**2bRAD 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. 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, either on its own or together with BcgI, is 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 duplicate-genotyping 3-5 samples in each 2bRAD experiment. Duplicates 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 genetic relatedness between samples.**

**Materials**

| **Reagent** | **Vendor/Cat #** |
| --- | --- |
| BcgI | NEB R0545S |
| SAM 320 µM | comes with BcgI |
| *(optional)* AlfI | Thermo Scientific ER1801 |
| NEBuffer #3 | NEB B7003S |
| T4 DNA ligase | NEB M0202L |
| Titanium Taq, with buffer | Clontech 639208 |
| dNTPs | NEB/N0447S |
| Eppendorf twin-tec 96-well PCR plate | Eppendorf  (VWR:95041-440): |
| Adhesive PCR plate Foil Seal | Fisher/UT Mkt:  AB-0626 |
| Quant\_IT Picogreen DS DNA Kit | Life Technologies P7589 |
| LMW DNA ladder | NEB N3233S |
| Ligated adaptor oligos \* # | IDTDNA |
| Barcode (index) oligos \* & | IDTDNA |
| P5 and P7 primers \* | IDTDNA |

\* see **2bRAD\_oligos\_order.xlsx** file; it lists oligos sufficient for a 576-sample experiment. If you have more samples per project, order more TruSeq\_Un oligos with additional indices (substitute GCCAAT, CAGATC, GATCAG, or CTTGTA for the sequence written in red font). Each additional TruSeq\_Un oligo adds capacity for 96 additional samples.

# Prepare ligated pseudo-double stranded adaptors by combining corresponding pairs of oligos (note: all oligo solutions mentioned here are in 10 mM Tris-HCl pH 8.0).

* For **Adaptor 1**, mix 5ILL-NNRW (10 µM) with the same volume of 10 µM Anti5ill-NNRW.
* For **Adaptor 2** (versions 1 to 12), combine 10 µM 3illBC(1-12) with the same volume of corresponding 10 µM antiBC.
* Incubate at 42°C for 5 minutes then keep at room temperature until ligation. Pre-mixed adaptors can be stored at -20oC for several months.

& Dilute ILL-BC and Truseq\_UN oligos to 2 µM in 10mM Tris-HCl, pH 8.0; store at -20oC for a few months. Each sample would require 0.125 µl of each of these stocks.

**Digest**

1. Dilute samples to contain **the** **same amount of DNA (50-100 ng) in 4 µl**. The DNA has to be high purity, with OD 260/230 ratio >1.8. It is very important to equalize input samples well: making sure there is no residual RNA, and using Qbit or picogreen (not just Nanodrop). Concentrating can be accomplished by drying under vacuum.   
   Pipet samples into 96-well plate(s).
2. Prepare a digestion master mix:

Number of samples x 1.1 =

| **Reagent** | **per sample** | **Volume in master mix** |
| --- | --- | --- |
| NEB Buffer #3 | 0.6 |  |
| SAM (included with BcgI) | 0.4 |  |
| H2O | 0.5 |  |
| BcgI (1 U µl-1) | 0.5 |  |

1. Pipet master mix into 8-tube strip (26 µl per tube per sample plate). Use multi-channel pipette to **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 60°C for 20 min** then let the thermocycler return to room temp while keeping the heated lid on. Hold samples on ice after this.

**Ligation**

1. Prepare 12 master mixes for ligations (one for each of the twelve Adaptor 2), in strip-tubes.  
  
 Number of rows in all 96-well sample plates x 1.1 =

| **Reagent** | **per sample** | **Volume in master mix** |
| --- | --- | --- |
| 10x T4 ligase buffer | 2 |  |
| Adaptor 1 | 0.5 |  |
| Adaptor 2 | 0.5 |  |
| H2O | 16 |  |
| T4 DNA ligase | 1 |  |

*NOTE: this is the stage at which reduced tag representation (RTR) can be achieved by using modified 5ILL-NNRW and 3illBC oligos, not with NN but with NG bases at their 3’-ends.*

1. Use multichannel pipette to combine **20 µl** master mix with digested DNA, **each master mix going into a specific column** on the plate. Mix by pipetting. Keep the plate on ice while mixing.

3. Incubate at **4°C** overnight.

4. Heat at **55oC for 30 min** to inactivate the ligase, in a thermocycler with a heated lid.

*Optional:* Test PCR for a random selection of 8 ligations :

Master mix:

| **Reagent** | **per sample** | **master mix** |
| --- | --- | --- |
| dNTPs 2.5 mM ea | 0.4 | 3.4 |
| H2O | 10 | 85 |
| 10 µM IC1-P5 | 0.4 | 3.4 |
| 10 µM IC1-P7 | 0.4 | 3.4 |
| 2 µM ILL-BC oligo | 1.2 | 20.4 |
| 2 µM TruSeq\_UN oligo | 1.2 | 20.4 |
| 10x Titanium buffer | 2 | 17 |
| Titanium Taq | 0.4 | 3 |

Add 16 µl of master mix to 8 strip-tubes, then add 4 µl of randomly chosen ligations.

Amplify as follows:

70°C 30 sec, then (95°C 20 sec, 65°C 3 min, 72°C 30 sec) X 15cycles

Load 3 µl on a 2% agarose gel alongside LMW ladder or other marker that has 150 and 200 bp bands. Confirm that all samples have a **visible band at ~180 bp** (See photo on next page).

5. Pool ligations by row in strip-tubes, using **3 µl from each well.** The 96-well plate is now reduced to 8 pooled samples, each corresponding to the original row.Store the ligations at 4oC.

