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Introduction

E.-Z 96™ Tissue RNA Kit is designed for the isolation of total RNA from animal tissues, e specially for some difficult fibrous tissues such as skeletal muscle, heart and aorta tissue. Those tissues normally make RNA isolation more difficult because they contain contractile proteins, connective tissue and collagen. The E-Z 96™ Tissue RNA Kit can also be used for isolation of other tissue samples. This kit allows simultaneous processing of up to 192 samples in less than 60 minutes. This procedure completely removes contaminants and enzyme inhibitors making RNA isolation fast, convenient, and reliable. RNA purified using the E-Z 96 Tissue RNA method is ready for applications such as RT-PCR*.

Principle

The E-Z 96™ Tissue RNA Kit uses reversible binding properties of HiBind® matrix, a new silica-based, time saving spin technology material. The sample is lysed first under highly denaturing buffer conditions so that the RNase will be inactivated, and the intact RNA is protected from degradation. After adjusting the buffer condition, the samples are treated with Proteinase K to digest the proteins. Then remove cell debris by centrifugation, the sample is mixed with ethanol to adjust the binding condition and loaded to a E-Z 96 RNA plate to bind RNA. With a brief centrifugation or vacuum, the samples pass through the RNA plate and the RNA is bound to the HiBind® matrix. Trace of the DNA that may co-purified with RNA will be removed by DNase treatment on the RNA spin column. After two washing steps, purified total RNA will be eluted with RNase-free water.

Storage and Stability

All components in the E-Z96™ Tissue RNA Kit except DNase I and Proteinase K should be stored at room temperature. DNase I and Proteinase K should be stored at -20°C 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 the 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

Product Number	R1088-01	R1088-02
Purification	2 x 96	8 x 96
E-Z 96™ RNA Plate	2	8
2.2 ml Deep-well Plate*	6	24
500µl Collection Plate	2	8
TRK Lysis Buffer	80 ml	240 ml
RWF Wash Buffer	250 ml	1000 ml
RNA Wash Buffer II Concentrate	80 ml	2 x 200 ml
Proteinase K	55 mg	220 mg
Proteinase Storage Buffer	3 ml	9 ml
DNase I**	300 µl	1.2 ml
DNase I Digestion Buffer	16 ml	70 ml
DEPC-ddH_2O	50 ml	200 ml
Instruction Manual	1	1

*2.2 ml Deep-Well Plate is reusable, see page 7 for instructions.
**DNase I is shipped separately by dry ice

Before Starting

IMPORTANT	Dilute **RNA Wash Buffer II** with **absolute ethanol** as follows **R1088-01** Add 320 ml ~96-100 % ethanol **R1088-02** Add 800 ml ~96-100% ethanol
	Reconstitute **Proteinase K** with DEPC Water as following **R1088-01** Add 2.2 ml Protease Storage Buffer **R1088-02** Add 8.8 ml Protease Storage Buffer Vortex vial briefly and aliquot into adequate portions. Store aliquots at -20°C.

Important Notes

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.
- Under cool ambient conditions, crystals may form in the TRK Lysis Buffer. This is normal and the bottle should be warmed to re-dissolve the salt.
- 2-mercaptoethanol (ß-mercaptoethanol) is key in denaturing the RNase and must be added to an aliquot of TRK Lysis Buffer before use. Add 20µl of 2-mercaptoethanol per 1 ml of TRK 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.

Note: Equilibrate samples and TRK Lysis buffer to room temperature before start. All steps must be carried out at room temperature. Work quickly, but carefully.

Materials supplied by user

- 96-100% ethanol
- RNase-Free DNase I (optional)
- β-Mercaptoethanol
- RNase-free filter pipette tips
- Centrifuge with swinging-bucket capable of 4000 x g
- Rotor and adapter for 96-well plate
- Water bath or heat block preset at 55°C
- 96 Deep-well pate

Starting Materials

Although the binding capacity for each well of the E-Z 96™ RNA plate is around 50µg, the maximum amount of starting material 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 with E-Z 96™ RNA Plate. For the first time user, we recommend to use less than 10 mg of tissue per sample. Depending on the yield and purity obtained, it may be possible to increase the starting material to 15 mg.**

Disruption & Homogenization of Tissues

A. Disrupt and Homogenize Tissue with Liquid Nitrogen

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 10ml of liquid nitrogen and pour the suspension into a pre-cooled 15ml 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 TRK Lysis Buffer to lyse the tissue sample by vortexing. Tissue lysate can also be homogenized with a E-Z 96 Homogenizer Plate (Product # HCR9601-02). **The lysate is loaded onto the Omega E-Z 96 Homogenizer Plate in a 2 ml collection plate**. Spin two minutes at a maximum speed in a microcentrifuge and collect the homogenized lysate. Use the Omega Homogenizer Spin Column, it is a fast and efficient way to homogenize the lysate without cross contamination of the samples. The alternative way to homogenize the lysate is to use the syringe and needle. High molecular-weight DNA can be sheared by passing the lysate through a narrow needle (19-21 gauge) 5-10 times.

B. Disruption and Homogenization with Rotor-Stator

Rotor-Stator is the most preferred method for disruption and homogenizing tissue samples if required the equipment is available. Rotor-stator homogenizers effectively homogenize most tissues in the present of TRK Lysis Buffer. 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. Disruption and Homogenization with Beads Mills

Tissue samples can also be effectively disrupted and homogenized by rapid agitation in the presence of beads and lysis buffer. Tissue samples are disrupted and simultaneously homogenized with the sheared and crushed action of the beads as they collide with cells.

