**2bRAD Protocol**

**Day 1: Digest**

1. Prepare samples each containing 200 ng of DNA in 4 µL, 50 ng/µL
2. Prepare a digestion master mix.

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| --- | --- | --- |
| **Component** | **Reaction Vol (µL)** | **Total Volume (µL) 96 rxn+10% error** |
| NEB Buffer #3 | 0.6 | 63.36 |
| 320 µM SAM | 0.4 | 42.24 |
| BcgI (2 U µL-1) | 1 | 105.6 |
| **Total** | **2** | **211.2** |

\***Note:** SAM [S-adenosyl-methionine] comes at 32 mM stock, add 198 µL of NFW to 2 µL aliquots of 32 mM SAM.

1. Add 2 µL of mastermix to each well, can be accomplished quickly and precisely with a 10 µL electronic pipette.
2. Use a multichannel pipette to combine 4 µL of the DNA sample with the 2 µL master mix (6 µL total volume).
3. Cover the plate with PCR film, spin down, and incubate at 37°C in a thermocycler with heated lid for 1 hr.
4. Inactivate the enzyme at 65°C for 10 min then hold samples at 4 °C.

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| **Digestion profile** | |
| **37ºC** | **60 min** |
| **65ºC** | **10 min** |
| **4 ºC** | **HOLD** |

* 1. **Hold samples on ice after this.**

**Day 1: Ligation**

In this step adaptors are ligated to the restriction fragments produced above.

1. Prepare double stranded adaptors by combining each pair of primers, Adaptor 1 (5ILL-NNRW, Anti5ill-NNRW) and the 12 different pairs of 3illBC, anti3illBC.
2. For a full plate, prepare Adaptor 1 in PCR tube, mix 60 µL of 5ILL-NNRW (10 µM) with 60 µL of Anti5ill-NNRW (10 µM).

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| **Adapter 1 Component** | **Reaction Vol (µL)** | **Total Volume (µL)** |
| 5ILL-NNRW | 0.5 | 60 |
| Anti5ill-NNRW | 0.5 | 60 |
| **Adapter 1** | **1** | **120** |

1. For Adaptor 2, set up 12 PCR tubes, to each tube mix 5 µL of 3illBC (1-12) (10 µM) with 5 µL Anti-ill-BC(1-12) (10 µM).

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| **Adapter 2 Component** | **Reaction Vol (µL)** | **Total Volume (µL)** |
| 3illBC(1-12) | 0.5 | 5 |
| Anti-ill-BC(1-12) | 0.5 | 5 |
| **Adapter 2** | **1** | **10** |

1. Incubate at **42°C for 5 minutes** then keep at room temperature until ligation.
2. Prepare 12 master mixes for ligations (one for each barcoded 3’ primer). This recipe is for a single reaction, so scale up as needed.

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| --- | --- |
| **Component** | **Reaction Vol (µL)** |
| NFW | 15 |
| 10x T4 ligase buffer w 10 mM ATP | 2 |
| 5 μM Adapter 1 | 1 |
| 5 μM Adapter 2 **\*Different for each plate column\*** | 1 |
| T4 DNA ligase | 1 |
| **Total** | **20** |

1. First make an initial master mix:

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| --- | --- | --- | --- |
| **Component** | **Reaction Vol (µL)** | **Total Vol (µL)** 8 rxn + 10% error | **Total Vol (µL)** for 12 MM + 10% error |
| 10x T4 ligase buffer w 10 mM ATP | 2 | 17.6 | 232.3 |
| 5 μM Adapter 1 | 1 | 8.8 | 116.2 |
| T4 DNA ligase | 1 | 8.8 | 116.2 |
| **Total** | **4** | **35.2** | **464.6** |

1. Into 12 separate 0.6 mL tubes add:

|  |  |
| --- | --- |
| **Component** | **Total Volume (µL)** 8 rxn+10% error |
| Master Mix | 35.2 |
| 5 μM Adapter 2  **(Different for each plate column)** | 8.8 |
| NFW | 132 |
| **Total** | **176** |

1. Use a 100 µL multi-channel pipette to combine **20 µL master mix** with digested DNA (~25 µL total volume). **Keep on ice while mixing**.
2. Incubate at 16 °C for BcgI for 12 hours.
3. Heat at 65 **°**C for at least 40 min to inactivate the ligase (in a thermocycler with heated lid).

