E.Z.N.A.® Ultra-Pure Total RNA Midi

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OMEGA bio-tek

Innovations in nucleic acid isolation

Introduction

The E.Z.N.A.™ Ultra-Pure Total RNA Midi Kit is designed for isolating total cellular RNA from tissues rich in fat such as brain adipose tissues. However, this kit can also be used for the isolation of total RNA from other type of tissues including cultured eukaryotic cells, animal tissues, or bacteria.

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

The E.Z.N.A.™ Ultra-Pure Total RNA Midi uses the reversible binding properties of the HiBind matrix, a new silica-based material. By combining the high lysis efficiency of RNA-Solv Reagent with Omega Bio-tek's innovative HiBind technology, this kit can extract total cellular RNA from all types of animal or human tissues including fatty tissues such as brain and adipose tissue. A specifically formulated high salt buffer system allows more than 1000µg of RNA molecules greater than 200 bases to bind to the matrix. Cells or tissues are first homogenized with RNA Solv Reagent that practically inactivates RNases. After adding chloroform, the homogenate is separated into aqueous and organic phase with centrifugation. The aqueous phase which contains the RNA is then adjusted with ethanol and then applied to the HiBind RNA column 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.

Binding Capacity

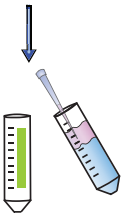
Each HiBind RNA Midi column can bind approximately 1000 µg of RNA. Using greater than 250 mg of tissue or 500 mg of adipose tissue is not recommended.

Spin Protocol

Add RNA Solv Buffer and Lyse

Homogenize

Add Chloroform



Transfer Aqueous Phase

Add Ethanol

Apply Sample to Column

Wash 3x

Dry

Elute

Kit Contents

E.Z.N.A. Ultra-Pure Total RNA Midi	2 Preps	10 Preps	25 Preps
Product Number	R6754-00	R6754-01	R6754-02
Purification	2	10	25
HiBind RNA Midi Columns	2	10	25
15 mL Collection Tubes	2	10	25
RNA-Solv Reagent	15 ml	3 x 20 ml	140 ml
RWC Wash Buffer	10 ml	50 ml	150 ml
RNA Wash Buffer II Concentrate	5 ml	12 ml	3 x12 ml
DEPC-ddH2O	1.0 ml	10 ml	40 ml
Instruction Manual	1	1	1

Storage and Stability

All components except RNA-Solv Reagent in the Ultra-Pure Total RNA Midi Kit should be stored at room temperature. RNA Solv should be store at 2-8° C. All Ultra-Pure Total RNA Midi components are guaranteed for at least 12 months from the date of purchase when stored indicated temperatures.

- Dilute RNA Wash Buffer II with absolute ethanol (96-100%) as follows:

Kit	Ethanol To Be Added
R6754-00	Add 20 mL absolute ethanol
R6754-01	Add 48 mL absolute ethanol
R6754-02	Add 48 mL absolute ethanol to each bottle

Store diluted RNA Wash Buffer II at room temperature.

- Please remember to always wear gloves whenever working with RNA. This will minimize RNase contamination. Use only clean RNase-free disposable plastic pipette tips when using the supplied reagents.

Quantification of RNA

Storage of RNA

Purified RNA can be stored at -70°C (RNase-free water). Under such conditions, RNA prepared with the E.Z.N.A.® Ultra-Pure Total RNA Midi Kit is stable for more than a year.

Quantification of RNA

To determine the concentration and purity of RNA, one should measure the absorbance at 260 nm and 280 nm in a spectrophotometer. 1 O.D. unit measured at 260 nm corresponds to 40µg of RNA per ml. DEPC treated 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 A_{260}/A_{280} ratio of pure nucleic acids is 2.0, while for pure protein is approximately 0.6. Therefore, a ratio of 1.8-2.0 corresponds to 90-100% pure nucleic acid. Phenol has a maximum absorbance at 275 nm and can interfere with absorbance readings of DNA or RNA.

RNA Quality

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

Expected Yields

For animal cell yields, see page 9.
For animal tissue yields, see page 14.

