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RNA extraction of *Escherichia coli* grown in Lysogeny Broth for use in RT-qPCR

Mariah Hay, Yu Michael Li, Yvonne Ma
Department of Microbiology and Immunology, University of British Columbia

Herein we describe a method used to effectively extract RNA from the Gram-negative bacterium *Escherichia coli* grown in Lysogeny Broth (LB). We employ a Proteinase K and enzymatic lysis step to facilitate the release of RNA from bacterial cells. A silica-based membrane extraction kit (Qiagen RNeasy) is used followed by an added DNase step (TURBO™ DNase Kit from Ambion). This kit includes guanidine-thiocyanate in the lysis buffer that inhibits RNases in the cell and in the solution. Ethanol is then added as a wash step and promotes binding of the RNA to the silica membrane column. To eliminate DNA contamination, our protocol includes two DNase treatment steps. In the first step, DNase is incubated with a RNA sample bound to a silica membrane during the extraction process. In the second added step, TURBO™ DNase is added to a RNA sample after extraction has been completed. Subsequently, the enzyme and contaminants are removed by high speed centrifugation. This paper outlines a method of RNA extraction of *E. coli* for later use in RT-qPCR.

INTRODUCTION

RNA extraction is a common molecular biology practice. It is a necessary step required for later applications such as reverse-transcription quantitative polymerase chain reaction (RT-qPCR), Northern hybridization, rapid amplification of cDNA ends (RACE), microarray analysis, and cDNA cloning (1). However, it remains a challenge to avoid RNA degradation because of the ubiquity of RNases in the environment and their natural occurrence within the cell (2). RNases are especially resistant due to their interchain disulfide bonds and the ability to refold after exposure to high temperatures and denaturation (2). Further, RNases do not require cofactors making them stable on their own (2).

Methods often used for RNA extraction include the use of organic solvents and phenol-chloroform such as an extraction with TRIZOL (3). Another method of RNA isolation is solid-phase nucleic extraction, which is efficient and also safer than using phenol-chloroform (3). In solid-phase RNA extraction. The desired samples are first lysed, often with Proteinase K to improve the purity (4). The lysis step is combined with a homogenization step that includes denaturation with a guanidine-thiocyanate containing buffer to inactivate the RNases released from the cell (5). Once lysed, the sample are mixed with ethanol and placed silica membrane lined column where the RNA can selectively bind (4). This is followed by multiple wash steps to remove the impurities including the unwanted nucleic acid (DNA), polysaccharides and proteins (4). Although, polysaccharides and proteins do not bind to the column in the first place (4). Another wash step that includes alcohol within the buffer, is used to get rid of excess salts (4). The final step is to elute the RNA in a low-salt buffer, which is often water (4). This paper describes a method of RNA extraction of *E. coli* for later use in RT-qPCR.

Key words: RNA, RNase, DNase, DNA contamination, enzymatic lysis, silica column, ethanol, proteinase K

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MATERIALS AND EQUIPMENT

Materials:

- RNeasy Mini Kit (Qiagen)
  - Includes Buffer RLT, Buffer RW1, Buffer RPE, RNeasy Mini Spin Column, RNase-free collection tubes
- RNaseZap® (Invitrogen)
- RNase-free DNase set (Qiagen)
  - Includes DNase I and Buffer RDD
- TURBO™ DNase Kit (Ambion)
  - Includes 10x TURBO™ DNase Buffer, TURBO™ DNase and DNase inactivation reagent
- Lysozyme (15 mg/ml)
- Tris-HCl
- EDTA
- Proteinase K (20 mg/ml)
- B-mercaptoethanol
- 100% ethanol

Equipment:

- Laboratory gloves
- Test tubes
- Culture media
- 37°C shaking incubator
- RNase-free tips (P2, P20, P100-200, P1000)
- RNase-free microcentrifuge tubes
- Vortex
- Spectrophotometer
- Cuvettes
- Sterile working space (ie. Biological safety cabinet)

Note: RNase-free equipment may not be available in MICB447/421 laboratories but are required to prevent RNA degradation.

PROTOCOL

Directions adapted from RNAprotect® Bacteria Reagent Handbook (Qiagen) for Gram-negative cells grown in complex media (7). Instruction for lysis are from on Protocol 4 and instructions for purifying total RNA from bacterial lysis are from Protocol 7 (7). Instruction for the optional on-column DNase treatment is from Appendix 2 (7). TURBO™ DNA-free kit (Ambion) instructions from manufacturer's instructions (8).

A. Cell culture preparation

This step is to grow *E. coli* cultures to the appropriate OD_{600} for RNA extraction. It is recommended in the manual to harvest cells in mid-logarithmic growth. This is also when the stressor (if applicable) can be applied.

