2bRAD Protocol (Eli Kallison Edits)

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). It is composed of 6 primary 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. Overnight ligation at +4°C, then heat-inactivate the ligase for 10 min at 65oC.
3. Pool ligations
    - Pool ligations with different 3' barcodes into groups of 12 (because we have 12 different 3' ligation adapters). Determine proportions from gel picture (regular pcr) or by qPCR.
4. Amplification and barcoding of pooled ligations.
    a. Adds row-specific barcoded primers while amplifying our DNA, and allows us to eventually pool all rows and columns of a plate into a single tube because each will have a unique pair of barcodes. This means 96 samples can be sequenced in 1 lane.
5. Purification
    - Purify the target 170 bp band by gel-electrophoresis (the only purification step in the whole procedure).
6. Quantification and mixing in equal proportions.

Step 1: Restriction Digest

1. Prepare a 4 µl high-quality genomic DNA sample containing 200-500 ng DNA.
    a. Calculate volume of DNA needed for 500ng DNA (ideally all samples should have the same amount of DNA).
    b. Vortex samples, and pipette volume calculated into a 96 well plate
    c. Place plate in speedvac with temperature turned off and using the V-AQ setting (usually 30 minutes - 1 hour)
    d. Rehydrate DNA with 4 ul PCR water and wait 20-30 minutes

2. Prepare a digestion master mix on gold super-cooling metal rack on ice. The following recipe is for a single reaction, so multiply by the number of samples plus some small amount for pipetting error.
    - For one plate (96 samples), make 110 reactions worth of master mix to account for loss.

Order	Reagent	x1	x110	Notes
1	NEB Buffer #3.1	0.6 uL	66 uL	Vortex before using. Thaw, then keep on ice.
2	H_2O	0.5 uL	55 uL	Put this in an autoclaved 1.5ml tube.
3	150uM SAM	0.4 uL	44 uL	Breaks down quickly, dispose after a few uses. Minimize freeze/thaw. Thaw, then keep on ice.
4	BcgI (1 U uM^{-1})	0.5 uL	55 uL	Enzyme, get this out last and mix by pipetting. Minimize freeze/thaw. Always keep on ice.

Note: reagents are found in fridge under 2nd bench in freezer. Use the box that says "prepared" not "stock, unless there is no "prepared" left.

    a. Get 2nd ice baths. 1 is for strip tubes in gold plate, 2 is for 1x row of 8 strip tubes to dole out your master mix (MM) into. Strip tubes are in front cabinets (far R cabinet on far L bottom drawer).
    b. Make the MM in autoclaved 1.5ml tube, label, and keep on ice.
    c. Add reagents in the order marked in table, and vortex MM before adding Enzyme.
    d. Invert to mix after adding Enzyme, quickly spin down on small tabletop centrifuge.

e. Dole out MM evenly to 1x8 strip tubes in ice bath 2.
    i. 220 µl (total volume of MM) / 8 = 27.5 µl into each

3. Combine 2 µl master mix with each 4 µl DNA sample (6 µl total volume) using the pink multi-channel micropipette. Change tips every time you add, and check after that everything is even.
    a. Cover this preparation with a drop of mineral oil (reduces evaporation). To do this, use P1000 set to a high capacity and just drip it out by gently depressing plunger.
    b. Seal the plate with PCR plastic strips seal (above 2nd bench). Place this covered plate in the centrifuge (next to PCR machines) for a few seconds to spin down.
    c. Set in PCR machine (make sure when closing you get the hood under the lip so that it is fully sealed, and close tightly).
    d. Click Saved Files -> Set to the ALF1_Digest protocol to Incubate at 37°C 1 hr. **Make sure volume is set to 6 ul.**
    e. To increase digestion efficiency add 0.5 µl of enzyme after the initial 1h incubation, spin down, and incubate for one more hour before heat-inactivation (2h total incubation).
    f. Inactivate the enzyme at 65°C for 20 min.
    g. Put samples in freezer if not using samples within 1 day.

Step 2: Ligation

In this step adaptors are ligated to the restriction fragments produced above.

