

Chapter 2

Isolation of Mycobacterial RNA

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Abstract This chapter describes two protocols for isolating total RNA from mycobacteria: one for extraction from *in vitro* cultures and one for extraction from *in vivo*. In these protocols, RNA is liberated from mycobacteria by disruption with small glass beads in the presence of Trizol to stabilize the RNA. The RNA is further purified with DNase treatment and RNeasy columns. This protocol leads to microgram quantities of RNA from log-phase cultures.

Keywords cell disruption · extraction · *in vivo* · *in vitro* · RNA

2.1 Introduction

In nearly all living cells, ribonucleic acid (RNA) exists simply to convert the information stored in DNA into a functional form, generally as proteins. Even though RNA is an indirect measure of proteins, RNA analysis is central to modern molecular biology. RNA-based methods such as Northern blotting, transcriptome profiling by microarray [1], and quantitative real-time PCR [2] provide an unparalleled opportunity to interrogate cellular processes such as development and responses to environmental perturbations. The speed and ease of these methods make RNA an indispensable material for high-throughput analysis.

RNA extraction is a relatively simple process that takes only a few hours of labor over 2 days. And yet, RNA extraction is often a dreaded chore, particularly in a lab that works with mycobacteria. Why? Because RNA is fragile, mycobacteria are not, and RNA released from bacilli faces a hostile world of thermal and enzymatic breakdown. RNases are extremely common in the environment, highly stable, and they can reduce months of careful experimental preparation to a tube of useless nucleotides in seconds.

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Success, readily achieved, depends on preparation. The simple protocol outlined below can be used to isolate large quantities of RNA from mycobacterial *in vitro* cultures using only a limited amount of specialized equipment and easily available materials. With a few modifications, this protocol can be used to isolate RNA from macrophages or infected animal tissue. Our laboratory has used this protocol or subtle variants hundreds of times over many experiments to isolate grams of mycobacterial RNA [3, 4, 5]. The primary key to success with this protocol is keeping it simple, quick, and consistent. As is traditional in this book, we will include many footnotes with observations, tips, and tricks.

2.2 Materials

2.2.1 *Isolation of Mycobacterium tuberculosis RNA from In Vitro Culture*

2.2.1.1 Log-Phase Culture of *Mycobacterium tuberculosis*

1. ADC (albumin-dextrose-catalase) supplement (Becton Dickinson, Franklin Lakes, NJ).
2. Middlebrook 7 H9 medium (Becton Dickinson) supplemented with 10% v/v ADC or other mycobacterial growth medium.
3. Roller apparatus (Stovall Life Science, Inc., Greensboro, NC).
4. 50-mL conical tubes (e.g., Falcon).
5. 250-mL, 500-mL, and 2-L roller bottles (*see Note 1*).

2.2.1.2 RNA Extraction

1. Trizol solution (Invitrogen Corporation, Carlsbad, CA).
2. Lysing matrix B supplied in 2-mL screw-cap tubes (QBiogene, Inc., Irvine, CA) (*see Note 2*).
3. Fastprep 120 homogenizer (Qbiogene).
4. Phase lock gel (Eppendorf North America, Inc., Westbury, NY).
5. Chloroform/isoamyl alcohol 24:1 (CIA).
6. Isopropanol.
7. High Salt Solution; 0.8 M Na citrate, 1.2 M NaCl.
8. RNase-free water.
9. 75% ethanol solution; make up with RNase-free water.

2.2.1.3 RNA Purification and On-Column DNase Digestion

1. RNeasy RNA purification kit (Qiagen Inc., Valencia, CA).
2. 95% ethanol solution (dilute with RNase-free water).

3. On-column DNase kit (Qiagen). Prepare DNase I stock by dissolving 1500 U DNase I in 550 μ L RNase-free water and mix gently by inverting the tube. Make into single-use aliquots and store at -20°C . A thawed aliquot is stable at 2°C to 8°C for 6 weeks.

2.2.1.4 RNA Quantification

1. TE buffer (10 mM Tris pH 7.5, 1 mM EDTA)
2. Spectrophotometer equipped to read at wavelengths 260 nm and 280 nm.
3. Gel electrophoresis equipment.

