ddRAD-Seq protocol V.2

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BEFORE STARTING

Quantify DNA and check its quality on an agarose gel. DNA should be of high molecular weight.

Purchase adaptors as described in:
1 Digest 300ng of DNA with the enzymes AvaI and MspI (New England Biolabs) in a total volume of 30 μl. Perform digestion in a PCR machine at 37°C for 3 hours, followed by inactivation of the enzymes at 65°C for 20 min. Confirm digestion is complete by running 4ul of the digest on a 2% agarose gel.

2 Ligate adaptors to the sticky ends of the digested DNA using T4 ligase (New England Biolabs) in 40 μl reactions by incubating for 2 hours at 23°C in a PCR machine, then inactivate the enzyme at 65°C for 20 min.

3 Perform a PCR on a subset of samples to test that the ligation was successful. PCR amplifications are performed in 12 μl reactions with 1× MyTaq reagent buffer (Bioline, Australia), 5 pmol of each primer (the oligo P2.2-Msp and the unique forward oligo used to make the adaptor used for that particular ligation). PCR thermocycling conditions are an initial denaturation of 2 min at 94°C, followed by 30 cycles of 94°C for 20 sec, 55°C for 20 sec and 72°C for 30 sec, then a final extension at 72°C for 5 min. Load 5ul of PCR product on a 2% agarose gel next to unamplified ligated DNA to confirm amplification.

4 Pool ligations for each index. Purify each index pool with a Qiaquick PCR purification kit (Qiagen), following the manufacturer’s instructions except elute in 35 μl of Buffer EB and leave on the column for 1 min prior to centrifugation. Leave the final elution in an open 1.7ml tubes on a heating block set at 40°C for 20 min to ensure no EtOH is present.

5 A gel rig for running the pooled index should be prepared by cleaning the gel apparatus with 70% ethanol. Each pooled index will be loaded into a single well so adjacent wells may need to be taped together to create sufficiently wide wells. An empty well should be left between each index, to avoid cross-contamination and a suitable ladder included at both sides on the gel. Fresh buffer should be used. Pour a 2% agarose and load the samples, then run at 80V for 80 minutes.

6 Prepare 1.7ml tubes by labeling them with the index and the size range to be collected. It is recommended to remove several gel slices per index (e.g., 300-500 bp, 500-700 bp) and to store the spares at -20°C as backups. Use a new razor blade for each index and try and minimize the time the gel is exposed to UV light during gel excision.

7 For the size range that will be processed, weigh the gel slice and subtract the weight of the tube. Process each index by putting it through a Qiaquick gel extraction kit (Qiagen), following manufacture’s instructions except use 30 μl buffer EB for the final elution and leave the elution buffer on the column for 4 min prior to centrifugation.

8 Quantify each index with a Qubit dsDNA HS (high sensitivity) assay kit (Thermo Fisher Scientific), following manufacturer’s instructions.

9 Perform 4-6 PCRs per index to add the Illumina sequences to the fragments. Perform PCRs in 20 μl reactions with 1x Phusion flash high fidelity PCR master mix (Thermo Scientific), 4 pmol of each primer (PCR1 and PCR-Index, with PCR-Index being a unique primer for each index), and 5 μl gel-extracted ligation.

10 Quantify one PCR of each index with a Qubit dsDNA HS (high sensitivity) assay kit (Thermo Fisher Scientific) to confirm that DNA concentration has increased with the PCR.

11 Combine the PCRs for each index and purify with a MinElute kit (Qiagen), eluting in 12 μl Buffer EB.

Quantify each index with a Qubit dsDNA HS (high sensitivity) assay kit (Thermo Fisher Scientific). Combine each index.
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