Generation of sequencing libraries for building immune cell methylomes

Stefan Floess[1*] and Jochen Huehn[1,2]

[1]Department of Experimental Immunology, Helmholtz Centre for Infection Research, 38124 Braunschweig, Germany
[2]Hannover Medical School, 30625 Hannover, Germany

*correspondence to: Stefan Floess, Department of Experimental Immunology, Helmholtz Centre for Infection Research, Inhoffenstr. 7, 38124 Braunschweig, Germany; Phone: +49 531 6181 3308; E-mail: stefan.floess@helmholtz-hzi.de

i. Running head: Immune cell methylome

ii. Abstract

The comparison of methylomes from immune cells enables the identification of differentially methylated regions and thereby region-associated gene loci. Those regions can be used to discriminate one immune cell population from the other, as well as help to identify key molecules and major pathways determining the unique phenotypes of immune cell lineages. The combination of bisulfite treatment of genomic DNA and next generation sequencing provides the basis for studying epigenetic changes in different immune cell populations. Further development of whole genome bisulfite sequencing resulted in a protocol for sequencing libraries that accepts both single- or double- stranded DNA from fixed or non-fixed cells, respectively. Therefore, researchers can include immune cell populations in their methylation studies whose isolation depends on the staining of intracellular molecules.

iii. Keywords: epigenetic regulation, methylome, sequencing library

1. Introduction

Although the methylation of the DNA building base cytosine was discovered more than 70 years ago (1), more than a quarter-century had passed before researchers set up the hypothesis that methylation of DNA is linked to transcriptional regulation (2, 3) and is inheritable during the process of DNA replication. Subsequent studies on regulatory DNA elements were able to confirm the relation between DNA methylation and transcriptional repression (4, 5) and demonstrated methylation-sensitive DNA binding of transcription factors. Nowadays, differential methylation of DNA belongs to the epigenetic mechanisms that regulate gene expression during embryonic development, cellular differentiation, and carcinogenesis, and is involved in genomic imprinting and X-chromosomal inactivation (6, 7).

An initial step for studying the methylation status of a DNA region of interest was the finding that bisulfite can attack the unmethylated base cytosine and convert it into uracil, whereas 5-methylcytosine remains unchanged (8). The protocol for bisulfite sequencing was developed by Frommer and colleagues (9), in which the bisulfite-converted DNA can be used as a template in a PCR, in which the uracil pairs with adenine and is progressively replaced by thymine. The PCR products can be cloned into plasmids and then subjected to sequencing. The C/T ratio at a given CpG motif can be directly translated into the methylation status. Further progress of the method was introduced by pyrosequencing (10, 11), conventional sequencing of the PCR product (12), and integrating amplification steps into quantitative real-time PCR (13). Finally, next generation sequencing approaches led to the development of methods like methyl-binding domain capture sequencing, reduced-representation-bisulfite sequencing or whole-genome bisulfite sequencing (WGBS).

In this method protocol, we focus on the generation of next generation sequencing libraries from bisulfite-converted genomes of immune cells. We provide an optimized WGBS protocol that can handle single- and double-stranded DNA as input. This flexibility enables researchers to use paraformaldehyde (PFA)-fixed, intracellularly stained cells for cell sorting and subsequent methylation analysis. Such approaches are necessary for cells that are difficult to define solely by surface markers, e.g. regulatory T cell or innate lymphoid cell subsets.

2. Material

H_2O (DNase-free)
Low Tris-EDTA (TE) buffer
Phosphate buffered saline (PBS)
99.8 % ethanol
70 % ethanol (fresh)
80 % ethanol (fresh)
50 % Chelex 100 (Biorad) slurry (5 g/10 ml H_2O)
Ampure XP beads (Beckman Coulter)
SPRI Beads (Beckman Coulter)
DNeasy Blood & Tissue Kit (Qiagen)
NucleoSpin Tissue Kit (Macherey-Nagel)
Genomic DNA Clean & Concentrator-10 Kit (Zymo Research)
EZ DNA Methylation-Lightning Kit (Zymo Research)
Accel-NGS Methyl-Seq DNA Library Kit (Swift Biosciences)
Indexing Kit (Swift Biosciences)
1.5 ml safe-lock reaction tubes
0.2 ml thin-walled PCR tubes
Strip tubes
MicroTubes AFA Fiber Pre-Slit Snap-Cap 6x 16mm (Covaris)
Filter-tips for pipettes
Pipette for small volumes (0.1-2.5 µl)
Magnetic NdFeB rack
Thermo mixer (suitable for 1.5 ml tubes)
Thermal cycler (incl. heated lid and suitable for 0.2 ml tubes)
Qubit (Thermo Fisher)
Bioanalyzer
Bioanalyzer high sensitivity RNA analysis Kit
Bioanalyzer high sensitivity DNA analysis Kit
Covaris S220
Bucket with ice

