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## Introduction

E-Z 96<sup>®</sup> Tissue RNA Kit is designed for isolation of total RNA from certain animal tissues such as cartilage, skeletal muscle and heart tissue. The kit allows simultaneous processing of up to 192 samples in less than 60 minutes. The procedure completely removes contaminants and enzyme inhibitors making RNA isolation fast, convenient, and reliable.

RNA purified using the E-Z 96<sup>®</sup> Tissue RNA method is ready for applications such as RT-PCR\*.

## Principle

The E-Z<sup>®</sup> Tissue RNA Kits use the reversible binding properties of HiBind<sup>®</sup> matrix, a new silica-based, time-saving spin technology material. Combined with the speed of 96-well spin or vacuum manifold technology, up to 192 samples can be processed at same time. The samples are lysed first under highly denaturing buffer conditions so that RNases will be inactivated, and the intact Tissue RNA is protected from degradation. After adjusting the binding conditions, the samples are loaded to the E-Z 96<sup>®</sup> RNA Plate. With a brief centrifugation or vacuum, the samples pass through the plate and the RNA binds to the Hibind<sup>®</sup> matrix. After two wash steps, purified Tissue RNA will be eluted with RNase-free water.

## Storage

All components in the E-Z 96<sup>®</sup>Tissue RNA Kit should be stored at room temperature . During shipping and storage, crystals may form in the QVL 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 <sup>®</sup> Tissue RNA Kits	4 x 96 Preps	12 x 96 Preps
Product Number	R1088-01	D1088-02
Purification	4	12
<b>Components</b>		
E-Z 96 <sup>™</sup> RNA Plates	4	12
Racked Microtubes (1.2ml)	4 x 96	12 x 96
8-Strip Microtube Caps	52 x 8	156 x 8
2 ml 96-well Collection Plates*	2	2
QVL Lysis Buffer	125 ml	600 ml
RNA Wash Buffer I	350 ml	900 ml
RNA Wash Buffer II Concentrate	2 x 50 ml	6 x 50 ml
OB Protease	4 x 30 mg	12 x 30 mg
DEPC-ddH <sub>2</sub> O	40 ml	120 ml
Instruction Manual	1	1

\* 2ml Deep well plates are reusable; see Page 7 for 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 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.
- Under cool ambient conditions, crystals may form in QVL Lysis Buffer. This is normal and the bottle should be warmed to redissolve the salt.
- 2-mercaptoethanol ( $\beta$ -mercaptoethanol) is key in denaturing RNases and must be added to an aliquot of QVL Lysis Buffer before use. Add 20  $\mu$ l of 2-mercaptoethanol per 1 ml of QVL Lysis Buffer. This mixture can be stored for 1 week at room temperature.

- All centrifugation steps must be carried out at 22°C-25°C.

## Before Starting

<b>IMPORTANT</b>	1. <b>Wash Buffer II Concentrate</b> must be diluted with absolute ethanol before use. R1088-01 Add 200 ml 100% ethanol per bottle R1088-02 Add 200 ml 100% ethanol per bottle
	2 Reconstitute OB Protease in 1.2 ml 10 mM Tris-HCl, pH 8. Vortex vial briefly prior to use.

## E-Z 96<sup>®</sup> Tissue RNA Protocol

### Materials supplied by user:

- 96-100% ethanol
- $\beta$ -Mercaptoethanol
- Multichannel pipet
- RNase-free filter pipette tips
- Reagent reservoirs for multichannel pipettor
- Centrifuge with rotor suitable for 96-well plate.
- Disposable latex gloves
- RNase-Free water
- RNase-Free 1.2 ml 96-well plate
- Adhesive sealing film for microplate (5 per plate)
- 2ml 96-well deep well plate
- Water bath or heat block preset at 55°C

**Note:** Equilibrate samples and QVL Lysis Buffer to room temperature before starting. All steps must be carried out at room temperature. Work quickly, but carefully.

### Homogenization of Tissues

#### A. Liquid Nitrogen Method

*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 QVL Lysis Buffer and continue with the procedure as outlined below. This is the preferred method of disrupting tissue samples.

#### B. Rotor-Stator Homogenizers

Rotor-stator homogenizers effectively homogenize most tissues. 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 of small volumes in microfuge tubes. Such homogenizers are available from:

- Tekmar Inc., Cincinnati, OH (Tissuemizers®)
- BIOSPEC Products, Bartlesville, OK (Tissue-Tearor™)
- Craven Laboratories, Austin, TX.

