TECHNICAL MANUAL

RNA Isolation Using the Total RNA Purification Kit

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1.0 Introduction

The successful isolation of intact RNA requires four essential steps: effective disruption of cells or tissue, denaturation of nucleoprotein complexes, inactivation of endogenous ribonuclease (RNase) activity, and removal of contaminating DNA and proteins. The most important step is the immediate inactivation of endogenous RNases that are released from membrane-bound organelles upon cell disruption.

The Total RNA Purification Kit combines the disruptive and protective properties of guanidine thiocyanate (GTC) and 1-thioglycerol to inactivate the ribonucleases present in cell extracts. GTC disrupts nucleoprotein complexes, allowing the RNA to be released into solution and to be isolated free of protein. Nucleic acids in lysates are bound to the minicolumns by centrifugation. The binding reaction occurs rapidly due to the disruption of water molecules by the chaotropic salts, thus favoring adsorption of nucleic acids to the column. RNase-free DNase I is applied directly to the membrane to digest contaminating genomic DNA. The bound total RNA is further purified from contaminating salts, proteins, and cellular components by simple washing steps. Finally, the total RNA is eluted from the membrane by the addition of nuclease-free water. This procedure yields an essentially pure fraction of total RNA after only a single round of purification, without organic extractions or precipitations. The procedure is easy to perform with small quantities of cultured cells, and it can be used to process multiple samples.

Processing Capacity

The Total RNA Purification Kit was developed and optimized for total RNA isolation from a wide range of input cell numbers (1×10^2 to 5×10^6 cultured cells) with a broad spectrum of RNA expression levels.

Downstream Applications

RNA purified using the Total RNA Purification Kit is suitable for many molecular biology applications, including RT-PCR, microarrays, and Northern blot hybridizations.

For all downstream applications, continue to protect your samples from RNases by wearing gloves and using solutions and centrifuge tubes that are RNase-free. The use of a ribonuclease inhibitor can help ensure protection from nucleases that may be introduced into purified RNA during downstream processing.

2.0 Materials, Reagents, and Equipment

2.1 Total RNA Purification Kit (Catalog #79040)

The components listed below are available as part of the Total RNA Purification Kit and are not available for individual sale.

Refer to the Product Information Sheet (PIS) for the Total RNA Purification Kit (Document #10000005437) for component storage and stability information; the PIS is also available at www.stemcell.com, or contact us to request a copy.

COMPONENT NAME	COMPONENT #	SIZE
RNA Minicolumns	79041	50 Columns
Collection Tubes	79042	50 Tubes
Column Wash Solution*	79043	5 mL
1-Thioglycerol*	79044	900 µL
DNase I	79045	1 vial
Nuclease-Free Water	79046	13 mL
$MnCl_2$	79047	250 µL
RNA Lysis Buffer*	79048	32.5 mL
Elution Tubes	79049	50 Tubes
RNA Wash Solution	79050	35 mL
DNA Digestion Buffer	79051	2.5 mL

*Please refer to the Safety Data Sheet (SDS) for hazard information.

2.2 Additional Required Materials and Reagents

PRODUCT	CATALOG #
100% Isopropanol, RNase-free	---
95% Ethanol, RNase-free	---
Microcentrifuge tubes, 1.7 mL	e.g. 38089
D-PBS (Without Ca++ and Mg++)	37350

2.3 Equipment

- Biosafety cabinet certified for Level II handling of biological materials
- Microcentrifuge
- Pipettor and sterile RNase-free tips
- -70°C freezer
- Refrigerator (2 - 8°C)

3.0 Protocol Diagram

4.0 Creating an RNase-Free Environment

Ribonucleases are extremely difficult to inactivate. Take care to avoid introducing RNase activity into your RNA samples during or after the isolation procedure. Two of the most common sources of RNase contamination are the user's hands and bacteria or molds that may be present on airborne dust particles. To prevent contamination from these sources, **wear gloves at all times** and use sterile technique when handling reagents. Whenever possible, use sterile disposable plasticware for handling RNA. These materials are generally RNase-free and thus do not require pretreatment to inactivate RNase. Autoclaved elution tubes are provided with the kit.