3. Method

3.1 gDNA preparation from non-fixed cells using the DNeasy Blood & Tissue Kit

Preparation of the cells of interest, including a proper comparison group, should be planned carefully (see Note 1). If one plans to include the allosomes in the analysis, one has to prepare the cells from male donors (see Note 2).

1. After sorting of at least 25.000 cells/sample (see Note 3), add PBS to reach a volume of 200 µl if necessary (see Note 4). If the volume is higher, generate multiple 200 µl samples.
2. Add 20 µl Proteinase K to the 200 µl cell suspension, followed by 200 µl buffer AL.
3 Mix by vortexing and incubate at 56°C for 10 min.
4. Carefully open the tube, add 200 µl ethanol (absolute, 99.8 %), mix by vortexing and quickly spin down all the fluid.
5. Transfer the solution into a DNeasy mini spin column placed in a 2 ml collection tube (see Note 5).
6. Centrifuge at ≥6000 g for 1 min and replace collection tube.
7. Add 500 µl buffer AW1 to the spin column.
8. Centrifuge at ≥6000 g for 1 min and replace collection tube.
9. Add 500 µl buffer AW2 to the spin column.
10. Centrifuge at 20000 g (or max. speed) for 3 min and place column into a clean 1.5 ml tube.
11. Add 200 µl buffer AE directly onto the column material and incubate for 1 min.
12. Centrifuge at 20000 g (or max. speed) for 1 min and place column into a clean 1.5 ml tube.
13. Add 200 µl buffer AE directly onto the column material and incubate for 1 min.
14. Combine the two eluates.

3.2 gDNA preparation from fixed cells using the NucleoSpin Tissue Kit

1. If the volume of the sorted cells is ≤ 30 µl (see Note 1-3), add buffer T1 to reach 200 µl. If the volume exceeds 30 µl, pellet the cells by centrifugation, carefully remove the supernatant and resuspend in 200 µl buffer T1.
2. Add 25 µl Proteinase K solution, 200 µl buffer B3 and mix by vortexing.
3. Incubate the samples at 70°C for 10-15 min.
4. Add 50 µl 50 % Chelex 100 (see Note 6).
5. Shake the samples at 1400 rpm at 95°C for 15 min.
6. Spin down the Chelex resin at 11000 g for 2 min.
7. Transfer the supernatant into a new tube, add 235 µl 99.8 % ethanol and mix.
8. Transfer the solution into a spin column placed into a collection tube.
9. Centrifuge at ≥11000 g for 1 min and replace collection tube.
10. Add 500 µl buffer BW to the spin column.
11. Centrifuge at ≥11000 g for 1 min, empty the collection tube and reuse.

12. Add 600 µl buffer B5 to the spin column.
13. Centrifuge at ≥11000 g for 1 min, empty the collection tube and reuse.
14. Dry column by centrifugation at ≥11000 g for 1 min and place into a new 1.5 ml tube.
15. Add 100 µl pre-warmed buffer BE (70°C) directly onto the column and incubate for 1 min.
16. Centrifuge at ≥11000 g for 1 min.
17. Add 100 µl pre-warmed buffer BE (70°C) directly onto the column and incubate for 1 min.
18. Centrifuge at ≥11000 g for 1 min.

