

Chapter 2

Profiling DNA Methylation Using Bisulfite Sequencing (BS-Seq)

Yun-Ru Chen, Sheng Yu, and Silin Zhong

Abstract

DNA cytosine methylation is one of the most abundant epigenetic marks found in the plant nuclear genome. Bisulfite sequencing (BS-Seq) is the method of choice for profiling DNA cytosine methylation genome-wide at a single nucleotide resolution. The basis of this technique is that the unmethylated cytosine can be deaminated to uracil by sodium bisulfite, while the methylated cytosine is resistant to the treatment. By deep sequencing of the bisulfite converted genomic DNA, the methylation level of each mappable cytosine position in the genome could be measured. In this chapter, we present a detailed 2-day protocol for performing a BS-Seq experiment and a simple bioinformatic workflow for wet lab biologists to visualize the methylation data.

Key words DNA methylation, Bisulfite sequencing

1 Introduction

Bisulfite sequencing (BS-Seq) is the most efficient method for the analysis of methylation status at single nucleotide resolution [1, 2]. As Sanger sequencing and most of the second generation sequencing methods cannot distinguish methylated from unmethylated cytosines, a bisulfite treatment is required to convert the unmethylated cytosine to uracil prior to sequencing. However, third generation sequencing such as PacBio is capable of detecting DNA modifications based on the pausing time of the polymerase when encountering a modified base, and could therefore eventually render the BS-Seq method obsolete.

The success of a BS-Seq experiment depends on the near complete conversion of the unmethylated cytosine to uracil. A small amount (1%) of exogenous DNA, such as lambda phage DNA, is often added before the bisulfite treatment, and the conversion rate can then be calculated from the spike-in unmethylated phage DNA. Plant DNA extract is often contaminated with organelle genomic DNA such as the chloroplast DNA, which lacks 5mC. Hence the

conversion rate could also be inferred from the chloroplast cytosine positions. For plant draft genomes without a chloroplast reference sequence, it might be necessary to include a lambda phage DNA spike-in. In addition to the conversion rate, sufficient sequence coverage is also essential for a successful BS-Seq experiment. Only cytosine positions with sufficient sequencing coverage will provide useful information and allow the determination of the methylation status, where a T indicates that the cytosine is unmethylated and a C that it is methylated. A typical BS-Seq experiment requires a sequencing depth of $10\times$ to $20\times$ genome coverage per strand, which is generally considered sufficient for calling differentially methylated regions (DMRs). However, nonunique alignments, such as the reads mapped to transposons and repeats, are discarded. Hence, in large repetitive plant genomes, the whole-genome coverage is often an underestimate of the actual cytosine coverage.

In this chapter, we provide a 2-day protocol for BS-Seq library preparation that is based on previously published methods [3] with some modifications to optimize for the current sequencing standards. In short, genomic DNA is first sonicated and the 300–500 bp fragments are recovered from agarose gel. The DNA fragments are end-repaired, dA-tailed and ligated to a fully methylated Illumina Y-shape adapter. Finally, the adapter ligated DNA fragments are bisulfite converted and PCR amplified prior to sequencing. In addition, we also provide a simple data analysis workflow that wet-lab biologist can perform to visualize the data. For advanced bioinformatic analysis, please refer to Chapter 4 [4] for details.

2 Materials

2.1 DNA Fragmentation and Size Selection

1. Agarose.
2. SYBR-Safe dye with compatible blue light transilluminator.
3. Optional: lambda phage DNA.
4. DNA loading buffer.
5. 1 kb DNA marker.
6. Gel running device.
7. Sonicator (e.g., Covaris M220 and Diagenode Bioruptor).
8. Purification kit for PCR product.
9. Purification kit for DNA extraction from agarose gel.

2.2 End Repair

1. DNA End Repair kit (e.g., from Enzymatics or NEB).
2. $10\times$ T4 ligase buffer with ATP (e.g., from Enzymatics or NEB).

3. 10 mM dNTP w/o dCTP (10 mM each dATP, dGTP, and dTTP).
4. Thermocycler.
5. Magnetic stand for 0.2 ml PCR tubes.
6. AMPure XP purification beads.
7. 80% EtOH.

