PRODUCT: **RNAzol® RT COLUMN KIT** April 2017
Catalog No: RC 290
Storage: Store at room temperature.

PRODUCT DESCRIPTION.

The RNAzol® RT Column Kit includes three procedures that can isolate either total RNA, large RNA or small RNA. This versatile and universal RNA isolation kit yields pure and undegraded RNA from solid or liquid samples of human, animal, plant, bacterial and viral origin. The isolated RNA is pure and ready for RT-PCR, qRT-PCR, microarrays, poly A+ selection or other molecular biology applications, without DNase treatment. In addition, the Kit allows for sequential isolation of RNA and DNA from the same biological sample. The isolation of total RNA or the large RNA fraction requires less than 30 minutes, and the isolation of small RNA requires less than 60 minutes. All procedural steps are performed at room temperature. The outstanding effectiveness and versatility of the RNAzol® RT Column Kit makes it the most advanced and reliable kit in this category.

RNA ISOLATION PROTOCOLS AVAILABLE WITH THE RNAzol® RT COLUMN KIT.
The column in this universal kit can be used for any of these three protocols to isolate all sizes of RNA.
1. Isolation of total RNA (Pages 3 - 4).
2. Isolation of large RNA containing large nuclear RNA, rRNA and mRNA > 150 - 200 bases (Pages 5 - 6).
3. Isolation of small RNA containing tRNA, small rRNA and microRNA < 150 - 200 bases (Pages 7 - 8).

Protocols 2 and 3 can be used to sequentially isolate large and small RNA in separate fractions from the same biological sample.

In the first step, a biological sample is homogenized in RNAzol® RT (US and international patents 1, 2). RNAzol® RT is an improved version of the single-step method that selectively solubilizes RNA and inactivates RNase. After mixing the homogenate with water, precipitated DNA, proteins, polysaccharides and other molecules are removed by centrifugation. RNA remains soluble in the aqueous supernatant. As an improvement of the original single-step method (3), no chloroform-induced phase separation is used to separate RNA from DNA and proteins. In the second step of the procedure, the RNA-containing aqueous supernatant is mixed with alcohol and applied on a column to further purify the RNA. Following alcohol washes, pure RNA is eluted from the column with water (4).

KIT COMPONENTS	**QUANTITY**
RNAzol® RT	50 ml
Columns with Collection tubes	50
Wash tubes	50
Elution tubes	50
RNase-free water	10 ml

Reagents required but not supplied: isopropanol and ethanol.

KIT SPECIFICATIONS.
- 50 isolations of either total, large or small RNA.
- A single column accommodates small (≤ 2 mg tissue or 10^5 cells) or large (up to 100 mg tissue) samples and can purify at least 300 µg of RNA.
- Column capacity is 800 µl.

STABILITY/STORAGE: RNAzol® RT Column Kit is stable at room temperature for at least two years from date of purchase.

SPECIAL HANDLING PRECAUTIONS.
RNAzol® RT contains phenol (corrosive liquid/poison) and guanidine thiocyanate (irritant). CAUSES BURNS. Can be fatal. When working with RNAzol® RT, use gloves and eye protection (face shield, safety goggles). Do not get on skin or clothing. Avoid breathing fumes. Read the warning note on the container and SDS. **In case of contact:** Immediately flush eyes or skin with a large amount of water for at least 15 minutes and seek medical attention if necessary.

NOTES TO THE PROTOCOLS.
1. Rapid removal and immediate homogenization of the tissue sample is critical for the integrity and yield of RNA. The most effective homogenization is with a Polytron-type homogenizer set at a high-speed. Please see MRC Technical Bulletin 10.
2. Brain samples cause excessive foaming. Use a glass-Teflon homogenizer for homogenization.
3. To estimate tissue weight used for homogenization, place the reagent in a tube on a balance and tare it. Drop fresh or frozen tissue into the reagent, quickly record the tissue weight and immediately homogenize the sample. After homogenization, supplement the homogenate with reagent to achieve the desired ratio of mg of tissue per ml of the reagent.

