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Introduction

E-Z 96[®] Total RNA Kit II is designed for isolating total cellular RNA from tissues rich in fat such as brain adipose tissues. However, This kit can also be used for the isolation of total RNA from other type of tissues include cultured eukaryotic cells, animal tissues, or bacteria. The kit allows simultaneous purification of 96 or 192 samples in less than 60 min. RNA purified using this kit is ready for applications such as RT-PCR*, Northern blotting, poly A⁺ RNA (mRNA) purification, nuclease protection, and *in vitro* translation..

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 96[®] 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 all type of animal or human tissues include 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 E-Z 96[®] RNA Plate 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

All components except RNA-Safer II reagent in Total RNA Kit II should be stored at room temperature. RNA-Safer Reagent II should be store at 4C after adding phenol for long term storage. All Total RNA Kit II components are guaranteed for at least 12 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 Kits	R6935-00	R6935-01	R6935-02
Purification	1 x 96	4 x 96	20 x 96
E-Z 96® RNA Plate	1	4	20
2 ml Collection Tubes	15	100	400
RNA-Safer Reagent II*	4 ml	22 ml	88 ml
RNA Wash Buffer I	5 ml	50 ml	200 ml
RNA Wash Buffer II Concentrate	5 ml	12 ml	50 ml
DEPC-ddH ₂ O	1.0 ml	10 ml	40 ml
Instruction Manual	1	1	1

*Note: RNA-Safer® Reagent contains Guanidine Thiocyanate, handle those reagents with extra care.

Before Starting

IMPORTANT	Dilute RNA Wash Buffer II with absolute ethanol before use. R6934-00 Add 20 ml 100% ethanol R6934-01 Add 48 ml 100% ethanol R6934-02 Add 200 ml 100% ethanol
	Prepare RNA-Safer Reagent II/Phenol mixture by adding water saturated phenol solution to RNA-Safer Reagent II R6934-00 Add 6 ml water saturated phenol R6934-01 Add 33 ml water saturated phenol R6934-02 Add 132 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.**
- **To freeze tissue for long term storage, flash-frozen liquid nitrogen and immediately transfer to -70 °C.** Tissue can be store for up to 6 months at -70 °C. To process the sample, do not thaw the sample during weighing or handing prior to the disruption with RNA-Safer® II/phenol solution. Homogenized tissue lysates can be store at -

70 °C for at least 6 months. To proceed the frozen tissue lysates, thaw the sample at 37 °C until all they are completely thawed and all salts in the lysis buffer are dissolved. Do not extend the treatment in 37 °C because it can cause chemical degradation of RNA.

- **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.
- **Prepare the water-saturated phenol solution:** Place the solid phenol into the water bath preset at 75 °C until phenol is completely dissolved. Add equal volume of molecular biology grade water and mix thoroughly by shaking. Store the solution at room temperature for 4 hours to overnight until the water phase (upper phase) and phenol phase (lower phase) are clearly separated. Remove the water phase with transfer pipettor. Use the water-saturated phenol (lower phase) to prepare the RNA-Safer® II Reagent (see instruction on page 3). Small amount of water will not effect the performance of RNA-Safer® II Reagent.

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.

A). 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 RNA-Safer Reagent II/phenol solution and continue with the procedure as outlined below. After phase separation by adding chloroform, the supernatant 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 two 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 lysate through a narrow needle (19-21 gauge) for 5-10 times..

B). 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.

C.) 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.

D). 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).

Total RNA Isolation Protocol

A. Isolating Total RNA from Animal Cells

Materials supplied by user

- 2-mercaptoethanol
- Chloroform
- Centrifuge with capable of at least 4,000 x g
- Centrifuge adapter for microplare
- 70% ethanol in DEPC-treated sterile distilled water
- Sterile RNase-free pipette tips and 1.5ml centrifuge tubes
- Disposable latex gloves

Procedure:

1. **Determine the proper amount of starting material:** This is critical to use correct number of cells to obtain optimal yield and purity with E-Z 96[®] RNA plate. The maximum number of cells that can be processed on a E-Z 96[®] RNA plate varies depends on the specific RNA contents and type of cell lines. The maximum binding capacity for each well in the E-Z 96[®] RNA plate is 100µg. The maximum number of the cells that RNA-Safer Reagent II/Phenol used in this protocol is 1×10^7 . Use following table as a guideline to select correct starting material.

Average Yield of Total cellular RNA

Source	Number of Cells	RNA Yield (µg)
IC21	1×10^6	12
Hela	1×10^6	15
293HEK	1×10^6	10
HIN3T3	1×10^6	15

2. **Harvest Cells:**

For cells grown in suspension: determine the number of cells. Pellet the appropriate number of cell by centrifuge at 500 x for 5 minutes. Aspirate the supernatant and continue the step 3 of this protocol.

