RNA extraction protocol No.1

**Benchwork prep**

1. Sterilize blue plastic pestles by scrubbing with bleach, rinsing with dH2O and subjecting to **15 minutes of UV light**.
2. Acquire **1.5 lbs** of **dry ice** from Entomology basement **and 0.5 liter** **of liquid nitrogen** from Spieth Hall basement. Ensure access to the key to the ice box available in the Entomology Administrative office (first floor) and access to the liquid nitrogen in the basement of Spieth Hall (acquire key from Brelsford lab). **Remember to return both keys** **once materials are acquired.** **Bring a pen** to record materials used. Especially for liquid nitrogen.
3. Thaw **glycogen and Qubit** solutions at room temperature.
4. Run the Eppendorf 5424r cold centrifuge for 15-20 minutes at **4°C** to get it cold for later runs.
5. Place the **isopropyl alcohol** in the dry ice for use in RNA precipitation.

**Tissue grinding and homogenization**

1. Submerge tubes with specimen in liquid nitrogen to render tissue brittle and facilitate grinding. Grind the sample with UV-sterilized plastic pestles that have been held in liquid nitrogen to maintain cold (held within sterilized collection tubes). Grind all samples for at least **45 seconds,** grinding in both clockwise and counterclockwise movements, and grinding the pestle against the walls and bottom of the tube. An ideal grinding should result in a fine white powder. Leave the pestle in the tube for the next step.
2. Add **400 μl of Trizol® reagent** to all samples. Be sure to wash the pestle with the Trizol so that all powdered tissue is washed into the bottom of the tube. The Trizol will likely freeze immediately. Let it sit for ~5 minutes at room temperature to allow it to melt. Remove the pestle. **Vortex for 5 seconds.**
3. Homogenize the solution by pipetting the lysate directly into a **QIAshredder spin column** placed in a 2 mL collection tube, and centrifuge **for 2 min at 14000 rpm**. Then incubate at room temperature for **5 minutes** for efficient tissue lysis. *Note: Unless centrifugation is specified to occur at 4***°***C, use the normal centrifuge, not the cold centrifuge.*
4. Transfer the supernatant to a new 1.5 uL microcentrifuge tube.

**Phase separation**

1. Add **80 μl** **chloroform to sample.** Or 200 μl of chloroform per 1000 μl of Trizol. For 400 μl, that would be 80 uL. Mix by **vortex (10 seconds)** and incubate the tube for **5 minutes** at room temperature.
2. Carefully **transfer** **the upper aqueous phase** without touching the interphase and the bottom of the solution to a new 1.5 mL RNase free tube. Setting a pipette to **200 uL** for this will suffice.

**RNA precipitation**

1. Add **10 μg of glycogen** to the aqueous phase. The use of glycogen as a nucleic acid carrier significantly increases the RNA yield.
2. Add **200 uL of isopropyl alcohol** to the aqueous phase.
3. Centrifuge at **14,000 rpm** for **30 minutes at 4°C.**
4. Place sample in **-20°C** conditions for 8 hours. **Allegedly, the RNA will drop to the bottom and form a pellet. However, this might not happen and if so, modify Step 15 by simply adding the 100 uL of 75% ethanol to the supernatant and incubating for 5 minutes at room temperature, then proceed to Step 17 and skip Step 16.**

Overnight incubation at -20**°C.** Place the 75% ethanol in the dry ice for Step 16.

1. If pellet is formed, add **100 uL of ultra cold 75% ethanol**, **incubate for 5 minutes at room temperature**, centrifuge **at 14,000 rpm for 5 minutes.**

**RNA purification (using RNeasy® Plus Mini Kit, Qiagen, from Step 5 of the kit handbook)**

1. Transfer up to **700 μl of the sample**, including **any precipitate** that may have formed, to a RNeasy spin column placed in a 2 mL collection tube. **Be especially careful to transfer the precipitate.** Centrifuge for **20 seconds** at **>10000 rpm**. Discard the flow through. Reuse the collection tube. If the sample volume exceeds 700 μl, centrifuge successive aliquots in the same RNeasy spin column. Discard the flow-through after each centrifugation. Use a kimwipe to blot the collection tube.
2. Add **700 μl Buffer RW1** to the RNeasy spin column. Centrifuge for **20 seconds** at **>10000 rpm** to wash the spin column membrane. Discard the flow-through. Reuse the collection tube. *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.*
3. Add **500 μl Buffer RPE** to the RNeasy spin column. Centrifuge for **20 seconds** at **>10000 rpm** to wash the spin column membrane. Discard the flow-through. Reuse the collection tube. *Note: Buffer RPE is supplied as concentrate. Ensure that ethanol is added to Buffer RPE before use.*
4. Add **500 μl Buffer RPE** to the RNeasy spin column. Centrifuge for **2 minutes at >10000** rpm 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. Discard the flow-through. *Note: After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carry-over of ethanol will occur.*
5. **Centrifuge at 14000 rpm for 1 minute**. Perform this step to eliminate any possible carryover of Buffer RPE.