2.2.2 Isolation of Mycobacterial RNA from Tissue

2.2.2.1 Tissue Homogenization

1. 13-mL round-bottom polypropylene tubes (e.g., Falcon).
2. Trizol solution (Invitrogen Corporation).
3. OMNI TH tissue homogenizer with serrated disposable tips (Omni International, Marietta, GA).

2.2.2.2 Extraction of *Mycobacterium tuberculosis* RNA

1. Trizol solution (Invitrogen Corporation).
2. Lysing matrix B supplied in 2-mL screw-cap tubes (QBiogene) (*see Note 2*).
3. Fastprep 120 homogenizer (Qbiogene).
4. RNase-free tubes.
5. Chloroform/isoamyl alcohol 24:1 (CIA).
6. Phase lock gel (Eppendorf North America, Inc.).
7. Isopropanol.
8. RNase-free water.
9. 75% ethanol solution; make up with RNase-free water.

2.2.2.3 Qiagen RNeasy Column Purification and On-Column Digestion

1. Qiagen RNeasy kit (Qiagen Inc.).
2. RNase-free tubes.

2.3 Methods

Isolation of high-quality RNA from mycobacteria depends on limiting the amount of time the RNA is susceptible to degradation. These protocols are therefore best done rapidly. Wherever possible, prepare tubes, tips, and equipment in advance. For pathogenic species of mycobacteria, the bulk of the first

part of this protocol is done inside a BL-3 (biosafety level 3) facility due to the risk of aerosols from the vortexing, centrifuging, and bead beating. However, because Trizol rapidly sterilizes the pellets, only the first step of the RNA extraction needs to be carried out in the safety cabinet.

2.3.1 Isolation of *Mycobacterium tuberculosis* RNA from In Vitro Culture

2.3.1.1 Log-Phase Culture of *Mycobacterium tuberculosis*

1. To obtain log-phase bacilli, inoculate 25 mL Middlebrook 7 H9-ADC broth with *M. tuberculosis* in a 250-mL roller bottle (see **Notes 1 and 3**).
2. Grow to early- to mid-log phase (optical density, or O.D., of 0.1 to 0.2) at 37°C with constant rolling.
3. Centrifuge cultures for 5 min at $2000 \times g$, at room temperature.
4. Pipette off supernatant and immediately process for RNA or freeze on dry ice (see **Note 4**).

2.3.1.2 RNA Extraction

1. Add 1 mL Trizol to the cell pellets and transfer to ice (see **Note 2**).
2. Resuspend pellets by vortexing until there are no visible clumps.
3. Add the suspension to a screw-top tube containing Lysing Matrix B and place on ice (see **Note 2**).
4. To disrupt the cells and liberate the RNA, shake for 30 s at maximum speed (6.5) in the Fastprep machine.
5. Place on ice for 30 s to cool sample. Repeat steps 3 to 4 two more times (see **Note 5**).
6. Centrifuge samples for 1 min at $16,000 \times g$ in a Microfuge.
7. Remove Trizol solution to a 2-mL Heavy Phase Lock Gel I snap cap tube containing 300 μ L chloroform/isoamyl alcohol.
8. Mix by inverting rapidly for 15 s, and place on ice. Once all the samples are transferred, continue inverting periodically for 2 min.
9. Centrifuge for 5 min at $16,000 \times g$ in a Microfuge, remove aqueous layer (above the phase lock, volume $\sim 540 \mu$ L), and add to a 1.5-mL tube containing 270 μ L isopropanol and 270 μ L high salt solution (see **Note 6**).
10. Invert several times and spray outside of tube with a mycobactericidal agent and remove from the BL-3. Precipitate at 4°C overnight (see **Note 7**).
11. Centrifuge for 10 min at 4°C and remove isopropanol.
12. Add 1 mL 75% EtOH, invert several times, centrifuge for 5 min at $16000 \times g$, and decant ethanol (see **Notes 8 and 9**).
13. Add 100 μ L RNase-free water.

2.3.1.3 RNA Purification and On-Column DNase Digestion (*see Note 10*)

This is a variant of the Qiagen protocol for RNA purification with the RNeasy columns, simplified slightly for our purposes.

