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## OPEN ACCESS

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# Reduced Representation Bisulfite Sequencing (RRBS) with NEB Reagents

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#### **ABSTRACT**

This protocol is for generating Reduced Representation Bisulfite Sequencing (RRBS) libraries. We recommend using 200ng input, but the protocol has worked with inputs as low as 50ng.

We recommend using a pippin prep to remove small library fragments prior to sequencing.

We see the best results when we size select between 180bp-2000bp and sequence using single end (at least 50 base) reads on an Illumina NovaSeq.

#### **NEB 10bp dual index sequences**

#### **ATTACHMENTS**

SMack\_Lab\_neb\_index\_pri mers.xlsx **Keywords:** RRBS, methylation, next-generation sequencing, next-gen, DNA, library, NEB

#### **MATERIALS**

- SPRI Beads
- Primers
- Mspl 25,000 units New England Biolabs Catalog #R0106L
- rCutSmart Buffer New England Biolabs Catalog
  #B6004S
- Unmethylated phage DNA (Sigma: #D3654-5UN)
- EB buffer Qiagen EB Buffer Mat. No. 1014609
- NEBNext Ultra II End Prep Reaction Buffer **New England Biolabs Catalog** #E7647
- NEBNext Ultra II End Prep Enzyme Mix **New England Biolabs Catalog** #E7646
- Blunt/TA Ligase Master Mix New England Biolabs Catalog #E7373 in Kit E73 or E7445
- USER Enzyme 250 units New England Biolabs Catalog #M5505L
- EpiMark Hot Start Taq and Buffer
- ZymoEZ-96 DNA Methylation-Lightning<sup>™</sup> MagPrep

#### Suggested schedule:

#### Day 1

- Afternoon: Steps 2-3 (end repair, ligation, and bead clean up)
- Freeze overnight at 【 -20 °C

#### Day 2

- Morning: start Step 4 (bisulfite conversion), which has a incubation at the beginning
- Afternoon: continue Step 4 (bead clean up)
- Set up Step 5 (PCR) run hold overnight at 4 °C in ThermoCycler or fridge after protocol finishes

#### Day 3

• Step 6 (bead clean up and amplification)

#### Notes:

- Do **not** vortex enzymes or mixtures that contain enzymes.
- Handle bisulfite converted DNA with care. Do not vortex or freeze-thaw. The DNA
  is single stranded, and therefore very fragile.
- Label all plates throughout the protocol



1 Prepare fragment master mix (fragment MM) in a 1.5 mL tube for n+1 samples.

Per sample, prepare 4 µL mixture containing:

- (Thaw) RCutSmart Buffer New England Biolabs Catalog
  #B6004S: △ 3 µL
- (On ice) 
  Mspl 25,000 units New England Biolabs Catalog
  #R0106L

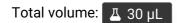
  L 1 µL

#### Invert to mix. DO NOT VORTEX.

1.1 Spin down samples before adding fragment MM. Add 4 µL of fragment MM to the template DNA.

#### Note

Template DNA = 200 ng template DNA + nuclease free  $H_2O$  for a total of  $\mathbb{Z}$  26  $\mu$ L



Cover and spin down samples before incubation.

1.2 Incubate samples at \$\mathbb{g}\$ 37 °C for \( \oldsymbol{O} \) 01:00:00



#### Note

Do not heat the lid higher than § 37 °C

SAFE STOPPING POINT: Leave digested DNA in ThermoCycler at 37 °C Overnight or freeze at -20 °C (cover with foil).

## Ligation

1h 35m

2 Prepare end repair master mix (end repair MM) in a 1.5 mL tube for n+1 samples.

Per sample, prepare 5 µL mixture containing:

- (On ice) NEBNext Ultra II End Prep Enzyme Mix New England Biolabs Catalog #E7646
- (Thaw) NEBNext Ultra II End Prep Reaction Buffer New England Biolabs Catalog #E7647
  - **Ξ** 3.5 μL

Invert to mix. DO NOT VORTEX.

2.1 Add A 5 µL of end repair MM to each well of fragmented DNA.

Total volume: A 35 µL

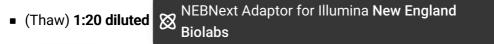
Cover and spin down samples before incubation.

- 2.2 Incubate samples for:
  - **③** 00:30:00 at **⑤** 20 °C
  - **(5)** 00:30:00 at **(5)** 65 °C
  - Hold at # 4 °C

1h

2.3 Prepare ligation master mix (ligation MM) in a 1.5 mL tube for n+1 samples.

Per sample, prepare 9.25 µL mixture containing:





Note

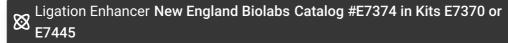
Dilute adapters 1:20 in nuclease-free water in a fresh tube

(On ice)



≟ 15 μL

(On ice)



 $\perp$  0.5  $\mu$ L

Invert to mix. DO NOT VORTEX.

