Thermocycler Programs

“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: Double Digestion

*Approximate time: 4 hr.*

1. Make Master Mix:

|  |  |
| --- | --- |
| **Component** | **x1** |
| 10X CutSmart Buffer | 5.7µl |
| EcoRI (20,000 units/mL) | 1µl |
| MspI (20,000 units/mL) | 1µl |
| Total | 7.7µl |

2. Mix MM with 50µl of DNA.

3. Incubate

37**°**C – 3 hrs

4**°**C – ∞

Part 3: Digestion AMPure Bead Cleanup

*Approximate time: 1.25 hr.*

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

\* Make 70% EtOH fresh \*

1. In each tube:

|  |  |
| --- | --- |
| **Component** | **x1** |
| Double Digest Mixture | 57.7 µl |
| AMPure Beads | 86.55 µl |
| Total | 144.25 µl |

1. Mix.
2. Incubate RT, 5 min.
3. Magnetic Stand – 2 min, RT.
4. Remove SUP (140 µl).
5. ON STAND – Add 200µl 70% EtOH.
6. Incubate RT, 30 sec.
7. Remove EtOH.
8. ON STAND – Add 200µl 70% EtOH.
9. Incubate RT, 30 sec.
10. Remove EtOH – ALL. (Remainder – use a 10µl pipet tip)
11. Remove from stand.
12. Resuspend 32µl water
13. Incubate RT – 2 min.
14. Magnetic Stand – 1 min, RT.
15. Take 30 µl SUP and transfer into a new tube.
16. Qubit 1 µl of each sample. (or make a 1:1 dilution, then take1 µl to Qubit using the HS kit)

SAFE STOPPING POINT – recommended to digest and cleanup (Parts 2 & 3) on the same day.

\* Optional: choose 7 samples per plate randomly using a random number generator (<https://www.random.org/>) to run out on a gel. Run a few samples that have not been digested as controls.

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

Working Solution for Ligations

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

Universal P2 adapter: 18.94 µl of 4µM stock, 31.06

1. Make Ligation Master Mix:

|  |  |
| --- | --- |
| **Component** | **x1** |
| 10X T4 DNA Ligase Buffer | 6.1 µl |
| T4 DNA Ligase (400,000 units/mL) | 2 µl |
| Universal P2 adapter (diluted) | 1 µl |
| Total | 9.1 µl |

In each tube:

|  |  |
| --- | --- |
| **Component** | **x1** |
| DNA (100 ng) | 50 µl |
| Barcode (unique; diluted) | 2 µl |
| Ligation MM | 9.1 µl |
| Total | 61.1 µl |

1. Mix.
2. Incubate at RT (~23ºC) for 30 min

4. Thermocycler: jrkill (Bio\_RAD)

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 61.1µl per sample into a 15 ml tube.

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

For 96 samples, a total of 5.8656 ml. Split this amount into **15 tubes** of 391µl.

For 60 samples, a total of 3.666 ml. Split this amount into **10 tubes** of 366µl

For 57 samples, a total of 3.4827 ml. Split this amount into **9 tubes** of 386.9µl.

1. AMPure Bead Cleanup
2. In each tube:

|  |  |  |  |
| --- | --- | --- | --- |
| **Component** | **96 samples (15 tubes)** | **60 samples (10 tubes)** | **57 samples (9 tubes)** |
| Ligated Mixture | 391.04 µl | 366 µl | 386.97 µl |
| AMPure Beads | 586.56 µl | 549.9 µl | 580.45 µl |
| Total | 977.6 µl | 916.5 µl | 950 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 5-10 min.
12. Resuspend 40µl water
13. Incubate RT – 2 min.
14. Magnetic Stand – 1 min, RT.
15. Take 40 µl SUP and transfer into a new tube.
16. Combine all the samples per plate into 1 tube.

For 96 samples, combine 40µl samples \* 15 tubes = 600 µl.

For 60 samples, combine 40µl samples \* 10 tubes = 400 µl.

For 57 samples, combine 40µl samples \* 9 tubes = 360 µl.

1. AMPure Bead Cleanup

1. In each tube:

|  |  |  |  |
| --- | --- | --- | --- |
| **Component** | **96 samples (15 tubes)** | **60 samples (10 tubes)** | **57 samples (9 tubes)** |
| Cleaned Ligated Mixture | 600 µl | 400 µl | 360 µl |
| AMPure Beads | 900 µl | 600 µl | 540 µl |
| Total | 1500 µl | 1000 µl | 900 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 5-10 min.
12. Resuspend 32µl water.
13. Incubate RT – 2 min.
14. Magnetic Stand – 1 min, RT.
15. Take 30 µl SUP and transfer into a new tube.
16. Qubit each sample.

Part 6: Library Amplification

*Approximate time: 1.5 hr*

Note: when choosing adapters for pooling:

From Illumina for 3 pools:

AD002 and AD007 and AD019

AD001 and AD010 and AD020

AD005 and AD006 and AD015

AD003 and AD009 and AD025

OR

2-plex options with any other adapter

AD005 and AD019

AD006 and AD012

\*We have AD006 and AD012, so we can do AD006, AD012, and any other adapter we have (AD001-AD011).\*

Library Amplification Master Mix

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

In each tube:

|  |  |
| --- | --- |
| **Component** | **x1** |
| Size Selected, Ligated DNA (~20 ng) | X µl |
| Water | Y µl |
| Library Amplification Master Mix | 7.2 µl |
| PCR Primer 2 Reverse (Index) (µM) | 1µl |
| Total | 20µl |

X+Y = 12.8 µl

1. Thermocycler: (11.3)

98**°**C – 30 sec

98**°**C – 10 sec

60**°**C – 30 sec

72**°**C – 30 sec

72**°**C – 5 min

4**°**C - ∞

(probably 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 \* # of PCR reactions) | 40 µl |
| AMPure Beads | 60 µl |
| Total | 100 µl |

1. Mix.
2. Incubate RT, 5 min.
3. Magnetic Stand – 2 min, RT.
4. Remove SUP (120 µl).
5. ON STAND – Add 200µl 70% EtOH.
6. Incubate RT, 30 sec.
7. Remove EtOH.
8. ON STAND – Add 200µl 70% EtOH.
9. Incubate RT, 30 sec.
10. Remove EtOH – ALL. (Remainder – use a 10µl pipet tip)
11. Remove from stand.
12. Resuspend 32µl 10mM Tris-HCl, pH 8.5.
13. Incubate RT – 2 min.
14. Magnetic Stand – 1 min, RT.
15. Take 30 µl SUP and transfer into a new tube.
16. Send for QC.

Part 8: Size Selection and Sequence

Send to the sequencing facility for size selection and sequencing

Target Range: 300-350 bp

Have them give me the concentration (or reQubit samples)