Empirical bioscience

Observe the Difference

TRUST THE OUTCOME

EB Pure Total RNA Kit

SS-EB-TRK-5	5 preps
EB-TRK-50	50 preps
EB-TRK-200	200 preps

Made in Michigan

www.Empiricalbioscience.com

REGISTERED ISO 13485

EB Pure Total RNA Kit

Table of Contents

Introduction

The EB Pure Total RNA Kit is an innovative system that radically simplifies the extraction and purification of RNA from a variety of sources. The key to this system is the use of the reversible binding properties of the binding matrix in combination with the speed of minispin columns which allows the user to process single or multiple samples at once. There is no need for phenol/chloroform extractions and time-consuming steps such as CsCl gradient ultracentrifugation, or precipitation with isopropanol or LiCl are eliminated. Purified RNA is ready for applications such as RT-PCR, Northern blotting, poly A+ RNA (mRNA) purification, nuclease protection, and *in vitro* translation.

The EB Pure Total RNA Kit can purify up to 100 µg total RNA from cultured eukaryotic cells or tissue. Normally, 1 x 10^6 - 1 x 10^7 eukaryotic cells, or 5-30 mg tissue, can be processed in a single experiment. Fresh, frozen, or RNALater® stabilized tissues can be used. Cell or tissue lysis occurs under denaturing conditions which inactivate RNases. After the homogenization process, samples are applied to the RNA Mini Column which binds total RNA. Cellular debris and other contaminants are effectively washed away after a few quick wash steps. High-quality RNA is eluted in DEPC Water. Total RNA greater than 200 nt is isolated using this kit.

Binding Capacity

Each RNA Mini Column can bind approximately 100 µg RNA. Using greater than 30 mg tissue or 1 x 10^7 cells is not recommended.

Illustrated Protocols

Centrifugation Protocol

Add TRK Lysis Buffer and Lyse

Homogenize

Add Ethanol
Apply Sample to Column

Wash 3X

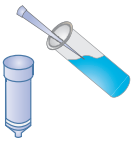
Dry

Elute

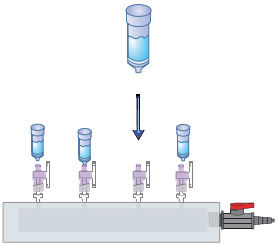
Vacuum Protocol

Add TRK Lysis Buffer and Lyse

Homogenize



Add Ethanol
Apply Sample to Column



Wash 3X

Dry

Elute

Kit Contents

Product	SS-EB-TRK-5	EB-TRK-50	EB-TRK-200
Purifications	5	50	200
RNA Mini Columns	5	50	200
2mL Collection Tubes	10	100	400
TRK Lysis Buffer	5 mL	40 mL	150 mL
RNA Wash Buffer I	5 mL	50 mL	200 mL
RNA Wash Buffer II	5 mL	12 mL	50 mL
DEPC Water	1.5 mL	10 mL	40 mL
User Manual	✓	✓	✓

Storage and Stability

All EB Pure Total RNA Kit components are guaranteed for at least 12 months from the date of purchase when stored at room temperature. During shipment, crystals or precipitation may form in the TRK Lysis Buffer. Dissolve by warming buffer to 37°C.

Important Notes

Please take a few minutes to read this booklet in its entirety to become familiar with the procedures. Prepare all materials required before starting to minimize RNA degradation.

- Whenever working with RNA, always wear gloves to minimize RNase contamination. Use only clean RNase-free disposable plastic pipette tips when using the supplied reagents.

- Equilibrate samples and reagents to room temperature before beginning this protocol. All steps should be carried out at room temperature unless otherwise noted. Work quickly, but carefully.

- Prepare all materials required before starting the procedure to minimize RNA degradation.

- Carefully apply the sample or solution to the center of the RNA Mini Columns. Avoid touching the membrane with pipet tips.

