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Standard Operating Procedure</td><td> </td></tr>
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<tr><td colspan="2">Author: Rachel Cheng, Alexa Cohn and Sean Guo</td><td colspan="2">Approved by: Martin Wiedmann</td></tr>
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Extraction and DNase treatment of RNA from Gram-negative bacteria (Salmonella)

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SECTION 1 INTRODUCTION

1.1 Purpose

The purpose of this document is to create a standard protocol for extracting RNA from *Salmonella* using the Qiagen RNEasy kit, performing a DNase treatment of the extracted RNA, and using qPCR to quantify DNA contamination levels.

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

Salmonella enterica contains approximately 2600 serovars, nearly all of which are pathogenic to humans and some of which can cause serious illness. *S. enterica* is a BSL-2 level organism, and all necessary precautions (personal protective equipment such as lab coat, gloves, and when dealing with concentrated amounts, face shields/eye protection, should be worn; all laboratory BSL-2 regulations must be followed, etc., see http://sp.ehs.cornell.edu/lab-research-safety/bios/research-with-microbes-and-cell-lines/Documents/Checklist_BSL2.pdf .

SECTION 2 MATERIALS

- **Acid phenol chloroform.** Ambion #AM9722. When the bottle is first opened, aliquot into 4 x 100 mL. 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. Be sure to shake bottle just prior to use to ensure that a homogenous solution is used.
- **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.** Ambion # AM9740. Store at room temperature.
- **Turbo DNase and 10x buffer.** Invitrogen #AM2238. Store at -20°C in Dumber freezer in RNA reagents cold box.
- **RNasin Plus.** Promega #N2615. Store at -20°C in Dumber freezer in RNA reagents cold box.
- **70% ethanol.** Prepare from 96-100% ethanol stock and nuclease free water. Store at -20°C in Dumber freezer.
- **10 mM Tris-HCl, pH 8.0, 1 mM EDTA.** Ambion #AM9858. Aliquot into 5mL portions, kept at RNA bench in 410.
- **1.5 mL tubes**. Available in the media stock room. Do not autoclave and use a fresh, unopened bag.
- **RNAprotect Bacterial Reagent.** Qiagen #76506.
- **Lysozyme.** ThermoFisher #89833.
- **Proteinase K.** Invitrogen #AM2544.
- **RNase Away.** Ambion #10328011.
- **SYBR Green Master Mix.** ThermoFisher #4309155.

SECTION 3 PROCEDURES

3.1 RNA Protect and Cell Collection

3.1.1 Culture cells to desired OD_{600} under test conditions.
3.1.2 Prepare tubes with RNA Protect Bacteria Reagent. Note that you will need to use a 2:1 ratio of RNA Protect Bacteria Reagent to bacteria (for example: 2 mL of RNA Protect per 1 mL of bacteria).
3.1.3 Add bacteria to tubes containing RNA Protect Bacteria Reagent. Vortex at speed 10 for 5 seconds and hold at room temperature for 5 minutes.
3.1.4 Collect bacterial cells by centrifuging cells. If using a microcentrifuge tube, the centrifugation step can be done at 10,000 rpm for 5 minutes at 4° C. If a 15 mL conical tube is used, the centrifugation step can be done at 4,000 rpm for 10 minutes at 4° C.
3.1.5 Decant RNA Protect Bacteria Reagent by pouring off supernatant into a labelled waste receptacle. Use a P1000 pipette to remove remaining RNA Protect reagent.
3.1.6 Store pellets at -20°C until using the RNEasy Kit.

3.2 RNEasy Kit Extraction of Nucleic Acids

3.2.1 Prepare lysis solution (note that you will need 200 µL per bacterial pellet to be processed). Add powdered lysozyme directly to TE buffer (10 mM Tris 1 mM EDTA). The following lists the amount of Proteinase K and Lysozyme to be added to 1 mL of TE buffer to make 1 mL of lysis solution.

