# RRBS library preparation

## Establishing libraries for reduced representation bisulfite sequencing from bovine tissues

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This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 815668. The content of this report reflects only the author's view. The European Commission is not responsible for any use that may be made of the information it contains.

**RRBS library preparation using TruSeq Nano DNA prep kit**

**Last update: 26-Feb-2020 (Nares Trakooljul)**

## Restriction Enzyme digestion

Consumables:

- QIAGEN DNeasy Kit
- DNA Sample (1-2 µg)
- Unmethylated cl857 Sam7 Lambda DNA (Promega, catalog # D1521)
- MspI (NEB, cat.no.R0106M)
- TaqI-v2 (NEB, cat.no.R0149S)
- MinElute Reaction Cleanup Kit (Qiagen, cat.no.28204)

1) Prepare an enzyme digestion reaction:

| **Reagent** | **Volume (µl)** |
|---|---|
| DNase free Water | fill up to 50 µl |
| CutSmart Buffer | 5 |
| 1-2 µg DNA | X |
| 0.5% Lambda DNA spike-in control (10 ng/µl) | 0.5-1 |
| MspI (100U/µl) | 0.5 |
| Total Volume | 50 |

3) Mix gently, but thoroughly and centrifuge briefly.

4) Incubate at 37°C for overnight (16 hours).

**Note:** **1)** High purity genomic DNA can be obtained using kits such as a QIAGEN DNeasy kit or equivalent.

**2)** In general, use MspI 20U/1µg DNA for an overnight digestion, use 50U/1µg DNA for a fast digestion.

**3) For double digestion (MspI and TaqI-v2/ TaqαI), perform MspI digestion for 7 hours and then add 20U of TaqI-v2 and further incubate at 65°C, overnight (16hours).**

## End Repair

Consumables:

- TruSeq Nano DNA library prep kit (LT)
- ERP2 (End Repair Mix) stored at -20°C, thaw at RT and then place on ice
- MinElute Reaction Cleanup Kit (Qiagen, cat.no.28204) or equivalent

Set the **ERP** program on the thermal cycler:

- Choose the preheat lid option and set to 100°C
- 30°C for 30 minutes
- Hold at 4°C

1) Add 10µl water to the 50µl enzyme digestion reaction.

2) Prepare the reaction mix on ice. Mix ERP gently and centrifuge briefly.

3) Add 40 µl ERP2 to 60 µl digested DNA fragments and pipette up and down.

4) Place on the thermal cycler and run the ERP program. Each reaction contains 100 µl.

5) Purify the reaction using a MinElute Reaction Cleanup Kit and elute with 21 µl of EB.

**Adenylate 3'-Ends**

Consumables:

•ATL (A-Tailing Mix)

Set the **ATAIL70** program on the thermal cycler:

• Choose the preheat lid option and set to 100°C

• 37°C for 30 minutes

• 70°C for 5 minutes

• 4°C for 5 minutes

• Hold at 4°C

1) Prepare the reaction mix on ice.

2) Mix ALT gently and centrifuge briefly.

3) Add 12.5 µl ALT to 17.5 µl blunt-ended DNA fragments and pipette up and down.

4) Place on the thermal cycler and run the ATAIL70 program. Each reaction contains 30 µl.

**Ligate Adapters**

Consumables:

•TruSeq Nano DNA library prep kit (LT)

•DNA Adapters (only LT but not HL kit contains methylated-Cs adapters)

•RBS (Resuspension Buffer)

•LIG (Ligation Mix 2) *thaw shortly before use and return immediately after use

•STL (Stop Ligation Buffer), thaw at RT and keep on ice

•MinElute Reaction Cleanup Kit (Qiagen, cat.no.28204)

Set the **LIG** program on the thermal cycler:

• Choose the preheat lid option and set to 100°C

• 30°Cfor 10 minutes

• Hold at 4°C

**1** Add the following reagents in the order on ice, and then mix thoroughly by pipette up and down.

| **Reagent** | **Volume (µl)** |
|---|---|
| DNA from A-Tailing reaction | 30 |
| RSB | 2.5 |
| LIG2 | 2.5 |
| DNA Adapter | 2.5 |
| Total Volume | 37.5 |

2) Centrifuge at 280 × g for 1 minute.

