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## Introduction

E-Z 96® Total RNA Kit is designed for isolation of cellular RNA from up to  $1 \times 10^6$  tissue cultured cells. The kit allows single or multiple, simultaneous processing of samples in less than 60 min. 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.

RNA purified using the E-Z 96® Total RNA method is ready for applications such as RT-PCR\*.

## Principle

The E-Z® Total RNA Kits use reversible binding properties of HiBind® matrix, a new silica-based, time saving spin technology material. Combined with the speed of mini-column spin technology or vacuum manifold, multiple samples can be processed at same time. The sample is lysed first under highly denaturing buffer conditions so that RNases will be inactivated, and the intact viral RNA is protected from degrading. After adjusting the buffer condition, the samples are loaded to the HiBind® RNA Plate. With a brief centrifugation or vacuum, the samples pass through the plate and the RNA binds to the HiBind® matrix. After two washing steps, purified viral RNA will be eluted with RNase-free water.

## Storage

All components in the E-Z 96® Total RNA Kit should be stored at room temperature. During shipping and storage, crystals may form in the TRK Lysis Buffer, simply warm to 37°C to dissolve. All kit components are guaranteed for at least 12 months from date of purchase.

\*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 96® Total RNA Kits	4 x 96 Preps	12 x 96 Preps
Product Number	R1034-01	D1034-02
Purification	4	12
<b>Components</b>		
HiBind™ RNA Plates	4	12
Square-Well Collection Plate	2	2
Racked Microtubes (1.2ml)	4 x 96	12 x 96
8-Strip Microtube Caps	52 x 8	156 x 8
TRK Lysis Buffer	60 ml	200 ml
RNA Wash Buffer I	200 ml	600 ml
RNA Wash Buffer II Concentrate	3 x 50 ml	4 x 100 ml
DEPC-ddH <sub>2</sub> O	40 ml	120 ml
Instruction Manual	1	1

\* 2ml Deep well plates are reusable, see page 7 for instruction.

### Important Notes

1. 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.
- Carefully Apply the sample or solution to the HiBind RNA column. Avoid touch the membrane with pipet tip.

2. Sample volume: HiBind® RNA resin can bind any RNA greater than 200nt. Yield will depend on the sample sources and conditions. The protocol is optimized for use with 150 µl samples. Smaller samples should be adjusted to 150 µl with PBS or DEPC water; lower titer samples should be concentrated to 150 µl before processing. For samples larger than 150 µl, the amount of QVL Lysis buffer and other reagents added to the sample before loading must be increased proportionally.

### Before Starting

<b>IMPORTANT</b>	<b>Wash Buffer II Concentrate</b> must be diluted with absolute ethanol before use.	
	R1034-01	Add 200 ml 100 % ethanol
	R1034-02	Add 400 ml 100% ethanol

## E-Z 96® Total RNA Protocol with Centrifugation

Materials supplied by user:

- 96-100% ethanol
- 1x Phosphate-Buffered Saline (PBS), sterile
- β-Mercaptoethanol
- Multichannel pipet
- RNase-free filter pipette tips
- Reagent reservoirs for multichannel pipets
- Centrifuge with suitable rotor for 96-well plate.
- Disposable latex gloves
- RNase-Free 1.2 ml 96-well plate
- Adhesive sealing film for microplate
- 2ml 96-well deep well plate

**Note:** All steps must be carried out at room temperature. Work quickly, but carefully.

### Procedure:

1. **A: Lysis of monolayer cultured cells grown in a 96 well tissue culture plate: Remove the medium, wash the cells once with sterile 1 x PBS. Add 150ul TRK Lysis Buffer directly to each well.**

**B: Lysis of suspension cultured cells:** Transfer aliquots of up to 1 x 10<sup>6</sup> cells into wells of a 96 microplate. Spin the plate at 300 x g for 5 minutes. Remove the medium and wash once with 1 x PBS. **Add 150ul TRK Lysis Buffer directly to each well.** Mix by pipetting.