- Optional: 2-mercaptoethanol is key in denaturing RNases and can be added to an aliquot of TRK Lysis Buffer before use. Add 20 µL 2-mercaptoethanol per 1 mL TRK Lysis Buffer. This mixture can be stored for 4 weeks at room temperature.

Quantification and Storage of RNA

To determine the concentration and purity of RNA, measure absorbance at 260 nm and 280 nm with a spectrophotometer. One OD unit measured at 260 nm corresponds to 40 µg/mL RNA. DEPC Water is slightly acidic and can dramatically lower absorbance values. We suggest that you dilute the sample in a buffered solution (TE) for spectrophotometric analysis. The A_{260}/A_{280} ratio of pure nucleic acids is 2.0, while an A_{260}/A_{280} ratio of 0.6 denotes pure protein. A ratio of 1.8-2.0 corresponds to 90%-100% pure nucleic acid. Phenol has a maximum absorbance at 270 nm and can interfere with spectrophotometric analysis of DNA or RNA. Store RNA samples at -70°C in water. Under these conditions, RNA is stable for more than a year.

Integrity of RNA

It is highly recommended that RNA quality be determined prior to beginning all downstream applications. The quality of RNA can be best assessed by denaturing agarose gel electrophoresis with ethidium bromide staining. The ribosomal RNA bands should appear as sharp, clear bands on the gel. The 28S band should appear to be double that of the 18S RNA band (23S and 16S if using bacteria). If the ribosomal RNA bands in any given lane are not sharp and appear to be smeared towards the smaller sized RNA, it is very likely that the RNA undergone degradation during the isolation, handling, or storage procedure. Although RNA molecules less than 200 bases in length do not efficiently bind to the column matrix, a third RNA band, the tRNA band, may be visible when a large number of cells are used.

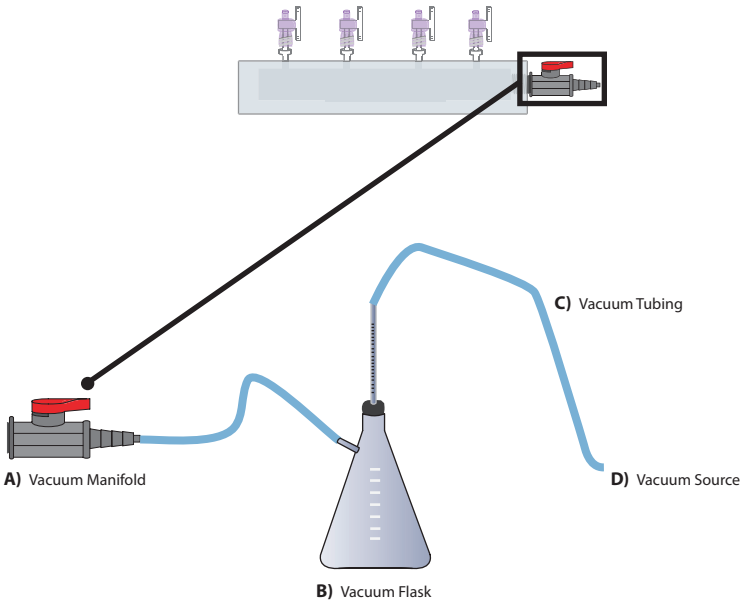
Guideline for Vacuum Manifold

The following is required for use with the Vacuum Protocol:

A) Vacuum Manifold
Compatible Vacuum Manifolds: Qiagen QIAvac24, Sigma AldrichVM20, Promega Vacman®, or manifold with standard luer connector
B) Vacuum Flask
C) Vacuum Tubing
D) Vacuum Source (review tables below for pressure settings)

Conversion from millibars:	Multiply by:
Millimeters of Mercury (mmHg)	0.75
Kilopascals (kPa)	0.1
Inches of Mercury (inchHg)	0.0295
Torrs (Torr)	0.75
Atmospheres (atmos)	0.000987
Pounds per Square Inch (psi)	0.0145

Illustrated Vacuum Setup:



- Dilute RNA Wash Buffer II with 100% ethanol as follows and store at room temperature.

