

Protocol 2: *B. anynana* Double Digest Restriction-site Associated DNA (RAD)-Tag

This protocol for *B. anynana* has been modified from Peterson *et al.*, in review:

Peterson, B.P., J.N. Weber, E.H. Kay, H.S. Fisher and H.E. Hoekstra. Double digest RADseq: an inexpensive, reference-free method for de novo discovery and genotyping of 10^2 - 10^6 SNPs in model and non-model species.

III. RAD-tag Library Construction

A. Double Restriction Enzyme Digestion

1. Double digest 50-100ng (0.05-0.1ug) concentrations of *B. anynana* genomic DNA with 10 Units of NlaIII and MluCI (4-cutters) in NEBuffer 4 (New England Biolabs) and add water to produce a final volume of 30uL. Initially, some samples may be single-digested with each enzyme to assess cut frequency and success in the target genome.
2. Digest on a thermal cycler using the following conditions:
 - a. 65°C for 3 hours
 - b. 4°C incubation (overnight)
3. Clean and purify the digested genomic DNA with an Agencourt AMPure XP PCR purification kit (Beckman Coulter) following the suggested protocol but modified to use a 1.5X volume of beads and a SPRIPlate Super Magnet Plate (Beckman Coulter). Elute the purified, digested genomic DNA in Buffer EB (Qiagen) to produce a final volume of 30uL.
4. Quantify the cleaned genomic DNA using a 2100 Bioanalyzer (Agilent Technologies) using the Bioanalyzer DNA High Sensitivity Kit (Agilent #5067-4626). Alternatively, DNA may be quantified using a Qubit fluorimeter (Invitrogen) using the protocol provided by the Quant-IT dsDNA High-Sensitivity Assay Kit for 0.2-100 ng (Invitrogen #Q32851).

B. Adapter Ligation

1. Determine the ligation efficiency for *B. anynana* using a “Ligation Molarity Worksheet” (Peterson *et al.*, in review).
 - a. Ligation efficiency is calculated with initial mass of double-digested DNA (0.05-0.1ug), concentrations of both Adapter 1 (P1; oligos 1.1 and 1.2; Peterson *et al.*, in review) and Adapter 2 (P2; oligos 2.1 and 2.2; Peterson *et al.*, in review), target adapter fold excess, target adapter volume per reaction, annealed adapter concentration, and desired volume of intermediate adapter dilution.
 - b. The end calculation is annealed adapter stock in addition to volume of 1X annealing buffer (diluted from 10X annealing buffer: 100mM Tris HCl (pH 8), 500mM NaCl, and 10mM EDTA).
 - c. Note, P1 and P2 adapter sequences can be found in “ddRAD oligo table” (Peterson *et al.*, in review). Of note, flex adapters were used for both P1 and P2 (adapter_P1-flex and adapter_P2-flex) which are compatible with NlaIII and MluCI.

2. Make adapters at 4uM (4 pmol/ul) concentration and then dilute to the proper ligation efficiency.
3. Combine dilutions of uniquely barcoded P1 adapters, the same P2 adapter, 50ng digested DNA, T4 DNA ligase (New England BioLabs), T4 DNA ligase buffer (New England BioLabs), and 1X annealing buffer to produce to 40uL total volume and mix thoroughly.
4. Incubate ligations on a thermal cycler using the following specifications:
 - a. 37°C for 30 minutes
 - b. Heat kill at 65°C for 10 minutes
 - c. Cool at 2°C every 90 seconds until room temperature.

C. Sample Pooling

1. Combine equal amounts of ligated genomic DNA from each sample to create a pool of individuals.
2. Clean and purify each pool as previously described in **A3** with the Agencourt AMPure XP PCR purification kit (Beckman Coulter) with a 1.5X ratio of bead solution to remove any unligated adapters and dual-adapter ligation products.
3. If multiple pools are made, combine each eluted pool of 40uL and repeat purification until a final volume of 30uL is achieved.

D. Size Selection

1. Determine the appropriate size of digested, ligated genomic DNA to use for amplification and sequencing. Here, 300bp was chosen.
2. Samples were loaded in 30uL amounts with 10uL loading solution (Sage Science). Additionally, 2% marker solution (Sage Science) was loaded in 40uL volume into 2% Pippin Prep cassette lanes with a fragment size range set between 262 and 308bp and run on the Pippin Prep apparatus (Sage Science)(Figure 5).
3. Samples were eluted in 40uL volumes of buffer solution (Sage Science).

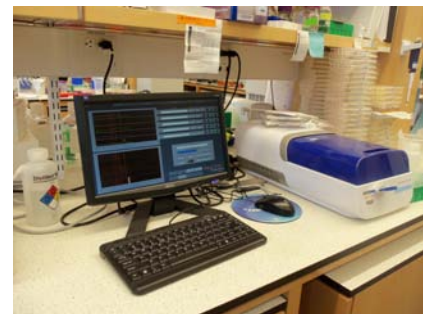


Figure 5: A Pippin Prep (Sage Science) apparatus was used to size select regions of ligated genomic DNA in the Hoekstra lab during library construction.

E. PCR of Ligated DNA products and Final Illumina Library Amplification

1. Aliquot size-selected sample DNA elutions into 8 PCR reactions totaling 20uL to attach to all fragments (Peterson *et al.*, in review):
 - a. Illumina-specific annealing sequences
 - b. Multiplexing indices (12 different unique P2 indices)
 - c. Sequencing primer annealing regions to all fragments
2. PCR reactions were composed of:
 - a. 2uL P1 primer (final concentration 2uM)
 - b. 2uL P2 primers with specific multiplexing indices (final concentration 2uM)

- c. 4uL 5X-HF Phusion™ buffer (New England BioLabs)
 - d. 0.4uL dNTPs
 - e. 6uL eluted, size-selected, ligated genomic DNA
 - f. 0.2 uL Phusion™ High Fidelity DNA polymerase (New England BioLabs #M0530L).
 - g. H2O to a final reaction volume of 20uL
3. Run samples at the following conditions:
 - a. 98C for 30s
 - b. 12 cycles with 98C for 40s, 65C for 30s, and 72C for 30s
 - c. 72C for 7 minute incubation (1 cycle)
 - d. Indefinite 4C incubation

F. Final Cleanup of Amplified Genomic DNA RAD-tagged Libraries

1. Combine amplified PCR products from all 8 reactions into one eppendorf tube.
2. Clean with Agencourt AMPure XP beads as previously described in **A2** and **C3**.
3. Elute in a final volume of 40uL.
4. Analyze RAD-tag libraries on a 2100 Bioanalyzer (Agilent Technologies) using the Bioanalyzer DNA High Sensitivity Kit (Agilent #5067-4626) to assess quantity of library and size of fragments.
5. Combine libraries in equimolar ratios to compose a final, pooled library for each sequencing lane, but using specific preferences from the sequencing facility.