4. RNA isolation can be interrupted and samples can be stored at Step 1 as a homogenate before addition of water, and at Step 2 as an aqueous supernatant. In both cases, samples can be stored overnight at 4 C or for at least two years at -70 C. To thaw the samples, incubate them at 37 - 40 C with periodic mixing until completely thawed.
5. For the isolation of small RNA from tissue samples with a high lipid content, such as lung or brain, it is recommended to use a modified small RNA isolation protocol that requires the use of two columns. For this modified protocol, obtain and use the water-supernatant as described in Step 2 of the Protocol for Isolation of Small RNA on Page 7. Transfer this supernatant to a clean tube and mix it with 0.4 volumes of isopropanol in the tube. Apply this mixture on a column, store for 5 minutes and centrifuge at 12,000 g for 20 seconds. Collect the pass-through solution that contains the small RNA fraction and transfer it to a clean tube. *(The column retains the large RNA fraction that can be washed, eluted and solubilized as described in Steps 3 - 5 on Pages 5 and 6.)* Add 0.5 volumes of isopropanol to the pass-through solution and mix it in the tube. Apply this mixture to a new clean column, store for 15 minutes and centrifuge for 20 seconds as described in Steps 4 - 6 on Page 8.
6. Another option when isolating small RNA from tissue samples with a high lipid content is to perform an optional phase separation step. For this modified protocol, homogenize the sample according to Step 1 of the Protocol for Isolation of Small RNA on Page 7. Add to the homogenate 0.4 ml water and 50 µl of BAN (4-bromoanisole, MRC Cat # BN 191) per 1 ml of RNAzol® RT used for homogenization. Shake the mixture vigorously by hand for 15 seconds, store for 5 minutes and centrifuge at 12,000 g for 15 minutes. Transfer the supernatant to a clean tube leaving at least a 2 mm layer of the supernatant above the DNA/protein pellet. Proceed with Steps 3 - 6 of the Protocol for Isolation of Small RNA on Pages 7 - 8.
7. For the isolation of RNA from blood samples, use RNAzol® BD.

RNA ISOLATION TROUBLESHOOTING GUIDE.

Low yield. a) incomplete homogenization or lysis of samples, insufficient elution of RNA from spin column.

260/280 ratio < 1.7. a) insufficient volume of the reagent was used for homogenization; b) proteoglycan or polysaccharide contamination; c) acidic water used for OD measurement; d) incomplete solubilization of RNA pellet; e) insufficient ethanol wash of RNA pellet.

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

DNA contamination. a) an insufficient volume of reagent was used for homogenization; b) samples used for RNA isolation contained organic solvents, strong buffers, salt or alkaline solution.

Removal of proteoglycans and polysaccharides. After homogenization (Step 1), perform an additional centrifugation at 12,000 g for 5 min to clarify the homogenate. Transfer the clear supernatant to a clean tube and add 0.4 volumes of water, shake vigorously and continue with RNA isolation as described in Step 2.

Removal of fat. After homogenization (Step 1), centrifuge samples with a high fat content at 12,000 g for 5 min. Excess lipid forms a layer at the top of the tube. Remove most of the fat layer with a pipette or syringe. Mix the remaining homogenate with 0.4 volumes of water, shake vigorously and continue with RNA isolation as described in Step 2.

RECOVERY OF DNA.

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

DNAzol®. Remove any remaining supernatant without disturbing the pellet obtained in Step 2. 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. Remove any remaining supernatant without disturbing the pellet obtained in Step 2. Solubilize the pellet with a minimum of 10 volumes of 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.

REFERENCES.

1. Chomczynski, P. (2010) Reagents and methods for isolation of purified RNA. US Patent 7,794,932. International Patents.
2. Chomczynsk, P. (2013) Reagents for isolation of purified RNA. US patent, 8,3677,817. International Patents.
3 Chomczynski, P. and Sacchi, N. (1987) Single step method of RNA isolation by acid guanidinium thiocyanate-phenol - chloroform extraction. Anal. Biochem. 162, 156 - 159.
4. Gribanov, OG, et. al. (1997) A simple method for RNA isolation and purification. Bioorg. Khim. 23(9): 763 - 765.

Cite this protocol by referring to the RNAzol® RT Column Kit Brochure, 2017. Molecular Research Center, Inc. Cincinnati, OH.

RNAzol® RT COLUMN KIT
PROTOCOL FOR ISOLATION OF TOTAL RNA.