Kit	100% Ethanol to be Added
SS-EB-TRK-5	20 mL
EB-TRK-50	48 mL
EB-TRK-200	200 mL

Optional: As a preparation step add 20 µL 2-mercaptoethanol (β-mercaptoethanol) per 1 mL TRK Lysis Buffer. This mixture can be stored for 4 weeks at room temperature.

Homogenization Techniques

Efficient sample disruption and homogenization is essential for successful total RNA isolation. Cell wall and plasma membrane disruption is necessary for the release of RNA from the sample and homogenization is necessary to reduce the viscosity of the lysates. Homogenization shears genomic DNA and other high-molecular-weight cell components creating a homogeneous lysate. Incomplete homogenization can cause the RNA Mini Columns to clog resulting in low or no yield.

Liquid Nitrogen Method

1. Wear appropriate gloves and take great care when working with liquid nitrogen.

2. Excise tissue and promptly freeze in a small volume of liquid nitrogen.

3. Grind tissue with a ceramic mortar and pestle under approximately 10 mL liquid nitrogen.

4. Pour the suspension into a pre-cooled 15 mL polypropylene tube.

   Note: Unless the tube is pre-cooled in liquid nitrogen, the suspension will boil vigorously and may cause loss of tissue.

5. Allow the liquid nitrogen to completely evaporate and add TRK Lysis Buffer.

6. Proceed to one of the homogenization steps below.

Homogenization with Syringe and Needle

- Shear high-molecular-weight DNA by passing the lysate through a narrow needle (19-21 gauge) 5-10 times.

- Proceed to Step 1 of the "Animal Cell Protocol" on Page 11 or to Step 1 of the "Animal Tissue Protocol" on Page 15.

Rotor-Stator Homogenizer: Sample Disruption and Homogenization

Using a rotor-stator homogenizer for sample disruption and homogenization can simultaneously disrupt and homogenize most samples. The process usually takes less than a minute depending on sample type. Many rotor-stator homogenizers operate with differently sized probes or generators that allow sample processing in 50 mL tubes.

Bead Milling: Sample Disruption and Homogenization

By using bead milling, cells and tissue can be disrupted and homogenized by rapid agitation in the presence of glass beads and a lysis buffer. The optimal size of glass beads to use for RNA isolation are 0.5 mm for yeast/unicellular cells and 4-8 mm for animal tissue samples.

Syringe Needle: Sample Disruption and Homogenization

High-molecular-weight DNA is responsible for the viscosity of cell lysates and can be shredded by passing the sample 10-20 times through a narrow gauge needle (19-21 G).

EB Pure Total RNA Kit Protocol - Cultured Cell Protocol

All centrifugation steps used are performed at room temperature.

Materials and Equipment to be Supplied by User:

- Microcentrifuge capable of at least 14,000 x *g*
- RNase-free pipette tips and 1.5 mL microcentrifuge tubes
- 100% ethanol
- 70% ethanol in sterile DEPC-treated water
- Homogenization equipment
  - Needle and Syringe
  - Mortar and pestle
  - Glass Beads
  - Rotor-stator Homogenizer
- Optional: 14.3M 2-mercaptoethanol (β-mercaptoethanol)

Before Starting:

- Prepare RNA Wash Buffer II according to the "Preparing Reagents" section on Page 8.
- Optional: Add 20 µL 2-mercaptoethanol per 1 mL TRK Lysis Buffer.

1. Determine the proper amount of starting material.
   Note: It is critical to use the correct amount of cultured cells in order to obtain optimal yield and purity with the RNA Mini Columns. The maximum amount of cells that can be processed with the Total RNA Protocol is dependent on the cell line and its RNA content. The maximum binding capacity of the RNA Mini Columns is 100 µg. The maximum number of cells that TRK Lysis Buffer can efficiently lyse is 1 x 107. Use the following table as a guideline to select the correct amount of starting material. If no information regarding your starting material is available, begin with 1 x 10^6 cells. Based on RNA yield and quality obtained from 1 x 10^6 cells, the starting amount can be adjusted for the next purification.

