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.

General remarks

The procedure described below is particularly suited for biopsies from heart/skeletal muscle with a high proportion of fibrous tissue.

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.

Because of the potential contamination of the biopsy specimen with infectious agents, particular care should be taken when working with disposable hypodermic needles (see below).

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

Material

- Dry ice or liquid nitrogen (lN_2) for transport of biopsies from lN_2 tank or –80°C freezer to laboratory.
- Hypodermic needles (e.g. 21 G 1 ½; 0,8x40 no 2; then 23 G 1 1/4; 0,6x30 no14): Alternatively: Mixer mill (e.g., Qiagen, type MM300, cat. no. 85120, Adapter sets, cat. no. 69998, sterile steel balls, 3 mm, cat. no. 69997).
- TRIzol (Invitrogen, cat. no. 15596-018)
- Chloroform
- Polyacryl-Carrier (Molecular Research Center Inc., order no. PC 152; www.mrcgene.com; distributed by Fermentas)
- 2-propanol (isopropanol)
- 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)
- Qiagen RNeasy Micro Kit (cat. no. 74004)
- Set of variable pipettes: 10, 200, 1000 µl.

Methods

A. Procedure using TRIzol

Procedure (Steps 1-10: ~75 min, steps 1b-10: ~60 min)

1. Without thawing, transfer the frozen biopsy to a microfuge tube (Eppi) containing 100 µl TRIzol. If the biopsy is stored in RNAlater: spin down briefly, remove the RNAlater solution and add 100 µl TRIzol.
2. Submerged in TRIzol, tear the biopsy into pieces with a disposable hypodermic needle, connected to a sterile 1 ml syringe. Add further 400 µl TRIzol and shear the biopsy by frequent passing through the needle, initially about 20x through one with wide, then about 20x through one 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.

An easier and less harmful alternative for the initial homogenization of the biopsies is the use of a special mixer mill (e.g. Qiagen, type MM300).

1b. Prepare in advance a 2 ml Eppi containing a sterile steel ball and 500 µl TRIzol.

2b. Transfer the biopsy to the Eppi, close thoroughly and homogenize with mixer mill for 60 sec at a frequency of 30 Hz.

3. Add 100 µl chloroform, homogenize thoroughly by vortexing and leave at room temperature for 2 min.
4. Centrifuge at RT for 3 min.
5. Transfer the aqueous upper phase to a new Eppi, avoid co-transfer of the inter- and lower organic phase.
6. Add 2 µl Polyacryl-Carrier and 250 µl 2-propanol, mix and place on ice for 10 min.
7. Centrifuge at 4 C for 20 min.
8. Remove the supernatant completely and wash the pellet with 200 µl 75% ethanol.
9. Centrifuge briefly and remove the supernatant completely.
10. Air dry the pellet for 3-5 min and dissolve in 20 µl RNase-free H_2O; pipette several times up and down if necessary. Store the RNA at –80°C.

Check quality and quantity by gel electrophoresis as recommended in the chapter "**RNA quality and quantity control".** *If complete removal of DNA is necessary for downstream applications, directly continue with DNase I treatment as described below.*

B. RNA clean-up using Qiagen RNeasy Micro Kit

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

Important notes and Material.

- Buffer RLT may form a precipitate upon storage. If necessary, redissolve by warming, and then place at room temperature.
- Buffer RLT contains a guanidine salt and is therefore not compatible with disinfecting reagents containing bleach.
- All steps of the protocol, including centrifugation, should be performed at room temperature.
- 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.
- Recommended: For on-column DNase digestion, prepare DNase I stock solution before using the RNase-free DNase 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 reconstituted 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: Adding β -Mercaptoethanol (-ME) to Buffer RLT may be helpful when cleaning up crude preps of RNA (e.g., after salting-out methods) or samples that contain large amounts of RNases.