**Note: Add 20 µl Buffer/β-mercaptoethanol with each 1ml QVL buffer before use.**

2. Keep the microplate flat on the bench, shake vigorously back and forth for 30 seconds. Rotate the plate 90° and shake the plate for another 30 seconds.
3. Add 150µl of 70% ethanol to the sample, mix thoroughly by pipetting.
4. **Carefully** apply entire samples from step 3 (including any precipitate) to each well of the HiBind® RNA plate.
5. Seal the HiBind® RNA plate with sealing film. Load the HiBind® RNA plate with 2ml square-well plate into the plate holder, and place the whole assembly into the rotor bucket of the centrifuge. Spin at 5000 x g for 5 minutes at room temperature.
6. Remove the sealing film. Wash plate with Wash Buffer I by pipetting 750 µl directly into the each well of the HiBind® RNA plate. Seal the plate with a new sealing film. Centrifuge at 5000 x g for 5 minutes at room temperature.
7. **DNase 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. Remove the sealing film and add 500 µl Wash Buffer II diluted with ethanol to each well of HiBind® RNA plate. Seal the plate with a new sealing film. Centrifuge at 5000 x g for 5 minutes at room temperature.

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

9. Remove the sealing film. Add another 500ul of RNA Wash Buffer II to each well of HiBind® RNA plate. Seal the plate with a new sealing film. Centrifuge at 5000 x g for 10 minutes at room temperature.

Note: It is very important to dry the HiBind® RNA plate before the elution because the residual ethanol might interfere with downstream applications.

10. Elution of RNA: Remove the sealing film and place the HiBind® RNA plate onto the microtube rack containing 1.2ml microtubes (supplied with kit).

11. Add 50-70 µl of DEPC-treated water to each well, and seal the HiBind® RNA plate with new sealing film(supplied with kit). Make sure to add water directly onto RNA matrix. Incubate for 1 minute at room temperature. Centrifuge at 5500 x g for 5 minutes at room temperature to elute RNA.

12. Remove the sealing film. Repeat step 13 and 14 for second elution.

## E-Z 96® Total RNA Vacuum Protocol

Materials supplied by user:

- 96-100% ethanol
- 1x Phosphate-Buffered Saline (PBS), sterile
- β-Mercaptoethanol
- Multichannel pipets
- RNase-free filter pipette tips
- Reagent reservoirs for multichannel pipets
- Vacuum Manifold (Product# Vac-03)
- Vacuum source capable of generating a vacuum pressure of -900 mbar
- Disposable latex gloves
- 800 µl or 1.2 ml 96-well plate
- 2ml 96-well deep well plate
- 800 µl microplate

**Note:** All steps must be carried out at room temperature. Work quickly, but carefully. Become familiar with the manifold by reading the instructions for the manifold before starting this vacuum protocol.

### Cell Lysis:

**A. Lysis of monolayer cultured cells grown in a 96 well tissue culture plate: Remove the medium, wash the cells once with sterile 1 x PBS. Add 150ul TRK Lysis Buffer directly to each well. Go to step 2.**

**B: Lysis of suspension cultured cells:** Transfer aliquots of up to 1 x 10<sup>6</sup> cells into wells of a 96 microplate. Spin the plate at 300 x g for 5 minutes. Remove the medium and wash once with 1 x PBS. **Add 150ul TRK Lysis Buffer directly to each well.** Mix by pipetting. Go to step 2.

**Note: Add 20 µl Buffer/β-mercaptoethanol with each 1ml QVL buffer before use.**

1. **Prepare the vacuum manifold: Place the 2ml 96-well deep well plate inside the vacuum manifold base. Place the top plate squarely over the base. Place the HiBind® RNA plate on the top plate, making sure that the HiBind® RNA plate is seated tightly on the rubber ring of the top plate. Connect the Vacuum manifold to the vacuum source. Keep the vacuum switch off.**
2. Add 150µl of 70% ethanol to the sample, mix thoroughly by pipetting up and down 3-4 times.
3. Apply entire sample from step 2 into wells of HiBind® RNA plate, switch on the vacuum source. Apply vacuum until all sample contents pass through the membrane.
4. Option DNase I Digestion: (See instruction on page 5)
5. Wash plate with Wash Buffer I by pipetting 500 µl directly into the each well of the HiBind® RNA plate. Apply the vacuum until transfer is complete. Switch off the vacuum, and ventilate the manifold.
6. Add 750 µl RNA Wash Buffer II to each well of the of HiBind® RNA plate, and apply the vacuum until transfer is complete. Switch off the vacuum, and ventilate the manifold.

