Protocol

Purification of Total RNA from Mammalian Cells and Tissues

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This protocol describes the use of a monophasic lysis reagent to isolate total RNA from mammalian cells (grown as either a monolayer or in suspension) or tissues. An alternative protocol for the isolation of RNA from a small quantity of cells (10^2 – 10^4) or tissue (1–10 mg) is also included. In the latter case, glycogen is added to the monophasic lysis reagent and is used as a carrier to increase the recovery of RNA.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

Reagents

Cell samples grown in suspension or monolayer, or tissue samples

Chloroform or bromochloropropane

Bromochloropropane is a preferable alternative to chloroform because it is less toxic and less volatile. Moreover, bromochloropropane gives better phase separation, which results in improved removal of genomic DNA from total RNA preparations and, therefore, better purification of RNA.

Ethanol (75%) diluted in diethylpyrocarbonate (DEPC)-treated water

Glycogen (20 mg/mL stock) (optional; see Step 6)

Glycogen, RNase-free (e.g., from Life Technologies) (for the alternative protocol only; see Steps 12–15)

Isopropanol

Monophasic lysis reagent (commercially available [e.g., TRIzol] or made from scratch [Chomczynski and Sacchi 1987; Weber et al. 1998])

Monophasic lysis reagents contain phenol, which is poisonous, and guanidine thiocyanate, which is an irritant. When working with monophasic lysis reagent, use gloves and eye protection (shield, safety goggles). Avoid contact with skin or clothing. Use in a chemical fume hood; avoid breathing vapor.

Phosphate-buffered saline (PBS)

Prepare 1× solution.

RNase-free water

To prepare RNase-free water, draw water into RNase-free glass bottles. Add DEPC to 0.01% (v/v). Let stand overnight and autoclave.

Equipment

Glass-Teflon or power homogenizer (e.g., Polytron, or Tekmar's TISSUMIZER or equivalent)

Needles, 26-gauge (for the alternative protocol only; see Steps 12–15)

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METHOD

*This protocol has been adapted from Life Technologies/Invitrogen's protocol for using TRIzol reagent. Unless otherwise stated, the procedure is performed at room temperature (15°C–30°C), and reagents are at 15°C–30°C. Normal precautions to avoid RNase contamination should be taken (see Introduction: **How to Win the Battle with RNase** [Green and Sambrook 2019]).*

Homogenization of Sample

The sample volume should not exceed 10% of the volume of monophasic lysis reagent used for homogenization.

1. Prepare and homogenize cell monolayers, suspension cultures, or tissue samples as described below.

For Cells Grown in Monolayers

- i. Remove the medium and rinse the cells once with 1–2 mL of ice-cold PBS.
- ii. Remove PBS and lyse the cells directly in the culture dish by adding 1 mL of monophasic lysis reagent per 100-mm culture dish and scraping with a cell scraper.
- iii. Pass the cell lysate through a pipette several times. Vortex thoroughly.

The amount of monophasic lysis reagent added is based on the area of the culture dish (1 mL per 10 cm²) and not on the number of cells present. An insufficient amount of monophasic lysis reagent may result in DNA contamination of the isolated RNA.

- iv. Proceed with Step 2.

Samples can be stored for at least 1 mo at –60°C to –70°C.

For Cells Grown in Suspension

- i. Harvest the cells by centrifugation at 200–1900g (1000–3000 rpm in a Sorvall H1000 rotor) for 5–10 min at room temperature in a benchtop centrifuge.
- ii. Remove medium and resuspend the cells in ice-cold PBS. Pellet cells by centrifugation at 200–1900g for 5 min.
- iii. Add 1 mL of monophasic lysis reagent per 5×10^6 to 10×10^6 cells, and lyse the cells by repetitive pipetting or by passing the cells through a syringe and needle.
- iv. Proceed with Step 2.

Samples can be stored for at least 1 mo at –60°C to –70°C.

For Tissues

- i. Homogenize the tissue sample in 1 mL of monophasic lysis reagent per 50–100 mg of tissue using a glass–Teflon or power homogenizer.

For samples with high content of proteins, fat, polysaccharides, or extracellular material, such as muscle and fat tissue, you may perform the following optional steps. Otherwise proceed to Step 2.

Samples can be stored for at least 1 mo at –60°C to –70°C.

- ii. Following homogenization, centrifuge the sample at 12,000g for 10 min at 2°C–8°C to remove insoluble material from the homogenate.

The resulting pellet contains extracellular membranes, polysaccharides, and high-molecular-weight DNA, whereas the supernatant contains RNA.

