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Introduction

E-Z 96® Total RNA Kits are designed for isolation of cellular RNA from up to 5 x 10^5 cultured cells. The kits allow 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*, qPCR*, differential display, microarrays, etc..

Principle

The E-Z 96® Total RNA Kits use reversible binding properties of HiBind® matrix, a new silica-based, time saving spin technology material. Utilizing centrifugation or a vacuum manifold, the system allows multiple samples to be processed simultaneously. The sample is lysed first under highly denaturing buffer conditions so that RNases will be inactivated, and the intact RNA is protected from degrading. After adjusting the binding conditions, the samples are loaded into 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 wash steps, purified RNA is 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 in cool ambient conditions, crystals may form in the TRK Lysis Buffer. Simply warm the buffer to 37°C and gently shake its container 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	R1034-00	R1034-01	R1034-02
E-Z 96® RNA Plates	1	4	12
Square-Well Collection Plate*	1*	2*	4*
Racked Microtubes (1.2ml)	1 x 96	4 x 96	12 x 96
8-Strip Microtube Caps	12 x 8	48 x 8	144 x 8
Aera Sealing Film	5	20	60
TRK Lysis Buffer	20 ml	65 ml	200 ml
RWF Wash Buffer	60 ml	250 ml	750 ml
RNA Wash Buffer II Concentrate	40 ml	3 x 50 ml	2 x 200 ml
DEPC-ddH_2O	10 ml	40 ml	120 ml
Instruction Manual	1	1	1

* 2 ml Square-well plates are reusable. See Page 11 for cleaning instructions.

Important Notes

1. Please take a few minutes to read this booklet thoroughly to become familiar with the protocol. Prepare all materials required before starting the procedure 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.
- Carefully apply the sample or solution to the HiBind® RNA membrane. Avoid touching the membrane with pipet tips.

Before Starting

IMPORTANT	Dilute **Wash Buffer II** Concentrate with **absolute ethanol** before use **R1034-00** Add 160 ml 96%-100% ethanol **R1034-01** Add 200 ml 96%-100% ethanol **R1034-02** Add 800 ml 96%-100% ethanol

E-Z 96® Total RNA Protocol with Centrifugation

Materials supplied by user

- 96%-100% ethanol
- 70% ethanol
- Optional: β-Mercaptoethanol
- Multichannel pipet
- RNase-free filter pipette tips
- Reagent reservoirs for multichannel pipets
- Centrifuge with rotor for 96-well plates
- Disposable latex gloves
- 2ml 96-well deep well plate

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

Procedure:

1. A. LYSIS OF MONOLAYER CULTURED CELLS GROWN IN A MULTI-WELL TISSUE CULTURE PLATE: Remove the medium by pipetting. Add 150 µl TRK Lysis Buffer directly to each well.

   B. LYSIS OF SUSPENSION CULTURED CELLS: Transfer aliquots of up to 5 x 10^5 cells into the wells of a 96-well microplate. Spin the plate at 300 x g for 5 minutes. Remove the medium completely by pipetting. Add 150 µl TRK Lysis Buffer directly to each well. Mix by pipetting.

   Optional: As a preparation step add 20µl of 2-mercaptoethanol (β-mercaptoethanol) per 1 ml of TRK Lysis Bufferf or samples with a high amount of Rnase Activity. This mixture can be stored for 1 week at room temperature

2. Keep the microplate flat on the bench. Shake vigorously, end to end and side to side, for a total of one minute.

   Note: *If the multi-well plate used has volume less than 300 µl, reduce volume of the TRK lysis buffer to 100µl, as the total volume would be 200 µl after addition of 100µl ethanol in Step 3.*

3. Add one volume (150 µl) of 70% ethanol to the sample; mix throughly by pipetting up and down 3 to 4 times.

4. Place the E-Z 96® RNA plate atop the Square-Well Collection Plate

and carefully add entire sample from Step 3 (including any precipitate) to each well of the HiBind® RNA plate.

