

Burford Reiskind Lab Double Digest RAD sequencing Protocol
(Either digest over night or start in the morning for 3 hours same day):

Quantify your samples and dilute to 200 ng of DNA in a 26 uL vol (with molecular grade water)

1. 30 uL reaction volume:

26 uL DNA

3 uL buffer (CutSmart Buffer)

0.5 uL *SphI* (diluted enzyme down to 10 units per uL)

0.5 uL *MluCI* (diluted enzyme down to 10 units per uL)

30 uL Total Vol.

- a. Make a master-mix of **buffer and two REs** multiplied by the number of samples
 - b. ADD 4 uL of the master mix to the 26uL DNA sample for a total vol. of 30 uL
 - c. Incubate @ 37° C for 3 hours on a thermocycler, hold at 4° C until ready for next step (Thermocycler = *Aegytpi*; Program = *digest01*)
- 2. MagBead Purification step AMPure XP beads for Serial Digestions (NOTE: SKIP this step for main protocol, this step is for serial digests before the bioanalyzer. Alternatively can also use a PCR cleanup kit instead of the AMPure XP beads):**
- a. Add 45 uL of AMPure beads to each well (you add 1.5 x the vol from step one). Pump each up and down until the beads are well mixed
 - b. Incubate with beads for 5 mins @ RT
 - c. Collect beads in a magnetic plate, wait until the solution is clear
 - d. Pull of the solution (for this rxn set it to 75+ uL) and toss
 - e. Wash 1 x with 200 ul of freshly made 70% EtOH (note: leave the plate on the magnetic plate to avoid beads floating too high in the plate well if using the peg plate)
 - f. Pull up EtOH until you have an air bubble to dry (for this rxn set it to 200 uL)
 - g. Elute 35 uL H₂O, take off the magnetic stand and pump up and down to mix the beads well (note: if using the peg mag plate, make sure the beads are in solution, which may require some manual labor)
 - h. Place back on magnetic stand and recover 20 uL (this leaves a little in the bottom to avoid taking any beads, & set up ligation in second half of the plate)
 - i. Optional step: Quantify post-digest DNA concentrations on a plate reader (GSL) (this is a good idea for making sure you fall within the measurements required for the ligation step. Note: you will do this at least once per species to configure concentration of adapter per rxn [see * below])

3. *Ligation rxn vol of 45 uL:

25 uL of digestion

5 uL of adapter mix (combined P1 [0.02 uM] & P2 [3uM])

4.5 uL of 10x NEB Ligase Buffer (with 10 mM ATP)

10 uL molecular grade H₂O

0.5 uL of T4 DNA ligase

- a. Make a master mix of **buffer, water** and **ligase**
- b. To 25 uL of restriction digest add **5 uL** of adapter mix to each well
- c. Then add **15 uL** of master mix made in step **a** to each well
- d. Ligate for 1 to 2 hours @ Room Temperature (RT)
- e. Heat on a thermocycler @ 65°C for 20 minutes. Cool to 12°C forever

Thermocycler = *Aegypti*; Program = *ligation1* (22°C for 2h, 65°C for 20m, Hold 12°C ∞)

Note: Completed ligation can be stored at -20°C

4. Pooling library and purification step:

- a. Pool all 5uL of the 48 samples into a single 1.5 mL tube (total vol of 240 uL. This is your un-purified library that you will now purify).
- b. Make **two** purified library pools above (Lib#a & Lib#b)
- c. Follow the Protocol for the Qiaquick PCR cleanup kit doing two washes and elute to 34 uL (use 4uL to spec samples on Qubit) (in some cases you can do two elutions first to 100 uL then to 34 uL). You will spin for 30 seconds each until the elution, than spin for 60 seconds.
- d. You will have two 30 uL tubes of purified library for size selection.
- e. Make sure to label this as your **purified library** so you do not confuse it with the unpurified library plate. Place the unpurified plate in the freezer for storage.

