Contents

Introduction

E.Z.N.A.® Total RNA Kit 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 10^7 eukaryotic cells, up to 1 x 10^9 bacterial cells, or 30 mg tissue (amounts depend on the tissue used) can be used in a single experiment. There is no need for phenol/chloroform extractions, and time-consuming steps such as CsCl gradient ultracentrifugation, and precipitation with isopropanol or LiCl, are eliminated. 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 # R6814) 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 Kits use the reversible binding properties of HiBind® matrix, a new silica-based material. This is combined with the speed of mini-column spin technology. 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 lysed under denaturing conditions that practically inactivate RNases. Samples are 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

E.Z.N.A.® Total RNA Kits should be stored at room temperature. During shipment crystals may form in the TRK Lysis Buffer. Warm to 37°C to dissolve. All E.Z.N.A.™ Total RNA Kit 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

Product Number	R6834-00	R6834-01	R6834-02
Purification times	5	50	200
HiBind™ RNA Mini column	5	50	200
2 ml Collection Tubes	15	150	600
TRK Lysis Buffer	5 ml	30 ml	125 ml
RNA Wash Buffer I	5 ml	40 ml	4X40 ml
RNA Wash Buffer II	2 ml	12 ml	4X12 ml
DEPC water	1 ml	5 ml	20 ml
Instruction Manual	1	1	1

Before Starting

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.

IMPORTANT	RNA Wash Buffer II must be diluted with absolute ethanol (96-100%) before use and store at room temperature.
	R6834-00 Add 8 ml absolute ethanol
	R6834-01 Add 48 ml absolute ethanol
	R6834-02 Add 48 ml absolute ethanol

- 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.
- Under cool ambient conditions, crystals may form in TRK Lysis Buffer. This is normal and the bottle should be warmed to redissolve the salt.
- 2-mercaptoethanol (ß-mercaptoethanol) is key in denaturing RNases and must be added to an aliquot of TRK Lysis Buffer before use. Add 20µl of 2-mercaptoethanol per 1 ml of TRK Lysis Buffer. This mixture can be stored for 1 week at room temperature.
- All centrifugation steps must be carried out at 22°C-25°C.

Disruption and Homogenization of Tissues

A. Disruption Tissue with 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 TRK Lysis Buffer and continue with the procedure as outlined below. After disruption of tissue, the lysate can be homogenized with an Omega Homogenizer Spin Column. The lysate is loaded into an Omega Homogenizer Spin Column inserted into a 2ml collection tube and centrifuged two minutes at maximum speed to collect the homogenized lysate. Use of the Omega Homogenizer Spin Column is a fast and efficient way to homogenize the lysate without cross contamination of samples. An alternate method for homogenizing the lysate is by use of a syringe and needle, can be sheared by passing the lysate through a narrow needle (19-21 gauge) six to ten times.

B. Disruption and Homogenization with Rotor-Stator

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:

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

C. Disruption and Homogenization with Beads Milling Method

Tissue sample can also be effectively disrupted and homogenized by rapid agitation in the presence of beads and TRK Lysis Buffer. Tissue samples are disrupted and simultaneously homogenized by the shearing and crushing action of the beads as they collide with cells.

E.Z.N.A.® Total RNA Kit I protocol

A. Eukaryotic Cells and Tissues
Materials supplied by user:

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

Procedure of cells:

1. Lyse cells with TRK Lysis Buffer in a microfuge tube. **For pelleted cells, loosen the cell pellet thoroughly by flicking the tube. Add the appropriate volume of TRK Lysis Buffer and vortex or pipet to mix.** *Remember to add 20 µl of 2-mercaptoethanol per 1 ml of TRK Lysis Buffer before use.*

   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 TRK Lysis Buffer directly to the cells. Use 600 µl for T35 flasks or 10 cm dishes, and 350 µl for smaller vessels. Pipette buffer over entire surface of vessel to ensure complete lysis. Transfer lysate to a clean 1.5 ml 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 (400xg) for 5 min. Discard supernatant, for <5 x 10^6 cells, add 350µl of TRK Lysis Buffer; For <1x 10^7 cells, add 600µl of TRK Lysis Buffer, lyse by vortexing or pipetting up and down, and transfer to a clean 1.5 ml tube. Proceed to step 2.

