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Introduction

E.Z.N.A.® Total RNA Midiprep Kit provides a rapid and easy method for the isolation of up to 600 µg of total RNA from cultured eukaryotic cells, tissues, or bacteria. The kit allows single or multiple, simultaneous processing of samples in less than 40 min. Normally, 5 x 10^6 - 1 x 10^8 eukaryotic cells, 5 x 10^8-1 x 10^{10} bacterial cells, or 25- 200 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 you use the E.Z.N.A.® Blood RNA Kit (product # R6614/R6615) 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 Midiprep 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 600 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. After the homogenization process by either bead-milling or rotor-stator homogenizer, samples are then applied to the HiBind® Midi spin columns to which total RNA binds, after few quick washing step, 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 Midi 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 Midiprep 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	R6664-00	R6664-01	R6664-02
Purification times	2 preps	10 preps	25 preps
HiBind™ RNA Midi Columns	2	10	25
15 ml Collection Tubes	4	20	50
TRK Lysis Buffer	10 ml	50 ml	100 ml
RNA Wash Buffer I	10 ml	50 ml	2 X50 ml
RNA Wash Buffer II	5 ml	12 ml	3 X12 ml
DEPC water	1 ml	10 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 Concentrate must be diluted with absolute ethanol before use.	
	R6664-00	Add 20 ml 100% ethanol
	R6664-01	Add 48 ml 100% ethanol
	R6664-02	Add 48 ml 100% 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 20 µl 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 samples

Efficient disruption and homogenization of the sample is essential for successfully isolating total RNA. Completely disruption of the cell walls and plasma membrane is very important for releasing all the RNA containing in the samples. The purpose of homogenization is to reduce the viscosity of the cell lysates produced by cell disruption. Homogenization shears the genomic DNA and other high molecular weight cell components to create a homogeneous lysate. Incompletely homogenization will reduce the binding of RNA to the RNA column and sometimes will clog the RNA column thus cause lower yield or no yield.

Disruption of sample 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 with the procedure as outlined below. This is the preferred method of disrupting tissue samples.

Disruption & homogenization of sample with Rotor-Stator Homogenizers

Rotor-stator homogenizers can effectively simultaneously disrupt and homogenize most samples. 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.

Disruption & homogenization of sample using Bead Milling

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

Homogenization of lysate with 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).

E.Z.N.A.® Total RNA Midi Kit Protocol

A. Eukaryotic Cells and Tissues

Materials supplied by user:

- 2-mercaptoethanol
- Swinging-bucket centrifuge capable of 5000 x g with adaptor for 15 ml tube.
- 70% ethanol in DEPC-treated sterile distilled water
- Sterile RNase-free pipette tips and 15 centrifuge tubes
- Disposable latex gloves

Procedure:

1. Disrupt cells or tissues with 2 ml of TRK Lysis Buffer. **Remember to add 20 µl of 2-mercaptoethanol per 1 ml of TRK Lysis Buffer before use.** Homogenize cells with a rotor-stator homogenizer or vortex.

   2 ml of TRK Lysis Buffer is sufficient for 10^8 cells or approximately 200 mg disrupted tissue (~3 mm cube). For difficult tissues, more than 10^8 cells, or greater than 200 mg tissue, use 4 ml of TRK Lysis Buffer. However, use no more than 300 mg tissue.

   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 800 µl for each T35 flasks or 10 cm dishes, and 400 µl for each smaller vessels. Pipette buffer over entire surface of vessel to ensure complete lysis. Transfer lysate from all flask to a clean 15 or 50 ml centrifuge 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 pipetting up and down, and transfer to a clean 15 or 50 ml microfuge tube. Proceed to step 2.

   For **tissue** samples, homogenize using one of the methods discussed on page 4. Unless using liquid nitrogen, disrupt and homogenize samples directly in TRK Lysis Buffer/2-mercaptoethanol and proceed to step.
   Note: incomplete homogenization of the sample will cause lower yields and clogging of the column. It is recommended to homogenize the sample with rotor-stator homogenizers since it normally produce better yield.

2. Spin at 5,000 x g for 10 min at room temperature. Transfer the supernatant into a new tube and add an equal volume (2ml or 4ml) 70% Ethanol to the lysate and mix thoroughly by vortexing.

3. Apply sample onto HiBind® RNA Midi column. The maximum capacity of the Midi column is 3.5 ml. (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 the 15 ml collecting tube (supplied with kit), centrifuge at 4,000-5,000x g for 5 min **at room temperature**. Discard flow-through and proceed to step 4.

4. Wash column with RNA Wash Buffer I by pipetting 3.5 ml directly into the spin column. Centrifuge as above and **discard the 15 ml collection tube**.
   Note: This the starting point if on-membrane Dnase I digestion (page 6) is desired.

