

METHODS PAGE

Isolation of Pancreatic RNA

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Abstract

Measurement of gene expression usually involves isolation of total RNA which includes mRNAs. Isolation of high quality intact RNA from the pancreas can be a challenge because of the very high levels of RNase. This protocol describes modifications to the widely used guanidium thiocyanate-phenol-chloroform extraction method, using commercial preparations of extraction reagent. The major modification to existing protocols is to increase the ratio of extraction reagent to tissue wet weight. This procedure yields high quality total RNA suitable for downstream measurement of gene expression such as quantitative RT-PCR and microarray transcriptome analysis.

Keywords: Pancreas, mRNA, TRIZOL.

Measuring gene expression has become an essential approach in most physiological studies. Key to obtaining reliable results for gene expression is the isolation of high quality, intact total RNA. Total RNA can then be employed in a

variety of techniques to measure gene expression. Isolation of RNA from the pancreas can be a challenge because the pancreas is one of the richest sources of RNase, one of the digestive enzymes produced by the exocrine pancreas.

Because of its ease of use and generally high quality results, probably the most commonly used technique to isolate RNA is the guanidium thiocyanate-phenol-chloroform extraction method of Chomczynski and Sacchi (1). Use of these reagents has been commercialized and mixtures of guanidium thiocyanate and phenol are sold, for example, as TRIZOL. When we used TRIZOL in my laboratory to isolate RNA from the mouse pancreas, following the protocol given by the supplier, the results were not optimal, as judged by analysis of the total RNA by gel electrophoresis. We explored changes to the standard protocol and achieved a modified protocol that is still easy to use and reliably yields high quality total RNA from rodent pancreas. The changes were to use a higher ratio of TRIZOL to tissue wet weight and to perform more steps at 4°C than in the supplier's protocol. We found that

samples homogenized in TRIZOL can be stored indefinitely at -70°C before further processing, which allows numerous samples to be harvested in a relatively short time. We have successfully used total RNA isolated with this protocol for measurement of pancreatic gene expression by quantitative RT-PCR (6), Northern blot (2), and Affymetrix microarray analysis (5). The protocol was also successful in isolating high quality total RNA from mice with caerulein-induced pancreatitis, where the tissue is undergoing damage that includes inappropriate digestive enzyme activation (6). In addition, the protocol was readily adopted by a colleague (3) who replaced the very laborious technique he had used previously that involved many steps over a 2 day period (4).

1. Materials

1.1. Chemicals (Note 1)

1. Guanidinium thiocyanate-phenol solution. This can be purchased from several sources, including TRIZOL (www.lifetechnologies.com) or Tri-Reagent (www.sigmaaldrich.com).
2. Diethylpyrocarbonate- (DEPC) treated water. To water in a RNase-free glass bottle, add DEPC to 0.01% (v/v). Let stand overnight, autoclave, and let cool before use.
3. Chloroform
4. Isopropyl alcohol
5. 75% Ethanol (in DEPC-treated water)
6. Reagents for agarose gel electrophoresis

1.2 Disposable consumables

1. Microfuge tubes, 1.7 mL
2. Tubes for homogenization that allow the homogenizer probe to reach the bottom of the tube. We use Falcon #352017, round-bottom, 14 mL polystyrene tubes or Falcon #352097, 15 mL conical bottom culture tubes with caps.
3. Filtered pipet tips for 20 μL , 200 μL , 1000 μL pipettors
4. Gloves

5. Filter paper (e.g., Whatman grade 2, #1002 070)

1.3 Equipment

1. Pipettes (20 μL , 200 μL , 1000 μL)
2. High speed blade homogenizer, such as a Polytron
3. Microcentrifuge
4. Vortex
5. A desiccator attached to a water vacuum source
6. Spectrophotometer or other instrument capable of measuring absorbance of small volumes at UV wavelengths (e.g. Nanodrop or Agilent Bioanalyzer)
7. A fume hood
8. Gel electrophoresis apparatus and UV light box with camera. Image analysis software to quantify intensities of 28S and 18S ribosomal RNA bands.
8. A benchtop balance with a readout of 0.01 g
9. Optional: Turbomix vortex attachment (#SI-0564, www.scientificindustries.com) which makes it convenient to process up to 12 TRIZOL homogenates at a time
10. Optional: A swinging bucket microcentrifuge

