

1. **mRNA purification** (you will need two heat blocks, one at 80⁰C and one at 65⁰C. Vortex beads vigorously in each step to resuspend and wash, do not allow beads to dry. The Illumina mRNA-Seq sample prep guide has more guidance as to how to work with the beads.)
 - 1.1 Dilute >80 ng total RNA with nuclease-free H₂O to 50 μ L in a 1.5 mL RNase free non-stick tube.
 - 1.2 Heat at 65⁰C for 5 minutes to disrupt the secondary structures, and place on ice.
 - 1.3 Meanwhile, vortex well and aliquot 15 μ L of Sera-mag oligo(dT) beads into a 1.5mL RNase free non-stick tube.
 - 1.4 Wash the beads twice with 100 μ L Bead Binding Buffer.
 - 1.5 Resuspend the beads in 50 μ L Bead Binding Buffer, and add the 50 μ L of total RNA sample from step 1.2; rotate at RT for 5 minutes.
 - 1.6 Remove the supernatant and wash the beads twice with 200 μ L of Bead Washing Buffer. Vortex well to re-suspend beads.
 - 1.7 Add 50 μ L of 10mM Tris-HCl to the beads, heat at 80⁰C for 2 minutes to elute mRNA from the beads
 - 1.8 Meanwhile aliquot 50 μ L of Bead Binding Buffer to a fresh 1.5mL RNase free non-sticky tube.
 - 1.9 After heating the bead and mRNA at 80⁰C for 2 minutes, immediately put on the magnet stand and transfer the supernatant (mRNA) to the binding buffer tube from step 1.8.
 - 1.10 Heat the samples in the binding buffer at 65⁰C for 5 minutes to disrupt the secondary structures, and place on ice.
 - 1.11 Meanwhile Wash the bead twice with 200 μ L of Bead Washing Buffer(Vortex Well)
 - 1.12 Add 100 μ L of mRNA sample from step 1.9 to bead and vortex to resuspend beads; rotate at RT for 5 minutes.
 - 1.13 Remove the supernatant and wash the beads twice with 200 μ L of Bead Washing Buffer. Vortex well to resuspend beads.
 - 1.14 Add 17 μ L of 10mM Tris-HCl to the beads, heat at 80⁰C for 2 minutes to elute mRNA from the beads. Immediately put on the magnet stand and transfer the supernatant (mRNA) to a fresh 200 μ L thin wall PCR tube, and there should be ~16 μ L of mRNA.

2. Fragment mRNA

2.1 Assemble the following reaction:

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|---------------------------|------------|
| ▪ 5x fragmentation buffer | 4 μ L |
| ▪ mRNA | 16 μ L |

2.2 Incubate the tube in a PCR thermocycler at 94°C for 12 minutes, put the tubes on ice and add 2 μ L of fragmentation stop solution.

2.3 Add 80 μ L of 100% Ethanol to fragmented RNA sample, then transfer RNA/Ethanol mix to a 1.5 mL tube containing 60 μ L of SPRI beads. Elute the RNA in 12 μ L of EB. Perform SPRI purification by following “**SPRI purification protocol**” shown at the end of this protocol.

3. First strand cDNA synthesis

3.1 Assemble the following reaction:

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|--------------------------------------|--------------|
| ▪ Random Primer (3 μ g/ μ L) | 1 μ L |
| ▪ mRNA | 11.1 μ L |

3.2 Incubate the tube in a PCR thermocycler at 65°C for 5 minutes, and put the tubes on ice.

3.3 Mix the following in order, make 10% extra reagent for multiple samples:

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|--|-------------|
| ▪ 5 \times 1 st strand buffer | 4 μ L |
| ▪ 100mM DTT | 2 μ L |
| ▪ dNTP mix (25mM) | 0.4 μ L |
| ▪ RNaseOUT (40U/ μ L) | 0.5 μ L |

