UNIVERSITY OF PENNSYLVANIA SCHOOL OF MEDICINE
Functional Genomics Core

RNA Extraction from Mammalian Tissues

Reagents

Choose the most appropriate kit for your sample. Consult the Qiagen web site for more specifics, or call Qiagen technical support (1 (800) 362-7737):

- **Qiagen RNeasy® Mini Kit (12)**: Qiagen (Cat. No. 74104). Yields <100 µg RNA from 0.5 - 30 mg tissue or $1x10^5$ to $1x10^7$ cells.
- **Qiagen RNeasy® Midi Kit (12)**: Qiagen (Cat. No. 75142). Yields <1 mg RNA from 20 - 250 mg tissue or $5x10^6$ - $1x10^8$ cells.
- **Qiagen RNeasy® Maxi Kit (12)**: Qiagen (Cat. No. 75162). Yields <6 mg RNA from 150 mg – 1 g of tissue or 5 x 10^7 – 5 x 10^8 cells.

TRIzol® Reagent (a ready to use mixture of phenol, guanidine isothiocyanate, red dye and other proprietary components): Invitrogen (Cat. No. 15596-026)

TRIzol® LS Reagent (recommended for liquid samples e.g. FACS sorted cells): Invitrogen (Cat. No. 10296-010)

Chloroform: Sigma (Cat. No. C-2432)

RNaseZap® RNase Decontamination Solution, 250 ml: Ambion (Cat. No. 9780)

RNase-free Water: Ambion (Cat. No. 9932 or 9922)

100 % (200 proof) Ethanol: Pharmco (Cat. No. 111ACS200)

70% Ethanol (in RNase-free H_2O)

50 ml Falcon Tubes (BD Cat. No. 352070). 9400 RCF rating. Required for Maxi Kit.

15 ml Falcon Tubes (BD Cat .No. 352097). 6000 RCF rating. Required for Midi Kit.

1.7 ml Microcentrifuge Tubes (Denville Scientific, Cat. No. C-2170). Required for Mini Kit.

Agilent 2100 bioanalyzer: (Agilent Technologies, Cat. No. G2940CA)

Agilent RNA 6000 Nano Kit: (Agilent Technologies, Cat. No. 5067-1511). For RNA in the concentration range of 25–500 ng μl^{-1}

Agilent RNA 6000 Pico Kit: (Agilent Technologies, Cat. No. 5067-1513). For RNA in the concentration range of 50–5000 pg μl^{-1}

NOTE: We do not recommend the use of the RNAlater RNA stabilization reagent (this should not be confused with Buffer RLT). Direct disruption of the tissue or cells in Buffer RLT or Trizol yields the best results in our experience. If the tissue has been stored in RNAlater, the tissue must first be removed and placed into Buffer RLT and immediately disrupted.

NOTE: We do not recommend DNase treatment of RNA samples for microarray analysis.

NOTE: If you are not experienced with RNA isolation, please read the literature and tips found on Ambion's website: http://www.ambion.com/techlib/basics/rnaisol/index.html.

Protocol A – Cells (<10^7), Islets or Small Amounts of Tissue (<30 mg)

Sample preparation and homogenization

Gloves should be worn at all times and follow standard RNA handling techniques. Lysis of the sample in **TRIzol®** works well and may give higher yields due to better lysis than can be achieved with the Buffer RLT provided in the Qiagen RNeasy kits.

a. Islets. Prepare the islets according to standard protocols, working quickly, and at the last step remove any residual wash buffer. Resuspend the islet pellet (nor more than 500 mouse islets) in a 2.0 ml microcentrifuge tube in 1 ml **TRIzol** and vortex vortex well to ensure complete lysis.

b. Cells. To a pellet of cells (<1 x 10^7 cells) add 1 ml **TRIzol** or for direct lysis of cells grown in a monolayer, add 1 ml **TRIzol** (<10 cm diameter dish) and collect cell lysate with a rubber policeman, transferring to a 2.0 ml microcentrifuge tube.

