Notes:

“Mix” translates to pipetting up & down 10-15X (or more if necessary).

Part 1: DNA extraction

Use the ThermoScientific GeneJET Plant Genomic DNA Purification Mini Kit

#K0791, #K0792

A few modifications:

* Warm Elution Buffer to 55ºC prior to elution.
* Elute into 2 different tubes.

Store DNA samples at -20ºC until ready to use.

Part 2: Quantify samples with Qubit

Qubit Kit: Qubit dsDNA HS Assay Kit (Q32854)

Qubit all samples. Use 1µl of sample.

Dilute all samples to 200 ng in 12.5 µl of 10 mM Tris-HCl, pH 8.0.

Qubit all samples again before double digestion. Use 1µl of sample.

Dilute all samples to 100 ng in 17 µl of 10 mM Tris-HCl, pH 8.0.

Part 3: Double Digestion

*Approximate time: 4 hr.*

1. Make Master Mix:

|  |  |
| --- | --- |
| **Component** | **x1** |
| 10X CutSmart Buffer | 2 µl |
| EcoRI-HF (20,000 units/mL) – 20 U/µl | 0.5µl |
| MspI (20,000 units/mL) – 20 U/µl | 0.5µl |
| **Total** | **3 µl** |

2. Mix MM with 17 µl of DNA (100 ng).

3. Incubate

37**°**C – 3 hrs

65**°**C – 20 min (heat inactivation for EcoRI)

4**°**C – ∞

4. Proceed to Adaptor Ligation as soon as possible.

\*Most samples have a 20 µl total reaction volume. A few samples (mostly from Plate 8) have different total reaction volumes. Check spreadsheet for more information.\*

Part 4: Adaptor Ligation

\* Do all steps on ice \*

Refer to Joanna’s protocol/write up to see how she calculated the amount of adapters to use per sample:

JR\_Ligation\_notes\_for\_Kate\_and\_Irene.docx

**Prep:**

P2 universal adaptors need to be made by ligating two oligos together (found in Joanna’s freezer box).

|  |  |
| --- | --- |
|  | **Reagents to make 40 µM Universal MspI adapter** |
| Oligo P2.1 (100µM) | 40 µl |
| Oligo P2.2 (100µM) | 40 µl |
| Water | 20 µl |
| Total | 100 µl |

Thermocycler:

97.5**°**C for 2.5 min

Cool to 21**°**C at no greater than 3**°**C per minute

Hold at 4ºC

Working Solution for Ligations

|  |  |  |
| --- | --- | --- |
|  | **Universal P2 adapter (Reverse)** | **Barcode Adapter (Forward)** |
| Adapter (diluted to 4 µM) | 18.94 µl | 1.97 µl |
| Water | 31.06 µl | 48.03 µl |

1. Make Ligation Master Mix:

|  |  |
| --- | --- |
| **Component** | **x1** |
| 10X CutSmart Buffer | 2 µl |
| T4 DNA Ligase (400,000 units/mL) – 400 U/µl | 0.5 µl |
| 10 mM ATP | 4 µl |
| Universal P2 adapter (diluted Working Solution) | 1 µl |
| 10 mM Tris-HCl, pH 8 | 10.5 µl |
| Total | 18 µl |

In each tube:

|  |  |
| --- | --- |
| **Component** | **x1** |
| Digested DNA | 20 µl |
| Barcode (unique; diluted Working Solution) | 2 µl |
| Ligation MM | 18 µl |
| Total | 40 µl |

1. Mix.

4. Thermocycler: jrkill (Bio\_RAD) – Heat inactivation

23**°**C – 30 min

65**°**C – 10 min

0.1**°**C/s cool to 23ºC

23ºC – 2 min

5. Store at 4**°**C to minimize freeze thaw.

\* Optional: choose 2-3 samples per plate to amplify using PCR amplification primers to make sure that samples were ligated. \*

Part 5: Pooling & AMPure Bead Cleanup

*Approximate time: 3 hr.*

\* Take out AMPure Beads at least 30 min prior to use\*

\* Make 70% EtOH fresh \*

1. Pool each plate. Mix well. Spin.

If there are 96 samples, then add all 40 µl per sample into a 15 ml tube.

1. Split this amount into tubes with less than 400µl.

For 96 samples, a total of 3.84 ml. Split this amount into **10 tubes** of 384 µl.