1. Add 350 μ L RLT buffer. *N.B.*: Remember to add 10 μ L β -mercaptoethanol per milliliter of RLT prior to use (*see Note 11*).
2. Vortex. Add 265 μ L 95% EtOH to each sample. Mix by vortexing briefly.
3. Transfer mixture to an RNeasy spin column, centrifuge for 15 s, and transfer column to a new 2-mL collection tube.
4. Add 350 μ L buffer RW1 (*see Note 11*), centrifuge for 15 s, and discard flow through.
5. Add 70 μ L buffer RDD to 10- μ L aliquot of DNase I stock solution and pipette directly onto the column membrane (*see Note 11*). Allow digestion to continue at room temperature for 15 min to 1 h (*see Note 12*).
6. Add 350 μ L buffer RW1, centrifuge for 15 s.
7. Add 500 μ L RPE buffer, centrifuge for 15 s, discard flow-through. Add 500 μ L additional RPE and centrifuge for 2 min. Discard flow-through and spin for 1 min to dry completely (*see Note 13*).
8. Transfer to a 1.5-mL collection tube, elute with 40 μ L RNase-free water, centrifuge 1 min (*see Note 14*) and recover eluate.

2.3.1.4 RNA Quantification

1. Dilute 1 μ L RNA sample in 74 μ L TE and measure absorbance at 260 and 280 nm to determine RNA concentration and purity (*see Note 15*).
2. Using the RNA concentrations estimated by the spectrophotometer reading, run 1 μ g RNA on a 2% w/v agarose gel.
3. Run gel for 45 min at 100 V. Ribosomal bands should be clear, concise, and relatively equal (*see Note 16* and Fig. 2.1).

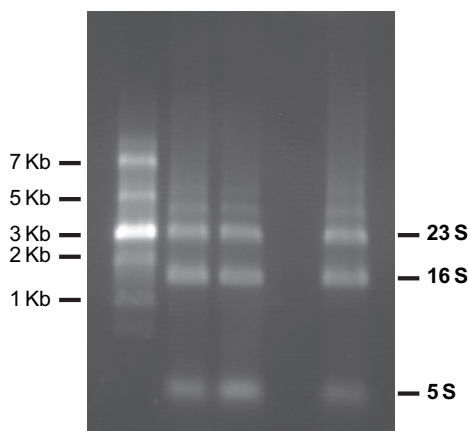
2.3.2 Isolation of Mycobacterial RNA from Tissue (*see Note 17*)

The initial steps of this protocol should be performed in a strict biosafety environment when processing pathogenic mycobacterial species due to the high probability of aerosolization during the tissue homogenization process.

2.3.2.1 Tissue Homogenization

1. Place infected tissue in a 13-mL round-bottom tube and weigh.
2. Add 1 mL Trizol reagent per 50 to 100 mg tissue. For less than 10 mg tissue, add 0.8 mL Trizol reagent.

Fig. 2.1 Nondenaturing “check” gel of *Mycobacterium bovis* RNA isolated from infected rabbit tissue samples



3. Thoroughly homogenize up to 5 mL of sample using the OMNI TH with serrated disposable tips (*see Note 18*) in 13-mL round-bottom tubes. For amounts >5 mL, it is recommended that the sample be split into two. Make sure the tissue is completely homogenized before proceeding.
4. Centrifuge homogenate at $3200 \times g$ for 10 min at room temperature (RT) to separate out the intact bacteria from eukaryotic cell lysate.
5. Decant the supernatant to a fresh tube and save if eukaryotic RNA extraction is desirable. Take care not to disturb the pellet containing the bacteria.

2.3.2.2 Extraction of *Mycobacterium tuberculosis* RNA

The steps below are very similar to the protocol for extraction of RNA from *in vitro* culture above.

1. Add 0.2 mL Trizol per milliliter of Trizol used in the tissue homogenization step to the bacterial cell pellet. Transfer up to 1 mL Trizol suspension to a 2-mL screw-cap tube of Lysing Matrix B (*see Note 2*).
2. Homogenize the cells 3 times using intervals of 30 s at speed 6.5 in a Fastprep 120. Incubate samples 30 s on ice in between disruptions to allow tubes to cool (*see Note 5*).
3. Centrifuge for 1 min at $>10,000 \times g$ to pellet the beads.
4. Transfer supernatant to a 1.5-mL RNase-free tube containing 300 μ L chloroform per mL of Trizol used in the bead-beating step.
5. Shake tube vigorously for 15 s and transfer contents to a (precentrifuged) 2 mL Heavy Phase Lock Gel.
6. Continue to vigorously invert tube for 2 min. At this point, the sample tube can be decontaminated and removed from the biosafety area.

