2b-RAD Protocol July 2019

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(Adapted from 2b-RAD Protocol, June 2017 Mikhail Matz and Galina Aglyamova)

Protocol: Restriction Digest

Use type 2b restriction enzyme to digest out genomic fragments that will make up our RAD library

Note: For best results, proceed only with 100-200 ng (see Pico green protocol) intact, high-quality genomic DNA in 4 uL (as little as 25 ng/uL). This concentration can be accomplished by vacuum drying or ethanol precipitation. We have successfully prepared samples from as little as 50 ng of DNA.

- Materials: 96 well plate or PCR strip tubes, ice bucket, waste container
- Reagents: NEB Buffer #3, SAM (320 µM), Bcg1 (2,000 U/ml), milliQ water
- Equipment: PCR tube spinner, thermocycler

1) Using the quantifications from the Pico green assay to prepare DNA samples each containing a total of 100-200 ng in 4 µl of milliQ water.

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.

Reagent	Vol. (µl)
NEB Buffer #3	0.6
320 µM SAM	0.4
BcgI (2,000 U/ml)	1.0

- SAM concentrations vary by vendor. Adjust your amount accordingly. For 150 µM SAM, add 0.12 µL per reaction

3) Combine **2 µl** of master mix with **4 µl** of each DNA sample (6 µl total reaction volume). Spin down tubes and ensure that they are sealed.
Avoid bubbles at this step! Don't pipet to the second stop.

4) Incubate in thermocycler for **1 hour at 37°C** for digestion reaction.

5) If using a heat-inactivated enzyme (e.g. BcgI) add 20 minutes at 65°C to heat inactivate the restriction enzyme*.

6) Return samples to ice

Protocol: DNA Ligation
Ligate adapters onto the fragments produced by the restriction digest.

*Note the number and amount of differently barcoded Adapters you will need depends on how you plan to pool your samples. Be sure to plan out pooling strategy before continuing.

- Materials: P20 multichannel pipet, PCR strip tubes, ice bucket, waste container

- Reagents: milliQ water, 10x T4 ligase buffer, 10mM ATP, 5 µM Adapter 1 (see step 1 below), 5 µM Adapter 2, T4 DNA ligase

- Equipment: Heat block or thermocycler, PCR tube spinner

1) The adapters are double stranded oligonucleotides (oligos) with sticky ends that will hybridize with the restriction fragments. In this step, prepare the double stranded adapters by mixing the correct pairs of single stranded oligos.
    a. For Adapter 1 (the generic adapter), mix equal volumes of 5Ill-NNRW (10 µM) and anti-IllRW (10 µM) for a final concentration of 5 µM. The final volume should be enough Adapter 1 to run all ligation reactions (0.5 µl per reaction).
    b. For Adapter 2 (barcoded adapter), mix equal volumes of 3IllBC(1-12) (10 µM) and antiBC(1-12). The final volume of each Adapter 2 solution should be enough to run ligation reactions for all samples that will use the particular barcode. These will be the barcodes for your columns.

2) Prepare a ligation master mix for each barcoded adapter (Adapter2). The following recipe is for a single reaction. Multiply each volume by the number of samples (+ a little extra ~.5 µl/ sample). **Avoid bubbles!!** Don't pipet to the second stop. Adapter 1* and Adapter 2* are the mixtures of the forward and reverse adaptors.

Reagent	Vol. (µl)
milliQ water	15.5
10x T4 ligase buffer + ATP	2.5
5 µM Adapter 1*	0.5
5 µM Adapter 2*	0.5
T4 DNA ligase	1.0

Master mix preparation tips:
- ✓ Add reagents to master mix in order of least expensive to most expensive to minimize the cost of making a mistake.
- ✓ Make sure your total volume will fit in the tube you plan to mix it in BEFORE you start adding reagents. You may have to use two tubes.