### C. Syringe Method

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

#### Starting Material:

Although the binding capacity for each well of the EZ-96® RNA Plate is around 100 µg, the maximum amount of starting material to be used depends on the type of tissue being processed and the corresponding RNA content. **It is essential to begin with the correct amount of tissue to get optimal RNA yield and purity using the E-Z 96® RNA plate. For the first time user, we recommend beginning with less than 10 mg tissue. Depending on yield and purity obtained, it may be possible to increase the starting material to 20 mg.**

#### Procedure:

1. **Excise the tissue sample from animal or from storage.**
2. Weigh 10 mg tissue and place it into suitable vessel for disruption and homogenization.
3. Add 250 µl QVL Lysis Buffer and disrupt tissue and homogenize lysate in TRK Lysis Buffer by using rotor-stator or mix mill.
4. Pipet 490 µl RNase-Free water to each homogenate. Add 10 µl OB Protease and mix thoroughly by pipetting.
5. Incubate at 55 °C for 10 minutes.
6. Centrifuge at 5,000-10,000 x g (depending on the vessel containing the sample) for 10 minutes at room temperature. A small pellet of tissue debris will form and a thin layer or film can be seen on top of the supernatant.
7. Transfer the supernatant into a RNase-Free 2 ml 96-well plate (not provided). Avoid transferring any part of the pellet. Hold the pipet tip under the thin layer of film on top of the supernatant, if present. This layer will usually adhere to the exterior of the tip; it should not be transferred.
8. Add 375µl of absolute ethanol (96-100%) to the cleared lysate, mix

thoroughly by pipetting.

9. **Carefully** apply 500µl solution from Step 8 above (including any precipitate) to each well of the E-Z 96® RNA plate.
10. Seal the E-Z 96® RNA plate with sealing film. Load the E-Z 96® RNA plate placed atop the 2ml 96-well collection plate into the plate holder; place the whole assembly into the rotor bucket of the centrifuge. Spin at 4,500 x g for 5 minutes at room temperature.
11. Remove the sealing film and repeat Steps 9 and 10 using solution from Step 8.
12. Remove the sealing film. Wash samples with RNA Wash Buffer I by pipetting 300 µl directly into the each well of the E-Z 96® RNA plate . Seal the plate with a new sealing film. Centrifuge at 4,500 x g for 5 minutes at room temperature.
13. Place the E-Z 96® RNA plate on top of another clean 2ml 96-well plate. Optional DNase digestion procedure can be started at this point. Otherwise, proceed at Step 15.
14. DNase digestion (Optional)

Since HiBind® RNA resin and spin-plate technology actually remove most DNA without the DNase treatment, Dnase digestion is not necessary for most downstream applications. However, certain sensitive RNA applications might require further DNA removal. Use the following steps for on-membrane DNase I digestion. (See DNase I Cat #E1091 for more detailed information.)

- a. For each well of E-Z 96® RNA plate, 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 and subject 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 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 the HiBind® RNA resin in each well. Make sure to pipet the DNase I digestion mixture directly onto the membrane. DNase I digestion will not be complete if some of the mixture sticks to the wall or the O-ring of the E-Z 96® RNA plate.

c. Incubate at room temperature(25-30°C) for 15 minutes

15. Add 500 µl Wash Buffer I to each well of E-Z 96™ RNA plate. Seal the plate with a new sealing film. Centrifuge at 4,500 x g for 5 minutes at room temperature.

16. Remove the sealing film. Add 500ul of **RNA Wash Buffer II** to each well of the E-Z 96® RNA plate. Centrifuge at 4,500 x g for 5 minutes. Remove the film and discard the flow-through. Reuse the 2ml collection plate.

17. Add another 500ul of RNA Wash Buffer II to each well of the HiBind® RNA plate. Seal the plate with new sealing film. Centrifuge at 4,500 x g for **15 minutes** at room temperature to dry the membrane.

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

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

19. Add 50-70 µl of DEPC-treated water to each well, and seal the E-Z 96® RNA plate with new sealing film. Make sure to add water directly onto RNA matrix. Incubate for 1 minute at room temperature. Centrifuge at 4,500 x g for 5 minutes at room temperature to elute RNA.

## 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 E-Z 96® 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
Little or no RNA eluted	Carrier RNA not added to TRK 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>Avoid warming the TRK/Carrier RNA frequently.</li> </ul>
	RNA remains on the plate	<ul style="list-style-type: none"> <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>
	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>