2.3 dA-Tailing

1. 10× T4 ligase buffer with ATP.
2. 10 mM dATP.
3. Klenow 3'–5' exo- (e.g., from Enzymatics or NEB).
4. Thermocycler.
5. Magnetic stand for 0.2 ml PCR tubes.
6. AMPure XP purification beads.
7. 80% EtOH.

2.4 Adapter Ligation and Double AMPure XP Cleanup

1. 25 µM TruSeq 5mC double-stranded adapter:

Adapter Oligos:

Methylated TruSeq adapter oligo A (100 µM in TE buffer):

A[5Me~dC]A[5Me~dC]T[5Me~dC]TTT[5Me~dC]
[5Me~dC][5Me~dC]TA[5Me~dC]A[5Me~dC]GA
[5Me~dC]G[5Me~dC]T[5Me~dC]TT[5Me~dC][5Me~dC]
GAT[5Me~dC]*T

* indicates the phosphorothioate modification.

Methylated TruSeq adapter oligo B (100 µM in TE buffer):

[Phos]GAT[5Me~dC]GGAAGAG[5Me~dC]A[5Me~dC]A
[5Me~dC]GT[5Me~dC]TGAA[5Me~dC]T[5Me~dC]
[5Me~dC]AGT[5Me~dC]A[5Me~dC]

Anneal the adapter oligos first, to obtain a double-stranded adapter: Mix 25 µl of both adapter oligos (100 µM) in a 0.2 ml PCR tube. Heat to 90 °C in a thermocycler, and slowly chill to room temperature at a ramp rate of –1 °C/min. The annealed adapter (50 µM) can then be diluted to 25 µM with TE and stored in small aliquots at –20 °C.

2. 2× ligase buffer with ATP and PEG.
3. T4 Ligase HC (high concentration; e.g., from Enzymatics or NEB).
4. Thermocycler.
5. Magnetic stand for 0.2 ml PCR tubes.
6. AMPure XP purification beads.
7. 80% EtOH.
8. TE buffer: 10 mM Tris–HCl, 1 mM EDTA.

2.5 Bisulfite Conversion

1. Fluorometer (e.g., Invitrogen Qubit)
2. Fluorescent dye (e.g., Invitrogen Qubit dsDNA HS Assay Kit Q32851 or Promega QuantiFluor dsDNA Dye E258A)
3. Bisulfite conversion kit (e.g., Invitrogen MethylCode Bisulfite Conversion Kit or ZYMO EZ DNA Methylation-Gold Kit)
4. Thermocycler.
5. 100% EtOH.
6. Nuclease-free water.

2.6 PCR Amplification

1. Thermocycler.
2. DNA polymerase (e.g., KAPA HiFi Uracil+ or NEB Epimark Hotstart Taq).
3. TruSeq Adapter and index PCR primers:
Primers:
TruSeq universal PCR primer 1.0
AATGATACGGCGACCACCGAGATCTACACTCTTTCCC
TACACGACGCTCTTCCGATC*T
TruSeq index PCR primer 2.x
CAAGCAGAAGACGGCATAACGAGAT[NNNNNN]
GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T
* indicates the phosphorothioate modification.
[NNNNNN] indicates the 6 nucleotide index sequence in the TruSeq PCR primer 2.
4. Thermocycler.
5. Magnetic stand for 0.2 ml PCR tubes.
6. AMPure XP purification beads.
7. 80% EtOH.
8. Nuclease-free water.

2.7 Analysis of BS-Seq Data

To perform BS-Seq data analysis, a computer with Linux/UNIX operating system is required, as well as installation of the here listed software [5, 6]. The instruction on how to download and install these packages can be found via the provided URLs.

1. BSMAP (<https://github.com/zyndagi/BSMAP>).
2. IGV Browser (<http://software.broadinstitute.org/software/igv/>).
3. Tabix (<https://github.com/samtools/tabix>).
4. R version 3.0.2 or higher (<https://www.r-project.org/>).
5. Bioconductor R package DSS (<http://bioconductor.org/packages/release/bioc/html/DSS.html>).
6. Samtools (<http://www.htslib.org/>).

