Studying DNA methylation genome-wide by bisulfite sequencing from low amounts of DNA in mammals

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Abstract

DNA methylation is extensively reprogrammed during mammalian embryogenesis and germ cell development. Protocols for genome-wide bisulfite sequencing enable the quantification of DNA methylation with high precision and single base-pair resolution, however they can be limited by the necessity for high amounts of DNA. Here we describe optimized Reduced Representation Bisulfite Sequencing (RRBS) and Whole Genome Bisulfite Sequencing (WGBS) protocols for low amounts of DNA, which include steps to estimate the minimal number of PCR cycles needed for the final library preparation to minimize PCR biases. These protocols require no more than 5 ng DNA and can easily be applied to mammalian cells available in small quantities such as early embryos or primordial germ cells.

Key words:

DNA methylation, DNA methylome, Epigenome, Bisulfite conversion, Whole genome bisulfite sequencing, Reduced representation bisulfite sequencing, WGBS, RRBS.

Running head:

Genome-wide bisulfite sequencing from low amounts of DNA

1. Introduction

DNA methylation in mammals occurs on cytosines mostly in the context of CpG dinucleotides. DNA methylation plays essential roles to maintain genome integrity and regulate embryonic development and cell specification [1]. Studies over the last decades have shown that genome wide DNA methylation profiles are dynamic during development and undergo a global reprogramming during the early phases of embryogenesis and germ cell development [2].

Among the variety of methods to assess DNA methylation in the genome, bisulfite sequencing has the advantage of providing quantitative methylation measurements at single nucleotide resolution and is currently considered as the gold standard for mapping DNA methylation throughout the genome. Treatment of DNA with sodium bisulfite converts unmethylated cytosines to uracil and subsequently to thymine after PCR whereas methylated cytosines remain unchanged. Combined with whole genome sequencing, this allows the quantification of methylation at almost all the cytosines in CpG and non-CpG contexts in the human or mouse genome. In the classical protocols, DNA fragmentation and adapter ligation precede bisulfite conversion and PCR amplification [3]. However, it is well known that sodium bisulfite treatment leads to degradation of up to 90% of the adapter-ligated DNA fragments, which strongly reduces the efficiency of library amplification. In post-bisulfite protocols such as the one presented in this chapter, DNA fragmentation and bisulfite treatment are performed before adapter ligation, which minimizes DNA loss for applications on low amounts of cells. In all cases, the final PCR amplification of the library should be done with as few cycles as possible to minimize PCR bias and PCR duplicates.

In order to overcome the high costs of sequencing required for Whole Genome Bisulfite Sequencing (WGBS) in large genomes, protocols have been developed to perform high-throughput bisulfite sequencing on a sample of the whole genome. This can be achieved by coupling bisulfite sequencing with targeted capture of a pre-defined set of genomic sequences [4-7]. Alternatively, Reduced Representation Bisulfite Sequencing (RRBS) uses restriction with the MspI enzyme (CCGG) to enrich for CpG-rich sequences of the genome followed by high-throughput sequencing [8,9]. RRBS efficiently captures most of the CpG islands and gene promoters while sequencing less than 5% of the genome, therefore providing methylation information of relevant regulatory regions at reduced sequencing costs [10]. Ultimately, the choice of assays between WGBS and RRBS depends on the best compromise between sequencing costs and genome coverage adapted to each project.

Here we describe optimized RRBS and post-bisulfite WGBS protocols adapted for low amounts of cells in mammals. These protocols are suitable for 5 to 100 ng DNA and include steps to estimate the minimal number of cycles for the final PCR amplification to preserve the library complexity and minimize PCR bias and PCR duplicates.

2. Materials

2.1 Reduced Representation Bisulfite Sequencing (RRBS)

1. Programmable thermocycler.
2. Methylated adapter oligos with double high-performance liquid chromatography (*see Table 1*)