5. Seal the E-Z 96® RNA plate RNA plate with sealing film. Load the E-Z 96® RNA /2 ml square-well plate into a microplate holder, and place the whole assembly into the rotor bucket of the centrifuge. Spin at 5,000 x g for 5 minutes at room temperature. Discard flow-through after centrifugation.

This is the point to begin the optional DNase I digestion treatment. If DNase I digestion is required, go to Step 6; otherwise skip to Step 7.

6. **DNase I Digestion Spin Protocol (Optional)**

Because the HiBind® RNA technology eliminates most DNA, DNase I digestion is not necessary for most downstream applications. However, certain sensitive RNA applications might require further DNA removal. Follow the steps below for on-membrane DNase I digestion. (See DNase I Digestion Set, Cat # E1091 for further information).

A. Follow the standard protocol until the samples **completely** pass through the HiBind™ RNA Plate (Steps 1-5). Complete DNase I digestion using the steps following:

1. Add 300 µl RWF Wash Buffer to each well of the E-Z 96® RNA Plate and centrifuge at ≥4,000 x g for 1 min.

2. For each RNA sample, prepare the DNase I digestion mixture as follows:

OBI DNase I Digestion Buffer	73.5 µl
RNase-free DNase I (20 Kunitz units/µl)	1.5 µl
Total volume	75 µl

Note:

a. DNase I is very sensitive and prone 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 beginning the RNA isolation procedure.

b. Standard DNase buffers may not be compatible with Omega Bio-Tek's DNase I Digestion Set.

3. Pipet 75 µl DNase I digestion mixture directly onto the surface of the membrane in each well of the E-Z 96® RNA Plate. Be certain to pipet the mixture directly onto each membrane, as DNA digestion will not be complete if some of the mixture is retained on the walls or the O-rings of the HiBind® RNA Plate.

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

B. Place the E-Z 96® RNA Plate on top of a clean 2ml collection plate and add 300 µl RWF Wash Buffer. **Incubate 3 minutes at room temperature.** Centrifuge at 4,000 x g and discard flow-through. Re-use the collection plate.

If the Dnase I digestion procedure was used, skip Step 7 and continue procedure at Step 8.

7. Remove the sealing film. Add 500µl RWF Wash Buffer directly into the each well of the E-Z 96® RNA plate, Seal the plate with new sealing film and centrifuge at 5,000 x g for 5 minutes at room temperature.

8. Remove the sealing film and add 600 µl RNA Wash Buffer II diluted with ethanol to each well of the E-Z 96® RNA plate. Seal the plate with new sealing film. Centrifuge at 5,000 x g for 5 minutes at room temperature Discard flow-through after centrifugation

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 600 µl of RNA Wash Buffer II to each well of E-Z 96® RNA plate. Seal the plate with a new sealing film. Centrifuge at 5,000 x g for 10 minutes at room temperature. The prolonged centrifugation is necessary to dry the HiBind RNA plate matrices.

Note: *It is very important to dry the E-Z 96® RNA plate completely before the elution step to remove residual ethanol that might otherwise interfere with downstream applications.*

10. Elution of RNA: Remove the sealing film and place the E-Z 96® RNA plate onto the microtube rack containing 1.2 ml microtubes (supplied with kit).

11. Add 50-75 µl DEPC-treated water to each well, and seal the E-Z 96® RNA plate with new sealing film(supplied with kit). Make sure to add water directly onto RNA matrix. Incubate for 3 minutes at room temperature. Centrifuge at 5,000 x g for 5 minutes at room temperature to elute RNA.

12. Remove the sealing film. Repeat Steps 10 and 11 for second elution.

Note: *Elution volume and number can vary according to user preference. To maintain a higher RNA concentration, first eluate can be used for second elution.*

E-Z 96® Total RNA Vacuum Protocol

Materials supplied by user

- 96-100% ethanol
- 70% ethanol
- Optional: 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
- 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 manufacturer's instructions before starting this vacuum protocol.

