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Introduction

The E.Z.N.A.® Total RNA Maxiprep Kit uses HiBind® matrix spin-column technology to isolate up to 5 mg total cellular RNA from a variety of sources including 10^{10} bacterial cells, 1 g tissue, and up to 5×10^8 cultured cells. Samples are lysed and homogenized in denaturing conditions to ensure degradation of proteins and inactivation of endogenous RNases. Binding conditions are then optimized to favor RNA isolation before the resulting cell lysate are passed through HiBind® RNA Maxi spin-columns. Subsequent wash steps remove cellular debris and trace salt contaminants to allow elution of pure RNA in DEPC-treated dH_2O . No organic solvents are used and only short centrifugation steps using a standard centrifuge equipped with a swinging-bucket rotor at room temperature are needed. The kit can also be used for RNA clean-up prior to any downstream application. Purified RNA is suitable for mRNA isolation (Product R6511, mRNA Enrichment Kit), Northern analysis, RNase protection assays, differential display, and reverse transcription.

Storage and Stability

All components of the Total RNA Maxiprep Kit must be stored at room temperature. During shipment or storage in cool ambient conditions, crystals may form in some buffers. Simply warm in a 37°C incubator to redissolve.. **All E.Z.N.A.™ Total RNA Maxiprep Kit components are guaranteed for at least 24 months from the date of purchase when stored at 22-25°C.**

HiBind® RNA Maxi Column Performance

	Amount To Use	Typical Yield (mg)
Bacteria	1 x 10 ¹⁰ cells	1.5 - 3.0
Cultured Cells	1 x 10 ⁷ - 5 x 10 ⁸ cells	4.0 - 5.0
Animal Tissue		
• Liver	1.0 g	2.5 - 3.5
• Spleen	1.0 g	2.3 - 3.0
• Brain	0.2 - 0.5 g	0.2 - 0.5
• Intestine	0.1 - 0.3 g	0.1 - 0.5

Kit Contents

Product	R6693-01	R6693-02
HiBind® RNA Maxi Columns	5	20
50 ml Collection Tubes	5	20
TRK Lysis Buffer	40 ml	160 ml
RNA Wash Buffer I	80 ml	2 x 160 ml
RNA Wash Buffer II Concentrate	25 ml	2 x 50 ml
DEPC Water	5 ml	20 ml
User Manual	1	1

Before Starting

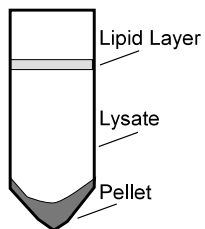
IMPORTANT	<p>All steps must be performed at room temperature. Centrifugation steps are carried out using a laboratory centrifuge equipped with a swinging-bucket rotor at 3,500 x g. The centrifuge must be maintained at 22-25°C.</p>
	<p>RNA Wash Buffer Concentrate must be diluted with absolute (100%) ethanol before use:</p> <p>R6693-00, 5 preps - Add 100 ml absolute ethanol. R6693-02, 20 preps - Add 200 ml absolute ethanol.</p> <p>Diluted, RNA Wash Buffer II must be stored at room temperature.</p>

Materials Provided by User

- 2-mercaptoethanol (β-mercaptoethanol, β-ME) is required and must be added to TRK Lysis Buffer before use. Add 20 µl β-ME per 1 ml TRK Lysis Buffer. The mixture should be stored at room temperature and is stable for about 1 week.
- 70% ethanol (in water) and absolute ethanol.
- RNase-free 50 ml conical centrifuge tubes.
- Centrifuge with swinging-bucket rotor at room temperature capable of 3,500 x g.
- RNase-free pipet tips and disposable gloves.
- DEPC-treated deionized water.
- Note that all centrifugation are performed using a centrifuge with a swinging-bucket rotor at 4,000 -6,000 x g at ROOM TEMPERATURE.

