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Introduction

The E.Z.N.A.® HP Total RNA Kit is designed for the isolation of up to 100 ug of total RNA from wide range cultured cells or tissues. This Kit allows for the simultaneous processing of multiple samples in less than 30 min. By using a specially designed DNA Removal Column, genomic DNA contamination is significantly reduced. Up to 1 x 107 eukaryotic cells or 30 mg tissues can be processed in 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.® HP Total RNA Kit uses 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.® HP Total RNA Kits should be stored at room temperature. During shipment crystals may form in the Buffer GTC. 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	R6812-00	R6812-01	R6812-02
Purification Times	5 Preps	50 Preps	200 Preps
HiBind™ RNA Mini column	5	50	200
2 ml Collection Tubes	15	150	600
gDNA Removal Column	5	50	200
Buffer GTC	5 ml	30 ml	125 ml
RNA Wash Buffer I	5 ml	45 ml	4 x 45 ml
RNA Wash Buffer II	2 ml	12 ml	4 x 12 ml
DEPC water	-	15 ml	40 ml
Instruction Manual	1	1	1

Before Starting

IMPORTANT		
	RNA Wash Buffer II must be diluted with absolute ethanol (96-100%) before use and store at room temperature.	
	R6812-00	Add 8 ml absolute ethanol
	R6812-01	Add 48 ml absolute ethanol
	R6812-02	Add 48 ml absolute ethanol to each bottle

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.
- Under cool ambient conditions, crystals may form in Buffer GTC. 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 Buffer GTC before use. Add 20µl of 2-mercaptoethanol per 1 ml of Buffer GTC. This mixture can be stored for 1 week at room temperature.

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 Mortar and Pestle

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 Buffer GTC and continue with the procedure as outlined below. After interrupt tissue, lysate can be homogenized with Homogenizer Spin Column (Product # HCR 002). The lysate is loaded onto Homogenizer Spin Column in a 2 ml collection tube. Spin for 2 minutes at a maximum speed in a micro centrifuge and the homogenized lysate is collected. Use the Omega Homogenizer Spin Column is a 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 lyrate through a narrow needle (19-21 gauge) for 5-10 times.

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 sample in 50ml tubes.

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 Needle Method

High molecular weight DNA is responsible for the viscosity of cell lysates and can be shredded by passing the sample 10-20 times through a narrow needle (19-21 gauge).

E.Z.N.A.® HP Total RNA Kit Spin Protocol

A. Eukaryotic Cells and Tissues

Materials supplied by user

- 2-mercaptoethanol
- absolute ethanol (96-100%)
- Sterile RNase-free pipette tips and microcentrifuge tubes

Determine the proper amount of starting material

This is critical to use correct amounts cells or tissues to obtain optimal yield and purity with HiBind® RNA column. The maximum amounts of cells or tissues that can be processed on a HiBind® RNA column varies depends on the specific RNA contents and type of cell lines or tissues. The maximum binding capacity of the HiBind® RNA column is 100µg. The maximum amount of the cells or tissues that Buffer GTC used in the this protocol is 1 x 10^7 or 30 mg.

Procedure of Cells

1. Lyse cells (≤1 x 10^7) with 500 µl of Buffer GTC in a microfuge tube. **For pelleted cells, loosen the cell pellet thoroughly by flicking the tube. Add the appropriate volume of Buffer GTC and vortex or pipet to mix.** *Remember to add 20 µl of 2-mercaptoethanol per 1 ml of Buffer GTC 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 Buffer GTC directly to the cells. Use 700 µl for T35 flasks or 10 cm dishes, and 500 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, add Buffer GTC, 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 or 2b.**

   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.

3. **Transfer the lysate into a gDNA Removal Column placed in 2 ml collection tube. Centrifuge at 14,000 x g for 3 minutes at room temperature**. Transfer the flow-through lysate into a new 1.5 ml tube. Proceed Step 4.

Procedure of Tissues

1. Lyse tissues (≤30 mg) with 500 µl of Buffer GTC in a microfuge tube. **Remember to add 20 µl of 2-mercaptoethanol per 1 ml of Buffer GTC before use.**

   500µl of Buffer GTC is sufficient approximately 30 mg disrupted tissue (~3 mm cube). For difficult tissues, greater than 20 mg tissue, use 700 µl of Buffer GTC. However, use no more than 40 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 Buffer GTC/2-Mercaptoethanol and proceed to Step 2.

2. **Centrifuge at maxi speed (>14,000 x g) for 5 min at room temperature.**

3. **Transfer the supernatant into a gDNA Removal Column placed in 2 ml collection tube. Centrifuge at 14,000 x g for 2 minutes at room temperature**. Transfer the flow-through lysate into a new 1.5 ml tube.

   Note: In some preparations, a fatty upper layer will form after the centrifugation, will form, transferring any pellet or fatty layer may reduce the RNA yield or clog the column.

Total RNA Isolation

4. **Add 0.5 volume (250µl or 350µl) of absolute ethanol (room temperature) to the lysate and mix well by pipetting.**

5. **Apply the 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 4. Vortex and add the entire mixture to the column. With the spin column inside a 2 ml collection tube (supplied with kit), ***centrifuge at 10,000 x g for 30-60 seconds min at room temperature.*** Discard flow-through and proceed to step 6.