1. Prepare double stranded adaptors by combining each pair of primers (illBC-ii, antiBC-ii) in a large tube rack.
    a. Get ice bath, plates, a 96 well tube rack, and a 12 rack gold block
    b. Put adapters for each column (Adapter 2) and Adapter 1 tubes into rack (lots of tubes) into 96 well tube rack
    c. Label 12 empty tubes MM 1-12 (1 per column), which will be used to mix MM (Adapters will eventually be added). Put these on ice in a 12 rack gold block.

    d. For generic **Adaptor 1**, mix 5Ill-NN (10 µM) with the same volume of anti-Ill (10 µM).
        - For 96 well plate of samples use **70uL 5Ill-NN + 70uL anti-Ill** and combine in 1 tube labeled Adapter 1.
    e. For barcoded **Adaptor 2** (adds column-specific barcoded adaptors), mix each pair: 3IllBC(1-12) (10 µM) with

the same volume of antiBC(1-12) (10 µM).
   i. For 96 well plate of samples use for each pair **6uL 3IllBC (1-12) + 6uL = 12uL antiBC (1-12**).
f. Incubate both combined adapters at 42°C in thermocycler or heat block for 10 minutes then keep at room temperature until ligation.

2. Prepare 12 tubes of master mix on ice for ligations (one for each barcoded 3' primer / one per column). This recipe is for a single reaction, so scale up as needed. For 1 plate, make 10 reactions worth per master mix (8 samples +2 extra).
   a. Thaw reagents and then keep on ice

Order	Reagent	1X rxns	10X rxnx	Notes
1	NFW (nuclease-free H20)	14.5 µl	145 µl	
2	10X T4 ligase buffer	2.5 µl	25 µl	Do not confuse with T4 DNA Ligase
3	5 µM Adaptor 2(1-12)	1.0 µl	10 µl	Vortex; column-specific adapter
4	5 µM Adaptor 1	1.0 µl	10 µl	Vortex; general adapter for all columns
5	T4 DNA ligase (enzyme)	1.0 µl	10 µl	Get out last, mix by inversion and spin down quickly. Always keep on ice.

Note: you will have a total of 120 reactions worth total for one plate of samples (x96)

3. a) Combine 20 µl master mix with 6 µl of digested DNA (26 µl total volume). Can use multichannel pipette to transfer MM 1-8 to each row in columns 1-8. Then, transfer MM 9-12 to each row in columns 9-12.

----------OR----------

4. b) Place 20 µl master mix into a new plate and then mix DNA from digestion into the plate using multichannel pipette for both (26 µl total volume).

3. Run the 2bRAD ligate program in thermocycler to: Incubate at 16°C for 16 hours then reduce to 4°C. To end 4C and end ligation, click "Skip step" which will start heating to 65C for 10 min and inactivate the ligase.

---------- 16 hours ----------

4. Store ligation plate in -24°C

Step 3: Test amplification for pooling ligations into 12-plex groups

Here, we test whether our last steps worked by amplifying our samples and running them on a gel.

1. Get gold ice bath and place Phusion Mastermix w/ HF buffer in to thaw (thaws on ice). Each tube of Phusion MM has 1.2ml when new.
2. Put a new 96 well plate on the ice and label as test amplification.
3. Make ready all barcode adapters into the 96 tube rack to thaw.
4. Prepare master mix on ice. Prepare for 100x reactions in a 2ml tube. Mix everything into master mix before distributing for test amplification.
   a. Note: tubes with blue caps are stock concentration.

