Total RNA Isolation

Good practices:

- Clean work area (bench, pipettes, and any racks) with 70% ethanol then RNase away before starting RNA extraction.
- Always keep samples on ice.
- Perform all centrifugation steps at 4°C.
- Since this protocol involves moving samples into new tubes multiple times, record the original tube information and always double check you are transferring to the correctly labeled tube.

Sample homogenization:

Whether you are extracting RNA from cell pellets, tissue, or sperm samples this step is slightly different. Follow the appropriate homogenization step below before proceeding to phenol-chloroform extraction.

Tissue samples:

1. Estimate the approximate volume of tissue you have and add 1 × volume of 1 mm beads to a 2 mL screw top tube.
    a. Typically, a mouse cauda epididymis is ~50 µL.
2. Add 3-5 × volume of Trizol to the tube with beads and tissue (For 50 µL add 150-250 µL of Trizol).
3. Transfer tissue to screw top tube (can do this before step 1 too). Use forceps if necessary but make sure to clean first and in between samples.
4. Place screw top tubes in cold aluminum block. Try and balance tubes. Place block in the homogenizer and run for 2 min at 2,000 rpm (program A).
5. Turn off homogenizer. Open shield, remove samples and place on ice. Remember to place the placeholder block back in the homogenizer, tighten and place shield down.

Proceed to phenol-chloroform extraction.

Cells (pellet in tube):

1. Thaw cells on ice.
2. Add appropriate volume of Trizol (see below) to the cell pellet and pipette up and down to resuspend cells.
3. Vortex for 1 min to homogenize cells.
4. Make sure cell pellet is completely broken up. If not pipette with P200 and vortex until resuspended.

Proceed to phenol-chloroform extraction.

Cells (adherent on cell culture plate):

1. Once cells are confluent and you are ready to extract RNA, wash cells with PBS twice by pipetting up and down across the bottom of the flask, removing PBS and adding fresh PBS and repeating.
2. Add 1 mL Trizol to flask (this volume will change based on cell number, see below).
3. Mix by placing on rocker at 4°C for 5 min.
4. Pipette volume up and down along the bottom of the dish before transferring to a fresh 1.5 mL tube.

Proceed to phenol-chloroform extraction.

Sample type	Starting material per 1 ml of TRIzol reagent
Cells grown in monolayer	1×10^5–1×10^7 cells grown in monolayer in a 3.5–cm culture dish (10 cm^2)
Cells grown in suspension	5–10 $\times 10^6$ cells from animal, plant, or yeast origin or 1 $\times 10^7$ cells of bacterial origin

Sperm cells:
Due to the specialized make-up of the sperm nucleus and chromatin they require an additional more rigorous homogenization/lysis prior to extraction. See https://doi.org/10.1095/biolreprod.116.142190 for more details.

1. For cauda sperm pellets, add enough water to sample so that the total volume is 120 µL (I.e., if there is 40 µL of sample, add 80 µL of water). For caput sperm pellets, add enough water so that the total volume is 60 µL.
2. Add Sperm Lysis Buffer, Proteinase K, and 1M DTT as below.

Sample Type	Sperm Lysis Buffer (µL)	20 mg/ml Proteinase K (µL)	1M DTT (µL)
Caput & Testis	33.3	3.3	3.3
Cauda	66.6	6.6	6.6

3. Mix with pipette until pellet is completely broken down. Pipette, vortex, spin down, and repeat until the sample is close to liquefied.
    a. Note: Set pipette to full volume of sample to break down sample faster
4. Place samples in mixer and allow samples to shake and incubate for 15 min at 60˚C.
5. Add 1 × volume of water to each sample (200 µL for cauda sperm samples and 100 µL for caput sperm samples) and mix well (pipette, vortex, spin down, and repeat) so full sample is resuspended.

Proceed to phenol-chloroform extraction.