3 Methods

3.1 DNA Fragmentation and Size Selection (TIMING: ~2 h)

Fragmentation of DNA creates small inserts for library preparation, and is the first step in next generation sequencing workflows. The current sequencing read length for the Illumina HiSeq X ten machine is paired end 150 bp. Hence, the ideal BS-Seq library insert should be slightly over 300 bp.

1. Prepare a 1% agarose gel with SYBR-Safe dye in advance.
2. Determine the concentration of the DNA sample; dilute 2 µg of DNA into 130 µl of water.
3. Optional: add 2 ng of lambda phage DNA as spike-in.
4. Fragment the DNA in a sonicator. We use the Covaris M220 sonicator, and select the 300 bp fragment peak size protocol (*see Note 1*).
5. Transfer the sample into a snap cap microTUBE and sonicate the DNA.
6. Optional: confirm the sonication efficiency by running 50 ng of the sonicated DNA in an agarose gel.
7. Concentrate the DNA using a PCR purification kit and elute in 25 µl elution buffer.
8. Perform gel electrophoresis and recover the DNA fragments in the range between 300 and 500 bp using a gel purification kit. Elute the DNA with 25 µl water.
9. Determine the DNA concentration, around 300 ng is required.

3.2 End Repair (TIMING: ~50 min)

The fragmented DNA contains different types of overhangs, and these need to be blunt-ended and phosphorylated for adapter ligation. This is achieved by the combined action of the T4 DNA polymerase and the T4 polynucleotide kinase in the End Repair enzyme mix.

1. Assemble the end repair reaction in a PCR tube as follows:

Components	Volume (µl)
300–500 ng of purified DNA in water	21
10× T4 Ligase buffer with ATP	2.5
10 mM dNTP w/o dCTP (<i>see Note 2</i>)	0.5
End Repair Enzyme Mix	1

2. Mix by pipetting, incubate in a thermocycler at 20 °C for 30 min.

3. Add 25 μ l AMPure XP beads into the end-repair reaction, mix by pipetting (at least ten times) and incubate at room temperature for 10 min.
4. Place the End-Repair reaction with AMPure XP on a magnetic stand for ~1 min or until the beads are fully attached to the magnetic side of the tube. Carefully pipette out the solution without touching the beads. Do not remove the tube from the magnet.
5. Add 150 μ l 80% EtOH without disturbing the beads and wait for 30 s, carefully remove the ethanol using the pipette.
6. Repeat the ethanol wash one more time. Do not remove the tube from the magnet throughout the process.
7. Use a new pipette tip to remove all residual ethanol from the bottom and the side of the PCR tube. Do not overdry the AMPure XP beads.
8. Add 20.5 μ l of water to elute the end-repaired DNA, mix well by pipetting.
9. Place the PCR tube on the magnetic stand for ~1 min or until the beads are fully attached to the magnetic side of the tube. Carefully pipette out the end-repaired DNA to a new PCR tube without touching the beads.

3.3 dA-Tailing (TIMING: ~50 min)

To prevent ligation between the blunt and phosphorylated DNA fragments and also self-circularization, a 3' dA overhang is added by the Klenow Fragment (3' exo-) of the *E. coli* DNA polymerase I.

1. Prepare the dA-tailing reaction as follows:

Components	Volume (μ l)
End-repaired DNA in water	20.5
10 \times T4 Ligase buffer with ATP	2.5
10 mM dATP	1
Klenow 3'-5' exo-	1

2. Place the tube in a thermocycler and incubate at 37 °C for 30 min.
3. Add 25 μ l AMPure XP beads into the tube, mix by pipetting (at least ten times) and incubate at room temperature for 10 min.
4. Place the tube on a magnetic stand for ~1 min or until the beads are fully attached to the magnetic side of the tube. Carefully pipette out the solution without touching the beads. Do not remove the tube from the magnet.

5. Add 150 µl 80% EtOH without disturbing the beads and wait for 30 s, carefully remove the ethanol using the pipette.
6. Repeat the ethanol wash one more time. Do not remove the tube from the magnet throughout the process.
7. Use a new pipette tip to remove all residual ethanol from the bottom and the side of the PCR tube. Do not overdry the AMPure XP beads.
8. Add 22 µl of water to elute the dA-tailed DNA, mix well by pipetting.
9. Place the PCR tube on the magnetic stand for ~1 min or until the beads are fully attached to the magnetic side of the tube. Carefully pipette out all the DNA to a new PCR tube without touching the beads.