[Insert Table 1 here] : OK

3. DNA low binding microtubes 1.5 ml.
4. 0.2 ml PCR tubes.
5. H₂O, sterile-filtered.
6. Low TE buffer: 10 mM Tris-HCl pH 8.0, 0.1 mM EDTA.
7. Sodium Chloride, NaCl 5 M.
8. MspI enzyme (10 U/μL) with its buffer (this method is optimized for MspI enzyme and Tango 10X buffer from ThermoFisher Scientific).
9. Klenow Fragment exo- (5 U/μL).
10. dNTP mix for end-repair and A-tailing: 10 mM dATP, 1 mM dCTP, 1 mM dGTP.
11. dNTP mix for PCR (10 mM each nucleotide, dATP, dCTP, dGTP and dTTP),
12. ATP (100 mM).
13. T4 DNA ligase (30 U/μL).
14. 3% agarose gel in TBE 0.5X, containing 0.08 μg/ml of Ethidium Bromide.
15. Bromophenol blue DNA loading dye: 10 mM Tris-HCl pH 7.6, 0.03% bromophenol blue, 60% glycerol, 60 mM EDTA.
16. 20bp and 50bp DNA ladders.
17. Gel Extraction Kit for low elution volume (*e.g.* MinElute Gel Extraction kit from Qiagen).
18. Qiagen EpiTect Bisulfite kit.
19. PfU Turbo Cx hotstart DNA polymerase (2.5 U/μL).
20. Agencourt AMPure XP magnetic beads (60 mL).

21. Magnetic rack for 1.5 ml tubes.
22. 70% ethanol solution, molecular biology grade.
23. Precast 5% Criterion TBE polyacrylamide gel.
24. SYBR Green I Nucleic Acid Gel Stain 10,000 X.
25. Instrument for determining DNA concentration, *e.g* Qubit fluorometer (ThermoFisher Scientific) and Quant-iT ds DNA HS Assay Kit (ThermoFisher Scientific).
26. Tris-HCl Buffer: 10 mM Tris-HCl, pH 8.5 (*e.g.* EB Buffer, Qiagen).
27. Orbital shaker

2.2 Whole Genome Bisulfite Sequencing (WGBS)

1. Programmable thermocycler.
2. DNA low binding microtubes 1.5 ml.
3. 0.2 ml PCR tubes.
4. Covaris E220 sonicator.
5. 3% agarose gel in TBE 0.5X, containing 0.08 µg/ml of Ethidium Bromide
6. Bromophenol blue DNA loading dye: 10 mM Tris-HCl pH 7.6, 0.03% bromophenol blue, 60% glycerol, 60 mM EDTA.
7. 20bp and 50bp DNA ladders.
8. EZ DNA Methylation-Gold kit (Zymo research).
9. Agencourt AMPure XP magnetic beads (60 mL).
10. Magnetic rack for 1.5 ml tubes.
11. 70% ethanol solution, molecular biology grade.
12. Accel-NGS Methyl-Seq DNA Library Kit (Swift Biosciences) containing low EDTA TE buffer, buffer G1, reagent G2, reagent G3, enzyme G4, enzyme G5, enzyme G6, reagent Y1, enzyme Y2, buffer B1, reagent B2, enzyme B3, buffer R1, reagent R2, enzyme R3.
13. Methyl-Seq Set A Indexing Kit (Swift Biosciences).
14. Precast 5% Criterion TBE polyacrylamide gel.
15. SYBR Green I Nucleic Acid Gel Stain 10,000 X.
16. Instrument for determining DNA concentration, *e.g* Qubit fluorometer (ThermoFisher Scientific) and Quant-iT ds DNA HS Assay Kit (ThermoFisher Scientific).

3. Methods

3.1 Reduced Representation Bisulfite Sequencing (RRBS)

This RRBS protocol is adapted from the original protocol described by Gu et al. [8] and is suitable for input DNA ranging from 5 to 100 ng for mammalian species.