3.3. Sample concentration using the Genomic DNA Clean & Concentrator-10 Kit

1. Start with step a or b.
a: Add 800 µl ChIP DNA Binding buffer to 400 µl sample generated from non-fixed cells and mix.
b: Add 1000 µl ChIP DNA Binding buffer to 200 µl sample generated from fixed cells and mix.
2. Transfer 900 µl sample to a Zymo-Spin IC-XL column placed into a collection tube.
3. Centrifuge at ≥11000 g for 1 min, empty the collection tube and reuse.
4. Transfer the residual sample to the column.
5. Centrifuge at ≥11000 g for 1 min, empty the collection tube and reuse.
6. Add 200 µl DNA wash buffer to the column.
7. Centrifuge at ≥11000 g for 1 min.
8. Add 200 µl DNA wash buffer to the column.
9. Centrifuge at ≥11000 g for 1 min and transfer the column into a new 1.5 ml tube.
10. Add 22 µl pre-warmed DNA elution buffer (70°C) directly to the column and incubate for 1 min.
11. Centrifuge at ≥11000 g for 1 min.
12. Measure the DNA concentration using a Qubit (see Note 7).

3.4 Bisulfite conversion using the EZ DNA Methylation-Lightning Kit

1. Use 50 ng sample gDNA, transfer to a PCR tube and add H_2O to reach 20 µl, if necessary.
2. Add 130 µl of lightning conversion reagent, mix carefully by pipetting, without creating droplets at the tube wall.
3. Place the tube in a thermal cycler (with heated lid) and start the following program: 98°C for 8 min, 54°C for 60 min, 4°C for up to 20 hours.
4. Take out the tube, transfer the sample to a 1.5ml tube and mix with 600 µl M-binding buffer.
5. Transfer the solution to a Zymo-Spin IC column placed into a collection tube.
6. Centrifuge at ≥11000 g for 1 min, empty the collection tube and reuse.
7. Add 100 µl M-wash buffer to the column.

8. Centrifuge at ≥11000 g for 1 min, empty the collection tube and reuse.
9. Add 200 µl L-desulphonation buffer to the column, carefully close the lid and incubate for 15-20 min.
10. Centrifuge at ≥11000 g for 1 min, empty the collection tube and reuse.
11. Add 200 µl M-wash buffer to the column.
12. Centrifuge at ≥11000 g for 1 min, empty the collection tube and reuse.
13. Add 200 µl M-wash buffer to the column.
14. Centrifuge at ≥11000 g for 1 min and transfer the column to a new 1.5 ml tube.
15. Add 10 µl of M-elution buffer directly to the column and incubate for 30 sec.
16. Centrifuge at ≥11000g for 1 min to elute the converted gDNA.

3.5. Fragmentation

1. Add 40 µl low TE to each sample and mix.
2. Transfer to a Covaris microTube.
3. Sonicate the sample using the following parameter: target peak: 350, duty cycle: 10 %, intensity: 5, peak incident power: 175 watts, cycles per burst: 200, processing time: 120 sec (see Note 8).
4. Transfer the sample to a strip tube and wait until the solution reaches RT.
5. Add 90 µl Ampure XP beads (RT) and mix 10 times by pipetting (see Note 9).
6. Incubate for 5 min.
7. Transfer the strip tube into a magnetic rack and incubate for 3 min.
8. Carefully remove the supernatant.
9. Add 200 µl 70 % ethanol and incubate for 30 sec.
10. Carefully remove the supernatant.
11. Add 200 µl 70 % ethanol and incubate for 30 sec.
12. Carefully remove the supernatant.
13. Dry for 60 sec.
14. Transfer the strip tube into a non-magnetic rack, add 16 µl low TE and resuspend the beads by 20 times pipetting.
15. Incubate for 2 min.
16. Put the strip tube into a magnetic rack and incubate for 3 min.
17. Transfer the supernatant containing the fragmented DNA into a new PCR tube.
18. Control fragment size on a Bioanalyzer (high sensitivity RNA analysis).

3.6. Library preparation using the Accel-NGS Methyl-Seq DNA Library Kit

1. Let the reagents G1, G2, G3, G4, G5, G6 and low TE thaw/temper on ice.
2. Incubate the sample tube in a thermal cycler (with heated lid) at 95°C for 2 min.