Procedure:

1. A. LYSIS OF MONOLAYER CULTURED CELLS GROWN IN A MULTI-WELL TISSUE CULTURE PLATE: Remove the medium by pipetting. Add 150 ul TRK Lysis Buffer directly to each well.

   B. LYSIS OF SUSPENSION CULTURED CELLS: Transfer aliquots of up to 5×10^5 cells into the wells of a 96-well microplate. Spin the plate at 300 x g for 5 minutes. Remove the medium completely by pipetting. Add 150 ul TRK Lysis Buffer directly to each well. Mix by pipetting.

   Optional: As a preparation step add 20µl of 2-mercaptoethanol (β-mercaptoethanol) per 1 ml of TRK Lysis Bufferf or samples with a high amount of Rnase Activity. This mixture can be stored for 1 week at room temperature

2. Keep the microplate flat on the bench. Shake vigorously, end to end and side to side, for a total of one minute.

   ***Note:** If the multi-well plate used has volume less than 300µl, reduce volume of the TRK lysis buffer to 100µl, as the total volume would be 300µl after addition of 150µl ethanol in Step 3.*

3. Add one volume (150µl) of 70% ethanol to the sample; mix throughly by pipetting up and down 3 to 4 times.

4. PREPARE THE VACUUM MANIFOLD: Place the 2ml 96-well deep-well plate or a waste collection tray inside the vacuum manifold base. Place the manifold's top section squarely over its base. Place the E-Z 96® RNA plate on the manifold's top section, making sure the E-Z 96® RNA plate is seated tightly on the rubber ring. Connect the vacuum manifold to the vacuum source. Keep the vacuum switch off.

5. Carefully add entire sample from Step 3 (including any precipitate) to each well of the E-Z 96® RNA plate. Seal the un-used wells with sealing film. Switch on the vacuum source. Apply vacuum until all the sample contents pass through the well membranes.

   This is the point to begin the optional DNase I digestion treatment. If DNase I digestion is required, go to Step 6; otherwise skip to Step 7.

6. **DNase I Digestion Vacuum Protocol (Optional)**

   Because the HiBind® RNA technology eliminates most DNA, DNase I digestion is not necessary for most downstream applications. However, certain sensitive RNA applications might require further DNA removal. Follow the steps below for on-membrane DNase I digestion. (See DNase I Digestion Set, Cat # E1091 for further information).

   A. Follow the standard vacuum protocol until the samples **completely** pass through the HiBind™ RNA Plate (Steps 1-5). Complete DNase I digestion using the steps following:

      1. Add 300 µl RWF Wash Buffer to each well of the E-Z 96® RNA Plate. Apply the vacuum until RNA Wash Buffer I passes through.

      2. For each RNA sample, prepare the DNase I digestion mixture as follows:

OBI DNase I Digestion Buffer	73.5 µl
RNase-free DNase I (20 Kunitz units/µl)	1.5 µl
Total volume	75 µl

Note:

a. DNase I is very sensitive and prone 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 beginning the RNA isolation procedure.

b. Standard DNase buffers may not be compatible with Omega Bio-Tek's DNase I Digestion Set.

3. Pipet 75 µl DNase I digestion mixture directly onto the surface

of the membrane in each well of the E-Z 96® RNA Plate. Be certain to pipet the mixture directly onto each membrane, as DNA digestion will not be complete if some of the mixture is retained on the walls or the O-rings of the HiBind® RNA Plate.

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

B. Add 300 µl RWF Wash Buffer to each well. **Incubate an additional 3 minutes at room temperature.** Apply vacuum until all the liquid passes through the membranes.

If the Dnase I digestion procedure was used, skip Step 7; continue at Step 8.

7. Add 500 µl RWF Wash Buffer directly into each well of the E-Z 96® RNA plate. Apply the vacuum until transfer is complete. Switch off the vacuum, and ventilate the manifold.

