Protocol for Illumina 2b-RAD sample preparation

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Overview
This is a modification of the protocol described in Wang et al, Nature Methods 2012 2b-RAD paper (doi:10.1038/nmeth.2023) re-designed for Illumina HiSeq and BcgI enzyme (https://www.neb.com/products/R0545-BcgI). BcgI is a relatively frequent cutter, with 75-100k sites per genome, and in our experience is one of the most efficient of the IIb-type restriction enzymes. It produces 36-base fragments with two-base overhangs and is fully heat-inactivatable, both of which facilitate ligation of adaptors.

The protocol involves the following steps, of which steps 1-3 are performed within the same tube (or well of a 96-well plate) by consecutively adding reagents:

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. Overnight ligation at 16 °C.

3. Amplification and barcoding. 2b-RAD tags are amplified for a small number of cycles to incorporate sample-specific barcodes.

4. Purification of the final product by gel-electrophoresis (the only purification step in the whole procedure).

5. Quantification and mixing in equal proportions.

Digest

1. Prepare intact, high-quality genomic DNA samples each containing a total of 100-200 ng in 4 µl. This can be accomplished using by ethanol precipitation or by drying under vacuum.
Note: we have successfully prepared samples from as little as 25 ng.

2. 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.

NEB Buffer #3	0.6 µl
150 µM SAM	0.4 µl
BcgI (2 U µl-1)	0.5 µl
NFW	0.5 µl (NFW: nuclease-free water)

**Note: SAM [S-adenosyl-methionine] and enzyme concentrations may differ depending on the manufacturer. If they are different in your case, re-calculate the volumes, make up the difference with nuclease free water if necessary, but keep the single-reaction volume at 6 µl.*

3. Combine 2 µl master mix with each 4 µl DNA sample (6 µl total volume). Incubate at 37°C 1 hr, then add one extra unit (0.5 µl + 0.5 µl NFW if the concentration is 2 U µl-1) of enzyme and incubate for one more hour. Then heat-inactivate the enzyme at 65°C for 20 min. Hold samples on ice, proceed to ligation immediately.

Ligation

In this step adaptors are ligated to the restriction fragments produced above. Note that this is the stage at which reduced tag representation (RTR) must be applied by the choice of adaptor sequences.

1. Prepare double stranded adaptors by combining each pair of adaptors. The oligonucleotide combination used for each alternative 2b-RAD preparations listed in the following table, and sequences of each oligo are provided at the end of this document.
For Adaptor 1, mix 5Ill-NN (10 µM) with the same volume of anti-Ill (10 µM). For Adaptor 2, mix 3Ill-NN (10 µM) with the same volume of anti-Ill (10 µM). No further procedures are necessary to prepare the adaptors. Store at -20 C.

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

10 mM ATP	0.5 µl
10X T4 ligase buffer	2.0 µl
5 µM Adaptor 1	1.0 µl
5 µM Adaptor 2	1.0 µl
T4 DNA ligase	1.0 µl #NEB M0202L
NFW	14.5 µl

3. Combine 20 µl master mix with the remaining 5 µl of digested DNA (25 µl total volume). Incubate at 16°C overnight.

Amplification

In this step, the constructs produced by ligation are amplified using a set of four primers that introduce sample-specific barcodes and the annealing sites for HiSeq amplification and sequencing primers.
Very Important: if you find that it takes more than 14 cycles to amplify a visible target band, consider optimizing previous steps (DNA isolation, digestion, and ligation), since over-amplified RAD samples tend to lose heterozygosity.

1. For each reaction prepare the following master mix:

NFW	15.6 µl
2.5 mM dNTP	7.5 µl
10 µM Mpx primer	0.9 µl
10 µM ILL-Lib-P1/20 (or IC1-P5)	1.2 µl
10 µM ILL-Lib-P2 (or IC2-P7)	1.2 µl
5X HF buffer	12 µl
Phusion polymerase	0.6 µl #NEB M0530L (or M0530S)

2. Combine 39 µl master mix with 12 µl ligation, add 9 µl of 1 µM Ill-Rad-bc (barcode-bearing) primer individually to each reaction, and amplify as follows: 70°C 30 sec then (95°C 20 sec, 65°C 3 min, 72°C 30 sec) X 12 cycles.

3. Prepare a 100 mL 2% agarose gel using TAE, run 5 µl of the amplified product together with 1 µl of 6x loading dye, alongside a 25 bp ladder until the bands at 100-200 bp are resolved (100 V for ca. 40 minutes should be enough). View the gel in an UV transilluminator, photograph and note the relative strengths of the 170 bp band and the 130 bp primer-dimer band.

Purification

In this step the target band is gel-extracted to exclude high-molecular weight fragments and any chaff that may emerge during PCR (e.g., primer dimers).

1. Prepare a 500 mL 2% agarose gel using TAE. Use a wide comb that can accommodate 55 µl sample loading plus 11 µl 6x loading dye, or tape together two wells if required.

2. Load the entire volume of each reaction alongside a low-molecular weight ladder. Run gel at low voltage for a long time until bands at 150bp and 200bp will clearly resolve. In our experience 100V for 70 minutes produce good separation.

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 at ~170 bp and ~130 bp. Typically ~5 cm run distance is sufficient. Photograph.

4. Cut out target band of 170 bp in a narrow gel slice.

5. Transfer each gel slice into an 0.5 ml microcentrifuge tube.

6. Use a Qiagen MinElute gel extraction kit to extract the DNA from the gel band.

7. Quantify the DNA yield using a QuBit dsDNA high-sensitivity assay, then pool the barcoded samples equimolarly as needed (as the fragments are the same length, weight concentrations can be used as proxy for molarity).

Oligonucleotide sequences for Illumina HiSeq

Name	**Sequence (5' – 3')**
anti-ILL	AGATCGGAAGAGC/3InvdT[1]/
5ILL-NN	CTACACGACGCTCTTCCGATCTNN
3ILL-NN	CAGACGTGTGCTCTTCCGATCTNN
ILL-Mpx	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGAT
ILL-RAD-bc[2]	CAAGCAGAAGACGGCATACGAGAT [barcode][3] GTGACTGGAGTTCAGACGTGTGCTCTTCCGAT
ILL-Lib1-20 / IC1-P5	AATGATACGGCGACCACCGA
ILL-Lib-P2 / IC2-P7	CAAGCAGAAGACGGCATACGA

[1] InvdT: inverted dT to prevent extension by DNA polymerase.
[2] "bc" stands for "barcode", and is typically replaced by the barcode number (or other barcode- specific identifier) in the actual name of the oligo.
[3] barcode: a 6-base sequence easily distinguishable from other sequences on that same sequencing run.

BUT NOTE that the adapter and barcode sequences are in fact REVERSE COMPLEMENTS relative to what needs to be communicated with the sequencing center!!!!!