2. Methods

2.1 Preparation of materials (Notes 2, 3)

1. Pre-chill the homogenization probe in a tube of DEPC water on ice
2. Aliquot TRIZOL into homogenization tubes on ice. Use 2 mL TRIZOL for 40 mg or less pancreatic tissue (~20% or less of an adult mouse pancreas). (**Note 4**)
3. Label microfuge tubes and place on ice
4. Have ready a benchtop balance with a dry piece of filter paper or other absorbent paper

2.2 Harvesting tissue

1. Kill the mouse (use CO_2 followed by rapid decapitation or other IACUC-approved method).
2. Snip the skin near the base of the abdomen without piercing the abdominal wall muscle, and

peel the skin up towards the neck; Cut through the abdominal wall muscle and connective tissue to open the abdomen, and squirt inside with ice-cold PBS. Take care to avoid introducing fur hair.

3. As quickly as possible remove a small portion of the pancreas (<20% of the organ) and place it in ice-cold PBS on ice. Quickly: transfer the pancreatic tissue to the filter paper on the balance, tare the balance, and transfer the blotted tissue back to the ice-cold PBS. Record the change in mass value from the balance which equals the blotted wet weight of the tissue. It should be <40 mg when using 2 mL of TRIZOL. If not, cut the tissue smaller or increase the volume of TRIZOL in the homogenization tube to maintain a ratio of ≤ 20 mg tissue per mL TRIZOL. Then, put the tissue into the chilled TRIZOL. A second worker can collect the remainder of the pancreas for histological and/or protein analyses. **(Notes 5, 6)**

4. In a fume hood, immediately homogenize for 30 seconds on ice using a Polytron or equivalent homogenizer at full speed. (Remove excess water from the probe first.)

5. Aliquot the homogenate into labeled microfuge tubes, up to 1 mL per tube using a pipet.

6. Immediately store at -70°C until ready to process.

7. If preparing multiple samples, rinse the homogenizer probe between samples by running a few seconds in DEPC-treated water, three times, and shake or blot off excess water from the probe. Alternatively rinse probe in clean TRIZOL reagent.

2.3. Processing the TRIZOL homogenates

1. Have ready 2 sets of labeled microfuge tubes (label 3 sets of microfuge tubes if the TRIZOL homogenates were not stored in microfuge tubes, for example, if the entire pancreas was homogenized in a larger volume and stored in the homogenization tube)

2. Thaw samples on ice; Vortex to mix. If the TRIZOL homogenates are not already in

microfuge tubes, transfer 1 mL to labeled microfuge tubes.

3. Centrifuge at 12,000 x g x 10 min at 4°C.

4. Decant the supernatant into a fresh tube; the pellet is hard and will not dislodge; discard the pellet.

5. Add 200 μ L chloroform to each tube. Mix well, 15 seconds on a vortex at low-mid range setting.

6. Incubate 2 min at room temperature.

7. Centrifuge at 12,000 x g x 15 min at 4°C. If a swinging bucket microcentrifuge is available, centrifuge the samples for an additional 30 sec which flattens the interphase and makes for easier separation in the next step; this can be at room temperature.

8. Transfer the upper aqueous phase to fresh labeled tubes. Avoid disturbing the interface that separates the RNA in the clear upper phase from proteins (including RNase!) in the lower (red-colored) organic phase. Discard the lower chloroform phase in hazardous waste. Similarly treat used TRIZOL as hazardous waste.