This protocol yields all classes of RNA in one fraction including large RNA, rRNA, mRNA, small RNA and microRNA down to 10 bases.

Protocol Summary.

1. Homogenization	- 1 ml RNAzol® RT + up to 100 mg tissue or 10^7 cells.
2. DNA/protein precipitation	- homogenate + 0.4 ml water, shake well, store for 5 min, 12,000 g x 15 min.
3. Total RNA purification	- mix supernatant + 1 vol isopropanol, apply on column, store 2 - 5 min, 12,000 g x 20 sec.
4. Total RNA wash	- 2X: 0.3 ml 100% ethanol, 12,000 g x 20 sec (or 1 - 2 min for RNA yield < 10 µg).
5. Total RNA elution	- 2X: 30 - 50 µl water, store 1 min, 12,000 x 20 sec.

Isolation is performed at room temperature. Centrifugations are performed at 12,000 g 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 processing tissues with high DNA content such as spleen, use 50 mg of tissue/1 ml reagent.
The protocol describes isolation using 1 ml of RNAzol® RT.

B. Cells. Cells grown in monolayer should be lysed by the addition of RNAzol® RT to the culture dish. 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. Using 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. To process a liquid sample < 0.4 ml, mix the sample with 1 ml of RNAzol® RT and supplement the mixture with water to approach a total volume of 1.4 ml (RNAzol® RT + sample + water supplement = 1.4 ml). Shake the resulting mixture vigorously for 15 seconds and proceed with centrifugation as described in Step 2.

D. Samples with High Fat Content. After complete homogenization, centrifuge samples with a high fat content at 12,000 g for 5 minutes. Excess lipid forms a layer at the top of the tube. Remove most of the fat layer with a pipette or syringe. Use the remaining homogenate and proceed with addition of water and centrifugation as described in Step 2.
The homogenate can be stored at -20 or -70 for at least two years.

2. DNA, PROTEIN AND POLYSACCHARIDE PRECIPITATION.

Add to the homogenate 0.4 ml of water per 1 ml of RNAzol® RT used for homogenization. Shake the resulting mixture vigorously for 15 seconds, store for 5 minutes and centrifuge at 12,000 g for 15 minutes. Following centrifugation, DNA, proteins and most polysaccharides form a semisolid pellet at the bottom of the tube. The RNA remains soluble in the supernatant. Transfer the supernatant to a clean tube leaving at least a 2 mm layer of the supernatant above the DNA/protein pellet. It is generally safe to collect about 85% of the supernatant without dislodging the semisolid pellet at the bottom of the tube.
The supernatant can be stored at - 20 or -70 C for at least two years.
Determine the volume of aqueous supernatant left above the phenol layer by pipetting. This value will be used to calculate the total yield of RNA per sample.

3. COLUMN PURIFICATION OF TOTAL RNA.

After transferring the supernatant to a clean tube, add 1 volume of isopropanol and mix it in the tube.

Supernatant - isopropanol volumes ≤ 0.8 ml: Transfer the supernatant - isopropanol mixture to the column, cap the column and store for 2 - 5 min at room temperature. Centrifuge at 12,000 g for 20 seconds. The total RNA is retained on the column.

Supernatant - isopropanol volumes > 0.8 ml: Transfer 0.6 - 0.8 ml of the supernatant - isopropanol mixture to the column, cap the column and store for 2 - 5 minutes at room temperature. Centrifuge at 12,000 g for 20 seconds. Remove the column from the collection tube and discard the pass-through solution. Re-insert the column in the collection tube. Apply the next aliquot of the supernatant - isopropanol mixture on the column and centrifuge at 12,000 g for 20 seconds. It is not necessary to wait an additional 2 - 5 min between each application of more solution on the column. Repeat the procedure until the entire sample is processed. The total RNA is retained on the column.
The loading limit on the column is at least 300 µg RNA.
Excess supernatant - isopropanol mixture can be stored at -20 or -70 C for at least one year.

4. TOTAL RNA WASH.
Insert the column into a clean, labeled wash tube. Apply 0.3 - 0.4 ml of 100% ethanol to the column and centrifuge at 12,000 g for 20 seconds. Repeat the ethanol wash step a second time.
When isolating a small amount of RNA (< 10 µg) the second spin should last 1 - 2 minutes. This allows for evaporation of any residual ethanol from the column.

