**Supplemental material – Methods for Molecular Biology and Genetics & Genomics Labs**

*Molecular biology lab details*

\*All primers and sequences are listed in a table at the end of the protocol.

Week 1 (DNA extraction)

1. Extract DNA according to the protocol outlined in the DNeasy PowerSoil kit (Qiagen)

Week 2 (DNA fragmentation)

1. Perform genomic library construction according to the KAPA HyperPlus kit (Roche)

*1.1* Dilute the amount of dsDNA to be used for library construction as follows.  If the DNA preparation does not contain EDTA, dilute in 10 mM Tris-HCl (pH 8.0-8.5) in a total of 35μL.

*1.2* Mix by gentle vortexing or pipetting up and down.

*1.3* Assemble each fragmentation reaction on ice by adding the components in this order:

Component Volume (μl)

dsDNA 35

KAPA Frag Buffer (10X) 5

KAPA Frag Enzyme 10

*1.4* Vortex gently and spin down briefly. Return the plate/tube(s) to ice. Proceed immediately to the next step.

*1.5* Incubate in a thermocycler, pre-cooled to 4°C and programmed as outlined below. A heated lid is not required for this step. If used, set the temperature of the heated lid to ≤50°C. Aim for approximately 350bp, 10 minutes at 37°C.

*1.6* Transfer reactions to ice, and proceed immediately to End Repair and A-tailing.

1. Perform A-tailing.

*2.1* Mix the following components in a sterile nuclease-free tube (Total volume 65μl):

Component Volume (μl)

Fragmented DNA 55.5

End Prep Enzyme Mix 3

End Repair Reaction Buffer (10X) 6.5

*2.2* Set a 100 or 200μl pipette to 50μl and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect the liquid from the side of the tube.  Note: it is important to mix well. The presence of a small amount of bubbles will not interfere with the performance.

*2.3* Place in a thermocycler with the heated lid set to ≥75°C, and run the following program:

Temperature (°C) Time (min)

20 30

65 30

4 Hold

*2.4* Add the following components directly to the End Prep reaction mixture.  For best results, add adapter last and immediately mix well, or premix adapter and sample and then add the other ligation reagents.

Component Volume (μl)

Blunt/TA Ligase Master Mix 15

Ligation Enhancer 1

NEBNext Adapter for Illumina 2.5

*2.5* Incubate at 20°C for 15 minutes in a thermocycler.

*2.6* Add 3μl of USER™ enzyme (NEB) to the ligation mixture.

*2.7* Incubate at 37°C for 15 minutes.

1. We used magnetic bead cleanup, but other precipitation-based methods can be used. Allow the beads to warm to room temperature for at least 30 minutes before use. The following size selection protocol is for libraries with 300 bp inserts only.

*3.1* Add 13.5μl dH2O to the ligation reaction for a 100μl total volume.

*3.2* Add 40μl (0.4X) of resuspended beads to the 100μl ligation reaction.

*3.3* Mix well by pipetting up and down at least 10 times.

*3.4* Be careful to expel all of the liquid out of the tip during the last mix. Vortexing for 3-5 seconds on high can also be used. If centrifuging samples after mixing, be sure to stop the centrifugation before the beads start to settle out.

*3.5* Incubate samples on bench top for at least 5 minutes at room temperature.

*3.6* Place the tube on a magnetic stand to separate the beads from the supernatant. After 5 minutes (or when the solution is clear), carefully transfer the supernatant containing your DNA to a new tube (Caution: do not discard the supernatant). Discard the beads that contain the unwanted large fragments.

*3.7* Add 20μl (0.2X) resuspended beads to the supernatant and mix at least 10 times.

*3.8* Incubate samples on the bench top for at least 5 minutes at room temperature.

*3.9* Place the tube/plate on an appropriate magnetic stand for 5 minutes to separate the beads from the supernatant.

*3.10* After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant that contains unwanted DNA. (Caution: do not discard beads).

*3.11* Add 200μl of 80% freshly prepared ethanol to the tube/plate while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

*3.12* Repeat the previous step once. Be sure to remove all visible liquid after the second wash.  If necessary, briefly spin the tube/plate, place back on the magnet and remove traces of ethanol with a P10 pipette tip.

*3.13* Air dry beads for up to 5 minutes while the tube is on the magnetic stand with the lid open.

*3.14* Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry.

*3.15* Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads into 17μl of 10 mM Tris-HCl.