9. Add 0.5 mL isopropanol to each tube containing the upper phase. Invert several times and vortex to mix.

10. Incubate at room temperature for 10 min.

11. Centrifuge at 10,000 x g x 10 min at 4°C.

12. Discard the supernatant by carefully decanting into a hazardous waste container. Using a clean tissue, blot off excess fluid. The pellet may be loose, so be gentle while decanting and blotting the tube.

13. Add 150 μ L 75% ethanol to the pellet and pipet up-and-down to resuspend the RNA.

14. Add 1 mL more 75% ethanol and vortex.

15. Centrifuge at 7,500 x g x 5 min at 4°C.

16. Discard the supernatant by decanting. Using a clean tissue, blot off excess fluid. The pellet will be small and white, at the bottom corner of the microfuge tube.

17. Dry the samples under vacuum for a few min. This can be done using a desiccator attached to a water vacuum source. The dried pellet will be very small and translucent or clear, and may be difficult

to see. Over-drying may make it difficult to dissolve the pellet.

18. Add 100 μ L nuclease-free water. Flick the tube to dissolve the RNA.

19. Immediately store at -70°C until use.

2.4. Assessing the quality of the total RNA

1. Quantify the RNA yield by spectrophotometry. Dilute a small aliquot (1 μ L) of the RNA sample 50-100 fold in TE buffer (10 mM Tris, pH 8.0, 0.1mM EDTA) and place in a quartz microcuvette (required to pass UV wavelengths) in the spectrophotometer. Measure OD_{260} (nucleic acids) and OD_{280} (protein). 1 $\text{OD}_{260} = 40 \mu\text{g/mL}$ RNA. Using this protocol, from 20% of an adult mouse pancreas the $[\text{RNA}] = 1\text{-}2 \mu\text{g}/\mu\text{L}$ (100-200 μg total).

2. Assessing the purity of the RNA by spectrophotometry. Calculate the $\text{OD}_{260} / \text{OD}_{280}$ ratio which should be 1.6 - 1.8. Pure RNA without protein contamination has a ratio of 2.0.

3. Assessing the intactness of the RNA by gel electrophoresis. This procedure isolates total RNA which is mostly ribosomal RNA. The major RNA species in ribosomal RNA are the 28S and 18S bands, which consist of ~ 5000 bases and 1900 bases, respectively. Therefore, if the RNA is intact the intensity of the 28S/18S RNA bands on a gel should be >2 . If the RNA is degraded the ratio will be <2 because the 28S RNA is more easily degraded than the 16S RNA.

a. Pour a 1.2% agarose gel with formaldehyde buffer

b. Prepare the samples in denaturing buffer in microfuge tubes:

i. 6 μ L RNA ($\sim 5 \mu\text{g}$ RNA)

ii. 2.8 μ L 5x running buffer

iii. 4.8 μ L formaldehyde

iv. 13.6 μ L formamide

c. Heat the samples at 65°C for 15', cool on ice.

d. Add 1.4 μ L loading buffer to each, mix, briefly centrifuge, and load on the gel.

e. Electrophorese the gel at $75\text{V} \times \sim 1.5 \text{ h}$.

f. Rinse the gel 3 x 10 min with dH_2O to remove formaldehyde which interferes with staining.

g. Stain the gel 10 min with ethidium bromide or SYBR Green II, rinse in dH_2O , and image the gel using an instrument such as a ChemiDoc or Bio Gel Doc (from Bio-Rad, www.bio-rad.com), or an ImageQuant LAS 4000 (from GE Life Sciences, www.gelifesciences.com). (**Note 7**)

4. Example of high quality total RNA from pancreas [from reference (6)], as judged by 28S/18S ratio of >2 is shown in **Figure 1**. Five μg of total RNA was loaded per lane and electrophoresed, the gel was stained with SYBR Green II, and imaged with a Bio-Rad ChemiDoc XRS digital system. The time points indicated refer to the elapsed time after the first caerulein injection. WT: wild type mice; CF: *Cftr*^{-/-} mice.