B. Preparation of buffers and reagents

To ensure smooth experimental steps, some buffers and reagents used in the lysis and RNA purification steps can be prepared ahead of time.

C. Gram-negative bacterial cell lysis with enzymatic lysis and proteinase K digestion of bacteria

For Gram-negative cells such as *E. coli* grown in complex media (such as LB media), it is recommended by the manual to use a combination of enzymatic lysis and proteinase K for lysis.

D. RNA purification from bacterial lysate with on-column DNase treatment

Using the RNeasy Mini Kit's Spin Columns and wash buffers (Qiagen), RNA is purified from the bacterial lysate. To remove some DNA contamination, on-column DNase treatment with RNase-Free DNase (Qiagen) is performed prior to RNA elution.

E. TURBO™ DNase treatment

Based on our experience from Steps A-C, if there is remaining DNA contamination after RNA elution with on-column DNase treatment. Successful removal of DNA contamination is attained following the manufacturer's protocol for TURBO™ DNA-free kit (Ambion).

METHODS

For all steps, work in an aseptic environment with controlled airflow (such as biological safety cabinet) to reduce RNase contamination. Move microcentrifuge and vortexer into the biological safety cabinet. Spray

and wipe surfaces and gloved hands with RNaseZap® (Invitrogen) or other suitable RNase decontamination reagent.

A. Cell culture

1. Inoculate 5 ml cultures of *E. coli* of interest in LB media and incubate at 37 ˚C with shaking overnight.
2. About 2 hours (hr) prior to RNA extraction, inoculate 5 ml of LB media with overnight culture with 1/100 dilution. This is also the point when the stressor can be applied (in the example by Hay et al., final concentrations of 1.6 µg/ml of kanamycin is introduced per strain (6)).
3. Harvest cells at 0.5 OD_{600} (or other appropriate OD_{600} at mid-logarithmic growth) in LB media and aliquot 0.5 ml into a RNAse-free microfuge tube. The maximum binding capacity of the RNeasy Mini spin column is 100 µg RNA, which is the yield from 6 x 10^8 *E. coli* cells grown in LB medium (7). However, successful RNA extractions have been performed with fewer cells (6).

B. Preparation of buffers and reagents

1. Prepare TE Buffer pH 8.5 (30 mM Tris-HCl, 1 mM EDTA) with 15 mg/ml lysozyme.
2. Prepare mastermix of 1/5 volume buffer RPE and 4/5 volume of 10% ethanol in a RNase-free tube, given that each sample will require 1 ml of Buffer RPE/Ethanol mixture.
   - ➔ For 3 samples to be RNA extracted, add 600 µl Buffer RPE and 2.4 ml 100% ethanol to a RNase-free conical tube. If there are no RNase free conical tubes, prepare mastermixes in RNase-free microfuge tubes by adding 200 µl Buffer RPE and 800 µl 100% ethanol, with 1 tube per sample.

C. Gram-negative bacterial cell lysis with enzymatic lysis and proteinase K digestion of bacteria

All following steps are performed at room temperature (15-25 ˚C) and are adapted from Protocol 4 from RNAprotect® Bacteria Reagent Handbook (Qiagen) (7).

1. Centrifuge cells for 10 minutes (min) at 5000 x g.
2. Decant the supernatant. Remove residual supernatant by gently dabbing the inverted tube once onto a paper towel. Do not remove remaining supernatant by pipetting, as this may lead to loss of the pellet.
3. Add 200 µl of lysozyme-TE buffer to tubes, then add 10 µl of proteinase K (20 mg/ml) to each tube. Carefully pipet after both lysozyme and proteinase K are added to resuspend the pellet.
   - ➔ The handbook instructs to add the proteinase K to the lysozyme-TE buffer prior to addition to the pellet. Successful lysis has been done by adding proteinase K and lysozyme-TE buffer to the cell pellet separately if there are a small number of samples (6).
4. Mix by vortexing for 10 seconds (s). Incubate at room temperature (15-25 ˚C) for 10 min. During incubation, incubate on a shaker-incubator, or vortex for 10 s at least every 2 min.
5. Add 700 µl of Buffer RLT (no β-mercaptoethanol added) and 7 µl β-mercaptoethanol to each tube and vortex vigorously. If particulate material is visible, pellet it by centrifugation, and use only the supernatant in step 6.
   - ➔ The handbook instructs to add 10 µl β-mercaptoethanol per 1 ml Buffer RLT and mix. However, Buffer RLT is only stable for 1 month after the addition of β-mercaptoethanol. Successful lysis has been done by adding Buffer RLT and β-mercaptoethanol separately if there are a small number of samples (6).
6. Add 500 µl of 100% ethanol. Mix by pipetting. Do not centrifuge.
7. Proceed to purification of total RNA from bacterial lysate using RNeasy Mini Spin Columns (Qiagen).