3) Place on the thermal cycler and run the LIG program.

4) Mix and centrifuge STL at 600 × g for 5 seconds.

5) Add 5 µl STL to each reaction, and then mix thoroughly by pipette up and down followed by centrifuge at 280 xg for 1 min. Total reaction vol. is now 42.5 µl.

**Clean up ligated fragments**

Consumables:

•SPB (Sample Purification Beads) from TruSeq Nano DNA library prep kit (LT)

•RSB (Resuspension Buffer) from TruSeq Nano DNA library prep kit (LT)

1) Vortex SPB until well-dispersed.

2) Add 42.5 µl SPB (1:1 ratio) to each well, and then mix thoroughly by pipette up and down10 times.

3) Incubate at room temperature for 5 minutes.

4) Place on a magnetic stand and wait until the liquid is clear (5 minutes).

5) Remove and discard all supernatant from each well. Immediately proceed washing step 6.1-6.4.

6) Bead Washing:

6.1 Add 180 µl freshly prepared 80% EtOH to each well.

6.2 Incubate on the magnetic stand for 30 seconds.

6.3 Remove and discard all supernatant from each well.

6.4 Repeat step 6.1-6.3

7) Use a 10µl pipette to remove residual EtOH from the bottom of each well.

8) Air-dry on the magnetic stand for 2-3 minutes. Do not let the beads over-dry more than 3 min.

9) Add 52.5 µl RSB to each well and pipette up and down.

10) Incubate at room temperature for 2 minutes.

11) Place on a magnetic stand and wait until the liquid is clear (5 minutes).

12) Transfer 50 µl supernatant to a new tube.

13) Add 50µl SPB (1:1 ratio) to each well, and then mix thoroughly by pipette up and down10 times.

14) Incubate at room temperature for 5 minutes.

15) Place on a magnetic stand and wait until the liquid is clear (5 minutes).

16) Remove and discard all supernatant from each well. Immediately proceed washing step 6.1-6.6.

17) Add 22.5 µl RSB to each well and pipette up and down.

18) Incubate at room temperature for 2 minutes.

19) Place on a magnetic stand and wait until the liquid is clear (5 minutes).

20) Transfer 20 µl supernatant (adapter-ligated DNA sample) to a new tube.

***SAFE STOPPINGPOINT If you are stopping, store at -25°C to -15°C for up to 7 days.**

**Bisulfite Conversion**

Follow the manufacturer's instructions in the EZ DNA Methylation-Gold Kit (Catalog no. D5005 and D5006, Zymo Research) for bisulfite conversion.

**Reagent Preparation**

• Preparation of CT Conversion Reagent

The **CT Conversion Reagent** supplied within this kit is a solid mixture and must be prepared prior to first use.

1. Add 900 µl water, 300 µl of **M-Dilution Buffer**, and 50 µl **M-Dissolving Buffer** to a tube of **CT Conversion Reagent**.

2. Mix at room temperature with frequent vortexing or shaking for 10 minutes.

**Note:** It is normal to see trace amounts of undissolved reagent in the CT Conversion Reagent. Each tube of CT Conversion Reagent is designed for 10 separate DNA treatments.

**Storage:** The CT Conversion Reagent is light sensitive, so minimize its exposure to light. For best results, the CT Conversion Reagent should be used immediately following preparation. If not used immediately, the CT Conversion Reagent solution can be stored overnight at room temperature, one week at 4°C, or up to one month at -20°C. Stored CT Conversion Reagent solution must be warmed to 37°C, then vortexed prior to use.

**Bisulfite Conversion Reaction**

1) Add 130 µl of the **CT Conversion Reagent** to 20 µl of adapter-ligated DNA sample in a PCR tube. Mix the sample by flicking the tube or pipetting the sample up and down, then aliquot the reaction into 2 PCR tube of 75µl each and centrifuge the liquid to the bottom of the tube (Note: In general, the thermocycle block can accommodate only 100µl-reaction).