**Amplification and barcoding**

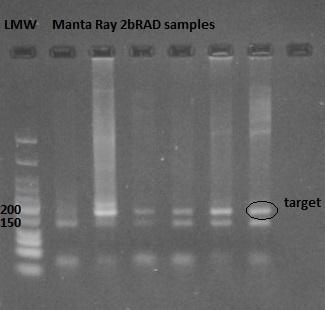
1. Prepare the following master mix:

Number of pooled samples x 1.1 =

| **Reagent** | **per sample** | **Volume in master mix** |
| --- | --- | --- |
| dNTPs 2.5 mM ea | 1 |  |
| H2O | 25 |  |
| 10 µM IC1-P5 | 1 |  |
| 10 µM IC1-P7 | 1 |  |
| 10x Titanium buffer | 5 |  |
| Titanium Taq | 1 |  |

1. Aliquot the master mix into strip-tubes, then use multi-channel to add **34 µl** to new 96-well plate. Each column in this plate corresponds to one whole original 96-well sample plate.
2. Pipet the eight 2 µM **ILL-BC oligos** into a strip tube. Use multi-channel pipette to add **3µl** of them to the plate with pooled ligations (**each oligo to its own row**).
3. Pipet as many 2 µM **TruSeq\_UN oligos** as there are columns in the **pooled** plate into a strip tube. Use multi-channel pipette to add **3 µl** of them to the pooled ligations plate (**each oligo to its own column**).
4. Add **10 µl** of pooled ligations.
5. Amplify as follows:

70°C 30 sec, then (95°C 20 sec, 65°C 3 min, 72°C 30 sec) X 15cycles

1. For a random subset of 4-6 samples. load 3 µl on a 2% agarose gel alongside LMW ladder or other marker that has 150 and 200 bp bands. 

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 ~170-180 bp** (actual target size is 176 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 176 b product is visible but barely, add two more cycles to the same reactions.

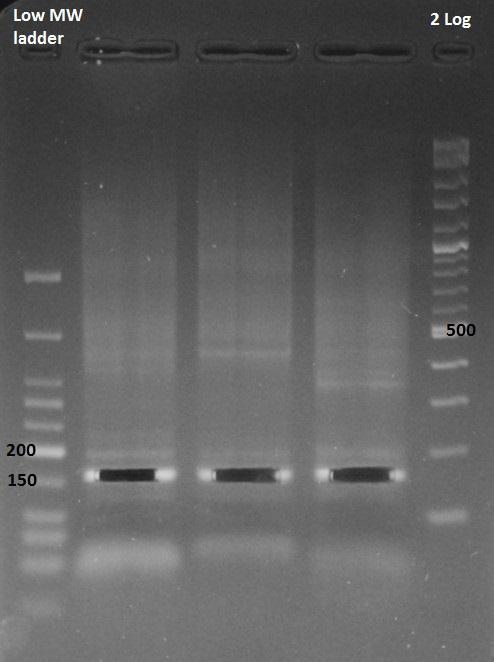
**If the 176 b product is not visible at all after 15 cycles, do not add more cycles**. Even though weak samples might be possible to amplify with more PCR cycles, such samples would have poor representation of allelic diversity (will show lower counts after removal of PCR duplicates and will often miss a second allele in a heterozygote). To troubleshoot, optimize previous steps. **Three common problems** are poor DNA quality (make sure OD 260/230 is 1.8 or higher), incorrect DNA quantification in presence of RNA, or expired enzymes (ligase and restriction enzyme).

**Pooling and concentrating**

1. Combine **2-5 µl** from each reaction into a single 1.5ml tube to have the final volume of about 100 µl.
2. Add **1.8x volume of AMPure beads** to the pooled sample; mix by pipetting sample until the beads are uniformly mixed. Incubate at room temperature for 15 minutes.
3. Place the tube on the magnet for 5 minutes to collect the beads. Carefully remove the supernatant without disturbing the beads. Wash the beads 2X with 100µl 80% EtOH. Discard the ethanol washes.
4. Dry the sample for 5 minutes. Do Not Overdry!
5. Resuspend the sample in **63µl** of water by uniformly mixing by pipetting up and down 10 times; incubate at room temperature for 2 minutes.
6. Place the tube on the magnet for approximately 5 min to collect the beads.
7. Transfer **60µl** of the purified cDNA into a new tube.

**Extract the target 180 bp band** with PippinPrep or BluePippin (170-190 bp range). Most sequencing core facilities would do it for you. If not, gel-purify the band as described below.

**Gel-purification**



1. Prepare a 2% agarose gel using TBE or TB. Use a wide comb that can accommodate 30-40 µl, or simply tape together two wells.
2. Load **35 µl** of sample (30 µl sample + 5 µl loading dye) alongside LMW ladder. Run gel at low voltage for a long time until bands at 150bp and 200bp will be clearly resolved. In our experience 100V for 70 minutes produces 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 ~170-180 bp and (possibly) below 150 bp. Typically ~5 cm run distance is sufficient. Photograph.
4. Cut out target 176 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.*

1. Transfer each gel slice into a 1.5 ml microcentrifuge tube and add 20 µl H2O.
2. 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.
3. The following day transfer the eluate (~15 µl) into a new tube. This material is now ready for sequencing. Minimal sequencing depth is 1-2M SE-50 reads per sample, which corresponds to about 100 samples per HiSeq lane. Sequence twice more if you have resources. The required number of reeds is tenfold less for the RTR version. Sequence on HiSeq 2500 if you have it; on HS 4000 add 20% phiX.

**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 terms 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 TruSeq\_Un oligo costs $97, so, for smaller projects, consider ordering less than six of them listed in the excel file.

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