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RN1easy Total RNA Isolation and Cleanup Protocol with Optional DNase Treatment

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A. Required Reagents:

DEPC-treated water (Ambion)

QIAshredder (Qiagen)

RNeasy RNA Isolation Kit (Qiagen)

RNase-Free DNase Set (Qiagen) *optional

ß-Mercaptoethanol (ß-Me) (Sigma)

70% and absolute ethanol (Ultrapure) (American Bioanalytical)

B. Equipment and Supplies:

1.5 ml microcentrifuge tubes

Micropipettors

Aerosol-barrier tips

Microcentrifuge with 1.5 ml tube rotor

Vortex mixer

Powder-free gloves

I. RNA ISOLATION:

* Maximum amount of starting material- animal cells: $1x10^7$, animal tissue: 30 mg.

* Maximum binding capacity of RNeasy mini spin column is 100 µg of RNA.

* Buffer RLT may form precipitate upon storage. If necessary, warm to redissolve.

* Add 10 µl ß-ME per 1 ml of buffer RLT just before use.

* Buffer RPE is supplied as a concentrate. Before using first time add 4 volumes of absolute ethanol as indicated on the bottle to obtain working solution.

* Prepare DNase I stock solution: dissolve the lyophilized DNase I (1500 Kunitz units) in 550 ul of RNase-free water. Inject water into vial using a needle and syringe. Mix gently by inverting the vial. Do not vortex. Aliquots can be stored at –20C. Do not refreeze aliquots.

1. Harvest Cells:

A. <u>Cells grown in suspension</u>

Spin the appropriate number of cells (<1x10^7) for 5 min at 300 x g in a centrifuge tube. Discard supernatant, completely removing all media. Continue with Step 2.

* Incomplete removal of the supernatant will inhibit lysis and dilute the lysate which will affect the conditions for binding RNA to the RNeasy membrane.

B. Cells grown in monolayer

Completely aspirate supernatant and immediately continue with Step 2.

* Incomplete removal of the supernatant will inhibit lysis and dilute the lysate which will affect the conditions for binding RNA to the RNeasy membrane.

2. Lyse cells by addition of Buffer RLT. Ensure ß-ME is added to buffer RLT before use.

A. For pelleted cells

Loosen cell pellet by flicking the tube and add Buffer RLT (according to table below). Vortex or pipette to mix. No cell clumps should be visible before proceeding to Step 3.

Buffer RLT (µl)	Number of pelleted cells
350	Up to 5 x 10^6
600	5 x 10^6 to 1 x 10^7

B. For monolayer cells

Add Buffer RLT (according to table below) to monolayer cells. Collect cell lysate with a cell scraper. Transfer to a 1.5 ml tube. Vortex or pipette to mix. No cell clumps should be visible before proceeding to Step 3.

Buffer RLT (µl)	Dish diameter (cm)
350	<6
600	6 -10

3. Pipette lysate directly onto a QIAshredder column sitting in 2 ml collection tube. Centrifuge for 2 min at maximum speed to homogenize.

* Incomplete homogenization will lead to significantly reduced yields and can cause clogging of the RNeasy spin column.

4. Add 1 volume (usually either 350 µl or 600 µl) of 70% ethanol to the homogenized lysate in the 2 ml QIAshredder collection tube. Mix well by pipetting 2 to 3 times.

II. TOTAL RNA CLEAN-UP

5. Apply 700 µl of sample, including any precipitate, to an RNeasy mini spin column sitting in a 2 ml collection tube. Centrifuge for 15 sec at full speed. If the volume of the mixture exceeds 700 µl, successively load aliquots onto the RNeasy column and centrifuge as above. Reuse the collection tubes but discard flow-through after each step.

*If performing optional DNase treatment, follow steps D1 – D4 after performing this step.

6. Pipette 700 µl Buffer RW1 onto the RNeasy column. Incubate column for 5 min. Centrifuge at maximum speed. Discard flow through and collection tube.

7. Transfer RNeasy column into a new 2 ml collection tube. Pipette 500 µl Buffer RPE onto RNeasy column, wait for 5 minutes and centrifuge for 15 sec at 10,000 rpm to wash. Discard flow through and reuse the collection tube in Step 8.

