General remarks

Efficient disruption and complete homogenization of the starting material ensures high yield and minimal degradation of RNA. Cultured cells may be disrupted by one passage through a QIAshredder column**,** but solid and fibrous tissues require rigorous disruption methods, such as grinding of the frozen tissue by mortar and pestle, douncing, Ultra Turrax or other means. Large frozen tissue pieces may be wrapped in sterile aluminum foil and mechanically disrupted into smaller pieces with a hammer.

Manipulations with TRIzol and phenol/chloroform should be carried out under a fume hood.

Wear laboratory gloves and safety goggles, particularly when working with TRIzol (phenol).

All laboratory material such as disposables and solutions which come in contact with RNA have to be RNase-free. Usually, autoclaving is sufficient. The working place should be clean and not be used for experiments with RNase.

Use of aerosol-barrier pipet tips is recommended.

Material

- Dry ice or liquid nitrogen (lN_2) for transport of biopsies from lN_2 tank or –80°C freezer to laboratory.
- Phosphate buffered saline PBS; RNase-free, autoclaved (137 mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4; pH 7.4)
- TRIzol (Invitrogen, cat. no. 15596-018)

  Chloroform

  2-propanol (isopropanol)

  Ethanol, abs., p.a.

  RNAse-free H_2O (e.g. autoclaved in the presence of 0.1% Diethylpyrocarbonat, DEPC)

  75% ethanol, p.a. (ethanol, abs., pa., diluted with DEPC-treated H_2O)
- QIAshredder columns (Qiagen, cat. no. 79654)

  RNeasy Midi Kit (Qiagen, cat. no. 75142 or 75144)

  RNeasy Mini Kit (Qiagen, cat. no. 74104)

  RNase-free DNase Set (Qiagen, cat. no. 79254)
- Dounce glass homogenizer, 2 ml; sterile and RNase-free

  Ultra Turrax T8 (hand held disperser); sterile and RNase-free
- mortar and pestle; sterile and RNase-free

  for cultured cells:

  Centrifuge with swing-out rotor, minimum speed 3000g, buckets and adapters for 12 ml centrifuge tubes; or centrifuge with fixed-angle rotor suitable for 12 ml centrifuge tubes.

  Sterile, disposable 12 ml centrifuge tubes.

  Set of variable pipettes: 10, 200, 1000 µl.

  Pipettes in the 1-10 ml range.

Methods

A. Procedure using TRIzol (an alternative using the RNeasy Midi Kit, Qiagen, is described below)

Cultured cells (prior to step 1: ~10-20 min)

After removing the medium by pipetting, adherent cells can be directly lysed in the culture dish by addition of 1 ml TRIzol/10 cm^2 and passing the cell lysate several times through a pipette.

Cells grown in suspension are pelleted and washed once with ice-cold PBS. The washed cell pellet is then resuspended in 1 ml of TRIzol per $2x10^6$ cells.

Tissue (prior to step 1: ~15 min)

Transfer frozen tissue to Dounce homogenizer containing 1ml TRIzol per 50 to 100 mg tissue. Disrupt tissue by douncing until the tissue is completely homogenized. Transfer to Eppi.

Alternative homogenization with Ultra Turrax T8:

Transfer frozen tissue to a 12 ml centrifuge tube containing 1ml TRIzol per 50 to 100 mg tissue. Homogenize with Ultra-Turrax T8, initially with low speed, then for 20-40 sec with highest speed until sample is completely homogenized. Transfer to Eppi.

Alternative homogenization with mortar and pestle:

Transfer frozen tissue to a mortar containing a small amount of liquid nitrogen (lN_2). Grind the tissue to powder in the permanent presence of lN_2. Transfer the still frozen powder to Eppi containing 1ml TRIzol per 50 to 100 mg tissue.

Cultured cells and tissue (Steps 1-7: ~45 min)

Optional: To optimize homogenization, the lysate may be loaded into a QIAshredder column sitting in a 2 ml collection tube and centrifuged for 2 min at room temperature (RT). Transfer lysate to new Eppi.

All centrifugations are performed in a microfuge with 12,000-14,000 rpm.