c. Sorted Cells. The issue with extracting RNA from sorted cells is the volume of cells after sorting must not dilute the denaturing agent beyond its ability to denature RNase. As such we recommend the use of **TRIzol LS** which remains active when diluted up to 1:3. If possible, sort cells directly into 750 µl of TRIzol LS (sample volume must not exceed 250 µl – if this is a problem use more reagent and adjust the protocol accordingly).

d. Tissue. This protocol should only be followed for use with small amounts of tissue (<30 mg); if using larger amounts of tissue follow **Protocol B**. The volume of lysis reagent should be at least 10 fold greater than the volume of tissue. Thus for <30 mg of tissue use 1ml **TRIzol**. The tissue must be completely disrupted by homogenization as detailed in **Protocol B,** using a homogenizer probe that is appropriate for small sample volume in a 2 ml microcentrifuge tube. If such a probe is not available you can attempt to chop the tissue directly in lysis reagent using very fine scissors (Fine Science Tools, Cat. No. 15012-12). Warning: incomplete lysis will reduce yield.

RNA isolation using Qiagen RNeasy® Mini Columns

1. For samples processed with **TRIzol**:
   a. Ensure that the sample is completely lysed: if working with cells or islets vortex well, or if working with tissue ensure complete homogenization. Samples can be stored at this point at <-70°C for at least 1 year.
   b. Incubate sample for 5 minutes in TRIzol at room temperature.
   c. Add 0.2 ml of chloroform for every 1ml of TRIzol used. Shake vigorously for 15 seconds and incubate at room temperature for 2-3 min.
   d. Centrifuge samples 5 min. at 12,000 x g at 4°C.

      Note: The 4°C spins are essential for phase separation. Room temperature spins may result in variable phase separation thus resulting in variable RNA yields.

   e. Transfer the aqueous phase to a fresh microcentrifuge tube. Proceed immediately to Step 2.

      Note: The aqueous phase is the colorless upper phase that corresponds to ~60% of the volume of TRIzol used. The interphase should be fairly well-defined.

2. Add **1 volume** (usually 600 µl) of **70% ethanol** to the cleared lysate, and mix immediately by pipetting. Do not centrifuge. Continue without delay with step 3.
3. Apply up to **700 µl of the sample**, including any precipitate that may have formed, to an **RNeasy mini column** placed in a 2 ml collection tube (supplied). Close the tube gently, and **centrifuge for 15 s** at ≥8000 x g (≥10,000 rpm). Discard the flow-through, but not the collection tube.

   Note: If the volume exceeds 700 µl, load aliquots successively onto the RNeasy column, and centrifuge as above. Discard the flow-through after each centrifugation step.
4. Add **700 µl Buffer RW1** to the RNeasy column. Close the tube gently, and **centrifuge for 15 s** at ≥8000 x g (≥10,000 rpm) to wash the column. Discard the flow-through and collection tube.
5. Transfer the RNeasy column into a new 2 ml collection tube (supplied). Pipet **500 µl Buffer RPE** onto the RNeasy column. Close the tube gently, and **centrifuge for 15 s** at ≥8000 x g (≥10,000 rpm) to wash the column. Discard the flow-through, but not the collection tube.

   Note: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to Buffer RPE before use.
6. Add another **500 µl Buffer RPE** to the RNeasy column. Close the tube gently, and **centrifuge for 2 min** at ≥8000 x g (≥10,000 rpm) to dry the RNeasy silica-gel membrane.

   Note: Following the centrifugation, remove the RNeasy mini column from the collection tube carefully so the column does not contact the flow-through as this will result in carryover of ethanol.
7. To **elute**, transfer the RNeasy column to a new 1.5 ml collection tube (supplied). Pipet 30–50 µl **RNase-free water** directly onto the RNeasy silica-gel membrane. Close the tube gently, wait 1 min, and **centrifuge for 1 min** at ≥8000 x g (≥10,000 rpm) to elute.

