Reduced representation bisulfite sequencing

Updated 5/19/16, R Kartzinel + K Brzeski
Updated 9/12/16, R Kartzinel
Updated 9/25/16, R Kartzinel

Notes:
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.

I. Digestion with MspI (purple tubes)

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.

High salt concentrations can inhibit digestion. Ideally, samples should be in AE or low-EDTA buffer, NOT 1xTE. If samples have been substantially concentrated and/or digestion is inhibited, clean with Ampure beads following the manufacturer's protocol, elute with water, and re-digest.

1. Quantify DNA and adjust volumes to have 100ng-1ug (ideally >500ng) in 40ul. Optional: include 1ng lambda DNA per sample as an internal control for conversion efficiency.
2. Set up the digestion reaction in strip tubes (total 50ul; see note above if using regular MspI). Reagents above the line can be made into a master mix:

   5ul FastDigest MspI
   5ul 10x FastDigest buffer

   40ul DNA (100ng - 1ug)

3. Combine **10ul of master mix** with 40ul DNA in strip tubes. Mix and quick spin strip tubes.
4. Incubate at 37° C (thermal cycler [program MspIdigestion-check conditions before running] or Thermomixer) for 20 minutes.
5. 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.
   - See note above if digests were not successful
6. Freeze the 50ul digested DNA or continue on to the next step.

II. NEBNext End Prep (green tubes)

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

3ul	**End Prep Enzyme Mix**
6.5ul	**10x End Repair Reaction Buffer**
5.5ul	**nuclease-free H2O (comes with kit)**
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50ul	**Digested DNA**

2. Add **15ul of master mix** directly to 50ul digested DNA. Mix well by pipetting and quick spin.
3. Incubate for 30 mins at 20° C, 30 mins at 65° C, & hold at 4° C. Use the hot top if incubating in the thermal cycler. Proceed immediately to the adapter ligation OR freeze (do not refrigerate).

III. Adapter ligation (red tubes)

1. Set up the adapter ligation (total 83.5ul). Reagents above the line can be made into a master mix:

2.5ul	**NEBNext Methylated Adapter for Illumina (15uM)**
15ul	**Blunt/TA Ligase Master Mix**
1ul	**Ligation Enhancer**
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65ul	**digested, end prepped product**

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 hot top turned off*.
4. Add **3ul USER Enzyme** directly to each tube, mix, spin, and incubate for 15 minutes at 37° C *with the hot top turned off*. Total volume: 86.5ul. Proceed immediately to the size selection OR freeze (do not refrigerate).

IV. 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..

1. Take AMPure beads out of the fridge to bring to room temperature and mix well. ALL STEPS ARE AT ROOM TEMPERATURE.
2. Make fresh 80% EtOH (~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. Keep the EtOH in a closed falcon tube until needed to avoid

evaporation. [To make 10mL 80% EtOH: combine 8.4mL 96% EtOH with 0.8mL molecular grade H2O].

3. Bring samples to a total volume of 100ul with nuclease-free water in 1.5ml tubes. 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.
4. Add 55ul room-temperature, well-mixed AMPure beads to each sample (1 DNA:0.55 beads), mix by pipetting 10 times and spin down.
5. Incubate 5min.
6. Incubate 5min on magnet, or until the solution has cleared.
7. While on magnet, carefully transfer the supernatant (approx. 155ul) to a new tube (**DISCARD THE BEADS**).
8. Add 25ul AMPure beads to the supernatant (1 DNA: 0.16 beads); mix by pipetting 10 times and spin down.
9. Incubate 5min.
10. Incubate 5min on the magnet, or until the solution has cleared, then discard supernatant (**KEEP BEADS**).
11. While on magnet, add 750 ul (180ul if in strip tubes) 80% EtOH, incubate for 30 seconds, remove EtOH.
12. Repeat step 11.
13. Air dry beads with lids open while on magnet for 5 mins (do not over- or under-dry beads; see note above).
14. Remove from magnetic stand, add 45ul of nuclease free water, mix by pipetting 10+ times and incubate 5min.
15. Place tubes on magnet and incubate 5min or until solution has cleared.
16. 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.
17. 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.