Disruption and Homogenization of Samples

Efficient sample disruption and homogenization is essential for successful Total RNA isolation. Complete cell wall and plasma membrane disruption is very important for the release of all of the RNA contained in the sample. The purpose of homogenization is to reduce the viscosity of the cell lysates produced by cell disruption. Homogenization shears genomic DNA and other high molecular weight cell components thereby creating a homogenous lysate. Incomplete homogenization will cause RNA binding to clog thus preventing efficient RNA binding result in low or no yield.

Mortar and Pestle: Sample Disruption

Sample disruption using a mortar and pestle followed the chosen of homogenization method:

Wear gloves, and take great care when working with liquid nitrogen.

1. Excise tissue and promptly freeze in a small volume of liquid nitrogen.

2. Grind tissue with a ceramic mortar and pestle under approximately 10 ml of liquid nitrogen. Pour the suspension into a pre-cooled 15ml polypropylene tube. The tube must be pre-cooled in liquid nitrogen or the suspension will boil vigorously possibly causing tissue loss.

3. Allow the liquid nitrogen to completely evaporate and add RNA-Solv Reagent.

Homogenization:

A) Homogenizer Spin column
Load the lysate into a homogenizer spin column pre-inserted into a 15 ml collection tube. Spin for five minutes at maximum speed in a micro centrifuge in order to collect homogenized lysate.

B) Syringe and Needle
Shear High molecular-weight DNA by passing the lysate through a narrow needle (19-21 gauge) 5-10 times.

Rotor-Stator Homogenizer: Sample Disruption and Homogenization

Using a rotor-stator homogenizer for sample disruption and homogenization can simultaneously disrupt and homogenize most samples. The process usually takes less than a minute depending on sample type. Many Rotor-stator homogenizers operate with differently sized probes or generators that allow sample processing in 50ml tubes.

Bead Milling: Sample Disruption and Homogenization

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

E.Z.N.A.® Ultra-Pure Total RNA Midi Animal Tissue Protocol

All centrifugation steps used are preformed at room temperature unless otherwise noted.

General Protocol Equipment:

- Chloroform
- Microcentrifuge capable of at least 5,000 x g and 4°C
- 70% ethanol in DEPC-treated sterile distilled water
- Sterile RNase-free pipette tips and 1.5ml centrifuge tubes
- Disposable latex gloves
- Absolute Ethanol

Sample Disruption and Homogenization Equipment:

- Liquid Nitrogen
- Needle and Syringe
- Mortar and pestle
- Glass Beads
- Rotor-stator Homogenizer

1. Determine the proper amount of starting material: It is critical to use the correct amount of tissue to obtain optimal yield and purity with the HiBind RNA Midi column. The maximum amount of tissue that can be processed on a HiBind RNA Midi column varies depending on the specific RNA contents and type of tissue. The maximum binding capacity of the HiBind RNA Midi column is 1000 µg. The maximum amount of tissue that can be used with RNA-Solv Reagent is 250 mg or 500 mg of adipose tissue. Use the following table as a guideline to select the correct amount of starting material. If you have no information about your starting material, use 100 mg as a starting amount, based upon the yield and quality of RNA obtained from 100 mg, adjust the starting amount in the next purification.

Average Yield of Total Cellular RNA From Mouse Tissue

Source	Amount of Tissue (mg)	RNA Yield (µg)
Sample Type : Mouse Tissue		
Brain	**100**	**100**
Kidney	**100**	**300**
Liver	**100**	**450**
Heart	**100**	**50**
Spleen	**100**	**330**
Lung	**100**	**120**
Pancreas	**100**	**400**
Thymus	**100**	**200**

To freeze tissue for long term storage, flash-freeze tissue in liquid nitrogen and immediately transfer to -70°C. Tissue can be store 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 store at -70°C for at least 6 months. To proceed with the frozen tissue lysates, thaw the sample at 37°C until they are 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.

2. Disrupt and homogenize the tissue in 5 mL of RNA Solv using one of the described methods on page 7.

   Note: Incomplete homogenization of the sample will cause lower yields and clogging of the column. It is recommended to homogenize the sample with rotor-stator homogenizers since it normally produce better yield.

3. Incubate the tube containing the homogenate at room temperature for 5 minutes.

4. Add 1000 µl of chloroform to the homogenate, close the cap of the tube incubate and shake vigorously for 15 seconds. Incubate at room temperature for 2-3 minutes.

5. Centrifuge at 5,000 x g for 15 minutes at 4°C to separate the aqueous and organic phase.

   Note: The sample should separate 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 3000 µL) into a new 15ml centrifuge tube. Add an equal volume of 70% ethanol and vortex to mix thoroughly. A precipitate may form at this point. This will not interfere with the RNA purification.

7. Apply 4 mL of sample into the HiBind RNA Midi column inserted into a 15 mL collection tube. Centrifuge at 5,000 x g for 5 minutes at room temperature. Discard the flow-through and reuse the collection tube in the next step.

8. Apply the remaining lysate from Step 6 to the HiBind RNA Midi Column inserted in a 15 mL Collection Tube. Centrifuge at 5,000 x g for 5 minutes at room temperature. Discard the flow-through and reuse the collection tube in the next step.

9. Add 4000 µL of RWC Wash Buffer by pipetting directly into the column matrix. Centrifuge at 5,000 x g for 5 minutes at room temperature. Discard the flow-through and reuse the collection tube in the next step.

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

10. Add 3000 µL of RNA Wash Buffer II to the Hibind RNA Midi Column. Centrifuge at 5,000 x g for 5 minutes at room temperature. Discard the flow-through and re-use the collection tube in the next step.

11. Add 2500 µL of RNA Wash Buffer II to the HiBind RNA Midi Column. Centrifuge at 5,000 x g for 5 minutes at room temperature. Discard the flow-through and reuse the collection tube in the next step.

12. With the HiBind RNA Midi column and collection tube emptied, centrifuge the spin cartridge for 10 minutes at 5,000 x g to completely dry the HiBind matrix.

13. Transfer the column to a clean 15 ml centrifuge tube (not supplied) and elute the RNA with 300 -700µl of DEPC-treated water (supplied). Make sure to add water directly onto the center of the column matrix. Centrifuge for 5 minutes at 5,000 x g. A second elution may be necessary if the expected yield of RNA > 500 µg.

    Alternatively, RNA may be eluted with a greater volume of water. While additional elutions increase total RNA yield, the concentration will be lowered since more than 80% of RNA is recovered with the first elution. Pre-heating the water to 70°C before adding to column and incubating the column for 5 minutes at room temperature before centrifugation may increase yields.

Optional DNase Digestion Protocol

E.Z.N.A.® Ultra-Pure Total RNA Midi DNase Digestion Protocol

For most downstream applications it is not necessary to do DNase digestion due to HiBind RNA resin and spin column technology removing nearly all DNA without the need for DNase Treatment. However, certain sensitive RNA applications might require further removal of DNA. In such case, we recommend that you please follow the outlined steps below using product E1091.

Note: After completing steps 1-8 of either of the centrifugation protocol (making sure that all of the sample has completely passed through the HiBind RNA Midi column), proceed with the following steps.

All centrifugation steps used are preformed at room temperature.

User Supplied Material:

- RNase Free DNase Set (E1091)

1. For each HiBind RNA Midi column, prepare the DNase I stock solution as follows:

Buffer	Volume per Prep
E.Z.N.A.® DNase I Digestion Buffer	367.5 µl
RNase Free DNase I (20 Kunitz/µl)	7.5µl
Total Volume	375µl

Note:

- DNase I is very sensitive to physical denaturation, therefore do not vortex this DNase I mixture. Please mix by GENTLY inverting the tube. Remember to freshly prepare DNase I stock solution right before RNA isolation.
- E.Z.N.A.® DNase I Digestion Buffer is supplied with Omega Bio-Tek, Inc.'s RNase-Free DNase Set (product no. E1091). Standard DNase Buffers are not compatible with on-membrane DNase digestion. The use of other buffers may affect the binding of RNA to the HiBind matrix, reducing RNA yields, and purity.

2. **Add 2000 µl of RWC Wash Buffer** by pipetting directly onto a new HiBind RNA Midi column inserted in a 2 ml collection tube. Centrifuge at 5,000 x g for 5 minutes and discard the flow-through and reuse the collection tube.

Optional DNase Digestion Protocol

3. Pipet 375µl of the DNase I stock solution directly onto the surface of the HiBind RNA resin in each column. Make sure to pipet the stock solution directly onto the center of membrane. DNase I Digestion will not go through completion if some of the stock solution remains stuck to the wall or the o-ring of the HiBind RNA column.

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

5. Add 2500 µl of RWC Wash Buffer. Place the column on a bench top for 2 minutes. Centrifuge at 5,000 x g for 5 minutes and discard flow-through and reuse the collection tube.

6. Add 3000 µl of RNA Wash Buffer II. Centrifuge at 5,000 x g for 5 minutes and discard flow-through and reuse the collection tube

   Important: RNA Wash Buffer II must be diluted with absolute ethanol before use. Refer to label for instructions. If refrigerated, RNA Wash Buffer II must be brought to room temperature before use.

7. Add another 2500 µl of RNA Wash Buffer II. Centrifuge at 5,000 x g for 5 minutes and discard flow-through and reuse the collection tube.

8. With the empty collection tube centrifuge the HiBind matrix for 10 minutes at 5,000 x g to completely dry the HiBind matrix.

9. Place the column in a clean 1.5 ml microcentrifuge tube (not supplied), and add 300-700 µl of DEPC-treated water (supplied). Make sure to add water directly onto the center of the column matrix. Let it sit for 1 minute, and then centrifuge for 2 minutes at maximum speed to elute the RNA. A second elution may be necessary if the expected yield of RNA > 500 µg.

   Alternatively, RNA may be eluted with a greater volume of water. While additional elutions increase total RNA yield, the concentration will be lower since more than 80% of RNA has been recovered in the first elution.

Troubleshooting Guide

Please use this guide to troubleshoot any problems that may arise. For further assistance, please contact the technical support staff, toll free, at **(800-832-8896).**

Possible Problems and Suggestions

Problem	Cause	Solution
Little or no RNA eluted	RNA remains on the column	Repeat elution step.
	Column is overloaded	Reduce quantity of starting material.
Problem	Cause	Solution
Clogged column	Incomplete homogenization	Completely homogenize sample.
		Increase centrifugation time.
		Reduce amount of starting material
Problem	Cause	Solution
Degraded RNA	Starting Culture Problems	Freeze starting material quickly in liquid nitrogen
		Do not store tissue culture cells prior to extraction unless they are lysed first.
		Follow protocol closely, and work quickly.
	RNase contamination	Ensure not to introduce RNase during the procedure.
		Check buffers for RNase contamination.

Troubleshooting Guide

Problem	Cause	Solution
Problem in downstream applications	Salt carry-over during elution	Ensure Wash Buffer II Concentrate has been diluted with 4 volumes of 100% ethanol as indicated on bottle.
		1 X RNA Wash Buffer II must be stored and used at room temperature.
		Repeat wash with RNA Wash Buffer II.
Problem	Cause	Solution
DNA contamination	DNA contamination	Digest with RNase-free DNase and inactivate DNase by incubate at 65°C for 5 min in the presence of EDTA.
Problem	Cause	Solution
Low Abs ratios	RNA diluted in acidic buffer or water	DEPC-treated water is acidic and can dramatically lower Abs_{260} values. Use TE buffer to dilute RNA prior to spectrophotometric analysis.

Ordering Information

The following components are available for purchase separately.
(Call Toll Free at 1-800-832-8896)

Buffer (Size)	Part Number
RNA-Solv Reagent	R6830-01
RNA-Solv Reagent	R6830-02
RNA Wash Buffer II (25 mL)	PR031
DEPC Water (100 mL)	PR032
RNase-free DNase Set (5o Mini/10 Midi preps)	E1091
RNase-free DNase Set (200 Mini/40 Midi preps)	E1091-02

Notes: