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Introduction

E-Z 96[®] Total RNA Kit II is designed for isolating total cellular RNA from all types of animal and human tissues. This kit allows simultaneous purification of 96 or 192 samples in less than 60 minutes. RNA purified using this kit is ready for applications such as RT-PCR*, Northern blotting, poly A⁺ RNA (mRNA) purification, nuclease protection, and *in vitro* translation..

RNA purified using the E-Z 96 Total RNA method is ready for applications such as RT-PCR*, Northern blotting, poly A⁺ RNA (mRNA) purification, nuclease protection, and *in vitro* translation.

Principle

The E.-Z 96[®] Total RNA Kit II provides a fast and easy method for high throughput RNA isolation. This kit integrates efficient lysis of phenol/guanidine buffer and the reversible binding properties of HiBind[®] matrix, a new silica-based material with the speed of the E-Z 96 plate format for fast processing of large number of samples. Cells or tissues are first homogenized with RNA-Solv Reagent that practically inactivates RNase. Add chloroform, then the homogenate is separated into aqueous and organic phase with centrifugation. The aqueous phase which contains RNA then adjusted with ethanol and then applied to the E-Z 96[®] RNA Plate to which total RNA binds, while cellular debris and other contaminants are effectively washed away. High quality RNA is finally eluted in DEPC-treated sterile water.

Storage

All components except RNA-Solv in the E-Z 96 Total RNA Kit II should be stored at room temperature. RNA-Solv should be store at 4°C for long term storage. All Total RNA Kit II components are guaranteed for at least 12 months from the date of purchase when stored at 22-25°C.

*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.N.A.™ Total RNA Kits	RNA Preps 1 x 96	RNA Prep 4 x 96	RNA Prep 20 x 96
Product Number	R6935-00	R6935-01	R6935-02
Purification	1 x 96	4 x 96	20 x 96
E-Z 96® RNA Plate	1	4	20
96 Deep-well Plate	1	4	20
Sealing Film	1	4	20
Aera Seal Film	3	12	60
RNA-SOLV	4 X 25ml	4 x 100 mL	10 x 200 ml
RNA Wash Buffer I	90 ml	360 ml	1850 ml
RNA Wash Buffer II Concentrate	35 ml	140 ml	4 x 160 ml
DEPC-ddH ₂ O	20 ml	80 ml	400 ml
Instruction Manual	1	1	1

*Note: RNA-Solv contains Guanidine Thiocyanate & Phenol, handle those reagents with extra care.

Before Starting

IMPORTANT	RNA Wash Buffer II must be diluted with absolute ethanol before use.	
	R6935-00	Add 140 ml 100% ethanol
	R6935-01	Add 560 ml 100% ethanol
	R6935-02	Add 640ml 100% ethanol

Please take a few minutes to read this booklet thoroughly and become familiar with the protocol. Prepare all of the 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.**
- **To freeze tissue for long term storage, flash-frozen liquid nitrogen and immediately transfer to -70°C.** Tissue can be stored for up to 6 months at -70°C. To process the sample, do not thaw the sample during weighing or handing prior to the disruption with RNA-Solv Reagent. Homogenized tissue lysates can be stored at -70°C for at

least 6 months. To proceed with the frozen tissue lysate, thaw the sample at 37°C until it is completely thawed and all salts in the lysis buffer are dissolved. Do not extend the treatment in 37°C because it can cause chemical degradation of RNA.

- **It is very important to determine the correct amount of starting material before the experiment.** If the maximum amount of the starting material is 100mg. The capacity of the HiBind® RNA column is 100µg. For samples containing high amounts of RNA, we suggest the usage of 30mg of tissue to start. For samples containing lower level RNA, the maximum amount of starting material (100mg) can be used.
- **Prepare the water-saturated phenol solution:** Place the solid phenol into the water bath preset at 75°C until phenol is completely dissolved. Add equal volume of molecular biology grade water and mix thoroughly by shaking. Store the solution at room temperature for 4 hours to overnight until the water phase (upper phase) and phenol phase (lower phase) are clearly separated. Remove the water phase with a transfer pipettor. Use the water-saturated phenol (lower phase) to prepare the RNA-Solv® Reagent (see instructions on page 3). Small amounts of water will not effect the performance of the RNA-Solv Reagent.