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

7. Repeat step 10 for second wash with RNA Wash Buffer II.
8. Remove the HiBind® RNA plate from top plate of vacuum manifold, and strike the bottle of the HiBind® RNA plate on a stack of paper towels. Repeat for few times until no liquid is released onto the paper towels.
9. Place the HiBind® RNA plate back to the top plate of the manifold. Apply vacuum for 15 minutes. Turn off the vacuum source and ventilate the manifold.
10. Replace the 2ml deep well plate with a microtube rack containing the 1.2ml microtubes. Reassemble the manifold. Place the HiBind® RNA plate on top plate of manifold.
11. Elution RNA: Add 50-70 µl of DEPC-treated water to each well, and seal the HiBind® RNA plate with new sealing film(supplied with kit). Make sure to add water directly onto RNA matrix. Incubate for 1 minute at room temperature. Switch on the vacuum source for 5 minutes. Switch off the vacuum, and ventilate the manifold.
12. Repeat the elution step once, with second volume of 50-70 µl of DEPC-treated water.

### Quantization and Storage of RNA

To determine the concentration and purity of RNA, measure absorbency 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. 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 absorbency maximum at 275 nm and can interfere with spectrophotometric analysis of DNA or RNA. However, the HiBind® RNA technology eliminates the use of phenol and avoids this problem.) Store RNA samples at -70°C in water. Under such conditions RNA is stable for more than a year.

### Clean the 2ml deep well plates:

Two 2ml deep well plate are supplied with each kit. If extra plates are needed, please call our customer service department for ordering information. To reuse the deep well plates, rinse them thoroughly with tap water, incubate overnight in 0.2M NaOH/1mM EDTA, rinse with distilled water and dry by air.

### Troubleshooting Tips

Problem	Cause	Suggestion
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Little or no RNA eluted	Carrier RNA not added to TRK Lysis Buffer or degraded	<ul style="list-style-type: none"> <li>Dissolve the carrier RNA with TRK Lysis Buffer and repeat the purification with new sample.</li> <li>Repeat elution.</li> <li>Pre-heat DEPC-water to 70° C prior to elution.</li> <li>Incubate for 5 min with water prior to elution</li> </ul>
	RNA remains on the plate	
	Plate is overloaded	<ul style="list-style-type: none"> <li>Reduce quantity of starting material.</li> </ul>
Clogged well	Incomplete lysis	<ul style="list-style-type: none"> <li>Mix thoroughly after addition of TRK Lysis Buffer..</li> <li>Reduce amount of starting material</li> </ul>
Degraded RNA	Source	<ul style="list-style-type: none"> <li>Do not freeze and thaw sample more than once.</li> <li>Follow protocol closely, and work quickly.</li> <li>Low concentration of virus in the sample</li> </ul>
	RNase contamination	<ul style="list-style-type: none"> <li>Ensure not to introduce RNase during the procedure.</li> <li>Check buffers for RNase contamination.</li> </ul>
Problem in downstream applications	Salt carry-over during elution	<ul style="list-style-type: none"> <li>Ensure Wash Buffer II has been diluted with 4 volumes of 100% ethanol as indicated on bottle.</li> <li>1 X Wash Buffer II must be stored at room temperature.</li> <li>Repeat wash with Wash Buffer II.</li> </ul>
	Inhibitors of PCR	<ul style="list-style-type: none"> <li>Use less starting material</li> <li>Prolong incubation with Buffer TRK to completely lyse cells</li> </ul>
DNA contamination		<ul style="list-style-type: none"> <li>Digest with RNase-free DNase and inactivate at 75°C for 5 min.</li> </ul>