1. PURPOSE

This protocol describes the extraction, purification, and assay of total RNA from human or animal tissue samples.

2. SCOPE

This procedural format is utilized by the human cancer projects under the supervision of John Quackenbush in the division of Mammalian Genomics. This protocol is optimized for the extraction of RNA from small samples of animal tissue and is applicable to both lipid-rich and lipid-poor tissues.

3. MATERIALS

3.1 TRIzol® Reagent (Invitrogen; Cat# 15596018)

3.2 Ethanol (200 proof) (Pharmco; Cat# 111000200)

3.3 Isopropanol (2-Propanol) (Fisher Scientific; Cat# A451-1)

3.4 Chloroform (Fisher Scientific; Cat# C298-500)

3.5 Culture Tube, Sterile, Disposable, 12 x 75 mm (VWR; Cat#60818-496)

3.6 50mL Falcon Centrifuge Tubes

3.7 RNAse-free water (DEPC treated) (ResGen; Cat# 750023)

3.8 RNAse ZAP™ (Invitrogen; Cat# 46-0434)

3.9 Qiagen RNEasy Mini Kit

3.10 Buffer TE, pH 8.0, RNAse-free (Ambion #9858)

3.11 Petri Dish, 100mm

3.12 Standard Forceps

3.13 Tissue Forceps, #5

3.14 Scalpel, #11 blade

3.15 Homogenizer (PRO Scientific PRO-250 rotary with 7mmX95mm stainless steel generator)

3.16 Spectrophotometer

3.17 RNAse-free 2mL tubes

3.18 Dry Ice, Ice

3.19 Agilent Bioanalyzer and Eukaryote Total RNA nano chip setup

4. PROCEDURE

4.1 Biohazard Considerations:

4.1.1 This protocol describes the extraction of RNA from animal tissues. It is important to consider the potential for infection or disease transmission by materials used in this procedure that contact the sample and/or the homogenate.

4.1.2 If using potentially infectious materials, conduct this work in an appropriate biosafety lab following OSHA and institutional protocols for the handling of biohazardous materials.

4.1.3 After working with biohazardous materials, thoroughly decontaminate the workspace and instruments according to institutional protocol. Consider wiping surfaces with undiluted bleach and/or ethanol after the protocol is complete.

4.1.4 Dispose of all biohazardous or potentially infectious waste according to institutional protocol.

4.2 Avoiding RNAse contamination:

4.2.1 To prevent microbial contamination (and subsequent RNAse contamination), always wear disposable gloves and practice good sterile technique when handling samples and reagents. Change gloves frequently.

4.2.2 Always use sterile, disposable plasticware and use pipettes which are dedicated only to RNA handling.

4.2.3 Before starting wipe down the work surface and pipettes with RNAse ZAP™ to promote an RNAse-free environment.

4.3 Prepare the following before starting the process:

4.3.1 Preparing for the samples (the list below gives the types and quantities of tubes, etc, PER SAMPLE):

- Sterile Tube for TRIzol and Tissue
  - Rinse a sterile culture tube (12 x 75 mm, VWR) with 2 x 5 mL aliquots of DEPC water.

  NOTE: This tube will contain the TRIzol® reaction and therefore does not need to be cleaned with RNAse Zap™. If RNAse contamination is an extreme concern, this tube can be prepared in a similar fashion to the 50mL conical tubes, as described below.

  NOTE: This particular tube works well with a 7 x 95mm homogenizer generator. If using a different size generator, select a similar tube with the appropriate dimensions.

- 2 mL tubes
  - Each 2mL sample will require two (2) 2mL RNAse-free tubes into which the TRIzol® reaction will be transferred. For larger samples, adjust the quantity of tubes accordingly.
  - Place these tubes on ice until needed.

- Each 2mL sample will require two (2) additional 2mL tubes (RNAse free) into which the aqueous phases (containing RNA) will be transferred.

4.3.2 Preparing the homogenizer

- RNAse-free 50mL conical tube (x4)

  - Pour 5mL of RNAse-ZAP™ into a 50mL conical tube (VWR).

  - Cap the tube and gently swirl the RNAse-Zap™ such that it completely contacts the tube walls and the inner surface of the cap.

  - Shake the tube vigorously for 30-45s (the RNAse-ZAP™ will foam).

  - Uncap the tube and vigorously dump the foam into the sink.

  - Rinse the tube with a 15mL aliquot of DEPC Water (be sure to recap the tube so that the inner surface of the cap is rinsed).

  - Repeat this rinse 2 more times (3 total rinses).

  - Vigorously expel any remaining DEPC Water from the tube.

4.3.3 Generator Rinses

- Into the above tubes, add the following:

  - Tube 1: DEPC Water, 30mL

  - Tube 2: 100% Ethanol, 30mL

  - Tube 3: DEPC Water, 30mL

  - Tube 4: DEPC Water, 30mL

- Into a new 50mL Falcon Tube (does not need to be RNAse free), add 20mL RNAse Zap™.