5. TOTAL RNA ELUTION.
Insert the column into a clean, labeled elution tube. Apply 30 - 50µl of RNase-free water near the bottom of the column. Store for 1 minute to hydrate the RNA and elute RNA from the column by centrifugation at 12,000 g for 20 seconds. Repeat the elution using a fresh volume of 30 - 50 µl of RNase-free water (total elution volume = 60 - 100 µl). Remove the column from the elution tube, cap the tube and vortex the eluted RNA solution. Measure the RNA content and store the RNA solution at -70 C.
Decreasing the total elution volume may increase the solubilized RNA concentration and/or diminish the RNA yield. Another option to increase the eluted RNA concentration is to reapply the eluted RNA to the column for a second elution pass. This option may work well for yields less than 10 µg RNA.

The purified total RNA contains all classes of RNA including high molecular weight untranslated RNA, rRNA, mRNA, small RNA and microRNA down to 10 bases long.
Expected total RNA yields: A) tissues (µg RNA/mg tissue): liver, 6 - 8 µg; kidney, spleen 3 - 4 µg; lung, 2 - 3 µg; skeletal muscle, brain, 0.5 - 1.5 µg; placenta, 1 - 3 µg: B) cells (µg RNA/10^6 cells): epithelial cells, 5 - 8 µg; fibroblasts, 3 - 5 µg.
The total RNA has a 260/280 of ratio 1.7 - 2.1and a 260/230 ratio of 1.2 to 1.8. The RIN value of RNA is > 8.5 when tissue is quickly excised and immediately processed, or immediately frozen in liquid nitrogen and stored at -70 for later processing.

To calculate total yield expressed as µg RNA/mg tissue sample:
In most cases, the entire aqueous supernatant containing RNA is not processed for RNA isolation. In order to account for this when determining RNA yield per mg of tissue or cell number, we suggest the following calculation.
1. Calculate the RNA yield for the volume of supernatant processed.
2. Calculate the RNA yield for the total volume of aqueous supernatant by including the volume of supernatant left behind above the phenol layer (Step 2).
3. Calculate the RNA yield per tissue weight or cell number of the sample.

See also NOTES TO THE PROTOCOLS, RNA ISOLATION TROUBLESHOOTING GUIDE and RECOVERY OF DNA on Pages 1 and 2 of this protocol.

Cite this protocol by referring to the RNAzol® RTColumn Kit Brochure, 2017. Molecular Research Center, Inc. Cincinnati, OH.

RNAzol® RT COLUMN KIT
PROTOCOL FOR ISOLATION OF LARGE RNA.

This protocol yields large RNA > 150 - 200 bases including long non-coding RNA, mRNA and rRNA.
Due to multiple splicing sites, some small RNA can be detected in both the large and small RNA fraction.

Protocol Summary.

1.	**Homogenization**	- 1 ml RNAzol® RT + up to 100 mg tissue or 10^7 cells.
2.	**DNA/protein precipitation**	- homogenate + 0.4 ml water, shake well, store 5 min, 12,000 g x 15 min.
3.	**Large RNA purification**	- mix supernatant + 0.4 vol isopropanol, apply on column, store 5 min, 12,000 g x 20 sec.
4.	**RNA wash**	- 2X: 0.3 ml 100% ethanol, 12,000 g x 20 sec (or 1 - 2 min for RNA yield < 10 µg).
5.	**RNA elution**	- 2X: 30 - 50 µl water, store 1 min, 12,000 g x 20 sec.

Isolation is performed at room temperature. Centrifugations are performed at 12,000 g 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. When processing tissues with high DNA content such as spleen, use 50 mg of tissue/1 ml reagent.
The protocol describes isolation using 1 ml of RNAzol® RT.

B. Cells. Cells grown in monolayer should be lysed by the addition of RNAzol® RT to the culture dish. Remove culture medium and add at least 1 ml of 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. Use 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. Using 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. To process a liquid sample < 0.4 ml, mix the sample with 1 ml of RNAzol® RT and supplement the mixture with water to approach a total volume of 1.4 ml (RNAzol® RT + sample + water supplement = 1.4 ml). Shake the resulting mixture vigorously for 15 seconds and proceed with centrifugation as described in Step 2.