1. Dissolve each methylated adapter oligo (*see Table 1*) in low TE buffer to generate 100 μM stocks.
2. Mix 30 μl of the top adapter oligo (100 μM stock), 30 μl of the bottom adapter oligo (100 μM stock), 2 μl of 5 M NaCl and 138 μl of low TE buffer in a 1.5 ml microtube. Incubate in a 98°C water bath for 5 min, then turn off the water bath and let the water cool down to room temperature. Aliquots of the annealed adapter solution (15 μM final) can be stored at -80°C for up to 2 years.
3. Prepare genomic DNA samples (between 5 and 100 ng) (*see Note 1*).
4. For MspI digestion: mix 5 to 100 ng of genomic DNA (maximum of 15 μl volume), 1.8 μL of Tango 10X Buffer, 1.2 μL of MspI enzyme (10 U/ μL) and H₂O (q.s. for 18 μL) in a 0.2 ml PCR tube. Briefly mix by pipetting and spin.
5. In a thermocycler, incubate at 37°C for 5 h, followed by 80°C for 20 min to inactivate MspI (*see Note 2*).
6. For end-repair and A-tailing, add in the same tube (18 μL volume from the previous step 5): 1 μL of Klenow Fragment exo- (5 U/ μL), 0.8 μL of dNTP (10 mM dATP, 1 mM dCTP, 1 mM dGTP) and 0.2 μL of Tango 10X Buffer (final volume: 20 μL).
7. Incubate at 37°C for 40 min in a thermocycler and inactivate the Klenow enzyme by incubating at 75°C for 15 min.
8. For adapter ligation, add to the 20 μL volume from the previous step in the same tube: 1 μL of T4 DNA ligase (30 U/ μL), 1 μL of methylated adapters (15 μM ; *see* step 2 in section 3.1), 0.5 μL of Tango 10X Buffer, 0.25 μL of ATP (100 mM), 2.25 μL of H₂O (final volume: 25 μL).
9. Incubate overnight (between 12 and 16h) in a thermocycler at 16°C and inactivate the DNA ligase by incubating at 65°C for 20 min.
10. Load the samples (one per well) with bromophenol blue DNA loading dye on a 3% agarose gel in TBE 0.5X containing 0.08 $\mu\text{g/ml}$ of Ethidium Bromide. Load a DNA ladder on each side of the gel (*see Note 3*).
11. Run the gel for 2 h at 120V until the bromophenol blue dye has migrated approximately 7.5 cm.
12. Excise the gel between 150 and 400 bp using a different razor blade for each sample to avoid cross contamination (*see* Fig. 1) (*see Note 4*).

[Insert Figure 1 here]

13. Extract DNA from the gel slice with the Gel Extraction Kit following the kit instructions. Each sample is loaded on two columns. DNA from both columns is eluted with 12 μ L of preheated (65°C) Tris-HCl Buffer (*e.g.* EB Buffer) in the same DNA Low Binding 1.5 mL microtube. The total volume of recovered DNA is around 20 μ L.
14. Perform two consecutive rounds of bisulfite conversion using the Qiagen EpiTect Bisulfite kit, following the manufacturer's protocol designated for DNA extracted from FFPE tissues. Two rounds are necessary to achieve a conversion rate of >99.5% (*see Note 5*). After the first conversion, elute the DNA twice with 11 μ L of preheated (65°C) EB Buffer in a DNA Low Binding 1.5 mL microtube, and use 20 μ L to perform the second conversion. After the second conversion, elute the DNA twice with 22 μ L of preheated (65°C) EB Buffer in a new DNA Low Binding 1.5 mL microtube. The volume of recovered DNA is around 40 μ L (*see Note 6*).
15. At this step, we recommend performing a test PCR to determine the minimal number of PCR cycles for the final library. Prepare the following reaction in a microtube: 4 μ L of PfU Turbo Cx hotstart DNA polymerase buffer (10X), 1 μ L of dNTP (10 mM each), 4.8 μ L of a mix containing PCR Primers PE1 and PE2 (*see Table 1*) diluted at 2.5 μ M each in low TE buffer, 0.8 μ L of PfU Turbo Cx hotstart DNA polymerase (2.5 U/ μ L), 0.8 μ L of converted DNA from the previous step (or H₂O for the negative control) and 28.6 μ L of H₂O (final volume: 40 μ L).
16. Dispense 10 μ L of the PCR reaction in four PCR tubes and perform four PCR reactions with varying number of cycles using the following program: 2 min at 95°C, 12/14/16/18 cycles of 30 s at 95°C, 30 s at 65°C, 45 s at 72°C, and a final extension step of 7 min at 72°C (*see Note 7*).
17. Load the entire PCR products on a 5% Criterion TBE polyacrylamide gel with a DNA Ladder and run at 120V in TBE 0.5X for 45 min until the bromophenol blue dye has migrated approximately 5 cm.
18. Stain the gel in a SYBR Green bath (1/10 000, TBE 0.5X) for 30 min on an orbital shaker and determine from the gel the lowest number of cycles for the final library preparation (*see Fig. 2 and Note 8*).

[Insert Figure 2 here]

19. Set up a large-scale PCR reaction for the library preparation as follows : 20 μ L of PfU Turbo Cx hotstart DNA polymerase buffer (10X), 5 μ L of dNTP (10 mM each), 24 μ L of a mix containing PCR Primers PE1 and PE2 (*see Table 1*) diluted at 2.5 μ M each in low TE buffer, 4 μ L of PfU

Turbo Cx hotstart DNA polymerase (2.5 U/ μ L), 24 μ L of converted DNA from the step 14 and 123 μ L of H₂O (final volume: 200 μ L) (*see Note 9*).

