Notes:

E.Z.N.A.® Ultra-Pure Total RNA Maxi Kit

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Manual Revision: March 2012

OMEGA bio-tek

Innovations in nucleic acid isolation

Introduction

Notes:

The E.Z.N.A.® Ultra-Pure Total RNA Maxi Kit is designed to isolate total cellular RNA from tissues rich in triglycerides and fatty acids such as brain and adipose tissues. However, this kit can also be used for the isolation of total cellular 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 Maxi method is ready for applications such as RT-PCR*, Northern blotting, poly A+ RNA (mRNA) purification, nuclease protection assay, and *in vitro* translation.

The E.Z.N.A.® Ultra-Pure Total RNA Maxi Kit utilizes 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 5 mg RNA molecules greater than 200 bases to bind to the matrix. Cells or tissue are homogenized with RNA-Solv® Reagent that inactivates RNases. After the addition of chloroform, the homogenate is separated into aqueous and organic phases. The aqueous phase, which contains the RNA, is adjusted with ethanol and applied to the HiBind® RNA Maxi Column. The HiBind® matrix binds total RNA while cellular debris and other contaminants are effectively washed away. High-quality RNA is eluted in DEPC Water.

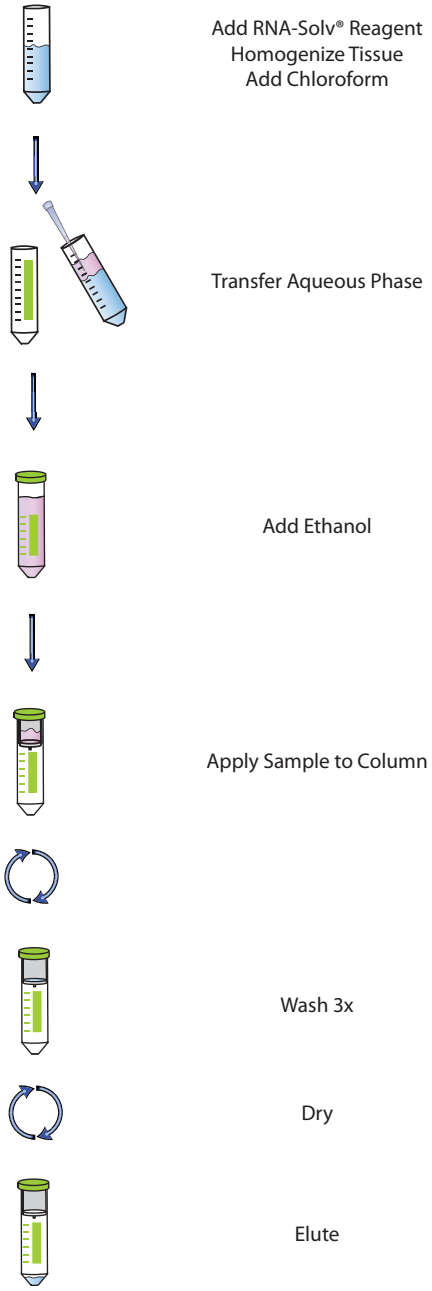
Binding Capacity

Each HiBind® RNA Maxi Column can bind approximately 5 mg total RNA. Using greater than 1 g tissue or 2 g adipose tissue is not recommended.

New in this Edition: This manual has been edited for content and redesigned to enhance user readability.

Notes:

Illustrated Protocol



Kit Contents

Product	R6755-00	R6755-01	R6755-02
Purifications	2	5	20
HiBind® RNA Maxi Columns	2	5	20
50 mL Collection Tubes	2	5	20
RNA-Solv® Reagent	2 x 25 mL	5 x 25 mL	3 x 200 mL
RWF Wash Buffer	40 mL	100 mL	350 mL
RNA Wash Buffer II	10 mL	25 mL	100 mL
DEPC Water	15 mL	40 mL	150 mL
User Manual	✓	✓	✓

Storage and Stability

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

Ordering Information

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

Product	Part Number
RNA-Solv® Reagent, 100 mL	R6830-01
RNA-Solv® Reagent, 200 mL	R6830-02
RNA Wash Buffer II, 25 mL	PR031
DEPC Water, 100 mL	PR032
RNase-free DNase Set, 1500 units	E1091
RNase-free DNase Set, 6000 units	E1091-02

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 **1-800-832-8896**.