5.0 Preparation of Reagents

Before starting cell lysis and the RNA purification protocol, prepare the following four reagents:

- DNase I Solution (section 5.1)
- RNA Lysis Buffer + TG (section 5.2)
- RNA Wash Buffer (section 5.3)
- Column Wash Buffer (section 5.4)

5.1 DNase I Solution

Prepare DNase I Solution as follows:

1. Add 275 µL of Nuclease-Free Water to the vial of DNase I. Gently mix by swirling. Do not vortex.
2. If not used immediately, aliquot DNase I Solution into sterile RNase-free microcentrifuge tubes and store at -20°C. After thawing the aliquots, use immediately. Do not freeze/thaw more than 3 times.

5.2 RNA Lysis Buffer + TG

Prepare RNA Lysis Buffer + TG (1-Thioglycerol) as follows:

1. Add 325 µL of 1-Thioglycerol to 32.5 mL of RNA Lysis Buffer. Mix thoroughly.
2. Label the bottle to indicate that 1-Thioglycerol has been added.
3. Store RNA Lysis Buffer + TG at 2 - 8°C for up to 1 month. Cap tightly between uses.

5.3 RNA Wash Buffer

Prepare RNA Wash Buffer as follows:

1. Add 60 mL of 95% ethanol to 35 mL of RNA Wash Solution. Mix thoroughly.
2. Label the bottle to indicate that ethanol has been added.
3. Store RNA Wash Buffer at 15 - 25°C, tightly capped.

5.4 Column Wash Buffer

Prepare Column Wash Buffer as follows:

1. Add 7.5 mL of 95% ethanol to 5 mL of Column Wash Solution. Mix thoroughly.
2. Label the bottle to indicate that ethanol has been added.
3. Store Column Wash Buffer at 15 - 25°C, tightly capped.

6.0 Preparation of Cell Lysate

The following protocols are for harvesting and lysing cultured cells from adherent or suspension cultures. Use from 1 x 10^2 to 5 x 10^6 cells per purification. The number of cells used may need to be adjusted depending on cell type, function, and RNA expression levels at the time of harvest.

For best results, use fresh cell samples. If you choose to store harvested cells as cell pellets, it is best to flash freeze the pellets in a dry ice/ethanol bath, then store at -80°C. RNA integrity depends on the rapid freezing of cells and direct thawing of the cell pellet into RNA Lysis Buffer + TG to ensure rapid lysis and inactivation of nucleases in the sample.

Due to the toxicity of the chemicals used in the RNA purification procedure and the prevalence of RNases, wear gloves throughout the lysis and purification procedures.

For harvesting and lysing adherent cells, proceed to section 6.1. For lysing cells in suspension, proceed to section 6.2.

6.1 Adherent Cells

1. Aspirate cell culture medium. Add ice-cold sterile D-PBS as indicated in Table 1. Aspirate D-PBS.

Table 1. Recommended Volumes of PBS, RNA Lysis Buffer + TG, and 100% Isopropanol for Various Cultureware

CULTUREWARE	VOLUME OF D-PBS	VOLUME OF RNA LYSIS BUFFER + TG	VOLUME OF 100% ISOPROPANOL
T-25 cm^2 flask	5 mL	500 µL	170 µL
6-well plate	2 mL	250 µL	85 µL
24-well plate	500 µL	100 µL	35 µL
48-well plate	250 µL	100 µL	35 µL
96-well plate	100 µL	100 µL	35 µL

2. Add RNA Lysis Buffer + TG as indicated in Table 1. Gently rock the plate or flask to completely cover the adherent cells with buffer. Pipette the lysate up and down over the cultureware surface 7 - 10 times.

   Note: For a T-25 cm^2 flask, scraping may increase yields due to the viscosity of the lysate. The maximum lysate volume that can be processed with a single minicolumn is 500 µL.

