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Reduced Representation Bisulfite Sequencing (RRBS) with NEB Reagents

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We use this protocol and it's working

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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

XLSX

SMack_Lab_neb_index_...

19KB

Materials

- SPRI Beads
- **Primers**
- MspI - 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 E7370 or E7445
- USER Enzyme - 250 units **New England Biolabs Catalog #**M5505L
- EpiMark Hot Start Taq and Buffer
- ZymoEZ-96 DNA Methylation-Lightning™ MagPrep

Protocol materials

- Blunt/TA Ligase Master Mix **New England Biolabs Catalog #**E7373 in Kit E7370 or E7445
- USER Enzyme - 250 units **New England Biolabs Catalog #**M5505L
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- Ligation Enhancer **New England Biolabs Catalog #**E7374 in Kits E7370 or E7445
- NEBNext Adaptor for Illumina **New England Biolabs**
- USER Enzyme - 250 units **New England Biolabs Catalog #**M5505L
- NEBNext Index Primers **New England Biolabs Catalog #**E7335 or E7500 or E7710 or E7730
- NEBNext Index Primers **New England Biolabs Catalog #**E7335 or E7500 or E7710 or E7730

Troubleshooting

Before start

Suggested schedule:

Day 1

- Morning: Step 1 (fragmentation), which incubates for 01:00:00
- 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 01:15:00 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

Fragment DNA

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) MspI - 25,000 units **New England Biolabs Catalog #**R0106L : 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 26 µL

Total volume: 30 µL

Cover and spin down samples before incubation.

1.2 Incubate samples at 37 °C for 01:00:00 1h

> 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 : 1.5 µL
- (Thaw)
  NEBNext Ultra II End Prep Reaction Buffer **New England Biolabs Catalog #**E7647 : 3.5 µL

Invert to mix. **DO NOT VORTEX.**

2.1 Add 5 µL of end repair MM to each well of fragmented DNA.
Total volume: 35 µL

Cover and spin down samples before incubation.

2.2 Incubate samples for: 1h
- 00:30:00 at 20 °C
- 00:30:00 at 65 °C
- Hold at 4 °C

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:
- (Thaw) **1:20 diluted** NEBNext Adaptor for Illumina **New England Biolabs** : 1.25 µL

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

- (On ice)
  Blunt/TA Ligase Master Mix **New England Biolabs Catalog #**E7373 in Kit E7370 or E7445 : 15 µL
- (On ice)
  Ligation Enhancer **New England Biolabs Catalog #**E7374 in Kits E7370 or E7445 : 0.5 µL

Invert to mix. **DO NOT VORTEX.**

2.4 Add 16.75 µL of ligation MM to each sample.
Total volume: 51.75 µL

Cover and spin down samples before incubation.

2.5 Incubate for 00:20:00 at 20 °C 20m

2.6 Add 1.5 µL of
USER 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 00:15:00 at 37 °C 15m

> 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 90 µL of 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**)
- Incubate for 00:00:30
- 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. 5m

Note

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

3.7 Remove plate from magnetic stand and add 22 µL of EB buffer. Pipette mix and incubate at Room temperature for 00:05:00 . 5m

3.8 Place plate back on magnetic stand and incubate at Room temperature for 00:05:00 . 5m

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

Add 130 µL of Lightning Conversion Reagent to each 20 µL sample and pipette mix.
Total volume: ~ 150 µL

Cover and spin down samples before incubation.

4.1 Incubate samples for: 21h 8m
- 00:08:00 at 98 °C
- 01:00:00 at 54 °C
- Hold at 4 °C (for up to 20:00:00)

4.2 Add 600 µL of M-Binding Buffer and 10 µL of MagBinding Beads to each well of a 2 mL 96 deep well plate

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. 5m

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.

4.7 Remove the plate from the magnetic stand. Add 200 µL of M-Wash Buffer to the beads. Pipette mix 5 times.

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 200 µL of L-Desulphonation Buffer to the beads. Pipette mix 5 times.

4.10 Incubate at Room temperature for 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 200 µL 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 00:20:00 to dry

4.16 Add 22 µL of M-Elution Buffer directly to the dried beads and pipette mix 5-10 times to re-suspend.

4.17 Heat the elution at 55 °C for 00:04:00 4m

4.18 Transfer the plate to the magnetic stand and incubate at Room temperature for 00:01:00 or until beads pellet. 1m

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: 5 µL
- (Thaw) 10 mM dNTP mix Catalog #N0447S: 0.5 µL
- (Leave in freezer and add last) EpiMark Hot Start Taq (2 units/ul): 0.125 µL

Invert to mix. **DO NOT VORTEX.**

5.1 Add 5.625 µL of PCR MM to each sample.

Total volume: ~ 25.625 µL

5.2 Add 1 µL of NEBNext Index Primer i7 to each well from the

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

.

5.3 Add 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. 68 °C for 00:05:00
4. Hold at 4 °C Overnight

Final Cleanup and Quantification 25m 30s

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Note

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

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

6.1 Incubate at Room temperature for 00:05:00 . 5m

6.2 Place plate on magnetic stand for 00:05:00 or until the solution is clear. Remove supernatant. 5m

6.3 While on the magnetic stand: 30s

- Add 200 µL 80% ethanol (**do not mix**)
- Incubate for 00:00:30 at Room temperature
- Remove ethanol

6.4 Repeat wash from 6.3

6.5 After removing the ethanol from the second wash, let the beads dry for 00:05:00 or until beads are no longer shiny. 5m

Note

Do not over dry the beads; this will reduce yield.**

6.6 Remove from magnetic stand and add 22 µL of nuclease-free H_2O. Pipette mix very well and incubate for 00:05:00 at Room temperature . 5m

6.7 Place tubes back on magnetic stand and incubate for 00:05:00 at Room temperature . 5m

6.8 Transfer all supernatant to new, sturdy skirted PCR plate for long-term storage at -80 °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 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!