5. **Place column in a clean 15 ml collection tube**, and add 3 ml RNA Wash Buffer II diluted with ethanol. Centrifuge at 4,000-5,000 x g for 5 minutes at room temperature. Discard flow-through and reuse the collection tube in step 6.
   Note: RNA Wash Buffer II Concentrate must be diluted with absolute ethanol before use. Refer to label on bottle for directions.

6. Wash column with a second 3 ml of RNA Wash Buffer II as in step 5. Centrifuge and discard flow-through. Then with the collection tube empty, centrifuge the spin cartridge for **10 min at 5000 x g** to completely dry the HiBind® matrix.

7. **Elution of RNA.** Transfer the column to a clean 15 ml centrifuge tube (Not supplied) and elute the RNA with 250-500µl of DEPC-treated water (supplied with kit). Make sure to add water directly onto column matrix. Centrifuge 5 min at 4,000-5,000 x g. A second elution may be necessary if the expected yield of RNA >500 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. DNase I digestion Protocol (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)

1. Follow the standard protocol until the samples **completely** pass through the HiBind RNA column (step1-4). Prepare the following:
   a. For each HiBind® RNA column, prepare the DNase I digestion reaction mix as follows:

OBI DNase I Digestion Buffer	176 µl
RNase-free DNase I (20 Kunitz unites/µl)	4 µl
Total volume	180 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 180 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

2. **Place column into a new 15 ml collection tube**, and add 2 ml RNA Wash Buffer I. **Place the column at benchtop for 5 minutes.** Centrifuge at 4,000-5,000 x g for 5 minutes and discard flow-through. Reuse the collection tube in step 3.

3. **Place column in the same 8ml collection tube**, and add 2ml RNA Wash Buffer II diluted with ethanol. Centrifuge at 4,000-5,000 x g for 5 minutes and discard flow-through. Reuse the collection tube in step 4.
   Note: Wash Buffer II Concentrate must be diluted with absolute ethanol before use. Refer to label on bottle for directions.

4. Wash column with a second 2 ml of Wash Buffer II as in step 3. Centrifuge and discard flow-through. Then with the collection tube empty, centrifuge the spin cartridge for **5 min at 5000 x g** to completely dry the HiBind® matrix.

5. **Elution of RNA.** Transfer the column to a clean 15 ml microfuge tube (not supplied with kit) and elute the RNA with 250- 500 µl of DEPC-treated water (supplied with kit). Make sure to add water directly onto column matrix. Let it stand for 1 minute. Centrifuge 3 min at 8000x g to elute RNA. 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.

C. 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/R6615).** All centrifugation steps must be carried out at room temperature.

Additional materials required by user

- RNase-free Proteinase K
- Water bath preheated to 65oc.

Prepare a Proteinase K solution by preparing 2ml 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 500 µl of blood into a sterile 15 ml tube.

2. Add 2 ml of TRK Lysis Buffer/ß-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 4000-5,000 x g for 10 min and transfer 2.5 ml supernatant to a sterile 15ml tube.

5. Add 1.25 ml of absolute ethanol to the mixture, vortex for 30 seconds, and proceed to step 3, (page 6) of main protocol (addition of sample to RNA HiBind® midicolumn/collection tube assembly).

D. 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 ~ 10^{10} cells per ml. This method is suitable for no more than 10^{10} cells.

Note : 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 500 µl TE/lysozyme and incubate at RT for 7 min.
   Centrifuge 10^{10} 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 2 ml of TRK Lysis Buffer and mix by pipetting several times. **Remember to add 20 µl of ß-mercaptoethanol per 1 ml of TRK Lysis Buffer.**

3. Add 1.5 ml 100% ethanol to lysate and mix by vortexing. A precipitate may form at this point. This will not interfere with RNA purification.

4. Apply sample (approximately 3.5 ml) from step 3 to an HiBind® RNA Midispin column. With the column mounted in a clean 15 ml collection tube (supplied with kit) centrifuge 5 min at 5000 x g (at room temperature) in a centrifuge. Discard flow-through and proceed to step 5. (This is the starting point for optional DNase I digestion treatment, see page 6 for protocol)

5. Wash column with 3ml RNA Wash Buffer I. Centrifuge 5 min at maximum speed and **discard both flow-through and collecting tube.**

6. Place spin column into **a 8 ml clean collection tube** (supplied) and add 3 ml 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.

7. Wash column with a second 3 ml 1 X Wash Buffer II.

Repeat step 6 and discard flow-through. Then empty the collection tube and centrifuge the spin cartridge for **10 min at 4000-5000 x g** to completely dry the HiBind™ matrix.

8. **RNA Elution.** Transfer column to a clean 15 ml collection tube (Not supplied) and elute RNA with 250-500µl DEPC-treated water (supplied with kit). Centrifuge column for 10 min at 4,000-5,000 x g. If the expected RNA yield > 500 µg the a second elution may be required. Elution with two 250 µl aliquots is no more efficient than with one 500 µl aliquot.

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	• 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.