2.4 Add  $\triangle$  16.75 µL of ligation MM to each sample.

Total volume: Δ 51.75 μL

Cover and spin down samples before incubation.

2.5 Incubate for (5) 00:20:00 at (1) 20 °C

20m

2.6 Add Δ 1.5 μL of WSER Enzyme - 250 units New England Biolabs Catalog #M5505L to

each sample and pipette mix.

Cover and spin down samples before incubation.

2.7 Incubate for (5) 00:15:00 at (5) 37 °C

#### Note

During incubation, take SPRI beads out of fridge to come to room temperature and prepare 50 ml of 80% ethanol.

## **Bead-Based Cleanup**

25m 30s

3

Add  $\underline{A}$  90  $\mu L$  of  $\underline{B}$  Room temperature SPRI beads to each sample and gently pipette mix ~5 times.

3.1 Incubate at Room temperature for 00:05:00

5m

3.2 Place plate on magnetic stand for 00:05:00 or until solution is clear.

5m

- **3.3** While on the magnetic stand, remove supernatant using a multichannel pipette.
- **3.4** While on the magnetic stand:

30s

- Add 🗸 200 µL of 80% ethanol (**do not mix**)
- Remove ethanol
- 3.5 Repeat wash from 3.4
- 3.6 Dry the beads for 00:05:00 or until beads are no longer shiny.

Note

Be careful to not over dry beads, as this will reduce yield.

- Remove plate from magnetic stand and add  $\perp$  22  $\mu$ L of EB buffer. Pipette mix and incubate at 8 Room temperature for  $\bigcirc$  00:05:00 .
- Place plate back on magnetic stand and incubate at Room temperature fo 00:05:00.
- **3.9** Transfer all of supernatant to into a new, sturdy PCR skirted plate.

SAFE STOPPING POINT: Freeze adaptor-ligated DNA at [ -20 °C (cover with foil).

### **Bisulfite Conversion**

4

Note

This section uses reagents from the Zymo EZ-96 DNA Methylation-Lightning MagPrep kit

Cover and spin down samples before incubation.

- **4.1** Incubate samples for:
  - 🚫 00:08:00 at 🖔 98 °C
  - (5) 01:00:00 at \$ 54 °C
  - Hold at **3** 4 °C (for up to ⑤ 20:00:00)
- 4.2 Add  $\triangle$  600  $\mu$ L of M-Binding Buffer and  $\triangle$  10  $\mu$ L of MagBinding Beads to each well of a 2 mL 96 deep well plate

21h 8m

- 4.3 Use multichannel to transfer samples to the 2 mL 96 deep well plate (containing M-Binding Buffer and MagBinding Beads) and pipette mix ~5 times.
- 4.4 Incubate at Room temperature for 00:05:00

5m

- 4.5 Transfer plate to a magnetic stand and incubate at Room temperature for 00:05:00 or until beads pellet and supernatant is cleared.
- **4.6** With the plate on the magnetic stand, remove the supernatant and discard.

#### Note

Some beads will adhere to the sides of the well. Remove supernatant slowly to allow these beads to be pulled to the magnet as the liquid level is lowered.

- 4.8 Place the plate on the magnetic stand for 00:03:00 or until beads pellet. Remove and discard supernatant.

3m

4.9 Remove the plate from the magnetic stand. Add Δ 200 μL of L-Desulphonation Buffer to the beads. Pipette mix 5 times.

4.10 Incubate at 8 Room temperature for 5 00:15:00

15m

While waiting, pre-heat a plate heating element to 55 °C. If using a ThermoMixer, put on 96-well attachment.

4.11 Place the plate on the magnetic stand for 00:03:00 or until beads pellet. Remove and discard supernatant.

3m

#### Note

\*\*Important: Take time for handling/re-suspension into account for the total incubation time. Adjust time as necessary to ensure that no sample remains in the L-Desulphonation Buffer for more than 20 minutes.\*\*

- 4.12 Remove plate from the magnetic stand. Add  $\underline{\mathbb{Z}}$  200  $\mu L$  of M-Wash Buffer to the beads. Pipette mix 5 times.
- 4.13 Place the plate on the magnetic stand for 00:03:00 or until beads pellet. Discard supernatant.

3m

**4.14** Repeat M-Wash Buffer wash (steps 4.12-4.13)

#### Note

\*\*Important: Remove as much buffer as possible after final wash to aid in the drying of the beads.\*\*

4.15 Transfer the plate to a heating element at 55 °C for 20-30 minutes to dry the beads and remove residual M-Wash Buffer.

20m

#### Note

Beads will change in appearance from glossy black when still wet to a dull brown when fully dry.