3.4 Adapter Ligation and Double AMPure XP Clean Up (TIMING: ~2 h or Overnight)

After dA-tailing, the DNA fragment with 3' dA overhang is ready to be ligated to the Illumina adapter, which contains a complementary 3' dT overhang. Compared to the regular Illumina adapter, the cytosines in the BS-Seq adapter are methylated, and will not be bisulfite converted to uridine.

1. Prepare the ligation reaction as follows:

Components	Volume (µl)
dA-tailed DNA in water	22
2× Ligase buffer with ATP and PEG	25
25 µM TruSeq 5mC double stranded adapter	2
T4 Ligase	1

2. The solution containing 2× ligase buffer is very viscous, pipette up and down at least ten times to mix after adding the T4 DNA Ligase.
3. Incubate the tube in a thermocycler at 25 °C for 15 min and hold at 4 °C for at least 1 h. Alternatively, perform the ligation overnight at 16 °C (*see Note 3*).
4. Add 50 µl AMPure XP beads into the tube, mix by pipetting (at least ten times) and incubate at room temperature for 10 min.
5. Place the tube on a magnetic stand for ~1 min or until beads are fully attached to the magnetic side of the tube. Carefully pipette out the solution without touching the beads. Do not remove the tube from the magnet.
6. Add 150 µl 80% EtOH without disturbing the beads and wait for 30 s, carefully remove the ethanol using the pipette.

7. Repeat the ethanol wash one more time. Do not remove the tube from the magnet during the process.
8. Use a new pipette tip to remove all residual ethanol from the bottom and the side of the PCR tube. Do not overdry the AMPure XP beads.
9. Add 50 μ l of TE to elute the adapter ligated DNA, mix well by pipetting.
10. Repeat the AMPure XP cleanup to achieve very pure DNA. After drying the beads, add 21 μ l of TE to elute the adapter ligated DNA, mix well by pipetting.
11. Place the PCR tube on the magnetic stand for ~1 min or until the beads are fully attached to the magnetic side of the tube. Carefully pipette out all the DNA to a new PCR tube without touching the beads.

**3.5 Bisulfite
Conversion (TIMING:
3 h)**

Sodium bisulfite can convert unmethylated cytosine into uracil, which can be subsequently converted to thymine during PCR. The 5-methylcytosines in the adapter and the DNA insert are protected from this conversion reaction. The following protocol describes a bisulfite conversion using the Invitrogen MethylCode Bisulfite Conversion Kit. We have also used conversion kits from QIAGEN and ZYMO with success following the condition recommended by the manufacturer.

1. Use 1 μ l of the library for DNA concentration measurement on a Qubit fluorometer. The expected DNA amount is around 300–500 ng (*see Note 4*).
2. Set up the bisulfite conversion reaction as follows:

Components	Volume (μ l)
Library DNA in TE	20
Freshly prepared CT conversion reagent	130

3. Mix the reaction carefully and incubate it in the PCR machine using the following cycle conditions:

Step	Time	Temp ($^{\circ}$ C)
Denature	10 min	98
Incubation	2.5 h	64
Hold	Indefinite	4

4. Purify the bisulfite converted library according to the kit's manual, except that the elution is performed using 2×20 μ L water instead of the EB buffer, which interferes with PCR.

5. **Critical step:** the purified library is unstable, so it should be PCR-amplified immediately. Do not store the purified libraries at -20°C . If storage is required, it can be kept in a 4°C fridge for 1 day without significant loss.

3.6 PCR Amplification (TIMING: ~120 min)

Cytosine deamination occurs naturally inside the cell, and is a major promutagenic event. Archaeal DNA polymerase such as *Pfu* can recognize the uracil in the template strand and stall polymerization. Hence, uracil tolerant polymerase must be used to amplify the bisulfite-converted library.

