PRODUCT: RNAzol® RT
Cat. No: RN 190

Store at room temperature. Keep tightly closed.

PROTOCOL FOR TOTAL RNA ISOLATION.
Reagents required but not supplied: ethanol, RNase-free water. Isopropanol is required for the isolation of small RNA. We recommend the use of disposable polypropylene tubes (see MRC catalog). Tubes from other sources should be tested to ensure integrity during centrifugation at 12,000 g with RNAzol®RT.
Abbreviated Protocol

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1. Homogenization - 1 ml RNAzol®RT + up to 100 mg tissue or 10^7 cells.
2. DNA/protein precipitation - homogenate + 0.4 ml water, wait 5 - 15 min, 12,000 g x 15 min.
3. RNA precipitation - 1 ml supernatant + 0.4 ml of 75% ethanol, wait 5 - 10 min, 12,000 g x 8 min.
4. RNA washes - 0.4 ml 75% ethanol, 4,000 g x 1 - 3 min; wash twice.
5. RNA solubilization - water or Formazol®.

All steps are performed at room temperature. Centrifugation can be performed at 4 - 28 C.

1. Homogenization.
A. TISSUES. Homogenize tissue samples in a Polytron-type homogenizer or glass-teflon homogenizer using up to 100 mg of tissue per 1 ml of RNAzol®RT.
For clarity of presentation, this protocol describes isolation of RNA using 1 ml of RNAzol®RT. The most economical isolation is to process samples using 0.6 - 0.8 ml of the reagent in 1.5 ml microcentrifuge tubes. For example, use a 0.88 ml aliquot(s) of the homogenate (0.88 ml = 0.8 ml reagent + 80 mg tissue) and freeze the rest of the homogenate for later use. The homogenate can be stored at -20 or -70 C for at least one year.
B. CELLS. Cells grown in monolayer should be lysed in a culture dish by the addition of RNAzol®RT. Remove culture medium and add at least 1 ml of the reagent per 3.5 cm culture dish (10 cm^2). Pass the lysate through a pipette several times to ensure lysis.
Cells grown in suspension should be sedimented first and then lysed by the addition of RNAzol®RT. Add at least 1 ml of RNAzol®RT per 10^7 cells and lyse cells by repeated pipetting.
Washing cells before the addition of RNAzol®RT is not recommended as it may contribute to RNA degradation.
For cells grown in monolayer, use the amount of RNAzol®RT based on the area of the culture dish and not on cell number. The use of an insufficient amount of RNAzol®RT will result in DNA contamination of the isolated RNA.
C. LIQUID SAMPLES. Homogenize/lyse liquid samples using 1 ml of RNAzol®RT per up to 0.4 ml of a liquid sample. For processing a small volume sample, mix the sample with 1 ml of RNAzol®RT and supplement the mixture with water to approach the sample + water volume of 0.4 ml.
D. SAMPLES WITH HIGH FAT CONTENT. Centrifuge homogenate of high-fat samples at 12,000 g for 5 min. After centrifugation, fat collects at the top of the tube. Pierce the fat layer with a pipette or syringe and transfer the supernatant into a new tube. Mix the transferred supernatant with 0.4 volume of water and process samples as described below in Step 2.
For better handling of samples, solidify the top fat layer by performing centrifugation at 4 - 10 °C.

2. DNA, protein and polysaccharide precipitation. Add to the homogenate/lysate 0.4 ml of water per 1 ml of RNAzol®RT used for homogenization. Shake the resulting mixture vigorously for 15 seconds and store it for 5 - 15 min. Samples with 100 mg tissue/ml RNAzol®RT require a 15 min storage at room temperature. Next, centrifuge samples at 12,000 g for 15 min. Following centrifugation, DNA, proteins and most polysaccharides form a semisolid pellet at the bottom of the tube. The RNA remains soluble in the supernatant.
Distilled water has sufficient purity to be used at this step.
Centrifugation at this and other steps of the protocol can be performed at 4 - 28 °C.
The DNA-protein pellet in a sample with 50 mg tissue/ml reagent constitutes about 5% of the total volume of the homogenate-water mixture (for 80 mg/ml it is about 8%).

