NEB RRBS Protocol

(*Reduced representation bisulfite sequencing protocol*)

Note: If this is done for a targeted genomic region, you may need to consider an RNase treatment of DNA during extraction! The RRBS selects for 200-350bp fragments and RNA may be a competing molecule. This is not a concern for genome-wide RRBS.

Ratios are presented as (**DNA : Beads**)

Fast Msp digestion

Msp1 digestion	
Component	Volume
DNA	100 ng -1 ug
FastDigest® enzyme	5 ul
10X FastDigest® buffer	5 ul
Nuclease-free H_2O	Variable
V_{tot}	50 uL

1. Quantify to have 100 ng-1ug of DNA in a 50ul reaction.
2. Make sure to add water to reaction for a total of 50ul.
3. Prepare the mixture at room temperature (add enzyme last).
4. Gently pipette mix & spin down.
5. Incubate at 37° C on a Thermomixer for 20 minutes.
    a. There is no need to stop the reaction at 4° C; the enzyme will tucker out before the reaction needs to be stopped.
6. [OPTIONAL] Run 100ng of digested DNA on a 1% agarose gel to check that genomic DNA is a {light} smear.
    a. If you run a gel, then add that same amount of water back into the original tube to keep the final volume of 50ul... this is critical!
    b. Use the 1Kb ladder
7. If you do not run a gel, you can either freeze the 50ul digested DNA or continue on to next step.

Note: Before any use of AMPure or Serapure beads throughout this protocol, the beads need to be warmed up to room temperature (RT) and resuspended extremely well by mixing!

Prepare 80% ETOH

NEBNext End Prep

Mix the following components:

End Repair	
Component	Volume
End Prep Enzyme Mix	3 ul
End Repair Reaction Buffer (10x)	6.5 ul
Fragmented DNA	50 ul
Nuclease-free H_2O	5.5 ul
V_{tot}	65 uL

1. Transfer to PCR strip tubes.
2. Incubate for 30 mins @ 20° C, 30 mins @ 65° C, & hold @ 4° C .

Adapter Ligation

Adapter Ligation	
Component	Volume
NEBNext Methylated Adapter for Illumina (15 um)	2.5 ul
Blunt/TA Ligase Master Mix	15 ul
Ligation Enhancer	1 ul
V_{tot}	83.5 uL

1. Incubate for 20 mins @ 20° C.
2. Add 3 ul USER Enzyme, mix, & incubate for 15 mins @ 37° C.

Bead-based size selection (200-350bp range)

1. Bring reaction up to 200 ul total volume using H_20.
2. Add (1 DNA : 0.7 beads)
   a. 140ul of AMPure beads to 200ul of adapter-ligated DNA
   b. Gently pipette mix and spin down briefly
3. Incubate 5min @ RT
4. Incubate 5min on the magnetic stand
5. While on magnetic stand, carefully transfer the supernatant to a new tube (DISCARD THE BEADS)
6. Add (1 DNA : 1.5 beads)
   a. ~488ul of AMPure beads to the ~ 325 ul newly transferred solution
   b. Gently pipette mix and spin down briefly
7. Incubate 5min @ RT
8. Incubate 5min on the magnetic stand and discard supernatant (**KEEP BEADS**)
9. While on magnetic stand, add ~1000 ul 80% ETOH, incubate for 30 seconds, remove ETOH.
10. Repeat Step 9.
11. Air dry beads with lids open while on magnetic stand for 10 mins (do not overdry beads).
12. Remove from magnetic stand and add 45ul of EB or AE buffer, mix very well and incubate 5min @ RT.
13. Place tube on magnetic stand and incubate 5min @ RT.
14. Transfer 40ul of supernatant to 2ml PCR strip tube.
15. You can either freeze or continue on to next step.
16. Measure concentration using Qubit or Nanodrop before beginning BS conversion (expected concentration = ~0.5-4 ng/ul).

