Reduced Representation Bisulfite Sequencing Libraries

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A few notes:

1. Do not vortex enzymes or mixtures containing enzymes
2. Be very careful with bisulfite converted DNA. Do not vortex it or freeze thaw it. The DNA is fragile and will break apart easily.
3. There are several steps in this protocol that involve bead cleanups. The times listed in the protocol are suggestions, but you should pay attention to your samples and use your best judgment. In general, a bead cleanup should proceed like this:
	1. Put DNA and beads together in a tube.
	2. Vortex briefly just to mix, you do not want to degrade your DNA
	3. Incubate for 15 min to ensure binding
	4. Place the tube on the magnetic rack and wait ~2min until the supernatant is clear
	5. Remove and discard the supernatant
	6. Wash the beads by adding enough 80% ethanol (freshly prepared) to cover the beads
	7. Remove the ethanol after ~30sec
	8. Repeat the wash step
	9. Air dry the beads for ~5-10 min until they are no longer shiny. Do not over dry the beads as this will reduce yield! Also, during this time use a pipette to remove any excess ethanol that has pooled at the bottom of the tube.
	10. Once the beads are dry, add EB buffer to the tube. Make sure the beads are immersed in EB buffer (not sticking to the side of the tube). You can do this by short vortex or using a pipette.
	11. Incubate for 3-5 min to allow the DNA to elute from the beads
	12. Place the bead mixture back on the rack and wait until the supernatant is clear
	13. Transfer the supernatant to a new tube, be careful not to get any beads in the supernatant (you can check this by holding the pipette tip up to the light).

DAY 1 – ENZYME DIGESTS

1. Add unmethylated phage DNA (about 0.1% relative to sample DNA, in terms of ng; note this is 1/10th of 1% so it will be a very very small amount) and 200ng of each sample into PCR tubes
	1. So, if you’re using 200ng of DNA, add 0.2ng phage DNA to each sample.Be very careful not to accidently add more phage DNA than necessary.
2. Add 3ul of 10X NEB Cutsmart buffer + 1ul MspI enzyme (20 U/μl) + enough water to bring total volume to 30ul
	1. Typically, it is easiest to make a master mix of Msp1 + buffer + lambda phage DNA and add this mixture (4ul) to each sample. This is more efficient and accurate than adding the components individually to each sample.
3. Digest the DNA at 37C overnight (do not heat the lid higher than 37C if using the thermocycler)

DAY 2 – FILLING IN AND A-TAILING, BEAD CLEANUP, ADAPTOR LIGATION

1. Perform end repair and A-tailing by adding: 1ul Klenow fragment + 1ul dNTP mix (containing 10mM dATP + 1mM dCTP + 1mM dGTP) to each sample. This should be done in PCR tubes.
2. Incubate at 30C for 20min then 37C for 20min in a thermocycler **without the heated lid** (this will inactivate the enzyme!)
	1. This is a good time to take AMPure beads out of fridge to come to room temperature
3. Once the program is complete, remove PCR tubes from thermocycler and spin down
4. Transfer each sample to a 1.5mL centrifuge tube for cleanup.
5. Add 64ul AMPure beads (2x the 32ul sample volume) to each sample and mix by pipette or short vortex.
6. Incubate at room temperature for 15min
7. Place plate on magnetic stand until the solution is clear
8. Remove supernatant
9. Wash beads twice with **freshly prepared** 80% ethanol (leaving each wash on for approximately 30sec)
10. After removing the ethanol from the second wash, let the beads dry for ~5-10min or until beads are no longer shiny (do not overdry the beads, this will reduce yield)
11. Add 20ul EB buffer to each sample
12. Cap samples, vortex very briefly to mix, and spin down
13. Put the tubes back on the magnet and transfer the ~20ul of sample to a new tube.
14. Dilute each adaptor 1:20 or get stock of already diluted adaptors
15. Ligate adaptors by adding: 3ul T4 ligation buffer (make sure this is not expired and is from the stock of aliquoted buffers) + 1ul T4 ligase (2,000,000 units/ml) + 4ul H20 + 2ul diluted adaptor to each well
16. Incubate reaction overnight at 16C **in an incubator** (thermocycler with heated lid may inactivate the enzyme; alternatively use the thermocycler without the heated lid)

