

Protocol: MTB RNA Isolation from MTB-Infected Lung Tissue (Covaris Method)

1. Preparation of Tissue Specimens

Preservation of RNA in lung tissues: the following method is optimized for MTB-infected lung tissues that have been dissected by a pathologist (immediately after removal from the patient or primate) into small pieces believed to contain caseous lesions, granulomas or other sites harboring MTB.

- Surgically-removed lung tissues believed to contain granulomas, caseous lesions or other MTB-containing segments are processed in the operating anteroom by a pathologist. Each block of tissue is dissected into pieces that are no bigger than 0.5 – 1.0 g on the overall.
- Tissue specimens not more than 1 gram should be flash-frozen in liquid nitrogen and stored in Covaris bags (Cat. No 520001, TT1) at -80°C. Ideally, all specimens received should arrive in these bags as standard operating procedure. This eliminates the extra step of transferring specimens from their original tubes to the Covaris bags.
- For tissue specimens > 1gram but < 10 grams, flash-freeze specimens in Covaris pouches (Cat. No 520008, TT3). Store specimens at -80°C.
- The samples are shipped in appropriate biohazard containers containing enough dry ice to ensure that they will remain frozen for several working days—estimated to provide sufficient time for samples to clear customs (if needed) in the United States and be delivered. Upon receipt, they are logged-in and immediately stored in the Stanford BL3. Prior to shipping, Miss Tran Van, BL3 technician, is notified. She will provide the current optimal delivery address, phone number and Federal permits for the importation of the samples. She may be reached at ttvan@stanford.edu

2. Covaris S2 Homogenization of Lung Tissue. The following steps are performed in the Stanford BL3 Facility

- Remove specimens from -80°C freezer and place on dry ice.
- Turn on Covaris S2 instrument, water bath chiller, and computer. Fill S2 water tank to the FILL LINE, approximately 1.6Liters.
Use only distilled or deionized water to fill water tank.
Gently lower the transducer down into the water tank. Check again to make sure the water level is sufficient.
Press the 'DEGAS - ON' button to start the degassing process. The water should degas for at least 30 minutes prior to system use.
- The Covaris Cryoprep instrument is used to pulverize tissue specimens prior to its use in the Covaris S2 system. Replace the rubber cap with a sterile 16mm glass borosilicate tube. Ensure that the tube is securely attached to the Covaris bag. Set the Cryoprep power to 4. Place tube/bag unit into the opening, press 'Green' button. Remove bag to make sure that specimen is completely pulverized. Repeat procedure, if necessary. Check for leaks or breakage in the bag before proceeding. For large specimens in Covaris pouches, pulverize tissue using the pre-cooled Covaris CP03 instrument.

- Add 2 ml RLT into the Covaris bag. Using a 1 ml disposable transfer pipet, mix and transfer contents into a sterile Covaris 16mm glass borosilicate tube. Make sure to keep tissue pieces submerged in the RLT and not on the sides of the tube.
- Make sure plastic cap is tightly secured to the tube. Place tube into the Covaris S2 instrument.
- Press 'START' button. As a safety feature, second 'User Alert' window will appear reminding the user to check that everything is in order before the process starts. Press 'START' button again. Once the program is completed, check to make sure that tissue sample is completely homogenized. If not, add an additional 30 sec. cycle.
- Using a 5 ml pipette, transfer homogenized contents to a 15ml screwcap tube. Place tube on ice. Continue Covaris process with the rest of the specimens.
- Centrifuge 15 ml screwcap tubes at 4000rpm for 10 min.

3. MTB RNA Isolation - Bead-Beating and Trizol Extraction

- Remove remaining supernatant and freeze (eukaryotic fraction). Add 1 ml Trizol containing 20µg/ml linear acrylamide to the pellet. Use pipette to resuspend pellet in Trizol.
- Transfer Trizol suspension to a 2 ml screw cap tube (*E&K Scientific, #640201-S*) containing 0.4 ml of 0.1mm zirconia/silica beads (*Biospec, #110791012*).
- Disrupt Mtb. cells for 30 sec at speed 6, 3x (*Fastprep24 bead-beater, MP Biomedicals*). Cool on ice between cycles.
- Continue to periodically invert tube(s) for 5 min, place on ice in between.
- Centrifuge for 1 min. at maximum speed (*Eppendorf 5415D*).
- Transfer supernatant to a 2 ml screw cap tube containing Heavy Phase Lock Gel (*Brinkmann, Eppendorf*). Add 300 µl chloroform. See note below if performing parallel DNA isolation.
- Vortex tube for 15 sec. Continue to vigorously invert tube for 2 min.
- Centrifuge for 10 min. at maximum speed (*Eppendorf 5415D*).
- Transfer the aqueous phase (~540 µl) to a 2.0 ml elution tube (*Ambion Cat#12480*). For DNA isolation: transfer the interphase and organic phase (bottom layer) to a new 1.5ml eppendorf tube. Refer to the DNA Isolation from Trizol protocol (see below).
- Place on ice.