*3.16* Mix well by pipetting up and down 10 times. Incubate for at least 2 minutes at room temperature.

*3.17* Place the tube/plate on a magnetic stand. After 5 minutes (or when the solution is clear), transfer 15μl to a new PCR tube for amplification.

*3.18* Samples can be stored at -20°C.

Week 4 (Amplification and clean-up)

1. Check concentration of post-clean-up libraries (e.g. using a NanoDrop).
2. Complete amplification reactions.

*2.1* Assemble each library amplification reaction as follows:

Component Volume (μl)

Adapter ligated fragments 15

NEBNext Q5 Hot Start HiFi PCR Master Mix 25

\* Index Primer i7 (each library gets its own) 5

\* Universal PCR primer i5 5

\*both primers from NEB Primer set 2 (<https://www.neb.com/products/e7500-neb-next-multiplex-oligos-for-illumina-index-primers-set-2#Product%20Information>)

*2.2* Mix thoroughly and centrifuge briefly.

*2.3* Amplify using the following cycling protocol:

Temperature (°C) Time Cycles

98 30 sec 1

98 10 sec 12

65 75 sec

65 5 min 1

4 Hold

*2.4* Proceed directly to Post-amplification cleanup

1. Post-amplification cleanup

*3.1* In the library amplification plate/tube(s), perform a 1X bead-based cleanup by combining 50μl of AMPure magnetic beads (Beckman Coulter) with 50μl of the PCR reaction.

*3.2* Mix thoroughly by vortexing and/or pipetting up and down multiple times.

*3.3* Incubate the plate/tube(s) at room temperature for 5 minutes to bind DNA to beads.

*3.4* Place the plate/tube(s) on a magnet to capture beads. Incubate until liquid is clear.

*3.5* Carefully remove and discard the supernatant.

*3.6* Keeping the plate/tube(s) on the magnet, add 200μL of 80% ethanol.

*3.7* Incubate the plate/tube(s) on the magnet at room temperature for ≥30 seconds.

*3.8* Carefully remove and discard the ethanol.

*3.9* Keeping the plate/tube(s) on the magnet, add 200μL of 80% ethanol.

*3.10* Incubate the plate/tube(s) on the magnet at room temperature for ≥30 seconds.

*3.11* Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.

*3.12* Dry the beads at room temperature for 3-5 minutes, or until all of the ethanol has evaporated. Caution: over-drying the beads may result in reduced yield.

*3.13* Remove the plate/tube(s) from the magnet.

*3.14* Thoroughly resuspend the beads in an appropriate volume of elution buffer (10 mM Tris-HCl, pH 8.0-8.5) or PCR-grade water. Always use PCR-grade water if proceeding to target capture.

*3.15* Incubate the plate/tube(s) at room temperature for 2 minutes to elute DNA off the beads.

*3.16* Place the plate/tube(s) on a magnet to capture the beads. Incubate until the liquid is clear.

*3.17* Transfer the clear supernatant to new plate/tube(s).

*3.18* Store purified, amplified libraries at 4°C for 1-2 weeks, or at -20°C.

1. Libraries were sequenced using an Illumina NextSeq 150bp paired end, 10 million reads per library.

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| --- | --- | --- |
| **Index Primer** | **Index Primer Sequence** | **Sequence Index** |
| NEBNext Adapter for Illumina | -/5Phos/GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CUA CAC TCT TTC CCT ACA CGA CGC TCT TCC GAT C-s-T-3 | N/A |
| NEBNext Index 13 Primer for Illumina | CAAGCAGAAGACGGCATACGAGATTGTTGACTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3 | AGTCAA |
| NEBNext Index 14 Primer for Illumina | CAAGCAGAAGACGGCATACGAGATACGGAACTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3 | AGTTCC |
| NEBNext Index 15 Primer for Illumina | CAAGCAGAAGACGGCATACGAGATTCTGACATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3 | ATGTCA |
| NEBNext Index 16 Primer for Illumina | CAAGCAGAAGACGGCATACGAGATGCGGACGGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3 | CCGTCC |
| NEBNext Index 18 Primer for Illumina | CAAGCAGAAGACGGCATACGAGATGTGCGGACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3 | GTCCGC |
| NEBNext Index 19 Primer for Illumina | CAAGCAGAAGACGGCATACGAGATCGTTTCACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3 | GTGAAA |
| NEBNext Index 20 Primer for Illumina | CAAGCAGAAGACGGCATACGAGATAAGGCCACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3 | GTGGCC |
| NEBNext Index 21 Primer for Illumina | CAAGCAGAAGACGGCATACGAGATTCCGAAACGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3 | GTTTCG |
| NEBNext Index 22 Primer for Illumina | CAAGCAGAAGACGGCATACGAGATTACGTACGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3 | CGTACG |
| NEBNext Index 23 Primer for Illumina | CAAGCAGAAGACGGCATACGAGATATCCACTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3 | GAGTGG |
| NEBNext Index 25 Primer for Illumina | CAAGCAGAAGACGGCATACGAGATATATCAGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3 | ACTGAT |
| NEBNext Index 27 Primer for Illumina | CAAGCAGAAGACGGCATACGAGATAAAGGAATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3 | ATTCCT |
| NEBNext Universal PCR Primer for Illumina 5 | AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC-s-T-3 | N/A |