2. **Homogenize the lysate according to step 2a, 2b or 2c.**

   See 'Disruption and Homogenization of samples' on page 4 for more details on homogenization. If processing ≤1x 10^5 cells, homogenize by vortexing for 1 min. Incomplete homogenization leads to significantly reduced RNA Yield and can cause clogging of column.

   2a. Homogenize the lysate for 30 seconds ***using a rotor-stator homogenizer***. Proceed to step 3.

   2b. Pass the lysate at least 5 times through ***a blunt 20-gauge needle*** (0.9 mm diameter) fitted to an Rase-Free syringe. Proceed to Step 3.

   2c. Transfer the lysis into **Omega Homogenizer Spin Column** and centrifuge at 10,000 x g for 1 minutes. Transfer the flow-through into a new tube and proceeds to step 3.

Procedure of Tissue:

1. Lyse tissues with TRK Lysis Buffer in a microfuge tube. **Remember to add 20 µl of 2-mercaptoethanol per 1 ml of TRK Lysis Buffer before use.**

   350µl of TRK Lysis Buffer is sufficient approximately 20 mg disrupted tissue (~3 mm cube). For greater than 20 mg tissue, use 600 µl of TRK Lysis Buffer. However, use no more than 30 mg tissue when the recommended maximum is exceeded.

   For tissue samples, homogenize using one of the methods discussed on page 4. Unless using liquid nitrogen, homogenize samples directly in TRK Lysis Buffer/2-Mercaptoethanol and proceed to Step 2.

2. **Centrifuge at maxi speed (>14,000 x g) for 5 min at room temperature. Transfer the supernatant into a new 1.5 ml tube and proceed to step 3.**

Purify RNA by using HiBind™ RNA Column

3. Add an equal volume (350µl or 600µl) 70% ethanol to the lysate and mix thoroughly by pipetting.

4. Apply sample onto HiBind® RNA Mini column. The maximum capacity of the spin cartridge is 750µl. (Larger volumes can be loaded successively.) A precipitate may form on addition of ethanol in step 3. Vortex and add the entire mixture to the column. With the spin column inside a 2ml collection tube (supplied with kit), centrifuge at 10,000 x g for 30-60 seconds **at room temperature**. Discard flow-through and proceed to step 5.

5. **Place column in a New 2ml collection tube**, and add 300µl RNA Wash Buffer I. Centrifuge at 10,000 x g for 30-60 seconds at room temperature and discard flow-through. Reuse the collection tube in step 6. If on-membrane DNase I digestion is desired, proceed to step 6, otherwise go to step 7.

6. **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 denaturaion, 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.

7. **Place column in the same 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 at 10,000×g for 30-60 seconds at room temperature and discard flow-through.

8. **Place column in a new 2ml collection tube**, and add 500µl RNA Wash Buffer II diluted with ethanol. Centrifuge at 10,000 x g for 30-60 seconds at room temperature and discard the flow-through. Reuse the collection tube in step 9.

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

9. Wash column with 500µl RNA Wash Buffer II as in step 8. Centrifuge at 10,000 x g for 30-60 seconds at room temperature and discard the flow-through. Then with the collection tube empty, centrifuge the spin cartridge at 10,000 x g for **2 min** at room temperature to completely dry the HiBind® matrix.

10. **Elution of RNA.** Transfer the column to a clean 1.5 ml microfuge tube (not supplied with kit) and elute the RNA with 30-50µl of DEPC-treated water (supplied with kit). Make sure to add water directly onto column matrix. Centrifuge at 10,000xg for 1 min at room temperature. A second elution may be necessary if the expected yield of RNA >30 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. Extraction of RNA from Blood

Note: This method yields adequate RNA for RT-PCR. For more efficient RNA isolation, we strongly recommend the E.Z.N.A.® Blood RNA Kit(Product # R6614) . All centrifugation steps must be carried out at room temperature.

