RNA isolation Using RNeasy Mini Kit

General Handling:

- keep purified RNA on ice when aliquots are pipetted for applications (e.g. PCR, RT-qPCR)
- use sterile, disposable propylene tubes
- nondisposable materials should be treated before to ensure RNAse free

Safety:

*****RLT, RLC, & RW1 ← DON'T clean spill w/ BLEACH – HIGHLY REACTIVE**

Notes:

- perform centrifugation steps @ 20-25 ^{o} C

Items Not Supplied in Kit (Gather/prepare before beginning procedure):

- 14.3 M β-mercaptoethanol (β-ME)
- Sterile, RNAse-free pipet tips
- Microcentrifuge (w/ rotor for 2 ml tubes)
- 96-100% ethanol (not denatured ethanol!) (put this into tube to have at bench)
- Disposable gloves
- Blunt Needle (20-gauge-0.9 mm) and Syringe
- 70% ethanol (make your own from stock and keep in tube @ bench)
- propylene centrifuge tube (for indirect lysis)
- microcentrifuge tube & rubber policeman (for direct lysis)

Before Starting:

- UNDER HOOD & with Protective Clothing: Add β-ME to Buffer RLT before use→ add 10 µL β-ME (14.3 M) per 1 mL Buffer RLT
    - This can be stored @ Room Temperature for 1 month (so make enough for the experiments you are doing within the month if you want)

- Buffer RPE is supplied as a concentrate. Before use for 1st time, add 4 volumes of 96-100% ethanol as indicated on the bottle to obtain working solution (there is a label on the cap with a box to "check" if you have added the ethanol…that way others will know if you already added it and will not add more)

*Determine the appropriate volume of cells to use for this isolation. This depends on size of plate, cell type, and whether the cell type has a typically low or high RNA yield. Reference page 25 and 26 of the RNEasy Mini Handbook to determine appropriate number of cells and what volume of Buffer RLT to use throughout the experiment, which depends on the number of cells you have.

Purification of Total RNA from Animal Cells Using Spin Technology

1. For cells grown in a monolayer, either lyse on plate or make pellet prior to lysis (pellet would have to be in polypropylene centrifuge tube)
   - **INDIRECTLY:** Aspirate medium, wash 1x with PBS, aspirate PBS, add 0.1% Trypsin in PBS. After the cells dtach, add medium, transfer to an RNAse-free polypropylene centrifuge tube & centrifuge @ 300xg for 5 min. Completely aspirate the supernatant, and proceed to step 2.
   - **Directly:** completely aspirate the medium & proceed to step 2.

2. Disrupt the cells by adding Buffer RLT

(For 10-cm dish=> add 600 μL of Buffer RLT)

(For pelleted cells 5 $x10^6$ -1 $x10^7$ = > add 600 μL Buffer RLT…any less add 350 μL)

   - Pelleted Cells: loosen the cell pellet thoroughly by flicking the tube. Add the appropriate volume of RLT. Then, vortex to mix, and proceed to step 3.
   - Direct lysis of cells grown in monolayer: Add the appropriate volume of RLT to the cell-culture dish. Collect the lysate with a rubber policeman. Pipet the lysate into a microcentrifuge tube. Vortex or pipet to mix, and ensure that no cell clumps are visible before proceeding to step 3.

3. Homogenize the lysate
   - Pass the lysate at least 5 times through a blunt 20-gauge needle (0.9 mm diameter) fitted to an RNase-free syringe. Proceed to step 4.

- *If homogenizing ≤ 1 x 10^5 cells, homogenize by vortexing for 1 minute.

*Important: incomplete homogenization leads to reduced RNA yields!

4. Add 1 volume of 70% ethanol to the homogenized lysate, and mix well by pipetting. Do Not Centrifuge!

5. Transfer up to 700 µL of the sample, including any precipitate that may have formed, to an RNeasy spin column placed in a 2 ml collection tube. Close the lid gently and centrifuge for 15 s @ ≥ 8000 x g (≥ 10,000 rpm). Discard the flow through (**contains RLT or RW1 = NOT COMPATIBLE WITH BLEACH!!!).** reuse the collection tube in step 6. (Don't exceed 700 uL in spin columns!!!)

   ****Optional DNAse Digestion Step: I recomment this***
   "Eliminating Genomic DNA Contamination" pg. 23. Follow Steps D1-D4 on page 69 of the manual after peforming this step.

6. Add 700 µL Buffer RW1 to the RNeasy spin column. Close lid gently, and centrifuge for 15 s @ ≥ 8000 x g (≥ 10,000 rpm), to wash the spin column membrane. Discard the flow-through **(NOT COMPATIBLE WITH BLEACH!!!)** Reuse the collection tube in step 7.
   - **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.**

7. Add 500 µL of Buffer RPE (that you added ethanol to!) to the RNeasy spin column. Close the lid gently, and centrifuge for 15 sec @ ≥ 8000 x g (≥ 10,000 rpm) to wash the spin column membrane. Discard the flow through. Reuse the collection tube in step 8.

8. Add 500 µL Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge 2 min @ ≥ 8000 x g (≥ 10,000 rpm) to wash the spin column membrane.

- This step dries the spin column membrane, sine residual ethanol can 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 ethanol will carryover.**

9. **Optional:** Place the RNeasy spin column in a new 2 ml collection tube, and discard the old collection tube with the flow-through. Close the lid gently, and centrifuge @ full speed for 1 min. (this step is to eliminate possible carryover of Buffer RPE, or if residual flow-through remains on the outside of the RNeasy spin column after step 8).
   - I would recommend this step*

10. Place the RNeasy spin column in a new 1.5 ml collection tube. Add 30-50 µL RNAse-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 minute @ ≥ 8000 x g (≥ 10,000 rpm) to elute the RNA

11. If the expected RNA yield is >30 µg, repeat step 10 using another 30-50 µL RNase-free water or using the eluate from step 10 (if high RNA conc. Is required). Reuse the collection tube from step 10.
    - If using the eluate from step 10, the RNA yield will be 15-30% less than that obtained using a second volume of RNAse-free water, but the final RNA concentration will be higher.