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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, or bacteria. The kit allows single or multiple, simultaneous processing of samples in less than 30 min. Normally, up to 1 x 10⁶ eukaryotic cells, up to 1 x 10⁹ bacterial cells, or 100 mg tissue 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-Safer II reagent /Phenol solution with OBI's 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-Safer II/Phenol solution 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.

New in this edition

- On-column DNase I digestion protocol included. (Page 5)
- Optional vacuum-spin (for V-Spin column) protocol include.(Page 9)

Storage

All components except RNA-Safer II reagent in E.Z.N.A.[®] Total RNA Kit II should be stored at room temperature. RNA-Safer II should be store at 4C after adding phenol for long term storage. All E.Z.N.A.[™] Total RNA Kit II components are guaranteed for at least 24 months from the date of purchase when stored at 22-25°C.

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

Kit Contents

E.Z.N.A.™ Total RNA Kit II	Trial Kit 5 Preps	RNA Prep 50	RNA Prep 200
Product Number	R6936-00	R6936-01	R6936-02
Purifications	5	50	200
Components			
HiBind™ Columns	5	50	200
2 ml Collection Tubes	10	100	400
RNA-Safer® Reagent II	6 ml	55 ml	220 ml
RNA Wash Buffer I	5 ml	40 ml	200 ml
RNA Wash Buffer II Concentrate	5 ml	12 ml	48 ml
DEPC-ddH ₂ O	1 ml	5 ml	20 ml
Instruction Manual	1	1	1

Note: RNA-Safer® Reagent contains Guanidine Thiocyanate, handle those reagents with extra care. Safety and risk phase: R20-24/25-32-34, S13-26-36/37/39-45. (See page 12 for detail)

Before Starting

IMPORTANT	Wash Buffer II Concentrate must be diluted with absolute ethanol before use.	
	Trial Sample (R6934-00)	Add 20 ml 100 % ethanol
	R6934-01	Add 48 ml 100% ethanol
	R6934-02	Add 192 ml 100% ethanol
	Prepare RNA-safer™ II /Phenol mixture by adding water saturated phenol solution to RNA-Safer Reagent II	
R6934-00	Add 6.0 ml water saturated phenol	
R6934-01	Add 37.5 ml water saturated phenol	

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.
- All centrifugation steps must be carried out at 22°C-25°C.

Disruption & Homogenization of Tissues

A. Disrupt & Homogenize Tissue with Liquid Nitrogen

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-Safer/Phenol solution and continue with the procedure as outlined below. After interrupt tissue, lysate can be homogenized with Omega Homogenizer Spin Column (Product # AC6681). The lysate is loaded onto Omega Homogenizer Spin Column in a 2 ml collection tube. Spin two minutes at a maximum speed in a microcentrifuge and the homogenized lysate collected. Use the Omega Homogenizer Spin Column is fast and efficient way to homogenize the lysate without cross contamination of samples. The alternated way to homogenize the lysate is to use the syringe and needle. High molecular-weight DNA can be sheared with by passing the lysate through a narrow needle (19-21 gauge) for 5-10 times.

B. Disruption and Homogenization with Rotor-Stator

Rotor-Stator is the most preferred method for disruption and homogenizing tissue samples if required equipments are available. Rotor-stator homogenizers effectively homogenize and homogenize most tissues in the present of RNA-Safer II/Phenol solution. 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. Disruption and Homogenization with Beads Mills

Tissue sample can also be effectively disrupted and homogenized by rapid agitation in the presence of beads and RNA-Safer II/Phenol Solution. Tissue samples are disrupted and simultaneously homogenized with the sheared and crushed action of the beads as they collide with cells.