D. RNA purification from bacterial lysate with optional DNase treatment

All following steps are performed at room temperature (15-25 ˚C) and are adapted from Protocol 7 and Appendix B from the RNAprotect® Bacteria Reagent Handbook (Qiagen) (7).

1. Transfer up to 700 µl lysate, including any precipitate that may have formed, to an RNeasy Mini spin column placed in a 2 ml collection tube. Close the lid gently, and centrifuge for 15 s at ≥ 8000 x g (≥ 10,000 rpm). Discard the flow-through. Repeat if there is lysate remaining. Reuse the collection tube in step 2.
2. Add 350 µl of Buffer RW1 to the RNeasy spin column and centrifuge for 15 s at ≥ 8000 x g. Discard the flow-through.
3. Add 10 µl DNase I stock solution to 70 µl of Buffer RDD in a RNAse-free microfuge tube. DNase I is sensitive to physical denaturation, thus mix by

gently inverting the tube, and centrifuge briefly to collect residual liquid from the sides of the tube.

➔ If there is more than one sample being processed, Buffer RDD and DNase I mixture can be scaled up to make a mastermix.

4. Add 80 µl of DNase I incubation mix directly to the RNeasy spin column membrane and incubate at room temperature for 15 min.
5. Add 350 µl Buffer RW1 to the RNeasy spin column, wait 5 min, then centrifuge for 15 s at ≥ 8000 x g
6. Place column in new collection tube. Add 500 µl Buffer RPE with ethanol to column. Close lid gently and centrifuge for 15 s at ≥ 8000 x g to wash the spin column membrane. Discard the flow-through. Reuse the collection tube in step 7.
7. Add 500 µl Buffer RPE to the column. Close lid gently and centrifuge for 2 min at ≥ 8000 x g to wash the spin column membrane.
8. Place column in a new 1.5 ml RNase-free collection tube, add 30 µl RNase-free water directly to the spin column membrane. Close lid gently and centrifuge for 1 min at ≥ 8000 x g. Repeat elution by adding 30 µl RNase-free water directly to the spin column membrane. Close lid gently and centrifuge for 1 min at ≥ 8000 x g.
9. Store RNA samples at -20 ˚C.

Note: the authors did not have success with the optional on-column DNase digestion using the RNase-Free DNase (Qiagen), as there was DNA contamination in the final RNA extract, and required an additional DNase treatment after RNA elution (6). If the on-column DNase digestion step is omitted, simply combine step 2 and 5 together by adding 700 µl of Buffer RW1 to the column at one time and skip step 3 and 4. In case of DNA contamination, the authors recommend using the TURBO DNA-free™ Kit (Invitrogen) to remove DNA contamination, as instructed in Step E, as DNase is removed by a centrifugation-based method rather than heat inactivation, which could potentially degrade the sample RNA.

E. TURBO™ DNase treatment

Instruction from manufacturer's instructions for TURBO™ DNA-free kit (8).

1. Add 0.1 volume of 10X TURBO™ DNase Buffer and 1 µl TURBO™ DNase to the RNA. Mix gently.
   ➔ For example, a 30 µl reaction volume will contain 26 µl of RNA extract, 3 µl of 10X TURBO™ DNase Buffer and 1 µl TURBO™ DNase.
2. Incubate for 20-30 min at 37˚C
3. Add resuspended DNase Inactivation Reagent (typically 0.1 volume) and mix well.