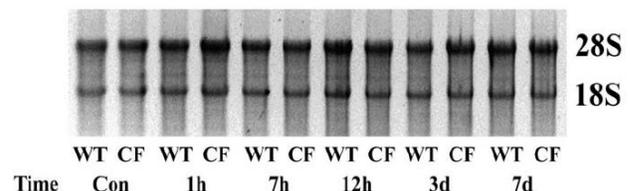


Figure 1. Agarose gel electrophoresis of pancreatic RNA stained with SYBR Green II.

2.5. Adaption of method to isolated acini

1. This protocol can also be used for preparing RNA from isolated pancreatic acini. In this case, the acini are suspended in ice-cold PBS and gently pelleted (centrifuged at $\sim 200 \times g \times 30 \text{ sec}$) in a microfuge tube. The supernatant is carefully aspirated off the pellet. Then ice cold TRIZOL is added to the pellet and immediately homogenized on ice. Assuming a reasonable sized pellet ($<100 \mu\text{L}$ packed volume), 1 mL of TRIZOL will be sufficient. A probe-type sonicator works well in this case because there is little connective tissue

in isolated acini as compared to the intact pancreatic tissue, and a small tipped probe fits easily into a microfuge tube.

2. For quantitative RT-PCR, 1 μ L of a 1,000X diluted sample (~1 ng total RNA) is sufficient for most mRNAs. My lab generally uses a one-tube RT-PCR kit (QuantiTect® SYBR® Green RT-PCR; www.qiagen.com) that employs the PCR minus strand primer annealed to the mRNA of interest as the template for the RT step of the reaction. Of course, cDNA can be prepared from a larger amount of the prepared total RNA which is then used for PCR.

3. Notes

Note 1: All reagents must be molecular biology grade and should be kept for RNA work only, separate from other uses. The same applies to pipet tips and tubes. Use of culture grade, sterile materials which are kept separate for RNA use minimizes the potential of RNase contamination.

Note 2: The protocol uses a commercial solution of guanidinium thiocyanate-phenol, such as TRIZOL® from www.lifetechnologies.com or Tri-Reagent® from www.sigmaaldrich.com. The 'Tri' in the names is an acronym for 'Total RNA Isolation' and for the fact that it is possible to isolate three different parts from the homogenates: RNA, protein, and DNA (1).

Note 3: Wear gloves throughout the procedure: phenol is very caustic and gloves prevent contamination of samples with RNase present on your skin.

Note 4: The amount of TRIZOL to pancreatic tissue is crucial as the guanidinium in the TRIZOL

has to denature and inactivate the high amount of RNase in the pancreas. Use 1 mL TRIZOL per 20 mg wet weight of pancreas, which is ~5X the volume recommended by the supplier. An adult mouse pancreas is ~200 mg. We usually process only ~ 1/5 of an adult mouse pancreas with 2 mL TRIZOL.

Note 5: As an alternative to round bottom homogenization tubes, 15 ml conical bottom tubes can be used as long as the homogenizer probe reaches far enough so a 2 mL volume will be adequately homogenized. A culture tube has the advantage that the sample does not need to be aliquoted after TRIZOL homogenization and the entire homogenate can be stored in a single capped tube in the freezer until processing. This is useful if the entire pancreas is to be processed to isolate RNA which will require ~ 10 mL of TRIZOL for a mouse pancreas.

Note 6: Some labs snap-freeze the pancreas in liquid nitrogen (LN₂) followed by storage either in LN₂ or at -70°C until processing to isolate RNA (7). The frozen sample is then ground in a mortar and pestle with LN₂ followed by processing. While this technique yields good results, it is cumbersome to grind the tissue in LN₂ and then transfer the frozen, powdered pancreas into the extraction reagent. Because TRIZOL homogenates of pancreas can be stored at -70°C until processing to isolate RNA, it is just as suitable as snap-freezing in LN₂ for processing a large number of samples in a short time.

Note 7: Care needs to be taken while using and disposing of ethidium bromide because it is carcinogenic. Less is known about the safety of SYBR Green II but, since it is a nucleic acid binding dye, caution should be used.

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