Place the tube on a magnetic rack for 2 min.

vi. Keep the tube on the magnetic rack, and remove the supernatant without disturbing the beads using a pipette.

Note: In this case a ratio of 0.88 (82 μL AMPure XP buffer/93 μL DNA) will capture fragments above 100 bp. The supernatant, containing unligated TruSeq adapters or DNA fragments below that size can be safely discarded.

vii. Keep the tube on the magnetic rack and add 200 μL of 80% (v/v) ethanol.

viii. Incubate for 30 s on the magnetic rack and use a pipette to remove the ethanol.

ix. Repeat steps vii and viii.

x. Evaporate ethanol by leaving the tube open on the magnetic rack for 5 min.

xi. Remove the tube from the magnetic rack and resuspend the beads by adding 22 μL of molecular grade water and pipette up and down until beads are fully resuspended.

xii. Leave the tube at room temperature for 5 min to allow the DNA to be released from the AMPure beads.

xiii. Place the tube in the magnetic rack and leave at room temperature for 2 min.

xiv. Transfer 20 μL of the supernatant to a clean 200 μL PCR tube. Make sure not to transfer the beads.

Storage: At this stage the size-selected samples can be stored until required for bisulfite treatment. For short-term storage keep at −20°C, for long-term store at −80°C.

4.7 Bisulfite conversion of size-selected library

DNA samples are bisulfite converted using the EZ DNA Methylation-Lightning Kit (Zymo Research).

i. Thaw samples completely (if stored in the freezer prior to bisulfite treatment), and centrifuge to bring droplets to the bottom.

ii. Add 130 μL of Lightning Conversion Reagent to the tube containing the 20 μL size-selected library.

Note: Mix and then centrifuge briefly to ensure there are no droplets in the cap or sides of the tube.

iii. Place the PCR tube in a thermal cycler and incubate using the following programme:

a. 98°C for 8 min[3]

[3] High temperature is used to achieve complete denaturation of the double stranded DNA molecule and to favor the forward reaction during the reversible sulphonation step.

b. 54°C for 60 min[4]

c. 4°C storage for up to 20 h[5]

iv. Add 600 μL of M-Binding Buffer to a Zymo-Spin™ IC Column, and place the column into the collection tube (provided by supplier).

Note: Do not touch the bottom of the column with a pipette tip; this may damage the filtering matrix.

v. Load the sample (from step iii) into the Zymo-Spin™ IC Column containing the M-Binding Buffer. Close the cap and mix by inverting the column 10 times.

Note: Do not touch the bottom of the column with a pipette tip; this may damage the filtering matrix.

vi. Centrifuge at full speed (>10,000 × *g*) for 30 s. Discard the flow-through.

Note: At this stage the DNA is captured in the column matrix and the flow-through liquid can be safely discarded.

vii. Add 100 μL of M-Wash Buffer[6] to the column. Centrifuge at full speed (>10,000 × *g*) for 30 s in benchtop centrifuge. Discard the flow-through.

Note: This is a wash step. At this stage, the DNA is still captured in the column matrix and the flow-through can be safely discarded.

viii. Add 200 μL of L-desulphonation buffer to the column, and leave at room temperature (20–30°C) for 15–20 min.

Note: This is an alkali desulphonation step that chemically removes the $SO3^2$ group added to unmethylated cytosines during the sulphonation step (**Figure 1**). At the end of this stage, cytosines that were originally unmethylated will be converted to uracils.

ix. After the incubation period, centrifuge at full speed for 30 s. Discard the flow-through.

Note: The aim of this centrifugation step is to remove the L-desulphonation buffer. At this stage the DNA is still captured in the column matrix.

x. Add 200 μL of M-Wash Buffer to the column. Centrifuge at full speed for 30 s. Discard the flow-through.

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[4] This step consists of two consecutive chemical reactions. First, a sulphonation step selectively adds a SO_3^- group to unmethylated cytosines leaving methylated cytosines unchanged. Then, a spontaneous hydrolytic deamination exchanges de amino group (NH_2) for an oxygen atom in the sulfonated cytosines during the sulphonation step (**Figure 1**).

[5] The 4°C storage step is optional. Ideally continue with the rest of the protocol right after the incubation. Longer storage at 4°C could result in DNA degradation.

[6] Ensure that molecular grade 100% ethanol is added to the M-DNA Wash Buffer as recommended by the manufacturer. For example, add 24 mL of ethanol to the 6 mL M-Wash Buffer concentrate (D5030) or 96 mL to the 24 mL M-Wash Buffer concentrate (D5031). M-DNA Wash Buffer included with D5030S and D5030T kits is supplied ready-to-use and does not require the addition of ethanol.

xi. Add 200 μL of M-Wash Buffer to the column. Centrifuge at full speed for 30 s. Discard the flow-through and collection tube. Keep the column matrix.