D. Samples with High Fat Content. After complete homogenization, centrifuge samples with a high fat content at 12,000 g for 5 minutes. Excess lipid forms a layer at the top of the tube. Remove the fat layer with a pipette or syringe. Use the remaining homogenate and proceed with addition of water and centrifugation as described in Step 2.
The homogenate can be stored at -20 or - 70 C for at least two years.

2. DNA, PROTEIN AND POLYSACCHARIDE PRECIPITATION.

Add to the homogenate 0.4 ml of water per 1 ml of RNAzol® RT used for homogenization. Shake the resulting mixture vigorously for 15 seconds, store for 5 minutes and centrifuge at 12,000 g for 15 minutes. Following centrifugation, DNA, proteins and most polysaccharides form a semisolid pellet at the bottom of the tube. The RNA remains soluble in the supernatant. Transfer the supernatant to a clean tube leaving at least a 2 mm layer of the supernatant above the DNA/protein pellet. It is generally safe to collect about 85% of the supernatant without dislodging the semisolid pellet at the bottom of the tube.
The supernatant can be stored at -20 or -70 C for at least two years.
Determine the volume of aqueous supernatant left above the phenol layer by pipetting. This value will be used to calculate the total yield of RNA per sample.

3. COLUMN PURIFICATION OF LARGE RNA FRACTION.

After transferring the supernatant to a clean tube, add 0.4 volumes of isopropanol and mix it in the tube.

Supernatant - isopropanol volumes ≤ 0.8 ml: Transfer the supernatant - isopropanol mixture to the column, cap the column and store for 5 minutes at room temperature. Centrifuge at 12,000g for 20 seconds. The large RNA fraction is retained on the column while the small RNA is in the pass-through solution. Collect and store the pass-through solution for isolation of small RNA.

Supernatant - isopropanol volumes > 0.8 ml: Transfer 0.6 - 0.8 ml of the supernatant - isopropanol mixture to the column, cap the column and store for 5 minutes at room temperature. Centrifuge at 12,000 g for 20 sec. Remove the column from the collection tube and transfer the pass-through solution to a clean tube. Re-insert the column in the collection tube. Apply the next aliquot of the supernatant - isopropanol mixture to the column and centrifuge at 12,000 g for 20 seconds. It is not necessary to wait an additional 5 minutes between each application of more solution to the column. Repeat the procedure until the entire sample is processed. The large RNA fraction is retained on the column. Collect and store the pass-through solution for isolation of small RNA.
The loading limit on the column is at least 300 µg RNA.
Excess supernatant - isopropanol mixture can be stored at -20 or -70 C for at least two years.
The pass-through solution containing small RNA and microRNA can be stored at -20 C or -70 C for at least one year.

4. LARGE RNA WASH.
Insert the column into a clean, labeled, wash tube. Apply 0.3 - 0.4 ml of 100% ethanol on the column and centrifuge at 12,000 g for 20 seconds. Repeat the ethanol wash step a second time.
When isolating a small amount of RNA (< 10 µg), extend the second spin for 1 - 2 minutes. This allows for evaporation of any residual alcohol from the column.

5. LARGE RNA ELUTION.
Insert the column into a clean, labeled elution tube. Apply 30 - 50 µl of RNase-free water near the bottom of the column. Store for 1 minute to hydrate the RNA and elute the RNA from the column by centrifuge at 12,000 g for 20 seconds. Repeat the elution using a fresh volume of 30 - 50 µl of RNase-free water (total elution volume = 60 - 100 µl). Remove the column after the second elution, cap the tube and vortex the eluted RNA solution. Measure the RNA content and store the RNA solution at -70 C.
Decreasing the total elution volume may increase the solubilized RNA concentration and/or diminish the RNA yield. Another option to increase the eluted RNA concentration is to reapply the eluted RNA to the column for a second elution pass. This option may work well for yields less than 10 µg RNA.