  Add 10 µl β -ME per 1 ml Buffer RLT. Dispense in a fume hood and wear appropriate protective clothing. Buffer RLT is stable at room temperature for 1 month after addition of β -ME.
- 80% ethanol, p.a. (ethanol, abs., pa., diluted with DEPC-treated H2O)

Procedure (Steps 11-21: ~50 min)

11. Adjust sample to a volume of 100 µl with RNase-free water. Add 350 µl Buffer RLT, and mix thoroughly.
12. Add 250 µl of ethanol, abs., p.a., to the diluted RNA, and mix thoroughly by pipetting. Do not centrifuge. Continue immediately with step 3.
13. Apply the sample to an RNeasy MinElute Spin Column in a 2 ml collection tube. Close the tube gently, and centrifuge for 15 s at ≥ 8000 x g (10,000 rpm). Discard the flow-through.
14. Pipet 350 µl Buffer RW1 into the RNeasy MinElute Spin Column, and centrifuge for 15 s at ≥ 8000 x g (10,000 rpm) to wash. Discard the flow-through. Reuse the collection tube in step 6.
15. Add 10 µl DNase I stock solution (see above) to 70 µl Buffer RDD. Mix by gently inverting the tube.
16. Pipet the DNase I incubation mix (80 µl) directly onto the RNeasy MinElute silica-gel membrane, and place on the benchtop at room temperature for 15 min.

    Note: Make sure to pipet the DNase I incubation mix directly onto the Rneasy MinElute silica-gel membrane. DNase digestion will be incomplete if part of the mix sticks to the walls or the O-ring of the RNeasy MinElute Spin Column.

17. Pipet 350 µl Buffer RW1 into the RNeasy MinElute Spin Column, and centrifuge for 15 s at ≥ 8000 x g. Discard the flow-through.
18. Transfer the spin column into a new 2 ml collection tube. Pipet 500 µl Buffer RPE onto the spin column. Close the tube gently, and centrifuge for 15 s at ≥ 8000 x g (10,000 rpm) to wash the column. Discard the flow-through. Reuse the collection tube in step 9.

19. Add 500 µl of 80% ethanol to the RNeasy MinElute Spin Column. Close the tube gently, and centrifuge for 2 min at ≥ 8000 x g (10,000 rpm) to dry the silica-gel membrane. Discard the flow-through and collection tube.

    Note: Following the centrifugation, remove the RNeasy MinElute Spin Column from the collection tube carefully so the column does not come into contact with the flow-through as this will result in carryover of ethanol.

20. Transfer the RNeasy MinElute Spin Column into a new 2 ml collection tube.

    Open the cap of the spin column, and centrifuge in a microcentrifuge at full speed for 5 min. Discard the flow-through and collection tube.

    To avoid damage to the caps, place the columns into the centrifuge with at least one empty position between each column. Place the caps so that they point in the opposite direction to the rotation of the rotor (i.e., if the rotor rotates in a clockwise direction, orient the caps in a counter-clockwise direction).

21. To elute, transfer the spin column to a new 1.5 ml Eppi. Pipet 15 µl RNase-free water directly onto the center of the silica-gel membrane. Close the tube gently, and centrifuge for 1 min at maximum speed to elute. Store the RNA at –80°C.

Check quality and quantity recommended in the chapter **"RNA quality and quantity control"**.

C. 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 11b-17b: ~60 min)

11b. Add 1 µl RNase inhibitor, 1 µl RNase-free DNase I and RNAse-free H_2O to a final volume of 30 µl. Incubate at 37°C for 30 min.

12b. Add 30 µl phenol/chloroform mix and vortex thoroughly.

13b. Centrifuge with v_{max} for 5 min at RT.

14b. Transfer the aqueous upper phase to a new Eppi and add 3 µl (1/10 vol.) 3M Sodium acetate, pH5.2, 1 µl Polyacryl-Carrier and 85 µl (2.5 vol.) ethanol, abs.

15b. Mix and centrifuge with v_{max} for 5 min at RT.

16b. Remove the supernatant completely, wash RNA precipitate with 100 µl 75% ethanol and centrifuge for 1 min.

17b. Remove the supernatant completely, air dry the RNA precipitate and disolve in 20 µl RNAse-free H_2O. Store the RNA at –80°C.

Check quality and quantity as recommended in the chapter "**RNA quality and quantity control".**

Version	Tracking of changes	Name	Date