Protocol for Illumina 2bRAD sample preparation

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Critical update: August 25 2014 – introduced NNRW adapters to discard PCR duplicates

Latest update (June 2018): removed all interim quantification steps. Instead, equalize your input DNA samples really well (based on Qbit or picogreen, not just Nanodrop) and ensure that their OD 260/230 ratio is >=2.

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 can be inactivated by heating, both of which facilitate ligation of adaptors. Another enzyme that can be used with this protocol is AlfI (http://www.thermoscientificbio.com/restriction-enzymes/alfi/)

The latest modifications include the use of barcoded ligated adaptors so the samples can be pooled by 12 after ligation, and use of degenerate 5'-adaptor that makes it possible to remove PCR duplicates.

The protocol involves the following steps:

1. Restriction digest. Genomic DNA is digested with a type IIB restriction enzyme to produce restriction fragments of uniform length.
2. Ligation. Adaptors, barcoded for 3' end and generic for 5', are ligated to the cohesive ends generated by restriction digest.
3. Heat-inactivate the ligase and pool the ligations with different 3' barcodes into groups of 12.
4. Amplification and barcoding of pooled ligations.
5. Purification of the target 165 bp band by Pippin or gel-electrophoresis (the only purification step in the whole procedure).

<u>NOTE on experimental design</u>: we strongly recommend including three pairs of genotyping replicates in each 2bRAD experiment. Replicates should be independently prepped DNA samples from the same individual. They will be extremely useful at the analysis step to assess the overall accuracy of genotyping, set quality filtering criteria, and quantify relatedness between samples.

Digest

1. Prepare samples each containing 100 ng of DNA in 4 µl. Equalize your input DNA samples well (based on qbit or picogreen, not just nanodrop) and ensure that their OD 260/230 ratio is >=2. Concentrating can be accomplished using by ethanol precipitation or by drying under vacuum.

2. Prepare a digestion master mix. The following recipe is for a single reaction, so multiply by the number of samples plus one (for pipetting error).

NEB Buffer #3	0.6 µl
150 µM SAM	0.4 µl
H_2O	0.5 µl
BcgI (1 U μl^{-1})	0.5 µl

**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 final single-reaction volume at 6 µl.*

3. Combine 2 µl master mix with each 4 µl DNA sample (6 µl total volume). Cover the plate with PCR film, incubate at 37°C (in air incubator or in a thermocycler with heated lid) for 1 hr.

4. If using a heat-sensitive enzyme (e.g. BcgI, AlfI), inactivate the enzyme at 65°C for 10 min then let the thermocycler return to room temp while keeping the heated lid on. Hold samples on ice after this.

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 primers (illBC-ii, antiBC-ii). The oligonucleotide combination used for each alternative 2b-RAD preparations and sequences of each oligo are provided at the end of this document. Pre-mixed adaptors can be stored just like normal oligos.

   For generic Adaptor 1, mix 5Ill-NNRW (10 µM) with the same volume of Anti5ill-NNRW (10 µM).

   For barcoded Adaptor 2, mix each par: 3IllBC(1-12) (10 µM) with the same volume of antiBC(1-12) (10 µM).

   Incubate at 42°C for 5 minutes then keep at room temperature until ligation.

2. Prepare 12 master mixes for ligations (one for each barcoded 3′ primer). 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(1-12)	1.0 µl
T4 DNA ligase (NEB M0202L)	1.0 µl
NFW (nuclease-free water)	14.5 µl

3. Combine 20 µl master mix with digested DNA (~25 µl total volume). For 96 well plate format we find it useful to prepare 12 master mixes, each with individual Adapter 2(BC), and distribute it with 8-channel pipette. Keep on ice while mixing.

   Incubate at 16°C for AlfI and BcgI, at 4°C for BsaXI, for at least 2 hours and up to overnight.

   Then heat at 65^{o}C for <u>at least</u> 20 min to inactivate the ligase (in thermocycler with heated lid to .

Pooling ligations into 12-plex groups

Samples barcoded at the 3′ end by different ligation Adapters 2 (1-12) are now pooled into groups of 12: each row of a 96-well plate, 3 µl from each well, is pooled into one sample. The result is 8 pooled samples for the initial 96 well plate.

Amplification

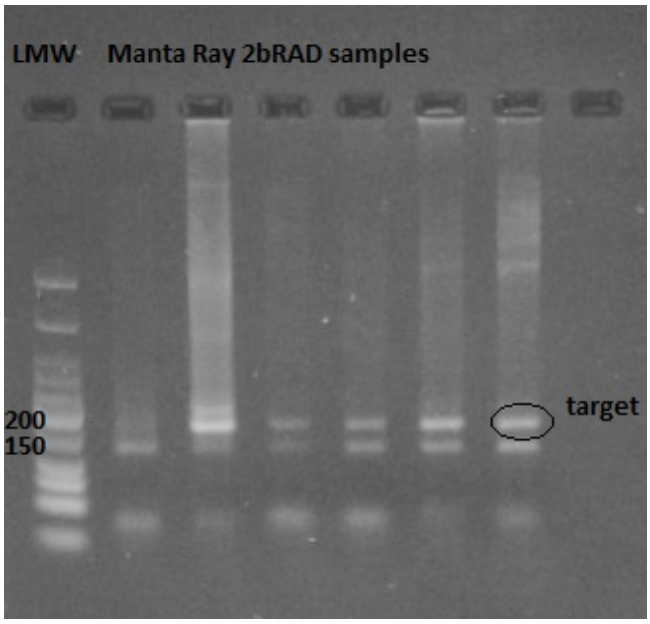
In this step, the constructs produced by ligation are amplified using a set of four primers that introduce pooled sample - specific barcodes and the annealing sites for HiSeq amplification and sequencing primers.