8. Add 800 µl RNA Wash Buffer II to each well of the of E-Z 96® 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.*

9. Repeat step 8 for a second wash with 800 µl RNA Wash Buffer II.

10. Remove the E-Z 96® RNA plate from top plate of vacuum manifold, and strike the bottom of the HiBind™ RNA plate on a stack of paper towels. Repeat for few times until no liquid is released onto the paper towels.

11. Place the E-Z 96® RNA plate back to the top plate of the manifold. Apply vacuum for 15 minutes. Turn off the vacuum and ventilate the manifold.

12. Replace the 2 ml 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.

13. Elution RNA: Add 50-75 µl of DEPC-treated water to each well, and seal the E-Z 96® 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.

14. Repeat the elution with a second volume of 50-75 µl DEPC-treated water.

Note: *Elution volume and number can vary according to user preference. To maintain a higher RNA concentration, first eluate can be used for second elution.*

E-Z 96® Total RNA Cleanup Protocol (Spin Protocol)

1. Adjust the volume of RNA sample to 150µl with RNase-free water.

2. Add one volume (150 µl) of 70% ethanol to the sample; mix throughly by pipetting up and down 3 to 4 times.

3. Place the E-Z 96® RNA plate atop the 2 ml deep-well plate and carefully add entire sample from Step 3 (including any precipitate) to each well of the HiBind® RNA plate.

4. Seal the E-Z 96® RNA plate with sealing film. Load the HiBind® RNA /2 ml square-well plate into a microplate holder, and place the whole assembly into the rotor bucket of the centrifuge. Spin at 5,000 x g for 5 minutes at room temperature. Discard flow-through and re-use the collection plate.

5. Remove the sealing film. Add 800µl RNA Wash Buffer II directly into the each well of the HiBind® RNA plate, Seal the plate with new sealing film and centrifuge at 5,000 x g for 10 minutes at room temperature.

6. Elution of RNA: Remove the sealing film and place the E-Z 96® RNA plate onto the microtube rack containing 1.2 ml microtubes (supplied with kit).

7. Add 50-75 µl DEPC-treated water to each well, and seal the E-Z 96® RNA plate with new sealing film(supplied with kit). Make sure to add water directly onto RNA matrix. Incubate for 3 minutes at room temperature. Centrifuge at 5,000 x g for 5 minutes at room temperature to elute RNA.

8. (Optional): Remove the sealing film. Repeat Steps 10 and 11 for second elution.

   Note: *Elution volume and number can vary according to user preference. To maintain a higher RNA concentration, first eluate can be used for second elution.*

Quantitation 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 plates 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 throughly with tap water, incubate overnight in 0.2M NaOH/1mM EDTA, rinse with distilled water and dry by air.

Troubleshooting Tips

Problem	Cause	Suggestion
Little or no RNA eluted	Carrier RNA not added toTRK Lysis Buffer or degraded	• Dissolve the carrier RNA with TRK Lysis Buffer and repeat the purification with new sample. • Repeat elution. • Pre-heat DEPC-water to 70° C prior to elution.
	RNA remains on the plate	• Incubate for 5 min with water prior to elution
	Plate is overloaded	• Reduce quantity of starting material.
Clogged well	Incomplete lysis	• Mix thoroughly after addition of TRK Lysis Buffer.. • Reduce amount of starting material
Degraded RNA	Source	• Do not freeze and thaw sample more than once. • Follow protocol closely, and work quickly. • Low concentration of virus in the sample • Add BME to Buffer TRK
	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 has been diluted with 4 volumes of 100% ethanol as indicated on bottle. • 1 X Wash Buffer II must be stored at room temperature. • Repeat wash with Wash Buffer II.
	Inhibitors of PCR	• Use less starting material • Prolong incubation with Buffer TRK to completely lyse cells
DNA contamination		• Digest with RNase-free DNase and inactivate at 37°C for 5 min.