1. Add 0.2 ml chloroform per 1 ml lysate, vortex thoroughly and incubate for 2 min at RT.

   Centrifuge for 5 min at RT
2. Transfer upper aqueous phase to new Eppi (carefully avoid contamination with interphase and lower organic phase)
3. Add 0.5 ml 2-propanol, mix and leave at RT for 10 min.
4. Centrifuge for 10 min at RT
5. Wash pellet twice with 0.5 ml 75% ethanol
6. Remove ethanol completely and air dry (or store under 75% ethanol at –80°C until further use)

7. Completely dissolve RNA in 100 µl RNase-free H_2O; pipette several times up and down if necessary.

The RNA clean-up (DNase I treatment) may be performed by one of the two procedures described below.

B. RNA isolation procedure with the RNeasy Midi Kit (Qiagen)

Important remarks before starting: β -Mercaptoethanol (β -ME) must be added to Buffer RLT before use. β -ME is toxic;dispense in a fume hood and wear appropriate protective clothing. Add 10 µl β –ME per 1 ml Buffer RLT. Buffer RLT is stable for 1 month after addition of β -ME.

- Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%), as indicated on the bottle, to obtain a working solution.
- Generally, DNase digestion is not required since the RNeasy silica-membrane technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be necessary for certain RNA applications that are sensitive to very small amounts of DNA (e.g., TaqMan RT-PCR analysis with a low-abundant target). Two alternative RNA clean-up protocols are described above.
- Buffer RLT may form a precipitate upon storage. If necessary, redissolve by warming, and then place at room temperature.
- Buffer RLT and Buffer RW1 contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach. Guanidine is an irritant. Take appropriate safety measures and wear gloves when handling.
- All steps of the RNeasy protocol should be performed at room temperature. During the procedure, work quickly.
- All centrifugation steps are performed at 20–25°C in a standard laboratory centrifuge with a swinging bucket rotor capable of ≥3000 x *g* (see page 24). Ensure that the centrifuge does not cool below 20°C.
- The RNeasy Midi Kit is suitable for $5x10^6$-$1x10^8$ cells or 20-250 mg of tissue.

Procedure

Cultured cells (prior to step 1b: ~10-20 min)

Cells are removed from the culture dish, pelleted in a and washed once with ice-cold PBS. The washed cell pellet is then resuspended in 2 ml RLT buffer.

Tissue (prior tp step 1c: ~15 min)

Transfer frozen tissue to Dounce homogenizer containing 4 ml RLT buffer. Disrupt tissue by douncing until the tissue is completely homogenized. Transfer to 12 ml centrifuge tube.

Alternative homogenization with Ultra Turrax T8:

Transfer frozen tissue to a 12 ml centrifuge tube containing 4 ml RLT buffer. Homogenize with Ultra-Turrax T8, initially with low speed, then for 20-40 sec with highest speed until sample is completely homogenized.

Alternative homogenization with mortar and pestle:

Transfer frozen tissue to a mortar containing a small amount of liquid nitrogen (lN_2). Grind the tissue to powder in the permanent presence of lN_2. Transfer the still frozen powder to a

12 ml centrifuge tube containing 4 ml RLT buffer.

Both cells and tissue (Steps 1b-7b: ~60 min)

1b. Suspensions are homogenized by passing 5-10x through a needle (connected to a 10 ml syringe) with a wide, then with a narrow bore (e.g. 21 G 1 ½; 0,8x40 no 2; then 23 G 1 1/4; 0,6x30 no14). In case of clogging, disconnect the needle from the syringe and reconnect; then try to push and pull again carefully, avoid spilling.

2b. Centrifuge 10 min. at 4,000 rpm and transfer supernatant to new tube.

3b. Add 1 vol. (2-4 ml) 70% ethanol and mix thoroughly; there should be no pellet left.

4b. Apply homogenate (max. 4 ml) to RNeasy Midi column sitting in a 12 ml tube and centrifuge 5 min. at 4,000 rpm. If necessary, apply the rest to the column and repeat the centrifugation. Discard the flow through.

5b. Wash the column by the applying 4 ml RW1 buffer, close the tube and centrifuge 5 min. at 4,000 rpm. Discard the flow through.

6b. Apply 2.5 ml RPE buffer to the column, close the tube and centrifuge 5 min. at 4,000 rpm. Discard the flow through. Transfer the column to a new tube.

7b. Elute RNA from the column by applying 100 µl RNase-free H_2O, let stand for 3 min, then centrifuge 3 min. at 4,000 rpm. Repeat the elution with further 100 µl RNase-free H_2O.

The RNA clean-up (DNase I treatment) may be performed by one of the two procedures described below.

