Detailed protocol for 2b-RAD sample preparation
Last updated: Tuesday, August 11, 2015 to reflect increased volume in initial steps.

Overview

Preparation of sequencing libraries for 2b-RAD genotyping involves three steps:

1. Restriction digest. Genomic DNA is digested with a type IIB restriction enzyme to produce restriction fragments of uniform length.
2. Ligation. Adaptors are ligated to the cohesive ends generated by restriction digest.
3. Amplification and barcoding. 2b-RAD tags are amplified for a small number of cycles to incorporate sample-specific barcodes.

Digest

*The protocol begins with **intact, RNA-free** genomic DNA. Three important precautions are necessary to achieve this: (1) avoid vortex mixing during DNA extraction; (2) minimize lysis/digestion times; and (3) treat the DNA with RNAse prior to purification. Before beginning this protocol, check the samples on a standard agarose gel – a single high molecular weight band should be visible.*

1. Prepare intact, high-quality genomic DNA samples each containing ~1.2 µg at a high concentration (125 ng μl^{-1}). Samples can be concentrated after purification by ethanol precipitation or by drying under vacuum. If the DNA is completely dried during this process (e.g. as in ethanol precipitation), be sure to dissolve the pellet overnight at 4°C before proceeding. Dilute all samples to the same volume (10 µl) with nuclease free water or 10 mM Tris – be sure not to use a buffer containing EDTA (e.g. TE).

2. (Optional) Reserve an additional 2 µl from each sample (in addition to the 10 µl above) for later comparison with the digested samples.

3. Prepare a digestion master mix. The following recipe is for a single reaction, so multiply by the number of samples plus some small amount for pipetting error.

10X buffer R	1.2 µl	
150 µM SAM	0.8 µl	
AlfI (2 U μl^{-1})	0.5 µl	(Thermo Scientific # ER1801)
NFW	1.5 µl	

(*NOTE: 1 Unit per reaction. If AlfI is at a different concentration, adjust recipe*)

4. Combine 4 µl master mix with each 8 µl DNA sample. Incubate at 37°C for at least 1 hr, or as long as overnight. If using a heat-inactivatable enzyme (e.g. AlfI), inactivate the enzyme at 65°C for 15 min. Hold samples on ice.

5. (Optional) For each sample, load 2 µl digested DNA on a 1% agarose gel alongside a comparable amount of intact DNA from the same sample to verify the effectiveness of the digest.

   The original gDNA should appear as a single high-molecular weight band (>10kb) while the digested samples should be visibly degraded. An effective AlfI digestion produces a slight downward shift in the original HMW band and a subtle smear trailing downward from that band. If initial gDNA is degraded, this test is not useful and may be skipped.

Ligation

In this step adaptors are ligated to the restriction fragments produced above. If reduced tag representation (RTR) is required, selective adaptors must be chosen at this stage.

1. Anneal the partially double stranded adaptors. Prepare these adaptors fresh each time you make 2bRAD libraries. The oligonucleotide combinations used for "full" (not reduced) preparations, and one example of a reduced representation reparation, are listed in the following table. Sequences of each oligo are provided at the end of this document.

Version	Strand	Adaptor 1	Adaptor 2
Standard AlfI	(+)	5ILL-NN	3ILL-NN
	(-)	Anti-ILL	Anti-ILL
RTR AlfI (1/16th)	(+)	5ILL-NG	3ILL-NG
	(-)	Anti-ILL	Anti-ILL

Prepare each adaptor separately by combining the appropriate oligonucleotides at a final concentration of 2 µM each. Hold 10 minutes at room temperature to anneal.

2. Prepare the following master mix for ligations. This recipe is for a single reaction, so scale up as needed.

10 mM rATP	1.0 µl	
10X T4 ligase buffer	4.0 µl	
2 µM Adaptor 1	5.0 µl	
2 µM Adaptor 2	5.0 µl	
T4 DNA ligase	1.0 µl	(New England Biolabs, #M0202L)

Nuclease-free water (NFW)	24 µl

a. Combine 40 µl master mix with the remaining 10 µl of digested DNA. Incubate (16°C for AlfI) for at least 1 hour or as long as overnight, then hold on ice.

The ligation product is highly temperature sensitive. Store on ice after ligation, and be careful never to expose this product to temperatures > 65°C prior to PCR.

Amplification

In this step, the constructs produced by ligation are amplified using a set of four primers that introduce sample-specific barcodes and the platform specific sequencing and cluster amplification primer binding sites. For the test-scale PCR a single pair of HT and BC oligos can be used. For the preparatory scale PCR, be sure to use a unique combination of HT and BC oligos, and record the oligos used for each sample.