1. For each reaction prepare the following master mix:

NFW	7.2 µl
2.5 mM dNTP	2.5 µl
10 µM IC1-P5	0.4 µl
10 µM IC2-P7	0.4 µl
10 µM Mpx primer	0.3 µl
10x Titanium buffer	2.0 µl
Titanium Taq	0.2 µl (#Clontech 639208)

2. Combine 13 µl master mix with 4 µl ligation, add 3 µl of 1 µM Ill-Rad-bc (barcode-bearing) primer 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 14 cycles

3. Load 5 µl on a 2% agarose gel alongside LMW ladder (NEB N3233S) or other marker that has 150 and 200 bp bands - see next page.



NB: There should be no substantial variation in product amount among samples at this stage (unlike this gel photo); if there is, something went wrong with DNA quantity/quality of some original samples.

Confirm that all samples have a visible band at ~160-170 bp. You might also see a band below 150 bp, which is an artifact from the carried-over ligase (make sure to heat-inactivate the ligase before mixing the PCR reaction!)

If the product looks over-amplified (as bright or brighter than the second lane in the gel photo above), re-do amplification with two less cycles. If the 165 b product is visible but barely, simply add two more cycles to the same reactions.

If the 165 b product is not visible at all after 14 cycles, do not add more cycles. Instead, optimize previous steps: make sure that ligase and restriction enzyme did not expire, consider adding an extra purification step during DNA isolation, and make sure you start with at least 50-100 ng on DNA. Even though weak samples could be amplified with more PCR cycles, such samples would have poor representation of allelic diversity, i.e. will show lower counts after removal of PCR duplicates, and will often miss a second allele in a heterozygote.

If all is well (all samples have visible 165 bp band of the same intensity), all reactions can be pooled together and the target band extracted with PippinPrep or BluePippin (155-170 bp range). Most sequencing core facilities would do it for you. If not, gel-purify the band as described below.

Gel-purification

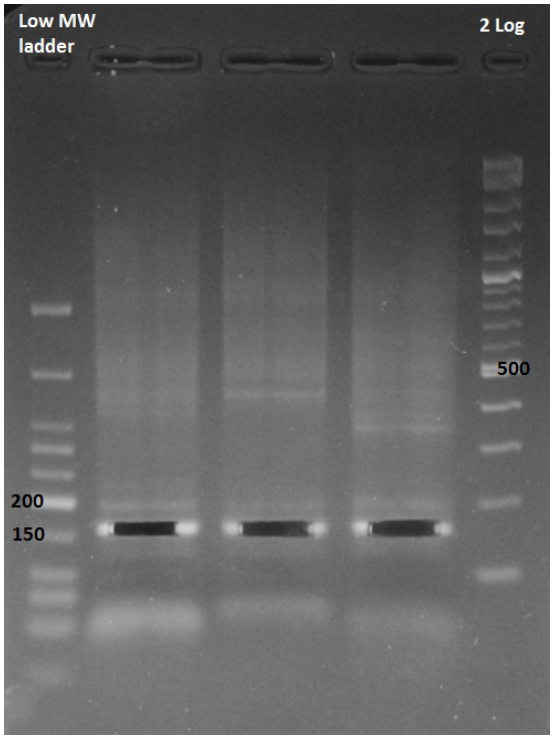
In this step the target band is gel-extracted to exclude high-molecular weight fragments and any chaff that may emerge during PCR (e.g., primer dimers).

1. Prepare a 2% agarose gel using TBE or TB. Use a wide comb that can accommodate 30-50 µl, or simply tape together two wells.

2. Load 30-50 µl of sample (40 µl sample + 10 µl loading dye) alongside LMW ladder. Run gel at low voltage for a long time until bands at 150bp and 200bp will clearly resolved. In our experience 100V for 70 minutes produce the good separation.

3. View the gel briefly (<20 seconds) on a UV or (possibly longer) on appropriate for your DNA dye black-light transilluminator set at low intensity to verify the presence of target band and adequate separation of molecular weight standards to resolve bands at ~165 bp and (possibly) below 150 bp. Typically ~5 cm run distance is sufficient. Photograph.

4. Cut out target ~165 bp band in a narrow gel slice, avoiding the edges of the lane (i.e., cut out the middle 70-75% of the band). Cut just inside the bottom boundary of the target band to avoid getting anything smaller.



**Note: at this stage a commercial gel-extraction kit can be substituted for the following three steps, if you feel more confident this way. In our practice, simply soaking the gel slice in water overnight, as described below, works just fine.*

5. Transfer each gel slice into a 1.5 ml microcentrifuge tube and add 20 µl NFW.

6. Make sure gel slice is in contact with water (cut or break it into a few, say 4-5, smaller pieces to make sure they sit comfortably at the bottom of the tube). Hold overnight at 4°C.

7. The following day transfer the eluate (~15 µl) into a new tube. This material is now ready for sequencing.

Oligonucleotide sequences for Illumina HiSeq

The repository includes the bulk-order excel file called **2bRAD_oligo_order.xlsx**, which can be directly uploaded to IDTDNA (the total cost as of January 2019 was $1845.36). These oligos can barcode 6 x 96 samples and should be sufficient for hundreds of 2bRAD projects in term of quantity. If you have more samples per project, order more TruSeq_Un oligos with additional indices (substitute TGACCA, ACAGTG, GCCAAT, CAGATC, GATCAG, or CTTGTA for the sequence written in red font). Each additional TruSeq_Un oligo adds capacity for 96 more samples. Note that each of TruSeq_Un oligos costs $97, so, for smaller projects, consider ordering less than six of them listed in the excel file.

For reduced-representation 2bRAD, all the NN in the excel file should be replaced with NG.