

**A. Total RNA QC**

1. Verify RNA quality by bioanalyzer.

**B. Day 1 Preparation**

1. Remove the following reagents:
  - RNA Purification Beads (4°C)
  - Bead Binding Buffer (4°C)
  - Bead Washing Buffer (4°C)
  - Elution Buffer (4°C)
  - Elute, Prime, Fragment Mix (-20°C)
  - First Strand Master Mix and SuperScript II Mix (Make sure that 55.7 ul of SuperScript II has been added to First Strand Master Mix tube, and labeled ‘added’ on First Strand Master Mix tube)
  - Resuspension Buffer 1.5 ml aliquot (-20°)
  - Second Strand Master Mix (-20°)
  - AMPure XP Beads
  - Freshly made 80% Ethanol

**C. Dilute total RNA**

1. Determine how much input (0.1-4ug) to use for up to eight samples in a 0.2ml labeled tube
2. Use nuclease-free water for dilution:

Sample Name	Owner	Tube Name	Conc. (ng/uL)	Input Vol (uL)	H2O up to 50 uL	Input Mass (ug)	Adapter

**D. Purify/Fragment the mRNA** – This process purifies the poly-A containing mRNA molecules using poly-T oligo attached magnetic beads.

1. Add 50 ul of **RNA Purification Beads** to each sample. Gently pipette entire volume up/down 6x to mix thoroughly.
2. Select Program “MRNA DEN” on thermal cycler to incubate samples at 65°C for 5 minutes. Remove samples when thermal cycler reaches (4°C) and place them on bench at RT.
3. Incubate samples at room temperature (RT) for 5 minutes to allow RNA to bind to the beads.
4. Place samples on the magnetic stand at RT for 5 minutes to separate the poly-A RNA bound beads from the solution.
5. Remove and discard all of the supernatant from each sample then remove samples from magnetic stand.
6. Wash the beads by adding 200 ul of **Bead Washing Buffer** to each sample, and gently pipette up/down 6x to mix thoroughly.
7. Place samples on magnet stand at RT for 3-5 minutes.
8. Remove and discard all of the supernatant from each sample then remove samples from magnetic stand.
9. Add 50 ul of **Elution Buffer** to each sample. Gently pipette entire volume up/down 6x to mix thoroughly. Store Elution Buffer tube in fridge.
10. Select Program “MRNA-ELU” on thermal cycler to incubate samples at 80°C for 2 minutes. Remove samples when thermal cycle reaches (25°C) and place them on bench at RT.
11. Add 50 ul of **Bead Binding Buffer** to each sample, and gently pipette up/down 6x to mix thoroughly.
12. Incubate samples at RT for 5 minutes. Store Bead Binding Buffer tube in fridge.

14. Remove and discard all of the supernatant from each sample then remove samples from magnetic stand.
15. Wash the beads by adding 200 ul of **Bead Washing Buffer** to each sample, and gently pipette up/down 6x to mix thoroughly.
16. Place samples on magnet stand at RT for 3-5 minutes.
17. Remove and discard all of the supernatant from each sample then remove samples from magnetic stand.
18. Add 19.5 ul **Elute, Prime, Fragment Mix**. Gently pipette entire volume up/down 6x to mix thoroughly. The Elute, Prime, Fragment Mix contains random hexamers for RT priming and serves as the 1<sup>st</sup> strand cDNA synthesis rxn buffer.
19. Select Program "Elution2" on thermal cycler to incubate samples at 94° for 8 minutes to elute, fragment, and prime the RNA. Remove samples when thermal cycler reaches (4°), and proceed immediately to Synthesis First Strand cDNA.

### **E. Synthesis of First cDNA**

1. Place samples on magnetic stand at RT for 5 minutes
2. Transfer 17 ul of the supernatant to a new 0.2 ml PCR tube.
3. Add 8 ul of First Strand Master Mix and SuperScript II Mix (working aliquots in strip tubes) to each sample. Gently pipette entire volume up/down 6x to mix thoroughly.
4. Select Program "1<sup>st</sup> STRA" on thermal cycler to incubate samples. (25° for 10 min, 42° for 50 min, 70° for 15 min, Hold at 4°). Remove samples when thermal cycler reaches (4°) and proceed immediately to Synthesis Second Strand cDNA.

