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Reduced representation bisulfite sequencing (RRBS)

Note: This protocol is based on the library preparation protocols in the NEBNext Multiplex Oligos for Illumina, methylated adaptor (NEB #E7535) instruction manual v2.0, with some modifications.

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.

This is a 3-day protocol
There are designated stopping points. Please adhere to them!

Section 1: DNA Standardization

1. All DNA will need to be run out on a gel to survey level (if any) of degradation and shearing. Aim to pool samples of similar quality together.
2. Qubit your DNA, with a minimum requirement of 20ng/ul.
    a. *If you need to concentrate your DNA, use a bead-clean to do so as drying down will increase salt concentrations, which can inhibit digestion. Ideally, samples should be in AE or low-EDTA buffer, NOT 1xTE.*
3. Standardize your high quality genomic DNA with 500ng-1ug in 40uL in a 96-well plate format.
4. Add 1ng of lambda DNA per sample as an internal control for conversion efficiency.

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Section 2: Digestion with *MspI* (purple tubes)

Materials Required

- ☐ Ice
- ☐ 10x Cutsmart Buffer
- ☐ *Msp1* Fast Digest Restriction Enzyme from ThermoFisher
- ☐ 8-well PCR strips
- ☐ AMPure beads rotating to warm up to room temperature (for use in size selection of Section 5)

Notes: This protocol uses FastDigest MspI (Thermo Scientific FD0544). Regular MspI can be used instead with the appropriate buffers and incubation times (e.g. NEB MspI (R0106) with Cutsmart buffer). Make sure you adjust the recipe accordingly depending on the enzyme + buffer concentrations.

1. Set up the digestion reaction in strip tubes
2. Reagents above the line can be made into a master mix on ice:

Item	**Volume (uL) per sample**
FastDigest Msp1	5
10X FastDigest buffer	5
DNA (min 20ng/ul)	40
Total	***50***

3. Add **10ul of master mix** to each sample. Pipette mix and quick spin strip tubes.
4. Incubate using "*RRBS_msp1_dig*" at:
   a. 37°C for 20 minutes.
5. Optional: Run ~3ul of digested DNA on a 1% agarose gel to check that genomic DNA is a light smear. After the gel, add the same amount of molecular grade water back into the original tube to keep the final volume of 50ul.

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Section 3: NEBNext End Prep (green tubes)

Materials Required

- ☐ End Prep enzyme mix
- ☐ 10x End repair reaction buffer
- ☐ Water
- ☐ Digested DNA

1. Set up the end prep reaction (total 65ul). Reagents above the line can be made into a master mix:

Item	Volume (uL) per sample
End Prep Enzyme Mix	3
10x End Repair Reaction Buffer	6.5
Water	5.5
Digested DNA	50
Total	***65***

2. Add **15ul of master mix** directly to 50ul of digested DNA. Mix well by pipetting and quick spin.
3. Incubate using "*RRBS_end_prep*":
   a. 30 mins at 20° C, 30 mins at 65° C, & hold at 4° C.
   b. Use a heated lid if incubating in the thermal cycler.

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Section 4: Adapter ligation (red tubes)

Materials Required

- ☐ Methylated adapters (15uM)
- ☐ Blunt/TA Ligase master mix
- ☐ Ligation enhancer
- ☐ Digested/end-repaired DNA

1. Prepare your master mix:

Item	Volume (uL) per sample
NEBNext methylated adapter for Illumina (15uM)	2.5
Blunt/TA ligase master mix	15
Ligation enhancer	1
Digested/end-repaired DNA	65
Total	***83.5***

2. Add **18.5ul of master mix** directly to 65ul end-prepped DNA. Mix well by pipetting and quick spin.
3. Incubate for 15 mins @ 20° C *with the heated lid turned off* using "*RRBS_adapt_lig1*"
4. Add **3ul USER Enzyme** directly to each tube, mix, spin, and incubate for another 15 minutes at 37° C *with the heated lid turned off* using "*RRBS_adapt_lig2*"
5. The total volume is now 86.5ul.

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Section 5: Bead-based size selection for fragments 200-350bp

Notes: Both over- and under-drying beads after the ethanol washes can reduce yield! Beads are over-dried when they look dry and cracked. Under-drying resulting in ethanol carryover of 5-10% can reduce elution efficiency. Set a timer for the drying steps, watch your beads carefully, and use small-volume pipets to pull the last drops of ethanol out of tubes.

