Total RNA Isolation from Cultured Cells with Qiagen RNeasy Midi Kit

Reference: RNeasy Midi/Maxi Handbook 6/2001, pg 17, pg 26 – 31 and pg 91 – 92

Harvest Cells

- Cells grown in suspension
  - Determine number of cells.
  - Pellet cells for 5 min., 300 x g, 4°C.
  - Carefully remove supernatant by aspiration.
  - Resuspend cell pellet in ice cold 1X PBS.
  - Pellet cells for 5 min., 300 x g, 4°C.
  - Carefully remove supernatant by aspiration.
  - Continue with RNA preparation or snap freeze the cell pellet in liquid nitrogen and store, -70°C.
- Cells grown in monolayer
  - Remove growth media. Add 5ml/dish ice cold 1X PBS. Scrape cells.
  - Pool scraped cells in cold PBS into polypropylene tube(s), sitting on ice.
  - Determine number of cells.
  - Pellet cells for 5 min., 300 x g, 4°C.
  - Resuspend cell pellet in ice cold 1X PBS.
  - Pellet cells for 5 min., 300 x g, 4°C.
  - Carefully remove supernatant by aspiration.
  - Continue with RNA preparation or snap freeze the cell pellet in liquid nitrogen and store, -70°C.

Isolation of RNA from Cultured Cells

Time Required:

- 1.5 Hr

Supplies:

- RNeasy Midi Kit – Qiagen Cat#75144 (50 isolations)

  <u>Buffers</u>
  RNase-free water
  Buffer RLT (Lysis Buffer)* contains guanidine isothiocyanate – use appropriate safety measures when handling
  Buffer RW1 (Wash Buffer)* contains guanidine isothiocyanate and alcohol – use appropriate safety measures when handling
  Buffer RPE (Wash Buffer)† supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (90-100%) as indicated on the bottle to obtain a working solution
  <u>Disposables</u>
  RNeasy midi columns in 15ml tubes
  Collection tubes for elution (15ml)
- 14.3 M β-mercaptoethanol (β-ME)
- Ethanol (96-100%)
- Ethanol (70% in water)
- RNase-Free DNase Set – Qiagen Cat#79254 (25 digestions), $67

- Sterile, RNase-free pipet tips
- Conventional rotor-stator homogenizer
- Centrifuge capable of attaining 3000-5000 x g equipped witwh a swing-out rotor and buckets to hold 15ml tubes
- Vortex mixer
- Disposable gloves

Set-up before beginning protocol:

- Label tubes:
  - 1set of Midi columns in 15ml tubes
  - 1set of elution tubes
  - 1set of (not supplied) microcentrifuge tubes
- Prepare DNase 1 stock solution before using the RNase-free DNase Set for the first time. Dissolve the solid DNase 1 (1500 Kunitz units) in 550µl RNase-free water provided. Take care that no DNase 1 is lost when opening the vial. Mix gently by inverting the tube. Do not vortex. For long-term storage of DNase 1, remove the stock solution from the glass vial, divide it into single-use aliquots, and store at -20^{o}C for up to 9 months. Do not refreeze the aliquots after thawing.
- Freshly prepare Buffer RLT by adding 10µl -βME/ml Buffer RLT (4 ml of Buffer RLT will be used per column)

All steps for the extraction of RNA are performed at room temperature.
All centrifugations are carried out using a swinging bucket rotor at 4000 x g (the maximum speed of 3500-5000 rpm corresponds to 3000-5000 x g for most rotors.)
During the procedure, work quickly.
Optional on-column DNase digestion step is included.

Protocol:

Hold pellets on ice. Frozen pellets will need to be thawed a little.

1. Loosen cell pellet thoroughly by flicking the tube. Add 4ml of Buffer RLT plus β-ME per column to lyse the cells (5 x 10^6 – 3 x 5 x 10^7 / column).
2. Homogenize cells using a rotor-stator homogenizer for at least 45 seconds at maximum speed until the sample is uniformly homogeneous.
3. Add an equal volume of 70% ethanol to the homogenized lysate, and mix by shaking vigorously. Do NOT centrifuge.
4. Apply 4 ml of the sample to an RNeasy midi-spin column sitting in a 15 ml (supplied) centrifuge tube. Close the tube lightly. Spin for 3 minutes. Discard the flow-through.
5. Repeat with the remaining lysate, if there was more than 4 ml. Re-use the tube in step 6.
6. Add 2 ml Buffer RWI to each column, close the centrifuge tube lightly and spin 5 minutes to wash the column. Prepare the DNase mix for the next step (20µl DNase 1 stock solution + 140µl Buffer RDD = 160µl / column. Mix only by gently inverting the tube). Discard the flow-through. Re-use tube in step 7.

7. Pipet 160µl DNase 1 mix directly onto the column, and place on the benchtop (20-30^{o}C) for 15 minutes.
8. Add 2 ml Buffer RW1 to the column, and place on the benchtop for 5 minutes. Then centrifuge for 5 min. Discard flow-through.
9. Add 2.5 ml of 1X Buffer RPE to the column, close lightly and spin 2 min. Discard flow-through.
10. Add another 2.5 ml of 1X Buffer RPE to the column, close tube lightly and spin 10 min. to dry the column membrane.
11. To elute, transfer RNeasy midi-spin column to a new 15 ml collection tube (supplied). Pipet 200µl of RNase-free water directly onto the column membrane. Close the tube lightly, let it stand for 1 minute and spin for 3 min.
12. Add another 150µl of RNase-free water and spin 5 min. To obtain a higher total RNA concentration, this 2nd elution step may be performed by using the 1st eluate. The yield might be 15-30% less than the yield obtained using a 2nd volume of RNase-free water, but the final concentration will be higher.
13. Mix and transfer the eluted RNA to a sterile 1.5 ml microcentrifuge tube.
14. Quantitate and store RNA at -70^{o}C.

For Agilent Commercial Arrays:
Aliquots of 5 µg between ~0.5 µg/µl (max of 1µg/µl) in RNase-free water, **and** one aliquot of 5µl at ~200ng/µl for Agilent BioAnalyzer.
RNA should be snap frozen and stored at -80^{o}C or over liquid nitrogen in a LN_2 freezer. Keep on ice when pulled out to use.

Check Quality of RNA on a gel

- Set up a 1.2% Formaldehyde Gel. Run 3µg of RNA per lane.
- For a mini-gel with 8 wells:
  0.3 g agarose
  18 ml RNase-free water
  Microwave to dissolve the agarose.
  Add 2.5 ml 10X FA Buffer
  4.45 ml formaldehyde
  0.075 µg EtBr/ml of gel
  Allow to cool to about 60^{o}C. Pour into the gel box with a comb.
- Add 1X FA Buffer to the gel box once the gel has set. Allow gel to equilibrate with buffer for about 30 min.
- Samples:

3 µg RNA	___ µl
water to 8 µl	___ µl
5X FA RNA Loading Buffer	2µl to each

- Denature samples at 60^{o}C for 10 minutes; sit on ice 2 - 5 minutes; briefly spin down; load. Run at 100 V for 0.8 - 0.9 hr.
- Photograph the gel on settings 8 and 2, orange filter, with Type 57 Polaroid film. 2 bands should be seen, 18 and 28 kD.