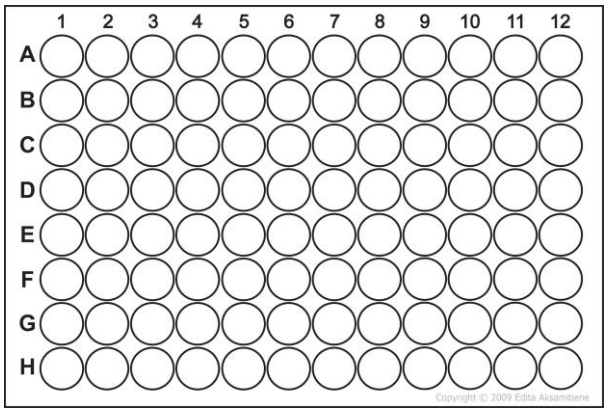
- ✓ Check off your reagents as you go to help keep track of what has been added.

- ✓ Make a master mix with every reagent except Adapter 2 first, pipette this mix into 12 tubes and then add the relevant Adapter 2 (BC 1-12) to respective tube.

3) Combine 20 µl of appropriate master mix with remaining 6 µl of digested DNA sample (25 µl final volume).
    a. Here it is easiest to assign each master mix to a column (each with a different 3Ill-BC) and use a multichannel pipet
    b. Keep ligations on ice while pipetting

4) Incubate at 4°C overnight
   Note: Try to keep it around 12 hours to avoid over-ligating.

5) After incubation, heat at 65°C for 10 minutes to inactivate ligase



96-well plate format

**Columns 1-12 will contain 3Ill-BC (1-12), such that the column number matches the barcode number. Later, a unique BC will be added to each row (A-H), so that every sample has a unique combination of both the 3Ill-BC and Ill-RAD-BC. For example, A1 might contain 3Ill-BC1 and Ill-BC67, whereas A2 would contain 3Ill-BC2 and Ill-BC67. This secondary barcode is added during the amplification.

Protocol: Test PCR amplification to check efficiency of ligation reactions
Run a test PCR on all samples, make sure to run samples that will be pooled later on the same gel.

- Materials (for PCR): 50X Titanium taq polymerase (we have found this to work better than phusion), MPX, P5, and P7 primers, any Ill-BC, dNTP (10 mM), 10X Buffer, sterile water

- Equipment: Thermocycler

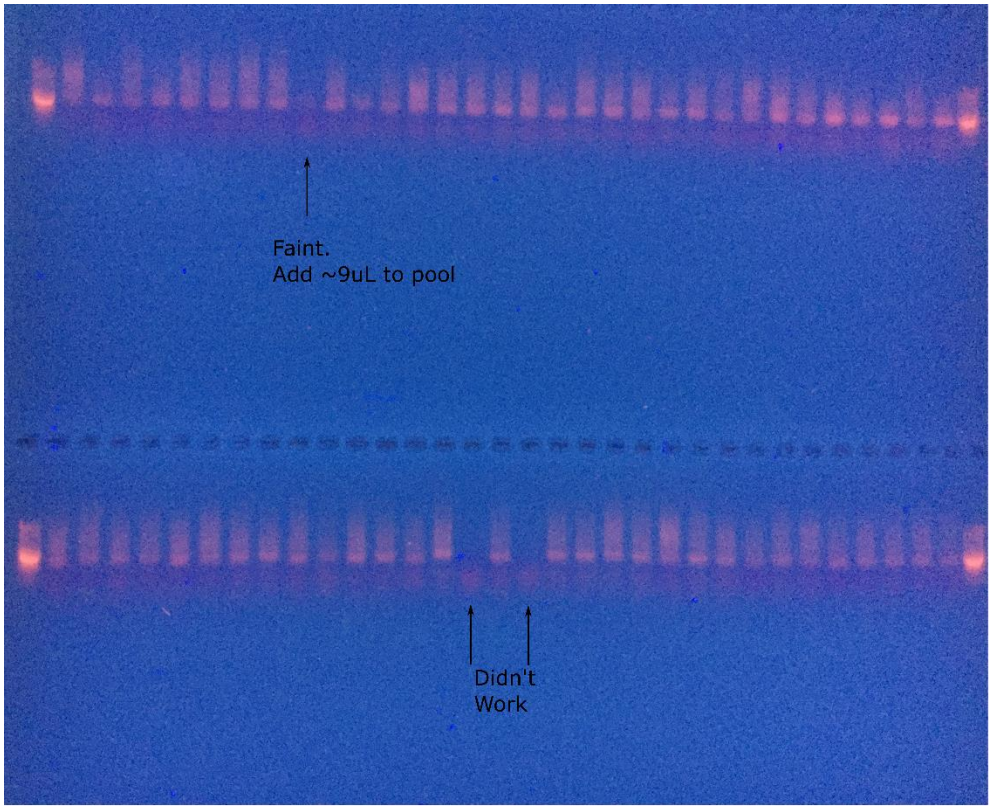
1) Prepare the master mix below. The following recipe is for a single reaction. Multiply each volume by the number of samples (+ a little extra).