EpiTect Qiagen kit

Protocol: Sodium Bisulfite Conversion of Unmethylated Cystosines in DNA from Low-Concentration Solutions

***Note: Check Qiagen handbook for any updates to protocol whenever a new kit is used!*

- Starts with 40ul of DNA (small fragments 200-350bp).

To do before staring:

- *Prepare buffers in kit if necessary (Add ETOH to Buffer BW & BD; add 310ul of RNase-free water to carrier RNA and prepare aliquots).*
- *Dissolve the Bisulfite Mix in 800ul RNase-free water; vortex.*
- *Each aliquot of Bisulfite Mix is sufficient for 8 conversion reactions*
  - *If <8 reactions, Bisulfite Mix can be stored at -20° C for up to 4 weeks for reuse*
- *Supplies storage*
  - *Buffer BW should be stored @ RT*
  - *Buffer BD should be stored at 2-8° C*
  - *DNA Protect buffer are stored at 4° C*
  - *Wash columns are stored at 4° C*
- *Carrier RNA should be prepared as a master mix of: 620ul Buffer BL and 6.2ul RNA carrier PER CONVERSION*
- *All centrifugation steps will be performed at RT.*
- *Equilibrate samples and buffers to RT.*

Day 1 Bisulfite DNA conversion

1. Vortex Bisulfite Mix until completely dissolved (may take up to 10min)
   a. Do not place dissolved Bisulfite Mix on ice!
   b. Can heat to 60° C to help dissolve.
2. Total reaction volume is 140ul (see table below).
3. Add DNA and each component IN THE ORDER LISTED to a 200ul PCR tube, close the tubes and mix the reactions thoroughly
4. Store at room temperature (15-25° C)
   a. DNA Protect Buffer should turn from green to blue color after adding the Bisulfite Mix, indicative of correct pH for the reaction
5. In thermocycler, complete the conversion (5 hour reaction)

Bisulfite reaction cocktail	
Component	Volume
DNA solution+ H_2O	40 ul
Bisulfite Mix (dissolved)	85 ul
DNA Protect buffer	15 ul
V_{tot}	140 ul

Bisulfite conversion PCR program		
Step	**Time**	**Temp**
1. Denature	5 min	95° C
2. Incubation	25 min	60° C
3. Denature	5 min	95° C
4. Incubation	85 min	60° C
5. Denature	5 min	95° C
6. Incubation	175 min	60° C
Hold	Forever	20° C

EpiTect Qiagen kit (continued) + bead clean-up

Day 2 Cleanup

1. Warm buffers and spin columns to RT before use; heat incubator to 56° C.
2. Briefly centrifuge tubes then transfer to 1.5ml epi tubes
3. Add 560ul freshly prepared Buffer BL containing 10ug/ml RNA carrier
    a. If you are doing <48 conversions, make up the exact amount of buffer/RNA carrier you need for those reactions (see table).

Number of Samples	1	4	8
Volume of Buffer BL	620 ul	2.5 ml	5 ml
Volume of carrier RNA solution	6.2 ul	25 ul	50 ul

4. Mix by vortexing then centrifuge briefly
5. Transfer mixture to Epitect spin columns and centrifuge at maximum speed for 1 min.
6. Discard flow-through and replace back in original spin column.
7. Add 500ul Buffer BW to the spin column, centrifuge at maximum speed for 1 min.
8. Discard flow-through and place the spin column back into the collection tube
9. Add 500ul Buffer BD to spin column (then place buffer back at 4° C) - keep lid closed until use!
    a. Close cap (the buffer will change pH if leave exposed to the air... be careful to keep the lid open only for as long as you need it!)
    b. Incubate for 15min @ RT
10. Centrifuge at max speed for 1min, then discard flow-through and place the spin column back onto the collection tube
11. Add 500ul Buffer BW to spin column and centrifuge at maximum speed for 1 min.
12. Discard flow-through and place the spin column back onto the collection tube
13. Repeat steps 11 and 12
14. Place spin columns into a new 2 ml collection tube and centrifuge at max speed for 1 min to remove any remaining wash buffer
15. Place spin columns into clean, labeled 1.5ml epi tubes
16. Open lids and dry at 56° C for 5min
17. Dispense 22ul of Buffer EB into spin column, wait 3min then spin at max speed for 2min
18. Repeat step 17 for a total elution volume of 40ul BS-converted DNA libraries
19. Add (1 DNA : 5 beads)
    1. 200ul of AMPure beads to 40ul of BS-converted DNA

