Problem	Cause	Suggestion
Little or no RNA eluted	RNA remains on the column	• Repeat elution. • Pre-heat DEPC-water to 65° C prior to elution. • Incubate column for 10 min with water prior to centrifugation.
	Column is overloaded	• Reduce quantity of starting material.
Clogged column	Incomplete homogenization	• Completely homogenize sample. • Increase centrifugation time. • Reduce amount of starting material
Degraded RNA	Source	• 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 in downstream applications	Salt carry-over during elution	• Ensure Wash Buffer II Concentrate has been diluted with 4 volumes of 100% ethanol as indicated on bottle. • 1 X Wash Buffer II must be stored and used at room temperature. • Repeat wash with Wash Buffer II.
DNA contamination		• Digest with RNase-free DNase and inactivate at 75°C for 5 min.
Low Abs ratios	RNA diluted in acidic buffer or water	• DEPC water is acidic and can dramatically lower Abs260 values. Use TE buffer to dilute RNA prior to spectrophotometric analysis.

Contents

Vacuum/Spin Protocol for RNA Extraction (V-Spin column only)

Carry out lysis, homogenization, and loading onto HiBind® RNA column as indicated previous protocols. Instead of continuing with centrifugation, follow steps blow.

Note: Please read through previous section of this book before using this protocol.

1. Prepare the vacuum manifold according to manufacturer' s instruction and connect the HiBind® RNA V-Spin column to the manifold.

2. **Load the samples from step 5 into HiBind® RNA V-spin column.**

3. Switch on vacuum source to draw the sample through the column and turn off the vacuum.

4. **(Optional): Perform on-membrane DNase I digestion steps if sensitive downstream application is desired.** (See previous section for details)

5. Wash the column by adding 500 µl **RNA wash buffer I**, draw the wash buffer through the column by turn on the vacuum source.

6. Wash the column by adding 700 µl **RNA wash buffer II**, draw the wash buffer through the column by turn on the vacuum source.

7. Assemble the column into a **2 ml collection tube** and transfer the column to a micro centrifuge. Spin at maxi speed for 2 minute to dry the column.

8. Place the column in a clean 1.5 ml microcentrifuge tube and add 30-50 µl RNase-free water. Stand for 1-2 minute and centrifuge 1 minute to elute RNA.

Kit Contents

Product Number	R6934-00	R6934-01	R6934-02
Purification times	5 Preps	50 Preps	200 Preps
HiBin® RNA Mini Column	5	50	200
2 ml Collection Tubes	10	100	400
RNA-Solv® Reagent	5 ml	60 ml	220 ml
RNA Wash Buffer I	5 ml	45 ml	2 x 90 ml
RNA Wash Buffer II **Concentrate**	2 ml	12 ml	4 x 12 ml
DEPC water	-	10 ml	40 ml
Instruction Manual	1	1	1

Note: RNA-Solv® Reagent contains Guanidine Thiocyanate and phenol, handle those reagents with extra care. Safety and risk phase: R20-24/25-32-34, S13-26-36/37/39-45.

Before Starting

IMPORTANT	**RNA Wash Buffer II Concentrate** must be diluted with absolute ethanol (96-100%) before use and store at room temperature.	
	R6934-00	Add 8 ml ~96-100% ethanol
	R6934-01	Add 48 ml ~96-100% ethanol
	R6934-02	Add 48 ml ~96-100% ethanol to each bottle

B. E.Z.N.A.® Protocol for Bacteria

Procedure

1. **Harvest Cells and resuspend in 100 µl TE/lysozyme and incubate at R.T. for 7 min**.

   Centrifuge 10^9 cells at 4,000 x g for 5 min. Discard supernatant and add 100 µl of TE buffer containing lysozyme **(0.5 mg/ml for Gram-negative and 4 mg/ml for Gram-positive bacteria)**. Resuspend cells completely and incubate at room temperature for 7 min.