3. Immediately put the tubes on ice and incubate for 2 min.
4. Carefully generate the adaptase mix in a fresh, cooled tube by mixing:
11.5 µl low TE
4 µl buffer G1
4 µl reagent G2
2.5 µl reagent G3
1µl enzyme G4
1µl enzyme G5
1µl enzyme G6
5. Mix sample with 25 µl adaptase mix.
6. Transfer the tube to a thermal cycler (37°C on hold and with heated lid).
7. Cycler steps: 37°C for 15 min, 95°C for 2 min and 4°C (on hold).
8. Put the tubes on ice.
9. Let the reagents Y1 and Y2 thaw/temper on ice.
10. Carefully generate the extension mix in a fresh, cooled tube by mixing:
42 µl enzyme Y2
2 µl reagent Y1
11. Mix sample with 44 µl extension mix.
12. Transfer the tube to a thermal cycler (98°C on hold and with heated lid).
13. Cycler steps: 98°C for 1 min, 62°C for 2 min, 65°C for 5 min and 4°C (on hold).
14. Transfer the sample into a strip tube and incubate for 5-10 min.
15. Add 101 µl SPRI beads (RT) and mix by pipetting 10 times.
16. Incubate for 5 min.
17. Place the strip tube into a magnetic rack and incubate for 3 min.
18. Carefully remove the supernatant (Note: ≤5 µl fluid may be left behind).
19. Add 190 µl 80 % ethanol and incubate for 30 sec.
20. Carefully remove the supernatant.
21. Add 190 µl 80 % ethanol and incubate for 30 sec.
22. Carefully remove the supernatant.
23. Dry for 1 min.
24. Transfer the strip tube into a non-magnetic rack, add 16 µl low TE and resuspend the beads by 20 times pipetting.
25. Incubate for 2 min.
26. Put the strip tube into a magnetic rack and incubate for 2 min.
27. Transfer the supernatant containing the DNA into a new PCR tube (Pipetting of beads has to be avoided) and put on ice.
28. Let the reagents B1, B2, and B3 thaw/temper on ice.

29. Carefully generate the ligation mix in a fresh, cooled tube by mixing:
3 µl buffer B1
10 µl reagent B2
2 µl enzyme B3
30. Immediately mix sample with 15 µl ligation mix.
31. Transfer the tube to a thermal cycler (25°C on hold and with heated lid).
32. Cycler steps: 25°C for 15 min, 4°C (on hold).
33. Transfer the sample into a strip tube and incubate (RT) for 5-10 min.
34. Add 30 µl SPRI beads (RT) and mix by pipetting 10 times.
35. Incubate for 5 min.
36. Place the strip tube into a magnetic rack and incubate for 3 min.
37. Carefully remove the supernatant (Note: ≤5 µl fluid may be left behind).
38. Add 190 µl 80 % ethanol and incubate for 30 sec.
39. Carefully remove the supernatant.
40. Add 190 µl 80 % ethanol and incubate for 30 sec.
41. Carefully remove the supernatant.
42. Dry for 1 min.
43. Transfer the strip tube into a non-magnetic rack, add 21 µl low TE and resuspend the beads by 20 times pipetting.
44. Incubate for 2 min.
45. Put the strip tube into a magnetic rack and incubate for 3 min.
46. Transfer the supernatant containing the DNA into a new PCR tube (Pipetting of beads has to be avoided), mix with 5 µl index primer (chosen by user; different index primer available) and put on ice.
47. Let the reagents R1, R2, R3 and low TE thaw/temper on ice.
48. Carefully generate the indexing mix in a fresh, cooled tube by mixing:
10 µl low TE
10 µl buffer R1
4 µl reagent R2
1 µl enzyme R3
49. Immediately mix sample with 25 µl indexing mix.
50. Transfer the tube to a thermal cycler (98°C on hold and with heated lid).
51. Cycler steps: 98°C for 30 sec, 10x [98°C for 10 sec, 60°C for 30 sec, 68° for 60 sec], 4°C (on hold), (see Note 10).
52. Transfer the sample into a strip tube and incubate (RT) for 5-10 min.
53. Add 42.5 µl SPRI beads (RT) and mix by pipetting 10 times.
54. Incubate for 5 min.