### **F. Synthesis of Second cDNA**

1. Add 25 ul **Second Strand Master Mix** (working aliquots in strip tubes) to each sample. Gently pipette entire volume up/down 6x to mix thoroughly.
2. Incubate sample on thermal cycler at 16°C for 1 hour (Program: Second).
3. Before adding the AMPure XP beads, vortex beads until they are well dispersed, then add 90 ul of beads (1.8x) to each sample containing 50 ul of ds cDNA. Gently pipette entire volume up/down 10x to mix thoroughly.
4. Incubate samples for 15 minutes.
5. Place samples on magnetic stand at RT for at least 3-5 minutes to ensure that all beads are bound to the side of the wells.
6. Remove and discard 135 ul of the supernatant from each sample.
7. With the samples still on the magnetic stand, add 200 ul of freshly prepared 80% EtOH to each sample without disturbing the beads.
8. Incubate at RT for 30 seconds, then remove and discard all of the supernatant from each sample.
9. Repeat steps 7 & 8 once for a total of two 80% EtOH washes. Use P10 pipettor to remove the rest of the EtOH.
10. Allow samples to air dry for about 5-10 minutes then remove the samples from the magnetic stand.
11. Add 52.5 ul Resuspension Buffer to each sample. Gently pipette entire volume up/down 6x to mix thoroughly.
12. Incubate samples at RT for 2 minutes.
13. Place samples on magnetic stand at RT for 3-5 minutes.
14. Transfer 50 ul of the supernatant (ds cDNA) to a new 0.2 ml PCR tube.

SAVE STOPPING POINT: Store samples at -15° to -25° for up to 7 days. This completes day 1 of 2 of library prepping.

### **G. Day 2 Preparation**

- End Repair Mix strip tube aliquots, 1rxn/well (-20°C)
- Resuspension Buffer 1.5 ml aliquot (-20°C)
- AMPure XP Beads
- A-Tailing Mix
- Adapter Indexes to be used
- Ligase Mix
- Stop Ligase Buffer
- Freshly made 80% Ethanol
- PCR Master Mix
- PCR Primer Cocktail

**H. End Repair** – This process converts the overhangs resulting from fragmentation into blunt ends, using an End Repair mix. The 3' to 5' exonuclease activity of this mix removes the 3' overhangs and the polymerase activity fills in the 5' overhangs.

1. Add 10 ul Resuspension Buffer to each sample that contains 50 ul ds cDNA.
2. Add 40 ul of End Repair Mix to each sample. Gently pipette entire volume up/down 10x to mix thoroughly.
3. Incubate on thermal cycler at 30°C for 30 minutes.
4. Remove samples from thermal cycler.
5. Add 160 ul of beads (1.6x) to each sample containing 100 ul End Repair Mix.
6. Incubate samples for 15 minutes.
7. Place samples on magnetic stand at RT for at least 3-5 minutes, until the liquid appears clear.
8. Remove and discard 127.5 ul of the supernatant from each sample.
9. Repeat step 8 once. Some liquid may remain.
10. With the samples still on the magnetic stand, add 200 ul of freshly prepared 80% EtOH to each sample without disturbing the beads.
11. Incubate at RT for 30 seconds, then remove and discard all of the supernatant from each sample.
12. Repeat steps 10 & 11 once for a total of two 80% EtOH washes. Use P10 pipettor to remove the rest of the EtOH.
13. Allow samples to air dry for about 5-10 minutes then remove the samples from the magnetic stand.
14. Add 17.5 ul of **Resuspension Buffer**. Gently pipette entire volume up/down 10x to mix thoroughly.
15. Incubate samples at RT for 2 minutes.
16. Place samples on magnetic stand at RT for at least 3-5 minutes.
17. Transfer 15 ul of the clear supernatant from each sample to a new 0.2 ml PCR tube.

SAVE STOPPING POINT: Store samples at -15°C to -25°C for up to 7 days.

**I. A-Tailing** – A single 'A' nucleotide is added to the 3' ends of the blunt fragments to prevent them from ligating to one another during the adapter ligation reaction. A corresponding single 'T' nucleotide on the 3' end of the adapter provides a complementary overhang for ligating the adapter to the fragment. This strategy ensures a low rate of chimera (concatenated template) formation.

1. Add 2.5 ul of **Resuspension Buffer** to each sample. Gently pipette entire volume up/down 10x to mix thoroughly.
2. Add 12.5 ul A-Tailing Mix to each sample. Gently flick tubes and quickly spin samples down.
3. Incubate on thermal cycler at 37°C (Instant program) for 30 minutes.
4. Remove samples from the thermal cycler, and proceed immediately to Ligate Adapters.

**J. Ligate Adapters** – This process ligates multiple indexing adapters to the ends of the ds cDNA, preparing them for hybridization onto a flow cell.