2. **Add 1ml of RNA-Solv® Reagent and mix by vortexing for 15 seconds.** Incubate the tube contains homogenate at room temperature for 3 minutes.

3. **Add 0.2 mL of chloroform per 1 mL of RNA-*Solv* Reagent.** Cap sample tubes securely and vortex vigorously for 15 seconds. Incubate on ice for 10 minutes.

4. **Centrifuge at 12,000 x g for 15 minutes at 4°C.** The mixture separates into a lower phenol-chloroform phase, an interphase, and an upper aqueous phase. RNA remains entirely in the aqueous phase.

5. **Transfer no more than 80% of the aqueous phase to a fresh tube. Add 1/3 volume of absolute ethanol (96-100%, room temperature)** and vortex at maximum speed for 15 seconds. A precipitate may form at this point. This will not interfere with RNA purification.

6. **Apply no more than 700 µl of sample from step 5 onto HiBind® RNA column.** (Larger volumes can be loaded successively.) A precipitate may form on addition of ethanol in step 5. Vortex and add the entire mixture to the column. With the spin column inside a 2 ml collecting tube (supplied with kit), centrifuge at 10,000 x g for 15-30 seconds **at room temperature**. Discard flow-through and reuse the collection tube.

7. Repeat step 6 by loading the remaining sample to the column, discard flow-through and collection tube.

8. Proceed as step 8-13 on page 6-7.

E.Z.N.A.® Total RNA Kit II Protocol

A. Eukaryotic Cells and Tissues

Procedure:

1. **Lyse cells or tissues with 1 ml of RNA-Solv® Reagent.**

   1ml of RNA-Solv® Reagent is sufficient for 10^7 cells or approximately 100 mg disrupted tissue (~30 mm cube).

   For tissue culture cells grown in **monolayer** (fibroblasts, endothelial cells, etc.), lyse the cells directly in the culture vessel as follows. Aspirate culture medium completely and add RNA-Solv® Reagent directly to the cells. Pipette buffer over entire surface of vessel to ensure complete lysis. Transfer lysate to a clean 1.5 ml microfuge tube and proceed to step 2 below. (This method is preferable to trypsinization followed by washing because it minimizes RNA degradation by nuclease contamination.)

   For cells grown in **suspension cultures**, pellet cells at no greater than 1,500 rpm (400 x g) for 5 min. Discard supernatant, add RNA-Solv® Reagent, lyse by vortex or pipetting up and down, and transfer to a clean 1.5 ml microfuge tube. Proceed to step 2.

   For **tissue** samples, determine the size of the samples and homogenize by using one of the methods discussed on page 4. Unless using liquid nitrogen, homogenize samples directly in RNA-Solv® Reagent and proceed to step 2.

2. Incubate the tube contains homogenate at room temperature for 2-3 minutes.

3. **Add 0.2 mL of chloroform per 1 mL of RNA-*Solv* Reagent.** Cap sample tubes securely and shake vigorously for 15 seconds. Incubate on ice for 10 minutes.

4. **Centrifuge at 12,000 x g for 15 minutes at 4°C.** The mixture separates into a lower phenol-chloroform phase, an interphase, and an upper aqueous phase. RNA remains entirely in the aqueous phase.

5. **Transfer no more than 80% of the aqueous phase to a fresh tube and add 1/3 volume of absolute ethanol (~96-100%, room temperature)**. Vortex at maximum speed for 15 seconds.

   Note: All of the centrifugation step below should be carried out at room temperature.

Introduction

E.Z.N.A.® Total RNA Kit II provides a rapid and easy method for the isolation of up to 100 µg of total RNA from cultured eukaryotic cells, tissues, bacteria, plant or fungal. The kit allows single or multiple, simultaneous processing of samples in less than 30 min. Normally, up to 1 x 10^6 eukaryotic cells, up to 1 x 10^9 bacterial cells, 100 mg tissue or 100 mg plant samples can be used in a single experiment. While this kit may be used for isolation of RNA from whole blood, we recommend you use the E.Z.N.A.® Blood RNA Kit (product # R6614) as it is specifically designed for effective hemolysis and hemoglobin removal and gives higher RNA yields.