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3. RNA precipitation. Transfer 1 ml of the supernatant (75% of total supernatant volume) to a fresh tube, leaving a layer of the supernatant above the DNA/protein pellet. Precipitate RNA by mixing the transferred 1 ml of supernatant with 0.4 ml of 75% ethanol (v/v). Store samples for 5 - 10 min and centrifuge them at 12,000 g for 5 - 8 min. RNA precipitate forms a white pellet at the bottom of a tube. Transfer the supernatant to a new tube and store it at 4 °C for isolation of small RNA (Step 6).
It is safe to collect up to 85% of the supernatant.
The 0.4 volume of 75% ethanol effectively precipitates RNA molecules longer than 200 bases.
4. RNA washes. Wash twice the RNA pellet by mixing it with 75% ethanol (v/v) followed by centrifugation at 4,000 - 8,000 g for 1 – 3 min. For samples in 1.5 ml tubes, use 0.4 ml of 75% ethanol. For samples in larger tubes use 0.5 ml of 75% ethanol per 1 ml of the supernatant used for precipitation. Remove the 75% ethanol wash using a micropipette.
Firm pellets can be washed by decanting. After the second decanting, briefly centrifuge the samples and remove residual ethanol.
5. RNA solubilization. Dissolve the RNA pellet, without drying, in water or Formazol® (MRC, FO 121) to approach the RNA concentration of 1 - 2 g/ml. For solubilization in water, hydrate the RNA pellet at room temperature for 5 min followed by vortexing and/or repetitive pipetting. Tubes and water used for the RNA solubilization should be RNase-free. Solubilization in Formazol requires incubation of RNA for 10 - 20 min followed by repetitive pipetting.
Drying the RNA pellet is not recommended as this greatly decreases its solubility.
Solubilization of RNA can be facilitated by incubation at 55 °C for 5 - 15 min.

The isolated RNA consists of RNA molecules >200 bases. This RNA fraction constitutes 80 - 85% of cellular RNA and contains ribosomal RNA and mRNA. The remaining small RNA fraction can be isolated as described below in Step 6. Expected yields: A) tissues (g RNA/mg tissue): liver, spleen, 4 - 6 g; kidney, 3 - 4 g; skeletal muscle, brain, lung 0.5 - 1.5 g; placenta, 1 - 3 g: B) cells (g RNA/10^6 cells): epithelial cells, 5 - 10 g; fibroblasts, 4 - 6 g.
The isolated RNA has a 260/280 ratio of 1.7 to 2.0 and a 260/230 ratio 2 to 3. The RIN value of a tissue removed from animal (or frozen in liquid nitrogen and stored at -70) and immediately processed is >9.
For accurate OD measurement, use water with a slightly alkaline pH (4). 1 mM NaOH or a buffer with pH >8 (MRC Phosphate Buffer for Spectrophotometry, SP 130) can be used for this purpose. Typically, distilled water has an acidic pH.

6. Precipitation of small RNA. Precipitate small RNA from the supernatant obtained after precipitation of RNA in Step 3 by mixing it with 0.8 volume of isopropanol. Store the samples for 30 min at 4 C and sediment the precipitated RNA at 12,000 g for 15 min at 4 - 28 C. Wash the precipitated RNA twice by mixing it with 70% isopropanol (v/v) followed by centrifugation at 12,000 g for 3 min. After the last wash, remove the residual alcohol with a micropipette and dissolve RNA, without drying, in water or Formazol.
The isolated small RNA contains small ribosomal RNA, tRNA, siRNA and miRNA ranging in size from 200 to10 bases.
Samples dissolved in Formazol should be diluted at least 5 fold with buffer before application on Small RNA Chip (Agilent Technology) and at least 50 fold for use in RT-PCR.

Abbreviated Protocol for small RNA isolation

Small RNA precipitation - 75% ethanol supernatant + 0.8 volume isopropanol, 30 min at 4 C, 12,000 g x 15 min.
Wash - 70% isopropanol, 12,000 g x 3 min, twice.
Solubilization - water or Formazol.