Disruption and Homogenization of samples

Efficient disruption and homogenization of the sample is essential for successfully isolating total RNA. Complete disruption of the cell walls and plasma membrane is very important for releasing all the RNA contained in the samples. The purpose of homogenization is to reduce the viscosity of the cell lysates produced by cell disruption. Homogenization shears the genomic DNA and other high molecular weight cell components to create a homogeneous lysate. Incomplete homogenization will reduce the binding of RNA to the RNA column and sometimes will clog the RNA column thus causing lower yield or no yield.

A). Disruption of Sample with Mortar and Pestle

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 RNA-Solv Reagent and continue with the procedure as outlined below. After phase separation by adding chloroform, the supernatant can be homogenized with Homogenizer Spin Column (Product # HCR 002). **The lysate is then loaded onto**

the Homogenizer Spin Column in a 2 ml collection tube. Spin for two minutes at the maximum speed in a micro centrifuge and the homogenized lysate is collected. Use the Omega Homogenizer Spin Column it's a fast and efficient way to homogenize the lysate without cross contamination of samples. The alternative approach to homogenizing 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 & homogenization of sample with Rotor-Stator Homogenizers

Rotor-stator homogenizers can effectively and simultaneously disrupt and homogenize most samples. 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 samples in 50ml tubes.

C.) Disruption & homogenization of sample using Bead Milling

By using bead milling, cells and tissues can be disrupted and homogenized by rapid agitation in the presence of beads and lysis buffer. The optimal use for RNA isolation are 0.5mm glass beads for yeast and unicellular cells, 4-8 mm beads for animal tissue samples.

D). Homogenization of lysate with Syringe Needle Method

High molecular weight DNA is responsible for the viscosity of cell lysate's and can be shredded by passing the sample 10-20 times through a narrow needle (19-21 gauge).

Total RNA Isolation Protocol

A. Isolating Total RNA from Animal Cells

Materials supplied by user:

- 2-mercaptoethanol
- Chloroform
- Centrifuge capable of at least 4,000 x g
- Adapter for 96-well plate
- Centrifuge adapter for micro-plate
- 70% ethanol in DEPC-treated sterile distilled water
- Sterile RNase-free pipette tips
- 96 deep-well plate (1 ml or 2 ml)
- Disposable latex gloves

Procedure:

1. **Determine the proper amount of starting material:** It is critical to use the correct number of cells to obtain optimal yield and purity with the E-Z 96[®] RNA plate. The maximum number of cells that can be processed on a E-Z 96[®] RNA plate varies, it depends on the specific RNA contents and type of cell lines. The maximum binding capacity for each well in the E-Z 96[®] RNA plate is 100µg. The maximum number of the cells that RNA-Solv Reagent used in this protocol is 1×10^7 . Use the following table as a guideline to select the correct starting material.

Average Yield of Total cellular RNA

Source	Number of Cells	RNA Yield (µg)
IC21	1×10^6	12
Hela	1×10^6	15
293HEK	1×10^6	10
HIN3T3	1×10^6	15

2. **Harvest Cells:**

For cells grown in suspension: determine the number of cells. Pellet the appropriate number of cell by centrifuge at 500 x for 5 minutes. Aspirate the supernatant and continue to step 3 of this protocol.

For cells grown in a mono-layer: Cells grown in a mono-layer in a

cell cultured dish can be directly lysed in the dish or trypsinized and collected the cell pellet before lysis. Cells grown in a cell culture flask should be trypsinized and the cell pellet collected prior to lysis.

3. **Disrupt cells (do not use more than 1×10^7 cells) with RNA-Solv:** For pelleted cells, loosen the cell pellet thoroughly by vortexing or pipetting up and down 5-10 times. Add the 800 μ l of RNA-Solv Reagent directly to lyse the cell in the culture plate, add the 800 μ l of RNA-Solv Reagent directly to the dish. Remember to add 20 μ l of 2-mercaptoethanol per 1 ml of RNA-Solv before use.

4. **Incubate the plate that contains the homogenate at room temperature for 5 minutes.**

5. **Add 160 μ l of chloroform to each homogenate, mix the sample thoroughly by pipetting. Incubate at room temperature for 2-3 minutes.**

6. **Seal the plate with Sealing film, centrifuge at $\geq 4,000 \times g$ at $4^\circ C$ for 20 minutes to separate the aqueous and organic phase.**

Note: The sample should be separated into 3 phases: an upper colorless aqueous phase, which contains RNA; a white interphase and a lower blue organic phase.