**RNA elution**

1. Place the RNeasy spin column in a new 1.5 mL collection tube. Add between **30 and 50** (we chose 45µL) **μl of RNase-free water** directly to the spin column membrane (amount of water depends on the quantity of tissue used and the required RNA concentration). Incubate for **1 minute** at room temperature.
2. Centrifuge for **1 minute at >10000 rpm** to elute the RNA.

**RNA quantification**

1. Prepare Qubit® working solution by diluting the RNA BR Reagent 1:200 in RNA BR Buffer in a clean plastic tube.
2. Add **190 μl** of Qubit working solution to each tube used for standards. Or use standards prepared previously from other quantifications. Use Qubit clear tubes for standards and sample preparation.
3. Add **10 μl** of each Qubit standard to the appropriate tube, then mix by **vortexing 2-3** seconds, being careful not to create bubbles.
4. Add Qubit working solution to individual assay tubes so that the final volume in each tube after adding sample is **200 μl.** *Note: Your sample can be anywhere from 1-20* μl. *Add a corresponding volume of working solution to each assay tube: anywhere from 180-199* μl.
5. Add each sample to the assay tubes containing the correct volume of working solution, then mix by vortexing **2-3 seconds.** The final volume in each tube should be **200 μl.**
6. Allow tubes to incubate at room temperature for **2 minutes.**

Table 1: Qubit Working Solution & Sample Prep

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Number of Samples | Qubit Buffer  (200 μL) | Qubit Reagent  (1 μL) | Qubit Working Solution  (180-199 μL) | Sample  (1-20 μL) |
| 5 | 1,000 μL | 5 μL | 190 μL | 10 μL |
| 10 | 2,000 μL | 10 μL |  |  |
| 15 | 3,000 μL | 15 μL |  |  |
| 20 | 4,000 μL | 20 μL |  |  |

**Clean-Up**

1. Clean salt build-up on Eppendorf 5424r cold centrifuge with a kimwipes. Turn off centrifuges.
2. Place RNA extractions in **-80°C freezer.** Return glycogen and Qubit reagents to the freezers.
3. Dispose of waste in trash cans.

RNA extraction protocol No.2

**Benchwork prep**

1. Sterilize blue plastic pestles by scrubbing with bleach, rinsing with dH2O and subjecting to **15 minutes of UV light**.
2. Acquire **2 lbs** of **dry ice** from Entomology basement **and 1 liter** **of liquid nitrogen** from Spieth Hall basement. Ensure access to the key to the ice box available in the Entomology Administrative office (first floor) and access to the liquid nitrogen in the basement of Spieth Hall (acquire key from Brelsford lab). **Remember to return both keys** **once materials are acquired.** **Bring a pen** to record materials used. Especially for liquid nitrogen.
3. Thaw **glycogen and Qubit** solutions at room temperature.
4. Run the Eppendorf 5424r cold centrifuge for 15-20 minutes at **4°C** to get it cold for later runs.

**Tissue grinding and homogenization**

1. Submerge tubes with specimen in liquid nitrogen to render tissue brittle and facilitate grinding. Grind the sample with UV-sterilized plastic pestles that have been held in liquid nitrogen to maintain cold. (held within sterilized collection tubes). Grind all samples for at least 1.5 minutes, grinding in both clockwise and counterclockwise movements, and grinding the pestle against the walls and bottom of the tube. Leave the pestle in the tube for the next step.
2. Add **400 μl of Trizol® reagent** to all samples. Be sure to wash the pestle with the Trizol so that all powdered tissue is washed into the bottom of the tube. Remove the pestle.
3. Homogenize the solution by pipetting the lysate directly into a **QIAshredder spin column** placed in a 2 mL collection tube, and centrifuge **for 2 min at 14000 rpm**. Then incubate at room temperature for **5 minutes** for efficient tissue lysis.