Component	Amount of Stock Added	Final Concentration in 1 mL Lysis Solution
Proteinase K (20 mg/mL)	10 – 20 µL	200 – 400 µg/mL
Lysozyme	15 mg	15 mg/mL

3.2.2. Prepare RLT buffer by adding 10 µL of a 1 M stock of DTT per mL of RLT buffer. Since the solution is only stable for 1 month after adding DTT, you should only prepare as much RLT+DTT buffer as you envision using in a month. Prepare solution by aliquoting RLT to a new, labelled 15 mL conical tube, and then add DTT.
3.2.3 Retrieve bacterial pellets from the -20°C freezer and allow pellets to thaw on ice.
3.2.4 Resuspend thawed bacterial pellets in 200 µL of lysis solution. Mix by vortexing pellets for 10 seconds (speed 10).

Note: If pellets are in 15 mL conical tubes, use a P1000 pipette to transfer resuspended pellets to a clean 1.5 mL Eppendorf tube.

3.2.5 Incubate pellets in lysis solution at room temperature for 10 min. Vortex for 10 seconds every 2 minutes.
3.2.6 Add 700 µL of RLT buffer containing DTT to each lysis solution, and vortex for 10 seconds.

Note: if particulate material is visible, pellet by centrifugation and transfer the supernatant to a new tube. For tubes up to 2 mL centrifuge for 2 min at maximum speed in a bench top microcentrifuge.

3.2.7 Add 500 µL of 96-100% ethanol (at room temperature). Mix by pipetting with a P1000 pipette tip. Do not centrifuge.
3.2.8 Transfer up to 700 µL of lysate, including any precipitate that may have formed, to an RNEasy mini spin column in a 2 mL collection tube. Close the lid gently, and centrifuge for 15-30 seconds ≥8,000 x g (>10,000 rpm). Discard the flow through by pipetting with a P1000 pipette.
3.2.9 Add the remaining volume of lysate to the same column, centrifuge for 15-30 seconds and discard flow through with a P1000 pipette.
3.2.10 Add 700 µL Buffer RW1 to the RNeasy Mini spin column. Close the lid gently, and centrifuge for 15-30 seconds at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane.
3.2.11 Discard the flow-through and collection tube.
3.2.12 Place the RNeasy Mini spin column in a new 2 mL collection tube (supplied). Add 500 µL Buffer RPE to the RNeasy Mini spin column. Close the lid gently, and centrifuge for 15 - 30 seconds at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through.
3.2.13 Add 500 µL Buffer RPE to the RNeasy Mini spin column. Close the lid gently, and centrifuge for 2 min at ≥ 8000 x g (≥10,000 rpm) to wash the spin column membrane. This long centrifugation ensures that no ethanol is carried over during elution in step 5 (residual ethanol may interfere with downstream reactions). Note: After centrifugation, carefully remove the RNeasy Mini spin column from the collection tube, so that the spin column does not touch the flow through. Otherwise, carryover of ethanol will occur.
3.2.14 Centrifuge the empty RNeasy Mini spin column for an additional 2 minutes at ≥ 8,000 x g (≥ 10,000 rpm).
3.2.15 Place the RNeasy Mini spin column in a new 1.5 ml collection tube (supplied). Add 100 µL of RNase-free water (supplied in kit) directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at ≥8000 x g (≥10,000 rpm) to elute the RNA.
3.2.16 Repeat the elution step by transferring the spin column to a clean tube, add 100 µL of nuclease-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 minute at >8,000 x g (≥ 10,000 rpm).
3.2.17 Use the nanodrop to measure the concentration of total nucleic acids in the eluted samples. Be sure to change the nucleic acid type to "RNA." Use water as the blanking solution.
3.2.18 Store samples at -80°C, or proceed to section 3.3.

3.3 DNase I Treatment and Phenol-Chloroform Extraction (Day 1)

3.3.1 Turn on bench-top centrifuge (5415R in room 350C) and use the "fast temp" function to chill the rotor to 4°C. It may take up to 30 minutes to reach 4°C. Pre-heat the heat block to 37°C.
3.3.2 Wipe down all pipettors and bench top with RNAse Away RNAse inhibitor.
3.3.3 Collect RNA samples from sample box (-80°C) and thaw on ice.
3.3.4 Take out the 10x Turbo DNase Buffer and nuclease-free dH_2O and let thaw on ice.
3.3.5 Label 1.5 mL Eppendorf tubes with sample name and store on ice.
3.3.6 Calculate how many microliters of each sample are needed to create a 50 µL volume containing a total of 10 µg of total RNA sample (final concentration 200 ng/µL). If the concentration is < 200 ng/ µL, do not dilute RNA sample.