Materials Required

- ☐ Mixed AMPure beads at room temp
- ☐ Freshly made 80% ethanol (~1.5mL per sample if using 1.5mL tubes; ~400ul per sample if using strip tubes) using nuclease-free water and 95% ACS grade ethanol
    - For total 5ml: 4.17mL of 96% ethanol, 833uL molecular grade water

1. Bring samples to a total volume of 100ul with nuclease-free water. A simple way to do this is to add ~14ul H2O to each sample, and then transfer 100ul to a 1.5ml tube. Alternatively, transfer the same volume to 0.2mL strip tubes, and perform the bead clean with a multichannel pipet and a plate magnet. This can be faster but requires extra care not to overflow the tubes or overdry the beads.
2. Add 55ul room-temperature, well-mixed AMPure beads to each sample (1 DNA:0.55 beads). Mix by pipetting 10 times and spin down.
3. Incubate 5 minutes.
4. Incubate 5 minutes on magnet.
5. While on magnet, carefully transfer the supernatant (approx. 155ul) to a new tube (**DISCARD THE BEADS**).
6. Add 25ul AMPure beads to the supernatant (1 DNA: 0.16 beads). Mix by pipetting 10 times and spin down.
7. Incubate 5 minutes.
8. Incubate 5 minutes on the magnet, then discard supernatant (**KEEP BEADS**).
9. While on magnet, add 750ul (180ul if in strip tubes) 80% EtOH. Incubate for 30 seconds, remove EtOH.
10. *Repeat*! While on magnet, add 750ul (180ul if in strip tubes) 80% EtOH. Incubate for 30 seconds, remove EtOH.
11. Air dry beads with lids open while on magnet for 5 mins (do not over- or under-dry beads; if using strip tubes, dry for ~3minutes).
12. Remove from magnetic stand, add 45ul of nuclease free water, mix by pipetting 10 times and incubate 5 minutes.
13. Place tubes on magnet and incubate 5 minutes.
14. Transfer 42ul of each sample to new, labeled strip tubes. DNA will be stable in the fridge for short-term storage, otherwise freeze at -20.
15. Quantify 2ul each sample with Qubit (final sample volume remaining: 40ul). Expected concentration may be low, <2ng/ul. Carry forward all samples, even those with very low/undetectable concentrations, as the final PCR step will usually recover enough library to submit.

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Section 6: Bisulfite conversion with Qiagen Epitect kit, Day 1 (conversion)

Notes: This is the Qiagen protocol "Sodium bisulfite conversion of unmethylated cytosines in DNA from low-concentration solutions." Check the Qiagen Epitect manual for any changes whenever a new kit is used! It is best to set this up in the afternoon and let the conversion run overnight.

DNA Protect buffer is stored in the reagent fridge. Dry bisulfite mix is at room temperature. Leftover rehydrated bisulfite mix from previous preps are stored in the -20 and are good for up to 4 weeks; thaw and reuse these before rehydrating new tubes.

1. Prepare bisulfite mix: Each tube of dry bisulfite mix is sufficient for 8 conversion reactions. Add 800ul molecular-grade water to each tube and vortex until completely dissolved (this may take several minutes). Heat on the thermomixer at 60° C to help dissolve. Unused rehydrated bisulfite mix can be stored at -20 for 4 weeks.
2. Set up the conversion reaction at room temperature. Add in the order listed to DNA tubes carried over from size selection step: do not mix the bisulfite mix and DNA Protect buffer before adding to samples. The reaction should turn from green to blue after adding the DNA Protect buffer, which indicates that it is at the correct pH. Total volume: 140ul.

40ul	**DNA**
85ul	**rehydrated bisulfite mix**
15ul	**DNA Protect buffer**

3. Mix reaction and quick spin tubes. If 140ul exceeds the maximum volume for the thermal cycler (the Eppendorf Mastercycler has a max volume of 70ul per reaction), divide each reaction into 2 tubes of 70ul each after mixing. This will ensure that the reactions reach the proper temperatures during cycling.
4. Run a 5-hour conversion reaction using "*RRBS_BSconv*"

Step	Time	Temp
denature	5m	95° C
incubation	25m	60° C
denature	5m	95° C
incubation	85m	60° C
denature	5m	95° C
incubation	175m	60° C
hold	forever	20° C

END OF DAY 1

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Section 7. Bisulfite conversion with Qiagen Epitect kit, Day 2 (cleanup)

Notes: Buffer BD and the spin columns are kept at 4°C. BD should be kept in the fridge until use. Buffer BL+carrierRNA solution should be made fresh immediately before use. Do not leave BL open to air for very long, as it will start to crystallize.