**Phase separation**

1. Centrifuge the tube for **1 minute at 14000 rpm** to pellet the powder or the digested tissue and pipette all surnatant to a new 1.5mL RNase free tube.
2. Add 200 μl of chloroform per 1000 μl of Trizol. For 400 μl, that would be **80 μl** **chloroform**. Mix by **vortex (10 seconds)** and incubate the tube for **5 minutes** at room temperature.
3. Centrifuge the tubes for **15 minutes** at **12000 rpm** and 4°C.
4. Carefully transfer the upper aqueous phase without touching the interphase and the bottom of the solution to a new 1.5 mL RNase free tube.

**RNA precipitation**

1. Add **10 μg of glycogen** to the aqueous phase. The use of glycogen as a nucleic acid carrier significantly increases the RNA yield.
2. Add an equivalent volume of 70% ethanol to the isolated upper aqueous phase (about ~60% of Trizol used in Step 2). For 400 μl Trizol, **add 240 μl 70% ethanol**. *Note: the volume of lysate may be less than 350 μl or 600 μl due to loss during homogenization.*
3. Mix by pipetting (20x).

**RNA purification (using RNeasy® Plus Mini Kit, Qiagen, from Step 5 of the kit handbook)**

1. Transfer up to **700 μl of the sample**, including any precipitate that may have formed, to a RNeasy spin column placed in a 2 mL collection tube. Centrifuge for **15 seconds** at **>10000 rpm**. Discard the flow through. Reuse the collection tube. If the sample volume exceeds 700 μl, centrifuge successive aliquots in the same RNeasy spin column. Discard the flow-through after each centrifugation.
2. Add **700 μl Buffer RW1** to the RNeasy spin column. Centrifuge for **15 seconds** at **>10000 rpm** to wash the spin column membrane. Discard the flow-through. Reuse the collection tube. *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.*
3. Add **500 μl Buffer RPE** to the RNeasy spin column. Centrifuge for **14 seconds** at **>10000 rpm** to wash the spin column membrane. Discard the flow-through. Reuse the collection tube. *Note: Buffer RPE is supplied as concentrate. Ensure that ethanol is added to Buffer RPE before use.*
4. Add **500 μl RPE** to the RNeasy spin column. Centrifuge for **2 minutes at >10000** rpm 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, carry-over of ethanol will occur.*
5. Place the RNeasy spin column in a new 2 mL collection tube and discard the old collection tube with the flow-through. **Centrifuge at 14000 rpm for 1 minute**. Perform this step to eliminate any possible carryover of Buffer RPE.

**RNA elution**

1. Place the RNeasy spin column in a new 1.5 mL collection tube. Add between **30 and 50** **μl of RNase-free water** directly to the spin column membrane (amount of water depends on the quantity of tissue used and the required RNA concentration). Incubate for **1 minute** at room temperature.
2. Centrifuge for **1 minute at >10000 rpm** to elute the RNA.

**RNA quantification**

1. Prepare Qubit® working solution by diluting the RNA BR Reagent 1:200 in RNA BR Buffer in a clean plastic tube.
2. Add **190 μl** of Qubit working solution to each tube used for standards.
3. Add **10 μl** of each Qubit standard to the appropriate tube, then mix by **vortexing 2-3** seconds, being careful not to create bubbles.
4. Add Qubit working solution to individual assay tubes so that the final volume in each tube after adding sample is **200 μl.** *Note: Your sample can be anywhere from 1-20* μl. *Add a corresponding volume of working solution to each assay tube: anywhere from 180-199* μl.
5. Add each sample to the assay tubes containing the correct volume of working solution, then mix by vortexing **2-3 seconds.** The final volume in each tube should be **200 μl.**
6. Allow tubes to incubate at room temperature for **2 minutes.**

References

1. Gayral, P., Weinert, L., Chiari, Y., Tsagkogeorga, G., Ballenghein, M., & Galtier, N. (2011). Next-generation sequencing of transcriptomics: a guide to RNA isolation in nonmodel animals. *Molecular Ecology*, **11**, 650-661.
2. Martinez-Ruiz, C., Pracana, R., Stolle, E., Ivon Paris, C., Nichols, R. A., & Wurm, Y. (2020). Genomic architecture and evolutionary antagonism drive allelic expression bias in the social supergene of red fire ants. *eLife*, 9;e55862. [dx.doi.org/10.17504/protocols.io.bi8fkhtn](https://dx.doi.org/10.17504/protocols.io.bi8fkhtn)
3. RNeasy® Mini Handbook (2019). Qiagen. Protocol: Purification of Total RNA from Animal Cells Using Spin Technology, p. 27-34.