Standard operating procedure for RRBS library preparation from bovine embryo genomic DNA

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RRBS library preparation

RRBS (Reduced Representation Bisulfite Sequencing) combines restriction enzyme digestion with bisulfite sequencing to enrich for a CpG-dense fraction of the genome and profile DNA methylation at single-nucleotide resolution. DNA methylation occurs predominantly in CpG contexts, and these CpG dinucleotides are more abundant in select regions of the genome. By enriching for CpG-dense regions and sequencing only the fragments pertaining to those regions, the RRBS platform allows for the capture of a significant amount of methylation data while reducing the amount of sequencing, leading to a substantially decreased cost.

Samples

- DNA isolated from bovine embryo were used for RRBS library preparation.

Equipment

- NextSeq 2000 Sequencing System plattform (Illumina).
- Biometra Thermocycler, T1- (Analytik Jena)
- Benchtop microcentrifuge (Hettich, Centrifuge Mikro 120)
- Agilent 2100 Bioanalyzer (Agilent Technologies)
- Qubit fluorometer (Invitrogen, Thermo Fisher Scientific)

Plastics

- RNase/DNase-free tubes

Kits & Chemicals

- Zymo-Seq RRBS Library Kit D5460/D5461
- NextSeq 2000 P3 Reagents (200 Cycles) (Illumina Cat # 20040560)
- Quant-it Qubit dsDNA HS Assay kit (Invitrogen, Cat # Q32854Thermo Fisher Scientific)
- Agilent High Sensitivity DNA Kit and reagents (Agilent Technologies, Cat # 5067-4626 and Cat # 5067-4627)
- Roti Nucleic acid free solution for removal of nucleic acid contamination from surfaces (Roth, Cat # HP69.1)

Preparation

- Add 24 mL 100 % ethanol (or 26 mL 95 % ethanol) to the 6 mL DNA Wash Buffer (concentrate) (D4003-2-6)
- Add 24 mL 100 % ethanol (or 26 mL 95 % ethanol) to the 6 mL M-Wash Buffer (concentrate) (D5001-4).

- Components that are stored at -20 °C or -80 °C should be thawed and kept on ice when they are needed
  Centrifuge briefly to collect contents that are potentially caught at the tube lids or the inner sidewalls.
- Avoid multiple freeze-thaws of the Zymo-Seq™ UDI Primer Sets. Make aliquots as necessary.
- Ensure the thermal cycler's lid temperature is set to 100-105 °C for each program.
- For Section 1, Step 1 and Section 2, Step 3 and 5, a master mix of the reagents is recommended when processing multiple samples in parallel.

- When mixing the components, pipet up and down or flick the tube to ensure sufficient mixing. Centrifuge briefly to collect the contents.
- All centrifugation steps should be performed at ≥ 10,000 x g.

Section 1: MspI Digestion

Preparation

- Thaw 10x RRBS Buffer and Mspl (20U/µl) from -20 °C on room temperature and store on ice.

Procedure

1. Mix the following components in a 0.2 ml PCR tube for each sample:
    (1) Genomic DNA in TE buffer/ H2O X µl (X = whole preparated DNA)
    (2) 10 x RRBS Buffer 4 µl
    (3) Mspl (20 U/µl) 0.5 µl
    (4) Fill up with DNase/RNase-free water until 40 µl in total.
2. Incubate the tube in a thermal cycler according to the following program:
    - Step 1: 4 h at 37 °C.
    - Step 2: Hold at 4 °C.

Section 2: Adapter Ligation

Preparation

- Thaw rATP (10 mM), RRBS Adapters, T4 DNA Ligase (400 U/µl) and 5-methylcytosine dNTP Mix (10 mM) from -20 °C and store on ice.
- When used, the polymerase is placed on ice and put it back to -20 °C after use.

Procedure

1. Add the following components to the tube from Section 1 after incubation:
    (1) 10x RRBS Buffer 1 µl
    (2) rATP (10 mM) 0.5 µl
    (3) RRBS Adapters (10 µM) 0.5 µl
    (4) Mspl (20 U/µl) 1 µl
    (5) T4 DNA Ligase (400 U/µl) 1 µl
    (6) DNase/RNase-free Water 6 µl
    (7) Product from Section 1
    - In total 50 µl
2. Mix well by pipetting and incubate the tube in a thermal cycler according to the following program:
    - Step 1: 3 h at 21 °C.
    - Step 2: 1 h at 37 °C.
    - Step 3: 1 h at 20 °C.
    - Step 4: Repaet steps 2-3.
    - Step 5: Hold at 4 °C.
3. Add the following components to the tube:
    (1) Taq DNA Polymerase (2 U/µl) 0.5 µl
    (2) 5-methylcytosine dNTP Mix (10 mM) 1.5 µl
    (3) Product (50 µL) from step 1.
    - In total 52 µl.