   Note: If not used immediately, store lysates at -20 to -80°C for up to 3 months.

3. Collect the lysate and transfer to a new microcentrifuge tube.
4. Add 100% isopropanol as indicated in Table 1. Mix by vortexing for 5 seconds.
5. Proceed to RNA purification (section 7.0).

6.2 Cells in Suspension

If starting with a frozen cell pellet, begin at step 3 by adding RNA Lysis Buffer + TG to the frozen pellet.

1. In a sterile centrifuge tube, centrifuge cell suspension at 300 x *g* for 5 minutes. Remove and discard supernatant.
2. Add ice-cold, sterile D-PBS to wash cells. Centrifuge at 300 x *g* for 5 minutes. Remove as much supernatant as possible and discard.
3. Add RNA Lysis Buffer + TG as indicated in Table 2 . Mix well by pipetting 7 - 10 times, or by vortexing.

   Note: For > 2 x 10^6 cells, pass the lysate through a 20 gauge needle 4 - 5 times to shear the genomic DNA. Expel the lysate into a 1.5 mL microcentrifuge tube. The maximum lysate that can be efficiently processed is 500 µL per column.

   Note: If not used immediately, store lysates at -20 to -80°C for up to 3 months.

Table 2. Recommended Volumes of RNA Lysis Buffer + TG and Isopropanol

CELL INPUT RANGE	VOLUME OF RNA LYSIS BUFFER + TG	VOLUME OF 100% ISOPROPANOL
1 x 10^2 to 5 x 10^5	100 µL	35 µL
> 5 x 10^5 to 2 x 10^6	250 µL	85 µL
> 2 x 10^6 to 5 x 10^6	500 µL	170 µL

4. Add 100% isopropanol as indicated in Table 2. Mix by vortexing for 5 seconds.
5. Proceed to RNA purification (section 7.0).

7.0 RNA Purification

Prepare sample as described in section 6.0. Prepare reagents as described in section 5.0. Wear gloves throughout the protocol, and change gloves frequently. Keep tubes closed whenever possible.

1. Open packs of Collection Tubes and minicolumns. Remove one minicolumn, two Collection Tubes, and one Elution Tube for each sample to be processed. Place the Collection Tubes in a microcentrifuge tube rack, and place the minicolumn into a collection tube. Label all tubes.
2. Transfer the lysate (prepared in section 6.0) to a minicolumn assembly. Centrifuge at 12,000 - 14,000 x *g* for 30 seconds at room temperature (15 - 25°C).
3. Remove the minicolumn from the Collection Tube, and discard the liquid in the Collection Tube. Place the minicolumn back into the Collection Tube.
4. Add 500 µL of RNA Wash Buffer to the minicolumn. Centrifuge at 12,000 - 14,000 x *g* for 30 seconds.
5. Remove the minicolumn from the Collection Tube, and discard the liquid in the Collection Tube. Place the minicolumn back into the Collection Tube. Place the tube in the microcentrifuge tube rack.
6. In a sterile microcentrifuge tube, prepare DNase I Incubation Mix by combining the reagents below, **in the order listed**. Volumes given are for one sample; for multiple samples, adjust volumes accordingly. Prepare only the amount needed; use immediately.
   - 24 µL DNA Digestion Buffer
   - 3 µL $MnCl_2$
   - 3 µL DNase I Solution (prepared in section 5.1)

   Mix by gentle pipetting; **do not vortex**. Store on ice.
7. Add 30 µL of fresh DNase I Incubation Mix directly to the membrane inside the minicolumn. Ensure the solution is in direct contact with and completely covers the membrane.
8. Incubate at room temperature (15 - 25°C) for 15 minutes.
9. Add 200 µL of Column Wash Buffer to the minicolumn. Centrifuge at 12,000 - 14,000 x *g* for 15 seconds.
10. Add 500 µL of RNA Wash Buffer. Centrifuge at 12,000 - 14,000 x *g* for 30 seconds.
11. Remove the minicolumn and transfer to a new Collection Tube. Discard the Collection Tube containing wash solution.
12. Add 300 µL of RNA Wash Buffer to the minicolumn. Centrifuge at high speed for 2 minutes.
13. Transfer the minicolumn to an Elution Tube. Add nuclease-free water to the membrane as indicated in Table 3. Ensure to completely cover the membrane surface with the water. Incubate at room temperature (15 - 25°C) for 1 minute.