1. Ligase Mix to be on cold block at all times when removed from storage: add 2.5 ul of **Ligation Mix** to each sample. Return Ligase Mix back to storage immediately after use.
2. Add 2.5 ul of **Resuspension Buffer** to each sample
3. Add 2.5 ul of each **Adapter Index** to corresponding sample. Gently pipette entire volume up/down 10x to mix thoroughly.
4. Incubate samples on thermal cycler at 30°C for 10 minutes.
5. Remove samples from thermal cycler.
6. Add 5 ul of **Stop Ligation Buffer** to each sample to inactivate the ligation mix. Gently pipette entire volume up/down 10x to mix thoroughly.
7. Add 42 ul of beads to each sample. Gently pipette entire volume up/down 10x to mix thoroughly.
8. Incubate samples for 15 minutes.
9. Place samples on magnetic stand at RT for at least 3-5 minutes, until the liquid appears clear.
10. Remove and discard 70 ul of the supernatant from each sample.
11. With the samples still on the magnetic stand, add 200 ul of freshly prepared 80% EtOH to each sample without disturbing the beads.
12. Incubate at RT for 30 seconds, then remove and discard all of the supernatant from each sample.
13. Repeat steps 11 & 12 once, total of two washes.
14. Allow samples to air dry for about 5-10 minutes then remove the samples from the magnetic stand.
15. Add 52.5 ul **Resuspension Buffer**. Gently pipette entire volume up/down 10x to mix thoroughly.
16. Incubate samples at RT for 2 minutes.

18. Transfer 50 ul of the supernatant to a new 0.2 ml PCR tube.
19. Add 50 ul of beads to each sample. Gently pipette entire volume up/down 10x to mix thoroughly.
20. Incubate samples for 5 minutes.
21. Place samples on magnetic stand at RT for at least 3-5 minutes, until the liquid appears clear.
22. Remove and discard 95 ul of the supernatant from each sample.
23. With the samples still on the magnetic stand , add 200 ul of freshly prepared 80% EtOH to each sample without disturbing the beads.
24. Incubate at RT for 30 seconds, then remove and discard all of the supernatant from each sample.
25. Repeat steps 23 & 24 once, total of two washes.
26. Allow samples to air dry for about 5-10 minutes then remove the samples from the magnetic stand.
27. Add 22.5 ul **Resuspension Buffer**. Gently pipette entire volume up/down 10x to mix thoroughly.
28. Incubate samples at RT for 2 minutes.
29. Place samples on magnetic stand at RT for at least 3-5 minutes, until the liquid appears clear.
30. Transfer 10 ul of the clear supernatant to a new 0.2 ml PCR tube. Transfer another 10 ul of the clear supernatant to a new 1.5 ml tube for storage.

SAVE STOPPING POINT: Store samples at -15°C to -25°C for up to 7 days.

**K. Enrich DNA Fragments** – This process uses PCR selectively enrich those DNA fragments that have adapter molecule on both ends to amplify the amount of DNA in the library. The PCR is performed with a PCR primer cocktail that anneals to the ends of the adapters. The number of PCR cycles should be minimized to avoid skewing the representation of the library.

1. Add 10 ul water to each sample (if 10 ul ligation is used, from step 30 above).
2. Add 5 ul of PCR Primer Cocktail to each sample.
3. Add 25 ul of PCR Master Mix to each sample. Gently pipette entire volume up/down 10x to mix thoroughly.
4. Incubate samples on the thermal cycler with PCR program: Lib 10 – Lib 12 (internal use)
  - 98°C for 30 seconds
  - 10-12 cycles of:
    - 98°C for 10 seconds
    - 60°C for 30 seconds
    - 72°C for 30 seconds
  - 72°C for 5 minutes
  - Hold at 4°C
5. Add 50 ul of beads to each sample. Gently pipette entire volume up/down 10x to mix thoroughly.
6. Incubate samples for 15 minutes.
7. Place samples on magnetic stand at RT for at least 3-5 minutes, until the liquid appears clear.
8. Remove and discard 95 ul of the supernatant from each sample.
8. With the samples still on the magnetic stand , add 200 ul of freshly prepared 80% EtOH to each sample without disturbing the beads.
9. Incubate at RT for 30 seconds, then remove and discard all of the supernatant from each sample.
10. Repeat steps 8 & 9 once, total of two washes.
11. Allow samples to air dry for about 5-10 minutes then remove the samples from the magnetic stand.
12. Add 32.5 ul of Resuspension Buffer. Gently pipette entire volume up/down 10x to mix thoroughly.
13. Incubate samples at RT for 2 minutes.
18. Place samples on magnetic stand at RT for at least 3-5 minutes, until the liquid appears clear.
19. Transfer 30 ul of the clear supernatant to a new 1.5ml centrifuge tube, properly labeled.

### **L. Validate Library**

Use DNA High Sensitivity bioanalyzer supplies to check the size and purity of the library. Dilute library up to 5 ng/ul for bioanalyzer assay.

Products Information

Vendor	Product Description	Catalog/Product No.
Illumina	TruSeq™ RNA Sample Prep Kit v2 –Set A (48 rxn)	RS-122-2001
Agencourt Bioscience Corporation	AMPure® XP Beads 5 ml	A63880
	AMPure® XP Beads 60 ml	A63881
	AMPure® XP Beads 450 ml	A63882