Phenol-Chloroform Extraction and RNA Precipitation:

1. Spin down Phase Lock Gel Heavy 2 mL tube (QuantaBio: 2302830) at 14,000 rpm for 1 minute at 4°C. Make sure to place the lid hinge to the outside of the centrifuge rotor.
2. Pipette homogenized sample into the prespun Phase Lock tube.
3. Add 0.2 × volume of 1-bromo-2 chloropropane (BCP; (MOLECULAR RESEARCH CENTER, INC.: BP151) to the liquid in the Phase Lock tube. I.e., if the total volume of the homogenized sample is about 200 µL, add 40-50 µL. Err towards more volume of BCP than less volume.
4. Invert samples 10-15 time (Important!).
    a. Note: Be sure not to vortex the phase lock tubes to avoid dislodging the grease.
5. Centrifuge samples at 4°C for 4 minutes at 14,000 rpm. Invert samples again immediately before placing in the centrifuge.
6. Pipette the aqueous layer of the phase separation into an Eppendorf low-adhesion tube. Be careful not to pipette any of the pink, Trizol layer or grease.

7. Add 1.1 × volume of cold isopropanol to aqueous layer and immediately invert to mix (i.e. if you had 100 µL of aqueous layer, add 110 µL of isopropanol. Always use an aliquot of isopropanol, do no pipette from stock container.
8. Add 1 µL of glycoblue to each sample.
9. Vortex to mix and place tubes at -20°C. Let precipitate for at least 1 hour. RNA is most stable at this step, so this is a good stopping point.

If you wish to also extract DNA after RNA extraction keep Phase lock tubes from Step 6 and proceed with DNA extraction below.

RNA wash and reconstitution:
Do this step on the day you wish to use the RNA (i.e. purify small RNA, DNase treat or quantitate). It is best to avoid freeze thaw of RNA samples and as mentioned above, RNA is most stable in isopropanol. For this step it is important to remove all ethanol without over drying, if you have not done this before it is best to watch someone else first!

1. Centrifuge at 14,000 rpm for 15 minutes at 4°C.
2. Remove supernatant and wash pellet with 1 mL cold 70% ethanol. Centrifuge at 14,000 rpm for 5 minutes at 4°C.
3. Remove supernatant while leaving pellet in place. Make sure all ethanol is removed by spinning down the tubes in a benchtop centrifuge and removing liquid using a gel-loading tip 2 ×. It is important to ensure no ethanol is left and not to over dry pellets so do this step with at most 2 tubes at a time.
4. Reconstitute the RNA pellet in 8 µL of nuclease-free water. This volume may differ depending on the final application.

For small RNA proceed to purification of small RNA (different protocol) using total RNA. For mRNA (low input and bulk) DNase treat RNA and aliquot the appropriate amount of RNA ready for sequencing.

DNA Extraction (optional):
1. With a clean pipette (P1000) gently break a hole in the grease layer. It is easiest to do this down the side of the tube.
2. With a new pipette, pipette the below layer into a new 1.5 mL tube. Do not transfer any grease.
3. Add 500 µL Back Extraction Buffer (BEB) for every 1 mL of Trizol used in the initial reaction. Pipette to mix 2-3 times. Do not vortex!
4. Place tubes on shaker for 10 min.
5. Centrifuge for 30 min at room temperature at 12,000 x g.
6. Transfer aqueous layer to new 1.5 mL tube.
7. Add 1 µL of glycoblue to each sample.
8. Add 1 × volume of isopropanol to each sample and mix.
9. Place tubes at -20°C.

Sperm Lysis Buffer (50mL):
- Guanidine HCl - 6.4M - 30.56g
- Tween-20 - 5% - 2.5mL
- Triton-X 100 - 5% - 2.5mL

- EDTA - 120mM - 12mL of 0.5M stock
- Tris-HCl pH 8.0 - 120mM - 6mL of 1M stock

Make up to 50mL with nuclease free water.

Back Extraction Buffer (BEB) (50mL):
23.64g Guanidine thiocyanate (4M)
0.74g sodium citrate tribasic dihydrate (50mM)
6.06g Tris base (1M)
Add Di water up to 50 mL. Add volume slowly and invert to mix as you won't need 50mL of water.

1M DTT:
616.8 mg in 4mL water – Store at -20°C in 100ul aliquot