**The procedure followed for samples 18-25. For more info, see Sophia’s lab book.**

**Procedure:**

* Harvest tissue sample (flash frozen or preserved in RNAlater)
  + If using RNAlater wait 24 hours at room temperature before freezing
  + To extract RNA Wash in PBS after samples have thawed

**Prepare Materials:**

* 10 uL of B-ME per 1 mL buffer RLT (in the RLT in Falcon tube)
* 1 mL of buffer RPE per sample (in Falcon tube)
* 700 uL of buffer RW1 per sample (in Falcon tube)
* Wear gloves as you label your final tube with Name and date
  + Blur Pipette set to 450 uL
  + Yellow Pipette set to 50 uL

**RNA Extraction Protocol**

* **Add 450 uL RLT/B-ME** (B-ME found in fridge) **to each tube with 3 grinding beats** (max of 100 mg plant) 3/13/18
* Pour RLT/B-ME solution with beads into frozen sample tube (ASAP)
* **GenoGrinder for 5 min**
* **Add 225 uL of 100% ethanol** to supernatant (in sample tube), mix with pipette
  + Spin for 15s 10, 000 rpm (*to pellet plant tissue*)
* **Transfer sample supernatant** (~650 uL) to RNeasy spin column (pink)
  + Spin for 15s at 10,000 rpm
  + Discard flow through
* Add 700 uL RW1 to spin column
* Spin for 15s at 10,000 rpm
  + Discard flow through
* Add 500 uL RPE (**1st wash!**)
  + Spin for 15s at 10,000 rpm
  + Discard flow through
* Add 500 uL RPE (**2nd wash!**)
  + Spin for 15s at 10,000 rpm
  + Discard flow through (swap out the bottom collection tube)
* Spin for 2 min at 10,000 rpm
  + Discard flow through
  + Dry membrane by centrifuging in new 2 mL collection tube for 1 min (full speed)
* Place RNeasy spin column in sterile 1.5 mL Eppendorf tube
* **Add 50 uL RNase-free water** to membrane
  + Spin for 15s to elute RNA
* Pipette 50 uL elute onto membrane, use the same tube
  + Spin for 1 min 30 sec
* **Freeze RNA at -800C**

**TURBO DNase**

* If the nucleic acid solution concentration is >200 ug/mL, dilute to 10 ug nucleic acid/50 uL
* Add 10X TURBO DNase Buffer to 1X concentration in the RNA sample
* Add 1 uL TURBO DNase (2U) for up to 10 ug RNA in a 50 uL reaction
* Incubate at 37 C for 30 minutes

**Phenol:Chloroform Extraction and Isopropanol Precipitation**

* Add equal volume of Phenol:Chloroform mix to sample
  + Vortex Phenol:Chloroform/sample mixture
  + Centrifuge at max speed for a minute
* Label new tubes
* Add cold isopropanol (2-propanol) at double the amount of sample
* Move top layer (aqueous) to new tube with isopropanol
  + Vortex aqueous phase and isopropanol
  + Centrifuge at max speed for 10 minutes
* Decant isopropanol
* Add 500 uL of 70% ethanol to pellet
  + Vortex
  + Centrifuge at max speed for 10 minutes
* Decant ethanol
* Dry pellet in 370 C incubator until all the ethanol evaporates

Resuspend pellet in 50 uL DEPC-treated water (RNAase free water)

**Calculating RNA Quality 6/6/18**

* Keep samples on ice
* Use autoclaved water to clean NanoDrop machine with a tissue
* Start NanoDrop software
  + RNA setting, not DNA setting
  + Test blank with autoclaved water
* Pipette 1 uL of RNA onto NanoDrop
* Close NanoDrop lid and run the measurement
* Record concentrations and other numbers
* Print
  + Go to “Records”, print off numbers
* Clean the machine when finished with autoclaved water

**Luna® Universal Probe One-Step RT-qPCR Kit Protocol (E3006)**

* Prepare RNA of interest using desired RNA extraction and purification methods. Determine concentration by OD260 absorbance.
* Make dilutions of RNA to be used for the standard curve. These should be prepared fresh before each experiment and can be diluted in either water or TE.

**Reaction Setup:** For best results, we recommend running each RNA standard and sample in triplicate.

| **COMPONENT** | **20 µl REACTION** | **FINAL CONCENTRATION** |
| --- | --- | --- |
| Luna Universal Probe One-Step Reaction Mix (2X) | 10 µl | 1X |
| Luna WarmStart® RT Enzyme Mix (20X) | 1 µl | 1X |
| Forward primer (10 µM) | 0.8 µl | 0.4 µM |
| Reverse primer (10 µM) | 0.8 µl | 0.4 µM |
| Probe (10 µM) | 0.4 µl | 0.2 µM |
| Template RNA | variable | < 1 µg (total RNA) |
| Nuclease-free Water | to 20 µl |  |

**QPCR Protocol**

1. Thaw Luna Universal Probe One-Step Reaction Mix and other reaction components at room temperature, then place on ice. After thawing completely, briefly mix each component by inversion, pipetting or gentle vortexing.
2. Determine the total volume for the appropriate number of reactions, plus 10% overage and prepare assay mix of all components except RNA template accordingly. Mix thoroughly but gently by pipetting or vortexing. Collect liquid to the bottom of the tube by brief centrifugation.
3. Aliquot assay mix into qPCR tubes or plate. For best results, ensure accurate and consistent pipetting volumes and minimize bubbles.
4. Add RNA template to qPCR tubes or plate. Seal tubes with flat, optically transparent caps; seal plates with optically transparent film. Care should be taken to properly seal plate edges and corners to prevent artifacts caused by evaporation.
5. Spin tubes or plates briefly to remove bubbles and collect liquid (1 minute at 2,500–3,000 rpm).
6. Program real-time instrument with indicated thermocycling protocol (see table below). Ensure a plate read is included at the end of the extension step.

Confirm selection of appropriate detection channel(s) for the fluorophore(s) present in the assay.

**For faster results, the “Fast” ramp speed mode can be used where available (e.g., Applied Biosystems StepOnePlus®, QuantStudio®, 7500 Fast instruments).**

| CYCLE STEP | TEMPERATURE | TIME | CYCLES |
| --- | --- | --- | --- |
| Reverse Transcription | 55°C\* | 10 minutes | 1 |
| Initial Denaturation | 95°C | 1 minute | 1 |
| Denaturation  Extension | 95°C  60°C | 10 seconds  30 seconds\*\*(+plate read) | 40-45 |

\* **A 55°C RT step temperature** is optimal for Luna WarmStart Reverse Transcriptase. To ensure best performance and full WarmStart activation, avoid using a temperature of < 50°C.

\*\* For Applied Biosystems real-time instruments use a 60 second extension step.