	FOOD SAFETY LAB / MILK QUALITY IMPROVEMENT PROGRAM *Standard Operating Procedure*		
Title: RNA Extraction from Salmonella			
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RNA extraction from Salmonella

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TABLE OF CONTENTS

SECTION 1 INTRODUCTION

1.1 Purpose

The purpose of this protocol is to obtain high-quality RNA from *S. enterica* cultures for use in RNA sequencing.

1.2 Scope

This SOP applies to the Food Safety Lab, including the Laboratory for Food Microbiology and Pathogenesis of Foodborne Diseases.

1.3 Definitions

1.4 Safety

Wear gloves and work in a chemical hood when using TriReagent, phenol, and chloroform.

SECTION 2 MATERIALS

- **TriReagent.** Zymo #2050-1-50. Store at 4°C.
- **Acid phenol chloroform.** Ambion #AM9722. When the bottle is first opened, aliquot into 4 x 100mL. Use Corning 100mL glass bottles designated for this purpose. The phenol comes with a water layer over the phenol, be sure to add some of the water layer to each aliquot. Wrap bottles in foil. Store the working stock at 4°C and the other aliquots at -20°C until needed.
- **Chloroform.** Located under the chemical hood in 350C.
- **Nuclease free water.** Qiagen #129114. Stocks are kept in room 350C.
- **DEPC-treated water.** Add 1 mL of 0.1% diethylpyrocarbonate (DEPC) to 1000 mL distilled water. Let spin overnight and autoclave for 20 minutes at 121°C to inactivate DEPC.
- **3M Sodium acetate pH 5.5.** Dissolve 246.1 g of sodium acetate in 500 mL sodium acetate. Adjust pH to 5.5 with glacial acetic acid. Autoclave for 20 minutes at 121°C.
- **Turbo DNase and 10x buffer.** Invitrogen #AM2238. Store at -20°C in Dumber freezer.
- **RNasin Plus.** Promega #N2615. Store at -20°C in Dumber freezer.
- **75% and 70% ethanol.** Prepare from ethanol stock and nuclease free water.
- **Isopropanol.** Stock kept in room 410 at RNA bench.
- **10 mM Tris-HCl, pH 8.0, 1 mM EDTA.** Ambion #AM9858. Aliquot into 5mL portions, kept at RNA bench in 410.
- **Bioanalyzer RNA Nano Kit.** Agilent #5067-1511.
- **Bioanalyzer RNA Pico Kit.** Agilent #5067-1513.
- **1.5 mL tubes**. Available in the media stock room. Do not autoclave.
- **2.0 mL tubes.** Available in the media stock room. Do not autoclave.
- **Ribo-Zero rRNA Removal Kit.** Illumina #MRZMB126.
- **RNAprotect Bacterial Reagent.** Qiagen #76506.
- **Lysozyme.** ThermoFisher #89833.
- **Proteinase K.** Invitrogen #AM2544.
- **RNase Away.** Ambion #10328011.
- **SYBR Green.** ThermoFisher #4309155.

SECTION 3 PROCEDURES

3.1. Prepare for RNA Extraction

(1) Prepare a stock of 15 mg/mL lysozyme by dissolving 15 mg of lysozyme in 1 mL Tris-HCl buffer.
(2) Prepare a stock of 50 mg/mL proteinase K by dissolving 50 mg of proteinase K in 1 mL Tris-HCl buffer.
(3) Add 10 µL of proteinase K stock to lysozyme stock and keep on ice.
(4) Place all ethanol and isopropanol reagents in -80°C freezer to cool prior to extraction.

3.2. Cell collection.

(1) Add 2 volumes of RNAprotect to 1 volume of cell culture and vortex. Let sample incubate at room temperature for 5 minutes.
(2) Centrifuge at 4,000 rpm for 10 minutes 4°C. Pour off all supernatant. Cell pellets can be stored at -80°C for up to one year for extraction at a later date.

3.3. RNA Extraction

(1) Suspend cell pellet in 200 µL of lysozyme/proteinase K. Incubate at room temperature for 10 minutes, flicking tubes every 2 minutes.
(2) Add 2 volumes of TriReagent for every 1 volume of cell culture used. Mix by inverting tube five times and incubate at room temperature for 5 minutes.
(3) Centrifuge mixture for 1 minute at 4,000 rpm. Move supernatant to a new, clean tube.
(4) Add 200 µL of chloroform per 1 mL of TriReagent used and mix by inverting tube 5 times. Incubate at room temperature for 2-3 minutes.
(5) Centrifuge samples at 12,000 x g for 15 minutes at 4°C.
(6) The sample will separate into a clear, upper aqueous layer, an interphase, and a pink, lower organic layer. Pipet aqueous layer into a new, 2 mL tube.
(7) Add 0.5 mL of ice cold 100% isopropanol to aqueous layer and incubate on ice for 30 minutes.
(8) Centrifuge tubes at 12,000 x g for 10 minutes at 4°C.
(9) Remove supernatant and add 1 mL of ice cold 75% ethanol per mL of TriReagent used, to the tube.
(10) Centrifuge tubes at 7,600 x g for 5 min at 4°C.
(11) Remove supernatant and resuspend pellet in 50 µL nuclease-free water. Measure concentration on NanoDrop.