DAY 3 – SAMPLE POOLING, BEAD CLEANUP, qPCR

1. We currently use OPTION 1 to prepare libraries, but it is possible to do Option 2 (qPCR) before pooling.
2. OPTION 1: If you **are not** quantifying each sample, do this:
	1. Pool all samples according to lane
	2. Add a 1.5x concentration of beads to each pool of samples. Mix and incubate at room temperature for 15 min to ensure binding.
	3. Put samples on magnetic stand and incubate until solution is clear.
	4. Remove supernatant
	5. Wash beads twice with **freshly prepared** 80% ethanol.
	6. After removing the second ethanol wash, dry the beads (~5-10min)
	7. Elute DNA from the beads by adding 25ul EB buffer
	8. Vortex to mix and spin down. Wait 3-5 min.
	9. Place tubes back on the magnetic stand and transfer elutant to a new microcentrifuge tube.
3. OPTION 2: If you **are** quantifying each sample, do this:
	1. Put each sample in a 1.5mL tube
	2. Add a 1.5x concentration of beads to each sample. Mix and incubate at room temperature for 15 min to ensure binding.
	3. Put samples on magnetic stand and incubate until solution is clear.
	4. Remove supernatant
	5. Wash beads twice with **freshly prepared** 80% ethanol.
	6. After removing the second ethanol wash, dry the beads (~10min)
	7. Elute DNA from the beads by adding 20ul EB buffer
	8. Vortex to mix and spin down. Wait 3-5 min.
	9. Place tubes back on the magnetic stand and transfer elutant to a new microcentrifuge tube.
	10. Use this cleaned DNA to do some sort of quantification on each sample (either qPCR or Qubit, see KAPA qPCR protocol in the Dropbox for this method)
	11. Pool samples (by lane) in an equimolar way and vacufuge the pool of samples down to 20-25 ul.

DAYS 3 and 4 – BISULFITE CONVERSIONS

1. Perform the bisulfite conversion as specified for FFPE samples. A few notes on the Qiagen protocol:
	1. Do the optional/recommended steps.
	2. Check the date on the tube of bisulfite mix (they only last for 3-4 weeks after being mixed with water and stored at -20 C)
	3. Ensure that DNA, bisulfite mix, and DNA protect buffer are added in the order indicated
	4. When you are ready for the elution, do this:
		1. Get EB preheated to 50-60C
		2. Add 24ul of EB directly to the column membrane (be careful to pipette directly onto the membrane)
		3. Incubate for 5min
		4. Spin at full speed for 1min
		5. Take the flow through (~21ul) and put it back on the membrane for a second elution
		6. Incubate for 3-5min
		7. Spin at full speed for 1min
2. Perform another round of bisulfite conversion as specified for FFPE samples, using ~20ul of sample and following the same tips provided above.

DAY 4 – PCR, FINAL LIBRARY QC

1. Perform 4 PCRs (15 cycles, program = PCR15) for each sample as follows: 5ul Pfu buffer + 0.5ul 100mM dNTP mix (or 5ul of 10mM dNTP mix, containing all 4 dNTPs) + 2.5 ul of the F primer at 10uM + 2.5 ul of the R primer at 10uM + 1ul Pfu enzyme + 5 ul DNA + water to 50ul
	1. The forward and reverse primers are AATGATACGGCGACCACCGA\*G and CAAGCAGAAGACGGCATACGA\*G
	2. Check the cycling conditions!
		1. 95C for 2min
		2. 15 cycles of 95C for 30s, 65C for 30s, 72C for 45S
		3. 72 for 7min
2. Pool all 4 PCRs for each sample into a 1.5ml tube
3. Add 240ul AMPure beads to each 200ul pool and mix
4. Incubate at room temperature for 15min
5. Place samples on magnetic stand until supernatant is clear
6. Remove supernatant
7. Wash beads twice with freshly prepared 80% ethanol
8. After removing the second ethanol was, dry the beads for ~5-10min
9. Elute in 40ul EB buffer by adding EB buffer to dried beads, mixing, waiting a few minutes, placing tube back on the magnetic stand, and removing the elutant.
10. Repeat the bead cleanup using 60ul AMPure beads + the 40ul resuspended sample
11. Elute the final library in 25ul of EB buffer.
12. Run each library on a High Sensitivity Bioanalyzer chip.