# ddRAD Library Preparation Protocol (96-Well Plate Format)

This protocol is adapted from ddRAD protocols written in the laboratories of Catherine Linnen (University of Kentucky) and Adam Leaché (University of Washington), and is based on the methods of Peterson *et al.* 2012 (*PLoS ONE*). This version was written at the University of Kansas Biodiversity Institute by Paul Hime and Pietro de Mello, with input and edits from Jenny Hackett at the KU GSC.

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- This protocol assumes that 96 gDNA samples are arranged in one 96-well plate.
- The gDNA samples are assumed to be intact, and standardized to 50 µl volumes at 20 ng/µl.
- This protocol starts with 10 µl of each sample transferred to a new 96-well plate.
- This protocol can be completed in 2–3 days. Store any library prep products at 4 C overnight, do not freeze until the very end of the protocol.
- Please keep each set of 24 samples (4 sets per plate) separate at the end. After each of these are quantified and run through the TapeStation, we will decide how to pool.

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## Step 0. Setup

Day 1

- Make 13 mL 70% ethanol, keep cold
- Take AmpureXP beads out of fridge, bring to room temperature

Day 2

- Make 3 mL 70% ethanol, keep cold
- Take AmpureXP beads out of fridge, bring to room temperature

## Step 1. Restriction Enzyme Double Digestion

Restriction enzyme (RE) digestions are performed in 20 µl volumes.

- Make master mix 1 (MM1)

MM1

| | | |
|---|---|---|
| 2 µl NEB CutSmart 5X Buffer | **x 110** | 220 µl NEB CutSmart 5X Buffer |
| 7.2 µl $H_2O$ | | 792 µl $H_2O$ |
| 0.4 µl *Sbf*I RE | | 44 µl *Sbf*I RE |
| 0.4 µl *Msp*I RE | | 44 µl *Msp*I RE |
| | | **1100 µl total** |

- Add 10 µl MM1 to each sample, pipette mix
- Incubate at 37 C for 3 hr, heat-kill enzymes at 65 C for 10 min, hold at 4 C

## Step 2. Adapter Ligation

Adapter ligations are performed in 26 µl volumes.

- Add 1 µl P1.X adapter (4 µM) to each sample, pipette mix
- Make master mix 2 (MM2)

MM2

| | | |
|---|---|---|
| 3.125 µl NEB T4 Ligase Buffer<br>0.625 µl $H_2O$<br>0.625 µl P2 Adapter (40 µM)<br>0.625 µl T4 Ligase | **x 110** | 343.75 µl NEB T4 Ligase Buffer<br>68.75 µl $H_2O$<br>68.75 µl P2 Adapter (40 µM)<br>68.75 µl T4 Ligase |

**550 µl total**

- Add 5 µl MM2 to each sample, pipette mix
- Incubate at 23 C for 1 hr, then 65 C – 23 C touchdown (2 C in 90 sec intervals), hold at 4 C
- After incubation, make 4 pools of 24 samples each (A1–H3, A4–H6, A7–H9, A10–H12)
- NOTE: There are 24 unique P1 adapters (P1.1–P1.24), each with a different inline barcode.
- See Figure 1 below.