If using the ThermoMixer:

- Use the 96-well plate attachment
- Rest the deep well plate on top
- Check on beads frequently; they may take less than (5) 00:20:00 to dry
- 4.16 Add  $\underline{\mathbb{Z}}$  22  $\mu L$  of M-Elution Buffer directly to the dried beads and pipette mix 5-10 times to re-suspend.

4m

- 4.18 Transfer the plate to the magnetic stand and incubate at Room temperature for 00:01:00 or until beads pellet.
- **4.19** Transfer all supernatant into to a new unskirted PCR plate.

Note

It is okay if **some** beads are removed with the elution.

## **PCR Amplification (Indexing)**

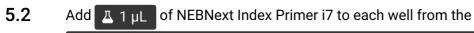
5 Prepare PCR master mix (PCR MM) in a 1.5 mL tube for n+1 samples.

Per sample, prepare 5.625 µL mixture containing:

- (Thaw) 5X EpiMark HS Taq Reaction Buffer Catalog #B0490S:
- (Thaw) 10 mM dNTP mix Catalog #N0447S: 🚨 0.5 µL

Invert to mix. DO NOT VORTEX.

5.1 Add  $\pm$  5.625  $\mu$ L of PCR MM to each sample.



NEBNext Index Primers New England Biolabs Catalog #E7335 or E7500 or E7710 or

5.3 Add I 1 µL of NEBNext Index Primer i5 to each well from the

> NEBNext Index Primers New England Biolabs Catalog #E7335 or E7500 or E7710 or E7730

and pipette mix.

Note

\*\*Important: Ensure all wells are unique combinations.\*\*

Cover and spin down samples.

5.4 Incubate samples for:

11m 45s 1. § 95 °C for 🕙 00:00:30

- 2. 16 cycles of:
- **₿** 95 °C **for ♦** 00:00:15
- § 61 °C for 💮 00:00:30
- \$ 68 °C for ♠ 00:00:30
- 3. \$\cdot 68 \cdot \text{for } \cdot 00:05:00
- 4. Hold at 🐉 4 °C (C) Overnight

## **Final Cleanup and Quantification**

25m 30s

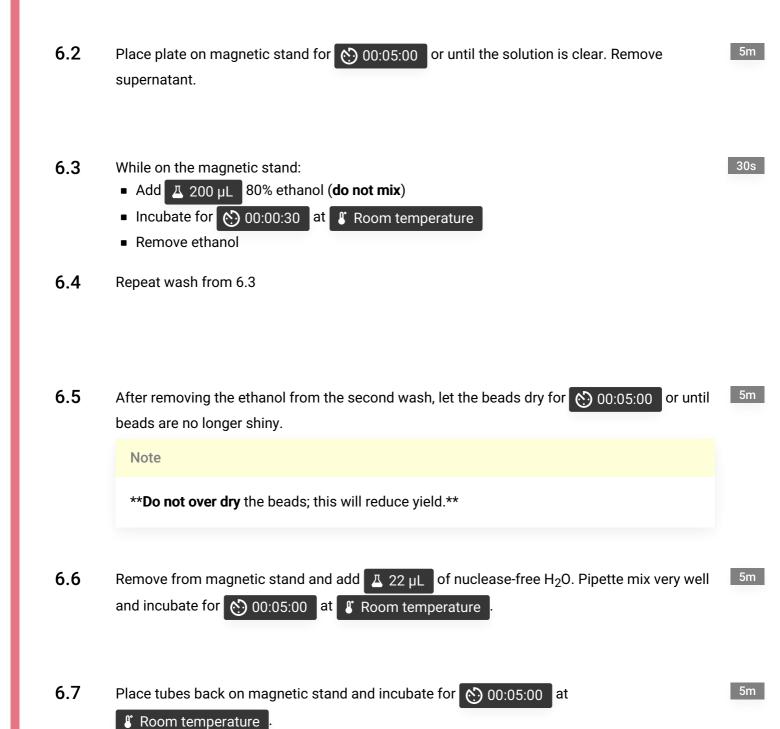
6

Note

Before you begin, take SPRI beads out of fridge to come to room temperature and prepare 50 ml of 80% ethanol.

Add A 50 uL of SPRI Beads to each sample. Gently pipette mix.

6.1 Incubate at | Room temperature | for ( 00:05:00



6.8 Transfer all supernatant to new, sturdy skirted PCR plate for long-term storage at \$\mathbb{E}\ -80 \cdot \mathbb{C}\$.

#### Note

If any beads transfer with the supernatant, place plate on magnetic stand when using samples in future protocols.

- **6.9** Quantify samples on instrument of choice.
- **6.10** Notes on Pooling:
  - Find sample with highest DNA conc. and calculate conc. for adding only Δ 1 μL to pool
  - Then, calculate the rest of the samples so that they all have the same concentration going into the pool
  - Run pool on pippen rep to remove anything under 190 bp
  - Now, it's ready for sequencing!