2) Place the sample tube in a thermal cycler and perform the following steps*:

1. 98°C for 10 minutes
2. 64°C for 2.5 hours
3. 4°C storage up to 20 hours. (Note: continue proceed step 3 may result a better library yield)

3) Add 600 µl of **M-Binding Buffer** to a **Zymo-Spin™ IC Column** and place the column into a provided Collection Tube.

4) Load the sample (from Step 2) into the **Zymo-Spin™ IC Column** containing the **M-Binding Buffer**. Close the cap and mix by inverting the column several times.

5) Centrifuge at full speed (>10,000 x g) for 30 seconds. Discard the flow-through.

6) Add 100 µl of **M-Wash Buffer** to the column. Centrifuge at full speed for 30 seconds.

7) Add 200 µl of **M-Desulphonation Buffer** to the column and let stand at room temperature (20-30°C) for 15-20 minutes. After the incubation, centrifuge at full speed for 30 seconds.

8) Add 200 µl of **M-Wash Buffer** to the column. Centrifuge at full speed for 30 seconds. Add another 200 µl of **M-Wash Buffer** and centrifuge for an additional 30 seconds.

9) Place the column into a 1.5 ml microcentrifuge tube. Add 10 µl of **M-Elution Buffer** directly to the column matrix. Centrifuge for 30 seconds at full speed to elute the DNA. Repeat elution with another 10 µl of **M-Elution Buffer**. Total elution volume is now 20µl.

**Enrich DNA Fragments**

Consumables:

- PfuTurbo Cx Hotstart DNA Polymerase Kit
- PCR Primer Cocktail (PPC) from TruSeq Nano DNA Sample Prep Kit

Note: Two independent PCR reactions are performed for each sample to maximize the library yield and diversity.

Set the **PCRNano** program on the thermal cycler:

- Choose the preheat lid option and set to 100°C
- 95°C for 3 minutes
- 15 cycles of:
- 98°C or 20 seconds
- 60°C for 15 seconds
- 72°C for 30 seconds
- 72°C for 5 minutes
- Hold at 4°C

1) Prepare the reaction mix on ice:

| **Reagent** | **Volume (µl)** |
|---|---|
| DNA | 20 |
| Ultra Pure Water | 17.75 |
| Pfu Turbo Cx Reaction Buffer | 5 |
| 10 mM dNTP Mix (10mM each) | 1.25 |
| PCR Primer Cocktail (PPC) | 5 |
| PfuTurbo Cx Hotstart DNA Polymerase | 1 |
| Total Volume | 50 |

2) Mix gently and centrifuge briefly.

3) Place on the thermal cycler and run the PCRNano program.

**Clean up amplified fragments**

Consumables:

- SPB (Sample Purification Beads) from TruSeq Nano DNA library prep kit (LT)
- RSB (Resuspension Buffer) from TruSeq Nano DNA library prep kit (LT)
- freshly prepared 80% EtOH

1) Vortex SPB until well-dispersed.

2) Add 50 µl SPB (1:1 ratio) to each well, and then mix thoroughly by pipette up and down10 times.

3) Incubate at room temperature for 5 minutes.

4) Place on a magnetic stand and wait until the liquid is clear (5 minutes).

5) Remove and discard all supernatant from each well. Immediately proceed washing step 6.1-6.4.

6) Bead washing:

6.1 Add 180 µl freshly prepared 80% EtOH to each well.

6.2 Incubate on the magnetic stand for 30 seconds.

6.3 Remove and discard all supernatant from each well.

6.4 Repeat step 6.1-6.3

7) Use a 10µl pipette to remove residual EtOH from each well.

8) Air-dry on the magnetic stand for 2-3 minutes. Do not let the beads over-dry the beads.