For cells grown in a monolayer: Cells grown in a monolayer in cell culture dish can be directly lysed in the dish or trypsinnized and collet the cell pellet before lysis. Cells grown in cell culture flask should be trypsinized and collect cell pellet prior to lysis.
3. **Disrupt cells (do not use more than 1×10^7 cells) with RNA-Safer Reagent II/phenol solution:** For pelleted cells, loose the cell pellet throughly by flicking the tube and add the appropriate amount of **RNA-Safer Reagent II/phenol solution** based on table blow. **To directly lyse the cell in the culture dish, add the appropriate amount of RNA-Safer Reagent II/phenol solution directly to the dish.** Remember to add 20 µl of 2-mercaptoethanol per 1 ml of RNA-Safer Reagent II/phenol solution before use. .

RNA-Safer Reagent II Volume for E-Z 96 RNA Plate

Number of Cells	Amount of RNA-Safer Reagent II/phenol solution (µl)
< 5 x 10 ⁶	500
5 x 10 ⁶ - 1 x 10 ⁷	1000

3. **Disrupt and homogenize cells by using one of the methods described in page 4-5. If samples are prepared in individual tubes, transfer the sample into a 96-well deep well plate (not supplied).**
4. **Incubate the plate contains homogenates at room temperature for 5 minutes.**
5. **Add 100 (for < 5 x 10⁶ cells) or 200µl (for 5 x 10⁶ - 1 x 10⁷ cells) chloroform to the homogenate, close the cap of the tube and vortex for 20 seconds. Incubate at room temperature for 2-3 minutes.**
6. **Centrifuge at ≥4,000 x g at 4° C for 15 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.
7. **Transfer the upper aqueous phase (around 250µl or 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.
8. **Apply sample onto HiBind® RNA spin column. The maximum capacity of the spin column is 700 µl. (Larger volumes can be loaded successively.)** A precipitate may form on addition of ethanol. Vortex and add the entire mixture to the column. With the spin column inside the 2ml collecting tube (supplied with kit), centrifuge at 10,000 x g for 1 min **at room temperature**. Discard flow-through and re-use the collection tube in next step.
9. **Add 500µl RNA wash Buffer I directly into the HiBind® RNA spin**

column. Centrifuge at 10,000 xg for 30 seconds. Discard the flow-through and collecting tube.

Note: This the starting point if on-membrane DNase I digestion (page 12).

10. **Place column in a clean 2 ml centrifuge tube (supplied), and add 500µl RNA Wash Buffer II diluted with ethanol. Centrifuge at 10,000 x g for 1 minutes at room temperature. Discard flow-through and reuse the collection tube in next step.**

Note: Wash Buffer II Concentrate must be diluted with absolute ethanol before use. Refer to label on bottle for directions.
11. **Wash column with a second 500µl of Wash Buffer II.** Centrifuge at 10,000 xg for 1 minutes and discard flow-through. Then with the collection tube empty, centrifuge the spin column for **2 min at maximum speed** to completely dry the HiBind® matrix.
12. **Elution of RNA.** Transfer the column to a clean 1.5 ml centrifuge tube (Not supplied) and elute the RNA with 45-75µl of DEPC-treated water (supplied with kit). Make sure to add water directly onto column matrix. Centrifuge 1 min at 10,000 xg. 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. Isolating Total RNA from Animal Tissues

Materials supplied by user

- 2-mercaptoethanol
- Chloroform
- Microcentrifuge capable of at least 14,000 xg
- 70% ethanol in DEPC-treated sterile distilled water
- Sterile RNase-free pipette tips and 1.5ml centrifuge tubes
- Disposable latex gloves

1. **Determine the proper amount of starting material:** This is critical to use correct number of cells to obtain optimal yield and purity with HiBind® RNA column. The maximum amount of tissue that can be processed on a HiBind® RNA column varies depends on the specific RNA contents and type of tissue. The Maximum binding capacity of the HiBind® RNA column is 100µg. The maximum tissue that TRK lysis buffer used in the Total RNA protocol is 30 mg. Use following table as a guideline to select correct starting material. **If you have no information about the your starting material, use 10 mg as starting amount, base ob the yield and quality of RNA obtained from 10 mg, adjust the starting amount in the next purification.**

Average Yield of Total cellular RNA

Source	Amount of Tissue (mg)	RNA Yield (µg)
Brain	10	10
Kidney	10	30
Liver	10	45
Heart	10	5
Spleen	10	33
Lung	10	12
Pancreas	10	40
Thymus	10	20

2. **Disrupt Tissue and homogenize the tissue in 1 ml RNA-Safer Reagent II /Phenol solution using one of the described methods on page 4.** (Do not use more than 30mg tissue). Remember to add 20 µl of 2-mercaptoethanol per 1 ml of RNA-Safer Reagent II/Phenol solution before use.

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.

