

Protocol: Directional RNA Sequencing of rRNA-depleted MTB RNA

Adapted from Illumina Directional mRNA Sequencing protocol

rRNA-depleted RNA is obtained by using the Epicentre Ribo-zero RNA Removal Gram-Positive kits.

1. RNA Fragmentation: start with rRNA-depleted mRNA

1.1. Chemical RNA fragmentation is performed using 10x Ambion Fragmentation buffer

- 1 μ l 10x Ambion Fragmentation buffer
- 9 μ l mRNA - ~100 ng (rRNA-depleted RNA using Ribo-zero Removal kit)
- Incubate in a thermocycler at **70°C for 5 min.**
- Immediately place sample(s) on ice
- Add 1 μ l Ambion 10x fragmentation stop solution

1.2. Purify fragmented mRNA using a Qiagen RNeasy minelute kit

- Transfer samples to a new 1.5 ml Eppendorf tube, adjust volume to 100 μ l using nuclease-free water.
- Add 350 μ l RLT buffer, mix well.
- Add 675 μ l of 100% EtOH, mix well.
- Load sample on a RNeasy minelute column
- Wash with 500 μ l RPE buffer, centrifuge column
- Wash with 750 μ l of 80% EtOH, centrifuge column
- Use new collection tube, centrifuge column for 2 min.
- Transfer to new 1.5 ml Eppendorf tube. Elute in 17 μ l nuclease-free water.

2. Phosphatase Treatment of Fragmented mRNA

2.1. Phosphatase reaction

Fragmented mRNA	16 μ l
10x phosphatase buffer	56 μ l
Antarctic phosphatase	1 μ l
<u>RNAse Out</u>	<u>1 μl</u>

2.2. Incubate in a thermalcycler:

- a. 37°C – 30 min.
- b. 65°C – 5 min.
- c. 4°C – HOLD

3. PNK Treatment of Fragmented mRNA

3.1. PNK reaction: add the following to the sample tube above

Nuclease-free water	17 μ l
10x phosphatase buffer	5 μ l
ATP (10mM)	5 μ l
RNase Out	1 μ l
<u>PNK</u>	<u>2 μl</u>

3.2. Incubate in a thermocycler:

- a. 37°C – 60 min.
- b. 4°C – HOLD

3.3. Purify sample using QIAquick PCR kit

- Transfer samples to a new 1.5 ml Eppendorf tube, adjust volume to 100 μ l using nuclease-free water.
- Add 350 μ l RLT buffer, mix well.
- Add 675 μ l of 100% EtOH, mix well.
- Load sample on a RNeasy minelute column
- Wash with 500 μ l RPE buffer, centrifuge column
- Wash with 750 μ l of 80% EtOH, centrifuge column
- Use new collection tube, centrifuge column for 2 min.
- Transfer to new 1.5 ml Eppendorf tube. Elute in 17 μ l nuclease-free water.

4. Ligate 3' and 5' RNA Adapters:

4.1. Ligation reaction

RNA sample	6 μ l
<u>Diluted v1.5 sRNA 3' Adaptor</u>	<u>2 μl</u>

4.2. Incubate at 70°C for 2 min, then store on ice.

4.3. Add the following to the sample tube above:

10x T4 RNL2 truncated reaction buffer	17 μ l
100mM MgCl ₂	0.8 μ l
<u>RNase Out</u>	<u>0.5 μl</u>

Mix well and spin down prior to adding enzyme.

4.4. Add 1.5 μ l T4 RNA Ligase 2, truncated

4.5. Incubate at 22°C for 1 hr. in a thermalcycler.

- 4.6. With 5 min. remaining in the above incubation, determine the required amount of 5' adaptor from the stock and heat an aliquot at 70°C for 2 min. Transfer tube to ice.
- 4.7. Add the following reagents to the ligation mixture above:

10mM ATP	6 μ l
<u>SRA 5' Adaptor</u>	<u>2 μl</u>

Mix well and spin down prior to adding enzyme.

- 4.8. Incubate at 20°C for 1 hr. on a thermalcycler.

5. Reverse Transcribe and Amplify the Adapter-ligated RNA

- 5.1. Combine the following: total volume is 5 μ l

5' and 3'ligated RNA	4 μ l
<u>Diluted SRA RT primer</u>	<u>1 μl</u>

- 5.2. Heat the mixture at 70°C in a thermalcycler for 2 min. Centrifuge tube and place on ice.

- 5.3. Dilute the 25mM dNTP Master Mix: make a master mix using a 1:1 ratio of nuclease-free water (1 μ l) and 25mM dNTP mix (1 μ l). The final dNTP concentration is 12.5mM.

- 5.4. Prepare RT Reaction Master Mix:

5x first-strand buffer	17 μ l
12.5mM dNTP	0.5 μ l
100mM DTT	2 μ l
<u>RNAse Out</u>	<u>0.5 μl</u>

- 5.5. Incubate at 48°C for 3 min, then add 1 μ l of Superscript II RT (Invitrogen).

- 5.6. Incubate at 44°C for 1 hr.

- 5.7. Prepare the PCR Reaction Master Mix: total volume is 40 μ l

5x Phusion HF buffer	17 μ l
Primer GX1	1 μ l
Primer GX2	1 μ l
25mM dNTP mix	0.5 μ l
Phusion DNA Polymerase	0.5 μ l
<u>Nuclease-free water</u>	<u>27 μl</u>

- 5.8. PCR Amplification:

5.8.1 Add 40 μ l of PCR master mix to the 10 μ l of product from the reverse

transcription reaction above.

5.8.2 PCR parameters:

- a. 37°C – 30 min.
- b. 12 cycles:
 - 98°C – 10 sec.
 - 60°C – 30 sec.
 - 72°C – 15 sec.
- c. 72°C – 10 min.
- d. 4°C – HOLD

6. Agencourt AMPure XP Purification of PCR Product: Round 1

- 6.1. Add 65 µl AMPure XP beads to the 50 µl pcr reaction from above.
- 6.2. Pipette 10x to mix.
- 6.3. Place tube(s) on the magnetic stand for 5 min.
- 6.4. Pipette off supernatant.
- 6.5. Wash beads with 500 µl 70% EtOH and pipette off supernatant. Repeat twice.
- 6.6. Air dry tube(s) for 15 min. Place tube back on magnetic stand and carefully pipette off any residual EtOH.
- 6.7. Add 30 µl Qiagen EB buffer to the pellet. Pipette 10x to mix.
- 6.8. Let tube(s) stand for 2 min.
- 6.9. Place tube(s) on magnetic stand for 5 min.
- 6.10. Transfer the supernatant to a new 1.5mL Eppendorf tube.

7. Agencourt AMPure XP Purification of PCR Product: Round 2

- 7.1. Add 39 µl AMPure XP beads to the 30 µl pcr reaction from above.
- 7.2. Pipette 10x to mix.
- 7.3. Place tube(s) on the magnetic stand for 5 min.
- 7.4. Pipette off supernatant.
- 7.5. Wash beads with 500 µl 70% EtOH and pipette off supernatant. Repeat twice.
- 7.6. Air dry tube(s) for 15 min. Place tube back on magnetic stand and carefully pipette off any residual EtOH.
- 7.7. Add 10 µl Qiagen EB buffer to the pellet. Pipette 10x to mix. Let tube(s) stand for 2 min.
- 7.8. Place tube(s) on magnetic stand for 5 min.
- 7.9. Transfer the supernatant to a new 1.5mL Eppendorf tube.
- 7.10. Run an aliquot of the library on the Agilent bioanalyzer to QC before sequencing.