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 material problems	Freeze starting material quickly in liquid nitrogen.
		Follow protocol closely, and work quickly.
	RNase contamination	Ensure not to introduce RNase during the procedure.
		Check buffers for RNase contamination.
Problem	Cause	Solution
Problem in downstream applications	Salt carry-over during elution	Dilute RNA Wash Buffer II with 100% ethanol as instructed on Page 5.
		RNA Wash Buffer II must be stored and used at room temperature.
		Repeat wash step with RNA Wash Buffer II.
Problem	Cause	Solution
DNA contamination	DNA contamination	Follow the DNase I Digestion Protocol on Page 13.
Problem	Cause	Solution
Low Abs ratios	RNA diluted in acidic buffer or water	DEPC Water is acidic and can dramatically lower Abs_{260} values. Use TE buffer to dilute RNA prior to spectrophotometric analysis.

Preparing Reagents

- Dilute RNA Wash Buffer II with 100% ethanol as follows and store at room temperature.

Kit	100% Ethanol to be Added
R6755-00	40 mL
R6755-01	100 mL
R6755-02	400 mL

Quantification and Storage of RNA

To determine the concentration and purity of RNA, measure absorbance at 260 nm and 280 nm with a spectrophotometer. One OD unit measured at 260 nm corresponds to 40 µg/mL RNA. 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 A_{260}/A_{280} ratio of pure nucleic acids is 2.0, while an A_{260}/A_{280} ratio of 0.6 denotes pure protein. A ratio of 1.8-2.0 corresponds to 90%-100% pure nucleic acid. Phenol has a maximum absorbance at 270 nm and can interfere with spectrophotometric analysis of DNA or RNA. Store RNA samples at -70°C in water. Under these conditions, RNA is stable for more than a year.

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.

Integrity of RNA

It is highly recommended that RNA quality be determined prior to beginning all downstream applications. The quality of RNA can be best assessed by denaturing agarose gel electrophoresis with ethidium bromide staining. The ribosomal RNA bands should appear as sharp, clear bands on the gel. The 28S band should appear to be double that of the 18S RNA band (23S and 16S if using bacteria). If the ribosomal RNA bands in any given lane are not sharp and appear to be smeared towards the smaller sized RNA, it is very likely that the RNA undergone degradation during the isolation, handling, or storage procedure. 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.

16. Centrifuge the empty column at 5,000 x *g* for 10 minutes to completely dry the HiBind® matrix.

    Note: It is important to dry the HiBind® RNA Maxi Column matrix before elution. Residual ethanol may interfere with downstream applications.

17. Transfer the HiBind® RNA Maxi Column to a clean 50 mL centrifuge tube (not supplied).

18. Add 1-3 mL DEPC Water.

    Note: Make sure to add water directly onto the HiBind® RNA Maxi Column matrix.

19. Centrifuge at 5,000 x *g* for 5 minutes and store eluted RNA at -70°C.

    Note: Any combination of the following steps can be used to help increase RNA yield.

    - Preheat the DEPC Water to 70°C before adding to the column.
    - Let sit at room temperature for 5 minutes after adding DEPC Water.
    - Increase the elution volume.
    - Repeat the elution step with fresh DEPC Water (this may increase the yield, but decrease the concentration).
    - Repeat the elution step using the eluate from the first elution (this may increase yield while maintaining elution volume).

3. Add 8 mL RWF Wash Buffer.

4. Centrifuge at 5,000 x *g* for 10 minutes.

5. Discard the filtrate and reuse the collection tube.

6. Add 1.5 mL DNase I digestion mixture directly onto the surface of the membrane of the HiBind® RNA Maxi Column.

   Note: Pipet the DNase I directly onto the membrane. DNA digestion will not be complete if some of the mixture is retained on the wall of the HiBind® RNA Maxi Column.

