RNA Isolation (GeneJet RNA Purification Kit) – Insect Total RNA Purification Protocol

Before beginning, review entire protocol and confirm you have all reagents and supplies, clean your prep space and wipe down your pipettors using ETOH and RNase Out. Sterile plastic (tips, tubes, inside of Ziploc bag) should *be RNase free. All liquid waste from this protocol goes in the hood.*

1. Weigh the tissue (use up to 30 mg of fresh or frozen tissue), take the insect and disrupt the material by using a mortar and pestle, as follows:
   a) Dip <30 mg of insect (~3 bees) into liquid nitrogen, then grind thoroughly with a mortar and pestle. (Do this step at the liquid nitrogen station, then return to your bench.)
   b) Transfer the ground tissue immediately into a 1.5 mL microcentrifuge tube containing 300 µL of Lysis Buffer supplemented with β-mercaptoethanol or DTT. Vortex for 10 s to mix thoroughly.
      Notes to prevent RNA degradation
      - Transfer the insect powder to the Lysis Buffer as quickly as possible.
      - Mix all grinded material thoroughly with the Lysis Buffer, leaving no dry material on the walls of the tube.
   c) Pass the lysate through a QIAshredder column placed in a new 1.5 ml RNase free tube (not included in kit): Pipette entire lysate sample into QIAshredder column, centrifuge, and discard the column, saving the flowthrough.
2. Add 600 µl of diluted Proteinase K (10 ul of the included Proteinase K diluted in 590 ul of TE buffer). Vortex to mix thoroughly and incubate at room temperature for 10 min.
3. Centrifuge for 10 min at ≥12000 x g. Being careful not to disturb the bee pellet, transfer the supernatant (lysate) into a new 1.5 ml microcentrifuge tube.
4. To the lysate, add 450 µL of 96-100% ethanol and mix by pipetting.
5. Transfer up to 700 µL of lysate to the GeneJET RNA Purification Column inserted in a collection tube (included in Ziploc). Centrifuge the column for 1 min at ≥12000 x g. Discard the flowthrough and place the purification column back into the collection tube. Repeat this step until all of the lysate has been transferred into the column and centrifuged. Discard the collection tube containing the flow-through solution. Place the GeneJET RNA Purification Column into a new 2 mL collection tube (included in the Ziploc).
6. Add 700 µL of ethanol-supplemented Wash Buffer 1 to the GeneJET RNA Purification Column and centrifuge for 1 min at ≥12000 x g. Discard the flow-through and place the purification column back into the collection tube.
7. Add 600 µL of ethanol-supplemented Wash Buffer 2 to the GeneJET RNA Purification Column and centrifuge for 1 min at ≥12000 x g. Discard the flowthrough and place the purification column back into the collection tube.
8. Final rinse:
   a) Add 250 µL of ethanol-supplemented Wash Buffer 2 to the GeneJET RNA Purification Column and centrifuge for 2 min at ≥12000 x g.
   b) Empty the collection tube and re-spin the column for 1 min at maximum speed to remove any excess ethanol.
   c) Discard the collection tube containing the flow-through solution and transfer the GeneJET RNA Purification Column to a sterile 1.5 mL RNase-free microcentrifuge tube (included in Ziploc). Label this tube neatly and carefully with your sample ID.
9. Add 100 µL of "Water, nuclease-free" (included in Ziploc) to the center of the GeneJET RNA. Purification Column membrane. Centrifuge for 1 min at ≥12000 x g to elute RNA.
10. Discard the purification column. Immediately continue to cDNA synthesis protocol. Use the purified RNA for cDNA synthesis and place remainder of RNA in ice bucket by PCR machine for later storage at -80°C.

cDNA Synthesis using TaqMan Reverse Transcription (Invitrogen)

1. A PCR tube with the TaqMan Reverse Transcription (Invitrogen) master mix containing the following reagents will be prepared for you by the TAs according to the manufacturer's instructions (see pages"2-11" and "2-17" of the manufacturer's protocol) and stored on ice:

Reagent	**Volume (µL)**
10X TaqMan RT buffer	2
25 mM $MgCl_2$	4.4
deoxyNTP mix (2.5 mM each)	4
Random hexamer primers (50 µM)	1
RNase Inhibitor (20 U/µL)	0.4
RNase-free water	5.7
Multiscribe Reverse transcriptase (50 U/µL)	0.5
Total	*18*

2. Add 2 µL of RNA to 18 µL of master mix to create a 20 µl reaction volume. Mix gently by inverting the tube several times. Place your tubes on ice until the TAs are ready for you to place them in the thermal cycler. Keep your RNA tube on ice for eventual long term storage at -80C.

3. cDNA synthesis thermal "cycling" conditions are as follows:

Step	**Temperature (°C)**	**Time (min)**
1. Primer annealing	25	10
2. Reverse transcription	48	30
3. Reverse transcriptase inactivation	95	5
4. Hold at 4C for overnight if necessary	4	infinity

4. cDNA will be stored at –20C, ready for future use for PCR. After PCR, transfer the remainder of your cDNA samples to a newly labeled 1.5 ml tube for long term storage at –80.

Lab Wrap-up

1. Give your RNA tube sample to the TAs for storage at -80.

2. Then clean up your entire station. Take your mortar and pestle to the sink and wash carefully and thoroughly, carefully inverting the mortar to dry on the drain board.