Materials Required

- ☐ Bring spin columns to room temperature before starting
- ☐ Make fresh buffer BL + carrier RNA solution:
    - For 96 samples: Add 310ul RNAse-free water to lyophilized carrier RNA and dissolve by vortexing. Previously rehydrated carrier RNA will be stored in the -20. Use this first (it is good for up to 12 months)!
    - For fewer samples, use the table below (this table includes 10% extra):

Number of Samples	1	4	8	16	24
Buffer BL	620uL	2.5mL	5mL	9.92mL	14.88mL
carrier RNA	6.2uL	25uL	50uL	99.2uL	148.8uL

1. Transfer converted DNA into 1.5mL tubes and preheat the thermomixer to 56°C.
2. Add 560ul BL+carrier RNA to each sample; mix well and quick spin.
3. Transfer to Epitect spin columns and centrifuge at maximum speed for 1 min.
4. Discard flow-through and place column back in the collection tube.
5. Add 500ul Buffer BW to the spin column and centrifuge at maximum speed for 1 min.
6. Discard flow-through and place the spin column back into the collection tube.
7. Add 500ul Buffer BD to each spin column and incubate for 15 min <u>at room temperature</u>. NOTE: The buffer will change pH when exposed to air. Close spin column caps immediately, and only keep the buffer bottle open as long as you need it. Return the buffer to the fridge immediately after use.
8. Centrifuge at max speed for 1 min, then discard flow-through and place the spin column back onto the collection tube.
9. Add 500ul Buffer BW to spin column and centrifuge at maximum speed for 1 min.
10. Discard flow-through and place the spin column back onto the collection tube.
11. Repeat steps 12 and 13.
12. Place spin columns into new 2 ml collection tubes and centrifuge at max speed for 1 min to remove any remaining wash buffer.
13. Place spin columns into clean, labeled 1.5ml tubes.
14. Open lids and dry <u>at 56°C</u> for 5min.
15. Add 22ul of Buffer EB into spin column, incubate for 3 min then spin at max speed for 2 min.
16. Repeat elution step with 22ul (3min incubation and spin for 2 min) for a total elution volume of 42ul of BS-converted DNA.

END OF DAY 2

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Section 8. PCR and doubple AMPure bead clean up

Notes: Indexes (barcodes) are added to BS-converted fragments in the PCR step to allow for demultiplexing of pooled samples after sequencing. Ensure that unique index primers are used for each sample in a pool! Use index primers that have the most mismatches to each other.

Materials Required

- ☐ 5x EpiMark Hot Start Taq Reaction Buffer
- ☐ dNTP (10mM)
- ☐ NEBNext Universal PCR primer (10uM)
- ☐ EpiMark Hot Start Taq (2U/uL)
- ☐ Mixed AMPure beads at room temp
- ☐ Freshly made 80% ethanol (~1.5mL per sample if using 1.5mL tubes; ~400ul per sample if using strip tubes) using nuclease-free water and 95% ACS grade ethanol
    - For total 5ml: 4.17mL of 96% ethanol, 833uL molecular grade water
- ☐

1. Set up the PCR reaction. Primers are in blue-capped tubes.

Item	Volume (uL) per sample
5x EpiMark Hot Start Taq Reaction Buffer	10
dNTP (10mM)	1
NEBNext Universal PCR primer (10uM)	2.5
EpiMark Hot Start Taq (2U/uL)	0.25

2. Separately add 2.5ul of the unique index primers (**NEBNext Index (X) Primer at 10uM)** directly to strip tubes of 33.75ul of adapter-ligated converted DNA. Save the unused DNA template, as it can be used for an additional PCR if your final library is low concentration!
3. **Add 13.75ul of master mix** to each sample. Mix well, quick spin.
4. Run a PCR using the program "*RRBS_epimark_PCR*":

Step	Time	Temp
denature	30s	95° C
15 cycles of:		
denature	15s	95° C
anneal	30s	61° C
extension	30s	68° C
final extension	5min	68° C
hold	forever	4° C

5. Add 45ul well-mixed, room-temperature beads to each sample (1:0.9 DNA:bead ratio), pipet mix 10 times and spin down briefly. Incubate 5min.
6. Incubate on plate magnet for 5min or until solution clears, then discard supernatant (**KEEP BEADS**).