Note: These are wash steps. At this stage the DNA is still captured in the column matrix, and the flow-through can be safely discarded.

xii. Place the column into a 1.5 mL microcentrifuge tube, and add 12 μL of M-Elution Buffer directly to the column matrix. Centrifuge for 30 s at full speed to elute the DNA.

Storage: Ideally use bisulfite-treated DNA immediately after treatment. After bisulfite conversion of non-methylated cytosines into uracils, genomic DNA does not maintain its original base pairing. This typically leads to single-stranded A-, U-, and T-rich DNA that is more susceptible to degradation. Long-term storage of bisulfite-converted DNA will lead to loss of sample concentration. If long-term storage is required, place in an ultralow freezer (−80°C).

4.8 PCR amplification of bisulfite-converted library

i. Prepare the PCR Master Mix: 25 μL Q5® High-Fidelity 2× Master Mix, 2.5 μL Forward and Reverse Library Amplification Primer Mix at 10 μM and 12.5 μL molecular grade water.

Note: When preparing Master Mixes, prepare 10% extra to account for pipetting errors and allow enough reaction mix for all samples.

ii. Thaw samples completely (if stored prior to bisulfite treatment) and centrifuge to bring droplets to the bottom.

iii. Transfer 10 μL of the bisulfite-treated library to a new 200 μL PCR tube.

iv. Add 40 μL of PCR Master Mix to each tube.

v. Place the PCR tube/tubes in a thermal cycler and incubate using the following program:

98°C for 30 s
98°C for 30 s
60°C for 30 s
Go to step 2: 7–12 times[7]
72°C for 4 min
72°C for 10 min
4°C hold[8]

vi. Centrifuge the PCR tube for a few seconds to ensure there are no droplets in the cap or sides of the tube due to condensation generated during PCR amplification.

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[7] Maintain the number of cycles as low as possible to minimize DNA polymerase base substitution errors.

[8] After PCR amplification, bisulfite-treated DNA recovers its base pairing. This stabilizes the DNA molecule making long-term storage possible.

vii. Add 45μL of beads to the PCR product, pipette up and down to achieve a homogenous mix, and leave at room temperature for 5 min.

viii. Place the tube on a magnetic rack for 2 min.

ix. Keep the tube on the magnetic rack, and remove the supernatant without disturbing the beads using a pipette.

Note: In this case a ratio of 0.9 (45 μL AMPure XP buffer/50 μL PCR product) will capture fragments above 100 bp. The supernatant containing unused PCR primers or DNA fragments below that size can be safely discarded.

x. Keep the tube on the magnetic rack and add 200 μL of 80% (v/v) ethanol.

xi. Incubate for 30 s on the magnetic rack and use a pipette to remove the ethanol.

xii. Repeat steps ix and x.

xiii. Air-dry any ethanol by leaving the tube open on the magnetic rack for 5 min.

xiv. Remove the tube from the magnetic rack and resuspend the beads by adding 22 μL of molecular grade water and pipette up and down until beads are fully resuspended.

xv. Leave the tube at room temperature for 5 min to allow the DNA to be released from the AMPure beads.

xvi. Place the tube in the magnetic rack and leave at room temperature for 2 min.

xvii. Transfer 20 μL of the supernatant to a clean 500 μL tube. Make sure not to transfer the beads.

xviii. Check sequencing library concentration using Qubit and fragment size distribution using the Agilent Fragment Analyzer, Agilent Bioanalyzer (Agilent Technologies) or the Bio-Rad Experion (Bio-Rad).

Note: A good WGBS library should show a fragment distribution between 150 and 500 bp (**Figure 3** Box B). Smaller peaks in the electropherogram would be indicative of sequencing adapters or PCR primers (**Figure 3** Box A). The presence or primers will reduce the quality and yield of the sequencing run. If present, they can be removed by repeating the AMPure XP bead clean-up described in steps vii to xvii of the PCR amplification of bisulfite-converted library protocol. Make sure that molecular grade water is added to the library to adjust to a final volume of 50 μL before adding the 45 μL of AMPure beads. Once the library passes the QC, it can be stored until sequenced. For short-term storage, keep at −20°C, for longer-term keep at −80°C.

xix. Sequence the final library using the HiSeq Illumina platform.

xx. Analyse sequencing results.