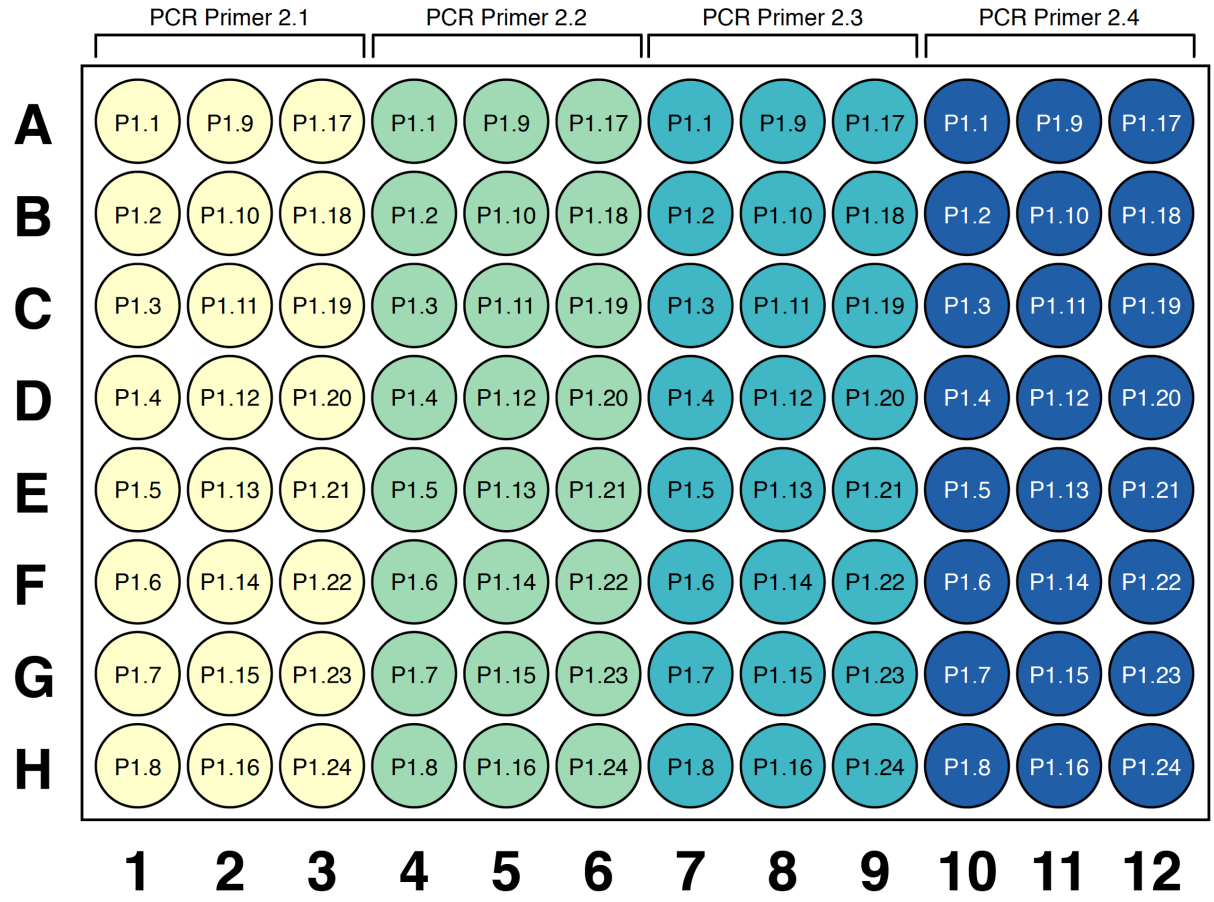


**Figure 1.** Arrangement of 96 samples in the plate. Samples are grouped into four batches of 24 (three consecutive columns). Each group of 24 gets the same set of 24 unique P1 adapters containing inline

barcodes (A1–H3, A4–H6, A7–H9, A10–H12). Each group of 24 samples in each plate gets a unique PCR primer (PCR Primer 2.X) containing a unique Illumina index. For a set of three plates, plate 1 gets PCR Primers 2.1–2.4, plate 2 gets PCR Primers 2.5–2.8, and plate 3 gets PCR Primers 2.9–2.12.

**Step 3. Post-Ligation Bead Clean (bead clean in 1.5X ratio of beads to sample)**

For each pool of 24 samples (624 µl each):

- Add 936 µl Ampure XP beads
- Pipette mix
- Incubate 5 min
- Place on magnet rack 5–10 min (until clear)
- Remove supernatant
- Add 1.4 ml 70% ethanol, incubate 30 sec, remove supernatant
- Add 1.4 ml 70% ethanol, incubate 30 sec, remove supernatant
- Optionally
    - Spin briefly for 3 sec
    - Place on magnet rack ~30 sec (until clear)
    - Remove supernatant
    - Repeat as needed
- Dry beads on 37 C heat block ~3–5 min (until beads have a matte finish and start to crack)
- Resuspend in 52 µl $H_2O$ (you will probably only recover 50 µl)
- Incubate 5 min
- Place on magnet rack 5–10 min (until clear)
- Collect eluate

**Step 4. Post-Ligation Quantification**

- Quantify each sample with a Qubit HS assay (use 1 µL for quantification)

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**SAFE PAUSE POINT**

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**Step 5. Size Selection**

- Load each sample into its own lane of the Blue Pippin 2% agarose cartridge
- Run on protocol with 541 bp target, using internal standards.
    - Blue Pippin Protocol:
        - Protocol name: Hime_ddRad_Internal
        - Cassette type: 2% DF Marker V1
        - Use internal standards
        - BP Target 541 bp (487–595 bp) in "Tight" Mode
- Collect samples, bring to 40 µl with $H_2O$ (if below that volume)

**Step 6. Post-Size Selection Bead Clean (bead clean in 1.5X ratio of beads to sample)**

For each sample pool (40 µl each):