Starting Material:

Although the binding capacity for each well of the HiBind™ RNA column is around 100 µg, however, the maximum amount of starting material depend on the type of the tissue being processed and the corresponding RNA content. **It is essential to begin with correct amount of tissue to get optimal RNA yield and purity with E.Z.N.A™ RNA spin column. For the first time user, we recommend to use less than 10 mg tissue. Depend the yield and purity obtained, it may be possible to increase the starting material to 20 mg.**

E.Z.N.A.® Total RNA Isolation Protocol

A. Eukaryotic Cells and Tissues

Materials supplied by user:

- 70% ethanol in DEPC-treated sterile distilled water
- Sterile RNase-free pipette tips and microcentrifuge tubes
- Disposable latex gloves

Time Considerations:

With E.Z.N.A.® Total RNA Kits, 10 simultaneous samples can be prepared in approximately 25 min.

Procedure:

1. Lyse cells or tissues with 1ml of RNA-Safer/Phenol solution.

1ml of RNA-Safer/Phenol solution is sufficient for 10⁷ 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-Safer II/Phenol solution 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-Safer/Phenol solution, lyse the sample 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-Safer/Phenol solution and proceed to step 2.

2. Incubate the tube contains homogenate at room temperature for 5 minutes.
3. Add 200µl chloroform to the homogenate and vortex for 20 seconds.
4. Centrifuge at 12,000 x g for 10 minutes to separate the aqueous and organic phase.
Note: The sample should be separated into 3 phases: an upper colorless aqueous phase, which contains RNA; a white interphase and a lower blue organic phase.
5. Transfer the upper aqueous phase (around 500ul) into a new 1.5ml centrifuge tube. Add equal volume of 70% ethanol and vortex to mix thoroughly. A precipitate may form at this point. This will not interfere with RNA purification.
6. Apply 700 µl sample onto HiBind® RNA spin column. (Larger volumes can be loaded successively.) A precipitate may form on addition of ethanol in step 2. Vortex and add the entire mixture to the column. With the spin column inside a 2ml collecting tube (supplied with kit), centrifuge at 10,000 x g for 15 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. **Place column in a clean 2ml collection tube**, and add 400 µl RNA Wash Buffer I. Centrifuge and discard flow-through. Reuse the collection tube for step 9. If on-membrane DNase I digestion is desired, proceed step 9, otherwise go to step 10.
9. DNase 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.# E1091 for 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:

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

- Place column in a clean 2ml collection tube**, and add 500 µl RNA Wash Buffer I. **(If on-membrane DNase digestion was performed in the previous step, wait at least 5 minutes before proceeding).** Centrifuge and discard flow-through.
- Place column in the same 2ml collection tube**, and add 600 µl RNA Wash Buffer II diluted with ethanol. Centrifuge and discard flow-through. Reuse the collection tube in step 12.
Note: Wash Buffer II Concentrate must be diluted with absolute ethanol before use. Refer to label on bottle for directions.
- Wash column with a second 500 µl of Wash Buffer II as in step 11. Centrifuge and discard flow-through. Then with the collection tube empty,

centrifuge the spin cartridge for **1 min at full speed** to completely dry the HiBind® matrix.

- Elution of RNA.** Transfer the column to a clean 1.5 ml microfuge tube (not supplied with kit) and elute the RNA with 50-100 µl of DEPC-treated water (supplied with kit). Make sure to add water directly onto column matrix. Centrifuge 1 min at maximum 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.

B. E.Z.N.A.® Protocol for Bacteria

The E.Z.N.A.® Total RNA Kit II can be modified for isolation of RNA from bacterial cultures. Only cells growing at log phase should be used. Measured at 600 nm, an OD of 0.5-1.0 corresponds to ~ 10⁹ cells per ml. This method is suitable for no more than 10⁹ cells. **Note that all centrifugation steps must be carried out at room temperature.**

Additional materials to be supplied by user

- RNase-free Lysozyme
- TE buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA)

Procedure

- Harvest Cells and resuspend in 100 µl TE/lysozyme and incubate at RT for 7 min.