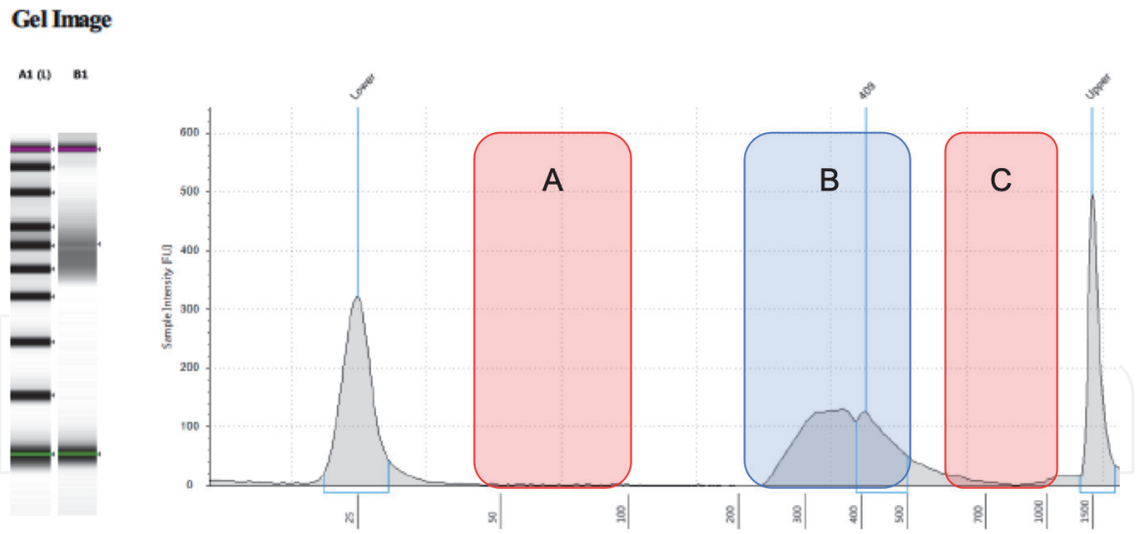

Figure 3.
Example electropherogram of successful WGBS library. Gel image on the left of the figure includes the gel images for (A1) the internal ladder and (B1) the WGBS library. The electropherogram on the right shows the lower and upper fragments of the internal ladder and the fragment size distribution for the WGBS (highlighted in blue in box B). The presence of peaks below 100 bp in the electropherogram is indicative of sequencing adapters or PCR primers. The presence of DNA fragments over 500 bp (Box C) indicates large fragments of DNA that could reduce the quality and output of the sequencing run. Both types of fragments should be removed using AMP XP beads size selection.

5. Data analysis and results

i. Perform FastQC Analysis to remove low-quality sequences.

ii. Use Trim Galore! (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore) to trim sequencing adapters and to remove low-quality sequence.

iii. Perform FastQC Analysis to remove low-quality trimmed sequences.

iv. Map trimmed reads using Bismark aligner.

v. Remove PCR duplicates with Bismark Deduplicate function.

vi. Obtain methylation calls and methylation percentages per each CpG site using the Bismark Methylation Extractor function.

6. Conclusion

By following the protocol described herein, you have have a single-base resolution methylome for your sample. The quality of this methylome will depend on two main factors: (a) the sequencing depth of the produced methylome and (b) the number of replicates included in your experiment. With this data, you can infer methylation density at different genomic levels (i.e. along chromosomes; in different genomic features like genes, transposable elements, etc.) and within specific genomic features like promoters and gene bodies. If you are trying to identify changes in DNA methylation associated to a specific variable (e.g. growing environment, stress, tissue/cell type, age, disease, etc.), then you can identify

differentially methylated cytosines (DMCs) or differentially methylated regions (DMRs) between groups of samples (i.e. control vs treatment). Methods such as Fisher's exact test can be used in the absence of replicates [34]. However, this approach does not consider the possibility of biological variability which is of great importance on a plastic trait such as DNA methylation. Linear or logistic regression-based methods are better suited to capture biological variability since they can compare methylation levels between groups of samples. One example of linear regression method is BSmooth [35] which assumes that data follows a binomial distribution and uses linear regression and t-tests to identify methylation differences for each site. One issue with linear regression is overfitting of DNA methylation levels beyond the 0 to 1 range that methylation proportion/fraction values regenerate. Logistic regression methods, implemented by software such as methylKit can deal better with data restricted to a 0 to 1 range by correcting to data dispersion.

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Conflict of interest

The authors declare no conflict of interest.

Author details

Kendall R. Corbin and Carlos M. Rodriguez Lopez*
Environmental Epigenetics and Genetics Group, Department of Horticulture, College of Agriculture, Food and Environment, University of Kentucky, Lexington, KY, USA

*Address all correspondence to: cmro267@uky.edu

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