milliQ water	12.4 µL
10X buffer	2 µL
dNTP (10 mM)	0.5 µL
50X Titanium Taq	0.4 µL
10 µM MPX primer	0.15 µL
10 µM Ill-BC	0.15 µL
10 µM P5	0.2 µL
10 µM P7	0.2 µL

2) Aliquot 16 µL of the master mix and 4 µL of the ligation template.
3) Amplify with the following PCR program:
    a. 95 °C 1 min (95 °C 40 sec, 63 °C 1 min, 72 °C 30 sec) x 14 cycles
4) Run 3 uL on a 1.5% gel to check for equal brightness across samples. (If using NEB low molecular weight ladder dilute ladder as follows: 1 µL ladder, 1 µL gel loading dye purple 6x no SDS, 4 µL H20. Add 1 µL dye to each sample. Avoid ladder smear by running at low voltage for longer- for 150 uL gel ran for ~42 minutes at 130V).
5) If the bands are too faint to see, add another 2 cycles and run on the gel again.

GOAL is to minimize PCR duplicates, not necessarily by reducing PCR cycles but by reducing the ratio between the number of reads and number of original DNA molecules – increasing purity of DNA sample

If you don't get a band after 15 test PCR cycles, you will likely want to re-extract/re-purify the sample; otherwise, additional PCR cycles will only add more duplicates
You want a 170 bp band for all samples after 12-15 PCR cycles
Usually not worth sequencing more than 6-10 million reads per sample
Coral genome typically 500 million bp


Faint.
Add ~9uL to pool
Didn't
Work

Protocol: Pooling ligations into groups

The purpose of this step is to equally pool samples with different 3' barcode primers into groups of (up to) 12. You can have up to 8 groups of 12 on a 96 well plate. Each group serves as an individual sample in the following Amplification and Purification steps.

Ideally, if the same amount of DNA went into the digest and ligation, ligation can be pooled together in equal proportions. Don't pool all the material! Mix 3 µl of each ligation with **different 3' barcodes (!)** into one tube (usually one row on the 96 well plate). Seal the rest of the plate and store at -20C in case you will need to redo some samples.

Use the test pcr results to determine how much to pool (e.g. 3 µl for a bright band, 9 µl for a faint band).

Optionally, perform Qpcr to numerically equalize ligation before pooling (amendment)

Ligations always stay on ice when not in the -20C! NEVER keep them at room temperature!

Protocol: Amplification

Now you are operating with ligations mixed together as new samples. From this point on in this protocol, samples refer to groups of pooled ligations.

- Materials: Nuclease-free water, **2.5 mM** dNTPs, 10 IC1-P5, 10 uL IC2-P7, 10 uM Mpx primer, 10X Titanium Taq buffer, 50X Titanium Taq Polymerase
- Equipment: Thermocycler

1. Prepare the PCR master mix. The following recipe is for a single reaction. Multiply each volume by the number of samples (+ a little extra).

Reagent	**Vol (µl)**
Milli Q water	23.15
2.5 mM dNTP	6.00
10 µM Mpx Primer	0.35
10 µM IC-P5	0.50
10 µM IC-P7	0.50
10X Buffer	5.00
50X Titanium Taq	1.0

2. Combine 36.5 µL master mix with 10 µL of pooled ligations

3. Add 3.5 µL of 1 µM Ill-Rad-BC (barcoding-bearing) primer to each reaction. Total reaction volume will be 50 µL.

4. Amplify in a thermocycler using the following profile:

   95 °C 1 min (95 °C 40 sec, 63 °C 1 min, 72 °C 30 sec) for 14 cycles (or optimal # of cycles based on test pcr)

Protocol: Size selection and DNA purification

- Materials: Agarose, gel buffer, SYBR Green I nucleic acid stain, low molecular weight ladder, 6X loading dye, nuclease-free water
- Equipment: Gel box, UV light, balance, blue light

1. Add ~12 uL of 6X loading dye to each tube/well after PCR is complete and samples have cooled.

2. Centrifuge the tubes/plate briefly

3. Mix the samples with the loading dye by aspirating and dispensing the sample a few times before loading the well.

4. Run 50 uL of each reaction + ~12 uL loading dye on a large 2% agarose gel with SYBR Green nucleic acid stain (add 6 uL of SYBR for a 60 uL gel) .

5. Run the gel slowly at 90 V for ~60 minutes or until the bands between 150 - 200 bp are clearly resolved

6. Use a blue light gel illuminator to safely cut the 170 bp band with a clean razor blade
   Note: Be careful to cut only the 170 bp band. There might be another band at approximately 130 bp. This is a result of excess primers and should be avoided.
   Note: Use picture below to determine correct region for cutting.

7. Transfer the cut band to a clean 1.5 mL centrifuge tube

8. Add 15 uL of nuclease-free water to each tube. Make sure the water and gel pieces are in contact and incubate overnight at 4C to let DNA diffuse into the water from the gel.

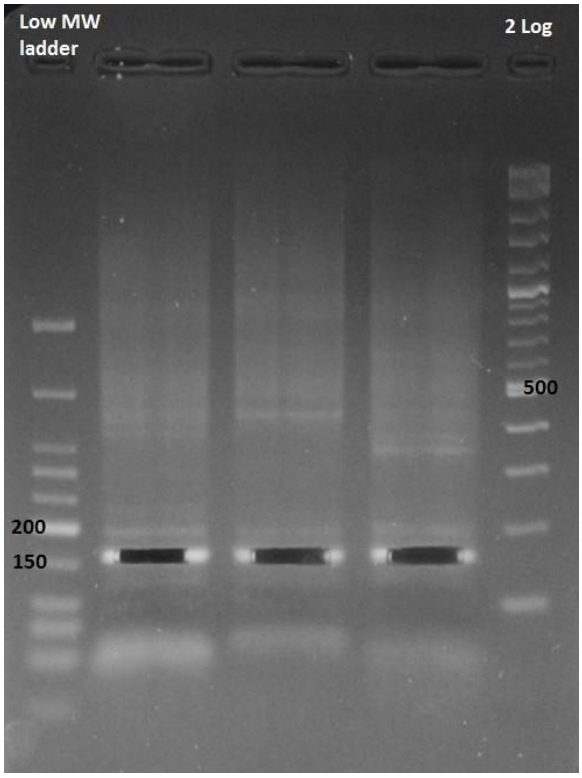
9. The following day, centrifuge each tube for 2 minutes at max speed.

10. Remove as much liquid as possible and dispense it into fresh strip tubes/96 well plate

11. Alternatively, use a gel-extraction kit like QIAGEN QIAquick Gel Extraction Kit CatN-28704 for example.

12. Run Quant-IT picogreen DS DNA assay (Life Technologies P7589) to determine the final concentrations of the eluted product in order to mix libraries in equal proportions. Total amount of DNA per sample should be 5 ng.

13. Store barcoded and size-selected DNA at -20C until further use



Note: Cut at black bar

Primers:

oligo	use	Sequence, 5'-3'	notes
3ILL-30TV	cDNA synthesis and amplification	ACGTGTGCTCTTCCGATCTAATTTTTTTTTTTTTTTTTTTTTTTTTTTTV	V=[ACG]
S-Ill-swMW	cDNA synthesis	ACCCCAUGGGGCUACACGACGCUCUUCCGAUCUNNMWGGG	RNA oligo; M=[AC], W=[AU]
5ILL	cDNA amplification	CTACACGACGCTCTTCCGATCT	
ILL-BC23	Barcoding	CAAGCAGAAGACGGCATACGAGATCCACTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGAT	the barcode is underlined
ILL-BC24	Barcoding	GCTACC	only the barcode
ILL-BC25	Barcoding	ATCAGT	only the barcode
ILL-BC26	Barcoding	GCTCAT	only the barcode
ILL-BC27	Barcoding	AGGAAT	only the barcode
ILL-BC28	Barcoding	CTTTTG	only the barcode
ILL-BC29	Barcoding	TAGTTG	only the barcode
ILL-BC30	Barcoding	CCGGTG	only the barcode
ILL-BC31	Barcoding	ATCGTG	only the barcode
ILL-BC32	Barcoding	TGAGTG	only the barcode
ILL-BC33	Barcoding	CGCCTG	only the barcode
ILL-BC34	Barcoding	GCCATG	only the barcode
ILL-BC35	Barcoding	AAAATG	only the barcode
ILL-BC36	Barcoding	TGTTGG	only the barcode
ILL-BC37	Barcoding	ATTCCG	only the barcode
ILL-BC79	Barcoding	ACGCGG	only the barcode
ILL-BC80	Barcoding	AGGGCG	only the barcode
ILL-BC81	Barcoding	CTGCAG	only the barcode
ILL-BC82	Barcoding	AACTTC	only the barcode
ILL-BC83	Barcoding	GGGTGC	only the barcode
ILL-BC84	Barcoding	TCCTGC	only the barcode
ILL-BC85	Barcoding	CGCGGC	only the barcode
ILL-BC86	Barcoding	ACCGCC	only the barcode
ILL-BC87	Barcoding	TAATAC	only the barcode
ILL-BC88	Barcoding	CACGTA	only the barcode
ILL-BC89	Barcoding	ATGTGA	only the barcode
ILL-BC90	Barcoding	TATAGA	only the barcode
ILL-BC91	Barcoding	TTTGCA	only the barcode
ILL-BC92	Barcoding	GTGCCA	only the barcode
ILL-BC93	Barcoding	CTAACA	only the barcode
ILL-BC94	Barcoding	ATAGAA	only the barcode
TruSeq-Mpx-2n	Barcoding	AATGATACGGCGACCACCGAAAAATACACTCTTTCCCTACACGACGCTCTTCCGAT	extends the linker at the 5' of the cDNA

TruSeq_Un1	Barcoding	AATGATACGGCGACCACCGAGATCTACAC ATCACG ACACTCTTTCCCTACACGACGCTCTTCCGATCT
TruSeq_Un2	Barcoding	AATGATACGGCGACCACCGAGATCTACAC ACTTGA ACACTCTTTCCCTACACGACGCTCTTCCGATCT
TruSeq_Un3	Barcoding	AATGATACGGCGACCACCGAGATCTACAC TAGCTT ACACTCTTTCCCTACACGACGCTCTTCCGATCT
TruSeq_Un4	Barcoding	AATGATACGGCGACCACCGAGATCTACAC GGCTAC ACACTCTTTCCCTACACGACGCTCTTCCGATCT
IC-P7	Final check	CAAGCAGAAGACGGCATACGA
IC-P5	Final check	AATGATACGGCGACCACCGA