4. Incubate the tube in a thermal cycler at 74 °C for 30 min.
5. Transfer the product to another 1.5 ml tube.
6. Add 364 µl DNA Binding Buffer to the product.
7. Mix well by pipetting and transfer the mixture to a Zymo-Spin IC Column in a Collection Tube.
8. Centrifuge for 30 sec at 10.000 x g.
9. Add 200 µl DNA Wash Buffer to the column and centrifuge for 30 sec at 10.000 x g.
10. Discard the flow-through and repaet step 9.
11. Transfer the Zymo-Spin IC Column to a clean 1.5 ml tube.
12. Add 11 µl DNA Elution Buffer directly to the column matrix.
13. Incubate for 1 min at room temperature.
14. Centrifuge for 30 sec at 10.000 x g to elute.
15. Repeat the steps 12 to 14.

Section 3: Bisulfite Conversion

Procedure

1. Mix the following components in a new 0.2 ml PCR tube:
   (1) Lightning Conversion Reagent 130 µl
   (2) Product Section 2 20 µl
   - In total 152 µl.
2. Split the sample in two new different 0.2 ml tubes (2 x 75 µl).
3. Incubate the tubes in a thermal cycler according to the following program:
   - Step 1: 8 min at 98 °C
   - Step 2: 1 h at 54 °C.
   - Step 3: Hold at 4°C (no more than 20 h!)
4. Pipette the tubes together again.
5. Add 600 µl of M-Binding Buffer to a Zymo-Spin IC Column in a Collection Tube.
6. Add the sample to the column, close the cap and invert 8 times to mix.
7. Centrifuge for 30 sec at 10.000 x g.

NOTE!: Thaw LibraryAmp Master Mix (-20 °C) and UDI Primer Set (-80 °C) and store on ice!
(Preparation for Section 4: Index Primer Amplification)

8. Discard the flow-through from the Collection Tube and add 100 µl of M-Wash Buffer to the column.
9. Centrifuge for 30 sec at 10.000 x g.
10. Add 200 µl of L-Desulphonation Buffer to the column and incubate for 20 min at room temperature.
11. Centrifuge for 30 sec at 10.000 x g.
12. Add 200 µl of M-Wash Buffer to the column and centrifuge for 30 sec at 10.000 x g.
13. Discard the flow-through.
14. Repeat the wash steps 12-13.
15. Transfer the Zymo-Spin IC Column to a new 1.5 ml tube.
16. Add 22 µl DNA Elution Buffer directly to the column matrix and incubate for 1 min at room temperature.
17. Centrifuge for 30 sec at 10.000 x g.

Section 4: Index Primer Amplification

Preparation

- Preheat the thermal cycler
- Thaw the components for this section, follow the advice from Section 3 after step 7!

Procedure

1. Mix the following components in a new 0,2 ml PCR tube:
   (1) LibraryAmp Master Mix (2x) 25 µl
   (2) UDI Primer Set (2,5 µM) 4 µl
   (3) Product from Section 3 21 µl
   - In total 50 µl
2. Incubate the tube in the thermal cycler according to the following program:
   - Step 1: 30 sec at 94 °C.
   - Step 2: 30 sec at 94 °C.
   - Step 3: 30 sec at 55 °C.
   - Step 4: 1 min at 68 °C.
   - Step 5: Go to step 2.

   } 15 cycles
   - Step 6: 5 min at 68°C.
   - Step 7: Hold at 4°C.
3. Transfer the product to a new 1.5 ml tube and add 350 µl of DNA Binding Buffer.
4. Mix well with the pipette and transfer the mixture to a Zymo-Spin IC Column in a Collection Tube.
5. Centrifuge for 30 sec at 10.000 x g.
6. Add 200 µl DNA Wash Buffer to the column and centrifuge for 30 sec at 10.000 x g.
7. Discard the flow-through.
8. Repeat the wash steps 6-7.
9. Centrifuge the column in a Collection Tube for 30 sec at 10.000 x g for an additional drying step.
10. Transfer the Zymo-Spin IC Column to a clean 1.5 ml tube and add 16 µl DNA Elution Buffer directly to the column matrix.
11. Incubate for 1 min at room temperature.
12. Centrifuge for 30 sec at 10.000 x g.

Take 3 µl for Qubit and Bioanalyzer!
The eluate is your final RRBS library. Store at 4 °C overnight or at -20 °C for long-term storage.

Sequencing of the libraries

For sequencing, either the NextSeq2000 System (Illumina), NextSeq 2000 P3 Reagents (200 Cycles) with following the manufacturer's protocols was used.

Library dilution for NextSeq 2000

- EB Buffer, 10 mM Tris-HCl, pH8.5 (Qiagen, Cat # 9086) with 0.1% Tween 20 for dilution
- Library Dilution Calculator

1. Use the Library Dilution Calculator to convert the library from ng/µl into nM.
2. Insert the library concentration in the calculator and define the base pair length. Calculate the molar concentration (nM) for the library.

3. Dilute the library with EB buffer contains 0,1% Tween to 10 nM.
4. Afterwards dilute from 10 nM to 2 nM with the same buffer.
5. Continue with pooling.