8. Pipette 500 µl RPE buffer onto RNeasy column. Centrifuge for 2 min at maximum speed to dry the RNeasy membrane.

9. Place the RNeasy spin column in a new 2 ml collection tube. Discard the old collection tube with the filtrate.

10. Open the column cap and spin at full speed (maximum speed) for 5 minutes with the caps open.

> *It is important to dry the RNeasy membrane since residual ethanol may reduce the recovery of RNA and also may interfere with subsequent reactions.

11. Transfer RNeasy column into a new 1.5 ml collection tube. Let sit with lid open for 5 to10 min to completely evaporate ethanol.

12. Add 40 µl of DEPC-treated water directly on to the RNeasy membrane. Wait 5 min. Centrifuge for 1 minute at full speed to elute.

13. Elute a second time by pipetting the flow-through in the collection tube (~36 ul) back onto the Spin Column membrane. Place the spin column back into the collection tube and incubate at room temperature for two minutes. Spin at maximum speed for 1 minute.

14. Use spectrophotometric analysis to measure RNA yield. Dilute 1 µl of RNA with 39 µl of DEPC-treated water (1:40 dilution). Using a 10 µl microcuvette, take OD at 260 nm and 280 nm to determine sample concentration and purity. The A_{260}/A_{280} ratio should be above 1.6. Apply the convention that 1 OD at 260 equals 40 µg/ml RNA.

15. Run 0.5-1 µg of RNA on native 1 % agarose gel or 0.1-0.5 µg on an Agilent Bioanalyzer chip to assess the quality of RNA.

16. Important: It is extremely important to start microarray experiments with very good quality of RNA. The A_{260}/A_{280} ratio should be above 1.8. The gel electrophoresis pattern should reveal two major bands of 28 S and 18 S RNA and no smear from genomic DNA (See Figure 1 below).

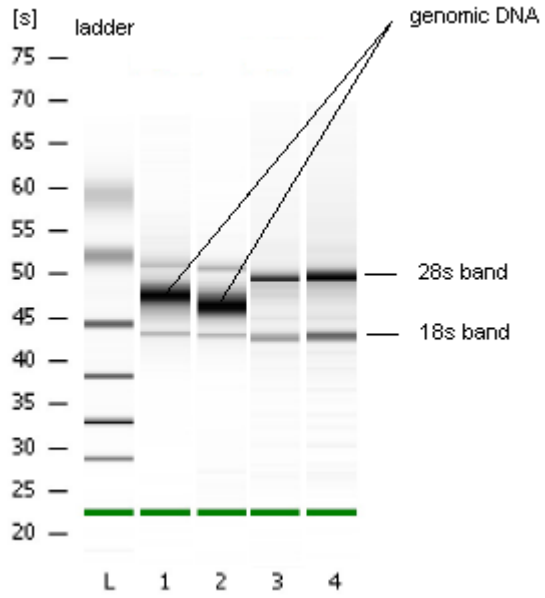

Figure 1: Bioanalyzer gel-image pattern of total RNA

On column DNase digestion

D1. Add 350 ul Buffer RW1 to column. Centrifuge for 15s at ≥10,000 rpm. Discard flow-through.

D2. Add 10 ul DNase I stock solution to 70 ul Buffer RDD. Mix by gently inverting the tube, and spin briefly. Do not vortex DNase.

D3. Add the DNase I incubation mix (80 ul) to column membrane. Incubate at room temperature for 15 min.

D4. Add 350 ul Buffer RW1 to column. Centrifuge for 15 s at ≥10,000 rpm. Discard the flow-through. Repeat RW1 wash (step-D4) one more time.

D5. Transfer RNeasy column into a new 2 ml collection tube. Pipette 500 µl Buffer RPE onto RNeasy column and centrifuge for 15 sec at 10,000 rpm to wash. Discard flow through and reuse the collection tube in Step 8.

D6. Repeat step D5 (RPE wash) two more times.

Continue with the step 9 above (column centrifugation for 5 minutes with caps open) .