3.3.7 Prepare 50 µL of 200 ng/µL total RNA sample in each tube with nuclease-free dH20 and store on ice. Return any remaining RNA samples to the -80°C freezer.
3.3.8 Retrieve the Turbo DNase and RNasin from -20°C freezer (Dumb, in blue freezing box).
3.3.9 Prepare a Master Mix containing DNase I, RNasin, and 10X DNAse Buffer for each reaction:

Reagent	Amount per reaction	Amount in Master Mix
DNase I	2 uL	
RNasin	2 uL	
10X DNase Buffer	6 uL	

Use a pipette to mix (add the 10X DNase Buffer last and use this same pipette tip to pipette up and down 5 times to mix).

3.3.10 Aliquot 10 µL of Master Mix to each sample tube (containing 50 µL of RNA sample). Pulse vortex (3 pulses, 1 second each) to mix. *Do not vortex for more than this, as extensive vortexing will result in shearing of nucleic acids.*
3.3.11 Incubate tubes in pre-heated heat block at 37°C for 15 minutes.
3.3.12 Flick tubes 5 times to mix, and continue to incubate tubes at 37°C for an additional 15 minutes.
3.3.13 Retrieve Acid Phenol from 4°C refrigerator (Pedro's Friend) and chloroform (stored beneath the fume hood) and place both in the fume hood in room 350C. Shake the Acid Phenol Chloroform to mix. *Note: Inhalation of phenol is associated with significant health concerns; always work with phenol and chloroform in a fume hood.*
3.3.14 Remove the samples from the 37°C heat block and transfer them to an ice box in the fume hood.
3.3.15 Add 60 µL of Acid Phenol Chloroform and 40 µL of Chloroform to each sample. Pulse vortex tubes (3 times, 1 second each) to mix.
3.3.16 Centrifuge samples at 4°C for 10 minutes at 13,000 rpm.
3.3.17 During the 10 minute centrifugation step, label clean 1.5 mL Eppendorf tubes and add 100 µL of chloroform to each newly labeled tube. Retain on ice.
3.3.18 Remove sample tubes from the centrifuge and transfer them to the ice box in the fume hood.
3.3.19 Use a 200 µL pipette to transfer the **top** aqueous layer to the tubes containing 100 µL of chloroform.
3.3.20 Pulse vortex tubes (3 times, 1 second each) to mix, and centrifuge samples at 4°C at 13,000 rpm for 10 minutes.
3.3.21 During the 10 minute centrifugation step, pipette the left over liquid from the organic phase from the original sample tubes to the waste bottle in the fume hood labelled "phenol chloroform waste."
3.3.22 Label new 1.5 mL Eppendorf tubes and add 250 µL of ice cold 100% ethanol (stored in the -20°C freezer) and 10 µL of 3M sodium acetate pH 5.5 to each tube. Keep these tubes on ice.
3.3.23 Remove samples from the centrifuge and place them on ice in the box in the fume hood.
3.3.24 Transfer the **top** aqueous layer to the new tubes containing ethanol and sodium acetate.

3.3.25 Pulse vortex (3 times, 1 second each) to mix. Place samples in the -80°C freezer overnight.
3.3.26 Pipette the remaining organic phase to the waste bottle in the fume hood and return the acid phenol to the 4°C incubator.