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7. Keeping tubes on the magnet, add 200ul 80% EtOH, incubate for 30s, then discard EtOH.
8. Repeat step 5.
9. Air dry beads with lids open while on the magnet for 5 mins (do not over-dry beads).
10. Remove from the magnet and add 53ul of 0.1X TE, mix well and incubate for 5min.
11. Place tubes on magnet and incubate for 5min or until the solution clears.
12. Transfer 50ul of supernatant to a new set of strip tubes.
13. Add 50ul well-mixed, room-temperature beads to each sample (1:1 DNA:bead ratio), pipet mix 10 times and spin down briefly. Incubate 5min.
14. Incubate on magnet for 5min or until solution clears, then discard supernatant (**KEEP BEADS**)
15. Keeping tubes on the magnet, add 200ul 80% EtOH, incubate for 30s, then discard EtOH.
16. Repeat EtOH wash.
17. Air dry beads with lids open while on the magnet for 5 min (do not over- or under-dry beads; see note in Section IV).
18. Remove from magnet and add 33ul of 0.1X TE, pipet mix 10+ times and incubate for 5min.
19. Place tubes on magnet and incubate for 5min or until the solution clears.
20. Transfer supernatant to new LoBind DNA tubes: **this is your library!**
21. Quantify concentration on Qubit using 1-2ul of library DNA. Final concentrations will depend on (among other things) input DNA quantity, adapter ligation efficiency, and efficiency of DNA recovery from bead cleans. If some samples are low concentration, don't worry: you don't need much to submit for sequencing. If necessary, run another PCR with any remaining template. Putting the final library through a second PCR to increase yield is not recommended.
22. Confirm that the libraries are the expected size range using Bioanalyzer. You should have a peak in the expected size range of 200-350bp. If your libraries have plenty of DNA, run 70-100ng of DNA per sample. If your libraries are low concentration you can load less, even as little as ~5-10ng.
23. For each pool, combine equal amounts (**in ng**, NOT equal volumes) of each sample library into a single 1.5mL DNA LoBind tube and submit for sequencing. An ideal pool is 50ul in total volume, aiming to pool 50ng per sample (which is ~8ng/ul of each sample). There is a minimum requirement of 10ul at 2ng/ul for submission to the Princeton Genomics core facility.

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Reagents and supplies needed

FastDigest MspI (Thermo Scientific FD0544)
or regular MspI (e.g. NEB R0106)

NEBNext Ultra DNA Library Prep Kit for Illumina (NEB E7370), includes:
NEBNext End Prep Enzyme Mix
NEBNext End Repair Reaction Buffer
Blunt/TA Ligase Master Mix
NEBNext Ligation Enhancer
NEBNext Q5 Hot Start HiFi PCR Master Mix *(not used here)*

**NEB also sells E7445, NEBNext Ultra Ligation Module (end prep enzyme mix + end repair reaction buffer) and E7442, NEBNext Ultra End Repair/dA-Tailing Module (Blunt/TA ligase master mix + ligation enhancer). With Princeton institutional pricing, buying the two modules separately is cheaper but only if you get the 96-reaction size. We have always bought the library prep kit (E7370) so I would check with NEB first to confirm that the modules are proper replacements for the kit.*

NEBNext Multiplex Oligos for Illumina (Methylated Adaptor, Index Primers Set 1 (NEB E7535), includes:
NEBNext Methylated Adaptor for Illumina
USER enzyme
NEBNext Universal PCR Primer for Illumina
NEBNext Index Primers for Illumina, 1-12

**If more than 12 unique indexes are needed, NEB makes a second set of index primers (Set 2). Currently, the only Set 2 index kit listed in the catalog comes with an unmethylated adapter that cannot be used in this protocol (E7500, NEBNext Multiplex Oligos for Illumina, Index Primers Set 2). NEBNext kits with Set 2 primers and a methylated adaptor can be custom ordered by contacting NEB technical support.*

EpiMark Hot Start *Taq* DNA Polymerase (NEB, M0490)

dNTPs (e.g. NEB, N0447 or N0446S)

EpiTect Bisulfite Kit (Qiagen, 59104)

Agencourt AMPure XP beads (Beckman Coulter, A63880/A63881/A63882)

DNA Lo Bind Tubes 1.5mL (Eppendorf, 022431021)

ACS grade ethanol

Molecular-grade water

Magnetic rack

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NEBNext Index 1–12 Primers for Illumina

Description: 12 Index Primers are included for producing barcoded libraries.