Order	Reagent	1X rxns	100X rxnx	Notes
1	Nuclease-Free Water	1.9 µl	190 µl	
2	10 µM IC1-P5	0.4 µl	40 µl	Vortex. Actual primer.
3	10 µM IC2-P7	0.4 µl	40 µl	Vortex. Actual primer.
4	10 µM Mpx primer	0.3 µl	30 µl	Vortex. Actual primer.
5	1-8 barcoded 1µM ILL RAD	3.0 µl	300 µl	Mix 1-8 primers in even proportions *
6	Phusion mastermix w/ HF buffer	10.0 µl	1 ml	Keep on ice. #NEB M0531S

**We could use just 1 barcoded primer for test amplification, but we don't want to draw them down unevenly so we mix all 8 in even proportions*

5. Distribute 16 µl master mix to each well of the new plate on ice.
6. Add 4 µl of ligated DNA fragments from each well of ligation plate and amplify as follows: (program: "2BRAD15CYCLE" and make sure volume is correct):

70°C (30 sec) -> 95°C (20sec) -> 65°C (3 min) -> 72°C (30sec) -> 4°C (Hold)
^---------- x14 ----------<

7. While PCR is running prepare a large 2% agarose gel (must fit 96 samples + a few ladders).
    a. Gel: 250mL TBE + 5g agarose + 12.5uL EtBr with two 50 cell combs
8. Add 5uL of Orange loading dye (in an 8 strip tube) directly into each well of your PCR plate using multichannel pipette, seal tubes with plastic, vortex, and spin down.
9. Add 12.5uL from PCR plate to each well of the large gel (slow multichannel pipette dispense speed down to 4).
10. For the ladder add 6uL of Low Molecular Weight ladder (LMW).
11. Add 15uL of ethidium bromide to the buffer on the RED side (+) of the gel rig and mix before starting.
12. Run at 105 volts for ~65-70mins (Check a few times while running)
13. Take image of gel.
    a. Put gel into machine
    b. Open ImageLab on gel computer (password: Garden2016)
    c. New protocol -> application -> nucleic acid gels -> ethidium bromide
    d. Image exposure -> faint bands -> unclick highlight saturated pixels
    e. Click position gel
    f. Position gel correctly, adjust zoom, and check on software
    g. Run protocol
14. Estimate the brightness of the bands on a gel.
    a. Score band brightness from 5-20 in increments of 5, with 20 being the LEAST bright (you will have four possible scores 5, 10, 15, 20; see FIGURE 1).
    b. Make a plate map in excel with scores assigned to corresponding plate positions. The scores correspond to the volume (uL) of DNA from your ligation plate you will transfer from each well when pooling samples (i.e. if you have 5 you will take 5uL from well).
15. Pool samples by row using values from above.
    a. Using your map to guide you, all samples in a row will become a pool (add every sample from all wells in 1 row into 1 tube). Samples should be pooled into an 8 well strip tube. Use a normal pipette to manually transfer volumes 1 by 1.
    b. Note: Each such group (you will end up with 8 such groups in a 96 well plate) works as a single sample in the subsequent Amplification and Purification.

