Reduced Representation Bisulfite Sequencing Libraries with EZ-96 DNA Methylation-Lightning™ MagPrep

SMack Lab (adapted from Amanda Lea)

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Reagents:

- AMPure XP Beads
- NEB Methylated Illumina Indexes Adaptors

***USE ONLY DILUTED adapters in subsequent protocol steps. Aliquot a small amount of adapters into a FRESH tube and dilute 1:20 such that the final concentration of diluted adapter is ~1 µM.**

****(MAKE SURE this is the NEB Methylated Illumina adapter, product number E7535S/L****

- Msp1 enzyme (NEB: #R0106)
- 1X NEB CutSmart buffer
- Unmethylated phage DNA (Sigma: #D3654-5UN)
- EB buffer
- NEBNext end prep enzyme + buffer
- NEBNext Blunt/TA Ligase Master Mix _ enhancer
- NEB User Enzyme
- EpiMark Hot Start Taq and buffer
- ZymoEZ-96 DNA Methylation-Lightning™ MagPrep

A few notes:

*Do **not** vortex enzymes or mixtures that contain enzymes

*Be very careful with bisulfite converted DNA. **Do not vortex it or freeze thaw it**. The DNA is single stranded, and therefore very fragile.

*Deep well plates will be needed for lightning conversion steps (included in kit)

Step 1: Fragment DNA

1. Prepare the following mix:

Mix	Storage Location	Volume per Sample	Volume for Mix
10x NEB Cut Smart Buffer		2.5 µL	
MspI Enzyme		1 µL	
0.2 ng/ul unmethylated phage DNA		0.5 µL	

1.1. ☐Add 4 µL of mix to the template DNA (template DNA = 300ng template DNA + nuclease free H2O for a total of 26 µL)

1.2. ☐Incubate the DNA at 37°C overnight with (do not heat the lid higher than 37°C if using the thermocycler)

***Safe Stopping Point: Freeze the digested DNA at -20°C**

Step 2

2. Prepare the following mix**:**

Mix	Storage Location	Volume per Sample	Volume for Mix
End Prep Enzyme Mix		1.5 µL	
End Repair Reaction Buffer (10x)		3.25 µL	

2.1. ☐Add 4.75 µL of mix to each well of the fragmented DNA (DNA volume is ~30 µL)

2.2. ☐Incubate for 30 minutes @ 20°C, 30 minutes @ 65°C and hold @ 4°C (Run "Neb endprep" program)

2.3. ☐Add the following mix to the reaction:

****Make sure that adaptors are diluted 1:20 prior to use in a fresh tube!****

Mix	Storage Location	Volume per Sample	Volume for Mix
NEBNext Methylated Adapter for Illumina (*1:20 diluted adapter*) **		1.25 µL	
Blunt/TA Ligase Master Mix (Ultra II)		7.5 µL	
Ligation Enhancer		0.5 µL	

2.4. ☐Incubate for 20 minutes @ 20°C (Run "NEB Adapter Ligate" program which will hold/pause for User Enzyme Incubation)

2.5. ☐Take AMPure beads out of fridge to come to room temperature

2.6. ☐Add 1.5 µL USER Enzyme to each sample, pipette to mix, and incubate for 15 minutes @ 37°C (Press enter on "NEB Adapter Ligate program" to end pause and continue the program)

Step 3:Bead-Based Cleanup (1.5x)

3.1. ☐Add 66 µL AMPure beads to each sample and gently mix by pipetting up and down

3.2. ☐Incubate at RT for 5min

3.3. ☐Place plate on magnetic stand until the solution is clear (5 mins)

3.4. ☐Remove supernatant **(KEEP BEADS)**

3.5. ☐While on the magnetic stand, add 200 µL 80% ethanol, incubate for 30 seconds remove ethanol

3.6. ☐Repeat wash: while on the magnetic stand, add 200 µL 80% ethanol, incubate for 30 seconds remove ethanol

3.7. ☐After removing the ethanol from the second wash, let the beads dry for ~5-10min or until beads are no longer shiny (**do not over dry** the beads, this will reduce yield)

3.8. ☐Remove from magnetic stand and add 21 µL of EB buffer, mix well and incubate 5 minutes @ RT

3.9. ☐Place tubes back on magnetic stand and incubate for 5 min @ RT

3.10.☐Transfer 20 µL of supernatant to new PCR strip tube or plate

**Qubit a few samples to check that some still have DNA before proceeding with bisulfite conversion

SAFE STOPPING POINT: Freeze the adapter-ligated DNA at -20°C

Step 4:Bisulfite Conversion (EZ-96 DNA Methylation-Lightning™ MagPrep)

4.1. ☐Add 130 µL of Lightning Conversion Reagent to 20 µL of a DNA sample in a Conversion Plate. Pipette mix 5 times.