E-Z 96™ Tissue RNA Protocol

1. **Excise the tissue sample from an animal or from storage**.

2. **Weigh 10mg of tissue and place it into a suitable vessel for disruption and homogenization.** *Do not use more than 15mg of tissue. For more information, see page 4 of Starting material.*

3. **Add 300µl of TRK lysis Buffer/2-ME and disrupt and homogenize tissue in TRK lysis buffer as described in previous section.** Rotor-Stator or the Bead Mill normally can result in higher RNA yield because they provide better disruption and homogenization.

   Note:2-mercaptoethanol (ß-mercaptoethanol) is key in denaturing the RNase and must be added to an aliquot of TRK Lysis Buffer before use. Add 20µl of 2-mercaptoethanol per 1 ml of TRK Lysis Buffer. This mixture can be stored for 1 week at room temperature.

4. Transfer the homogenized lysate into a 2.2 ml Deep-Well plate (Supplied).

5. **Pipet 590µl of RNase-Free water to each sample and add 10µl of Proteinase K solution and mix thoroughly by pipetting.** Incubate at 55°C for 10 minutes.

6. **Centrifuge at 4,000 x g for 15 minutes at room temperature**. A small pellet of tissue debris will form at the bottom of each well and a thin layer or film can be seen on top of the supernatant.

7. Transfer the supernatant into a new 2.2 ml Deep-Well plate (Supplied).

   Note: Avoid transferring any of the pellet. Hold the pipette tip under the thin layer of film on top of the supernatant, if present. This layer will usually adhere to the outside of the tip and should not be transferred.

8. **Add ½ volume (around 450µl) of absolute ethanol (96-100%) to the cleared lysate, mix thoroughly by pipetting or vortexing.**

9. Place a **E-Z 96™ RNA Plate** on top of the 2.2 ml Deep-well plate from step 4.

10. **Carefully apply 700µl of samples from step 8 (including any precipitate) to E-Z 96™ RNA Plate.**

11. Spin at 4000 x g for 5 minutes at room temperature.

12. Transfer the remaining samples to the E-Z 96™ RNA Plate. Note: After transferring the lysate keep the 2.2 ml Deep-Well Plate for the use of collection in step 14.

13. Centrifuge at 4000 x g for 5 minutes.

14. Remove the E-Z 96™ RNA Plate and place it onto a 2.2 ml Deep-Well plate.

15. **Pipet 350µl of RWF Wash Buffer into the column. Centrifuge at 4,000 x g for 5 minutes.**

16. **Prepare the DNase I mixture:** For 96 samples, in a 15ml microtube, add 150µl of DNase I to 7.35 ml of DNase I Digestion Buffer. Mix gently by inverting the tube. Do not vortex, DNase I is especially sensitive with physical denaturation.

17. Add 75µl of the DNase I mixture directly onto the center of the membrane in each well of E-Z 96™ RNA Plate.

18. Incubate the plate at room temperature for 15 minutes.

19. Place the E-Z 96™ RNA Plate on top of a new 2.2 ml Deep-Well Plate. Add 800µl of RWF Wash Buffer to each well of the E-Z 96™ RNA Plate. Wait 5 minutes and Centrifuge at 4000 x g for 5 minutes at room temperature.

20. Add 800µl of RNA Wash Buffer II to each well of the E-Z 96™ RNA Plate. Centrifuge at 4000 x g for 5 minutes at room temperature.

21. Place the E-Z 96™ RNA Plate on top of a new 2.2 ml Deep-Well Plate. Add another 800µl of RNA Wash Buffer II to each well of the E-Z 96™ RNA Plate. Centrifuge at 4000 x g for 10 minutes at room temperature.

22. Place the E-Z 96™ RNA Plate on top of a new 500µl collection plate (supplied).

23. Add 75µl of DEPC-Treated water onto the center of membrane in each well of the plate. Centrifuge at 4000 x g for 4 minutes at room temperature.

24. Repeat the elution step (step 23) once with second 75 µl of DEPC-Treated water.

25. Seal the plate containing purified RNA and store at -70°C.

Reuse of 2.2 ml Collection Plate

To reuse the 2.2 ml Deep-Well Plates, rinse them thoroughly with tap water, incubate for 2 hours overnight in 0.1 N NaOH/1mM EDTA, rinse in distilled water, and dry at 50C. Do not use bleach.

Quantification 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 44 µg of RNA per ml. If it is necessary to dilute RNA sample, use a buffer with neutral pH. 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 1.8-2.1 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.

Integrity of RNA

Run a denatured gel to determine the integrity and the size distribution of the RNA. The respective riborsomal RNA bands should appear as sharp and clear bands on the gel. The 28S bands should be presented twice amounts of 18S RNA band. If the ribosomal RNA bands in a given lane are not sharp and it shows to be smeared towards the smaller sized RNA, it is very likely that the isolated RNA suffered major degradation during the isolation procedure.

Troubleshooting Guides

Problem	Cause	Suggestion
Little or no RNA eluted	RNA remains on the membrane	• Repeat elution. • Pre-heat DEPC-water to 70° C prior to elution. • Incubate for 5 minutes with water prior to elution.
	overloaded sample	• Reduce the quantity of the starting material.
Clogged wells	Incomplete lysis	• Mix thoroughly after the addition of TRK Lysis Buffer. • Reduce the 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
	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		• Make sure to perform RNase-free DNase Digestion correctly
Abnormal OD reading on A260/A280	DEPC residue remains in the DEPC-water	• use different RNase-free water. • Remove DEPC by Autoclave • Use 10mM Tris-HCl, not the DEPC water to dilute the sample before measuring purity.