20. Dispense 25 μ L of the PCR reaction in eight PCR tubes and perform eight PCR reactions using the same program as described in step 16 with the number of cycles determined in step 18.
21. Quickly spin and pool the PCR products in a DNA Low Binding 1.5 mL microtube.
22. Add 360 μ L of AMPure magnetic beads and mix well by pipetting up and down. Leave at room temperature for 15 min (tap the tube every 5 min).
23. Place the tube 5 min on a magnetic rack until the solution is clear and carefully discard the aqueous phase.
24. Add 1 mL of fresh 70% EtOH, flip the tube horizontally in the magnetic rack and wait 5 min until the solution is clear. Remove the ethanol and repeat this step.
25. Let the magnetic beads air-dry completely.
26. Remove the tube from the magnetic rack. Add 40 μ L of Tris-HCl Buffer (*e.g.* EB Buffer) to eluate the DNA and vortex. Place the tube back to the magnetic rack and transfer the DNA solution to a DNA Low Binding 1.5 mL microtube.
27. Use 1 μ L of the library to quantify the DNA concentration for example with the Quant-iT ds DNA HS Assay Kit. Our protocol typically yields between 30 and 80 ng of final library, which is sufficient for several sequencing reactions on an Illumina sequencer.
28. Optional: verify the size of the library and the removal of primers by loading 5-10 ng of the library on a 5% Criterion TBE polyacrylamide gel. Run at 120V in TBE 0.5X for 45 min until the bromophenol blue dye has migrated approximately 5 cm and stain the gel in a SYBR Green bath (1/10 000, TBE 0.5X) for 30 min on an orbital shaker (*see Fig. 3*).

[Insert Figure 3 here]

3.2 Whole Genome Bisulfite Sequencing (WGBS)

This protocol is based on the use of the Accel-NGS Methyl-Seq DNA Library Kit (Swift Biosciences). Its post-bisulfite library preparation based on the Adaptase technology maximizes DNA recovery during the library preparation and generates very good yields from low input DNA. Moreover, according to our tests and other publications [11], this kit has good performance in terms of library complexity and coverage. The protocol described here is suitable for input DNA ranging from 5 to 100 ng for mammalian species.

1. Prepare genomic DNA sample (*see Note 1*).
2. Fragment the DNA (5 to 100 ng in a final volume of 20 μ L) to 350 bp with a Covaris E220 sonicator using the following settings: 85 s, 140 W, 200 cycles/burst, duty cycle: 15% (for input DNA > 30 ng) or 10% (for input DNA < 30 ng).
3. When possible, verify the sonication by loading 5 to 10 ng of sonicated DNA on a 2.5% Agarose gel in TBE 1X and run at 180V for 30 min.
4. Perform bisulfite conversion with the EZ DNA Methylation-Gold kit according to the manufacturer's instructions (*see Note 10*). Briefly, mix 20 μ L of fragmented DNA with 130 μ L of CT conversion reagent and incubate 10 min at 98°C, 150 min at 64°C, then hold at 4°C. Perform washing and desulfonation steps on a Zymo-Spin IC Column following the manufacturer's instructions and elute with 15 μ L M-elution buffer preheated at 65°C in a 1.5 mL Low Binding microtube (*see Note 11*).
5. Transfer the bisulfited DNA in a 0.2 ml PCR tube. Denature in a thermocycler at 95°C for 2 min and place the tube 2 min on ice.
6. Prepare a mix containing 11.5 μ L of low EDTA TE, 4 μ L of buffer G1, 4 μ L of reagent G2, 2.5 μ L of reagent G3, 1 μ L of enzyme G4, 1 μ L of enzyme G5 and 1 μ L of enzyme G6 (*see Note 12*). Add this mix to the 15 μ L of denatured DNA from the step 5 in section 3.2 (final volume: 40 μ L) and incubate in a thermocycler at 37°C for 15 min, 95°C for 2 min, then hold at 4°C.
7. Prepare a mix containing 2 μ L of reagent Y1 and 42 μ L of enzyme Y2 (*see Note 13*). Add this mix to the sample from the previous step (final volume: 84 μ L) and incubate in a thermocycler at 98°C for 1 min, 62°C for 2 min, 65°C for 5 min, then hold at 4°C.
8. Quickly spin and transfer the sample from the PCR tube to a 1.5 mL Low Binding microtube. Add 101 μ L of AMPure beads first into the empty PCR tube (to recover the leftover liquid) and then add it to the sample in the 1.5 mL microtube and mix by pipetting up and down. Incubate 5 min at room temperature.
9. Spin down the sample. Place the tube on a magnetic rack and wait 5 min until the solution is completely clear.
10. Discard the supernatant (<5 μ L may be left behind) and leave the tube on the magnetic rack. Add 500 μ L of 70% EtOH and wait 1 min before removing the EtOH.
11. Repeat the previous wash step (step 10).
12. Spin down the sample, put the tube back on the magnetic rack and discard the residual EtOH.
13. Air dry the pellet for 4-5 min (do not over-dry the beads). Remove the tube from the magnetic rack.
14. Add 15 μ L of low EDTA TE to the beads and mix by pipetting up and down. Incubate at room temperature for 2 min.
15. Place the tube back on the magnetic rack and wait 5 min until the solution is completely clear.