RNA-Safer Reagent II/Phenol Volume for RNA Mini-Column

Amount of Tissue (mg)	Amount of TRK Lysis Buffer (µl)
≤ 15	500
20- 30	1000

3. **Incubate the tube contains homogenate at room temperature for 5 minutes.**
4. **Add 200µl chloroform to the homogenate, close the cap of the tube and vortex for 20 seconds. Incubate at room temperature for 2-3 minutes.**
5. **Centrifuge at ≥ 12,000 x g at 4 °C for 15 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.

6. **Transfer the upper aqueous phase (around 250µl or 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 sample onto HiBind® RNA spin column. The maximum capacity of the spin column is 700 µl. (Larger volumes can be loaded successively.)** A precipitate may form on addition of ethanol. Vortex and add the entire mixture to the column. With the spin column inside the 2ml collecting tube (supplied with kit), centrifuge at 10,000 x g for 1 min **at room temperature**. Discard flow-through and re-use the collection tube in next step.

- Wash column with RNA Wash Buffer I by pipetting 500µl directly into the spin column. Centrifuge as above and **discard the 2 ml collecting tube.**

Note: This the starting point if on-membrane DNase I digestion (page 12).

- Place column in a clean 2 ml centrifuge tube**, and add 500µl RNA Wash Buffer II diluted with ethanol. Centrifuge at 10,000 xg for 1 minutes at room temperature. Discard flow-through and reuse the collection tube in next step.
- Wash column with a second 500µl of Wash Buffer II. Centrifuge at 10,000 xg for 1 minutes and discard flow-through. Then with the collection tube empty, centrifuge the spin cartridge for **2 min at maximum speed** to completely dry the HiBind® matrix.
- Elution of RNA.** Transfer the column to a clean 1.5 ml centrifuge tube (Not supplied) and elute the RNA with 45-70 µl of DEPC-treated water (supplied with kit). Make sure to add water directly onto center of column matrix. Centrifuge 2 min at 10,000 xg. A second elution may be necessary if the expected yield of RNA > 30µg.

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. 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)

- Follow the standard protocol until the samples **completely** pass through the HiBind RNA column (step1-6). Prepare the following:
- 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 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.**
- OBI DNase I digestion buffer is supplied with OBI RNase-free Dnase set.**
- Standard Dnase buffers are not compatible with on-membrane Dnase digestion.**

- 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.
- Incubate at room temperature(25-30°C) for 15 minutes.
- Place column into a new 2 ml centrifuge tube**, and add 400µl RNA Wash Buffer I. **Place the column at benchtop for 2 minutes.** Centrifuge at 10,000 xg for 1 minutes and discard flow-through. Reuse the collection tube in step 6.
- Place column in the same 2 ml centrifuge tube**, and add 500 µl RNA Wash Buffer II diluted with ethanol. Centrifuge at 10,000 xg for 1

minutes and discard flow-through. 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 500µl of Wash Buffer II. Centrifuge and discard flow-through. Then with the collection tube empty, centrifuge the spin cartridge for **2 min at maximum speed** to completely dry the HiBind® matrix.
8. **Elution of RNA.** Transfer the column to a clean 1.5 ml microfuge tube (not supplied with kit) and elute the RNA with 40- 70 µl of DEPC-treated water (supplied with kit). Make sure to add water directly onto column matrix. Let it stand for 1 minute. Centrifuge 2 min at 10,000 x g to elute RNA. 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.

D). Total RNA Kit II Vacuum/Spin Protocol

Carry out lysis, homogenization, phase separation steps as indicated previous protocols. Instead of continuing with centrifugation, follow steps below.

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. **Transfer the upper aqueous phase (around 250µl or 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.
3. **Load the homogenized sample onto HiBind® RNA V-spin column.**
4. Switch on vacuum source to draw the sample through the column and turn off the vacuum.
5. Wash the column by adding 300 µl **RNA wash buffer I**, draw the wash buffer through the column by turn on the vacuum source.
6. **(Optional): Perform on-membrane DNase I digestion steps if sensitive downstream application is desired.** (See previous section for

details)

7. Wash the column by adding 500 µl **RNA wash buffer I**, draw the wash buffer through the column by turn on the vacuum source.
8. Wash the column by adding 700 µl **RNA wash buffer II**, draw the wash buffer through the column by turn on the vacuum source.
9. Assemble the column into a **2 ml collection tube** and transfer the column to a micro centrifuge. Add 200 µl of **RNA wash buffer II** into the column. Spin 2 minute to dry the column. Try to Avoid carrying over the RNA Wash Buffer II when remove the spin column from the collection tube.
10. Place the column in a clean 1.5 ml microcentrifuge tube and add 50-100µl RNase-free water. Stand for 1-2 minutes and centrifuge for 1 minute to elute RNA.

DNA Contamination

No RNA extraction procedure can completely remove genomic DNA. For sensitive work (such as RT-PCR or differential display) we suggest that you either to do the on-membrane DNase I digestion treatment or treat the eluted RNA with RNase-free Dnase I. 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.

Quantitation 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.