**Protocol for Illumina 2bRAD sample preparation** Galina Aglyamova (aglyamova@austin.utexas.edu) Mikhail Matz (matz@utexas.edu)

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 Qubit or picogreen, not just Nanodrop) and ensure that their OD 260/230 ratio is >=2.

Latest update – March 2019 – normalized oligoniucleotide names in the document to match oligo order list (**2bRAD\_oligo\_order.xlsx**).

**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).](http://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 ~180 bp band by Pippin or gel-electrophoresis (the only purification step in the whole procedure).

NOTE on experimental design: 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.

**Day 1, Digest**

1. Prepare samples each containing 100-200 ng of DNA in 4 µl, 25-50 ng/uL
   1. Equalize your input DNA samples well (based on qubit or picogreen, not just nanodrop) and ensure that their OD 260/280 ratio is >=1.8 And 260/230 ratio is ~2 or higher.
   2. Concentrating can be accomplished using by ethanol precipitation or by drying under vacuum.
2. Prepare a digestion master mix.

|  |  |  |
| --- | --- | --- |
| **Component** | **Reaction Vol (uL)** | **Total Volume (uL) 96 rxn+10% error** |
| NEB Buffer #3 | 0.6 | 63.36 |
| 320 µM SAM | 0.4 | 42.24 |
| H2O | 0.4 | 42.24 |
| BcgI (2 U µl-1) | 0.6 | 63.36 |
| **Total** | **2** | **211.2** |

\***Note:** SAM [S-adenosyl-methionine] comes at 32 mM stock, add 198 uL of NFW to 2 uL aliquots of 32 mM SAM.

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

|  |  |
| --- | --- |
| **Digestion PCR profile** | |
| **37ºC** | **60 min** |
| **65ºC** | **10 min** |
| **20 ºC** | **Continuously** |

**Day 1, 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).

* For a full plate, prepare Adaptor 1 in PCR tube, mix 60 µl of 5ILL-NNRW (10 µM) with 60 µl of Anti5ill-NNRW (10 µM).

|  |  |  |
| --- | --- | --- |
| **Adapter 1 Component** | **Reaction Vol (uL)** | **Total Volume (uL)** |
| 5ILL-NNRW | 0.5 | 60 |
| Anti5ill-NNRW | 0.5 | 60 |
| **Adapter 1** | 1 | 120 |

* For Adaptor 2, set up 12 PCR tubes, to each tube mix 5 µl of 3illBC(1-12) (10 µM) with 5 µl Anti-ill-BC(1-12) (10 µM).

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| --- | --- | --- |
| **Adapter 2 Component** | **Reaction Vol (uL)** | **Total Volume (uL)** |
| 3illBC(1-12) | 0.5 | 5 |
| Anti-ill-BC(1-12) | 0.5 | 5 |
| **Adapter 2** | 1 | 10 |

* 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.

|  |  |
| --- | --- |
| **Component** | **Reaction Vol (uL)** |
| NFW | 15 |
| 10x T4 ligase buffer w 10 mM ATP | 2 |
| 5 μM Adapter 1 | 1 |
| 5 μM Adapter 2  **\*Different for each column\*** | 1 |
| T4 DNA ligase | 1 |
| **Total** | **20** |

First make an initial master mix:

|  |  |  |  |
| --- | --- | --- | --- |
| **Component** | **Reaction Vol (uL)** | **Total Volume (uL)** 8 rxn+10% error | **Total Volume for 12 MM** |
| 10x T4 ligase buffer w 10 mM ATP | 2 | 17.6 | 232.32 |
| 5 μM Adapter 1 | 1 | 8.8 | 116.16 |
| T4 DNA ligase | 1 | 8.8 | 116.16 |
| **Total** | **4** | **35.2** | **464.64** |

Into 12 separate 0.6 mL tubes add:

|  |  |
| --- | --- |
| **Component** | **Total Volume (uL)** 8 rxn+10% error |
| Initial Master Mix | 35.2 |
| 5 μM Adapter 2  **\*Different for each column\*** | 8.8 |
| NFW | 132 |
| **Total** | **176** |

1. Combine **20 µl master mix** with digested DNA (~25 µl total volume).

* Note: 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**.

1. Incubate at **16°C** for BcgI for at least 2 hours and up to overnight.
2. Heat at 65oC for at least 20 min to inactivate the ligase (in a thermocycler with heated lid).