16. Transfer the supernatant to a 0.2 ml PCR tube.
17. Prepare a mix containing 3 μ L of buffer B1, 10 μ L of reagent B2 and 2 μ L of enzyme B3. Add this mix to the sample from the previous step 16 (final volume: 30 μ L) and incubate in a thermocycler at 25°C for 15 min, then hold at 4°C.
18. Repeat steps 8 to 16 from section 3.2 with the following changes: add 30 μ L of AMPure beads in step 8 and perform final elution in 20 μ L low EDTA TE in step 14.
19. At this step, we recommend performing a test PCR to determine the minimal number of PCR cycles for the final library. Prepare the following reaction in a microtube: 1 μ L of DNA from the previous step 18 (or 1 μ L of H₂O for the negative control), 1 μ L of indexed oligos (from the Methyl-Seq Set A Indexing Kit), 5 μ L of low EDTA TE, 2 μ L of buffer R1, 0.8 μ L of reagent R2, 0.2 μ L of enzyme R3 (total volume: 10 μ L).
20. Divide the PCR reaction in 2 PCR tubes and perform 2 PCR reactions with varying number of cycles using the following program: 30 s at 98°C, between 4 and 10 cycles of 10 s at 98°C, 30 s at 60°C, 60 s at 68°C, then hold at 4°C (*see Note 14*).
21. Load the entire PCR products on a 5% Criterion TBE polyacrylamide gel with a DNA Ladder and run at 120V for 1 h.
22. Stain the gel in a SYBR Green bath (1/10 000, TBE 0.5X) for 30 min on an orbital shaker and determine from the gel the lowest number of cycles for the final library preparation (*see Fig. 4 and Note 15*).

[Insert Figure 4 here]

23. For the final PCR, add 5 μ L of indexed oligos (from the Methyl-Seq Set A Indexing Kit) directly to the sample from step 18 in section 3.2.
24. Prepare a mix containing 10 μ L of low EDTA TE, 10 μ L of Buffer R1, 4 μ L of reagent R2, 1 μ L of enzyme R3. Add the mix to the sample from the previous step (final volume: 49 μ L) and perform a PCR reaction using the following program: 30s at 98°C, 4 to 10 cycles (accordingly to the adequate number of cycles chosen in step 22) of 10 s at 98°C, 30 s at 60°C, 60 s at 68°C, then hold at 4°C.
25. Repeat steps 8-16 with the following changes: add 42.5 μ L of AMPure beads in step 8 and perform final elution in 20 μ L of low EDTA TE in step 14.
26. Quantify 1 μ L of the library for example with a Qubit and Quant-iT ds DNA HS Assay Kit. The protocol should yield between 30 and 50 ng of final library (*see Note 16*).

27. Optional: verify the size of the library and the removal of primers by loading 5-10 ng of the library on a 5% Criterion TBE polyacrylamide gel. Run at 120V for 1 h and stain the gel in a SYBR Green bath (1/10 000, TBE 0.5X) for 30 min on an orbital shaker (*see* Fig.5).