7. Centrifuge for 10 min at $16,000 \times g$ for 10 min at 2°C to 8°C (*see Note 19*).
8. Transfer the aqueous phase ($\sim 540\ \mu\text{L}$ from 1 mL Trizol) to a 1.5-mL RNase-free tube.
9. Precipitate RNA by adding 0.5 mL isopropanol per milliliter Trizol used to resuspend the bacterial pellet.
10. Mix the sample by repeated inversion and centrifuge *briefly* to bring down any contents from the lid.
11. Incubate sample at 15°C to 30°C for 10 min.
12. Centrifuge the sample at $16,000 \times g$ for 10 min at 2°C to 8°C .
13. The RNA pellet should be visible on the side and bottom of tube. Carefully aspirate the supernatant
14. Add 0.2 mL Trizol per milliliter Trizol used in the initial tissue homogenization and gently pipette up and down to dissolve the RNA pellet.
15. Add $300\ \mu\text{L}$ chloroform per milliliter Trizol and shake tube vigorously for 15 s.
16. Transfer contents to a (precentrifuged) 2 mL Heavy Phase Lock Gel.
17. Continue to vigorously invert tube for 2 min.
18. Centrifuge at $16,000 \times g$ for 10 min at 2°C to 8°C .
19. Transfer the aqueous phase to a 1.5-mL RNase-free tube.
20. Add 1 volume 70% EtOH or 0.7 volume 100% EtOH. Invert tube immediately to mix.

2.3.2.3 Qiagen RNeasy Column Purification and On-Column Digestion

1. Apply the entire sample (up to $700\ \mu\text{L}$) to an RNeasy minicolumn placed in a 2-mL collection tube.
2. Centrifuge for 15 s at $16,000 \times g$ and discard the flow-through.
3. Add $350\ \mu\text{L}$ RW1 buffer and centrifuge at $16,000 \times g$ for 15 s (*see Note 11*). Discard flow-through.
4. Add $500\ \mu\text{L}$ RPE and wash the column by centrifuging at $16,000 \times g$ for 15 s.
5. Discard flow-through and add another $500\ \mu\text{L}$ RPE to the column. Centrifuge at $16,000 \times g$ for 15 s and discard flow-through.
6. Thoroughly dry the column by centrifuging at full speed for 1 min.
7. Add $10\ \mu\text{L}$ DNase I stock to $70\ \mu\text{L}$ RDD (Qiagen) and mix by gently inverting the tube. Pipette all $80\ \mu\text{L}$ of the mix directly onto the column and incubate at RT for 15 min.
8. Add $350\ \mu\text{L}$ RW1 and centrifuge at $16,000 \times g$ for 15 s. Discard flow-through.
9. Add $500\ \mu\text{L}$ RPE and wash the column by centrifuging at $16,000 \times g$ for 15 s.
10. Discard flow-through and add another $500\ \mu\text{L}$ RPE to the column.
11. Centrifuge at $16,000 \times g$ for 15 s and discard flow-through, or place column into a new 2-mL collection tube.

12. Thoroughly dry the column by centrifuging at full speed for 1 min.
13. Transfer column to a 1.5-mL RNase-free tube and keep cap open to allow membrane to air dry for 2 min.
14. To elute the RNA, add 50 μ L RNase-free water to the center of the membrane (do not close column cap). Allow the membrane to soak for 2 min. Close cap and centrifuge at max speed for 1 min. Store RNA at -80°C .
15. Quantify RNA as in Section 2.3.1.4.