V. 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 (check PCR program before running):

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

VI. Bisulfite conversion with Qiagen Epitect kit, Day 2: conversion cleanup [& optional bead clean]

Notes: Buffer BD and the spin columns (yellow) are kept in the reagent fridge. Bring the columns to room temperature before starting. BD should be kept in the fridge until use.

Buffer BL + carrier RNA solution should be made fresh immediately before use. Do not leave BL open to air for very long, as it will start to crystallize. Leftover carrier RNA can be kept in the freezer for up to 1 year.

BISULFITE CONVERSION CLEANUP:

1. Transfer converted DNA into 1.5mL tubes and preheat the thermomixer to 56°C. Bring the spin columns to room temperature.
2. Make fresh buffer BL + carrier RNA solution:
   a) 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.
   b) If processing 48 samples at once, add all 310ul carrier RNA to an unopened bottle of buffer BL. If processing fewer than 48 samples, make up only

enough as required according to the following table (enough for 560ul per sample + 10% extra):

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

5. Add 560ul BL+carrier RNA to each sample; mix well and quick spin.
6. Transfer to Epitect spin columns and centrifuge at maximum speed for 1 min.
7. Discard flow-through and place column back in the collection tube.
8. Add 500ul Buffer BW to the spin column and centrifuge at maximum speed for 1 min.
9. Discard flow-through and place the spin column back into the collection tube.
10. Add 500ul Buffer BD to each spin column and incubate for 15 min at room temperature. 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.
11. Centrifuge at max speed for 1 min, then discard flow-through and place the spin column back onto the collection tube.
12. Add 500ul Buffer BW to spin column and centrifuge at maximum speed for 1 min.
13. Discard flow-through and place the spin column back onto the collection tube.
14. Repeat steps 12 and 13.
15. Place spin columns into new 2 ml collection tubes and centrifuge at max speed for 1 min to remove any remaining wash buffer.
16. Place spin columns into clean, labeled 1.5ml tubes.
17. Open lids and dry at 56°C for 5min.
18. Add 22ul of Buffer EB into spin column, incubate for 3 min then spin at max speed for 2 min.
19. Repeat step 18 for a total elution volume of 42ul BS-converted DNA.
20. *If doing the bead clean (steps 22-30):* Transfer 40ul (42ul if doing Qubit) of eluted BS-converted DNA into a new tube to be precise on 40ul volume.
21. (optional) Quantify 2ul with Qubit. Loss of DNA is normal here, as the bisulfite conversion damages and destroys DNA.

BEAD CLEAN (optional):

Note: This is part of NEB's library prep protocol, but according to NEB tech support, the protocol was not developed using a column-based conversion cleanup and the bead clean was necessary to remove the bisulfite reagent. However, the Qiagen Epitect kits are intended to produce PCR-ready DNA without additional cleaning steps. Qiagen's tech support told RYK in Aug 2016 that this bead clean is not necessary, since it is redundant with (and less effective than) the column cleanup. Therefore this bead clean is optional but may produce slightly cleaner template for PCR. We have not tested this

extensively but have had success amplifying + sequencing libraries without this bead clean.

22. Before starting the bead clean, take AMPure beads out of the fridge to bring to room temperature. Make fresh 80% EtOH (~800ul per sample) using nuclease-free water and 95% ACS grade ethanol. Keep the EtOH in a closed falcon tube until needed to avoid evaporation. [To make 10mL 80% EtOH: combine 8.4mL 96% EtOH with 0.8mL molecular grade H2O].
23. Add 200ul well-mixed, room temperature AMPure beads (1 DNA:5 beads) to each sample, mix gently and spin down.
24. Incubate for 5 min.
25. Incubate 5min on the magnet, or until the solution has cleared, then discard supernatant (**KEEP BEADS**)
26. With tube on magnet, add ~400 ul 80% EtOH, Incubate 30 seconds, and remove EtOH.
27. Repeat step 25.
28. Air dry beads with lids open while on the magnet for ~5 mins (do not over- or under-dry beads; see note in Section IV).
29. Remove tubes from magnet and add 25ul of 0.1X TE or 10mM Tris-HCL pH 8, mix very well and incubate 5min. [50ml 0.1X TE= 5ml 1X TE + 45ml molecular grade H2O]
30. Place tube on magnet and incubate 5 min, or until the solution has cleared.
31. Transfer 20ul of supernatant to PCR strip tube. You can either freeze or continue on to next step.