C. RNA clean-up using Qiagen RNeasy Mini Kit and RNase-free DNase Set

An alternative RNA clean-up using DNase I and phenol/cloroform is described below this paragraph.

Prepare DNase I stock solution when using the RNase-Free DNase Set for the first time. Dissolve the solid DNase I (1500 Kunitz units) in 550 µl of the RNase-free water provided. Take care that no DNase I is lost when opening the vial. Mix gently by inverting the tube. Do not vortex.

For long-term storage of DNase I, remove the stock solution from the glass vial, divide it into single-use aliquots, and store at –20°C for up to 9 months. Thawed aliquots can be stored at 2–8°C for up to 6 weeks. Do not refreeze the aliquots after thawing.

Optional: Determine concentration of RNA by OD measurement if yield is expected to exceed 100 µg (maximum capacity of RNeasy Mini).

Procedure (Steps 8-17: ~50 min)

8. To 100 µl RNA (maximum 100 µg) add 350 µl RLT (before use, RLT has to be supplemented with 10 µl ß-Mercaptoethanol per ml) and 250 µl ethanol, mix thoroughly by pipetting.
9. Load the sample (700 µl) to an RNeasy Mini spin column sitting in a collection tube and centrifuge for 15 sec at 10,000 rpm, discard flow-through.
10. Pipet 350 µl RW1 buffer into the spin column and centrifuge for 15 sec at 10,000 rpm, discard flow-through.
11. Add 70 µl of buffer RDD to a 10 µl aliqot of DNase I, mix gently by tapping. Pipet DNaseI incubation mix (80 µl) directly onto the spin column membrane, incubate at RT for 15 min.
12. Pipet 350 µl RW1 buffer into the spin column and centrifuge for 15 sec at 10,000 rpm, transfer spin column to a fresh collection tube.
13. Pipet 500 µl Buffer RPE into the spin column and centrifuge for 15 seconds at 10,000 rpm at RT, discard flow-through.
14. Pipet another 500 µl Buffer RPE into the spin column and centrifuge for 2 min at 12,000 rpm to dry the column membrane.
15. Transfer the spin column into a new Eppi and pipet 30 µl of RNase-free H_2O directly onto the membrane, leave at RT for 1 min..
16. Elute by centrifugation for 15 seconds with 10,000 rpm at RT.
17. Repeat elution with another 30 µl of RNase-free H_2O and join the eluates. Store the RNA at –80°C.

Determine RNA concentration and purity as recommended in the chapter "**RNA quality and quantity control".**

D. RNA clean-up with RNase-free Dnase I and phenol/chloroform extraction

Material:

RNase inhibitor (e.g., Fermentas, 40 u/µl, cat. no. EO0311)

RNase-free DNase I (e.g. Amersham, 10 u/µl, cat. no.27-0514-01)

Phenol/cloroform/isoamyl alcohol (25:24:1; e.g., Roth, cat. no. A156.1)

Polyacryl-Carrier (Molecular Research Center Inc., cat. no. PC 152; distributed by Fermentas)

3 M Sodium Acetate pH 5.2; RNase-free, autoclaved

Ethanol, abs., p.a.

RNAse-free H_2O (e.g. autoclaved in the presence of 0.1% Diethylpyrocarbonat, DEPC)

75% ethanol, p.a. (ethanol, abs., pa., diluted with DEPC-treated H_2O)

Procedure (Steps 8b-14b: ~60 min)

8b. To 100 µl RNA solution, add 1 µl RNase inhibitor and 1 µl RNase-free DNase I. Incubate at 37°C for 30 min.

9b. Add 100 µl phenol/chloroform mix and vortex thoroughly.

10b. Centrifuge with vmax for 5 min at RT.

11b. Transfer the aqueous upper phase to a new Eppi and add 10 µl (1/10 vol.) 3M Sodium acetate, pH5.2, 1 µl Polyacryl-Carrier and 250 µl (2.5 vol.) ethanol, abs.

12b. Mix and centrifuge with vmax for 5 min at RT.

13b. Remove the supernatant completely, wash RNA precipitate with 100 µl 75% ethanol and centrifuge for 1 min.

14b. Remove the supernatant completely, air dry the RNA precipitate and disolve in 100 µl RNAse-free H_2O. Store the RNA at –80°C.

Determine RNA concentration and purity as recommended in the chapter "**RNA quality and quantity control".**

Version	Tracking of changes	Name	Date