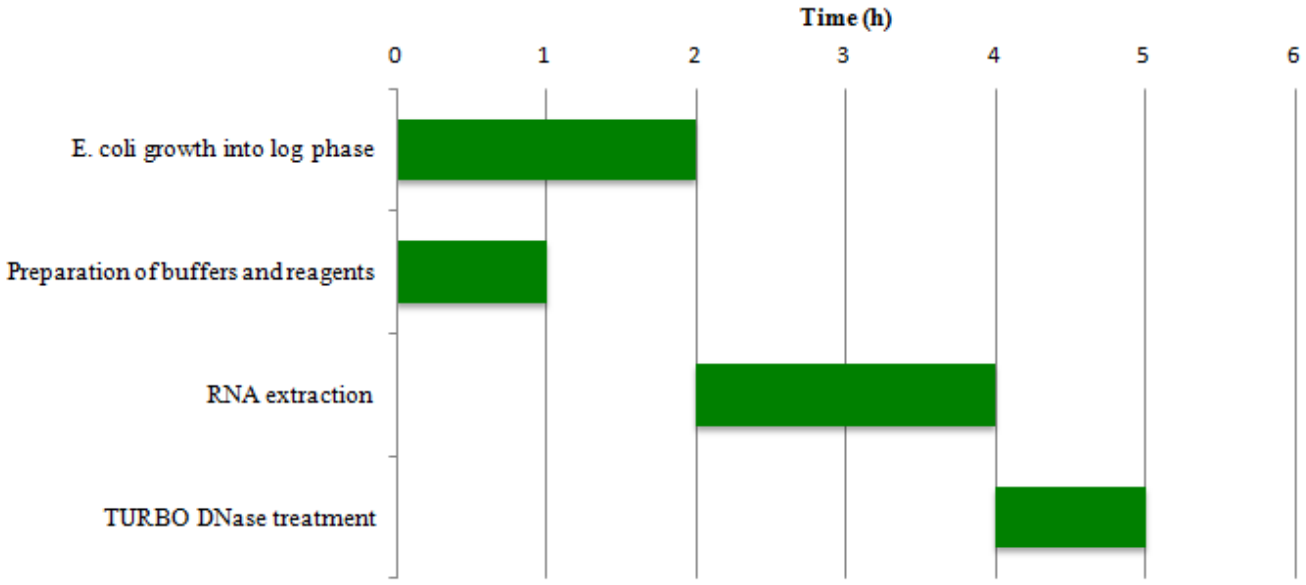

FIG 1 Gantt chart of the expected timeframe on the day of RNA Extraction.

TABLE 1. Troubleshooting guidelines for RNA extraction

Observation/Issue	Possible Explanation	Solution
Ct values less than 35 (or less than the Ct value of the negative control) when RNA samples used for qPCR.	DNA contamination in RNA extraction.	- Use less cells in the RNA extraction - Use TURBO™ DNase to degrade DNA contamination
Ct values less than 35 (or less than the Ct value of the negative control) when RNA samples treated with DNAse are used for qPCR.	DNA contamination in RNA extraction. This may be due to too many cells used in the RNA extraction such that the DNase was not able to target all of the genomic DNA.	- Use fewer cells in the RNA extraction - Check to see that the DNase is working properly using a control sample containing DNA treated with DNase - Use an alternative DNase
Ct values greater than 35 (or Ct value greater than the negative control) when cDNA samples are .used for qPCR	RNA was not isolated properly possibly due to RNA degradation from surrounding RNases in surfaces and/or released from the cell after lysis. RNA samples were exposed to high temperature. There may be missing components in the reverse transcription reaction or qPCR.	- Make sure to use RNase-free tubes and tips. - Spray surfaces thoroughly with RNase decontamination reagent. - Check that the positive control for qPCR worked. - Check that all reagents were included in the reverse transcription and qPCR

➔ For example, if the reaction volume previously was 30 µl, add 3 µl of DNase inactivation reagent.

4. Incubate for 5 min at room temperature, mixing occasionally. Flick the tube 2–3 times during the incubation period to redisperse the DNase Inactivation Reagent. (Note: If room temperature cools below 22–26 ˚C, move the tubes to a heat block or oven to control the temperature. Cold environments can reduce the inactivation of the TURBO™ DNase, leaving residual DNase in the RNA sample).
5. Centrifuge at 10,000 x g for 1.5 min and transfer the RNA to a fresh tube. For 96-well plates, centrifuge at 2000 x g for 5 min. This centrifugation step pellets the DNase Inactivation Reagent.
6. After centrifuging, carefully transfer the supernatant, which contains the RNA, into a fresh tube. Avoid introducing the DNase Inactivation Reagent into solutions that may be used for downstream enzymatic reactions, because it can sequester divalent cations and change the buffer conditions.
7. Store RNA samples at -20 ˚C.

ANTICIPATED RESULTS AND CONTROLS

If the entire protocol is performed properly and effectively, there should not be any DNA contamination in the extracted samples but there should be pure RNA. Following RNA extraction, it is necessary to include a method to ensure that there is not any DNA contamination with the sample. For example, the use of the RNA samples as template for qPCR using SsoAdvanced™ Universal SYBR® Green Supermix (BioRad) can be used to check for DNA contamination (6). The protocol above includes DNase treatment while the sample is in the column and another treatment afterwards. A small sample should be taken after elution from the column and after treatment with the TURBO™ DNase in which case, the eluted sample may contain some DNA contamination but the second DNase treatment should produce negative results. A positive control and negative control (no template) should also be performed to ensure that the qPCR is working properly. When followed by RT-qPCR, this will provide support that a positive result was due to pure RNA rather than DNA contaminants. RNA extracts using this protocol are expected to be suitable for use in RT-qPCR. As shown in another study, RNA samples are successfully reverse transcribed to cDNA using SuperScript™ II Reverse Transcriptase (Invitrogen) for use in qPCR (6).

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