- These tubes will serve as the washes for the generator and the instruments used in the extraction process.

  NOTE: These tubes will be used to rinse the generator between samples. As such, they may be contaminated with biohazardous materials and must be handled with care and disposed of properly.

4.3.4 Generator

NOTE: This protocol uses a 7mm x 95mm stainless steel generator in conjunction with a PRO Scientific PRO-250 rotary homogenizer.

NOTE: Homogenizer generators should NEVER be run dry for ANY length of time. Always start and stop the generator while in liquid. Failure to do so will result in damage to the lower bearings and require that the generator be serviced or replaced.

- Before each BATCH of samples, do the following:

  - Disassemble and clean the generator per manufacturer specifications.

  - Autoclave in a metal box on dry cycle.

- In preparation for homogenizing a batch of samples, do the following

  - Tighten the set screw at the top of the generator to ensure that the generator will not fall apart during homogenization (consider checking this between samples if loose generator shaft becomes a problem).

  - Attach the generator securely to the PRO-250 and test to ensure that the connection is secure.

- ❖ Start the generator at the lowest speed and slowly increase RPM's to maximum.

NOTE: that the generator will be shaken loose if it is not completely secure, and the falling generator can be damaged. Attempt to prevent this, but be aware that the spinning blades will easily cause injury.

- ❖ When the generator is securely fastened, proceed to washing the generator head.

- Wash the generator head by running it at maximum speed in the generator wash tubes (50mL Falcon, prepared above) as follows:
  - ➢ RNAse ZAP™: 30s
  - ➢ DEPC Water: 30s
  - ➢ 100% Ethanol: 30s
  - ➢ DEPC Water: 30s

4.3.5 Setup for Sample Dissection

- Collect a container of ice for holding the following:
  - ➢ 2mL tubes (2 per sample)
  - ➢ Sterile culture tube containing 1.5ml TRIzol®
- Collect a container of Dry Ice for holding the following instruments for sample dissection:
  - ➢ Scalpel, with #11 blade
  - ➢ Tissue Forceps, #5 (i.e. Dumont Dumoxel)
  - ➢ Standard Forceps
  - ➢ Petri Dishes (100mm), one per sample

4.3.6 Setup for Sample Purification

- Buffer RLT with Beta-Mercaptoentanol
  - For each 5mm x 5mm x 5mm tissue block to be used (~100ug total RNA), prepare one reaction worth of Qiagen buffer RLT for subsequent RNA purification using the Qiagen RNEasy Mini kit (350µL RLT + 3.5µL B-ME).
- 75% Ethanol
  - For each sample, prepare 2 mL of 75% Ethanol by combining 1.5mL of 100% EtOH with 0.5mL of DEPC-Water.

4.4 RNA Extraction

NOTE: This procedure was originally optimized for extracting total RNA from tissue samples of approximately 5mm x 5mm x 5mm. If isolating RNA from a different size, consult the table of sample size/mass and adjust accordingly using Form No. 18057N (which accompanies TRIzol® Reagent) to make adjustments.

NOTE: TRIzol® Reagent is very toxic and should only be used with caution. Work only in a fume hood with proper personal protective equipment (gloves, eye protection, etc.). TRIzol® waste is hazardous and should be handled and disposed of according to institutional hazardous waste protocols.

4.4.1 Dissect a 5x5x5mm sample tissue block

NOTE: The key to the dissection is FAST and COLD. Take as little time as possible from the time the sample is removed from cryostorage and the time the homogenization starts. Prolonging this step favors the activity of endogenous RNAses in the tumor block (simply being in TRIzol will NOT prevent this).

NOTE: Conduct dissection and homogenization under a tissue culture hood. Cover all exposed equipment with plastic bags to prevent the accumulation of potentially infectious material on

equipment. Wear protective clothing, etc, as the spinning homogenizer sprays tissue sample (potentially infectious material).

- Remove the sample from liquid nitrogen and place it quickly onto a cold Petri dish using cold forceps.

- Close the cryo freezer.

- Using a cold knife and cold tissue forceps, dissect the block using the cold Petri dish as a stage.

NOTE: It is best to wait until the tissue is just thawed enough to cut with the knife. Trying to cut too soon will fracture the tissue, diminishing precision and potentially spreading infectious tissue throughout the hood.

- Quickly place the dissected sample into the Sterile culture tube/TRIzol® (1.5mL).

- Replace the remainder of the tumor block in the cryofreezer quickly.

- As soon as possible, proceed with the following homogenization process.

4.4.2 Homogenize the tissue block

- Start the homogenizer and accelerate quickly to full speed. Verify that the generator is securely in place.

- Homogenize the sample for 1 minute, 30s, and 30s.