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| **Ligation profile** | |
| **16ºC** | **12 hr** |
| **65ºC** | **40 min** |
| **4 ºC** | **HOLD** |

**Day 2, OPTIONAL STEP: qPCR to ensure amplification success**

1. Prepare a qPCR Plate, for each plate you can run three rows of samples in duplicate and three negative control wells, you will need to run three qPCR plates for every ligation plate.

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| **Component** | **Reaction Vol (µL)** | **Total Volume (µL) (75 rxn+10% error)** |
| NFW | 5.53 | 456.23 |
| SYBR Green Mastermix | 7.5 | 618.75 |
| 10 uM TruSeq | 0.07 | 5.78 |
| 1 uM any ILLBC Primer | 0.7 | 57.75 |
| 10 uM P5 | 0.1 | 8.25 |
| 10 uM P7 | 0.1 | 8.25 |
| **Total** | **14** | **1484** |

1. Add 14 µL of mastermix to each well, avoid bubbles
2. Add 1 µL of ligation to each well
3. Centrifuge plate
4. Turn on qPCR machine, ensure the correct reaction volume (15 µL) is inputted, follow the 2bRAD template and ensure the plate is inserted correctly.
5. Rank samples from highest to lowest CT score.
6. *Optional:* Select 4 sample ligations with relatively low CT scores and 4 samples with relatively high CT scores to use in a test PCR.

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| --- | --- | --- | --- | --- | --- |
| **qPCR Profile** | | | | | |
| **Step** | **Temperature** | **Acquisition** | **Time** | **# Cycles** |
| Pre-Incubation | 95 ºC | None | 10 min | 1 x |
|  | 95 ºC | None | 15 s |  |
| Amplification | 60 ºC | None | 30 s | 40 x |
|  | 72 ºC | Single | 30 s |  |
|  | 95 ºC | None | 5 s | 1x |
| Melting Curve | 65 ºC | None | 1 min |  |
|  | 97 ºC | Continuous |  |  |
| Cooling | 40 ºC | None | 10 s | 1x |

**Day 3, OPTIONAL STEP: Test PCR**

*Optional:* Test PCR for a set of 8 ligations, recommended to use 4 with low Ct values and 4 with high Ct values if qPCR was conducted.

Master mix:

|  |  |  |
| --- | --- | --- |
| **Reagent** | **Volume (µL)** | **Total vol. (µL) 8 samples + 10% error** |
| dNTPs 2.5 mM ea | 0.4 | 3.52 |
| H2O | 9.7 | 85.36 |
| 10 µM IC1-P5 | 0.4 | 3.52 |
| 10 µM IC1-P7 | 0.4 | 3.52 |
| 1 µM ILL-BC oligo | 2.4 | 21.12 |
| 10 µM TruSeq\_UN oligo | 0.3 | 2.64 |
| 10x Titanium buffer | 2 | 17.6 |
| Titanium Taq | 0.4 | 3.52 |
| **Total** | 16 | 140.8 |

1. Add 16 µL of master mix to 8 strip-tubes, then add 4 µL of ligation and amplify:

|  |  |  |
| --- | --- | --- |
| **Amplification PCR profile** | | |
| 70 °C | 30 s |  |
| **95 °C** | **20 s** |  |
| **65 °C** | **3 min** | **X 15 Cycles** |
| **72 °C** | **30 s** |  |
| 20 °C | Continuously |  |

1. Load 5 µL on a 2% agarose gel alongside LMW ladder or other marker that has 150 and 200 bp bands. Confirm that all samples have a **visible band at ~180 bp**
2. Pool ligations by row in strip-tubes, using **6 µL from each well.** The 96-well plate is now reduced to 8 pooled samples, each corresponding to the original row. **Store the ligations at -20 oC.**

**Day 3: Amplification**

In this step, the constructs produced by ligation are amplified using a set of four primers that introduce pooled sample - specific barcodes and the annealing sites for HiSeq/NovaSeq amplification and sequencing primers.