55. Place the strip tube into a magnetic rack and incubate for 3 min.
56. Carefully remove the supernatant (Note: ≤5 µl fluid may be left behind).
57. Add 190 µl 80 % ethanol and incubate for 30 sec.
58. Carefully remove the supernatant.
59. Add 190 µl 80 % ethanol and incubate for 30 sec.
60. Carefully remove the supernatant.
61. Dry for 1 min.
62. Transfer the strip tube into a non-magnetic rack, add 21 µl low TE and resuspend the beads by 20 times pipetting.
63. Incubate for 2 min.
64. Put the strip tube into a magnetic rack and incubate for 3 min.
65. Transfer the supernatant containing the DNA into a new PCR tube (Pipetting of beads has to be avoided) and put on ice.
66. Control fragment size and purity on a Bioanalyzer (high sensitivity DNA assay). A peak should appear between 300-400 base pairs.
67. If you see primer contamination in your analyzer FU/bp plot you can try to purify the library by using Ampure XP beads or Illumina Free Adapter Blocking Agent according to the manufacturer's instruction.

4. Notes

1. Strategic project planning should be the first step in starting a methylome sequencing experiment (Table 1). This includes correlating a hypothesis or question to the methylome data set that originates from the comparison of at least two sample groups. Since epigenetic changes reflect cellular development, it might be useful to implement sample groups with related, well-defined populations. An analysis containing different $CD4^+$ memory T cells or subpopulations of dendritic cells (DCs) might be easier and more meaningful to interpret than the comparison of $CD4^+$ T helper 1 cells and plasmacytoid DCs. A sample group should consist of at least three biological replicates to ensure proper statistical calculations.
2. One caveat for mammals is the involvement of the X and Y chromosome: If the studies should cover all chromosomes, only material from male donors should be used. The inactivation of one X chromosome in female mammals increases the methylation signal and impedes detection of all differentially methylated regions.
3. It is necessary to perform some test experiments before using precious material (Table 2). This protocol requires 50 ng genomic DNA (gDNA) as starting material. We strongly recommend performing a test experiment without sequencing to make sure that the workflow produces a proper sequencing library. During this test phase, one can use non-valuable gDNA in different concentrations (e.g. 5, 10, 25, 50 ng). The amount of gDNA prepared from

a given number of cells can vary in different laboratories depending on the gDNA isolation protocol chosen. Therefore, we recommend establishing the preparation from at least 25.000 non-fixed or 40.000 fixed cells. After the first preparations, one can adjust the required cell number to reach at least 50 ng gDNA.

4. Direct sorting into 1.5 ml safe-lock reaction tubes is recommended.

5. If you have more than one tube from the same sample and if you do not exceed $5x10^6$ cells, you can reload the column after the next step (just empty the collection tube and reuse it).

6. Use prepared 20-200 µl Tips; cut off at least 5 mm from the tip to increase the inlet.

7. Please note that gDNA from non-fixed cells is double stranded, whereas the protocol for fixed cells produce single stranded gDNA.

8. Another critical step is the fragmentation of the gDNA, if it is not possible to use a Covaris S220 device. Other devices should be tested to produce a fragment peak between 200 and 500 nucleotides (Figure 1).

9. It is recommended to include a purification step with Ampure XP beads before analyzing the fragments with a Bioanalyzer. If the fragment size is correct, one can continue and produce a sequencing library.

10. The amplification step is adjusted to 50 ng input gDNA. If one plans to use more or less gDNA one might have to adjust the number of PCR cycles. Finally, the library should produce a peak between 300-400 base pairs (Figure 2).

References

1. Hotchkiss RD (1948) The quantitative separation of purines, pyrimidines, and nucleosides by paper chromatography. J Biol Chem 175:315-332
2. Holliday R, Pugh JE (1975) DNA modification mechanisms and gene activity during development. Science 187:226-232
3. Riggs AD (1975) X inactivation, differentiation, and DNA methylation. Cytogenet Cell Genet 14:9-25
4. Ben-Hattar J, Jiricny J (1988) Methylation of single CpG dinucleotides within a promoter element of the Herpes simplex virus tk gene reduces its transcription in vivo. Gene 65:219-227
5. Iguchi-Ariga SM, Schaffner W (1989) CpG methylation of the cAMP-responsive enhancer/promoter sequence TGACGTCA abolishes specific factor binding as well as transcriptional activation. Genes Dev 3:612-619
6. Greenberg MVC, Bourc'his D (2019) The diverse roles of DNA methylation in mammalian development and disease. Nat Rev Mol Cell Biol 20:590-607
7. Millar DS, Holliday R, Grigg GW (2003) Five not four: History and Significance of the Fifth Base. In: Beck S, Olek A (eds) The Epigenome. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany, p 3-20
8. Hayatsu H, Wataya Y, Kai K et al. (1970) Reaction of sodium bisulfite with uracil, cytosine, and their derivatives. Biochemistry 9:2858-2865
9. Frommer M, Mcdonald LE, Millar DS et al. (1992) A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. Proc Natl Acad Sci U S A 89:1827-1831
10. Colella S, Shen L, Baggerly KA et al. (2003) Sensitive and quantitative universal Pyrosequencing methylation analysis of CpG sites. Biotechniques 35:146-150
11. Uhlmann K, Brinckmann A, Toliat MR et al. (2002) Evaluation of a potential epigenetic biomarker by quantitative methyl-single nucleotide polymorphism analysis. Electrophoresis 23:4072-4079
12. Lewin J, Schmitt AO, Adorjan P et al. (2004) Quantitative DNA methylation analysis based on four-dye trace data from direct sequencing of PCR amplificates. Bioinformatics 20:3005-3012
13. Huang TH-M, Plass C, Liang G et al. (2003) Epi Meets Genomics: Technologies for Finding and Reading the 5th Base. In: Beck S, Olek A (eds) The Epigenome. Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany, p 41-64

Figures

Strategic project planing
Develop hypothesis or question that can be linked to the methylome data set.
Define at least two sample groups (minimal scenario).
Well-defined cell populations facilitate a deeper analysis.
Generate triplicates within a sample group to enable meaningful data set statistic.

Table 1: Strategic planning before starting the experimental part.

Essential requirements for the protocol
Sufficient amount of input gDNA.
➤ Establish gDNA preparation from purified cells.
Correct size of the gDNA fragments.
➤ Test several sonication conditions.
Sufficient amount of library fragments.
➤ Test and/or optimize library amplification step.

Table 2: Requirements for the protocol.

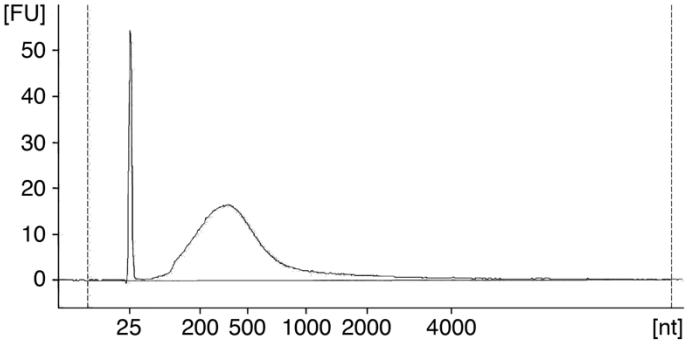

Figure 1: gDNA fragmentation after bisulfite conversion. gDNA was analyzed on an Agilent Bioanalyzer 2100 by using the high sensitivity RNA kit. Fluorescence units were plotted against fragment size (nucleotides).

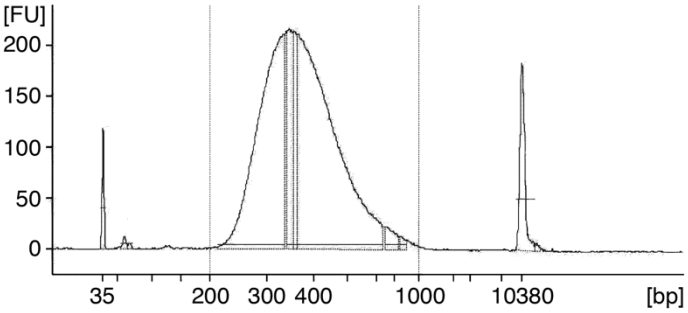

Figure 2: Analysis of a prepared sequencing library. Bead-purified library was analyzed on an Agilent Bioanalyzer 2100 by using the high sensitivity DNA kit. Fluorescence units were plotted against fragment size (base pairs (bp)).