NEB #	Product	Index Primer Sequence	Expected Index Primer Sequence Read
#E7311A: 0.010 ml **#E7311AA: 0.040 ml**	**NEBNext Index 1 Primer for Illumina**	5´-CAAGCAGAAGACGGCATACGAGAT-**CGTGAT**GTGACTGGAGTTCAGACGT-GTGCTCTTCCGATC-s-T-3´	ATCACG
#E7312A: 0.010 ml **#E7312AA: 0.040 ml**	**NEBNext Index 2 Primer for Illumina**	5´-CAAGCAGAAGACGGCATACGAGAT-**ACATCG**GTGACTGGAGTTCAGACGT-GTGCTCTTCCGATC-s-T-3´	CGATGT
#E7313A: 0.010 ml **#E7313AA: 0.040 ml**	**NEBNext Index 3 Primer for Illumina**	5´-CAAGCAGAAGACGGCATACGAGA-T**GCCTAA**GTGACTGGAGTTCAGACGT-GTGCTCTTCCGATC-s-T-3´	TTAGGC
#E7314A: 0.010 ml **#E7314AA: 0.040 ml**	**NEBNext Index 4 Primer for Illumina**	5´-CAAGCAGAAGACGGCATACGAGAT-**TGGTCA**GTGACTGGAGTTCAGACGT-GTGCTCTTCCGATC-s-T-3´	TGACCA
#E7315A: 0.010 ml **#E7315AA: 0.040 ml**	**NEBNext Index 5 Primer for Illumina**	5´-CAAGCAGAAGACGGCATACGAGAT-**CACTGT**GTGACTGGAGTTCAGACGT-GTGCTCTTCCGATC-s-T-3´	ACAGTG
#E7316A: 0.010 ml **#E7316AA: 0.040 ml**	**NEBNext Index 6 Primer for Illumina**	5´-CAAGCAGAAGACGGCATACGAGA-T**ATTGGC**GTGACTGGAGTTCAGACGT-GTGCTCTTCCGATC-s-T-3´	GCCAAT
#E7317A: 0.010 ml **#E7317AA: 0.040 ml**	**NEBNext Index 7 Primer for Illumina**	5´-CAAGCAGAAGACGGCATACGAGA-T**GATCTG**GTGACTGGAGTTCAGACGT-GTGCTCTTCCGATC-s-T-3´	CAGATC
#E7318A: 0.010 ml **#E7318AA: 0.040 ml**	**NEBNext Index 8 Primer for Illumina**	5´-CAAGCAGAAGACGGCATACGAGA-T**TCAAGT**GTGACTGGAGTTCAGACGT-GTGCTCTTCCGATC-s-T-3´	ACTTGA
#E7319A: 0.010 ml **#E7319AA: 0.040 ml**	**NEBNext Index 9 Primer for Illumina**	5´-CAAGCAGAAGACGGCATACGAGA-T**CTGATC**GTGACTGGAGTTCAGACGT-GTGCTCTTCCGATC-s-T-3´	GATCAG
#E7320A: 0.010 ml **#E7320AA: 0.040 ml**	**NEBNext Index 10 Primer for Illumina**	5´-CAAGCAGAAGACGGCATACGAGAT-**AAGCTA**GTGACTGGAGTTCAGACGT-GTGCTCTTCCGATC-s-T-3´	TAGCTT
#E7321A: 0.010 ml **#E7321AA: 0.040 ml**	**NEBNext Index 11 Primer for Illumina**	5´-CAAGCAGAAGACGGCATACGAGA-T**GTAGCC**GTGACTGGAGTTCAGACGT-GTGCTCTTCCGATC-s-T-3´	GGCTAC
#E7322A: 0.010 ml **#E7322AA: 0.040 ml**	**NEBNext Index 12 Primer for Illumina**	5´-CAAGCAGAAGACGGCATACGAGAT-**TACAAG**GTGACTGGAGTTCAGACGT-GTGCTCTTCCGATC-s-T-3´	CTTGTA

Where -s- indicates phosphorothioate bond.

Note: If fewer than 12 indexes are used in a lane for sequencing, it is recommended to use the following indexes:
Pool of 2 samples: Index #6 and 12
Pool of 3 samples: Index #4, 6 and 12
Pool of 6 samples: Index #2, 4, 5, 6, 7 and 12