1. For each reaction prepare the following master mix:

|  |  |  |
| --- | --- | --- |
| **Reagent** | **per sample** | **master mix (8 pooled samples + 10% error)** |
| dNTPs 2.5 mM ea | 1 | 8.8 |
| H2O | 12 | 105.6 |
| 10 µM IC1-P5 | 1 | 8.8 |
| 10 µM IC1-P7 | 1 | 8.8 |
| 10x Titanium buffer | 5 | 44 |
| Titanium Taq | 1 | 8.8 |
| **Total** | **21** | **184.4** |

1. Set up 8 PCR tubes, combine:

|  |  |
| --- | --- |
| **Component** | **Volume (µL)** |
| Mastermix | 21 |
| Pooled Ligation | 20 |
| 1 µM ILL-BC primer **\*Different for each tube\*** | 6 |
| 2 µM TruSeq **\*Different for each set of 8 tubes\*** | 3 |
| **Total** | **50** |

Amplify as follows:

|  |  |  |
| --- | --- | --- |
| **Amplification PCR profile** | | |
| 70°C | 30 sec |  |
| **95°C** | **20 sec** |  |
| **65°C** | **3 min** | **X15 Cycles** |
| **72°C** | **30 sec** |  |
| 20 °C | Continuously |  |

1. Load 5 µL on a 2% agarose gel alongside LMW ladder (NEB N3233S) or other marker that has 150 and 200 bp bands.
   1. NB: There should be no substantial variation in product amount among samples at this stage

|  |  |
| --- | --- |
| *Gel* | 2% Agarose Gel 300 mL (large) |
| 6g Agarose |
| 285 ml DI H2O |
| 15 ml 20X SB |
| 6 µL EtBr |
| Single well combs |
| *Load* | 3 µL DNA ladder |
| 2 µL loading dye on parafilm |
| 5 µL product |
| *Run* | 150 V for 15–20 min |

1. Confirm that all samples have a **visible band at ~180 bp**. You might also see a band below 150 bp, which is an artifact from the carried-over ligase (if this is an issue you can heat inactivate the ligase for longer
2. If the 180 bp product is visible but barely, add two more cycles to the same reactions, do not continue to amplify the samples if no band is visible at 15 cycles, go back and troubleshoot the samples within that pool.

**Gel-purification**  
In this step the target band is gel-extracted to exclude high-molecular weight  
fragments and any chaff that may emerge during PCR (e.g., primer dimers)

1. Prepare a 2% agarose gel using TBE or TB. Use a wide comb that can accommodate 30-50 μl, or simply tape together two wells.
2. Load 30-50 μl of sample (40 μl sample + 10 μl loading dye) alongside LMW ladder. Run gel at low voltage for 70 minutes at 100 V or until bands at 150bp and 200bp will be clearly resolved.
3. View the gel briefly a blue-light transilluminator to verify the presence of target band and adequate separation of molecular weight standards to resolve bands at ~180 bp and (possibly) below 150 bp. Photograph.
4. Cut out target ~180 bp band in a narrow gel slice, avoiding the edges of the lane (i.e., cut out the middle 70-75% of the band). Cut just inside the bottom boundary of the target band to avoid getting anything smaller.
5. Follow QiaQuick gel extraction protocol.

**Combining Libraries for Sequencing**

Pool libraries in equimolar ratios to try to equalize coverage across sample pools.

1. Prepare a 1:100 dilution of each pooled library by combining 2 µL of eluted library with 198 µL of NFW, can use multi-channel pipette.
2. Prepare qPCR mastermix, run each library in duplicate and have 2–3 wells reserved for negative controls.

|  |  |  |
| --- | --- | --- |
| **Reagent** | **Single RXN** | **21 RXNs** (8 RXNs in duplicate and three negative controls + error) |
| NFW | 4.3 | 90.3 |
| Sybr Green qPCR MM | 7.5 | 157.5 |
| 10 uM P5 | 0.6 | 12.6 |
| 10 uM P7 | 0.6 | 12.6 |
| **Total** | **13** | **273** |
| **Diluted PCR Product (1:100)** | **2** |  |

1. Conduct qPCR following previously described qPCR profile and calculate CT values for each sample.
2. To determine volumes of each library for the combined pool:
   1. Rank samples from lowest to highest CT and identify reference sample (sample with the highest CT)
   2. Calculate the proportion of each library to sequence as:

PL = 2[CT(sample) – CT (reference)]

* 1. Calculate the volume of each library to use as:

V = PL \* 28 µL

1. Combine libraries and follow Zymo Clean and Concentrator Protocol to concentrate final libraries in ~25 µL of NFW.

Note: For PCR products you must use a 5:1 ratio of binding buffer to sample.

1. Qubit the final pool with a high-sensitivity kit to determine if further concentration is needed.