REED LABORATORY - qRT-PCR PROTOCOL
UIC College of Dentistry

A.1 mRNA isolation from tissue using QIAzol:

1. Remove RNA later from tubes containing tissues treated with **RNA later**.
2. Snap freeze tissue in **liquid nitrogen**.
3. Pulverize tissue with a chilled **mortar and pestle**.
4. Place pulverized tissue into RNase/DNase Free 1.5ml centrifuge tube with **1 ml Trizol** at room temperature.
5. Vortex for 1 min; let sit at room temperature for 10 min. Repeat 3 times.
6. Proceed to Trizol purification (or *store at -20°C overnight*).

B. mRNA purification using TRIzol (Cat No. 15596-018, Invitrogen)

1. Add 0.2 ml **chloroform**; vortex 3 min.
2. Incubate 2-3 mins at room temperature.
3. Centrifuge samples at 14,000 ref for 15 mins at 4 °C.
4. Samples are then separated into lower red phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. **RNA remains in aqueous phase.**
5. Transfer aqueous phase (0.6+ ml) to separate RNase/DNase Free 1.5ml centrifuge tube.
   Save the other phases if DNA or protein isolation is needed.
6. Precipitate RNA by adding 0.6 ml **isopropyl alcohol**. Make sure tubes are even; then invert multiple times to mix.
7. Incubate 5 mins at 15-30°C (*or at -20C ON*)
8. Centrifuge at 14,000 ref for 15mins at 4C, RNA pellet will be gel-like on side and bottom
9. Remove (pour or pipette off) supernatant, wash pellet once with at least 1 ml **75% ethanol.**
10. Mix the sample by vortexing.
11. Centrifuge at 14,000 ref for 10mins at 4 °C.
12. Pour off ethanol.
13. Dry the RNA pellet with the cap open 30+ min. Turn on 55°C water bath.
14. Do not let the sample dry completely- it will decrease its solubility. Partially dissolved RNA samples have an A260/280 ratio <1.6.
15. Dissolve the RNA in **100uL RNAase-free (Ultra-filtered) water** for 5 min in 55°C water bath.
16. Fast spin down.
17. Proceed to mRNA cleanup (or *store at -80°C*).

C. mRNA cleanup using RNeasy Mini Kit by Qiagen

1. FOR TRIZOL EXTRACTION: Adjust the sample to a volume of 100 µL with RNase-free water (should already be 100ul). Add **350 µL Buffer RLT**, and mix well. Incubate for 5 min.

   OTHERWISE: Add homogenized tissue to **350 µL Buffer RLT**, and mix well. Incubate for 5 min.

2. Add to **QIAShredder** column (Cat No./ID: 79654); spin 14000 g for 2 min

3. Transfer **flow through of QIAshredder** to new 1.5 ml collection tube

4. Add **250 µL 95% ETOH** to the flow through (diluted RNA), and mix well by pipetting. Do not centrifuge. *Proceed immediately to step 5.* **Keep cold on ice.**

5. Transfer the sample (RNA + ETOH) to an RNeasy Mini spin column with 2 ml collection tube (in kit). Close the lid gently. Centrifuge for 1 min at >8000 ref. Discard flow-through. **Reuse collection tube in step 6.**

   Note: After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Be sure to empty the collection tube completely.

6. Add 700 ul **Buffer RW1** to the RNeasy spin column. Close the lid gently, and centrifuge for 1 min >8000. rcf to wash the spin column membrane. Discard flow-through. **Reuse collection tube in step 7.**

7. add **10 ul DNase I** stock solution to **70 ul Buffer RDD (per tube)**. Mix by gently inverting the tube. Centrifuge briefly

8. Add **80 ul DNase/Buffer RDD mix** over the RNeasy column membrane. Incubate on ice 15 min at RT

9. Add 350 ul **Buffer RW1** to the RNeasy spin column. Close the lid gently, and centrifuge for 1 min >8000. rcf to wash the spin column membrane. Discard flow-through. **Reuse collection tube in step 7.**

10. Add 500 µL **Buffer RPE** to the RNeasy spin column. Close the lid gently, and centrifuge for 1 min >8000. rcf to wash the spin column membrane. Discard flow-through. **Reuse collection tube in step 7.**

    Note: When using a new kit from Qiagen make sure to add 100% ETOH to Buffer RPE before use (4x volume)

11. Add 500 µL **Buffer RPE** to the RNeasy spin column. Close the lid gently, and centrifuge for **2 min at> 8000 ref** to wash the spin column membrane. Discard flow-through. Reuse collection tube in step 6.

12. Centrifuge spin column at max speed for 1 min, to completely remove any remaining Buffer RPE on the column. Discard flow through. At this point you may discard the

collection tube as well, but have a labeled 1.5 mL collection tube waiting to place the spin column in.

> *Note: The long centrifugation dries the spin column membrane, ensuring that no ethanol is carried over during RNA elution. Residual ethanol may interfere with downstream reactions. After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.*

13. Place the RNeasy spin column in a new 1.5 ml collection tube (in kit). Add 30 µL RNase-free water directly to the spin column membrane. Let sit for 10 min. Centrifuge for 1 min at >11,000 ref to elute the RNA.

14. Proceed to mRNA quantification (or *store at -80°C*).

D. mRNA Quantification

1. Open the Nano Drop software
2. Choose Nucleic Acid setting
3. Change drop-down menu on the right to RNA
4. Load 2 uL of RNase free water to the reader, change label header to BLANK, hit BLANK function on the left
5. Load 2 uL of total RNA to the reader, change label header to sample, hit measure
6. Write the concentration and the 260/280 ratio on the tube
   Note: the 260/280 ratio should be between 1.9-2.1. Out of this range means contamination. Also look for a distinct peak on the output wave. If there is no peak, make a note of this
7. Dilute to a standardize the rna concentration

E. cDNA synthesis using High Capacity cDNA Reverse Trans Kit (Applied Biosystems; REF 4368814)

8. Add 1 ug RNA in 10 ul solution to 10 ul of cDNA master mist
   a. Yellow = 2.0 ul
   b. White = 2.0 ul
   c. Blue = 0.8 ul
   d. Purple = 1.0 ul
   e. dH2O = 4.2 ul
   - *eg for 300 ng/ul → add 3.33 ul rna plus 7 ul mol water → 1 ug rna*
9. Run PCR machine cycler → "CDNA…"

F. qRT-PCR using SYRB green and Primers

10. Number of primers x 18 = TOTAL/17 = n
    a. 10 ul SYBR green x n
    b. 7 ul dH2O x n
11. Pipette 54 into microtube, Add 3 ul F Primer and 3 ul R Primer, 3 ul cDNA
12. Pipette 20 ul into three wells in 96 well plate → Repeat
13. Cover the plate in plastic, seal
14. Spin the 96 well plate at 1000 g for 3 minute
15. Power on the Biorad machine
16. Hit "saved files", then "FASTSYBRAB15" → run

17. Confirm that the read out says goto 2, 49x, also that the volume is 20 ul