Note: if performing parallel DNA isolation, do NOT use Heavy Phase Lock Gel for the chloroform separation step. Instead, transfer the trizol supernatant to a 2 ml screw cap tube containing 200 µl chloroform. Proceed as normal. After centrifugation, the aqueous phase contains the RNA and the interphase/organic phase contains the DNA.

4. Decontaminate tube(s) by wiping with Staphene and remove from BL3.

5. Purification and DNase Treatment of MTB RNA

A. Precipitation of aqueous phase:

- Add Glycoblue coprecipitant (*Ambion Cat#9515, 15mg/ml*) at 150µg/ml final concentration. Glycoblue consists of a blue dye covalently linked to glycogen; it improves visibility of RNA pellet upon precipitation.

i.e. 500 µl aqueous phase + 10 µl Glycoblue.

- Add 1:10 volume of 5M ammonium acetate. Mix well.
- Add 1:1 volume of isopropanol to aqueous phase. Mix samples repeatedly by inversion. *i.e. 500 µl aqueous phase + 500 µl isopropanol*
- Incubate at room temperature for 20min. Alternatively, samples may be placed at -20c overnight, if not processed immediately.
- Centrifuge at 4°C for 10min. Blue RNA pellet should be visible on the side of tube.
- Decant isopropanol. Wash twice in 70% ethanol. Spin for 5min. after each wash.

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

- Remove trace ethanol. Reconstitute by adding **100 µl RNase-free water**.
- **Column clean up:** to **100 µl** of sample, add **350 µl** of *fresh RLT* buffer (+ 10 µl/ml β-Mercapto-ethanol + 20 µg/ml linear acrylamide) and pipette thoroughly to mix. Add **250 µl** of **100% EtOH** and pipette thoroughly to mix.
 - Add to a new RNeasy column. Centrifuge for **30 sec**.
- Transfer RNeasy column to a new collection tube. Wash by adding **700 µl** of buffer **RW1** to RNeasy column and centrifuge for **30 sec**. Discard eluate. Repeat wash with **700 µl** of buffer **RW1** and centrifuge a second time for **30 sec**.
- Transfer RNeasy column to a new 2 ml collection tube. Wash by adding **500 µl** 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 **500ul** of **RPE** and centrifuge a second time for **2 min**.
- Transfer column to a new collection tube and centrifuge for **1 min** to dry column membrane.
- 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**.
- Elute sample by adding **50 µl** 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 µl** of **RNase-free water** and centrifuge for **2 min**. Total sample volume should be **80 µl**.
- **OFF-column RQ1 DNase:** add **10 µl** RQ1 buffer, **2.5 µl** Ribolock RNase inhibitor (Fermentas, Cat. No. EO0381), and **8 µl** RQ1 DNase (Promega, Cat. No. M6101). Mix by gently flicking side of tube until mixture is homogenous.
- Incubate in 37c water bath for 30 minutes.
- **Column clean up:** to **100 µl** of sample (from step 14), add **350 µl** of *fresh RLT* buffer (+ 10 µl/ml β-Mercapto ethanol + 20 µg/ml linear acrylamide) and pipette thoroughly to mix. Add **250 µl** of **100% EtOH** and pipette thoroughly to mix. Add to a new RNeasy column. Centrifuge for **30 sec**.
- Transfer RNeasy column to a new 2 ml collection tube. Wash by adding **500 µl** 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 500ul of RPE and centrifuge a second time for **2 min**.
- Transfer column to a new collection tube and centrifuge for **1 min** to dry column membrane.
- 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**.

- Elute sample by adding 50 μl 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 μl of **RNase-free water** and centrifuge for 2 min. Total sample volume should be 80 μl .
- **2nd OFF-column RQ1 DNase:** Repeat OFF-column RQ1 DNase treatment (total 2x DNase). Refer to procedures above.
- Elute sample by adding 30 μl 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 μl of **RNase-free water** and centrifuge for 2 min. Total sample volume should be 50 μl .
- Store in -80°C freezer.