NOTE: Long periods of homogenization can cause heating of the sample/TRIzol. If this becomes a problem, it is best to consider 1) doing more homogenizations but at shorter lengths each, and/or 2) placing the tube containing the sample in a beaker of ice while homogenizing.

- Allow the homogenized solution to stand for 5 minutes.

- Carry out the generator rinse protocol from section 4.3.4 during this waiting period.

- A second (or several additional) sample(s) may be dissected and homogenized while the first homogenate stands, but do not allow any homogenate to stand for more than 15-20 minutes before proceeding.

4.4.3 Extract the RNA from the homogenate

- After allowing the sample to stand for 5 minutes, pipette 1mL of the TRIzol®/Homogenate into each of the two (2) 2mL tubes that are currently on ice.

NOTE: The homogenized tissue will add volume to the final mixture. Be sure to consider this factor, as more 2mL tubes may be necessary.

- To each 1mL aliquot, add 200µL of chloroform.

- Shake the tubes (homogenate + chloroform) vigorously for 30s and allow to stand at room temperature for 3-5 minutes.

NOTE: If possible, conduct the remainder of the extraction (except where indicated) in a cold room.

- Centrifuge at 12,000g for 20 minutes at 4°C.

- Remove the aqueous phase from each tube and combine them all into one fresh, RNAse-free 2mL tube.

- Mix this tube briefly.

- Transfer half of this mix to the remaining 2mL tube.

- Add an equal volume of isopropanol to each tube and mix thoroughly.

- Let stand at ROOM TEMPERATURE for 15 minutes.

- Centrifuge at 12,000g for 20 minutes at 4°C.
- Carefully pipette off the supernatant.
- Rinse with 1 mL of 75% ethanol, gently dislodging the pellet.
- Centrifuge at 7,500g for 4 minutes at 4°C to re-pellet.
- Repeat the rinse with another 1 mL aliquot of ethanol, and then repeat the centrifugation to re-pellet.
- Again remove the ethanol. Centrifuge at 7,500g for 1 more minute at 4°C to collect residual ethanol. Carefully remove this ethanol using a 100µL or 200µL pipettor.
- Allow the pellet to air dry at room temperature for 5-10 minutes. Watch the pellet closely to avoid overdrying, as this will inhibit redissolving
- Redissolve the pellet in n/100µL of RNAse-free water (where n equals the number of aliquots into which the original aqueous phase was transferred – the goal is to have all of the RNA from the sample resuspended in a TOTAL of 100µL.
- Combine the aliquots. There will now be 100µL containing all of the RNA from the original sample.
- Measure the OD 260 and quantitate the RNA using the Nanodrop.
- Proceed with purification immediately.

4.5 RNA Purification

NOTE: The remainder of the protocol should be conducted at room temperature, NOT in a cold room.

4.5.1 To the Microfuge tube containing the RNA dissolved in 100µL of DEPC-Water, add 350µL of the Buffer RLT + Beta-Mercaptoethanol Mix.

4.5.2 Vortex for 15s to mix.

4.5.3 To this mixture, add 250µL of 100% EtOH. The final volume should now be 700µL.

4.5.4 Select the appropriate Qiagen column for RNA cleanup.

- For ≤ 45µg of RNA, use the Qiagen MinElute column.
- For > 45µg of RNA, use the Qiagen RNEasy Mini Column.

4.5.5 Transfer the 700µL to one Qiagen column (as selected above)

NOTE: Transfer as quickly as possible, as the RNA begins to precipitate immediately upon adding the EtOH.

4.5.6 Proceed with the RNA cleanup as directed by the appropriate Qiagen protocol.

4.6 RNA Assays

4.6.1 Dilute 1µL of the purified RNA product in 19µL of TE buffer, pH 8.0 (Ambion). Be sure to use an RNAse-free tube (0.5mL) for this 1:20 dilution.

4.6.2 Assay the RNA product for purity and quality as follows:

- Use 1µL of the 1:20 dilution to measure the OD230, OD260, and OD280 using the nano-drop.
- Use 1µL of the 1:20 dilution to run a Eukaryote Total RNA Nano assay using the Agilent Bioanalyzer 2100.

4.6.3 Using this system, RNA is considered pure and acceptable for use with the following parameters:

OD 260/230	>2.0
OD 260/280	>2.0
28S/18S	>2:1

4.7 RNA Storage

4.7.1 Storing the purified RNA

- Immediately after removing the 1µL aliquot from the purified RNA product, add 1u/µL Superase-IN (Ambion) RNAse inhibitor to each tube of purified RNA.
- Vortex to mix.
- Wrap the sealed tube with Parafilm and store at -80°C for future use.

4.7.2 Storing the Diluted RNA

- After running the RNA assays, store the diluted product in case additional assays are needed (the RNA with Superase-In will erroneously show protein contamination if future assays are run on these tubes. It is therefore important to store the diluted product in case additional purity assays are needed).
- Wrap the tubes with Parafilm and store at -80°C for future use.