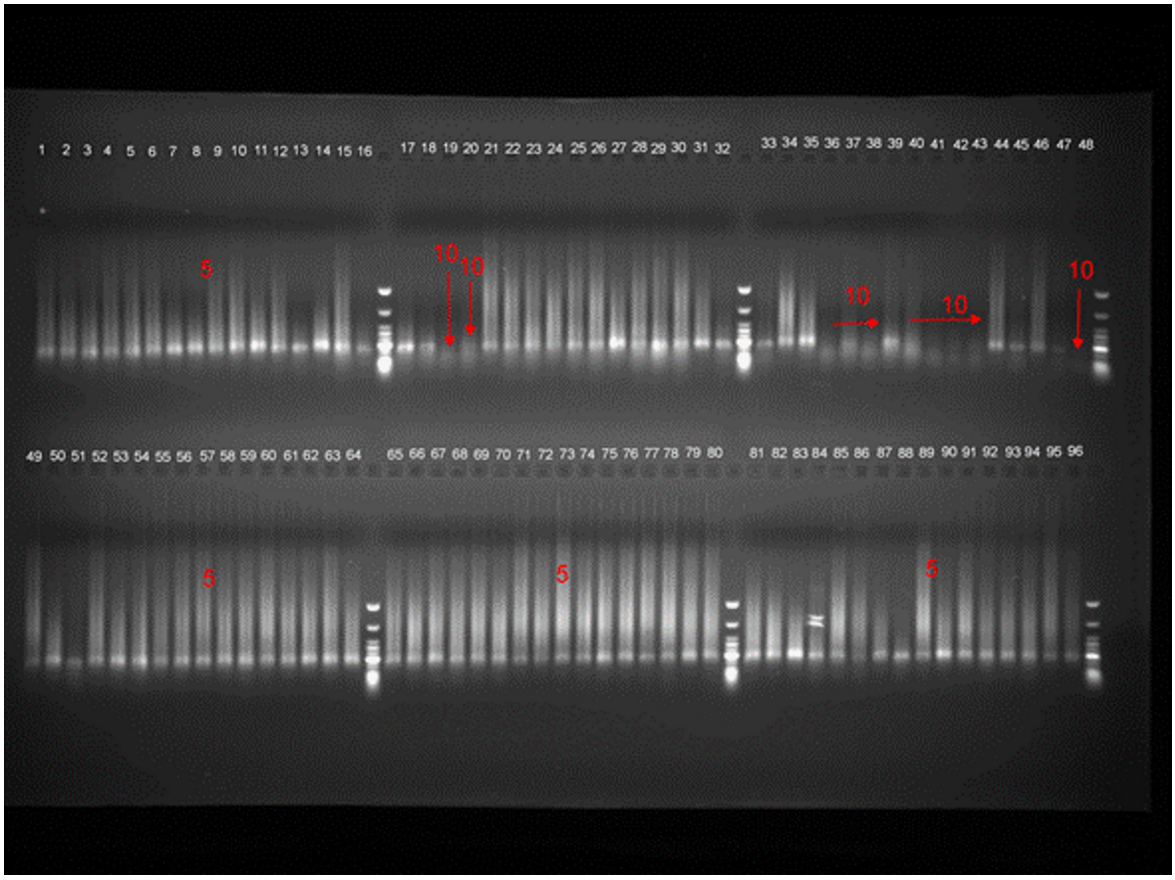

Figure 1.
96 samples run on a test PCR of 14 cycles (cycle at which all samples produced a clear band). All samples mark 5 or unmarked are of high efficacy (5uL added to pool). Samples marked 10 are of lower efficacy (10uL added to pool).

Step 4: Large Scale PCR
This step adds row-specific barcoded primers while amplifying our DNA, and allows us to eventually pool all rows and columns of a plate into a single tube because each will have a unique pair of barcodes. This means 96 samples can be sequenced in 1 lane.

1. Prepare the following master mix for 8 pools into a new 1.5ml tube

Order	Reagent	1x Pool	8x Pool	Notes
1	Nuclease-Free Water	4.75 µl	38 µl	
2	10 µM IC1-P5	1 µl	8 µl	Vortex. Actual primer.
3	10 µM IC2-P7	1 µl	8 µl	Vortex. Actual primer.
4	10 µM Mpx primer	0.75 µl	6 µl	Vortex. Actual primer.
6	Phusion mastermix w/ HF buffer	25 µl	200 µl	Keep on ice. #NEB M0531S

2. Combine 30 µl master mix with 10 µl pooled ligation (our samples, grouped 1-8) into a new 8 strip tube.
3. Add 7.5 µl of 1µM Ill-Rad-bc (barcode-bearing) primer individually to each reaction
4. Amplify in thermocycler using the program: "2BRAD15CYCLE" and make sure volume is correct (40ul):

70°C (30 sec) -> 95°C (20sec) -> 65°C (3 min) -> 72°C (30sec) -> 4°C (Hold)
^---------- x14 ----------<

Step 5: Purification

In this step the target band is gel-extracted to exclude high-molecular weight fragments and any primer dimers.