[Insert Figure 5 here]

4. Notes

1. For low amounts of cells, we recommend phenol/chloroform extraction and quantification of DNA concentration with a Qubit fluorometer.
2. It is recommended when possible to verify the digestion by loading 10 ng of the digested DNA alongside a non-digested control on a 1% Agarose Gel.
3. We recommend diluting the DNA Ladder in 1X Tango Buffer to ensure a uniform migration. Moreover, when preparing the gel, place additional wells at the bottom of the gel to facilitate precise gel excision.
4. Protect the samples from UV exposure as much as possible to avoid degradation.
5. Bisulfite conversion kits from other manufacturers (*e.g.* Zymo Research) might require only one round of conversion but induce more DNA fragmentation. In our tests, two rounds of conversion with the Qiagen EpiTect kit give the best results in terms of yield and conversion rate (>99.5 %).
6. For optimal DNA recovery, wait 5 min after adding the preheated (65°C) Tris-HCl Buffer (*e.g.* EB Buffer from Qiagen) to the column before spinning.
7. 14/16/18/20 cycles can be used for RRBS starting with less than 15 ng DNA.
8. In our conditions, we usually choose two cycles below the first cycle that generates a detectable signal on the gel (*see* Fig. 2). If the protocol works well, 12 cycles are sufficient when starting with 100 ng DNA and no more than 18 cycles should be used when starting with 5 ng DNA.
9. We usually use 24 µL out of the 40 µL of converted DNA for the final PCR and keep the rest as a backup. However, in the case of very small amounts of starting DNA, it is recommended to use the 40 µL in the final PCR and adjust the volume of H₂O in the PCR mix accordingly.
10. We did not test the Qiagen EpiTect Bisulfite kit for WGBS libraries.
11. The experiment can be stopped at this point by storing the DNA at -20°C for up to 1 week.
12. For each step of the Accel-NGS Methyl-Seq DNA Library Kit, thaw enzymes on ice 10 min before use.
13. Mix well enzyme Y2 by pipetting up and down before use.

14. We recommend 4 and 7 cycles when the amount of starting DNA is 50-100 ng, and 7 and 10 cycles when the amount of starting DNA is <20 ng.
15. If the protocol works well, 5-6 cycles should be sufficient when the amount of starting DNA is 50-100 ng, and 8-9 cycles when the amount of starting DNA is <20 ng
16. Important remark: after sequencing, it is necessary to trim the first five bases of reads R1 and first ten bases of reads R2 because the Adaptase reaction adds low complexity polynucleotide tails. If untrimmed, these tails reduce the mapping rates.

5. References

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Table 1: Sequences and specific modifications of oligos used in the RRBS protocol.

Name	Sequence (5' to 3')	Modifications
Methylated paired-end Adapter (top)	AcAcTcTTTcccTAcAcGAcGcTcTTccGATc*T	*: Phosphorothioate bond p: 5'-phosphate c: 5-methyl C in red: Illumina index (variable)
Methylated paired-end Adapter (bottom)	pGATcGGAAGAGcGGTTcAGcAGGAATGccGAG	
PCR primer PE1	AATGATACGGCGACCACCGAGATCTACAC TCTTTCCCTACACGACGCTCTTCCGATC*T	
PCR primer PE2	CAAGCAGAAGACGGCATAcGAGATNNNN NNCATTcCTGCTGAACCGCTCTTCCGATC*T	

Figure legends

Figure 1: Example of size selection of adapter-ligated DNA by electrophoresis on a 3% agarose gel. 20 bp and 50 bp DNA ladders are loaded on each side of the gel.

Figure 2. Examples of test PCRs for mouse RRBS libraries migrated on a 5% Criterion TBE polyacrylamide gel with a 50 bp DNA ladder and stained with SYBR Green. For each sample, PCR was performed with 12, 14, 16 and 18 cycles. A negative PCR control is loaded on the right of the gel. In these examples, we choose 14 cycles for the final PCR of the sample 1, and 12 cycles for the final PCR of the samples 2, 3 and 4.

Figure 3. Examples of final mouse RRBS libraries. 10 ng were migrated on a 5% Criterion TBE polyacrylamide gel with a 20 bp DNA ladder and stained with SYBR Green.

Figure 4. Examples of test PCRs for mouse WGBS libraries migrated on a 5% Criterion TBE polyacrylamide gel with 50 bp and 1 kb+ DNA ladders and stained with SYBR Green. For each sample, PCR was performed with 4 and 7 cycles. A negative PCR control is loaded on the right of the gel. In these examples, we choose 6 cycles for the final PCR.

Figure 5. Examples of final mouse WGBS libraries. 10 ng were migrated on a 5% Criterion TBE polyacrylamide gel with a 50 bp DNA ladder and stained with SYBR Green.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5