1. AMPure Bead Cleanup
2. In each tube:

|  |  |
| --- | --- |
| **Component** | **96 samples (10 tubes)** |
| Ligated Mixture | 384 µl |
| AMPure Beads | 576 µl |
| Total | 960 µl |

1. Mix.
2. Incubate RT, 5 min.
3. Magnetic Stand – 2 min, RT.
4. Remove SUP.
5. ON STAND – Add l mL 70% EtOH.
6. Incubate RT, 30 sec.
7. Remove EtOH.
8. ON STAND – Add 1 mL 70% EtOH.
9. Incubate RT, 30 sec.
10. Remove EtOH – ALL. (Remainder – use a 10µl pipet tip)
11. Remove from stand. DRY 5-15 min.
12. Resuspend 40µl 10 mM Tris-HCl pH 8.0.
13. Incubate RT – 2 min.
14. Magnetic Stand – 2 min, RT.
15. Take 40 µl SUP and transfer into a new tube.
16. Redo steps 15 & 16.
17. Combine all the samples per plate into 1 tube.

For 96 samples, combine 40µl samples \* 10 tubes = 400 µl.

1. AMPure Bead Cleanup
2. In each tube:

|  |  |
| --- | --- |
| **Component** | **96 samples (10 tubes)** |
| Cleaned Ligated Mixture | 400 µl |
| AMPure Beads | 600 µl |
| Total | 1000 µl |

1. Mix.
2. Incubate RT, 5 min.
3. Magnetic Stand – 2 min, RT.
4. Remove SUP.
5. ON STAND – Add l.5 mL 70% EtOH.
6. Incubate RT, 30 sec.
7. Remove EtOH.
8. ON STAND – Add 1.5 mL 70% EtOH.
9. Incubate RT, 30 sec.
10. Remove EtOH – ALL. (Remainder – use a 10µl pipet tip)
11. Remove from stand. DRY 5-15 min.
12. Resuspend 32µl 10mM Tris-HCL pH 8.0.
13. Incubate RT – 2 min.
14. Magnetic Stand – 2 min, RT.
15. Take 30 µl SUP and transfer into a new tube.
16. Redo steps 15 & 16.
17. Qubit each sample.

Part 6: Library Amplification

*Approximate time: 1.5 hr*

Make 4-8 20µl reactions for each pooled sample. In this case, make 5 20µl reactions in one tube with the pooled template, then separate them into 5 tubes for the thermocycler.

Library Amplification Master Mix

|  |  |  |
| --- | --- | --- |
| **Component** | **x1** | **x5** |
| 5X Phusion Buffer | 4 µl | 20 µl |
| 10mM dNTPs | 0.4 µl | 2µl |
| DMSO | 0.6 µl | 3µl |
| PCR Primer 1 Forward (10 µM) | 1µl | 5µl |
| PCR Primer 2 Reverse (Index) (10 µM) | 1µl | 5µl |
| Phusion Taq | 0.2 µl | 1 µl |
| Total | 7.2 µl | 36 µl |

In each tube:

|  |  |
| --- | --- |
| **Component** | **x5** |
| Ligated DNA (~20 ng/20µl PCR rxn) | X µl |
| Water | Y µl |
| Library Amplification Master Mix | 36 µl |
| Total | 100 µl |

X+Y = 64 µl

1. Thermocycler:

98**°**C – 30 sec

98**°**C – 10 sec

60**°**C – 30 sec

72**°**C – 30 sec

72**°**C – 5 min

4**°**C - ∞

(for 10 cycles)

3. Qubit. Make sure each concentration is relatively consistent with expectations.

Part 7: PCR Amplification AMPure Bead Cleanup & Pool

*Approximate time: 1.25 hr.*

\* Take out AMPure Beads at least 30 min prior to use\*

\* Make 70% EtOH fresh \*

Combine

1. In a tube:

|  |  |
| --- | --- |
| **Component** | **x1** |
| PCR Product Combined (20 µl \* 5 of PCR reactions) | 100 µl |
| AMPure Beads | 150 µl |
| Total | 250 µl |

1. Mix.
2. Incubate RT, 5 min.
3. Magnetic Stand – 2 min, RT.
4. Remove SUP (240 µl).
5. ON STAND – Add 600µl 70% EtOH.
6. Incubate RT, 30 sec.
7. Remove EtOH.
8. ON STAND – Add 600µl 70% EtOH.
9. Incubate RT, 30 sec.
10. Remove EtOH – ALL. (Remainder – use a 10µl pipet tip)
11. Remove from stand. DRY for 5-10 min.
12. Resuspend 32µl 10mM Tris-HCL pH 8.0.
13. Incubate RT – 2 min.
14. Magnetic Stand – 1 min, RT.
15. Take 30 µl SUP and transfer into a new tube.
16. Qubit.
17. Send for size selection.

Part 8: Size Selection and Sequence

Send to the sequencing facility for size selection and sequencing

Target Range: 300-500 bp

Have them give me the concentration (or reQubit samples)