1. Prepare a test-scale PCR to determine optimum cycle number and evaluate relative yield across samples.

a. For each reaction prepare the following master mix:

NFW	1.0 µl	
1 mM (each) dNTP	4.0 µl	
10 µM ILL-Lib1	0.4 µl	
10 µM ILL-Lib2	0.4 µl	
1 µM ILL-HT	1.0 µl	
1 µM ILL-BC	1.0 µl	
5X Q5 buffer	4.0 µl	
Q5 Taq polymerase	0.2 µl	(New England Biolabs #M0491L)

b. Combine 12 µl master mix with 8 µl ligation. Amplify on the following profile:

(98°C 5 sec, 60°C 20 sec, 72°C 10 sec) X *N* cycles

c. Sample 5 µl from each reaction at N=10, 15, 20, & 25 cycles. Load these products on a 2% agarose gel with a low molecular weight marker to confirm molecular weight of PCR product.

Note – for reduced schemes, depending on genome size, you may wish to add one cycle per 2-fold reduction. e.g. for a 16-fold reduction ("NG/NG" adaptors), add 4 cycles (test 14, 19, 24, & 29 cycles instead). If the original DNA was degraded or contained inhibitors, additional cycles may also be necessary but the number can only be determined empirically.

d. Identify the minimum number of cycles required to produce a visible product at 166 bp. Note that primer dimers are likely to be visible at ~70-90 and ~130 bp, so be sure to run the gel until these sizes can be resolved.

2. Run a preparatory-scale PCR based on the cycle numbers determined above.
   a. Prepare the following master mix for each sample:

NFW	5.0 µl
1 mM dNTP	20 µl
10 µM ILL-Lib1	2.0 µl
10 µM ILL-Lib2	2.0 µl
5X Q5 buffer	20.0 µl
Q5 Taq polymerase	1.0 µl

Note that the BC and HT oligos are not added to this master mix, but are added individually to each sample.

   b. Combine 50 µl master mix with 40 µl ligation.
   c. Add 5 µl of each barcode (HT and BC), so that each sample has a final volume of 100 µl and a unique combination of BC and HT barcodes.
   d. Amplify using the optimal cycle number determined above.

Purification

In this step the target band is gel-extracted to exclude any high-molecular weight DNA remaining after the AlfI digest, and any incorrect constructs that may emerge during PCR (e.g., primer dimers).

1. Prepare a 2% agarose gel using TAE, TA, or SB. Use a wide comb that can accommodate 100 µl sample loading, or tape together two wells if required.

2. Load the entire volume of each reaction alongside a low-molecular weight ladder and run until low molecular weight bands can be resolved.

3. View the gel briefly (<30 seconds) on a UV transilluminator set at low intensity to verify the presence of target bands and adequate separation of molecular weight standards to resolve bands between 90-150 bp. Typically ~5 cm run distance is sufficient.

4. Cut out the target band (~170 bp) in a narrow gel slice, being careful to avoid any primer dimers (70-90 and ~130 bp) that may be present.

Note: at this stage a commercial gel-extraction kit can be substituted for the following three steps.

5. Transfer each gel slice into a 2 ml microcentrifuge tube and add 40 µl NFW.

6. Centrifuge tubes 1 min at high speed to bring gel into contact with the water. Freeze at -80°C for at least 1 hour.

7. Centrifuge at maximum speed, 4°C for 10-20 minutes.

8. Press gel slice against side of tube using pipette tip, and withdraw supernatant (~50-60 µl should be recovered). Transfer the supernatant to a new tube.

9. Combine aliquots of multiple samples into a pooled sample for sequencing. Volumes of each sample may be based simply on relative intensity of the gel images, or may be based on qPCR using primers ILL-Lib1 and ILL-Lib2.

10. After pooling, sample volume is frequently too high for Illumina HiSeq (max sample volume ~19 µl). This can be resolved by purifying the pooled sample on a PCR cleanup column or simply by drying under vacuum.

11. The libraries are now ready to sequence.

Oligonucleotide sequences

Illumina HiSeq

Name	Sequence (5' – 3')
anti-ILL	AGATCGGAAGAGC(InvdT)
5ILL-NN	CTACACGACGCTCTTCCGATCTNN
3ILL-NN	CAGACGTGTGCTCTTCCGATCTNN
5ILL-NG	CTACACGACGCTCTTCCGATCTNG
3ILL-NG	CAGACGTGTGCTCTTCCGATCTNG
ILL-HT	AATGATACGGCGACCACCGAGATCTACAC**[BC2]**ACACTCTTTCCCTACACGACGCTCTTCCGATCT
ILL-BC	CAAGCAGAAGACGGCATACGAGAT**[BC1]**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC
ILL-Lib1	AATGATACGGCGACCACCGA
ILL-Lib2	CAAGCAGAAGACGGCATACGA

InvdT: inverted T to prevent extension by DNA polymerase that may lead to tandem ligations. BC1: barcode 1 (called "index 1" or "i7" by Illumina); may be any one of the standard 6-bp TruSeq barcodes or other custom 5- to 8-bp barcodes. BC2: barcode 2 (called "index 2" or "i5" by Illumina); custom 5- to 8-bp barcodes.

Example schemes for reduced tag representation.

Fraction of sites targeted	5' oligo	3' oligo
1	-NN	-NN
1/4	-NR	-NR
1/8	-NC	-NG
1/16	-NC	-NC
1/32	-RG	-YG
1/64	-RG	-RG