4.2. ☐Seal the plate with film. Transfer the Conversion Plate to a thermal cycler and perform the following steps:

i. 98°C for 8 minutes

1. 54°C for 60 minutes
2. 4°C storage (for up to 20 hours)

4.3. ☐Pre-heat a plate heating element to 55°C.

4.4. ☐Add 600 µL of M-Binding Buffer and 10 µL of MagBinding Beads to each well of a Collection Plate.

4.5. ☐Transfer the samples from the Conversion Plate into the Collection Plate (containing the M-Binding Buffer and MagBinding Beads). Pipette mix 5 times.

4.6. ☐Let plate stand at room temperature for 5 minutes, then transfer plate to a magnetic stand for an additional 5 minutes or until beads pellet and supernatant is cleared.

4.7. ☐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. ☐Remove the Collection Plate from the magnetic stand.

4.9. ☐Add 200 µL of M-Wash Buffer to the beads. Pipette mix 5 times.

4.10.☐Place the plate on the magnetic stand for 3 minutes or until beads pellet.

4.11.☐Remove and discard supernatant.

4.12.☐Remove the Collection Plate from the magnetic stand.

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

4.14.☐Let plate stand at room temperature (20-30°C) for 15 minutes.

4.15.☐After the incubation, replace the plate on the magnetic stand for 3 minutes or until beads pellet. Remove and discard supernatant. **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**

4.16.☐☐Remove the Collection Plate from the magnetic stand.

4.17.☐☐Add 200 µL of M-Wash Buffer to the beads. Pipette mix 5 times.

4.18.☐☐Place the plate on the magnetic stand for 3 minutes or until beads pellet. Discard supernatant.

4.19.☐Repeat M-Wash Buffer wash once.

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

4.20.☐Transfer the plate to a heating element at 55°C for 20-30 minutes to dry the beads and remove residual M-Wash Buffer. Note: Beads will change in appearance from glossy black when still wet to a dull brown when fully dry.

4.21.☐Add 21 µL of M-Elution Buffer directly to the dried beads and pipette mix 5-10 times to re-suspend.

4.22.☐Heat the elution at 55°C for 4 minutes then transfer the plate to the magnetic stand for 1 minute or until beads pellet.

4.23.☐Remove the 20 µL of the supernatant and transfer to a clean Elution Plate.

Note: It is OK if **some** beads are removed with the elution.

The DNA is ready for immediate analysis or can be stored at or below -20°C for later use. For long term storage, store at or below -70°C.

***Quantification should be performed on NanoDrop using RNA settings (Factor 40) or other similar spectroscopy methods using RNA absorbance (If yields are high a second conversion round can be performed)*

SAFE STOPPING POINT: Freeze the digested DNA at -20C

Step 5: PCR Amplification (Indexing) ****BE SURE TO USE NEBNext INDEXES****

5.1. ☐Make PCR Master Mix as follows:

Mix	Storage Location	Volume per Sample	Volume for Mix
5X EpiMark Hot Start Taq Reaction Buffer		5 µL	
10mM dNTPs		0.5 µL	
EpiMark Hot Start Taq (2 units/ul)		0.125 µL	

5.2. ☐Add 5.625 µL of Master Mix to each well of the Adapter Ligated, BS-treated DNA (has approx. 20 µL of DNA).

5.3. ☐Add 0.5 µLof NEBNext Index Primer i7 to each well.

5.4. ☐Add 0.5 µLofNEBNext Index Primer i5 to each well. Ensure all wells are unique combinations.

5.5. ☐Run the following PCR Program:

1. 95°C for 30 seconds
2. 16 cycles of:
    1. 95°C for 15 seconds
    2. 61°C for 30 seconds
    3. 68°C for 30 seconds
3. 68°C for 5 mins
4. Hold at 4°C

Step 6: Final cleanup and quantification

6.1. ☐2:1 bead clean up

a. Add 50 µL AMPure Beads to each 25 µL reaction (gently pipetting to mix)

6.2. ☐Incubate @ RT for 5 mins

6.3. ☐Place plate on magnetic stand until the solution is clear (5 mins)

6.4. ☐Remove supernatant **(KEEP BEADS)**

6.5. ☐While on the magnetic stand, add 200 µL 80% ethanol, incubate for 30 seconds remove ethanol

6.6. ☐Repeat wash: While on the magnetic stand, add 200 µL 80% ethanol, incubate for 30 seconds remove ethanol

6.7. ☐After removing the ethanol from the second wash, let the beads dry for ~5-10 min or until beads are no longer shiny (**do not over dry** the beads, this will reduce yield)

6.8. ☐Remove from magnetic stand and add 22 µL of nuclease-free H2O, mix very well and incubate 5 minutes @ RT

6.9. ☐Place tubes back on magnetic stand and incubate for 5 min @ RT

6.10. ☐Transfer 21 µL of supernatant to new “candy cane” tube or **plate**

6.11. ☐Quantify and visualize libraries on Fragment Analyzer