    Note: If more concentrated RNA is required, the elution volume can be decreased. This may result in decreased total yield of RNA, especially when elution volume is < 10 µL. Elution volumes below 7 µL are not recommended. Alternatively, RNA can be concentrated by vacuum drying and resuspending in a smaller volume of water.

Table 3. Recommended Volume of Nuclease-Free Water

CELL INPUT RANGE	VOLUME OF NUCLEASE-FREE WATER
1 x 10^2 to 5 x 10^5	15 µL
> 5 x 10^5 to 2 x 10^6	30 µL
> 2 x 10^6 to 5 x 10^6	50 µL

14. Place the elution tube in the centrifuge with the lid of the tube facing out. Centrifuge at 12,000 - 14,000 x *g* for 1 minute.

    Note: If maximum recovery of RNA is essential, a second elution is recommended. Place the minicolumn in a second elution tube, add 15 µL of nuclease-free water, and centrifuge at 12,000 - 14,000 x g for 1 minute. Depending on the number of input cells and RNA expression levels, a second elution may yield 10 - 20% more RNA.

15. Remove the minicolumn and discard. Cap the tube containing the purified RNA and store at -80°C.
16. Proceed to section 8.0 for determination of RNA yield and quality.

8.0 Determination of RNA Yield and Quality

The Total RNA Purification Kit can be used to isolate intact RNA from a variety of cell sources. The yield of total RNA obtained may be determined spectrophotometrically at 260 nm, where one absorbance unit (A_{260}) equals 40 µg of single-stranded RNA/mL. The purity may also be estimated by spectrophotometry from the relative absorbances at 230, 260, and 280 nm (i.e. A_{260}/A_{280} and A_{260}/A_{230}). If the number of cells estimated in the starting material is less than 5×10^4, spectrophotometric analysis will not yield accurate results due to the lack of sensitivity of this method for low concentrations of nucleic acid.

RNA isolated using the Total RNA Purification Kit is substantially free of DNA and contaminating protein and may be used directly for downstream applications including RT-PCR, microarrays, and Northern blot hybridizations. Pure RNA will exhibit an A_{260}/A_{280} ratio of 2.0. However, due to the variations between individual starting materials and in performing the procedure, the expected range of A_{260}/A_{280} ratios for RNA will be 1.7 - 2.1. If the RNA ratio is less than 1.7, refer to section 9.0 for possible causes and tips for improving the purity of the RNA. The RNA will usually have an A_{260}/A_{230} ratio of 1.8 - 2.2. A low A_{260}/A_{230} ratio may indicate guanidine contamination, which can interfere with downstream processing.

If sufficient quantities of RNA are available, determine the integrity of the purified RNA by denaturing agarose gel electrophoresis. The ratio of 28S to 18S eukaryotic ribosomal RNAs should be approximately 2:1 by ethidium bromide staining, indicating that no gross degradation of RNA has occurred. In RNA samples that have been degraded, this ratio will be reversed, since the 28S ribosomal RNA characteristically is degraded to an 18S-like species. Refer to sections 4.0 and 9.0 for tips on avoiding RNA degradation.