Source	Number of Cells	RNA Yield (µg)
IC21	1 x 10^6	12
HeLa	1 x 10^6	15
293HEK	1 x 10^6	10
HIN3T3	1 x 10^6	15

2. Harvest cells using one of the following methods. Do not use more than 1 x 10^7 cells.

   - For cells grown in suspension:

     1. Determine the number of cells.
     2. Centrifuge at 500 x *g* for 5 minutes.
     3. Aspirate and discard the supernatant.
     4. Proceed to Step 3 on Page 13.

   - For cells grown in a monolayer:
     Note: These cells can either be lysed directly in the cell culture dish or trypsinized and collected as a cell pellet prior to lysis. Cells grown in cell culture flasks should always be trypsinized.

     - For direct cell lysis:

       1. Determine the number of cells.
       2. Aspirate and discard the cell culture medium.
       3. Immediately proceed to Step 3 on Page 13.
          Note: Incomplete removal of the cell-culture medium will inhibit lysis and dilute the lysate. This will affect the RNA binding conditions of the RNA Mini Columns and may reduce RNA yield.

     - To trypsinize and collect cells:

       1. Determine the number of cells.
       2. Aspirate and discard the cell-culture medium and wash the cells with PBS.
          Note: Incomplete removal of the cell-culture medium will inhibit trypsin. Multiple washes may be necessary for cells that are difficult to detach.
       3. Add 0.1-0.25% Trypsin in a balanced salt solution.

4. Incubate for 3-5 minutes to allow cells to detach. Check cells for detachment before proceeding to the next step.

5. Add an equal volume of cell-culture medium containing serum to inactivate the trypsin.

6. Transfer cells to an RNase-free glass or polypropylene centrifuge tube (not supplied).

7. Centrifuge at 500 x *g* for 5 minutes.

8. Aspirate the supernatant.

9. Proceed to Step 3 below.
   Note: Incomplete removal of the cell-culture medium will inhibit lysis and dilute the lysate. This will affect the conditions for binding of RNA to the RNA Mini Columns and may reduce RNA yield.

3. Disrupt cells (do not use more than 1 x 10^7 cells) with TRK Lysis Buffer. Vortex or pipet up and down to mix thoroughly.

Note: For pelleted cells, loosen the cell pellet thoroughly by flicking the tube before adding the appropriate amount of TRK Lysis Buffer based on the table below. To directly lyse the cells in the culture dish, add the appropriate amount of TRK Lysis Buffer directly to the dish. Collect the cell lysate with a rubber policemen and transfer the cell lysate into a 1.5 mL microcentrifuge tube.

Optional: Add 20 µL 2-mercaptoethanol per 1 mL TRK Lysis Buffer before use.

Number of Cells	Amount of **TRK Lysis** Buffer (µL)
< 5 x 10^6	350 µL
5 x 10^6 - 1 x 10^7	700 µL

4. Homogenize the cells:

   - Syringe and Needle: Shear high molecular-weight DNA by passing the lysate through a narrow needle (19-21 gauge) 5-10 times.

   Note: Incomplete homogenization of the sample may cause the column to clog resulting in decreased yields.

5. Add 1 volume 70% ethanol. Vortex to mix thoroughly. Do not centrifuge.
   Note: A precipitate may form at this point. This will not interfere with the RNA purification. If any sample has lost its volume during homogenization, adjust the volume of ethanol accordingly.

6. Insert a RNA Mini Column into a 2mL Collection Tube.

7. Transfer 700 µL sample (including any precipitate that may have formed) to the RNA Mini Column.

8. Centrifuge at 10,000 x *g* for 1 minute.

9. Discard the filtrate and reuse the 2mL Collection Tube.

10. Repeat Steps 7-9 until all of the sample has been transferred to the column.

Optional: This the starting point of the optional on-membrane DNase I Digestion Protocol. Since the binding matrix of the RNA Mini Column eliminates most DNA, DNase I digestion is not necessary for most downstream applications. However, certain sensitive RNA applications may require further DNA removal. If an additional RNA removal step is required, please continue to the DNase I Digestion Protocol found on Page 24. (See DNase I Digestion Kit, (EB-DDK-Preps) for more information). If DNase I digestion is not required, proceed to Step 11.