The purified large RNA fraction contains rRNA, mRNA and high molecular weight untranslated RNA. Typically, this RNA fraction constitutes 80 - 85% of the cellular RNA.
Expected total RNA yields: A) tissues (µg RNA/mg tissue): liver, 5 - 7 µg; kidney, spleen, 3 - 4 µg; lung, 2 - 3 µg; skeletal muscle, brain, lung, 0.5 - 1.5 µg; placenta, 1 - 3 µg: B) cells (µg RNA/10^6 cells): epithelial cells, 5 - 8 µg; fibroblasts, 3 - 5 µg.
The large RNA fraction has a 260/280 ratio of 1.7 - 2.1 and 260/230 ratio of 1.8 to 2.3. The RIN value for large RNA is > 8.5 when tissue is quickly excised and immediately processed, or immediately frozen in liquid nitrogen and stored at -70 for later processing.

To calculate yield expressed as µg RNA/mg tissue sample:
In most cases, the entire aqueous supernatant containing RNA is not processed for RNA isolation. In order to account for this when determining RNA yield per mg of tissue or cell number, we suggest the following calculation.
1. Calculate the RNA yield for the volume of supernatant processed.
2. Calculate the RNA yield for the total volume of aqueous supernatant by including the volume of supernatant left behind above the phenol layer (Step 2).
3. Calculate the RNA yield per tissue weight or cell number of the sample.

See also NOTES TO THE PROTOCOLS, RNA ISOLATION TROUBLESHOOTING GUIDE and RECOVERY OF DNA on Pages 1 and 2 of this protocol.

Cite this protocol by referring to the RNAzol® RTColumn Kit Brochure, 2017. Molecular Research Center, Inc. Cincinnati, OH.

RNAzol® RT COLUMN KIT
PROTOCOL FOR ISOLATION OF SMALL RNA.

This protocol yields small RNA < 150 - 200 bases including tRNA, small rRNA, and microRNA down to 10 bases.

Protocol Summary.

1.	**Homogenization**	- 1 ml RNAzol® RT + up to 100 mg tissue or 10^7 cells.
2.	**DNA/protein precipitation**	- homogenate + 0.4 ml water, shake well, store 5 min, 12,000 g x 15 min.
3.	**Remove large RNA**	- mix supernatant + 0.4 vol isopropanol, pellet large RNA fraction (or apply on a column)
4.	**Small RNA purification**	- Step 3 supernatant + 0.5 vol isopropanol, mix, apply on column, 15 min, 12,000 g x 20 sec.
5.	**RNA wash**	- 2X: 0.3 ml 100% ethanol, 12,000 g x 20 sec (or 1 - 2 min for RNA yield < 10 µg).
6.	**RNA elution**	- 2X: 30 - 50 µl water, store 1 min, 12,000 g x 20 sec.

Isolation is performed at room temperature. Centrifugations are performed at 12,000 g 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. When processing tissues with high DNA content such as spleen, use 50 mg of tissue/1 ml reagent.
The protocol describes isolation using 1 ml of RNAzol® RT.

B. Cells. Cells grown in monolayer should be lysed by the addition of RNAzol® RT to the culture dish. Remove culture medium and add at least 1 ml of 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. Use 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. Using 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. To process a liquid sample < 0.4 ml, mix the sample with 1 ml of RNAzol® RT and supplement the mixture with water to approach a total volume of 1.4 ml (RNAzol® RT + sample + water supplement = 1.4 ml). Shake the resulting mixture vigorously for 15 seconds and proceed with centrifugation as described in Step 2.

D. Samples with High Fat Content. After complete homogenization, centrifuge samples with a high fat content at 12,000 g for 5 minutes. Excess lipid forms a layer at the top of the tube. Remove the fat layer with a pipette or syringe. Use the remaining homogenate and proceed with addition of water and centrifugation as described in Step 2.
The homogenate can be stored at -20 or - 70 C for at least two years.

2. DNA, PROTEIN AND POLYSACCHARIDE PRECIPITATION.

Add to the homogenate 0.4 ml of water per 1 ml of RNAzol® RT used for homogenization. Shake the resulting mixture vigorously for 15 seconds, store for 5 minutes and centrifuge at 12,000 g for 15 minutes. Following centrifugation, DNA, proteins and most polysaccharides form a semisolid pellet at the bottom of the tube. The RNA remains soluble in the supernatant. Transfer the supernatant to a clean tube leaving at least a 2 mm layer of the supernatant above the DNA/protein pellet. It is generally safe to collect about 85% of the supernatant without dislodging the semisolid pellet at the bottom of the tube.
The supernatant can be stored at -20 or -70 C for at least two years.
Determine the volume of aqueous supernatant left above the phenol layer by pipetting. This value will be used to calculate the total yield of RNA per sample.