Centrifuge 10⁹ 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.
- Add 1ml of RNA-Safer/Phenol Solution and mix by vortexing for 15 seconds.
- Incubate the tube contains homogenate at room temperature for 5 minutes.
- Add 200µl chloroform to the homogenate and vortex for 20 seconds.
Note: The sample should be separated into 3 phases: an upper colorless

aqueous phase, which contains RNA; a white interphase and a lower blue organic phase.

5. Centrifuge at 12,000 x g for 10 minutes to separate the aqueous and organic phase.
6. Transfer the upper aqueous phase (around 500ul) into a new 1.5ml centrifuge tube. Add equal volume of 70% ethanol and vortex to mix thoroughly. A precipitate may form at this point. This will not interfere with RNA purification
7. Apply 700 µl sample onto HiBind® RNA spin column. (Larger volumes can be loaded successively.) A precipitate may form on addition of ethanol in step 2. Vortex and add the entire mixture to the column. With the spin column inside a 2ml collecting tube (supplied with kit), centrifuge at 10,000 x g for 15 seconds **at room temperature**. Discard flow-through and reuse the collection tube.

Note: this is the point that on-column DNase I digestion can be started. Follow the steps shown on page 6-7 after performing this step.

8. Wash column with 650 µl RNA Wash Buffer I. Centrifuge 15 sec at maximum speed and **discard both flow-through and collecting tube.**
9. Place spin column into **a clean collection tube** (supplied) and add 500 µl RNA Wash Buffer II diluted with ethanol. Centrifuge and discard flow-through as above. Reuse the collection tube in step 7.

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

10. Wash column with a second 500 µl 1 X Wash Buffer II. Repeat step 6 and discard flow-through. Then empty the collection tube and centrifuge the spin cartridge for **1 min at full speed** to completely dry the HiBind™ matrix.
11. **RNA Elution.** Transfer column to a clean 1.5 ml microfuge tube (not supplied with kit) and elute RNA with 50-100 µl DEPC-treated water (supplied with kit). Centrifuge column for 1 min at maximum speed. If the expected RNA yield > 50 µg the a second elution may be required. Elution with two 50 µl aliquots is no more efficient than with one 100 µl aliquot.

The expected yield varies depending on type and strain of bacteria used as well on number of cells and phase of growth at which cells are harvested. 10⁹ cells of *E.coli* typically yield 50-60 µg RNA with an absorbance ratio of 1.7-2.0.

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 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 details0
5. Wash the column by adding 750 µl **RNA wash buffer I**, draw the wash buffer through the column by turn on the vacuum source.
6. Wash the column by adding 500 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 1 minute to dry the column.
8. Place the column in a clean 1.5 ml microcentrifuge tube and add 50-100µl RNase-free water. Stand for 1-2 minute and centrifuge 1 minute to elute RNA.

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-column 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 band 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.

Troubleshooting Tips

Problem	Cause	Suggestion
Little or no RNA eluted	RNA remains on the column	<ul style="list-style-type: none"> Repeat elution. Pre-heat DEPC-water to 70° C prior to elution. Incubate column for 10 min with water prior to centrifugation.
	Column is overloaded	<ul style="list-style-type: none"> Reduce quantity of starting material.
Clogged column	Incomplete homogenization	<ul style="list-style-type: none"> Completely homogenize sample. Increase centrifugation time. Reduce amount of starting material
Degraded RNA	Source	<ul style="list-style-type: none"> 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	<ul style="list-style-type: none"> Ensure not to introduce RNase during the procedure. Check buffers for RNase contamination.
Problem in downstream applications	Salt carry-over during elution	<ul style="list-style-type: none"> 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		<ul style="list-style-type: none"> Digest with RNase-free DNase and inactivate at 75°C for 5 min.
Low Abs ratios	RNA diluted in acidic buffer or water	<ul style="list-style-type: none"> DEPC-treated water is acidic and can dramatically lower Abs₂₆₀ values. Use TE buffer to dilute RNA prior to spectrophotometric analysis.