7. Let sit at room temperature for 15 minutes.

8. Add 8 mL RWF Wash Buffer.

9. Let sit at room temperature for 2 minutes.

10. Centrifuge at 5,000 x *g* for 5 minutes.

11. Discard the filtrate and reuse the collection tube.

12. Add 10 mL RNA Wash Buffer II.

    Note: RNA Wash Buffer II must be diluted with ethanol before use. Please see Page 5 for instructions.

13. Centrifuge at 5,000 x *g* for 5 minutes.

14. Discard the filtrate and reuse the collection tube.

15. Repeat Steps 12-14 for a second RNA Wash Buffer II wash step.

Efficient sample disruption and homogenization is essential for successful total RNA isolation. Cell wall and plasma membrane disruption is necessary for the release of RNA from the sample and homogenization is necessary to reduce the viscosity of the lysates. Homogenization shears genomic DNA and other high-molecular-weight cell components creating a homogenous lysate. Incomplete homogenization can cause the HiBind® RNA Maxi Column to clog resulting in low or no yield.

Liquid Nitrogen Method

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

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

3. Grind tissue with a ceramic mortar and pestle under approximately 10 mL liquid nitrogen.

4. Pour the suspension into a pre-cooled 15 mL polypropylene tube.

   Note: Unless the tube is pre-cooled in liquid nitrogen, the suspension will boil vigorously and may cause loss of tissue.

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

6. Proceed to one of the homogenization steps below.

Homogenization - Choose one method below

1. Homogenizer Spin Columns and 15 mL Collection Tubes

   - Load the lysate into a homogenizer spin column pre-inserted into a 15 mL Collection Tube (not provided).
   - Spin for 5 minutes at maximum speed in a centrifuge in order to collect homogenized lysate.
   - Proceed to Step 1 of the "Animal Tissue Protocol" on Page 9.

2. Syringe and Needle

   - Shear high-molecular-weight DNA by passing the lysate through a narrow needle (19-21 gauge) 5-10 times.
   - Proceed to Step 1 of the "Animal Tissue Protocol" on Page 9.

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 50 mL 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 size of glass beads to use for RNA isolation are 0.5 mm for yeast/unicellular cells and 4-8 mm for animal tissue samples.

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

Since the HiBind® matrix of the RNA Maxi Column eliminates most DNA, DNase I digestion is not necessary for most downstream applications. However, certain sensitive RNA applications may require further DNA removal. (See DNase I Digestion Set, Cat # E1091 for further information).

After completing Steps 1-13 of the Animal Tissue Protocol (Pages 9-11) , proceed with the following protocol.

User Supplied Material:

- DNase I Digestion Set (Cat# E1091)

1. For each HiBind® RNA Maxi Column, prepare the DNase I stock solution as follows:

Buffer	Volume per Prep
E.Z.N.A.® DNase I Digestion Buffer	1.47 mL
RNase-free DNase I (20 Kunitz/µL)	30 µL
Total Volume	1.5 mL

Important Notes:

- DNase I is very sensitive and prone to physical denaturing. **Do not vortex the DNase I mixture.** Mix gently by inverting the tube.
- Freshly prepare DNase I stock solution right before RNA isolation.
- Standard DNase buffers are not compatible with on-membrane DNase I digestion. The use of other buffers may affect the binding of RNA to the HiBind® matrix and may reduce RNA yields and purity.
- All steps must be carried out at room temperature. Work quickly, but carefully.

2. Insert the HiBind® RNA Maxi Column containing the sample into a 50 mL Collection Tube.

18. Centrifuge at 5,000 x *g* for 5 minutes.

19. Discard the filtrate and reuse the collection tube.

20. Repeat Steps 17-19 for a second RNA Wash Buffer II wash step.

21. Centrifuge the empty column at 5,000 x *g* for 10 minutes to completely dry the HiBind® matrix.

    Note: It is important to dry the HiBind® RNA Maxi Column matrix before elution. Residual ethanol may interfere with downstream applications.