E.Z.N.A.[®] Total RNA Maxiprep Protocol

A. Animal Tissues



Quantity of Starting Material: In general the binding capacity of the HiBind[®] RNA Maxi column (~5 mg RNA) will not be exceeded and between 0.1g and 1.0 g of tissue can be used. However, some tissues such as brain and skeletal muscle tend to be rather difficult to lyse and homogenize and can block the column, leading to lower RNA yields. With such tissues, we recommend starting with 250-400 mg to ensure optimal column performance. Subsequently, the starting quantity may be increased to 1.0g if favorable yield and quality is obtained. *Overloading the column always leads to*

clogging and reduced yields.

Freezing Samples: Fresh tissue is best, but frozen samples may also be processed. To store tissues for future use, flash-freeze under liquid nitrogen and store at -70°C for 2-4 months. Frozen samples should be processed directly in TRK Lysis Buffer without prior thawing. Once homogenized in TRK Lysis Buffer, samples may be stored at -70°C for several months.

Disruption & Homogenization of Tissues

a. Disrupt & 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 10 ml of liquid nitrogen and pour the suspension into a pre-cooled 50 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 TRK Lysis Buffer and continue with the procedure as outlined below. After interrupt tissue, lysate can be homogenized with Omega Homogenizer Maxi Spin Column (Product # AC6684). The lysate is loaded onto Omega Homogenizer Spin Column in a 50 ml collection tube. Spin 10 minutes at a 5000 x G in a centrifuge and the homogenized lysate collected. Use the Omega Homogenizer Spin Column is fast and efficient way to homogenize the lysate without cross contamination of samples. The alternated way to homogenize the lysate is to use the syringe and needle. High molecular-weight DNA can be sheared with by passing the lysate through a narrow needle (19-21 gauge) for 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 equipments are available. Rotor-stator homogenizers effectively homogenize and 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 sample 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.

1. In almost every case, optimal yields are only obtained if the tissue sample is first completely and thoroughly disrupted and homogenized in TRK Lysis Buffer. We suggest using a mechanical rotor homogenizer. Wearing gloves, place 250-400 mg tissue in a sterile RNase-free 50 ml polypropylene centrifuge tube (not provided). Add 7.0 ml TRK Lysis Buffer containing 2-mercaptoethanol and homogenize for 2 minutes.

Note: Remember to add 2-mercaptoethanol to TRK Lysis Buffer before use. Add 20 µl 2-mercaptoethanol per 1 ml TRK Lysis Buffer before use.

Tip: For difficult tissues, use up to 14.0 ml of TRK Lysis Buffer with no more than 0.4 g tissue and extend homogenization period.

2. To obtain a cleared lysate centrifuge for 15 min at 5,000 x g. A loose pellet usually forms accompanied by an upper lipid layer.
3. Without disturbing the pellet or the lipid layer, carefully pipet only the cleared lysate to a fresh 50 ml polypropylene centrifuge tube (not provided).

Tip: Transferring contaminants from the pellet or the lipid layer will cause the spin column to clog resulting in significantly reduced yields. If necessary, sacrifice 1-3 ml lysate to ensure optimal yield and quality.

4. Add an equal volume (7.0 or 14.0 ml) of 70% ethanol to the cleared lysate from step 2. If some volume is lost in prior steps, add the appropriate amount of ethanol. Mix well by vortexing or vigorous shaking for 1 minute. Precipitates may form upon addition of ethanol. This will not interfere with the procedure so long as the mixture is thoroughly and immediately mixed. Proceed to step 4 immediately.
5. Apply sample to an HiBind[®] RNA maxi column assembled in a 50 ml collecting tube (provided). The spin column has a maximum capacity of approximately 25 ml. Cap the tube and centrifuge for 5 min at 5,000 x g at room temperature. If more than 0.4 g tissue was used, extend centrifugation time to 10-12 min. Discard flow-through liquid and reuse collecting tube in step 5.

Tip: If sample volume exceeds 25 ml, load the spin column in successive aliquots and centrifuge as above, discarding the flow-through liquid each time.

Note: this is the starting point for on-membrane DNase I digestion treatment. See page 10 for detail protocol.