Additional materials required by user

- Water bath or heat block preset at55°C
- RNase-free Proteinase K

Prepare a Proteinase K solution by preparing 450 µl TRK Lysis Buffer/2-mercaptoethanol containing 4mg/ml Proteinase K. This protocol has been tested successfully on fresh whole blood treated with all forms of anticoagulant. The Product is suitable for RT-PCR and detects RNA molecules ≥200nt. For more sensitive work we highly recommend the E.Z.N.A.® Blood RNA Kit which specifically lyses and removes erythrocytes prior to leukocyte lysis. This eliminates many inhibitors of PCR such as hemoglobin.

1. Pipette 100µl of blood into a sterile microcentrifuge tube.

2. Add 350 µl of TRK Lysis Buffer/2-ME containing 4mg/ml Proteinase K.

3. Incubate at 55°C for 10 minutes. Mix the sample twice by inversion during the incubation.

4. Centrifuge sample at 10,000xg for 3 min and transfer 450µl supernatant to a sterile microfuge tube.

5. Transfer the lysate directly into a Homogenization Spin Column. Placed in 2ml collection tube. Centrifuge at ≥12,000xg for 5 minutes at room temperature.

6. Add 225µl of absolute ethanol to the mixture, vortex for 10 seconds, and proceed to step 4, (page 6) of main protocol (addition of samples to RNA HiBind column collection tube assembly).

C. Extraction of RNA for Bacteria

Additional materials to be supplied by user

- RNase-free Lysozyme
- TE buffer (10mM Tris-HCl, PH7.6, 1mM EDTA)

1. Harvest cells and resuspend in 100µl TE Buffer/lysozyme and incubate at RT for 7 minutes.

Centrifuge 10^9 cells at 4000xg for 5 minute. Discard supernatant and add 100 µl of TE Buffer containing lysozyme(0.5mg/ml for Gram-negative and 4mg/ml for Gram-positive bacteria). Resuspend cells completely and incubate at room temperature for 7 min.

2. Add 350µl TRK Lysis Buffer and mix by pipetting several times. **Remember to add 20 µl of 2-mercaptoethanol per 1 ml of TRK Lysis Buffer**.

3. **Centrifuge at >10,000 x g for 3 min.** Transfer the flow-through lysate into a new 1.5 ml tube.

4. Add 250µl absolute ethanol to lysate and mix by vortexing. A precipitate may form at this point. This will not interfere with RNA purification.

5. **Apply sample (approximately 700 µl) from step 4 to a HiBind RNA Mini column.** With the column mounted in a clean 2ml collection tube(supplied with Kit) centrifuge for 1 min at maximum speed (at room temperature) in a microcentrifuge. Discard flow-through and proceed to Step 6.

6. Wash column with 750µl RNA Wash Buffer I. Centrifuge 15 second at maximum speed and discard both flow-through and collection tube.

7. Place spin column into a clean collection tube(supplied) and add 500µl RNA Wash Buffer II diluted with ethanol. Centrifuge and discard flow-through as above. Reuse the collection tube in Step 8.

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

8. Wash column with a second 500µl RNA Wash Buffer II; discard flow- through. Re-Using the same collection tube, centrifuge the spin cartridge for 1 min at full speed to completely dry the HiBind Matrix.

9. **RNA Elution:** Transfer the spin column to a clean 1.5ml microfuge tube (not supplied with Kit) and elute RNA with 50-100µl DEPC-treated water (supplied with Kit). Centrifuge column for 1 minute at maximum speed. If the expected RNA Yield >50 µg, then a second elution may be required. Elution with two 50 µl aliquots is no more efficient than with one 100 µl aliquot.

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

Carry out lysis, homogenize, and load onto HiBind® RNA Minicolumn as indicated previous protocols. Instead of continuing 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 instruction and connect the HiBind® RNA V-Spin column to the manifold.

2. **Load the homogenized sample onto 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 750µl **RNA wash buffer I**, draw the wash buffer through the column by turn on the vacuum source.

6. Wash the column by adding 500µl **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-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 40µg 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.

Trouble Shooting Guide

Little or no RNA eluted	RNA remains on the column	• 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	• 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-treated water is acidic and can dramatically lower Abs260 values. Use TE buffer to dilute RNA prior to spectrophotometric analysis.