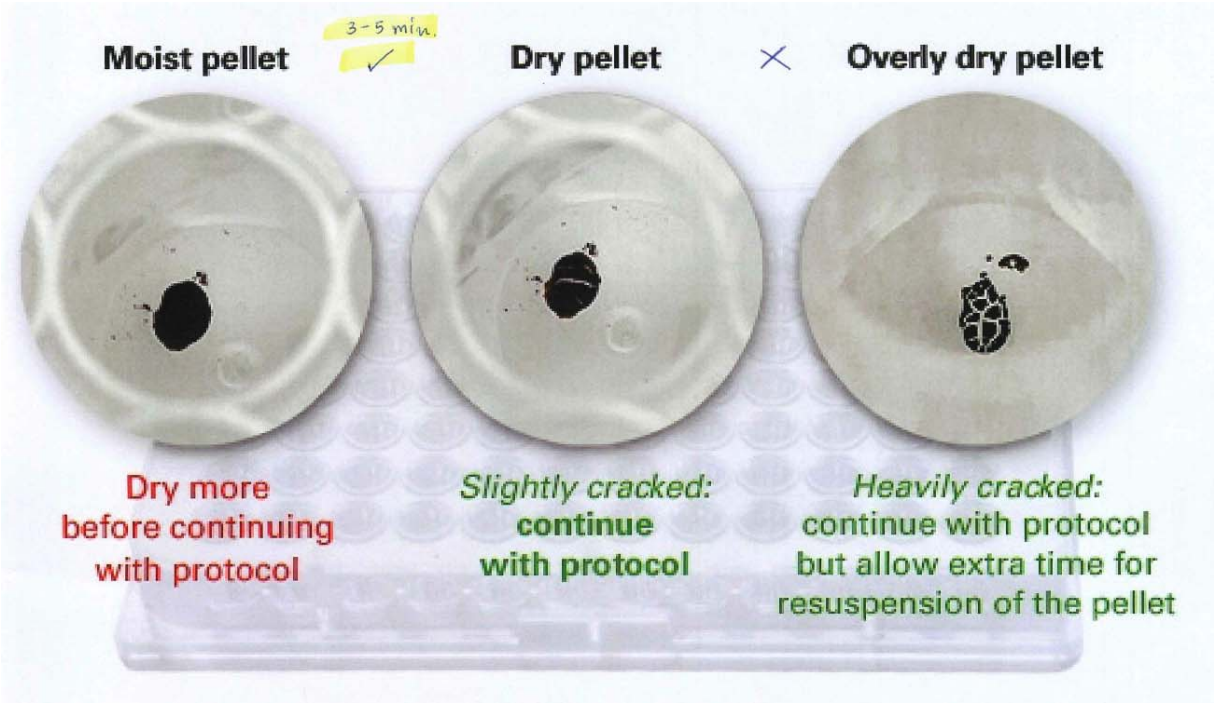
9) Add 22.5 µl RSB to each well and pipette up and down.

10) Incubate at room temperature for 2 minutes.

11) Place on a magnetic stand and wait until the liquid is clear (5 minutes).

12) Transfer 20 µl supernatant containing **RRBS library** to a new tube and mix well with 2 µl of 1% Tween20 buffer.

13) Aliquot 2.5 µl for QC (fragment size distribution) on an Agilent Technologies 2100 Bioanalyzer and concentration measurement by Qubit HS DNA Kit.



**Optional**: If necessary check the DNA ligation efficiency before bisulfite conversion by PCR

Prepare PCR mix on ice:

| Reagent | Volume (µl) |
|---|---|
| EPM (Enhanced PCR Mix) | 6.0 |
| PPC (PCR Primer Cocktail) | 1.0 |
| Water | 7.0 |
| Size-separated adapter-ligated DNA | 1.0 |
| Total Volume | 15 |

PCR program:
- 3 min at 98 °C
- 29 cycles of:
  - 20 seconds at 98 °C
  - 15 seconds at 60 °C
  - 30 seconds at 72 °C
- 5 min at 72 °C
- Hold at 4 °C

- Run PCR product on 1.5 % agarose gel. The amplified products should be within the range of size selection.

References

| Enzyme | Enzyme/ DNA | Reference |
|---|---|---|
| MspI (NEB) | 200U/ 5, 50, 1000ng | Akalin et.al. (2012) PLoS Genet 8(6) |
| MspI (NEB) | 100U/ 4 µg | Gao et al. BMC Genomics 2014 15:716 |
| MspI (NEB)<br>ApeKI (NEB) | 300U/100ng (7hrs, 37°C)<br>20U/100ng (double digestion)<br>overnight | Wang et al. BMC Genomics 2013, 14:11 |
| MspI | 20U/1µg (16 -20 hrs, 37°C) | Gu et al. Nat Methods 2010 7(2):133-136 |