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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 10⁷ eukaryotic cells, up to 1 x 10⁹ bacterial cells, or 30 mg tissue 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 use of 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 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 specially 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.

New in this edition

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

Storage

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

E.Z.N.A. [®] Total RNA Kit I	Trial Kit 5 Preps	RNA Prep 50	RNA Prep 200
Product Number	R6634-00 R6834-00 R6934-00	R6634-01 R6834-01 R6934-01	R6634-02 R6834-02 R6934-02
Purifications	5	50	200
HiBind [®] Columns	5	50	200
Homogenizer ZColumn	5	50	200
2 ml Collection Tubes	15	150	600
TRK Lysis Buffer	5 ml	40 ml	150 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
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Before Starting

	Wash Buffer II Concentrate must be diluted with absolute ethanol before use.	
IMPORTANT	Trial Sample (R6634-00/R6834-01/R6934-01)	Add 20 ml 100 % ethanol
	R6634-01/R6834-01/R6934-02 R6634-02/R6834-02/R6934-02	Add 48 ml 100% ethanol Add 192 ml 100% ethanol

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.

- 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 (β -mercaptoethanol) is key in denaturing RNases and must be added to an aliquot of TRK Lysis Buffer before use. Add 20 μ l 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.

Homogenization of Tissues

Disruption & Homogenization of Tissues

A. Disruption 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 TRK Lysis Buffer and continue 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 2 ml 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. High molecular-weight DNA, responsible for viscosity in cell lysates, 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 is the most preferred method for disrupting and homogenizing tissue samples, if available. Rotor-stator homogenizers effectively homogenize most tissues in the presence of TRK Lysis Buffer. The process usually takes less than a minute, depending on the type of tissue. Many Rotor-stator homogenizers operate with various sized probes or generators that allow for the 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 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 Isolation 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

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 and homogenize in 400 µl of TRK Lysis Buffer. **Remember to add 20 µl of 2-mercaptoethanol per 1 ml of TRK Lysis Buffer before use.**

400 µl of TRK Lysis Buffer is sufficient for 10⁷ cells or approximately 30 mg disrupted tissue (~3 mm cube). For difficult tissues, more than 10⁷ cells, or greater than 30 mg tissue, use 700 µl of TRK Lysis Buffer. However, use no more than 40 mg tissue when the recommended maximum is exceeded.

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 700 µl for T35 flasks or 10 cm dishes, and 400 µl for smaller vessels. 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 TRK Lysis Buffer, 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, 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. **Transfer the lysate directly into a Homogenization Spin Column, placed in 2ml collection tube. Centrifuge at $\geq 12,000 \times g$ for 5 min at room temperature.** Transfer the flow-through lysate into a new 1.5 ml tube.
3. Add an equal volume (400 µl or 700 µl) 70% Ethanol to the lysate and mix thoroughly by vortexing.
4. Apply sample to HiBind[®] RNA spin column. The maximum capacity of the spin cartridge is 800 µl. (Larger volumes can be loaded successively.) A precipitate may form upon addition of ethanol in Step 2. 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 15 seconds **at room temperature**. Discard flow-through and proceed to Step 5.
5. **Place column in a clean 2ml collection tube**, and add 300 µl RNA Wash Buffer I. Centrifuge and discard flow-through. Reuse the collection tube in Step 6. If on-membrane DNase I Digestion is desired, proceed to Step 5, otherwise go to Step 7.
6. **DNase Digestion (Optional)**

Since the HiBind[®] RNA resin and spin-column technology actually remove most 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. The following steps are to be followed for on-membrane DNase I Digestion. (See DNase I Cat.# E1091for detailed 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 and subject to physical denaturing, so do not vortex the 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 the HiBind® RNA membrane 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 sticks to the wall or to the O-ring of the HiBind® RNA column.

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

7. **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.

8. **Place column in the same 2ml collection tube** and add 500 µl RNA Wash Buffer II diluted with ethanol. Centrifuge and discard flow-through. Reuse the collection tube in Step 9.

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

9. Wash column with a second 500 µl of Wash Buffer II as in Step 8 . 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.

10. **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. 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

- RNase-free Proteinase K
- Water bath preheated to 65°C.

Prepare a Proteinase K solution by preparing 450 µl TRK Lysis Buffer/2-mercaptoethanol containing 4 mg/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 ≥200 nt. 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 100 µl of blood into a sterile microcentrifuge tube.
2. Add 350 µl of TRK Lysis Buffer/β-mercaptoethanol containing 4 mg/ml Proteinase K and **vortex for 30 seconds** to thoroughly mix.
3. Incubate at 70°C for 10 minutes. Mix the sample twice by inversion during the incubation.
4. Centrifuge sample at 10,000 x g for 3 min and transfer 450 µl supernatant to a sterile microfuge tube.
5. **Transfer the lysate directly into a Homogenization Spin Column, placed in 2ml collection tube. Centrifuge at ≥12,000 x g for 5 min at room temperature.** Transfer the flow-through lysate into a new 1.5 ml tube.
6. Add 225 µl of absolute ethanol to the mixture, vortex for 10 seconds, and proceed to step 4, (page 6) of main protocol (addition of sample to RNA HiBind® column/collection tube assembly).

C. E.Z.N.A.® Protocol for Bacteria

The E.Z.N.A.® Total RNA Kit 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

1. Harvest Cells and resuspend in 100 µl TE/lysozyme and incubate at RT 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 350 µl of TRK Lysis Buffer and mix by pipetting several times. **Remember to add 20 µl of β-mercaptoethanol per 1 ml of TRK Lysis Buffer.**
3. **Transfer the lysate directly into a Homogenization Spin Column, placed in 2ml collection tube. Centrifuge at $\geq 12,000$ x g for 5 min at room temperature.** Transfer the flow-through lysate into a new 1.5 ml tube.
4. Add 250 µl 100% 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 3 to an HiBind® RNA spin column. With the column mounted in a clean 2ml collection tube (supplied with kit) centrifuge 15 sec at maximum speed (at room temperature) in a microcentrifuge. Discard flow-through and proceed to Step 5.

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

6. Wash column with 750 µl RNA Wash Buffer I. Centrifuge 15 sec at maximum speed and **discard both flow-through and collection tube.**
7. 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.

8. Wash column with a second 500 µl 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 1.5 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, then 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 as number of cells and phase of growth at which cells are harvested. 10^9 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 in previous protocols. Instead of continuing with centrifugation, follow steps below.

Note: Please read through previous sections of this manual before using this protocol.

1. Prepare the vacuum manifold according to manufacturer's instructions 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 then 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 750 µl **RNA wash buffer I**, draw the wash buffer through the column by turning on the vacuum source.
6. Wash the column by adding 500 µl **RNA wash buffer II**, draw the wash buffer through the column by turning 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 remove most of the DNA without DNase treatment. However, no RNA extraction procedure can completely remove genomic DNA. For sensitive work (perhaps 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.