

## **Protocol: MTB RNA Isolation from *in vitro* MTB Cultures**

### **1. Harvest MTB cultures**

MTB *in vitro* cultures typically grown in 7H9 media supplemented with ADC, glycerol, and Tween80. Once cultures reach the designated OD<sub>600</sub> or timepoint, cells can be harvested for RNA isolation.

1. Transfer 20ml culture into a 50ml screwcap tube containing 20ml 4M GITC (Add 10 $\mu$ l  $\beta$ -mercaptoethanol per ml GITC right before use). Alternatively, Qiagen RLT can be used in lieu of GITC. Mix tube well.
2. Centrifuge at 4,000 rpm, 10min, 20 $^{\circ}$ c (Beckman Coulter Allegra15R).
3. Decant supernatant.
4. Resuspend the MTB pellet in 1ml Trizol.
5. Transfer Trizol suspension into a 2.0ml screwcap tube containing 0.4ml of 0.1mm zirconia/silica beads.
6. Lyse Mtb. cells by bead-beating in the FastPrep24 instrument (MP Biomedicals) for 30sec (speed 6) for a total of 3 cycles. Place tubes on ice in between cycles. Invert tubes periodically for 5 min.
7. Centrifuge tubes for 1min. at maximum speed.
8. Transfer Trizol supernatant to a tube containing Heavy-Phase Lock Gel.
9. Add 300 $\mu$ l chloroform.
10. Shake tubes vigorously for 15sec. Invert periodically for 2min.
11. Centrifuge tubes for 10min. at maximum speed.
12. Transfer aqueous phase into a new 1.5ml eppendorf tube.
13. Place on ice.
14. Spray the outside of tube with Staphene. Remove from P3.

### **2. RNA Purification and DNase Treatment**

#### **A. Precipitation of aqueous phase:**

1. Add 1:1 volume of isopropanol to aqueous phase. Mix samples repeatedly by inversion.
2. *Optional:* Add Glycoblue coprecipitant (*Ambion Cat#9515, 15mg/ml*) at 150 $\mu$ g/ml final concentration. Glycoblue consists of a blue dye covalently linked to glycogen and improves visibility of RNA pellet upon precipitation.
3. Incubate at room temperature for 10min.
4. Centrifuge at 4 $^{\circ}$ c for 10min.
5. Decant isopropanol. Wash twice in 70% ethanol. Spin after each wash.

#### **B. RNeasy column followed by OFF-column RQ1 DNase (1x DNase):**

For *in vitro* samples, typically 1x DNase treatment is sufficient for RT-PCR assays.

1. Remove trace ethanol. Reconstitute by adding 100  $\mu$ l **Rnase-free water**.
2. **Column clean up:** to 100  $\mu$ l of sample, add 350  $\mu$ l of *fresh* **RLT** buffer (+ 10 $\mu$ l/ml  $\beta$ -mercapto-ethanol + 20 $\mu$ g/ml linear acrylamide) and pipette thoroughly to mix. Add 250  $\mu$ l of **100% EtOH** and pipette thoroughly to mix.
3. Add to a new RNeasy column. Centrifuge for 30 sec.
4. Transfer RNeasy column to a new collection tube. Wash by adding 700  $\mu$ l of buffer **RW1** to RNeasy column and centrifuge for 30 sec. Discard eluate. Repeat wash with 700  $\mu$ l of buffer **RW1** and centrifuge a second time for 30 sec.
5. Transfer RNeasy column to a new 2 ml collection tube. Wash by adding 500  $\mu$ l of buffer **RPE** (ensure that EtOH has been added to this buffer) to RNeasy column and centrifuge for 30 sec. Discard eluate. Repeat wash with 500 $\mu$ l of **RPE** and centrifuge a second time for 2 min.
6. Transfer column to a new collection tube and centrifuge for 1 min to dry column membrane.
7. Transfer column to an Rnase-free 1.5ml eppendorf tube (from kit), open cap of column and allow membrane to air dry for 2 min.
8. Elute sample by adding 50  $\mu$ l of **RNase-free water**. Do not close cap, let membrane soak for 2 min. Close cap and centrifuge for 2min. Perform a second elution in the same collection tube with 34  $\mu$ l of **RNase-free water** and centrifuge for 2 min. Total sample volume should be 84  $\mu$ l.
9. **OFF-column RQ1 DNase treatment:** add 10  $\mu$ l RQ1 buffer, 2.5  $\mu$ l Ribolock inhibitor, and 4  $\mu$ l RQ1 DNase. Mix by gently flicking tube.
10. Incubate in 37c water bath for 30 minutes.
11. **Column clean up:** to 100  $\mu$ l of sample (from step 14), add 350  $\mu$ l of *fresh* **RLT** buffer (+ 10  $\mu$ l/ml  $\beta$ -Mercapto ethanol + 20  $\mu$ g/ml linear acrylamide) and pipette thoroughly to mix. Add 250  $\mu$ l of **100% EtOH** and pipette thoroughly to mix. Add to a new RNeasy column. Centrifuge for 30 sec.
12. Transfer RNeasy column to a new 2 ml collection tube. Wash by adding 500  $\mu$ l of buffer **RPE** (ensure that EtOH has been added to this buffer) to RNeasy column and centrifuge for 30 sec. Discard eluate. Repeat wash with 500 $\mu$ l of RPE and centrifuge a second time for 2 min.
13. Transfer RNeasy column to a new 2 ml collection tube. Wash by adding 500  $\mu$ l of buffer **RPE** (ensure that EtOH has been added to this buffer) to RNeasy column and centrifuge for 30 sec. Discard eluate. Repeat wash with 500 $\mu$ l of RPE and centrifuge a second time for 2 min.
14. Transfer column to a new collection tube and centrifuge for 1 min to dry column membrane.
15. Transfer column to an Rnase-free 1.5ml Eppendorf tube (from kit), open cap of column and allow membrane to air dry for 2 min.
16. Elute sample by adding 30  $\mu$ l of **RNase-free water**. Do not close cap, let membrane soak for 2 min. Close cap and centrifuge for 2min. Perform a second elution in the same collection tube with 20  $\mu$ l of **RNase-free water** and centrifuge for 2 min. Total sample volume should be 50  $\mu$ l.
17. Store in -80c freezer.