6. Add 15 ml RNA Wash Buffer I, cap the 50 ml collecting tube and centrifuge for 5 min at 5,000 x g at room temperature. Again discard flow-through liquid and reuse 50 ml tube in step 6.
7. Add 10 ml RNA Wash Buffer II diluted with absolute ethanol (see Before Starting, page 3) to the HiBind® RNA maxi column, cap the 50 ml tube and centrifuge for 3 min at 5,000 x g at room temperature. Discard flow-through liquid and reuse the 50 ml tube in step 8.

Note: RNA Wash Buffer II is supplied as a concentrate and must be diluted with absolute ethanol as described on page 3.

8. Repeat step 6 with another 10 ml RNA Wash Buffer II. Discard flow-through liquid and re-insert the spin column in the empty 50 ml collecting tube. Centrifuge the empty column for 10 min at 5,000 x g to dry the HiBind® matrix. *This step is critical for removing traces of ethanol that may otherwise interfere with subsequent downstream applications.* **Note: When a vacuum oven is available, place the maxi column into a vacuum oven which is preset at 60C for 10-15 minutes. This will ensure that the column can be completely dried before elution**
9. RNA elution. Transfer the HiBind® RNA maxi column to a new 50 ml centrifuge tube (not supplied) and pipet 850 µl DEPC-treated water directly onto the matrix. For expected RNA yields greater than 1 mg use 1.25 ml of water. Cap the tube and allow the matrix to soak for 1 min. Centrifuge for 5 min at 5,000 x g.

Tip: Yield will be increased by 30%-70% by repeating the elution with a second volume of DEPC-treated water. For a higher final RNA concentration, this second elution may be performed using the first eluate. However, this will result in a ~30% lower overall yield.

Note: For laboratory centrifuges not capable of achieving 5,000 x g step 7 may not completely dry the column. As a result traces of ethanol may remain on the HiBind RNA matrix and will be eluted with RNA. In this case it is best to further purify the RNA following elution by ethanol precipitation. Add 1/10 volume DEPC-treated 5 M ammonium acetate followed by 3 x vol absolute ethanol. In situations where expected RNA yields are less than 100µg or so, it is recommended to add 5 µg yeast tRNA or 10 µg glucogen as carrier prior to ethanol precipitation. Vortex to mix and incubate 2 hours at -70°C or overnight at -20°C. Centrifuge at 10,000 x g for 15 min at 4°C to pellet RNA. Aspirate supernatant and discard without disturbing pellet. Wash once with

75% ethanol and centrifuge as before. Discard supernatant and air-dry RNA pellet briefly before reconstituting in DEPC-treated water.

B. Animal Cells

Harvesting Cells

a. Cells grown in monolayer may be directly lysed with TRK Lysis Buffer/2-mercaptoethanol. Completely remove and discard culture medium and add an aliquot of TRK Lysis Buffer/2-mercaptoethanol to the adherent cells. Add 7.0 ml of TRK Lysis Buffer to no more than 5×10^8 cells. Pipette buffer over flask surface several times to ensure complete cell lysis. For more than 5×10^8 cells adjust the volume of TRK Lysis Buffer accordingly. Transfer lysate to an RNase-free 50 ml centrifuge tube and proceed to step 3 of tissue protocol on page 5.

b. Cells grown in suspension should be collected by centrifugation for 5 min at 1,000-2,000 x g. There is no need for washing cells as this may lead to RNase contamination. To no more than 5×10^8 cells add 7.0 ml TRK Lysis Buffer/2-mercaptoethanol. Pipette up and down several times to effect complete lysis and use one of the homogenization methods described on page 4 to homogenize the lysate. Proceed to step 4 on page 5 and complete the RNA isolation procedure.

Note: Remember to add 20 µl 2-mercaptoethanol per 1 ml of TRK Lysis Buffer before use.