7. **Transfer the upper aqueous phase (around 350-400 μ l) into a new 96 deep-well plate (Not supplied). Add an equal volume of 70% ethanol and mix thoroughly by pipetting up and down 20-30 times.** A precipitate may form at this point. This will not interfere with the RNA purification.

8. **Place the E-Z 96 RNA plate on top of a 2.2 ml Deep-well plate (supplied). Load sample onto each well of the E-Z 96 RNA plate and seal the plate with AeraSeal film.** Centrifuge at $4,000 \times g$ for 5 minutes at room temperature.

9. **Remove the sealing film and add 600 μ l of RNA wash Buffer I directly into the each well of the plate.** Centrifuge at $4,000 \times g$ for 5 minutes. Remove the sealing film. Discard the flow-through and re-use the deep-well plate in the next step.

Note: This the starting point if on-membrane DNase I digestion (page 12).

10. **Place the E-Z 96 RNA Plate back on top of the deep-well plate.** Add 750 μ l RNA Wash Buffer II into each well. Seal the plate with AeraSeal film. Centrifuge at $4,000 \times g$ for 5 minutes at room temperature.

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

11. **Remove the sealing film, add another 750 μ l of Wash Buffer II into each well of the plate.** Seal the plate with AeraSeal film. Centrifuge at $4,000 \times g$ for 20 minutes.

12. **Elution of RNA.** Transfer the E-Z 96 RNA plate on to a new microplate (supplied). Add 45-75 μ l of DEPC-treated water to the center of membrane in each well. Make sure to add water directly onto center of matrix. Incubate at room temperature for 1 minute at room temperature. Centrifuge 5 minutes at $4,000 \times g$ to elute the RNA from plate.

13. Add another 45-75 μ l of DEPC-treated water into each well. Centrifuge for 5 minutes at $4,000 \times g$.

Alternatively, RNA may be eluted with same elute from the first elution for higher concentration of RNA. Pre-heating the water to $70^\circ C$ before adding to plate and incubating the column for 5 minutes at room temperature before centrifugation may increase yields.

B. Isolating Total RNA from Animal Tissues

Materials supplied by user:

- 2-mercaptoethanol
- Chloroform
- Centrifuge with capable of at least 4,000 x g
- Adapter for 96-well plate
- 70% ethanol in DEPC-treated sterile distilled water
- 96 deep-well plate (1 ml or 2 ml)
- Disposable latex gloves

1. **Determine the proper amount of starting material:** This is critical to use correct number of cells to obtain optimal yield and purity. The maximum amount of tissue that can be processed in each well of E-Z 96 RNA plate varies depends on the specific RNA contents and type of tissue. The maximum binding capacity for each well of the E-Z 96 RNA plate is 100µg. We recommend to use 50mg tissues as starting material. For liver or spleen, only 25 mg tissue should be use. and Use following table as a guideline to select correct starting material. **If you have no information about the your starting material, use 20 mg as starting amount, base on the yield and quality of RNA obtained from 10 mg, adjust the starting amount in the next purification.**

Average Yield of Total cellular RNA

Source	Amount of Tissue (mg)	RNA Yield (µg)
Mouse Tissue		
Brain	10	7-20
Kidney	10	5-30
Liver	10	10-75
Heart	10	5-25
Spleen	10	15-80
Lung	10	5-45
Pancreas	10	15-80
Adipose Tissue	10	0.2-2

2. **Disrupt Tissue and homogenize the tissue in 800µL RNA-Solv using one of the described methods on page 4.** Do not allow the sample to be thawed before it is place into the RNA-Solv . We recommend using the bead beating method because it can simultaneously process 96 samples at one time. (Do not use more than 100mg tissue). Remember to add 20µl of 2-mercaptoethanol per 1 mL of RNA-Solv before use.

Note: Incomplete homogenization of the sample will cause lower yields and clogging of the well.

3. **Incubate the tube that contains the homogenate at room temperature for 5 minutes.**
4. **Add 160µl of Chloroform to the homogenate, close the cap of the tube and vortex for 20 seconds. Incubate at room temperature for 2-3 minutes.**
5. **Centrifuge at $\geq 4,000$ x g at 4 °C for 20 minutes to separate the aqueous and organic phase.**

Note: The sample should be separated into 3 phases: an upper colorless aqueous phase, which contains RNA; a white inter phase and a lower blue organic phase.

