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Introduction

E-Z 96® HP Total RNA Isolation Kits are designed for isolation of cellular RNA with low genomic DNA contamination. Lysates are first passed through a E-Z 96® DNA Clearance plate to selectively bind DNA and then through a E-Z 96® RNA plate to selectively isolate RNA. RNA purified using the E-Z 96® HP Total RNA method is ready for applications such as RT-PCR*, qPCR*, differential display, microarrays, etc. DNA purified from this kit is suitable for PCR, Souther blot, genotyping and ANP analysis.

Principle

The E-Z 96® HP Total RNA isolate kits combines reversible binding properties of the HiBind® RNA technology with a specially designed buffer system which selective binds DNA to a DNA Clearance Plate before RNA isolation. Samples are first lysed and homogenized in a specially designed denature buffer (GTC), which immediately inhibits the activity of RNase. The lyste is then passed through a E-Z 96® DNA Clearance Plate which will selectively bind genomic DNA. The flow-through lysate from E-Z 96® DNA Clearance Plate is then added ethanol to create proper RNA binding condition, the sample is then loaded into the E-Z 96® RNA Plate to bind RNA. 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 and Stability

All components in the E-Z 96® HP Total RNA should be stored at room temperature. During shipping and storage in cool ambient conditions, crystals may form in the GTC 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® HP Total RNA Kits	R6813-00	R6813-01	R6813-02
Purification	1 x 96	4 x 96	20 x 96
E-Z 96® DNA Clearance Plate	1	4	20
E-Z 96® RNA Plates	1	4	20
Square-Well Collection Plate*	1*	2*	6*
Racked Microtubes (1.2ml)	2 x 96	8 x 96	40 x 96
8-Strip Microtube Caps	8 x 24	8 x 96	8 x 480
Aera Sealing Film	8	32	160
GTC Lysis Buffer	30 ml	125 ml	600 ml
RWC Wash Buffer	60 ml	250 ml	1000 ml
RNA Wash Buffer II Concentrate	40 ml	3 x 50 ml	3 x 200 ml
DEPC-ddH_2O	10 ml	40 ml	200 ml
Instruction Manual	1	1	1

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

Important Notes

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.
- Dilute **RNA Wash Buffer II** with **absolute ethanol** before use, and store at room temperature.

R6813-00	Add 160 ml 96%-100% ethanol.
R6813-01	Add 200 ml 96%-100% ethanol per bottle.
R6813-02	Add 200 ml 96%-100% ethanol per bottle.

E-Z 96® HP Total RNA Protocol with Centrifugation

Materials supplied by user

- 96%-100% ethanol
- 70% ethanol
- ß-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 300 µl of GTC Lysis Buffer directly to each well.** Mix thoroughly by pipetting up and down 10-20 times.

   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 300µl of GTC Lysis Buffer directly to each well.** Mix thoroughly by pipetting up and down 10-20 times.

   Note: *Add 20µl of ß-mercaptoethanol per 1ml of GTC Lysis buffer before use. Complete removal of supernatant is critical for RNA isolation.*

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 GTC lysis buffer to 200µl, as the total volume would be 400 µl after addition of 200µl of ethanol in Step 6.*

3. **Place the E-Z 96® DNA Clearance Plate on top of the Square-Well Collection Plate and carefully add entire sample from Step 2 to each well of the E-Z 96® DNA Clearance Plate.**

4. Seal E-Z 96® DNA Clearance Plate with Aera Sealing Film. Load the E-Z 96® DNA clearance plate/2 ml square-well plate into a microplate holder, and place the whole assembly into the rotor bucket of the centrifuge. Spin at 3,000-5,000 x g for 5 minutes at room temperature.

5. Use the flow-through for RNA purification in steps 6-14.

6. **Add one volume (300 µl) of 70% ethanol to the flow-trhough;** mix thoroughly by pipetting up and down 3 to 4 times. Do no centrifuge.
   Note: *if some lysate was lost during previous DNA binding step, adjust the volume of ethanol accordingly.*

7. Place the E-Z 96® RNA plate atop the 2 ml deep-well plate and carefully transfer entire sample from Step 6 (including any precipitate) to each well of the E-Z 96® RNA plate.

8. Seal the E-Z 96® RNA plate with Aera 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. Remove the Aera Sealing Film and discard the flow-through.

9. Add 500µl of RWC Wash Buffer directly into the each well of the E-Z 96® RNA plate, Seal the plate with new Aera Sealing Film and centrifuge at 5,000 x g for 5 minutes at room temperature.

10. Remove the Aera Sealing Film and add 700 µl Wash Buffer II diluted with ethanol to each well of the E-Z 96® RNA plate. Seal the plate with new Aera Sealing Film. Centrifuge at 5,000 x g for 5 minutes at room temperature. Discard the flow-through and re-use the Square-Well Collection Plate.