   Note: Never elute with less than 30 µl water. If the expected RNA yield is >30 µg, repeat the elution step with a second volume of RNase-free water. Elute into the same collection tube.
8. Keep eluted RNA on ice at all times and **store at <-70°C**.

Simplified RNA Isolation Protocol for Experienced Users

1. Complete sample pre-processing for samples processed in **TRIzol**:
   f. Lyse / homogenize and incubate sample for 5 minutes in TRIzol at room temperature.
   g. Add 0.2 ml of **chloroform** for every 1ml of TRIzol used. Shake vigorously for 15 seconds and incubate at room temperature for 2-3 min.
   h. Centrifuge samples 5 min. at 12,000 x g at 4°C. Transfer the aqueous phase to a fresh microcentrifuge tube. Proceed immediately to Step 2.
2. Add **1 volume** (usually 600 µl) of **70% ethanol** to the cleared lysate, and mix immediately by pipetting.
3. Apply **up to 700 µl of the sample** to an **RNeasy mini column** placed in a 2 ml collection tube. Centrifuge for 15s and discard the flow-through. Repeat as necessary.
4. Add **700 µl Buffer RW1** to the RNeasy column. Centrifuge for 15 s as above.

5. Transfer the RNeasy column into a new 2 ml collection tube. Pipet **500 µl Buffer RPE** onto the RNeasy column. Centrifuge as above and discard the flow-through.

   Add another **500 µl Buffer RPE** to the RNeasy column. Centrifuge for 2 min to dry the RNeasy silica-gel membrane.

6. To elute, transfer the RNeasy column to a new 1.5 ml collection tube. Pipet 30–50 µl **RNase-free water** directly onto the RNeasy silica-gel membrane. Wait 1 min, and centrifuge for 1 min to elute.

7. Keep eluted RNA on ice at all times and **store at <-70°C**.

Protocol B– ANIMAL TISSUES (150 to 1000 mg)

Preparation

Clean the homogenizer probe by running it at maximum speed in the probe wash tubes (50 ml conical tubes) as follows:

I. RNAseZAP®: 30s
II. DEPC Water: 30s
III. 100% Ethanol: 30s
IV. DEPC Water: 30s

Sample preparation and homogenization

The tissue should be placed in at least 10 volumes of **TRIzol®** or **Buffer RLT** (or approximately 10 µl reagent per 1 mg tissue). Larger volumes can be used if necessary or desired. Smaller volumes may lead to RNA degradation during processing or storage. For the purposed of this protocol we will assume that the weight of tissue used is 1 g. If using 150-500 mg of tissue, use half the volumes used below - consult the Qiagen RNeasy® Handbook for further details.

1. Arrange appropriately labeled 50 ml conical tubes with 15 ml of **TRIzol** or **Buffer RLT** with 2-Mercaptoethanol in each on ice.
2. Quickly dissect out up to 150 mg to 1 g of tissue and place immediately into tubes containing cold **TRIzol** or **Buffer RLT** on ice.

Note: If working with the pancreas, the animal should be anesthetized and the pancreas removed while the animal is still living - immediately proceed to step 3, before processing any other samples.

3. Immediately homogenize the tissue using a conventional rotor–stator homogenizer for at least 45 s at maximum speed until the sample is uniformly homogeneous.
4. Place homogenate on ice and when all samples are complete proceed immediately to step 7. Alternatively, snap freeze the homogenate in liquid nitrogen and store at <-70°C for future RNA extraction (this may not be an option with pancreas).
5. Wash the homogenizer probe as above and repeat steps 2 to 4 for each sample. When finished, ensure that the probe is thoroughly cleaned.