RNA purified using the E.Z.N.A.® Total RNA method is ready for applications such as RT-PCR*, Northern blotting, poly A^+ RNA (mRNA) purification, nuclease protection, and *in vitro* translation.

Principle

The E.Z.N.A.® Total RNA Kit II use the reversible binding properties of HiBind® matrix, a new silica-based material. By combined the high lysis efficient of RNA-Solv® Reagent with OBI innovative HiBind® technology, this kit can extract total cellular RNA from different sources of samples specially for fatty tissues such as brain and adipose tissue. A specifically formulated high salt buffer system allows more than 100 µg of RNA molecules greater than 200 bases to bind to the matrix. Cells or tissues are first homogenized with RNA-Solv® Reagent that practically inactivate RNases. After add chloroform, the homogenate is separated into aqueous and organic phase with centrifugation. The aqueous phase which contains RNA then adjusted with ethanol and then applied to the HiBind® spin columns to which total RNA binds, while cellular debris and other contaminants are effectively washed away. High quality RNA is finally eluted in DEPC-treated sterile water.

Storage and Stability

All components except RNA-Solv Reagent in E.Z.N.A.® Total RNA Kits should be stored 22-25°C. RNA-Solv® Reagent should be store at 4°C for long term storage. All E.Z.N.A.™ Total RNA Kit components are guaranteed for at least 24 months from the date of purchase when stored as above.

*The PCR process is covered by U.S. Patents 4,683,195 and 4,683,202 (and international equivalents) owned by Hoffmann-LaRoche, Inc.

DNA Contamination

Generally HiBind® RNA spin column technology will efficiently removes most of the DNA without DNase treatment. However, no RNA extraction procedure can completely remove genomic DNA. For sensitive work (such as RT-PCR or differential display) we suggest that you perform on-membrane DNase I digestion (OBI cat# E1091) or treat the eluted RNA with RNase-free DNase. Also for RT-PCR, use intron-spanning primers that allow easy identification of DNA-contamination. A control PCR reaction containing the RNA as template will also allow detection of DNA contamination. For designing intron-spanning primers, call our technical staff at 800-832-8896 for assistance. We can help design primers suited to your needs.

Quantization and Storage of RNA

To determine the concentration and purity of RNA, measure absorbance at 260 nm and 280 nm in a spectrophotometer. 1 O.D. unit measured at 260 nm corresponds to 40 µg of RNA per ml. 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 ratio of A_{260}/A_{280} of pure nucleic acids is 2.0, while for pure protein it is approximately 0.6. A ratio of 1.8-2.0 corresponds to 90%-100% pure nucleic acid. (Phenol has an absorbance maximum at 275 nm and can interfere with absorbance readings of DNA or RNA. However, the E.Z.N.A.® Total RNA Kit eliminates the use of phenol and avoids this problem.) Store RNA samples at -70°C in water. Under such conditions RNA prepared with the E.Z.N.A.® system is stable for more than a year.

RNA Quality

It is highly recommended that RNA quality be determined prior to all analyses. The quality of RNA can best be assessed by denaturing agarose gel electrophoresis and ethidium bromide staining. Two sharp bands should appear on the gel. These are the 28S and 18S (23S and 16S for bacteria) ribosomal RNA bands. If these bands smear towards lower molecular weight RNAs, then the RNA has undergone major degradation during preparation, handling or storage. Although RNA molecules less than 200 bases in length do not efficiently bind the HiBind matrix, a third RNA band, the tRNA band, may be visible when a large number of cells are used.