6. **Transfer the upper aqueous phase (around 500ul) into a new deep-well plate. Add equal volume of 70% ethanol and vortex to mix thoroughly.** A precipitate may form at this point. This will not interfere with RNA purification.
7. **Place a E-Z 96 Apply sample onto a 2.2 ml deep-well plate (supplied). Transfer the entire sample from step 6 into each well of the E-Z 96 RNA plate. Centrifuge at 4,000 x g for 5 minutes at room temperature.**
8. **Add 600µl RNA wash Buffer I to each well of the plate. Centrifuge at 4000 x g for 3 minutes.** Discard the flow-through and re-use the collection plate.

Note: This the starting point if on-membrane DNase I digestion (page 12).

9. **Add 750µl of RNA Wash Buffer II into each well of the plate.** Centrifuge at 4,000 x g for 3 minutes at room temperature.
10. **Add another 750µl of Wash Buffer II into each well of the plate.**

Centrifuge at 4,000 x g for 20 minutes.

11. **Elution of RNA.** Place the E-Z 96 RNA plate on to a new 96-well microplate (supplied). Add 45-75µl of DEPC-treated water into each well. Make sure to add water directly onto the center of matrix in each well. Centrifuge for 5 minutes at 4,000 x g.
12. Add another 45-75µl of DEPC-treated water into each well. Centrifuge for 5 minutes at 4,000 x g.

Alternatively, RNA may be eluted with the same elute from the first elution for higher concentration of RNA. Pre-heating the water to 70°C before adding to plate and incubating column for 5 minutes at room temperature before centrifugation may increase yields.

C. DNase I digestion Protocol (Optional)

In most case, DNase I treatment is not necessary by using E-Z 96 Total RNA kit II protocol because most of DNA are effectively removed by the RNA-Solv extraction and column purification. However, if further DNA removal are desired, follow this protocol for On-Membrane DNase Digestion:(Product # DNase I cat.# E1091)

1. Follow the standard protocol until the cell lysate **completely** passes through the E-Z 96 RNA plate. Prepare the following:
2. 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:

- a. **DNase I is very sensitive for physical denaturaion, so do not vortex this DNase I mixture. Mix gently by inverting the tube. Prepare the fresh DNase I digestion mixture before RNA isolation.**
 - b. **DNase I digestion buffer is supplied with RNase-free DNase set.**
 - c. **Standard DNase buffers are not compatible with on-membrane Dnase digestion.**
3. Pipette 75µl of the DNase I digestion reaction mix directly onto the surface of HiBind® RNA resin in each well. Make sure to pipette 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 well.
 4. Incubate at room temperature(25-30°C) for 15 minutes.
 5. **Place plate into a 2.2 ml Deep-well plate**, and add 300µl of RNA Wash Buffer I. **Place the plate on the bench top for 2 minutes.** Centrifuge at 4,000 x g for 3 minutes.
 6. **Add 750µl of RNA Wash Buffer II** into each well and centrifuge at 4,000 x g for 3 minutes. Discard the flow-through and re-use the collection plate.

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

7. Add another 750 μ l of RNA Wash Buffer II into each well. Centrifuge at 4000 x g for 20 minutes.
8. **Elution of RNA.** Transfer the plate onto a new 96-well micro-plate (supplied). Add 40- 70 μ l of DEPC-treated water into each well. Make sure to add water directly onto the column matrix. Let it stand for 1 minute. Centrifuge for 3 minutes at 4,000 x g to elute RNA.
9. Add another 45-75 μ l of DEPC-treated water into each well. Centrifuge for 5 minutes at 4,000 x g.

Alternatively, RNA may be eluted with the same elute from the first elution for higher concentration of RNA. Pre-heating the water to 70°C before adding to plate and incubating the column for 5 minutes at room temperature before centrifugation may increase yields.

D). Total RNA Kit II Vacuum/Spin Protocol

Carry out lysis, homogenization, phase separation steps as indicated in previous protocols. Instead of continuing with centrifugation, follow steps below.

Note: Please read through previous sections of this book before using the protocol.