**Day 2, qPCR to ensure amplification success and quantification**

1. Prepare a qPCR Plate, for each plate you can run three rows of samples in duplicate and three negative control wells

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| --- | --- | --- |
| **Component** | **Reaction Vol (uL)** | **Total Volume (uL) (75 rxn+10% error)** |
| NFW | 5.53 | 456.23 |
| SYBR Green Mastermix | 7.5 | 618.75 |
| 10 uM TruSeq | 0.07 | 5.78 |
| 1 uM any ILLBC Primer | 0.7 | 57.75 |
| 10 uM P5 | 0.1 | 8.25 |
| 10 uM P7 | 0.1 | 8.25 |
| + ligation template | 1 | \_ |
| **Total** | **15** | **1155.00** |

1. Add 14 uL of mastermix to each well, avoid bubbles
2. Add 1 uL of ligation to each well
3. Centrifuge plate
4. Turn on qPCR machine, ensure the correct reaction volume (15 uL) is inputted, follow the 2bRAD template and ensure the plate is inserted correctly.

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| --- | --- | --- | --- | --- |
| **# Cycles** | **Step** | **Temperature** | **Acquisition** | **Time** |
| 1x | Pre-Incubation | 95C | None | 10 min |
| 40x | Amplification | 95C | None | 15 sec |
|  |  | 60C | None | 30 sec |
|  |  | 72C | Single | 30 sec |
| 1x | Melting Curve | 95C | None | 5 sec |
|  |  | 65C | None | 1 min |
|  |  | 97C | Continuous |  |
| 1x | Cooling | 40C | None | 1. sec |

1. Rank samples from highest to lowest CT score. If some CT scores are really high then pull them from the pool and use the next highest CT score as reference.
2. Ensure that these are not above a 25.5 threshold which are likely failed samples.

**Day 3, 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:

|  |  |  |
| --- | --- | --- |
| **Reagent** | **per sample** | **master mix (8 pooled samples + 10% error)** |
| dNTPs 2.5 mM ea | 1 | 8.8 |
| H2O | 14.4 | 193.6 |
| 10 µM IC1-P5 | 1 | 8.8 |
| 10 µM IC1-P7 | 1 | 8.8 |
| 10x Titanium buffer | 5 | 44 |
| Titanium Taq | 1 | 8.8 |
| **Total** | **31** | **272.8** |

|  |  |  |
| --- | --- | --- |
| **Reagent** | **per pool** | **8 pools (+error)** |
| dNTPs 2.5 mM ea | 1 | 8.8 |
| H2O | 14.4 | 126.72 |
| 10 µM IC1-P5 | 1 | 8.8 |
| 10 µM IC1-P7 | 1 | 8.8 |
| TruSeq\_Un1 | 0.6 | 5.28 |
| 10x Titanium buffer | 5 | 44 |
| Titanium Taq | 1 | 8.8 |
| **Total** | **24** | **211.2** |
|  |  |  |
| **To each sample add a different 1 uM ILL-BC oligo** | 6 |  |
| **To each sample add pooled ligation** | 20 |  |

Pool 6µL from each ligation across rows (e.g. 6µL each from A1-A12 into one pool)

1. Set up 8 PCR tubes, combine:

|  |  |
| --- | --- |
| **Component** | **Volume (uL)** |
| Mastermix | 31 |
| Pooled Ligation | 10 |
| 1 µM ILL-BC primer  **\*Different for each tube\*** | 6 |
| Add 20 uL NFW to 5 uL aliquot of 10 uM TruSeq primers to dilute to 2 uM  **\*Different for each set of 8 tubes\*** | 3 |
| **Total** | **50** |

1. Amplify as follows:

|  |  |  |
| --- | --- | --- |
| **Amplification PCR profile** | | |
| 70°C | 30 sec |  |
| **95°C** | **20 sec** |  |
| **65°C** | **3 min** | **X15-17 Cycles** |
| **72°C** | **30 sec** |  |
| 20 °C | Continuously |  |

1. Load 5 µl on a 2% agarose gel alongside LMW ladder (NEB N3233S) or other marker that has 150 and 200 bp bands.
   1. NB: There should be no substantial variation in product amount among samples at this stage

|  |  |
| --- | --- |
|  | |
| *Gel* | 2% Agarose Gel 300mL (large) |
| 6g Agarose |
| 285 ml DI |
| 15 ml 20X SB |
| 6ul EtBr |
| Single well combs |
| *Load* | 3ul DNA ladder |
| 2ul loading dye on parafilm |
| 5ul product |
| *Run* | 150 V for 30-35 min |

1. Confirm that all samples have a **visible band at ~180 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 180 bp product is visible but barely, add two more cycles to the same reactions.