Trouble shooting Tips

Please take a few minutes to read this booklet thoroughly and become familiar with the protocol. Prepare all materials required before starting to minimize RNA degradation.

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

- During the procedure work carefully but quickly.

- **It is very important to determine the correct amount of starting material before the experiment**. If the maximum amount of starting material is 100mg. The capacity of the HiBind® RNA column is 100µg. For samples contains high amount of RNA, we suggest to use 30mg tissue to start. For samples contains lower level RNA, the maximum amount of starting material (100mg) can be used.

Homogenization of Tissues

A. Liquid Nitrogen Method

Wear gloves and take great care when working with liquid nitrogen. Excise tissue and promptly freeze in a small volume of liquid nitrogen. Grind tissue with a ceramic mortar and pestle under approximately 10 ml of liquid nitrogen and pour the suspension into a pre-cooled 15 ml polypropylene tube. Unless the tube is pre-cooled (in liquid nitrogen), the suspension will boil vigorously possibly causing loss of tissue. When the liquid nitrogen has completely evaporated, add RNA-Solv® Reagent and continue with the procedure as outlined below. This is the preferred method of disrupting tissue samples.

B. Rotor-Stator Homogenizers

Rotor-stator homogenizers effectively homogenize most tissues. The process usually takes less than a minute depending on the tissue. Many Rotor-stator homogenizers operate with differently sized probes or generators that allow processing of small volumes in microfuge tubes. Such homogenizers are available from:

- Tekmar Inc., Cincinnati, OH (Tissuemizers ®)
- BIOSPEC Products, Bartlesville, OK (Tissue-Tearor™)
- Craven Laboratories, Austin, TX.

C. Syringe Method

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

C. Plant or Fungal samples

This protocol is suitable for most fresh or frozen tissue samples allowing efficient recovery of RNA. However, due to the tremendous variation in water and polysaccharide content of plants or fungal, sample size should be limited to ≤ 100 mg. Best results are obtained with young leaves or needles. The method isolates sufficient RNA for a few tracks on a standard Northern assay.

Wearing latex disposable gloves, collect tissue in a 1.5-ml or 2-ml microfuge tube and freeze by dipping in liquid nitrogen with a pair of tweezers to fill the tube. Grind the tissue using disposable pestles (available from OBI Cat# SS-1014-39 &1015-39) or equivalent. Alternatively, one can allow liquid nitrogen to evaporate and then store samples at -70°C for later use. **Do not allow samples to thaw**. Use disposable pestles only once. Alternatively, a small clean mortar and pestle can be used. The above methods for disrupting plant tissue **cannot** be replaced with mechanical homogenizers.

1. **Collect frozen ground tissue (up to 100 mg) in a microfuge tube and immediately add 1 ml of RNA-Solv® Reagent and mix by vortexing at maximum speed for 30 seconds.** Vortex vigorously to make sure that all clumps are dispersed. RNA cannot be effectively extracted from clumped tissue.

2. Incubate the tube contains homogenate at room temperature for 3 minutes.

3. **Add 0.2 mL of chloroform per 1 mL of RNA-*Solv*® Reagent.** Cap sample tubes securely and vortex vigorously for 15 seconds. Incubate on ice for 10 minutes.

4. **Centrifuge at 12,000 x g for 15 minutes at 4°C.** The mixture separates into a lower phenol-chloroform phase, an interphase, and an upper aqueous phase. RNA remains entirely in the aqueous phase.

5. **Transfer no more than 80% of the aqueous phase to a fresh tube. Add 1/3 volume of absolute ethanol (96-100%, room temperature)** and vortex at maximum speed for 15 seconds. A precipitate may form at this point. This will not interfere with RNA purification.

6. **Apply no more than 700 µl of sample from step 5 onto HiBind® RNA column**. (Larger volumes can be loaded successively.) A precipitate may form on addition of ethanol in step 5. Vortex and add the entire mixture to the column. With the spin column inside a 2 ml collecting tube (supplied with kit), centrifuge at 10,000 x g for 15-30 seconds **at room temperature**. Discard flow-through and reuse the collection tube.