3.4 Add 6.9 μ L mixture to the tube, mix well, and heat the sample at 25°C in a thermocycler for 2 min.

3.5 Add 1 μ L SuperscriptIII (200U/ μ L) to the sample, and incubate the sample in a thermocycler with following program:

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|----------|------|-------|
| ▪ Step 1 | 25°C | 10min |
| ▪ Step 2 | 50°C | 50min |
| ▪ Step 3 | 70°C | 15min |
| ▪ Step 4 | 4 °C | Hold |

4. Second strand cDNA synthesis

- 4.1 Put the tubes on ice.
- 4.2 Add 18 μ L of H₂O to the first strand cDNA synthesis mix.
- 4.3 Add the following reagents:
 - 10 \times second strand buffer 5 μ L
 - dNTP mix (25mM) 1 μ L
- 4.4 Mix well, incubate on ice 5 minutes and add:
 - RNaseH (2U/ μ L) 1 μ L
 - DNA pol I (10U/ μ L) 5 μ L
- 4.5 Mix well, and incubate at 16°C in a thermocycler for 2.5 hours.
- 4.6 Purify the DNA by adding 75 μ L of SPRI beads to 50 μ L of cDNA mix and elute in 33 μ L of EB solution. Perform SPRI purification by following “**SPRI purification protocol**” shown at the end of this protocol.

End of Day 1, store samples at -20°C. The rest of the protocol offers more opportunities to stop, basically anytime after SPRI purification, store at -20°C and be sure to use non-stick tubes for long term storage.

5. End repair

- 5.1 Prepare the following reaction mix:
 - Eluted DNA 32.4 μ L
 - 10X End-repair buffer 5 μ L
 - dNTP mix (25mM) 1.6 μ L
 - T4 DNA polymerase (3U/ μ L) 5 μ L
 - Klenow DNA polymerase (5U/ μ L) 1 μ L
 - T4 PNK (10U/ μ L) 5 μ L
- 5.2 Incubate at 20°C for 30min.
- 5.3 Purify the DNA by adding 75 μ L of SPRI beads to 50 μ L of End-repair-mix and elute in 33 μ L of EB solution.

6. Adenylation of 3' ends

6.1 Prepare the following reaction mix:

▪ Eluted DNA	32μL
▪ “A” Tailing Buffer	5μL
▪ dATP(1mM)	10μL
▪ Klenow 3' to 5' exo- (5U/μL)	3μL

6.2 Incubate at 37°C in for 30min.

6.3 Purify the DNA by adding 75 uL of SPRI beads to 50 μL of ‘A’ tailing-mix and elute in 23μL of EB solution. Perform SPRI purification by following “**SPRI purification protocol**” shown at the end of this protocol.

7. Adaptor ligation

We prefer the T4 DNA ligase available from Enzymatics (and the included 2x rapid buffer) for this step, as with small DNA concentrations, adapter concatimers can be a problem.

7.1 Prepare the following reaction mix:

▪ Elute DNA	23μL
▪ DNA rapid 2x ligase buffer	25μL
▪ PE Adaptor oligo mix (1:15 dilution)	1μL
▪ T4 DNA ligase (600U/μL)	1μL

7.2 Incubate at RT for 15min.

7.3 Purify the DNA by adding 50 μL of SPRI beads to 50 μL of adaptor-ligation-mix and elute in 30μL of EB solution.

8. PCR

8.1 Set up PCR master mix, make 10% extra reagent for multiple samples, and aliquot 20μL to each PCR tube:

▪ 5 × cloned Phu Buffer	10μL
▪ PCR primer 1.0	1μL
▪ PCR primer 2.0	1μL
▪ 25mM dNTP mix	0.5μL
▪ Phu polymerase	0.5μL
▪ H2O	7μL