- iii. In samples from fat tissue, remove the fat that collects as a top layer.
- iv. Transfer the cleared homogenate solution to a fresh tube.
- v. Proceed with Step 2.

Samples can be stored for at least 1 mo at –60°C to –70°C.

Separation of Phases

2. Incubate the homogenized samples for 5 min at room temperature to permit complete dissociation of nucleoprotein complexes.
3. Add 0.2 mL of chloroform per milliliter of monophasic lysis reagent used for the initial homogenization in Step 1. Cap tubes securely. Shake tubes vigorously by hand for 15 sec, and incubate them for 2–3 min at room temperature.

Do not vortex the samples because it will lead to DNA contamination.

4. Centrifuge the samples at no more than 12,000g for 15 min at 2°C–8°C. The mixture separates into a lower phenol:chloroform phase, an interface, and an upper aqueous phase. RNA remains exclusively in the upper aqueous phase.

Table-top centrifuges with a maximum speed of 2600g are suitable for use in this protocol if the centrifugation time is increased to 30–60 min in this step and Step 7.

5. Carefully transfer the upper, aqueous phase to a fresh tube without disturbing the interface. The volume of the aqueous phase should be ~600 µL per milliliter of monophasic lysis reagent used for the initial homogenization in Step 1.

Save the lower organic phase if isolation of DNA or protein is desired.

The volume of the aqueous phase should be ~60% of the volume of the monophasic lysis reagent used in Step 1. Although ~600 µL can be recovered per 1 mL of monophasic lysis reagent used, to prevent DNA contamination it is preferable to recover only 500 µL.

Isolation of RNA

6. Precipitate the RNA from the aqueous phase by adding 0.5 mL of isopropanol per milliliter of monophasic lysis reagent used in Step 1. Mix by inverting the tube several times. Incubate the samples for 10 min at room temperature.

Addition of 1 µL of a 20 mg/mL glycogen stock will facilitate visualization of the pellet formed in Step 7.

7. Collect the precipitated RNA by centrifugation at no more than 12,000g for 10 min at 2°C–8°C.

The RNA precipitate, often invisible before centrifugation, forms a gel-like pellet on the side and bottom of the tube.

8. Decant the supernatant and completely remove any traces of liquid by aspiration.
9. Wash the RNA pellet once with 75% ethanol, adding at least 1 mL of 75% ethanol per milliliter of monophasic lysis reagent used in Step 1. Mix the sample by vortexing, then centrifuge at no more than 7500g for 5 min at 2°C–8°C.

The RNA precipitate can be stored in 75% ethanol for at least 1 wk at 2°C–8°C, or at least 1 yr at –5°C to –20°C.

10. Remove all traces of ethanol. Air-dry or vacuum-dry the RNA pellet for 5–10 min.

After removing the ethanol, the pellet should be semitransparent; when dried, the pellet will be transparent and gel-like. Do not dry the RNA by centrifugation under vacuum. It is important not to let the RNA pellet dry completely because this will greatly decrease its solubility. Partially dissolved RNA samples have an A_{260}/A_{280} ratio of <1.6. The A_{260}/A_{280} ratio is a measure of the purity of the RNA sample.

11. Dissolve the RNA in 20 µL of RNase-free water by passing the solution a few times through a pipette tip, and then incubating the solution for 10 min at 55°C–60°C.

To assist in dissolving the RNA, use RNase-free water that has been warmed to ~40°C. The RNA can also be dissolved in 100% formamide (deionized) and stored at –20°C for up to 1 yr. Formamide provides a chemically stable environment that protects RNA against degradation by RNases.

Alternative Protocol: Preparing RNA from Smaller Samples

This protocol can be used to isolate RNA from a small quantity of cells (10^2 – 10^4) or tissue (1–10 mg). Glycogen is added directly to the monophasic lysis reagent and is used as a carrier to increase the recovery of RNA. In addition to

maximizing the precipitation of RNA, the glycogen adds mass to the RNA pellet, making it easier to see. Glycogen does not inhibit first-strand synthesis at concentrations up to 4 mg/mL and does not inhibit polymerase chain reaction (PCR). The glycogen remains in the aqueous phase and coprecipitates with the RNA.

12. Add 800 μ L of monophasic lysis reagent to the tissue or cells. Add 200 μ g of glycogen directly to the monophasic lysis reagent. Homogenize the sample as described above in Step 1.

The final glycogen concentration in monophasic lysis reagent is 250 μ g/mL.

13. Incubate the homogenized samples for 5 min at room temperature.
14. To reduce viscosity, shear the genomic DNA with two passes through a 26-gauge needle.
15. Add chloroform and proceed with the phase separation as described in Step 3.

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