Date: \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

Name: \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

Species/Tissue: \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

**Protocol: Purification of Total RNA from Fresh or Frozen Animal Tissues with gDNA elimination step**

**Things to do before starting:**

Ensure all materials are nuclease free before starting. Wipe off bench the nuclease free wipes (e.g. RNaseZAP) and ensure all consumables and reagents are nuclease free. Use nuclease-free filter pipet tips. Always wear gloves and do not hesitate to change gloves if you think you may have contaminated them!

**Equipment/Supplies:**

* Qiagen RNeasy **Plus** RNA extraction kit
* Tissuelyser, 2mL Tissuelyser tubes, beads
* Analytical Scale (precision to 0.01g)
* Centrifuge
* Vortex
* Nanodrop
* Qubit
* Gel rig and power supply
* Dry ice
* Ice buckets, ice
* Labeled liquid waste bin

**Consumables:**

* Latex or nitrile gloves
* Frozen tissue samples
* Nuclease-free forceps & scalpels (or razor blades)
* Microfuge tube racks
* Nuclease-free P100, P1000 Pipet and nuclease-free filter tips
* 2 mL Nuclease-free centrifuge tubes
* 2 mL spin columns (from kit)
* 2 mL gDNA removal spin columns
* 1.5 mL centrifuge tubes

**Make up Solutions (Note: our lab support tech will make up these solutions)**

* 70% ethanol
  + \_\_\_ samples X 600 μL per sample + 10% = \_\_\_\_\_\_
* Buffer RLT Plus w/ 2M DTT
  + \_\_\_ samples X 600 μL per sample + 10% = \_\_\_\_\_\_
* Buffer RPE
  + \_\_\_ samples X 1 mL per sample + 10% = \_\_\_\_\_\_
* Buffer RW1
  + \_\_\_ samples X 700 μL per sample + 10% = \_\_\_\_\_\_
* Nuclease free water
  + \_\_\_ samples X 50 μL per sample + 10% = \_\_\_\_\_\_
* Prepare the correct volume of 70% ethanol using 100 proof ethanol
  + Use C1V1 = C2V2; (100% ethanol)\*(V1) = (70% ethanol)\*(V2)
  + Use nuclease-free water to prepare ethanol
* Add 20 μL of 2 M DTT (0.31 g DTT in 1 mL RNase-free water) per 1 mL Buffer RLT Plus. The stock solution of 2 M DTT in water should be prepared fresh or frozen in single-use aliquots.
* Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%) as indicated on the bottle to obtain a working solution.
* 44 mL ethanol + 11 mL concentrated RPE = 55 mL total Buffer RPE
* Place 2 mL centrifuge tubes containing 1 stainless steel bead on dry ice for 15 min. Keep the Tissuelyser insert at room temperature.

**Protocol**

**The first step is the most critical in terms of the risk of RNA degradation. Be careful about contamination and keep the tissue frozen at ALL times. If the tissue thaws, your experiment may be ruined. For un-stabilized frozen tissues:**

* Add 600uL of Buffer RLT Plus into a labeled, round bottom 2ml tube
* Remove the coral tissue from the freezer using sterile forceps
* Place one coral fragment about the size of a pencil eraser into a sterile weigh boat
* Quickly blot away excess **stabilization solution** with an absorbent kimwipe and quickly move it into pre-filled 2ml tube with 600ul of RNA isolation lysis solution

and a stainless steel Tissuelyser bead

* Homogenize tissue promptly after placing it in lysis/ denaturation solution.

**The following procedures should be carried out as quickly as possible.**

1. **Disrupt the tissue and homogenize the lysate in Buffer RLT Plus.**

* Disrupt and homogenize using the TissueLyser for 8 min at 50Hz.

1. **Centrifuge the lysate for 3 min at full speed.**

* Remove supernatant by pipetting.
* Transfer supernatant to a new gDNA Eliminator spin column (PURPLE) placed in a 2 mL collection tube.
* Centrifuge for 30 s at >8000 x *g* (>10,000 rpm). Discard the column, but save the flow-through and collection tube.

1. **Add 1 volume (600 μL) of 70% ethanol to the cleared lysate.**

* Mix immediately by pipetting slowly up and down 3X. Do not centrifuge.

**Note: The volume of the supernatant may be less than 600 μL due to loss during homogenization and centrifugation in steps 3 and 4.**

1. **Transfer up to 700 μL of the sample, including any precipitate that may have formed, to an RNeasy spin column (PINK) placed in a 2 mL collection tube.**

* Centrifuge for 15 sec at full speed.
* Discard the flow-through into liquid waste.
* Reuse the collection tube in the next step.

**If the sample volume exceeds 700 μL, centrifuge successive aliquots in the same RNeasy spin column. Discard the flow-through after each centrifugation.**

1. **Add 700 μL Buffer RW1 to the RNeasy spin column.**

* Centrifuge for 15 sec at full speed to wash the spin column membrane.
* Discard the flow-through into liquid waste.
* Reuse the collection tube in the next step.

**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.**

1. **Add 500 μL Buffer RPE to the RNeasy spin column.**

* Centrifuge for 15 sec at full speed to wash the spin column membrane.
* Discard the flow-through into liquid waste.
* Reuse the collection tube in step 7.

1. **Add 500 μL Buffer RPE to the RNeasy spin column.**

* Centrifuge for 2 min at full speed to wash the spin column membrane.

**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.**

**Note: 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.**

1. **Place the RNeasy spin column in a new 2 mL collection tube.**

* Discard the old collection tube with the flow-through.
* Centrifuge at full speed for 1 min.

**Perform this step to eliminate any possible carryover of Buffer RPE, or if residual flow-through remains on the outside of the RNeasy spin column.**

1. **Place the RNeasy spin column in a new labeled 1.5 mL collection tube**.

* **Top of tube**: sample #
* **Side of tube**: species, sample #, initials, date (S. caur 6 – CAL 012916)
* Add 50 μL of RNase-free water directly to the spin column membrane.
* Centrifuge for 1 min at full speed to elute the RNA. ***NOTE: Your RNA has now been eluted from the membrane and the RNA is in the flow-through (do not discard)!***
* Make sure your tube is labeled (if not already labeled). Close tube and place on regular ice immediately. This tube contains your final total RNA.

* **Label a new 1.5 ml centrifuge tube and aliquot 10 μL of total RNA into this tube**. Ensure that the original tube (now with 90 μL) gets into the -80C freezer as soon as possible. We will use the 10 μL aliquot for the Qubit, agarose gels and Fragment Analyzer QC steps. This way we do not risk degrading the RNA that will be used for the cDNA library protocol.
* Assess RNA integrity and quantity: Run agarose gel, Qubit and Nanodrop for quantity and quality of total RNA **or freeze at -80C until next step.**
* Assess the quantity and quality of your RNA with 1 ul of your sample using the Nanodrop. Write down the concentration in ng/ul, the 268:280 ratio and the 260:230 ratio in your lab notebook. Also fill in the class spreadsheet with this information, including the original weight of your tissue.

**Lessons Learned** *(use this space AND your lab notebook to write down any deviations from the protocols, potential mistakes, or tips on what to remember for next time):*