3.4. DNase treatment of RNA with Turbo DNase for RNA Sequencing.

(1) To a 50 µL sample, add the following:
- 2 µL RNasin Plus
- 5 µL Turbo DNase buffer
- 1 µL Turbo DNase

(2) Mix by flicking tubes and incubate in 37°C hot plate for 30 minutes.
(3) Add 30 µL of acid phenol chloroform and 20 µL chloroform to sample. Flick tubes to mix.

(4) Centrifuge at 13,000 rpm for 10 minutes at 4°C. Pipet aqueous layer into a new 1.5 mL tube.
(4) Add 50 µL of chloroform to tube. Flick tubes to mix.
(5) Centrifuge at 13,000 rpm for 10 minutes at 4°C. Pipet aqueous layer into a new 1.5 mL tube.
(6) Add 5 µL 3M sodium acetate, pH 5.5, and 125 µL ice cold 100% ethanol to sample. Mix by flicking tubes and incubate at -80°C overnight.
(7) Centrifuge samples at 13,000 rpm for 60 minutes at 4°C. Remove supernatant and wash pellet with 1 mL ice cold 70% ethanol.
(8) Centrifuge at 13,000 rpm for 10 minutes at 4°C.
(9) Remove supernatant and resuspend pellet in 50 µL nuclease-free water. Measure concentration on NanoDrop.

3.5. qPCR for DNA Contamination

(1) Dilute an aliquot of each RNA sample to 50 ng/µL. This will be used as the template for the qPCR reactions.
(2) Prepare a SYBR Green master mix with the following reagents (µL per reaction):

- 12.5 µL SYBR Green master mix.
- 0.5 µL *rpoB* F1 primer.
- 0.5 µL *rpoB* R1 primer.
- 10.5 µL nuclease free water.

(3) Prepare two reactions for each RNA sample to be tested. Also include two reactions that will contain water as the template (no template controls) and two reactions with *Salmonella* genomic DNA as a positive control. Add 1 µL of sample to each reaction.
(4) Load into an optical 96-well PCR plate, seal with optical film, and spin briefly in the swinging bucket centrifuge. Load the plate into the QuantStudio 6 system and run using the standard run parameters. After the run, assemble to C_t values for each sample. The average C_t for a sample should be >35 to proceed. If the C_t is <35, the sample needs to be treated with DNase again.

3.6. Sample Assessment on Agilent BioAnalyzer

(1) Following the BioAnalyzer protocol for Nano 6000 chip, assess total RNA quality. Total RNA quality should have a RIN > 8.0 to be used for further analyses.

3.7. rRNA Depletion Using RiboZero Kit

(1) Follow protocol for RiboZero Kit to remove 16s and 23s rRNA.
(2) Following the final precipitation step, hydrate RNA in 30 µL nuclease-free water and measure concentration on NanoDrop.

3.8. Sample Assessment on Agilent BioAnalyzer

(1) Following the BioAnalyzer protocol for Pico 6000 chip, assess total RNA quality. Total RNA quality should have a RIN > 8.0 to be used for further analyses. rRNA depleted RNA should have little evidence of 16s and 23s peaks, indicating that these rRNAs were removed. If peaks are still present, repeat rRNA depletion.

SECTION 4 TROUBLESHOOTING

Problems previously encountered with this assay include:

(1) Make sure to keep ethanol and isopropanol reagents at -80°C until they are needed. Ice cold reagents ensure that RNA isn't degraded.
(2) Do not vortex samples. Vortexing causes RNA to shear and will yield low quality RNA.
(3) Always wipe bench and pipets down with RNase away prior to use.

SECTION 5 REFERENCES

Ambion TriReagent Protocol: https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Bulletin/t9424bul.pdf

Turbo DNase Protocol: https://www.thermofisher.com/order/catalog/product/AM2238

RNA 6000 Nano Kit Protocol: https://www.agilent.com/cs/library/usermanuals/public/G2938-90034_RNA6000Nano_KitGuide.pdf

RNA 6000 Pico Kit Protocol: https://www.agilent.com/cs/library/usermanuals/Public/G2938-90046_RNA600Pico_KG_EN.pdf

RiboZero rRNA Depletion Protocol: https://www.illumina.com/products/by-type/molecular-biology-reagents/ribo-zero-rrna-removal-bacteria.html