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

11. Remove the Aera Sealing Film. Add another 700 µl of RNA Wash Buffer II to each well of E-Z 96® RNA plate. Do not seal the plate with film. Centrifuge

at 5,000 x g for 10 minutes at room temperature. The prolonged centrifugation is necessary to dry the E-Z 96® RNA plate.

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.*

12. 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).

13. Add 75-100µl of DEPC-treated water to each well, and seal the E-Z 96® RNA plate with new Aera Sealing Film(supplied with kit). Make sure to add water directly onto the RNA matrix. Incubate for 3 minutes at room temperature. Centrifuge at 5,000 x g for 5 minutes at room temperature to elute RNA.

14. Remove the Aera Sealing Film. Repeat Steps 12 and 13 for second elution. ***Note:*** *Elution volume and numbers can vary according to user preference. To maintain a higher RNA concentration, first eluate can be used for second elution.*

E-Z 96® HP Total RNA Vacuum Protocol

Materials supplied by user

- 96-100% ethanol
- 70% ethanol
- ß-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 300ul of GTC 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 300 ul of GTC Lysis Buffer directly to each well. Mix by pipetting.

   Note: Add 20µl of ß-mercaptoethanol per 1ml of GTC Lysis buffer before use. Complete removal of supernatant is critical for RNA isolation.

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 a volume less than 300µl, reduce the volume of the GTC lysis buffer to 200µl, as the total volume would be 400µl after addition of 200µl ethanol in Step 3.*

3. PREPARE THE VACUUM MANIFOLD: Place the 2ml Square-Well Collection Plate inside the vacuum manifold base. Place the manifold's top section squarely over its base. Place the E-Z 96® DNA Clearance Plate on the manifold's top section, making sure the E-Z 96® DNA Clearance Plate is seated tightly on the rubber ring. Connect the vacuum manifold to the vacuum source. Keep the vacuum switch off.

4. Carefully transfer entire sample from Step 2 to each well of the E-Z 96® DNA Clearance Plate. Seal the un-used wells with sealing film. Switch on the vacuum source. Apply vacuum until all of the sample contents pass through the well membranes. Turn of the vacuum source and remove the E-Z 96® DNA Clearance Plate.

Note*: If some well of the E-Z 96® DNA Clearance Plate is clogged, remove the E-Z 96® DNA Clearance Plate and the Square-Well Collection Plate from manifold. Place E-Z 96® DNA Clearance Plate on top of the Square-Well Collection Plate, centrifuge at 5000 x g for 5-10 minutes.*

5. Use the flow-through for RNA purification in steps 6-16.

6. Add one volume (300µl) of 70% ethanol to the sample; mix thoroughly by pipetting up and down 3 to 4 times.

Note: *If some lysate was lost during previous DNA Clearance step, adjust the volume of ethanol accordingly.*

7. Place a E-Z 96® RNA plate on the top part of the vacuum manifold.

8. Carefully transfer the entire sample from Step 6 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 of the sample contents passes through the well membranes.

9. Add 500µl of RWC 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.

10. Add 700µl of 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.

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

11. Add another 700µl of 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.

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

13. 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.

14. Replace the Square-Well Collection Plate or waste collection tray with a microtube rack containing the 1.2ml microtubes. Reassemble the manifold. Place the E-Z 96® RNA plate on the top plate of the manifold.

15. Elution RNA: Add 75-100 µl of DEPC-treated water to each well, and seal 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 5-10 minutes. Switch off the vacuum and ventilate the manifold.

16. 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.*

17. Remove the eluted RNA from vacuum manifold and store at -80°C.

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 -80°C in water. Under such conditions RNA is stable for more than a year.

Clean the 2ml deep well plates:

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 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
Little or no RNA eluted	Add Carrier RNA to GTC Lysis Buffer RNA remains on the plate	• Dissolve the carrier RNA with GTC Lysis Buffer and repeat the purification with new sample. • Repeat elution. • Pre-heat DEPC-water to 70° C prior to elution. • Incubate for 5 min with water prior to elution
	Plate is overloaded	• Reduce quantity of starting material.
Clogged well on DNA Clearance Plate	Incomplete lysis	• Mix thoroughly after addition of GTC 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.
	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 RNA 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 GTC to completely lyse cells
DNA contamination	Too much starting material	• Reduce the cell number and repeat the extraction. • Perform DNase digestion
Little or no RNA eluted	Lost DNA during process	• Make sure DNA wash Buffer is diluted with absolute ethanol with correct amount of ethanol • use the pre-heated Elution Buffer (65°C) for DNA elution.
RNA contamination on genomic DNA elute		• Add 50µl of RNase A (20mg/ml) to the DNA wash Buffer.