2.4 Notes

1. Culture volumes of up to 20% of the total container volume can be aerobically grown in roller bottles.
2. It is possible to use screw-cap tubes with sterile 0.2- μm beads in the place of Lysing Matrix beads. However, we prefer to use the commercially available Lysing Matrix B tubes for convenience. Similarly, it is possible to formulate a noncommercial guanidium thiocyanate solution to replace Trizol as a stabilization reagent.
3. The protocol is written for isolation of RNA from log-phase culture to provide the highest yield. This protocol has been used to isolate RNA from late log, hypoxic, and many other culture conditions.
4. We routinely leave pellets frozen at -80°C , sometimes for as long as 2 years with no discernible degradation or loss of RNA, as long as they are frozen rapidly after centrifugation.
5. Samples will heat during processing, which may damage the RNA. Cooling the samples on ice between each round of bead beating minimizes RNA damage.
6. Note that this protocol is slightly different from the method used to extract RNA from tissue culture: the phase separation is performed in a room-temperature centrifuge, instead of at 2°C to 8°C , which we have found to be sufficient for *in vitro* isolation. However, if the phase separation is not clean, centrifugation in a refrigerated centrifuge may improve resolution of the phases.
7. We have found that precipitating in a freezer can decrease the yield of RNA. Precipitating for as long as 3 days at 4°C seems to result in little or no loss or degradation of the RNA.
8. Make certain that the pellet is not decanted with the supernatant. After the isopropanol extraction, a small, often translucent pellet may be visible. After the ethanol wash, a pellet is almost always visible. We typically decant by simply pouring off the supernatant. However, the risk of losing the pellet can be reduced by suctioning off the supernatant with a pipetman or RNase-free Pasteur pipette. When washing, it is not necessary to resuspend the pellet entirely.
9. RNA can be air dried for 5 to 15 min to remove the last traces of ethanol, resuspended in RNase-free water, and frozen at -80°C for later use. The RNA is not clean at this step, but for some experiments this crude extract is sufficient. Do not dry RNA extensively, as it can be very difficult to resuspend.
10. The RNeasy column purification is optional. If column purification is skipped, an additional chloroform extraction step can be added immediately after step 5 of Section 2.3.1.2, and any commercially available DNase can be used. We use the RNeasy columns for simplicity and consistently higher-purity samples, but the added expense of the columns may make them a dispensable step.
11. Supplied with RNeasy kit by manufacturer (Qiagen).
12. The protocol from Qiagen recommends a 15-min incubation. However, RT-qPCR results suggest that extending the incubation to an hour reduced DNA contamination.

The length of DNase treatment will depend on the downstream application and the RNA purity required.

13. Residual ethanol from RPE can interfere with downstream reactions.
14. For RT-qPCR, we have found that it is helpful to have an additional DNase step.
15. The 260/280 ratios are notoriously poor at detecting protein contamination [6]. If yields are very low, the 260/280 readings may be below the level of detection of spectrophotometers and will be thereby skewed.
16. Standard gel electrophoresis is usually sufficient. However, some degradation often occurs in the gel. If degradation is a serious concern, RNA can be analyzed on a denaturing gel containing 20% formaldehyde. Formaldehyde gels require buffer recirculation and ventilation. It is almost certain that there will be some amount of RNA degradation and DNA contamination in a typical sample. However, most applications allow for this fact, particularly microarray analysis where cDNA synthesis and lack of amplification reduce the effects of degradation and DNA contamination.
17. Isolation of good-quality mycobacterial RNA from infected tissue relies primarily on (1) the time it takes to get the tissue into an RNA stabilization reagent, (2) the type of stabilization reagent used, and (3) the method used to preserve the tissue. Different stabilization reagents include Trizol reagent (Invitrogen), RNAlater (Ambion, Austin, TX), or any of the various available guanidine-based solutions. Methods of tissue preservation include immediate freezing of the tissue with or without prior submersion of the tissue in a stabilization reagent. Ideally, infected tissue would be placed quickly into a stabilization reagent, homogenized, and immediately processed. Immediate processing of tissue is often not convenient or not possible due to technical restrictions. In these cases, the tissue should be submerged in stabilization reagent and frozen in liquid nitrogen or dry ice/ethanol for subsequent processing.
18. Adding too much Trizol will not adversely affect RNA stability, but adding too little can lead to stabilization failure. Typically, 5 mL Trizol can be used for tissues weighing 10 to 500 mg.
19. Phase separation in the presence of Trizol should be performed at 2°C to 8°C as indicated. Separations performed at higher temperatures may lead to DNA and protein contamination of the aqueous phase. This does not appear to be a problem with RNA extraction from *in vitro* cultures.

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