- Add 60 µl Ampure XP beads
- Pipette mix
- Incubate 5 min

- Place on magnet rack 5–10 min
- Remove supernatant
- Add 200 µl 70% ethanol, incubate 30 sec, remove supernatant
- Add 200 µl 70% ethanol, incubate 30 sec, remove supernatant
- Optionally
    - Spin briefly for 3 sec
    - Place on magnet rack ~30 sec (until clear)
    - Remove supernatant
    - Repeat as needed
- Dry beads on 37 C heat block ~1–3 min (until beads have a matte finish and start to crack)
- Resuspend in 34 µl $H_2O$ (you will probably only recover 32 µl)
- Incubate 5 min
- Place on magnet rack 5–10 min (until clear)
- Collect eluate in strip tubes

**Step 7. Post-Size Selection Quantification (and possibly pool subdivision)**

- Quantify each sample with a Qubit HS assay (use 1µL for quantification)
- If any samples exceed 10 ng total DNA, split these sample pools into equal subsets and continue through with one replicate of each sample (label and store any duplicates at -20 C).

**Step 8. PCR Amplification and Library Indexing**

PCR reactions are performed in 50 µl volumes.

- Make master mix 3 (MM3) (need to adjust the 4.5X factor below if any samples were split)

MM3

| | | |
|---|---|---|
| 10 µl NEB Phusion Buffer (5X) | **x 4.5** | 45 µl NEB Phusion Buffer (5X) |
| 4.5 µl $H_2O$ | | 20.25 µl $H_2O$ |
| 1 µl dNTPs (10 mM) | | 4.5 µl dNTPs (10 mM) |
| 2 µl PCR Primer 1 (10 µM) | | 9 µl PCR Primer 1 (10 µM) |
| 0.5 µl Phusion Polymerase | | 2.25 µl Phusion Polymerase |
| | | **81 µl total** |

- Add 2 µl PCR Primer 2.x (10 µM) to each sample pool, pipette mix
- Add 18 µl MM3 to each sample pool, pipette mix
- Incubate at 98 C for 30 sec, run 12 cycles of (98 C for 10 sec, 65 C for 20 sec, 72 C for 60 sec), hold at 72 C for 10 min, then hold at 4 C

**Step 9. Post-PCR Bead Clean (bead clean in 1.5X ratio of beads to sample)**

Note that if multiple, replicate PCR reactions were performed for any sets of 24, combine these in a single 1.5 mL tube prior to the bead cleaning (combine same sets of 24).

For each sample pool (50 µl each):

- Add 75 µl Ampure XP beads
- Pipette mix

- Incubate 5 min
- Place on magnet rack 5–10 min
- Remove supernatant
- Add 200 µl 70% ethanol, incubate 30 sec, remove supernatant
- Add 200 µl 70% ethanol, incubate 30 sec, remove supernatant
- Optionally
    - Spin briefly for 3 sec
    - Place on magnet rack ~30 sec (until clear)
    - Remove supernatant
    - Repeat as needed
- Dry beads on 37 C heat block ~1–3 min (until beads have a matte finish and start to crack)
- Resuspend in 27 µl $H_2O$
- Incubate 5 min
- Place on magnet rack 5–10 min (until clear)
- Collect eluate
- If working in BI's Molecular Lab, take 2 x 2 µl aliquots of each final library for quantification and fragment analysis at GSC.
- If working in GSC, directly take 2 x 1 µl aliquots of each final library for quantification and fragment analysis.
- Store final libraries at -80 C

**Step 10. Final Library Quantification and Fragment Analysis**

- Quantify each sample (set of 24 individuals) with a Qubit HS assay (use 1µL for quantification)
- Run each sample (set of 24 individuals) on a TapeStation HS D1000
- There will be four separate pools of 24 individuals each at the end. Users will do final pooling.

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**Reagent List (per 96-well plate)**

| | |
|---|---|
| Ampure XP beads (Agencourt) | 4.3 mL |
| *Sbf*I (NEB) | 44 µL |
| *Msp*I (NEB) | 44 µL |
| T4 Ligase (NEB) | 68.75 µL |
| Phusion polymerase (NEB) | 2.25 µL |
| NEB CutSmart Buffer (5X) | 220 µL |
| NEB T4 Ligase Buffer | 343.75 µL |
| NEB Phusion HF Buffer | 45 µL |
| NEB Phusion dNTPs (XX µM) | 4.5 µL |
| Fresh 70% ethanol | ~20 mL |
| | |
| P1.X adapters (4 µM) | 4 µL each |
| P2 adapter (40 µM) | 68.75 µL |
| PCR primer 1 (10 µM) | 9 µL |
| PCR primers 2.X (10 µM) | 8 µL each |