8.2 Add 30 μ L purified ligation mix to the PCR tube.

8.3 Run following PCR cycle:

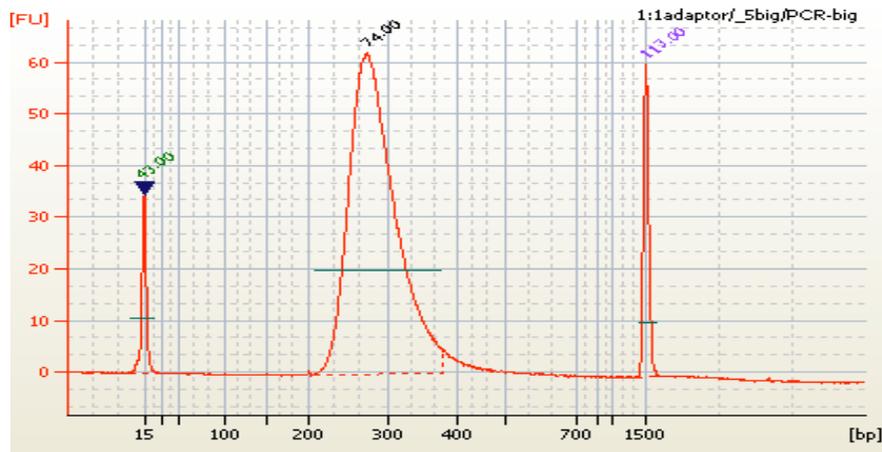
- 98°C 30 sec
 - 98°C 10 sec
 - 65°C 30 sec
 - 72°C 30 sec
 - 72°C 5 min
 - 4°C ∞
- } 15x

8.4 Purify the DNA by adding 50 μ L of SPRI beads to 50 μ L of PCR product and elute in 15-35 μ L of EB solution (depending on how small of a sample you started with, and which loading procedure you plan to use). Perform SPRI purification by following “**SPRI purification protocol**” shown at the end of this protocol.

8.5 Dry down in a vacuum pump for a few minutes, just to make sure all traces of ethanol and other contaminants are removed.

9. Library quantitation

Measure concentration with KAPA Library Quantification Kit (KAPA Biosystems, a qPCR assay, helpful for very low concentrations) or Agilent bioanalyzer. Reference image from Agilent bioanalyzer.



How to perform SPRI (Ampure XP) purification:

1. Transfer *** μL of well mixed SPRI beads (Ampure XP) from stock bottle to each 1.5 mL sample tube.
2. Pipette up and down for at least 8 times for mixing thoroughly.
3. Incubate at room temperature for 5 min.
4. Place 1.5 mL tubes on magnet stand for ~ 3 min until the liquid appears clear. (Don't rush this step, it's easier to remove the supernatant in the next step without disturbing the beads if you leave it on the magnet a bit longer than you think you should before the next step.
5. Discard the supernatant.
6. Add 180 μL of 70% Ethanol 1.5 mL tube without disturbing the beads. Leave the tubes on the magnet for all wash steps.
7. Wait for 30 seconds and discard the supernatant (70% Ethanol) by using pipette to transfer.
8. Repeat step 6 and 7 for a total of two 70% Ethanol washes.
9. Spin down for 10 seconds at 1000 rpm to spin down any ethanol from side of tube.
10. Place each 1.5 mL tube on magnet stand for 30 seconds then remove all the remaining Ethanol using 10 μl pipette. (There is a delicate balance here of wanting to remove all the ethanol and not wanting to dry the beads too much as this reduces elution efficiency.)
11. Add *** μL of EB to the dry pellet for eluting the DNA. Pipette mixing the pellet and then sit at room temp for 2 min and put on the magnet stand or plate.
12. Transfer clear supernatant to new tubes. Label with all the information and store in -20°C .

Materials:

Illumina RNA-seq sample prep kit

Non-Stick RNase-free Microfuge Tubes, 1.5mL (Ambion, AM12450)

Superscript III (Invitrogen, 18080-093) or Superscript II, just adjust PCR program accordingly

Ampure XP beads (Beckman-Coulter, A63880 or A63881)

Magnet stand (we recommend Invitrogen DynaMag-2 , 123-21D)

T4 DNA Ligase, Rapid (Enzymatics, L603-HC-L)

Ethanol

EB buffer