*Genetics and genomics lab details*

\*Associated worksheets and background reading available upon request.

Before module begins: Make reagents and grow donor and recipient cells as described in the Mulvey lab’s pSAM\_Ec supplemental information available at www.addgene.org/102939/.

Week 1 (Conjugation; adapted from the Mulvey lab protocol above)

1. Follow procedure as written by the Mulvey lab.
2. Incubate plates upright at 37°C for 5 hours. Move the plates to 4°C until next week.

Week 2 (Selecting conjugants)

1. Follow conjugant collection procedure as written by the Mulvey lab.  1:100 and 1:1000 dilutions are a good starting point for plating the conjugants on selective media.
2. Incubate plates at 37°C overnight. Move the plates to 4°C until next week.

Week 3 (Replica plating)

1. Choose original Kanamycin+ plate that has approximately 20-50 colonies and count for an exact number to determine conjugation efficiency.
2. Mark an edge of each plate used so that the plates can be lined up and colonies identified in following weeks.
3. Place sterile velvet over the wooden replicating block and secure with a rubber band.
4. Place original Kan+ plate with successful conjugants so that the bacterial colonies are touching the velvet.  Tap the plate gently to transfer bacteria to the velvet, then remove the plate quickly and directly upwards.
5. Place various appropriate antibiotic+ plates onto the velvet and gently tap the plates to transfer colonies. Choose antibiotics that you know the original bacteria of interest are resistant to.
6. Complete a final transfer to a clean Kan+ plate.
7. Incubate plates at 37°C overnight. Move the plates to 4°C until next week.
8. During the following week, start overnight cultures of bacteria in LB broth + Kan that grew on the Kan+ plate but not on the other selective media.

Week 4 (DNA preparation and restriction digest)

1. Prepare DNA from overnight cultures using a standard genomic or plasmid DNA prep kit.
2. Digest the DNA with the restriction enzyme MmeI, using the included protocol.

Week 5 (Tag ligation and PCR)

1. Set up the following ligation reaction in a microcentrifuge tube on ice.

Component Volume (μl)

T4 DNA Ligase Buffer (10X) 2

Adapter DNA\* 2

Digested bacterial DNA 10

Nuclease-free water 5

T4 DNA Ligase 1

\*Adapter sequence: -/5Phos/GAT CGG AAG AGC ACA CGT CTG AAC TCC AGT CUA CAC TCT TTC CCT ACA CGA CGC TCT TCC GAT C-s-T-3

1. Gently mix the reaction by pipetting up and down and centrifuge briefly.
2. Incubate at room temperature for 30 minutes, then heat inactivate at 65°C for 10 minutes.
3. Set up PCR as follows:

Component Volume (μl)

Ligation (from above) 10

10X buffer 2.5

10 μM Kan primer\* 1

10 μM Adapter primer\*\* 1

10 mM dNTPs 1

Taq polymerase 0.5

\*Kan primer sequence: CCT GAC GGA TGG CCT TTT TGC GTT TCT ACC

\*\* Adapter primer sequence: AAT GAT ACG GCG ACC ACC GAG ATC TAC ACT CTT TCC CTA CAC GAC GCT CTT CCG ATC-s-T-3

1. Run the PCR using the following parameters:

Temperature (°C) Time

95 2 min

95 30 sec

60 30 sec

72 1 min

72 5 min

8 Hold

1. Send reactions for Sanger sequencing.

Week 6 (Sequence analysis)

1. Query the returned sequences on BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine the interrupted genes and their potential relevance to antibiotic resistance.