11. Add 500 µL RNA Wash Buffer I to the RNA Mini Columns.

12. Centrifuge at 10,000 x *g* for 30 seconds.

13. Discard the filtrate and reuse the 2mL Collection Tube.

14. Add 500 µL RNA Wash Buffer II to the RNA Mini Columns.
    Note: RNA Wash Buffer II must be diluted with 100% ethanol before use. Please see Page 8 for instructions.

15. Centrifuge at 10,000 x *g* for 1 minute.

16. Discard the filtrate and reuse the 2mL Collection Tube.

17. Repeat Steps 14-16 for a second RNA Wash Buffer II wash step.

18. Centrifuge at maximum speed for 2 minutes to completely dry the RNA Mini Column.
    Note: It is important to dry the RNA Mini Column matrix before elution. Residual ethanol may interfere with downstream applications.

19. Transfer the RNA Mini Column to a clean 1.5 mL microcentrifuge tube (not provided).

20. Add 40-70 µL DEPC Water.

    Note: Make sure to add water directly onto the RNA Mini Column matrix.

21. Centrifuge at top speed for 2 minutes and store eluted RNA at -70°C.

    Note: Any combination of the following steps can be used to help increase RNA yield.

    - Heat the DEPC Water to 70°C before adding to the column.
    - Increase the incubation time to 5 minutes.
    - Increase the elution volume.
    - Repeat the elution step with fresh DEPC Water (this may increase the yield, but decrease the concentration).
    - Repeat the elution step using the eluate from the first elution (this may increase yield while maintaining elution volume).

EB Pure Total RNA Kit Protocol - Animal Tissue Protocol

All centrifugation steps used are performed at room temperature.

Materials and Equipment to be Supplied by User:

- Microcentrifuge capable of at least 14,000 x *g*
- RNase-free pipet tips and 1.5 mL microcentrifuge tubes
- 100% ethanol
- 70% ethanol in sterile DEPC-treated water
- Homogenization Equipment
  - Needle and Syringe
  - Mortar and pestle
  - Glass Beads
  - Rotor-stator Homogenizer
- Optional: 14.3M 2-mercaptoethanol (β-mercaptoethanol)

Before Starting:

- Prepare RNA Wash Buffer II according to the "Preparing Reagents" section on Page 8.
- Optional: Add 20 µL 2-mercaptoethanol per 1 mL TRK Lysis Buffer.

1. Determine the proper amount of starting material.

   Note: It is critical to use the correct amount of tissue in order to obtain optimal yield and purity with the RNA Mini Columns. The maximum amount of tissue that can be processed with the Total RNA Protocol is dependent on the tissue type and its RNA content. The maximum binding capacity of the RNA Mini Columns is 100 µg. The maximum amount of tissue that TRK Lysis Buffer can lyse in the this protocol is 30 mg. Use the table on the following page as a guideline to select the correct amount of starting material. If no information regarding your starting material is available, begin with 10 mg. Based on RNA yield and quality obtained from 10 mg, the starting amount can be adjusted for the next purification.

Average Yield of Total Cellular RNA from Mouse Tissue

Source	Amount of Tissue (mg)	RNA Yield (µg)
Brain	10	10
Kidney	10	30
Liver	10	45
Heart	10	5
Spleen	10	33
Lung	10	12
Pancreas	10	40
Thymus	10	20

2. Homogenize and disrupt the tissue according to one of the following methods described below:

Amount of TRK Lysis Buffer per Tissue Sample

Amount of Tissue	Amount of TRK Lysis Buffer (µL)
≤ 15 mg	350 µL
20-30 mg	700 µL

Note: For samples stored in RNALater® use 700 µL TRK Lysis Buffer.