I. NOTES

1. Fast removal from an animal and effective dispersion of tissue by homogenization is critical for the integrity and yield of RNA. The most effective method is homogenization for 2 - 3 min in a Polytron-type homogenizer set at a high-speed. Brain samples require the use of a glass-Teflon homogenizer because of excessive foaming.
2. To determine tissue weight used for the homogenization, place 1 - 5 ml of the reagent in a tube on a balance and tare it. Drop fresh or frozen tissue into the reagent, record the tissue weight and immediately homogenize. After dispersing the tissue, supplement the homogenate with the reagent to approach, for example, 80 mg tissue per ml of the reagent.
3. The RNA isolation can be interrupted and samples can be stored as indicated below:
- The sample homogenate, before addition of water (Step 1), can be stored overnight at 4 C or for at least one year at -20 C. To thaw the samples, incubate them at 37 - 40 C for 5 min.
- The RNA precipitate can be stored in 75% ethanol overnight at room temperature (Step 4, RNA second wash), for at least one week at 4 C, or at least one year at -20 C.

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II. RNA ISOLATION TROUBLESHOOTING GUIDE.

Low yield. a) incomplete homogenization or lysis of samples; b) incomplete solubilization of the final RNA pellet.

260/280 ratio<1.6. a) too small volume of the reagent was used for homogenization; b) acidic water was used for the OD measurement; c) incomplete solubilization of the RNA pellet; d) proteoglycan or polysaccharide contamination.

RNA degradation. a) tissues were not immediately processed or frozen in liquid nitrogen after removal from an animal; b) samples used for the isolation were stored at -20 C instead of at -70 C; c) cells were dispersed by trypsin digestion; d) aqueous solutions or tubes used for solubilization of RNA were not RNase-free.

DNA contamination. a) too much tissue or cells were used for the volume of the reagent used for homogenization; b) samples used for the isolation contained organic solvents, strong buffers, salt or alkaline solution.

The following modifications of the DNA precipitation (Step 2) further reduce DNA contamination:

- After addition of water (Step 2), extend the DNA precipitation time by storing samples for 15 min.
- Sediment the precipitated DNA at 16,000 g.

Fat, proteoglycan and polysaccharide contamination. Use additional spin of the homogenate (step 1D) and/or the phase separation described in the Section IV of the protocol.

III. MODIFICATION OF THE BASIC PROTOCOL. Two-step isolation employing phase separation.

A phase separation step can be incorporated into the basic protocol for total RNA isolation. This additional step can be beneficial for samples with a high content of extracellular material.

Phase separation. Transfer 1 ml of the supernatant obtained after the DNA/protein precipitation (Step 2) into a new tube and add 50 l (5% of the transferred supernatant volume) of 4-bromoanisole (MRC, BN 191). Shake the resulting mixture for 15 sec and centrifuge it at 12,000 g for 10 min at 4 - 25 C.

Nontoxic 4-bromoanisole can be substituted with bromochloropropane (MRC, BP 151) or chloroform.

RNA precipitation. Transfer the top aqueous phase into a new tube and mix it with 0.8 volume of isopropanol. Store the mixture for 10 min and centrifuge at 12,000 g for 8 min. Precipitated RNA forms a white pellet at the bottom of a tube. Wash the RNA pellet with 75% ethanol and solubilize it as described in the basic protocol (Steps 4 and 5).

The addition of 0.8 volume of isopropanol precipitates total RNA including large and small RNA molecules. Alternatively, large RNA can be selectively precipitated with 0.4 volume of 75% ethanol (as in Step 3 of the basic protocol). Then, the small RNA fraction can be isolated as described in Step 6.

IV. RECOVERY OF DNA.

For use in PCR, DNA can be recovered from the pellet obtained after centrifugation of the homogenate/lysate (Step 2), either by using either DNAzol® or DNAzol®Direct.

DNAzol®. Remove remaining supernatant without disturbing the pellet. Vigorously mix the pellet with 8 - 10 volumes of DNAzol® (MRC, DN 127) and perform DNA isolation as described in the DNAzol®brochure.

DNAzol®Direct. Mix the resulting pellet with DNAzol®Direct. Take an aliquot of the resulting mix, dilute it with water and use it for PCR. Heating the DNAzol®Direct - pellet mixture for 10 min at 60 C improves DNA recovery.

V. REFERENCES.

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2. Chomczynski P, and N. Sacchi (1987) Single step method of RNA isolation by acid guanidinium thiocyanate-phenol- chloroform extraction. Anal. Biochem. 162, 156-159.
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The RNAzol®RT method can be cited by referring to: RNAzol®RT Brochure, 2009, Molecular Research Center, Inc. Cincinnati, OH.

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