1. Assemble the PCR reaction as follows (*see Note 5*):

Components	Volume (μl)
BS-treated library	20
2 \times KAPA HiFi Uracil+ ReadyMix	25
TruSeq universal primer (10 μM)	2.5
TruSeq index primer (10 μM)	2.5

2. Perform the PCR in a thermocycler using the following program.

Cycle	Denature	Anneal	Extend	Hold
1	94 $^{\circ}\text{C}$ for 1 min	–	–	–
6–8	98 $^{\circ}\text{C}$ for 10 s	65 $^{\circ}\text{C}$ for 20 s	72 $^{\circ}\text{C}$ for 30 s	–
1	–	–	72 $^{\circ}\text{C}$ for 3 min	–
1	–	–	–	4 $^{\circ}\text{C}$

3. Purify the PCR product using 1 volume of AMPure XP as previously described. The library DNA is now ready for sequencing (*see Note 6*).

3.7 Analysis of BS-Seq Data: Read Mapping and Methylation Calling

In this example, we assume that there is a paired-end *Arabidopsis* BS-Seq Illumina dataset from sample ‘At_C1’. The fastq files are named At_C1_R1.fq.gz and At_C1_R2.fq.gz. We also assume that the reference genome sequence file TAIR10.fa is stored in the same directory.

The following script will invoke BSMAP to align the BS-Seq reads against the Arabidopsis genome. The number following option ‘-p’ in the script specifies the number of CPU cores to be used during the BSMAP mapping. The alignment BAM file is then used by the python program methratio.py supplied by BSMAP to call the methylation level for each cytosine position. The result (a bigwig file named AtC1_methratio.bw) can be directly viewed in the IGV browser.

Create a shell script (in the Unix-Shell command line interpreter):

```
$ cat >run_bsmmap.sh
#!/bin/bash

sample=At_C1
ref=./TAIR10.fa

bsmap -a ${sample}_R1.fq.gz -b ${sample}_R2.fq.gz -d $ref -o $sample.bam -r 0 -p
8 -v 5 -q 10 2>${sample}_bsmap.err

methratio.py -o ${sample}_methratio.txt -w ${sample}_methratio.wig -b 1 -d $ref
-z -r $sample.bam 2>${sample}_methratio.err

samtools view -H $sample.bam | grep '^@SQ' | cut -f 2- | sed -e 's/SN:/' -e 's/
LN:/' >genome.size

wigToBigWig ${sample}_methratio.wig genome.size ${sample}_methratio.bw

bgzip ${sample}_methratio.txt

tabix -b 2 -e 2 -S 1 ${sample}_methratio.txt.gz

Press Ctrl-C to save and exit, then execute the script:

$ nohup bash run_bsmmap.sh &
```

3.8 Analysis of BS-Seq Data: Differential Methylation Calling

Once the mapping and methylation calling are finished, one can call differentially methylated regions (DMRs) between two samples using DSS. In the following scenario, we assume that BS-Seq data from the control and treatment groups (with one biological replicate) have been processed as indicated above. The methylation file for two control samples, (At_C1_methratio.txt.gz and At_C2_methratio.txt.gz) and the treatment samples, (At_T1_methratio.txt.gz and At_T2_methratio.txt.gz) are in the same path.