22. Transfer the HiBind® RNA Maxi Column to a clean 50 mL centrifuge tube (not supplied).

23. Add 1-3 mL DEPC Water. Make sure to add the water directly to the center of the column matrix.

24. Centrifuge at 5,000 x *g* for 5 minutes. A second elution may be necessary if the expected yield of RNA > 2 mg.

    Note: Any combination of the following steps can be used to help increase RNA yield.

    - Preheat the DEPC Water to 70°C before adding to the column.
    - Let sit at room temperature for 5 minutes after adding DEPC Water.
    - Increase the elution volume.
    - Repeat the elution step with fresh DEPC Water (this may increase the yield, but decrease the concentration).
    - Repeat the elution step using the eluate from the first elution (this may increase yield while maintaining elution volume).

E.Z.N.A.® Ultra-Pure Total RNA Maxi Kit Protocols

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

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

Materials and Equipment to be Supplied by User:

- 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
- Nuclease-free 50 mL centrifuge tubes
- 100% ethanol
- Chloroform
- Homogenization Equipment
    - Liquid nitrogen
    - Needle and syringe
    - Mortar and pestle
    - Glass beads
    - Rotor-stator homogenizer

Before Starting:

- Prepare RNA Wash Buffer II according to the "Preparing Reagents" section on Page 5.

1. Determine the proper amount of starting material.

   Note: It is critical to use the correct amount of tissue in order to obtain optimal yield and purity with the HiBind® RNA Maxi Column. The maximum amount of tissue that can be processed with is dependent on tissue type and its RNA content. The maximum binding capacity of the HiBind® RNA Maxi Column is 5 mg. The maximum amount of tissue that can be used with RNA-Solv® Reagent is 1 g or 2 g adipose tissue. Use the table on the following page as a guideline to select the correct amount of starting material. If no information regarding your starting material is available, begin with 500 mg. Based on RNA yield and quality obtained from 500 mg, the starting amount can be adjusted for the next purification.

Average Yield of Total Cellular RNA From Various Mouse Tissues

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

2. Disrupt and homogenize the tissue in 20 mL RNA-Solv® Reagent according to one of the following methods described on Pages 7-8:

   Note: Incomplete homogenization of the sample may cause the column to clog thus resulting in decreased yield. It is recommended to homogenize the tissue sample with rotor-stator homogenizers since this method normally produces better yield.

3. Let sit at room temperature for 5 minutes.

4. Add 4 mL chloroform. Vortex or shake vigorously for 15 seconds.

5. Let sit at room temperature for 2-3 minutes.

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

   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.

7. Transfer the upper aqueous phase (approximately 12 mL) into a new 50 mL centrifuge tube (not provided).

E.Z.N.A.® Ultra-Pure Total RNA Maxi Kit Protocols

8. Add an equal volume of 70% ethanol. Vortex to mix thoroughly.

   Note: A precipitate may form at this point. This will not interfere with the RNA purification.

9. Insert a HiBind® RNA Maxi Column into a 50 mL Collection Tube.

10. Transfer 16 mL sample to the HiBind® RNA Maxi Column.

11. Centrifuge at 5,000 x *g* for 5 minutes.

12. Discard the filtrate and reuse the collection tube.

13. Repeat Steps 10-12 until all the sample has been transferred to the column.

Optional: This the starting point of the optional on-membrane DNase I Digestion Protocol. Since the HiBind® matrix of the RNA Maxi Column eliminates most DNA, DNase I digestion is not necessary for most downstream applications. However, certain sensitive RNA applications may require further DNA removal. If an additional RNA removal step is required, please continue to the DNase I Digestion Protocol found on Page 13. (See DNase I Digestion Set, Cat # E1091 for more information). If DNase I digestion is not required, proceed to Step 14.

14. Add 16 mL RWF Wash Buffer.

15. Centrifuge at 5,000 x *g* for 5 minutes.

16. Discard the filtrate and reuse the collection tube.

17. Add 10 mL RNA Wash Buffer II.

    Note: RNA Wash Buffer II must be diluted with ethanol before use. Please see Page 8 for instructions.