VII. PCR and double AMPure bead cleanup

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!
If you are pooling 6 or fewer samples, use the following combinations:

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

NEB has recently changed their primer concentrations to 10 μM (NEBNext Multiplex Oligos for Illumina, Methylated Adaptor #E7535). This PCR recipe uses 10uM primer concentrations. Adjust the recipe accordingly if you are using lot 0021402 or 0031407 (25 μM primer).

1. Set up the PCR reaction (total 50ul). Primers are in blue-capped tubes. Reagents above the line can be made into a master mix. Separately add 2.5ul of the unique index primers directly to strip tubes. Mix well, quick spin.

IF YOU SKIPPED THE PRE-PCR BEAD CLEAN AND HAVE ~40ul DNA:

10ul	**5x EpiMark Hot Start Taq Reaction Buffer**
1ul	**dNTPs (10mM)**
2.5ul	**NEBNext Universal PCR Primer (10uM)**
0.25ul	**EpiMark Hot Start Taq (2U/ul)**
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2.5ul	**NEBNext Index (X) Primer (10uM)**
33.75ul	**adapter-ligated, converted DNA**

Add 13.75ul of master mix to each sample. Save the unused DNA template, as it can be used for an additional PCR if your final library is low concentration!

IF YOU DID THE PRE-PCR BEAD CLEAN AND HAVE 20ul DNA:

13.75ul	**molecular grade H2O**
10ul	**5x EpiMark Hot Start Taq Reaction Buffer**
1ul	**dNTPs (10mM)**
2.5ul	**NEBNext Universal PCR Primer (10uM)**
0.25ul	**EpiMark Hot Start Taq (2U/ul)**
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2.5ul	**NEBNext Index (X) Primer (10uM)**
20ul	**adapter-ligated, converted DNA**

Add 27.5ul of master mix to each sample.

Run PCR with the following cycling conditions:

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

2. Before starting the bead clean, take AMPure beads out of the fridge to bring to room temperature. Make fresh 80% EtOH (~800ul per sample) using nuclease-free water and 96% ACS grade ethanol. Keep the EtOH in a closed falcon tube until needed to avoid evaporation. [To make 10mL 80% EtOH: combine 8.4ml 96% EtOH with 0.8ml molecular grade H2O].
3. 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.
4. Incubate on plate magnet for 5min or until solution clears, then discard supernatant (**KEEP BEADS**).
5. Keeping tubes on the magnet, add 200ul 80% EtOH, incubate for 30s, then discard EtOH.
6. Repeat step 5.

7. Air dry beads with lids open while on the magnet for 5 mins (do not over- or under-dry beads; see note in Section IV).
8. Remove from the magnet and add 53ul of 0.1X TE, mix well and incubate for 5min.
9. Place tubes on magnet and incubate for 5min or until the solution clears.
10. Transfer 50ul of supernatant to a new set of strip tubes.
11. 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.
12. Incubate on magnet for 5min or until solution clears, then discard supernatant (**KEEP BEADS**)
13. Keeping tubes on the magnet, add 200ul 80% EtOH, incubate for 30s, then discard EtOH.
14. Repeat step 13.
15. 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).
16. Remove from magnet and add 33ul of 0.1X TE, pipet mix 10+ times and incubate for 5min.
17. Place tubes on magnet and incubate for 5min or until the solution clears.
18. Transfer supernatant to new LoBind DNA tubes: **this is your library!**
19. 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.
20. Confirm that the libraries are the expected size range on a 2% w/v gel (or can run on a Bioanalyzer). Use TrackIt cyan/yellow loading dye and a low molecular weight ladder (e.g. 50bp or 2log). You should have a smear 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. To make the bands more visible with low-concentration samples, use combs that make small wells, and load only 1-2ul ladder.
21. 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. Pooled DNA must be a minimum of 10ul at 2ng/ul for submission to the Princeton Genomics core facility.

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

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