2. Gently pipette mix and spin down briefly

20. Incubate 5min @ RT
21. Incubate 5min on the magnetic stand and discard supernatant (**KEEP BEADS**)
22. Keep tube on magnetic, add ~400 ul 80% ETOH, Incubate 30 seconds, and remove ETOH.
23. Repeat step 22.
24. Remove from magnetic stand and add 25ul of 0.1X TE or 10mM Tric-HCL (pH 8), mix very well and incubate 5min @ RT.
25. Place tube on magnetic stand and incubate 5min @ RT.
26. Transfer 20ul of supernatant to PCR strip tube.
27. You can either freeze or continue on to next step.

0.1X TE	
Component	Volume
1 X TE	5 mL
H_20	45 mL
V_{tot}	50 mL

PCR Amplification & AMPure bead clean-up

Notes:
- *EBT Buffer: EB buffer + 0.1% Tween-20*

1. Run PCR using conditions described in tables below (add components in order listed).
2. Add (2 DNA : 1 beads)
   a. 100ul of AMPure beads to the 50ul reaction
   c. Gently pipette mix and spin down briefly
3. Incubate 5min @ RT
4. Incubate 5min on the magnetic stand and discard supernatant (**KEEP BEADS**)
5. Keep tube on magnet, add 200 ul 80% ETOH, incubate for 30 seconds, discard ETOH.
6. Repeat step 4.
7. Air dry beads with lid open for 10 mins while on magnetic stand (be careful not to overdry).
8. Remove from magnetic stand and add 33ul of 0.1X TE, mix very well and incubate 5min @ RT
9. Place tube on magnetic stand and incubate 5min @ RT
10. Transfer supernatant to new LoBind DNA tube --> **this is your library!!!**
11. Qubit, Nanodrop, or run Bioanalyzer (expect range from ~20-70ng/ul) using the Broad Range Qubit kit and 1ul of library DNA
12. 2% agarose gel
    a. Use high purity agarose
    b. Use low molecular weight DNA ladder
    c. Load ~70-100ng of DNA in gel
    d. Use **orange** loading dye
    e. Bands expected at 200-350bp (average 280bp)
13. Calculate molarity: http://www.molbiol.ru/eng/scripts/01_07.html
    a. Use kb (so 0.28kb is 280bp)
    b. Expect 100-400nM
    c. Goal: 10nM per sample
14. Dilute each sample to 10nM using EBT buffer (MAKE EBT BUFFER BEFORE HAND)
15. Pool samples
    a. If 4 samples per lane, then in a single 1.5ml epi tube, add 10ul of each final diluted library (with appropriate molarity) to give a final volume of 40ul
    b. 20ul is the minimum volume for Illumina sequencing.

PCR Set-up	
Component	Volume
Adapter ligated, BS-treated DNA	20 ul
NEBNext Universal PCR Primer (10 uM)	2.5 ul
NEBNext Index (X) Primer (10 uM)	2.5 ul
5X EpiMark Hot Start Taq Reaction Buffer	10 ul
10 mM dNTPs	1 ul
EpiMark Hot Start Taq (2 units/ul)	0.25 ul
Sterile H_20	13.75 ul

PCR program		
Step	**Time**	**Temp**
Initial Denature	30 sec	95° C
15 cycles		
Denature	*15 sec*	*95° C*
Anneal	*30 sec*	*61° C*
Extension	*30 sec*	*68° C*
Final extension	5 min	68° C
Hold	Forever	4° C