DNase I Treatment and Phenol-Chloroform Extraction (Day 2)
3.3.27 Turn on centrifuge (5415R), and use the “fast temp” function to chill the centrifuge to 4°C.
3.3.28 Remove samples from the -80°C freezer. Centrifuge samples at 4°C at 13,000 rpm for 60 minutes. Be sure to align the microcentrifuge tubes so that the hinge part is facing the outer rim of the centrifuge.
3.3.29 Transfer the ice cold 70% ethanol from the -80°C freezer to an ice box.
3.3.30 Remove the samples from the centrifuge and transfer them to an ice box in the fume hood.
3.3.31 Using a P1000 pipette, discard the supernatant into a waste receptacle, taking care not to dislodge the RNA pellet in the bottom of the tube.
3.3.32 Add 500 µL of ice cold 70% ethanol to each tube. Centrifuge samples at 4°C at 13,000 rpm for 30 minutes. Place the 70% ethanol back in the -80ºC freezer during this centrifugation step.
3.3.33 Remove the samples from the centrifuge and transfer them to an ice box in the fume hood.
3.3.34 Using a P1000 pipette, discard the supernatant into a waste receptacle, taking care not to dislodge the RNA pellet in the bottom of the tube.
3.3.35 Perform an additional rinse with 70% ethanol by adding 500 µL of ice cold 70% ethanol to each tube, and centrifuge tubes at 4°C at 13,000 rpm for 30 minutes.
3.3.36 Remove samples from the centrifuge and transfer them to an ice box in the fume hood. Remove the supernatant, leaving the RNA pellet undisturbed. Use a P10 pipette to remove all residual ethanol at the bottom of the tube.
3.3.37 Re-suspend the RNA pellet in 20 µL of nuclease free dH_2O and store samples on ice.
3.3.38 Quantify the amount of RNA recovered using the nanodrop on “RNA” setting.
3.3.39 Store samples at -80°C until the qPCR check for DNA contamination is performed.

3.4. qPCR Quantification of DNA contamination
For each project, a set of criteria should be developed that will guide whether or not a sample will be considered “of sufficient quality” for reverse transcriptase and subsequent qPCR. Consult with your project manager for these criteria.

3.4.1 Turn on centrifuge and use the “fast temp” option to chill the centrifuge to 4°C.
3.4.2 Collect the following supplies: nuclease free dH_2O, forward primer (10 µM stock), reverse primer (10 µM stock), and the 2X SYBR Green Master Mix (stored at 4°C in Pedro’s friend) and place on ice.
3.4.3 In the PCR prep room, prepare a master mix for each RNA sample to be tested (remember to prepare enough for testing each sample in duplicate); also include reagents for testing one positive and control and one negative control. It may be helpful to generate a map to assist with keeping track of which samples are assigned to which wells in the 96-well plate.

Component	µL per reaction	µL in Master Mix
Primer F 10 µM stock	1 µL	
Primer R10 µM stock	1 µL	
2X SYBR Green Master Mix	12. 5 µL	
Nuclease Free dH_2O	9.5 µL	

3.4.4 Aliquot 24 µL of master mix to wells in a clean 96 well plate (stored in the PCR prep room).
3.4.5 In the RNA prep room (350C) add 1 µL of sample to each reaction well using a P10 pipette.
3.4.6 Add 1 µL of gDNA (20-50 ng/µL) sample to each of the “positive control” reaction wells. Add 1 µL of nuclease free water to the negative control reaction wells.
3.4.7 Seal plate with clear optical film and centrifuge the plate at 4°C at 4,000 rpm for 3 minutes.
3.4.8 Load the plate into the QuantStudio 6 system for qPCR and follow instructions at the instrument for creating, saving and starting a run.

SECTION 4 TROUBLESHOOTING

(1) Make sure to keep ethanol reagents at -20°C until they are needed. The use of ice cold ethanol will help to reduce RNA degradation by RNAses (their activity is very low at cold temperatures).
(2) Always wipe bench and pipets down with RNase away prior to use.
(3) Acid-phenol can become oxidized. Always inspect the acid-phenol you are using for brownish-pink globules. If brownish-pink globules are present, discard the stock of acid-phenol and use a fresh one. Oxidized acid-phenol can degrade RNA.
(4) Keep vortexing to a minimum. Vortexing causes RNA to shear and will yield low quality RNA.

SECTION 5 REFERENCES

SECTION 6 METHOD VERSION & CHANGES

VERSION	DATE	EDITOR	COMMENTS
Version 1	02/20/2020	Rachel Cheng, Alexa Cohn, Sean Guo	Original SOP
Version 2	04/22/2020	Maureen Gunderson	Updated SOP to new standard lab template.