C. E.Z.N.A.® Total RNA Maxiprep Protocol for Bacteria

Additional Materials Required:

- TE Buffer - 10 mM Tris-HCl, pH 7.4; 1 mM EDTA pH 8.0
- Lysozyme - stock solution at 50 mg/ml

1. Harvest 1×10^{10} - 5×10^{10} fresh bacterial cells grown to early log-phase in the appropriate medium by centrifugation for 5 min at 5,000 x g. Completely resuspend cells in 2.5 ml TE by gentle pipetting. Add 200 µl (for Gram-positive bacteria) or 25 µl (for Gram-negative bacteria) of lysozyme solution at 50 mg/ml. Vortex briefly and incubate at room temperature for 15 min.
2. Add 8.0 ml TRK Lysis Buffer containing 2-mercaptoethanol and mix by pipetting several times. Remember to add 20 µl 2-mercaptoethanol per 1 ml TRK Lysis Buffer before use. Homogenize the lysate as described on page 4.
3. Add 6.0 ml absolute (100%) ethanol and immediately mix by vigorous shaking or vortexing for 1 min. Proceed to step 4 on page 6.

D. E.Z.N.A.® Maxiprep Protocol for RNA Clean-Up

1. A maximum of 5 mg of RNA may be used with this procedure. Adjust the sample volume to 2.5 ml with DEPC-treated water. Add 8.0 ml TRK Lysis Buffer/2-mercaptoethanol and vortex to mix.

Note: Remember to add 20 µl 2-mercaptoethanol per 1 ml TRK Lysis Buffer

before use.

2. Add 6.0 ml absolute (100%) ethanol and mix by vigorous shaking or vortexing for 1 min. Immediately proceed to step 4 of main protocol on page 6.

E. Modified Protocol for Heart, Muscle, Brain and Skin tissue:

Isolate total RNA from skeletal muscle, heart, brain, and skin tissue can be hard due to abundance of contractile proteins, connection tissue and collagen.

Note: Always use less starting material (<500mg) for the first time isolation to ensure a good result. If results obtained are satisfactory increase amount of starting material.

1. Disrupt the tissue and homogenize lysate in 6.0mL TRK lysis buffer with a rotor-stator homogenizer. Transfer the homogenate to a 50mL centrifuge tube which is capable of 3500 xg.
2. **Add 15ml ddH₂O to the homogenate and add 220 µl Proteinase K or OB Protease (20mg/ml). Vortex for 30 seconds to mix. Incubate at 60C for 15-30 minutes.**
3. **Centrifuge at 3500 x g for 10 minutes and transfer clear supernatant (around 22.0ml) to a new 50 ml tube. (Avoid any precipitate or film on top of the supernatant may formed during process of step 2.)**
4. **Add 0.5 volume of absolute ethanol (11ml) to the sample and mix with pipetting.**
5. **Transfer 13ml of the mixture including any precipitate may have formed in step 4 to the HiBind® RNA Maxi-column placed in a 50ml centrifuge tube (supplied). Closed the tube and centrifuge at 3500-5000 x g for 10 minutes. Discard the flow-through. Re-use the tube for next step.**
6. **Repeat step 5 twice until all the mixture from step 4 pass through the HiBind® RNA Maxi-column.**
7. Add 15 ml RNA Wash Buffer I, cap the 50 ml collecting tube and centrifuge for 5 min at 3,500-5,000 x g at room temperature. discard flow-through liquid and reuse 50 ml tube in step 6.
8. Add 15 ml RNA Wash Buffer II, cap the 50 ml collecting tube and centrifuge for 5 min at 3,500-5,000 x g at room temperature. Again, discard flow-through liquid and reuse 50 ml tube in step 6.
9. Repeat step 6 with another 15 ml RNA Wash Buffer II. Discard flow-through liquid and re-insert the spin column in the empty 50 ml collecting tube.