3. PRECIPITATION OF THE LARGE RNA FRACTION.

After transferring the supernatant to a clean tube, add 0.4 volumes of isopropanol and mix it in the tube. Store the mixture for 5 minutes at room temperature and centrifuge at 12,000 g for 10 minutes. Carefully collect the supernatant containing small RNA without dislodging any of the precipitated large RNA. Transfer this supernatant with small RNA to a clean tube and mix it with isopropanol to precipitate the small RNA as described in Step 4 below.
This protocol extends use of the kit to obtain 50 small RNA isolation procedures using columns, reducing your cost if your primary goal is small RNA isolation only.
To preserve the large RNA pellet, wash it two times with 75% ethanol and solubilize according to preference.

For the isolation of small RNA from tissue samples with a high lipid content, such as lipid, lung or brain, please see Notes 5 and 6 on Page 2 for alternative procedures to isolate or remove large RNA prior to small RNA isolation.

4. COLUMN PURIFICATION OF SMALL RNA FRACTION.
Supplement the supernatant collected in Step 3 with 0.5 volumes of isopropanol per 1 volume of the solution and mix in the tube. Apply up to 0.8 ml of the mixture to a new clean column and store for 15 minutes at room temperature. Centrifuge at 12,000 g for 20 seconds. Remove the column from the collection tube and discard the pass-through solution. Re-insert the column back into the collection tube. Apply the next aliquot of the solution - isopropanol mixture onto the column and repeat the procedure until the entire sample is processed. It is not necessary to wait an additional 15 minutes between each application of more solution on the column.

5. SMALL RNA WASH.
Insert the column into a clean, labeled, wash tube. Apply 0.3 - 0.4 ml of 100% ethanol on the column and centrifuge at 12,000 g for 20 seconds. Repeat the ethanol wash step a second time.
When isolating a small amount of RNA (< 10 µg), extend the second spin for 1 - 2 minutes. This allows for evaporation of any residual alcohol from the column.

6. SMALL RNA ELUTION.
Insert the column into a clean, labeled elution tube. Apply 30 - 50 µl of RNase-free water near the bottom of the column. Store for 1 minute to hydrate the RNA and elute the RNA from the column by centrifuge at 12,000 g for 20 seconds. Repeat the elution using a fresh volume of 30 - 50 µl of RNase-free water (total elution volume = 60 - 100 µl). Remove the column after the second elution, cap the tube and vortex the eluted RNA solution. Measure the RNA content and store the RNA solution at -70 C.
Decreasing the total elution volume may increase the solubilized RNA concentration and/or diminish the RNA yield. Another option to increase the eluted RNA concentration is to reapply the eluted RNA to the column for a second elution pass. This option may work well for yields less than 10 µg RNA.

The purified small RNA fraction contains small rRNA, tRNA and microRNA down to 10 bases. Expected yield is about 15 - 20 % of the total RNA yield for a sample.
The small RNA fraction has a 260/280 ratio of about 1.7 - 1.9 and a 260/230 ratio of about 1.5.

To calculate yield expressed as µg RNA/mg tissue sample:
In most cases, the entire aqueous supernatant containing RNA is not processed for RNA isolation. In order to account for this when determining RNA yield per mg of tissue or cell number, we suggest the following calculation.
1. Calculate the RNA yield for the volume of supernatant processed.
2. Calculate the RNA yield for the total volume of aqueous supernatant by including the volume of supernatant left behind above the phenol layer (Step 2).
3. Calculate the RNA yield per tissue weight or cell number of the sample.

See also NOTES TO THE PROTOCOLS, RNA ISOLATION TROUBLESHOOTING GUIDE and RECOVERY OF DNA on Pages 1 and 2 of this protocol.

Cite this protocol by referring to the RNAzol® RTColumn Kit Brochure, 2017. Molecular Research Center, Inc. Cincinnati, OH.