**Day 1**

1. Add 7 ul of working adapter stock to well
2. Add 250 ng DNA
3. #Spin, cover with Airpore tape, dry using SpeedVac
4. Or Cover with Airpore tape, briefly spin down, at set at 37 deg in thermocycler overnight

**Day 2**

1. Digestion Master Mix

|  |  |  |
| --- | --- | --- |
|  | 1x | 100x |
| NEB Buffer 3 | 2.μL | 200 |
| ApeKI | 1 μL | 100 |
| H2O | 17 μL | 2000 |

1. Add 20 μL to each well of plate
2. Spin down, cover with silicon mat
3. Incubate for 2 hr at 75 deg, hold at 4°
4. Make Ligation Master Mix

|  |  |  |  |
| --- | --- | --- | --- |
|  | 1x | 100x | half |
| T4 Ligase Buffer | 5 μL | 500 | 250 |
| T4 Ligase | 1.6 μL | 160 μL | 80 |
| H2O | 23.4 μL | 2340 μL | 1170 |

1. Add 30 μL to each well
2. Ligation Rxn
   1. 19° for 60 min
   2. 65° for 30 min
   3. Cool to 4°
3. Prepare vacuum manifold, put waste tray inside base and PCR plate on top
4. Pipet PCR samples onto plate
5. Apply vacuum at 800 mbar until completely dry.
6. **Remove the plate from the vacuum manifold.** Carefully tap the plate on a stack of clean Kimwipes to remove any liquid. If a large amount of liquid remains, it needs to be vacuum treated longer.
7. **Elute DNA from the membrane.** Add 25 uL Elution Buffer to each well. Shake plate side to side gently for 20 seconds (by tapping will work). Mix by pipetting up and down 20 times or more. For easier recovery of eluate, the plate can be placed at an angle on the vacuum manifold. (It is likely that only about ~23uL will be recovered by this method, which is taken into account in the pooling step).
8. **Transfer samples to a new PCR plate.** Check the wells of the purification plate to make sure no DNA was left behind.
9. **Quantify with Quibit**

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Conc | A1  4.56 | A2  6.48 | A3  6.9 | A4  9.5 | A5  6.48 | A6  7.64 | A7  5.96 | A8  8.28 | A9  10.6 | A10  4.86 | A11  0 | A12  4.08 |
| uLAdd |  |  |  |  |  |  |  |  |  |  |  |  |
|  | B1  5.7 | B2  10.9 | B3  2.96 | B4  5.24 | B5  6.3 | B6  6.98 | B7  11.7 | B8  6.32 | B9  5.78 | B10  5.24 | B11  4.8 | B12  0 |
|  |  |  |  |  |  |  |  |  |  |  |  |  |
|  | C1  5.64 | C2  0 | C3  0 | C4  4.72 | C5  3.34 | C6  3.02 | C7  2.62 | C8  3.9 | C9  2.5 | C10  0 | C11  3 | C12  0 |
|  |  |  |  |  |  |  |  |  |  |  |  |  |
|  | D1  4.52 | D2  5.1 | D3  2.9 | D4  6.12 | D5  5.94 | D6  7.92 | D7  6.32 | D8  6.62 | D9  6.20 | D10  4.44 | D11  5.18 | D12  3.64 |
|  |  |  |  |  |  |  |  |  |  |  |  |  |
|  | E1 | E2 | E3 | E4 | E5 | E6 | E7 | E7 | E9 | E10 | E11 | E12 |
|  |  |  |  |  |  |  |  |  |  |  |  |  |
|  | F1 | F2 | F3 | F4 | F5 | F6 | F7 | F8 | F9 | F10 | F11 | F12 |
|  |  |  |  |  |  |  |  |  |  |  |  |  |
|  | G1 | G2 | G3 | G4 | G5 | G6 | G7 | G8 | G9 | G10 | G11 | G12 |
|  |  |  |  |  |  |  |  |  |  |  |  |  |
|  | H1 | H2 | H3 | H4 | H5 | H6 | H7 | H8 | H9 | H10 | H11 | H12 |
|  |  |  |  |  |  |  |  |  |  |  |  |  |

1. **Pool Samples to Make Library**
   1. Find the sample with the lowest concentration in each planned pool and multiply by 22uL to get total ng of smallest sample in each pool.
   2. For the remainder of the samples in each pool, divide the total ng of the lowest concentration sample from the last step by the concentration of the current sample, resulting in the volume of that sample to add to the final pool.
   3. Combine calculated volumes in a single 1.5mL microcentrifuge tube per library (12 or 24)
2. Concentrate Libraries for size selection
   1. **Sum all volumes added to final pool and divide final volume by 4 (for 24 samples/pool) to get volume to add to each well on the vacuum plate.**
   2. **Add library to vacuum wells, turn on vacuum to -800 mbar, and wait until wells are completely dry.**
   3. Turn off the vacuum and detach the hose. **Re-suspend the DNA in 20-25uL of elution buffer, pipetting up and down 20+ times to recover it all.** With 24 samples per pool, you will need to perform 2 rounds of vacuum concentration to get a small final volume, using 4 wells and then 2 wells.
   4. When finished, quantify the library using the Nanodrop and record the concentration and quality.

**Day 3**

1. Pippin Prep

**Prepare PCR Master Mix**. Use 2mL Eppendorf tube or larger and be sure to prepare **on ice**. Vortex the mix.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Reagent | Volume/Rxn |  | Total Rxns (8) | Volume in Master Mix |
| **dH20** | 11.0 uL | **x** | 9.5 | 104.5 uL |
| **NEB 2X Taq Master Mix** | 25.0 uL | **x** | 9.5 | 237.5 uL |
| **PCR primer 1 (5uM)** | 2.0 uL | **x** | 9.5 | 19 uL |
| **PCR primer 2.1 (5uM)** | 2.0 uL | **x** | 9.5 | 19 uL |
| **TOTAL** | 40.0 uL | **x** | 9.5 | 380 uL |
| Reagent | Volume/Rxn |  | Total Rxns (8) | Volume in Master Mix |
| **dH20** | 11.0 uL | **x** | 9.5 | 104.5 uL |
| **NEB 2X Taq Master Mix** | 25.0 uL | **x** | 9.5 | 237.5 uL |
| **PCR primer 1 (5uM)** | 2.0 uL | **x** | 9.5 | 19 uL |
| **PCR primer 2.3 (5uM)** | 2.0 uL | **x** | 9.5 | 19 uL |
| **TOTAL** | 40.0 uL | **x** | 9.5 | 380 uL |

1. **Prepare adequate PCR strip tubes or a PCR plate on ice and at 40uL of the mix to each well**
2. **Add 10ul of the purified post-ligation product to the appropriate wells in the PCR plate**
3. **Protocol** is:
4. incubate 72C for 5 mins
5. 95C 1 min
6. 95C 30s
7. 65C 30s
8. 72C 1 min
9. cycle to step 3 16X
10. 72C 5 mins
11. 4C forever
12. Clean PCR reactions with Enzymax
13. Quantify