6. **Place column in a new 2 ml collection tube and add 300µl RNA Wash Buffer I.** Centrifuge as above and discard flow-through. Reuse the collection tube in step 7. If on-membrane DNase I digestion is desired, proceed to step 7, otherwise go to step 8.

7. **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.

8. **Place column in a new 2 ml collection tube and add 400µl RNA Wash Buffer I**. (If on-membrane DNase digestion was performed in the previous step, wait at least 5 minutes before proceeding). Centrifuge as above and discard flow-through.

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

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

10. **Wash column with a second 500 µl of RNA Wash Buffer II as in step 9.** Centrifuge as above 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**.

11. **Transfer the column to a clean 1.5 ml micro centrifuge 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. let it sit at room temperature for 2 minutes and centrifuge at 10,000 x g for 1 min. 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 65°C before adding to column and incubating column 5 min at room temperature before centrifugation may increase yields.

B. Extraction of RNA from Fibrous tissue

Tissues such as skeletal muscle, heart and aorta tissues normally make RNA isolation more difficult because they contains contractile proteins, connective tissue and collagen. For more efficient RNA isolation, we strongly recommend the E.Z.N.A.® Tissue RNA Kit (Product # R6688).

Additional materials required by user

- Water bath or heat block preset at 55°C
- RNase-free Proteinase K (20mg/ml)

1. Lyse tissues (≤25 mg) with 300 µl of Buffer GTC in a microfuge tube. **Remember to add 20 µl of 2-mercaptoethanol per 1 ml of Buffer GTC before use.** homogenize using one of the methods discussed on page 4. Unless using liquid nitrogen, homogenize samples directly in Buffer GTC/2-Mercaptoethanol and proceed to Step 2.

2. **Dilute the lysate with 590 µl of DEPC Water and vortex to mix well.**

3. **Add 10 µl of RNase-Free Proteinase K to the lysate and vortex to mix well.** Incubate at 55°C for 10 minutes. Mix the sample twice by inversion during the incubation.

4. **Centrifuge sample at 14,000 x g for 5 min at room temperature . Transfer the supernatant into a gDNA Removal Column placed in 2 ml collection tube. Centrifuge at 14,000 x g for 1 minutes at room temperature**. Transfer the flow-through lysate into a new 1.5 ml tube.

5. **Add 0.5 volume of absolute ethanol (room temperature) to the mixture and mix well by pipetting.**

6. Proceed to step 4, (page 6) of main protocol (addition of samples to RNA HiBind RNA column).

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 # R6814**)**. 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 (20mg/ml)

1. Pipette 100µl of whole blood into a sterile 1.5 ml microcentrifuge tube.

2. **Add 300 µl of Buffer GTC/2-mercaptoethanol. Vortex at maxi speed for 20 seconds.**

3. **Dilute the lysate with 590 µl of DEPC Water and vortex to mix well.**

4. **Add 10 µl of RNase-Free Proteinase K to the lysate and vortex to mix well.** Incubate at 70°C for 10 minutes. Mix the sample twice by inversion during the incubation.

5. **Centrifuge sample at 14,000 x g for 5 min at room temperature. Transfer the supernatant to a new 1.5 ml collection tube.**

6. **Add 0.5 volume of absolute ethanol (room temperature) to the mixture and vortex at maxi speed for 20 seconds**, and proceed to step 4, (page 6) of main protocol (addition of samples to RNA HiBind RNA column).

D. Extraction of RNA for Bacteria

The E.Z.N.A.® HP Total RNA Kit can be modified for isolation of RNA from bacterial cultures. Only cells growing at log phase should be used. Measured at 600nm, and OD of 0.5-1.0 corresponds to ~10^9 cells per ml. This method is suitable for no more than 10^9cells. Note that all centrifugation steps must be carried out at room temperature.

Additional materials to be supplied by user

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

Procedure

1. Harvest cells and resuspend in 100µl TE Buffer/lysozyme and incubate at RT for 10 minutes.

   Centrifuge 10^9 cells at 4000 × g 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 500µl Buffer GTC/2-ME and mix by pipetting up and down several times.** *Remember to add 20 µl of 2-mercaptoethanol per 1 ml of Buffer GTC.*

3. **Centrifuge at 14,000 x g for 5 min. Transfer the clear lysate into a gDNA Removal Column place in 2 ml collection tube. Centrifuge at 14,000 x g for 1 minute at room temperature.** Transfer the flow-through lysate into a new 1.5ml tube.

4. **Add 0.5 volume of absolute ethanol (96-100%, room temperature) to the mixture and mix well by pipetting.**

5. Proceed to step 4, (page 6) of main protocol (addition of samples to RNA HiBind RNA column).

E.Z.N.A.® HP Total RNA Kit Spin/Vacuum Protocol

Carry out lysis, homogenize, and load onto HiBind® RNA Mini column as indicated previous protocols. Instead of continuing centrifugation, follow steps blow. 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 turning on the vacuum source.
6. Wash the column by adding 1000µl **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 2 minute to dry the column.
8. Place the column in a clean 1.5 ml microcentrifuge tube and add 30-50µl RNase-free water. Stand for 1-2 minute and centrifuge 1 minute to elute RNA.

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.

Trouble Shooting Guide

Problem	Cause	Solution
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.