LARGE SAMPLE PROTOCOL
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**.