A. Rotor-Stator Homogenizer: Homogenize tissue with a rotor-stator homogenizer or until the sample is uniformly homogenized. See Page 10 for details.

B. Liquid Nitrogen Method: See Page 9 for detailed protocol.

- Syringe and Needle: Shear high molecular-weight DNA by passing the lysate through a narrow needle (19-21 gauge) 5-10 times.

Note: Incomplete homogenization of the sample may cause the column to clog resulting in decreased yield. It is recommended to homogenize the tissue samples with a rotor-stator homogenizer as this method normally produces better yields.

3. Centrifuge at maximum speed for 5 minutes.

4. Transfer the cleared supernatant to a clean 1.5 mL microcentrifuge tube (not supplied).
   Note: In some preparations, a fatty upper layer will form after centrifugation. Transfer of any of the fatty upper layer may reduce RNA yield or clog the column.

5. Add 1 volume 70% ethanol. Vortex to mix thoroughly. Do not centrifuge.
   Note: A precipitate may form at this point. This will not interfere with the RNA purification. If any sample has lost its volume during homogenization, adjust the volume of ethanol accordingly.

6. Insert a RNA Mini Column into a 2mL Collection Tube.

7. Transfer 700 µL sample (including any precipitate that may have formed) to the RNA Mini Column.

8. Centrifuge at 10,000 x *g* for 1 minute.

9. Discard the filtrate and reuse the 2mL Collection Tube.

10. Repeat Steps 7-9 until all of the sample has been transferred to the column.

Optional: This the starting point of the optional on-membrane DNase I Digestion Protocol. Since the binding matrix of the RNA Mini Column eliminates most DNA, DNase I digestion is not necessary for most downstream applications. However, certain sensitive RNA applications may require further DNA removal. If an additional RNA removal step is required, please continue to the DNase I Digestion Protocol found on Page 24. (See DNase I Digestion Kit, (EB-DDK-Preps) for more information). If DNase I digestion is not required, proceed to Step 11.

11. Add 500 µL RNA Wash Buffer I to the RNA Mini Column.

12. Centrifuge at 10,000 x *g* for 30 seconds.

13. Discard the filtrate and reuse the 2mL Collection Tube.

14. Add 500 µL RNA Wash Buffer II to the RNA Mini Column.
    Note: RNA Wash Buffer II must be diluted with 100% ethanol before use. Please see Page 8 for instructions.

15. Centrifuge at 10,000 x *g* for 1 minute.

16. Discard the filtrate and reuse the 2mL Collection Tube.

17. Repeat Steps 14-16 for a second RNA Wash Buffer II wash step.

18. Centrifuge at maximum speed for 2 minutes to completely dry the RNA Mini Columns.
    Note: It is important to dry the RNA Mini Column matrix before elution. Residual ethanol may interfere with downstream applications.

19. Transfer the RNA Mini Column to a clean 1.5 mL microcentrifuge tube (not provided).

20. Add 40-70 µL DEPC Water.

    Note: Make sure to add water directly onto the RNA Mini Column matrix.

21. Centrifuge at top speed for 2 minutes and store eluted RNA at -70°C.

    Note: Any combination of the following steps can be used to help increase RNA yield.

    - Heat the DEPC Water to 70°C before adding to the column.
    - Increase the incubation time to 5 minutes.
    - Increase the elution volume.
    - Repeat the elution step with fresh DEPC Water (this may increase the yield, but decrease the concentration).
    - Repeat the elution step using the eluate from the first elution (this may increase yield while maintaining elution volume).

EB Pure Total RNA Kit Protocol - Vacuum Method

All centrifugation steps used are performed at room temperature.