1. **Prepare the vacuum manifold according to manufacturer's instruction:** Place a waste collection tray inside the vacuum manifold. Place a E-Z 96 RNA plate on top part of the manifold.
2. **Transfer the sample mix (after addition of equal volume of 70% ethanol) into a each well of the E-Z 96 RNA plate.**
3. **Switch on vacuum source to draw the sample through the plate and turn off the vacuum.**
4. **Add 500 μ l of RNA wash buffer I into each well of the plate, draw the wash buffer through the plate by turning on the vacuum source.** Turn off the vacuum source and ventilate the vacuum.
5. **Add 750 μ l RNA wash buffer II to each well and draw the wash buffer through the column by turning on the vacuum source.** Turn off the vacuum source and ventilate the vacuum.
6. **Add another 750 μ l of RNA wash buffer II into each well of the plate. Seal the plate with AeroSeal film. Centrifuge at 4000 x g for 15 minutes.**
7. Place the E-Z 96 RNA plate on top of the new micro-plate (500 μ l). Add 45-75 μ l of DEPC-treated water onto the center of the membrane in each well. Incubate at room temperature for 3 minutes. Centrifuge at 4000 x g for 5 minutes to elute RNA.
8. Add another 45-75 μ l of DEPC-treated water into each well. Centrifuge at 4000 x g for 5 minutes to elute RNA.

DNA Contamination

No RNA extraction procedure can completely remove genomic DNA. For sensitive work (such as RT-PCR or differential display) we suggest that you either to do the on-membrane DNase I digestion treatment or treat the eluted RNA with RNase-free Dnase I. Also for RT-PCR, use intron-spanning primers that allow easy identification of DNA-contamination. A control PCR reaction containing the RNA as template will also allow detection of DNA contamination.

Quantitation and Storage of RNA

To determine the concentration and purity of RNA, measure absorbance 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. DEPC-water is slightly acidic and can dramatically lower absorbance values. We suggest that you dilute the sample in a buffered solution (TE) for spectrophotometric analysis. 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 absorbance maximum at 275 nm and can interfere with absorbance readings of DNA or RNA. Store RNA samples at -70°C in water. Under such conditions, RNA prepared with the E.Z.N.A.® system is stable for more than a year.

RNA Quality

It is highly recommended that RNA quality be determined prior to all analyses. The quality of RNA can best be assessed by denaturing agarose gel electrophoresis and ethidium bromide staining. Two sharp bands should appear on the gel. These are the 28S and 18S (23S and 16S for bacteria) ribosomal RNA bands. If these band smear towards lower molecular weight RNAs, then the RNA has undergone major degradation during preparation, handling, or storage. Although RNA molecules less than 200 bases in length do not efficiently bind the HiBind matrix, a third RNA band, the tRNA band, may be visible when a large number of cells are used.

Troubleshooting Tips

Problem	Cause	Suggestion
Little or no RNA eluted	RNA remains on the column	<ul style="list-style-type: none"> Repeat elution. Pre-heat DEPC-water to 70° C prior to elution. Incubate column for 10 min with water prior to centrifugation.
	Column is overloaded	<ul style="list-style-type: none"> Reduce quantity of starting material.
Clogged column	Incomplete homogenization	<ul style="list-style-type: none"> Completely homogenize sample. Increase centrifugation time. Reduce amount of starting material
Degraded RNA	Source	<ul style="list-style-type: none"> Freeze starting material quickly in liquid nitrogen or store the sample in RNA-Solv Do not store tissue culture cells prior to extraction unless they are lysed first. Follow protocol closely, and work quickly.
	RNase contamination	<ul style="list-style-type: none"> Ensure not to introduce RNase during the procedure. Check buffers for RNase contamination.
Problem in downstream applications	Salt carry-over during elution	<ul style="list-style-type: none"> Ensure Wash Buffer II Concentrate has been diluted with 4 volumes of 100% ethanol as indicated on bottle. 1 X Wash Buffer II must be stored and used at room temperature. Repeat wash with Wash Buffer II.
DNA contamination		<ul style="list-style-type: none"> Digest with RNase-free DNase and inactivate at 75°C for 5 min.
Low Abs ratios	RNA diluted in acidic buffer or water	<ul style="list-style-type: none"> DEPC-treated water is acidic and can dramatically lower Abs₂₆₀ values. Use TE buffer to dilute RNA prior to spectrophotometric analysis.