7. Repeat step 6 by loading the remaining sample to the column, discard flow-through and collection tube.

8. Proceed as step 8-13 on page 6-7.

6. Apply no more than 700 µl of the mixture from step 5 onto HiBind® RNA column. (Larger volumes can be loaded successively.) A precipitate may form on addition of ethanol in step 5. Vortex and add the entire mixture to the column. With the spin column inside a 2 ml collecting tube (supplied with kit), ***centrifuge at 10,000 x g for 30-60 seconds at room temperature.*** Discard flow-through and reuse the collection tube.

7. Repeat step 6 by loading the remaining sample to the column. Centrifuge as above and discard flow-through.

8. **Place column in a new 2 ml collection tube, and add 300 µl RNA Wash Buffer I.** Centrifuge as above and discard flow-through. Reuse the collection tube for step 10. If on-membrane DNase I digestion is desired, proceed step 9, otherwise go to step 10.

9. **DNase I digestion (Optional)**

   Since HiBind® RNA resin and spin-column technology actually removes most of DNA without the DNase treatment, it is not necessary to do DNase digestion for most downstream applications. However, certain sensitive RNA applications might require further DNA removal. Following steps provide on-membrane DNase I digestion:(see DNase I cat.# E1091for detail information)

   a. For each HiBind® RNA column, prepare the DNase I digestion reaction mix as follows:

OBI DNase I Digestion Buffer	73.5 µl
RNase-free DNase I (20 Kunitz unites/µl)	1.5 µl
Total volume	75 µl

Note:

1. **DNase I is very sensitive for physical denaturation, so do not vortex this DNase I mixture. Mix gently by inverting the tube. Prepare the fresh DNase I digestion mixture before RNA isolation.**

2. **OBI DNase I digestion buffer is supplied with OBI RNase-free DNase set**.

3. **Standard DNase buffers are not compatible with on-membrane DNase digestion.**

   b. Pipet 75 µl of the DNase I digestion reaction mix directly onto the surface of HiBind® RNA resin in each column. Make sure to pipet the DNase I digestion mixture directly onto the membrane. DNase I digestion will not be complete if some of the mix stick to the wall or the O-ring of the HiBind® RNA column.

   c. Incubate at room temperature(25-30°C) for 15 minutes

10. **Place column in the collection tube** and add 400 µl RNA Wash Buffer I. **(If on-membrane DNase digestion was performed in the previous step, wait at least 5 minutes before centrifugation).** Centrifuge as above and discard flow-through.

11. **Place column in the same 2 ml collection tube**, and add 500 µl RNA Wash Buffer II diluted with absolute ethanol. Centrifuge as above and discard flow-through. Reuse the collection tube in step 12.

    Note: RNA Wash Buffer II Concentrate must be diluted with absolute ethanol before use. Refer to label on bottle for directions.

12. **Wash column with a second 500 µl of RNA Wash Buffer II as in step 11**. Centrifuge and discard flow-through. Then with the collection tube empty, centrifuge the spin cartridge for **2 min at full speed** (≥13,000 x g) to completely dry the HiBind® matrix.

13. **Elution of RNA.** Transfer the column to a clean 1.5 ml microfuge tube (not supplied with kit) and elute the RNA with 30-50 µl of DEPC water (supplied with kit). Make sure to add water directly onto column matrix. let the column sit at room temperature for 2 minutes and centrifuge for 1 min at full speed. A second elution may be necessary if the expected yield of RNA >50 µg.

    Alternatively, RNA may be eluted with a greater volume of water. While additional elutions increase total RNA yield, the concentration will be lowered since more than 80% of RNA is recovered with the first elution. Pre-heating the water to 70°C before adding to column and incubating column 5 min at room temperature before centrifugation may increase yields.