RNA isolation using Qiagen RNeasy® Maxi Columns

6. If the lysate has been stored frozen, thaw quickly and **transfer to a new 50 ml tube** (storage at <-70°C reduces the integrity of the tube and they should never be directly centrifuged).
7. Dependant upon lysis method used, follow the appropriate procedure:
   a. For samples processed with **TRIzol**:
      i. **Incubate sample for 5 minutes** in TRIzol at room temperature.
      ii. Add 3 ml **chloroform** (0.2 ml for every 1ml of TRIzol used). Shake vigorously for 15 seconds and incubate at room temperature for 2-3 min.
      iii. Centrifuge samples for **15 min at 10,000 x g at 4°C** (9000 rpm in Sorval SLA-600TC rotor).

      Note: The 4°C spins are essential for phase separation. Room temperature spins may result in variable phase separation thus resulting in variable RNA yields.

iv. **Transfer the aqueous phase** to a fresh tube. Use only this aqueous phase in subsequent steps and proceed immediately to Step 8.

Note: The aqueous phase is the colorless upper phase that corresponds to ~60% of the volume of TRIzol used. The interphase should be fairly well-defined.

b. For samples processed with **Buffer RLT**:

i. Centrifuge the tissue lysate for **15 min at 10,000 x g at 4°C** (9000 rpm in Sorval SLA-600TC rotor). Carefully transfer the supernatant to a new 50 ml tube by pipetting. Use only this supernatant (lysate) in subsequent steps and proceed immediately to Step 8.

Note: In most preparations a small pellet will form, sometimes accompanied by a fatty upper layer. Transferring the pellet or the fatty layer may reduce the amount of RNA that binds to the membrane and cause the spin column to clog. To avoid transferring contaminants, hold the pipet tip under the fatty upper layer, and don't disturb the pellet.

8. Add 1 volume (9 to 15 ml) of **70% ethanol** to the lysate, and mix thoroughly by shaking vigorously. Do not centrifuge. Proceed immediately to step 9.
9. **Apply half of the sample**, including any precipitate that may have formed, to an RNeasy maxi column placed in a 50 ml centrifuge tube (supplied). Maximum loading volume is 15 ml. Close the tube gently, and **centrifuge for 5 min** at 3000–5000 x g (5000 rpm in Sorval SLA-600TC rotor). Discard the flow-through.
10. Repeat step 9 with the remaining sample from step 8.
11. Add **15 ml Buffer RW1** to the RNeasy column. Close the centrifuge tube gently and **centrifuge for 5 min** at 3000–5000 x g to wash the column. Discard the flow-through.
12. Add **10 ml Buffer RPE** to the RNeasy column. Close the centrifuge tube gently, and **centrifuge for 3 min** at 3000–5000 x g to wash the column. Discard the flow-through and replace column in centrifuge tube.

    Note: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to Buffer RPE before use.
13. Add another **10 ml Buffer RPE** to the RNeasy column. Close the centrifuge tube gently, and **centrifuge for 10 min** at 3000–5000 x g to dry the RNeasy silica-gel membrane. It is important to dry the RNeasy membrane since residual ethanol may interfere with downstream reactions.

    Note: This centrifugation ensures that no ethanol is carried over during elution. Following the centrifugation, remove the RNeasy column from the centrifuge tube carefully so the column does not contact the flow-through as this will result in carryover of ethanol.
14. To **elute**, transfer the RNeasy column to a new 50 ml collection tube (supplied). Pipet the appropriate volume of **RNase-free buffer** of water (0.8 to 1.2 ml) directly onto the RNeasy silica-gel membrane. Close the tube gently. Let it stand for 1 min, and then **centrifuge for 3 min** at 3000–5000 x g.
15. Repeat the elution step (step 14) as described with a second volume of **RNase-free buffer** or water. To obtain a higher total RNA concentration, this second elution step may be performed by using the first eluate (from step 14). The yield will be 15–30% less than the yield obtained using a second volume of RNase-free water, but the final concentration will be higher.
16. **Store at -80°C**.