1. Prepare a large 2% agarose gel using a deep (taped) 10 well comb. 250ml TBE + 5g Agarose + 12.5ul EtBr
2. Add 5ul of orange loading dye to each sample.
3. Load the entire volume of each reaction alongside 6 µl low-molecular weight ladder.
4. 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.
5. Image the gel in order to ensure amplification and good separation of bands
6. Prepare a tube rack with labeled 1-8 1.5ml tubes.
7. Using a DNA dye black-light transilluminator and scalpel (wear proper eye and skin protection), cut out target band of 170 bp in a narrow gel slice, avoiding the edges. Put in the appropriate prepared labeled tubes.
    a. On computer, selection Gel Imaging -> manually set exposure time to 999+ -> Run Protocol
8. Weigh each sample for next step, and turn heat block up to 50 C.
9. Run the slice through a MinElute Gel Extraction Kit (cat# 28604, Qiagen)
    a. Protocol Link: https://www.qiagen.com/us/resources/resourcedetail?id=0d0d73a7-e906-4a74-8f11-3b70311d7d66&lang=en
    b. Use purple MinElute Spin Column 50 in fridge

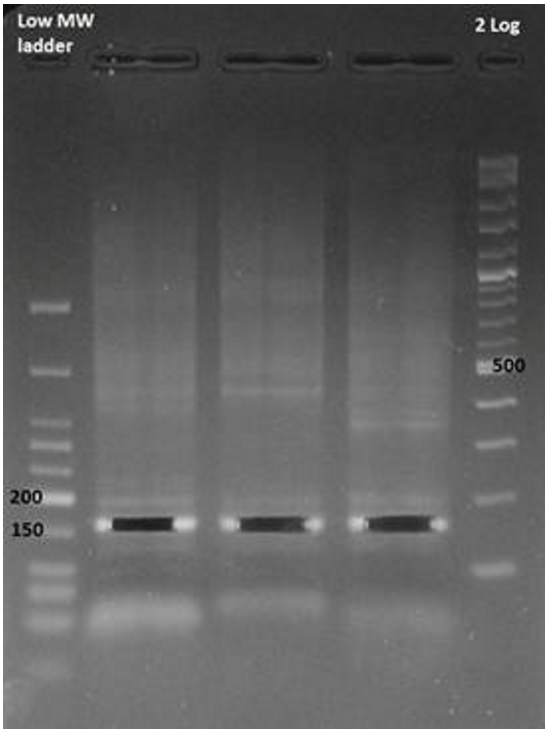

Figure 3. Gel Extraction

Step 6: Pooling for Sequencing

In this step samples pools are quantified, converted to nM, and pooled into a single library for sequencing on an Illumina platform.

1. Quantify the 8 sample pools using a Qubit fluorometer, record the concentration in ng/uL.
    a. Use 1uL of DNA from pooled samples instead of the normal 2uL. Fill to QuBit assay tubes to 200uL with 199uL working solution instead of 198uL.
2. Calculate the nM of the sample using the following equation (use the excel table below to calculate)

$$\left(\frac{Concentration\ (\frac{ng}{uL})}{\frac{660g}{mol}xfragment\ size}\right)x10^6 = nM$$

   a. Pools should be above 1ng/uL in order for the procedure below to work.
3. If pools are >1ng/uL, add 4uL of each sample to strip tube
4. Dilute with NFW to exactly 10nMol (calculated in table)
5. Take 3.75uL from each sample and add to a tube (8 pools * 3.75uL = 30uL library at 10nMol concentration)
   a. Or, as sequencing facility requires. This will ensure that all pools are represented equally.

Example Spreadsheet:
Weinmannia2020_Plate1.xlsx

Pool	ng/ul	nMol	total volume sample + h2O	add _ul water for 10nM
1	2.5	=(B7/(660*170))*10^6	=(C7*4)/10	=D7-4
2	1.67	=(B8/(660*170))*10^6	=(C8*4)/10	=D8-4
3	1.97	=(B9/(660*170))*10^6	=(C9*4)/10	=D9-4
4	3.46	=(B10/(660*170))*10^6	=(C10*4)/10	=D10-4
5	3.22	=(B11/(660*170))*10^6	=(C11*4)/10	=D11-4
6	2.82	=(B12/(660*170))*10^6	=(C12*4)/10	=D12-4
7	5.06	=(B13/(660*170))*10^6	=(C13*4)/10	=D13-4
8	3.78	=(B14/(660*170))*10^6	=(C14*4)/10	=D14-4

Original Protocol: