

Myers Lab RRBS Protocol

Reduced Representation Bisulfite Sequencing

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Last edited by: Dr. Flo Pauli, Dr. K-T Varley, Dr. Jason Gertz, Stephanie Parker, Dr. Richard Myers

Contact information:

Flo Pauli, Ph.D. or K-T Varley, Ph.D.

Myers Lab

HudsonAlpha Institute for Biotechnology

601 Genome Way

Huntsville, AL 35806

Telephone: 256-327-5220

Email: k-tvarley@hudsonalpha.org

Introductory information

This protocol is the one currently in use by the Myers Lab at the HudsonAlpha Institute for Biotechnology. Reduced representation bisulfite sequencing (RRBS) is a protocol for determining the methylation status of hundreds of thousands of CpG dinucleotides in an MspI-digested genome. Genomic DNA is first digested with MspI, which cuts at every CCGG site regardless of methylation status. The fragments are end repaired and an adenosine is added to 3' ends. Next, a methylated version of the illumina paired-end adapters is ligated onto the genomic DNA. Fragments of adapter-ligated DNA ranging from 105 to 185 basepairs are purified using gel electrophoresis and subsequently treated with sodium bisulfite, which converts unmethylated cytosines to uracils and leaves methylated cytosines unchanged. Bisulfite treated DNA is then amplified in a final PCR reaction (converting uracils to thymidines). The sample is then ready to be sequenced on the Illumina Genome Analyzer.

Reagents

Qiagen Dneasy Blood and Tissue Kit (Qiagen 69581)

ilAdap Methyl PE1 (IDT: ACACTCTTTCCCTACACGACGCTCTTCCGATC*T; all C's are methylated, *=phosphorothioate bond)

ilAdap Methyl PE2 (IDT: GATCGGAAGAGCGGTTCAGCAGGAATGCCGA*G; all C's are methylated, 5' phosphate, *=phosphorothioate bond)

MspI includes NEB Buffer 4 (NEB R0106L)

Klenow fragment (3' to 5' -exo) (NEB M0212L)

Qiagen MinElute Kit (Qiagen 28004)

T4 DNA ligase (400 U/μl) and buffer (NEB M0202L)

Lonza NuSieve GTG agarose (Fisher BMA50080)

Low MW DNA ladder (NEB N3233L)

Qiaquick Gel Extraction Kit (Qiagen 28704)

EZ DNA Methylation Gold Kit (ZymoResearch D5005)

Platinum Taq DNA Polymerase 5U/ μ l and buffer (Invitrogen 10966-026)

iPCR PE1 (IDT: AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC
GAC GCT CTT CCG ATC* T; *=phosphorothioate bond)

iPCR PE2 (IDT: CAA GCA GAA GAC GGC ATA CGA GAT CGG TCT CGG CAT TCC
TGC TGA ACC GCT CTT CCG ATC* T; *=phosphorothioate bond)

25 mM each dNTP mix (NEB N0447L)

5 M Betaine (Sigma B0300)

Lonza Metaphor Agarose (Fisher BMA50180)

I. Isolation of Genomic DNA

1. Prepare genomic DNA from cells or tissue by using Qiagen Dneasy Blood and Tissue Kit. Follow the manufacturer's specifications for the cell or tissue type used.
2. Dilute genomic DNA to 1 μ g in 16 μ l in dH₂O. The volume can be up to 44 μ l as long as it is not in EDTA buffer.

II. Illumina Sequencing Library Construction

1. Prepare pre-anneal methylated Illumina paired-end sequencing adapter oligonucleotides.
 - a. Set up the following reaction in a PCR tube:

5 μ l	10X T4 DNA ligase buffer
20 μ l	100 μ M ilAdap Methyl PE1
20 μ l	100 μ M ilAdap Methyl PE2
5 μ l	dH ₂ O
 - b. Incubate in a thermal cycler with the following program:

95°C	5 min.
70°C	1 min.
60°C	1 min.
50°C	1 min.
40°C	1 min.
30°C	1 min.
25°C	∞

- c. Store the annealed adapters at -30°C
2. Digest genomic DNA with Msp1
 - a. Incubate the following reaction at 37°C for 30 minutes:
 - 16 µl genomic DNA (1 µg)
 - 5 µl 10X NEB Buffer 2
 - 1 µl Msp1 (20U/ml - NEB R0106S)
 - 28 µl dH₂O
 - b. Heat the reaction to 80°C for 20 minutes to inactivate the Msp1.
3. Fill-in and create 3' A overhangs with Klenow fragment (3' to 5' exo-)
 - a. Incubate the following reaction at 37°C for 30 minutes:
 - 50 µl Msp1-digested genomic DNA from previous step
 - 1.8 µl dNTP Mix 1 mM each
 - 1 µl Klenow Fragment (3' to 5' exo-), 5U/µl
 - 2.2 µl dH₂O
 - b. Heat the reaction to 75°C for 20 minutes to inactivate enzyme.
 - c. Purify the DNA with a Qiagen MinElute Kit. Elute for 1 minute with 10 µl EB pre-warmed to 55°C.
4. Ligate methylated Illumina PE adapters to genomic DNA
 - a. Incubate the following reaction at 20°C for 15 min.
 - 10 µl purified genomic DNA sample
 - 2 µl 10X T4 DNA Ligase Buffer
 - 1 µl T4 DNA Ligase, 400U/µl
 - 1 µl 40 µM methyl adapt mix (from step 1)
 - 6 µl dH₂O
 - b. Heat to 65°C for 10 minutes to deactivate ligase.
5. Sequencing fragment size selection
 - a. Prepare a 2.5% NuSieve GTG agarose gel (Lonza) with EtBr (150 ml TAE + 7.5 µl Ethidium Bromide (10 mg/ml) + 3.75 g agarose).
 - b. Load samples skipping a lane in between each one as follows:
 - DNA ladder - 1 µl Low MW ladder + 4 µl dH₂O + 1 µl 6X Orange Loading Dye
 - Samples - 20 µl ligation reaction + 4 µl 6X Orange Loading Dye
 - c. Run gel at 105V for 1.5 hours.
 - d. Image gel before and after extraction.
 - e. For each sample, cut out the section of the gel corresponding to 105-185 bp fragments.
 - f. Purify DNA from the gel using Qiaquick Gel Extraction Kit. Elute DNA for 1 min. with 20 µl EB pre-warmed to 55°C.
6. Sodium Bisulfite Treatment

The sodium bisulfite treatment is performed with the ZymoResearch EZ DNA Methylation Gold Kit. Follow the manufacturer's instructions and elute with 10 μ l M-Elution buffer.

7. PCR Amplification and Size Selection of Illumina Sequencing Library

a. Amplify each sample with the following PCR reaction:

10 μ l Bisulfite-treated sample
1 μ l Platinum Taq DNA Polymerase 5 U/ μ l
5 μ l 10X PCR Buffer with no MgCl₂
2 μ l 50 mM MgCl₂
0.25 μ l 100 μ M iPCR PE1
0.25 μ l 100 μ M iPCR PE2
1 μ l 25 mM each dNTP mix
5 μ l 5 M Betaine
25.5 μ l dH₂O

b. PCR amplification cycle:

98°C - 1 min.
95°C - 30 sec.
62°C - 3 min.
19 more cycles of steps 2 and 3
4°C - ∞

8. Confirmation of correct size amplification

a. Prepare a 3% Metaphor Agarose Gel (50 mL 1X TAE + 1.5 g agarose + 2.5 μ l 10 mg/ml Ethidium Bromide) or use Invitrogen E-gel 2% with SYBR Safe.

b. Load samples as follows:

DNA ladder: 1 μ l Low MW ladder + 4 μ l dH₂O + 1 μ l 6X Orange Loading Dye (1 μ l Low MW ladder + 10 μ l dH₂O for E-gel)

Samples: 20 μ l PCR reaction + 4 μ l 6X Orange Loading Dye (10 μ l PCR reaction for E-gel)

c. Run gel at 105V for 1.5 hours (E-gel runs approximately 15 minutes)

d. Image the gel and check that the correct sized fragments were amplified (175 - 275 bp).

e. Cleanup the remaining PCR product with a QIAquick Spin Column and elute in 30 μ l of warmed (55°C) EB.

f. Quantify the DNA using Qubit HS kit. Sample is ready for Illumina sequencing.