Materials and Equipment to be Supplied by User:

- Microcentrifuge capable of at least 14,000 x *g*
- Vacuum Manifold
- Vacuum Source
- RNase-free pipet tips and 1.5 mL microcentrifuge tubes
- 100% ethanol
- 70% ethanol in sterile DEPC-treated water
- Homogenization Equipment
  - Needle and Syringe
  - Mortar and pestle
  - Glass Beads
  - Rotor-stator Homogenizer
- Optional: 14.3M 2-mercaptoethanol (β-mercaptoethanol)

Before Starting:

- Prepare RNA Wash Buffer II according to the "Preparing Reagents" section on Page 8.
- Assemble vacuum manifold (see Page 7)
- Optional: Add 20 µL 2-mercaptoethanol per 1 mL TRK Lysis Buffer.

Note: Please read through previous sections of this manual before proceeding with this protocol. Steps 1-5 from the Total RNA Animal Cell protocol should be completed or Steps 1-5 from the Total RNA Animal Tissue Protocol should be completed before loading the sample to the RNA Mini Column. Instead of continuing with centrifugation, follow the steps below. Do not use more than $1x10^6$ cells or 10 mg tissue for the vacuum protocol.

1. Prepare the vacuum manifold according to manufacturer's instructions.

2. Connect the RNA Mini Column to the vacuum manifold.

3. Load the homogenized sample onto the RNA Mini Column.

4. Switch on the vacuum source to draw the sample through the column.

5. Turn off the vacuum.

6. Add 500 µL RNA Wash Buffer I to the RNA Mini Column.

7. Switch on the vacuum source to draw the RNA Wash Buffer I through the column.

8. Turn off the vacuum.

9. Add 500 µL RNA Wash Buffer II to the RNA Mini Column.
   Note: RNA Wash Buffer II must be diluted with 100% ethanol before use. Please see Page 8 for instructions.

10. Switch on the vacuum source to draw the RNA Wash Buffer I through the column.

11. Turn off the vacuum.

12. Repeat Steps 9-11 for a second RNA Wash Buffer II wash step.

13. Insert a RNA Mini Column into a 2mL Collection Tube provided with this kit.

14. Centrifuge at maximum speed for 2 minutes to completely dry the RNA Mini Column.
    Note: It is important to dry the RNA Mini Column matrix before elution. Residual ethanol may interfere with downstream applications.

15. Transfer the RNA Mini Column to a clean 1.5 mL microcentrifuge tube (not provided).

16. Add 40-70 µL DEPC Water.

    Note: Make sure to add water directly onto the RNA Mini Column matrix.

17. Centrifuge at top speed for 2 minutes and store eluted RNA at -70°C.

    Note: Any combination of the following steps can be used to help increase RNA yield.

    - Heat the DEPC Water to 70°C before adding to the column.
    - Increase the incubation time to 5 minutes.
    - Increase the elution volume.
    - Repeat the elution step with fresh DEPC Water (this may increase the yield, but decrease the concentration).
    - Repeat the elution step using the eluate from the first elution (this may increase yield while maintaining elution volume).

EB Pure Total RNA Kit - DNase I Digestion Protocol

Since the binding matrix of the RNA Mini Column eliminates most DNA, DNase I digestion is not necessary for most downstream applications. However, certain sensitive RNA applications may require further DNA removal. (See DNase I Digestion Kit, Cat# EB-DDK-Preps for further information).

After completing Steps 1-10 of the Animal Cell Protocol (Pages 11-14) or Steps 1-10 of the Animal Tissue Protocol (Pages 17-19), proceed with the following protocol.

User Supplied Material:

- EB Pure DNase I Digestion Kit (EB-DDK-Preps)

1. For each RNA Mini Columns, prepare the DNase I stock solution as follows:

Buffer	Volume per Prep
DNase Digestion Buffer	73.5 µL
DNase I (20 Kunitz/µL)	1.5 µL
Total Volume	75 µL

Important Notes:

- DNase I is very sensitive and prone to physical denaturing. **Do not vortex the DNase I mixture.** Mix gently by inverting the tube.
- Freshly prepare DNase I stock solution right before RNA isolation.
- Standard DNase buffers are not compatible with on-membrane DNase I digestion. The use of other buffers may affect the binding of RNA to the binding matrix and may reduce RNA yields and purity.
- All steps must be carried out at room temperature. Work quickly, but carefully.