5. Size selection on the PippinPrep:**

- a. Qubit the pooled and purified libraries. Note a large quantity of total DNA can run on the PippinPrep (maximum quantity 10 ug of DNA per lane on the BluePippin).
- b. Aliquot your 30 uL purified library for size selection (Pool 1a and 1b).
- c. Each pool runs on its own lane of the PippinPrep cassette (2 lanes total).
- d. Run on the PippinPrep to the specific fragment size you want to select. For our purposes we target the 200+ bp region, and we set the size selection range from 350 to 500 bps to account for the barcode adapters.
- e. Elute each aliquot to 40 uL two times and run the 40 uL elutions on the bioanalyzer to quantify and check size selection (quantities were approximately 200pg/uL).
- f. Pool the two 40 uL per pooled library (80 uL per pool) & calculate average concentration per pool* (NOTE: if there is little in the second elution, then do not pool the samples as this will lower the concentrations for PCR, we rarely use second elutions).

- g. The size-selected sample will be used for the PCR and sequencing steps below. (Note: you now will have an unpurified library plate, 2 pre-size-selection (4 uL), and 2 size-selected samples).

6. Amplification of size selected library:

Make 4 PCR rxns per pooled library within a reverse index (8 total PCRs):

PCR rxn vol 25 uL

10 uL Library (@ approximately 100 pg/uL)

12.5 uL PCR Taq MasterMix (2x)

1.25 uL 10uM Forward Primer

1.25 uL 10uM Reverse Primer

- a. PCR Conditions: 72°C(5min); 98°C(30sec); 18x(98°C(10sec), 65°C(30sec), 72°C(30sec); 72°C(5min); 4°C(forever)**
- b. Set up two sets of PCR reactions for each pool (using reverse primers P2.1 or P2.2 with the same forward primer P1) [Thermo = *Aegypti*; Prog = *ddRAD_PCR*]
- c. Pool 4 PCR rxns per pool (100 uL total) and clean-up on Qiagen column (QIAquick PCR Purification Kit). Follow manufacturer's directions and re-suspend in 34 uL
- d. Run the two pools on the Qubit to quantify DNA (4 uL of sample for analysis)
- e. Now combine the two pools making a total of 60 uL of the original library

7. Sequencing set-up for the HiSeq 2000 Illumina platform at UNC or NCSU:

- a. Submit a 1 to 25 dilution of the 60 uL amplified library to the bioanalyzer
- b. From this use the pmol/L (molarity) to calculate the nmol/L of the amplified library
- c. Dilute the stock amplified library to 10 nmol/L into 20 uL and return to the Genomic Science Laboratory for sequencing.
- d. Provide the indices to the GSL for each library for post-sequencing library de-multiplexing if more than one library is run per lane.

Supporting Information:

*To calculate the amount of adapters and ligase to add to this step of the reaction go to the spreadsheet labeled “ddRAD ligation molarity calculator_Reiskind2013” and go to the bioanalyzer sheet. We followed the method established by Elshire et al. (2011). Do this only once per species and then use the proper ratio of adapters to digested DNA. Note, to do this step you have to quantify post-digestion above. The proper ratio of adapters to sample DNA “sticky” ends is critical to the success of this method. Too few adapters encourage chimera formation among sample DNA molecules while too many results in formation of adapter dimers. Adapter dimers will produce DNA sequence data and waste reagents and machine time. Remember we will size select on the PP so this should exclude some of the adapter dimers.

1. **Size Selection:**

Submit each 30 uL elution to the NCSU GSL for size selection, and run each pool on its own lane of a PippinPrep cassette (2 lanes total). Size select samples on 200 bp fragments (using narrow size selection window), elute each pool 2X (40 uL per each).

Unpurified LIBRARY of 48 individuals			
30 uL of Purified Pool 1		30 uL Purified Pool 2	
Size Selection		Size Selection	
40 uL	40 uL	40 uL	40 uL

EXAMINE ALL 40 uL on the Bioanalyzer then combine per Pool, unless the quantities are low in the second elution. In most cases the first of the 40 uL was sufficient, and the second 40 uL was too dilute to use.

2. The length of the PCR extension can be shortened or lengthened to produce the optimal distribution of fragments
3. Using Poland’s et al. 2012 (Supplemental Material) barcode setup and library-specific index we designed our 48 unique, variable-length barcodes. The barcodes used in our study are listed in the data archive. In addition, we designed a sequencing primer to capture the unique index we designed to allow us to multiplex the two sets of 48. This primer is spiked into the sequence ready library and only used if you have two sets of 48 individuals per lane (5'CGGAAGAGCGGTTTCAGCAGGAATGCCGAGACCG 3').