Create an R script:

```
$ cat >run_dss.R
library(DSS)
require(bsseq)

readData <- function(file, context="CG", skip=1){
  if(! file.exists(file)){stop(file, " doesn't exist !!!")}

  classes = c("character", "integer", "character", "character", "NULL", "NULL",
"integer", "integer", rep("NULL", 4))

  names = c("chr", "pos", "strand", "context", "NULL", "NULL", "X", "N", rep
(NULL, 4))
  data <- read.table(file, colClasses=classes, col.names=names, skip=skip)
  data <- data[data$context==context, c("chr", "pos", "N", "X")]
}

sample.c1 <- "At_C1"
sample.c2 <- "At_C2"
sample.t1 <- "At_T1"
```

```

sample.t2 <- "At_T2"
sample.c <- "At_C"
sample.t <- "At_T"

file.c1 <- paste(sample.c1, "methratio.txt.gz", sep="_")
file.c2 <- paste(sample.c2, "methratio.txt.gz", sep="_")
file.t1 <- paste(sample.t1, "methratio.txt.gz", sep="_")
file.t2 <- paste(sample.t2, "methratio.txt.gz", sep="_")

context.all <- c("CG", "CHG", "CHH")

for(context in context.all){
  data.c1 <- readData(file.c1, context)
  data.c2 <- readData(file.c2, context)
  data.t1 <- readData(file.t1, context)
  data.t2 <- readData(file.t2, context)
  BSobj <- makeBSseqData(list(data.c1, data.c2, data.t1, data.t2), c(sample.c1,
sample.c2, sample.t1, sample.t2))
  rm(data1, data2)

  dml <- DMLtest(BSobj, group1=c(sample.c1, sample.c2), group2=c(sample.t1,
sample.t2), smoothing=F)

  dml.r <- callDML(dml, delta=0.1, p.threshold=0.05)
  dmr <- callDMR(dml, delta=0.1, p.threshold=0.05, minCG=4, dis.merge=100, pct.
sig=0.75)

  prefix <- paste(paste(sample.c, sample.t, sep="-"), context, sep=".")
  save(dml, file=paste(prefix, "Rdata", sep="."))
  write.table(dml.r, file=paste(prefix, "DMC.txt", sep="."), sep="\t", quote=F,
row.names=F)
  write.table(dmr, file=paste(prefix, "DMR.txt", sep="."), sep="\t", quote=F,
row.names=F)
}

```

Type Ctrl-C to save and exit.
Then execute the script:

```
$ nohup Rscript run_dss.R &
```

The output will be two tables containing the DMR and DMC information that can be viewed directly in MS Excel.

4 Notes

1. We often use 1–2 µg genomic DNA in 130 µl as starting material. Some polysaccharide-rich DNA, such as those prepared from fruits, need to be diluted and sonicated in multiple batches. In this protocol, we choose to purify the 300 bp DNA fragments because we use HiSeq X ten for sequencing and the current read length is paired end 150 bp.

2. Due to the lack of dCTP, 5' overhang containing guanosine cannot be filled in, and, hence, those DNA fragments cannot be converted to library. If the starting DNA amount is limited (e.g., DNA from laser capture microdissection), we could add 1 μ L of mung bean nuclease and incubate for 10 min to remove the remaining 5' overhang after the end-repair reaction. One could also include dCTP in the end-repair reaction. The unmethylated dCTP added to the DNA ends could be excluded from the analysis, and the BSMAP package described in this chapter have an option “-trim-fillin N” supporting this type of end-repaired library.
3. Although a 15 min room temperature ligation reaction is often sufficient for RNA-Seq or ChIP-Seq library preparation, we found that for BS-Seq library preparation, an extended ligation at 4 °C or even overnight at 16 °C could significantly improve the library yield.
4. We do not recommend using more than 500 ng library DNA in each bisulfite reaction, as too much DNA can reduce conversion rate. If more DNA has to be used, the samples can be split into multiple bisulfite conversion reactions. On the other hand, too few DNA could lead to over bisulfite conversion, degradation of DNA fragments with low 5mC level and loss in the post-bisulfite column purification step. If DNA sample is limited, one could add some raw lambda DNA, which could not be PCR-amplified, to the bisulfite conversion reaction to bring the final DNA amount in the bisulfite reaction to the optimum range (300–500 ng).
5. The PCR cycle number depends on the input DNA amount, the adapter ligation efficiency and the loss during bisulfite conversion. In our hands, 100–200 ng of pre-bisulfite DNA requires no more than 8 cycles of PCR. The choice of a different polymerase could also affect the PCR cycle condition. For example, 2–3 more cycles are often required if Pfu Turbo Cx (Stratagene) is used. We found that the low cost NEB EpiMark Taq polymerase is as efficient as the KAPA Uracil+ enzyme. However, it will also amplify the adapter dimer if the AMPure XP cleanup step after adapter ligation failed to remove all adapters, and the PCR amplified libraries can be gel purified.
6. Each Illumina sequencing service provider has its preferred library format. The sequencing core facility we use requires that each library is to be submitted in TE solution with a concentration no <2 ng/ μ L and in a volume of no <10 μ L. Some service providers prefer the user to ethanol precipitate 50–100 ng of library DNA with glycogen as carrier and post the library in the form of a dry pellet.

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