2. Insert the RNA Mini Column containing the sample into a 2mL Collection Tube.

3. Add 250 µL RNA Wash Buffer I to the RNA Mini Column.

4. Centrifuge at 10,000 x *g* for 1 minute.

5. Discard the filtrate and reuse the 2mL Collection Tube.

6. Add 75 µL DNase I digestion mixture directly onto the surface of the membrane of the RNA Mini Column.
   Note: Pipet the DNase I directly onto the membrane. DNA digestion will not be complete if some of the mixture is retained on the wall of the RNA Mini Column.

7. Let sit at room temperature for 15 minutes.

8. Add 250 µL RNA Wash Buffer I to the RNA Mini Column.

9. Let sit at room temperature for 2 minutes.

10. Centrifuge at 10,000 x *g* for 1 minute.

11. Discard the filtrate and reuse the 2mL Collection Tube.

12. Add 500 µL RNA Wash Buffer II.
    Note: RNA Wash Buffer II must be diluted with 100% ethanol before use. Please see Page 8 for instructions.

13. Centrifuge at 10,000 x *g* for 1 minute.

14. Discard the filtrate and reuse the 2mL Collection Tube.

15. Repeat Steps 12-14 for a second RNA Wash Buffer II wash step.

16. Centrifuge at maximum speed for 2 minutes to completely dry the RNA Mini Column matrix.

    Note: It is important to dry the RNA Mini Column matrix before elution. Residual ethanol may interfere with downstream applications.

17. Place the column in a clean 1.5 mL microcentrifuge tube (not supplied).

18. Add 40-70 µL DEPC Water.

    Note: Make sure to add water directly onto the RNA Mini Column matrix.

19. Let sit at room temperature for 1 minute.

20. Centrifuge at maximum speed for 2 minutes and store eluted RNA at -70°C.

    Note: Any combination of the following steps can be used to help increase RNA yield.

    - Heat the DEPC Water to 70°C before adding to the column.
    - Increase the incubation time to 5 minutes.
    - Increase the elution volume.
    - Repeat the elution step with fresh DEPC Water (this may increase the yield, but decrease the concentration).
    - Repeat the elution step using the eluate from the first elution (this may increase yield while maintaining elution volume).

Troubleshooting Guide

Please use this guide to troubleshoot any problems that may arise.

Problem	Cause	Solution
Little or no RNA eluted	RNA remains on the column	Repeat the elution step.
	Column is overloaded	Reduce the amount of starting material.
Problem	Cause	Solution
Clogged column	Incomplete homogenization	Completely homogenize the sample.
		Increase the centrifugation time.
		Reduce the amount of starting material.
Problem	Cause	Solution
Degraded RNA	Starting culture problems	Freeze starting material quickly in liquid nitrogen.
		Do not store tissue culture cells prior to extraction unless they are lysed first.
		Follow protocol closely and work quickly.
	RNase contamination	Ensure not to introduce RNase during the procedure.
		Check buffers for RNase contamination.
Problem	Cause	Solution
Problem in downstream applications	Salt carry-over during elution	Ensure RNA Wash Buffer II has been diluted with 100% ethanol as indicated on bottle.
		RNA Wash Buffer II must be stored and used at room temperature.
		Repeat wash steps with RNA Wash Buffer II.
Problem	Cause	Solution
DNA contamination	DNA contamination	Digest with RNase-free DNase and inactivate DNase by incubation at 65°C for 5 minutes in the presence of EDTA.
Problem	Cause	Solution
Low Abs ratios	RNA diluted in acidic buffer or water	DEPC Water is acidic and can dramatically lower Abs_{260} values. Use TE Buffer to dilute RNA prior to spectrophotometric analysis.

Notes: