RNA extraction using TriReagent

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Authored by: Teresa M. Bergholz
Modified by: Veronica Guariglia
Last Modified on: 4-20-16
Approved by:

EFFECTIVE DATE: Date of Approval

APPROVED BY:

_____ _____
Dr. Martin Wiedmann (date)

AUTHORED BY:

_____ _____
(Name) (date)

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SECTION 1 INTRODUCTION

1.1 Purpose

The purpose of this protocol is to obtain high-quality RNA from *L. monocytogenes* 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.

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SECTION 2 MATERIALS

In this section one should list all materials that will be necessary to carry out the procedure. Each material should be bulleted, in bold, and followed by any necessary supplementary information such as directions for the preparation of a stock solution or the location of the material.

- **TriReagent.** Ambion #AM9738. 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 this water layer to each aliquot. Wrap bottles in foil. Store the working stock at 4°C, store the other aliquots at -20°C until needed.
- **Acid phenol chloroform in ethanol stop solution.** Used to stop RNA degradation before RNA extraction. Prepare fresh each day needed by adding 90mL ethanol to 10mL acid phenol chloroform and making aliquots of the appropriate volume for your samples.
- **Chloroform.** Located under the chemical hood in 415.
- **Nuclease free water**. Qiagen #129114. Stocks are kept in room 410.
- **Sodium acetate pH 5.5.** Ambion #AM9740. Aliquot into 1mL portions in 1.5mL tubes.
- **RQI DNase and 10x buffer.** Promega #M610A. Store at -20°C in freezer in 410.
- **RNasin.** Promega #N2615. Store at -20°C in freezer in 410.
- **75% and 70% ethanol.** Prepare from ethanol stock and nuclease free water.
- **Isopropanol.** Stock kept in room 410, at RNA bench.
- **BCP – bromo-chloro-propane.** Located under the chemical hood in 410.
- **10mM Tris-HCl, PH 8.0, 1mM EDTA.** Ambion #AM9858. Aliquot into 5mL portions, kept at RNA bench in 410.
- **Qiagen RNeasy mini kit.** Qiagen #74104. Use for RNA intended for microarrays only.
- **MicrobeExpress mRNA enrichment kit.** Ambion #AM9105. Components stored at 4C and at -20C.
- **Bioanalyzer RNA Nano kit.** Agilent #5067-1511.
- **8mL bead beating tubes.** Located at RNA bench in 410.
- **0.1mm acid washed zirconium beads.** Stock kept in room 410. to prepare a new batch of beads, add ~75mL of beads to designated bottle, add ~100mL 6N HCl and mix thoroughly. Let sit for 30 min, then wash beads with de-ionized water to remove HCl (pour out HCl, add water, shake to mix, then pour out water, repeat with water). Autoclave beads (with water removed) on the dry cycle, place in drying oven with cap off for a few days to dry completely. Very important that the beads are dry before use.
- **0.2mL PCR tubes.** Available in the media stock room.
- **1.5mL tubes.** Available in the media stock room.

SECTION 3 PROCEDURES

3.1. Preparation for cell collection.

Cells can be 'stopped' using one of two methods. One is by using RNA protect. The other method is to use phenol:ethanol stop solution, which is more cost-effective for large culture volumes. The methods described below are for cell collection with phenol:ethanol stop solution.

(1) Take a 250mL centrifuge bottle for each culture that will be collected, and rinse with chloroform and allow to dry in chemical hood.
(2) Prepare phenol:ethanol stop solution by adding 90mL ethanol to 10mL acid phenol chloroform. Aliquot 10.5mL into 14mL sarstedt tubes. Make one aliquot for each culture that will be 'stopped'.
(3) Prepare a 1% phenol:ethanol solution that will freeze in the 250mL centrifuge tubes. For 100mL 37°C cultures, 20mL of ice will chill samples. Add 2mL of phenol:ethanol stop solution to each centrifuge bottle, then add 18mL water to each centrifuge bottle. Swirl to mix and chill centrifuge bottles in -80°C freezer until time for cell collection.

3.2. Cell collection

Acid phenol:ethanol method

(1) Add 1/10 volume 10% phenol in ethanol stop solution to cells in culture (i.e. for a 100mL culture, add 10mL stop solution). Swirl flask to mix, pour contents into chilled 250mL centrifuge bottle. Vortex bottle for 5 seconds. Centrifuge immediately for 20min at 10,000rpm at 4°C.
(2) Pour off supernatant into phenol waste container, resuspend cells in remaining ~1mL of liquid. Transfer to a 2mL tube, centrifuge for 1 min to pellet cells. Remove all excess media with a pipet, then suspend cells in 1mL Tri Reagent (Ambion).
(3) Transfer cells to a 8mL bead-beating tube with ~ 3mL of 0.1mm zirconium beads. Total volume of Tri Reagent should be 5mL.

(a) If combining cells from multiple cultures into one extraction (useful for lag phase or low density cultures), then add each suspension in TriReagent to the bead beating tube, and make up the remaining volume with TriReagent.

RNA protect method

(1) Add culture to 1 volume RNA protect and vortex. NOTE: If using stationary phase cells, should use 2x volume RNA protect – for example, add 5mL RNA protect to 2.5mL culture. Let sample incubate at room temperature for 5 minutes.
(2) Centrifuge at 6,000 rpm for 10 minutes at 4°C. Pour off all supernantant. Cell pellets can be stored at -80°C for extraction at a later date.

OPTIONAL LYSIS: Resuspend pellet in 200uL lysozyme (20mg/ml in TE) and 100 uL Proteinase K (50mg/ml in TE) for better lysis.

3.3 RNA extraction with TriReagent

(1) Suspend cell pellet (or lysate) in 1mL of TriReagent and then transfer sample to a 8mL bead-beating tube with ~ 3mL of 0.1mm zirconium beads. Fill in to 5mL with TriReagent.

(2) Place samples in bead beater and run on maximum speed 2 times for 2 minutes minutes each, incubating on ice between runs. For the 8mL tubes, the largest side of the bead beater lid should face the caps of the tubes.
(3) Centrifuge samples for 10 minutes at 4,000 rpm at 4C in the swinging bucket eppendorf centrifuge.
(4) Pipet supernatant into 14mL Sarstedt tube, add 500µL BCP and vortex for 10s. Incubate samples at room temperature for 10min.
(5) Centrifuge samples at 12,000 rpm for 15 min at 4C.
(6) Pipet aqueous layer into a new 14mL tube, add 2.5mL ice cold isopropanol to precipitate RNA. Incubate on ice for 30 minutes; alternatively, can precipitate overnight at -20C.
(7) Centrifuge samples at 12,000 rpm, 4C, for 20 minutes to pellet RNA.
(8) Pour off supernatant and add 5mL ice cold 75% ethanol. Vortex sample briefly to wash pellet. Centrifuge samples again at 12,000 rpm, 4C, for 20 min.
(9) Pour off supernatant, spin briefly, and remove all liquid with a fine tip pipet. Let pellets air dry for ~ 5min.
(10) Suspend RNA pellet in 100µL nuclease free water and measure concentration on the Nanodrop. If the RNA is concentrated, it can be suspended in 200µL nuclease free water.

For RNA intended for RNA sequencing, follow steps 3.4, 3.5, 3.6 and 3.7.

For RNA intended for microarrays, follow step 3.8.

3.4 DNase treatment of RNA with RQ1 DNase (promega) for RNA sequencing *Alternatively can use Turbo DNAse (ambion) and follow their instructions)*

(1) To a 100µL sample, add the following:
- 10µL RNasin
- 2µL 0.1M DTT
- 20µL 10x RQ1 buffer
- 70µL RQ1 DNase

mix well and incubate in 37C waterbath for 1h.
(2) To a 200µL sample, add the following:
- 120µL acid phenol chloroform, 5:1
- 80µL chloroform, for a final ratio of 1:1 phenol: chloroform

(3) Vortex and centrifuge at 13,000 rpm, 4C, for 10 min. Remove aqueous layer and repeat extraction with equal volume of chloroform only.
(4) Add 1/10 volume 3M sodium acetate, pH 5.5, and 2.5 volumes ice cold 100% ethanol to sample. Mix well and incubate at -80C overnight.
(5) Centrifuge samples at 13,000 rpm, 4C, for 30-60 min. Remove supernatant and wash pellet with 1mL 70% ethanol.
(6) Centrifuge at 13,000 rpm, 4C, for 10min. Remove supernatant and wash pellet with 1mL 70% ethanol.
(7) Centrifuge at 13,000 rpm, 4C, for 10min.

(8) Remove all supernatant with a fine tip pipet and let pellet air dry for 5 min. Hydrate RNA pellet in 100µL TE (10mM Tris-HCl, PH 8.0, 1mM EDTA). Measure the concentration of RNA on Nanodrop.

3.5 Q-PCR to check for DNA contamination

(1) Dilute an aliquot of each RNA sample to 10ng/µL. This will be used as the template for the Q-PCR reactions.

(2) Prepare a Taqman master mix with the following reagents (µL per reaction):

- 12.5 µL 2x Universal master mix
- 4.5 µL rpoB TqMn F1 primer
- 4.5 µL rpoB TqMn R1 primer
- 0.25 µL rpoB Taqman MGB probe
- 0.75 µL nuclease free H2O

Alternatively can use SYBR green with rpoB primers and follow the SYBR green protocol

(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). Also include two reactions with 10403S genomic DNA as a positive control. Add 2.5 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 the 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 the Nano chip, assess total RNA quality as well as the removal of rRNA. Total RNA quality should have a RIN > 8.0 to be used for further analyses. The Bioanalyzer traces from enriched mRNA should have little evidence of 16S and 23S rRNA peaks, indicating that these rRNAs were removed during the enrichment process.

3.7 mRNA enrichment (microbe express kit) *For rRNA depletion and library construction follow ScrpitSeq protocol*

(1) Follow protocol for microbe express kit to remove 16S and 23S rRNA. Use only 5µg total RNA as input for each reaction – may need to perform two enrichment reactions for each sample to obtain a sufficient amount of rRNA depleted RNA.
(2) Following the final precipitation step, hydrate RNA in 25µL TE and measure on nanodrop.

3.8 DNase treatment of RNA for microarrays

NOTE: If your RNA concentration is high (1µg/ µL or greater), you should dilute the sample to 200µL, and only use 100µL for DNAse treatment.

(1) To a 100µL sample, add the following:

- 10µL RNasin
- 2µL 0.1M DTT
- 20µL 10x RQ1 buffer
- 70µL RQ1 DNase

mix well and incubate in 37C waterbath for 1h.

(2) Use the Qiagen RNeasy mini kit to clean up the RNA. Follow the protocol for RNA purification in the kit handbook.

(3) Measure concentration of RNA on Nanodrop.

(4) Following the Bioanalyzer protocol for the Nano chip, assess total RNA quality. Total RNA quality should have a RIN > 8.0 to proceed with cDNA synthesis and dye coupling.

SECTION 4 TROUBLESHOOTING

No problems have been experienced to date.

SECTION 5 REFERENCES

Ambion Tri Reagent Protocol
http://www.ambion.com/techlib/prot/bp_9738.pdf

Microbe Express Kit Protocol
http://www.ambion.com/techlib/prot/fm_1905.pdf