9.0 Troubleshooting

PROBLEM	POSSIBLE CAUSE	SOLUTION
RNA is degraded/ no RNA obtained	RNase contamination	Create an RNase-free working environment: Wear gloves during all steps of the protocol, and change gloves frequently; use sterile, disposable polypropylene tubes; keep tubes closed whenever possible. Glassware should be oven-baked for at least 2 hours at 250°C before use.
Poor RNA quality or yield	Reagents not applied or prepared properly	Prepare reagents as described in section 5.0. Ensure ethanol is added to wash solutions. Mix thoroughly. Ensure isopropanol is added after lysis (section 6.1 or 6.2 step 4); RNA binding to the membrane will only occur in the presence of isopropanol.
	Improper storage of kit components	Prepare and store reagents as indicated in section 5.0. Keep bottles tightly closed.
	Improper sample storage	Use fresh sample material when possible. Otherwise, flash freeze samples in liquid nitrogen and store at -80°C. Do not allow samples to thaw before adding Lysis Buffer.
	Insufficient disruption/homogenization of starting material	Ensure thorough sample disruption.
	Poor-quality sample RNA	Samples that were not lysed or frozen immediately upon isolation may have decreased amounts of RNA and/or reduced RNA integrity. If samples cannot be processed immediately, freeze in liquid nitrogen and store at -80°C. Samples in RNA Lysis Buffer + TG should be stored at -20 to -70°C.
	Binding capacity of minicolumn exceeded	If the lysate contains more RNA than the capacity of the minicolumn, the excess RNA will be washed away during the wash steps. Divide the lysate and perform multiple purifications; pool the resulting RNA solutions then determine total yield.
	RNA yield below the level of detection for spectroscopic quantitation	Spectroscopic methods may not be accurate when expected RNA yield is below ~2 ng/µL. Accurate quantitation may be possible only with fluorescent dye-based methods or by qPCR.
	Lysate was allowed to overheat during processing	Work as quickly as possible. Place lysates on ice during sample preparation. Use ice-cold RNA Lysis Buffer + TG to improve yield and stability.

PROBLEM	POSSIBLE CAUSE	SOLUTION
Low A_{260}/A_{230} ratio	Carry-over of guanidinium thiocyanate	• Carefully load the lysate on the column (section 7.0 step 2), avoiding contamination of the upper part of the column and the column lid. • Precipitate the RNA by adding NaCl to a final concentration of 0.1 M. Add 2.5 volumes of ethanol. Incubate for 30 minutes at -20°C. Collect the RNA by centrifugation at 10,000 x *g* for 15 minutes at 2 - 8°C. Resuspend the RNA in nuclease-free water.
Low A_{260}/A_{280} ratio	Protein contamination	• Several methods may be used for removing protein contamination, including phenol:chloroform extraction. This may yield higher A_{260}/A_{280} ratios but up to 40% RNA loss can be expected.
Clogged column	• Too much sample material used • Insufficient disruption/homogenization of sample material • Lysate too concentrated/difficult to pipette	• Reduce amount of sample material or use a higher volume of RNA Lysis Buffer + TG. Use no more than 500 µL of lysate per column. • Ensure thorough sample disruption. • If the lysate is too viscous, dilute it with RNA Lysis Buffer + TG before adding RNA Wash Buffer.
Genomic DNA contamination	DNase I not active	Prepare and store DNase I Solution as indicated in section 5.1.
	DNase I improperly applied	Pipette DNase I Incubation Mix directly onto the center of the membrane (section 7.0 step 7); ensure that the solution completely covers the membrane.
	Sample may contain too much genomic DNA	• For cultured cells, do not exceed 5×10^6 cells per column. • Perform a post-RNA isolation DNase treatment followed by phenol:chloroform extraction.
Suboptimal performance of RNA in downstream experiments	Carry-over of ethanol or salt	• Do not allow the flow-through to touch the column outlet after the second wash with RNA Wash Buffer (section 7.0 step 12). Centrifuge at the indicated speed and time to ensure wash buffer is completely removed. • Ensure RNA Wash Buffer is at room temperature (15 - 25°C) before use.
	Improper storage of isolated RNA	Store eluted RNA on ice, at -20°C for short-term storage, or at -80°C for long-term storage.

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DOCUMENT #10000005434 VERSION 00 AUG 2019