Centrifuge the empty column for 10 min at 5,000 x g to dry the HiBind® matrix. *This step is critical for removing traces of ethanol that may otherwise interfere with subsequent downstream applications.* **Note: When a vacuum oven is available, place the maxi column into a a vacuum oven which is preset at 60C for 10-15 minutes. This will ensure that the column can be completely dried before elution.**

10. RNA elution: transfer the HiBind® RNA maxi column to a new 50 ml centrifuge tube (not supplied) and pipet 850 µl DEPC-treated water directly onto the matrix. For expected RNA yields grater than 1 mg use 1.25 ml of water. Cap the tube and allow the matrix to soak for 1 min. Centrifuge for 5 min at 5,000 x g.
Tip: Yield will be increased by 30%-70% by repeating the elution with a second volume of DEPC-treated water. For a higher final RNA concentration, this second elution may be performed using the first eluate. However, this will result in a ~30% lower overall yield.

F. Optional On-Membrane DNase I digestion

Since HiBind® RNA resin and spin-column technology actually removes most of DNA without the DNase treatment, it is not necessary to do DNase digestion for most downstream applications. However, certain sensitive RNA applications might require further DNA removal. Following steps provide on-membrane DNase I digestion:(see DNase I cat.# E1091for detail information)

1. Follow the standard protocol until the samples **completely** pass through the HiBind® RNA Maxicolumn (step1-5). Prepare the following:

- a. For each HiBind® RNA column, prepare the DNase I digestion reaction mix as follows:

OBI DNase I Digestion Buffer	240 µl
RNase-free DNase I (20 Kunitz unites/µl)	10 µl
Total volume	250 µl

Note:

1. **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.**
 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 250 µl of the DNase I digestion reaction mix directly onto the surface of HiBind® RNA resin in each column. Make sure to pipet 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 HiBind® RNA column.

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

6. **Place column into a new 50 ml collection tube**, and add 15 ml RNA Wash Buffer I. **Place the column at benchtop for 5 minutes.** Centrifuge at 5000-8000 x g for 5 minutes and discard flow-through. Reuse the collection tube in step 7.
7. **Place column in the same 50ml collection tube**, and add 15ml RNA Wash Buffer II diluted with ethanol. Centrifuge at 5000-8000 x g for 5 minutes and discard flow-through. Reuse the collection tube in step 7.
Note: Wash Buffer II Concentrate must be diluted with absolute ethanol before use. Refer to label on bottle for directions.
8. Wash column with a second 15 ml of Wash Buffer II as in step 5. Centrifuge and discard flow-through. Then with the collection tube empty, centrifuge the spin cartridge for **5 min at 8000 x g** to completely dry the HiBind® matrix.
9. **Elution of RNA.** Transfer the column to a clean 50 ml microfuge tube (not supplied with kit) and elute the RNA with 850 µl- 1.20ml of DEPC-treated water (supplied with kit). Make sure to add water directly onto column matrix. Let it stand for 1 minute. Centrifuge 3 min at 8000xg to elute RNA. A second elution may be necessary if the expected yield of RNA >50 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 column 5 min at room temperature before centrifugation may increase yields.

Quantization 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 lower absorbance values. We suggest that you dilute the sample in a buffered solution (10 mM Tris-HCl, pH 7.6) 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 1.7-2.0 corresponds to 85%-100% pure nucleic acid. (Phenol has an absorbance maximum at 275 nm and can interfere with absorbance readings of DNA or RNA. However, the E.Z.N.A.® Total RNA Kits eliminate the use of phenol and avoid this problem.) 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

We suggest that RNA quality be determined prior to all analyses. The quality of RNA is best 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 prokaryotes) ribosomal RNA bands. If these bands

smear towards lower molecular weight RNAs, then the RNA has undergone significant degradation during preparation, handling, or storage.

Troubleshooting Tips

Problems	Suggestions
Poor Yields	<ul style="list-style-type: none"> • Inadequate homogenization/lysis: Start with less tissue, increase amount of TRK Lysis Buffer, or increase homogenization time. • RNA not eluted from HiBind® Maxi column: repeat elution and extend incubation time to 10 minutes with DECP-water before centrifuging. • Column overloaded: Too much starting material reduces column performance and overall yield. Start with 250 mg of material.
Blocked Column	<ul style="list-style-type: none"> • Incomplete homogenization/lysis. First try increasing centrifugation g-force and time. Otherwise reduce amount of starting material, increase amount of TRK Lysis Buffer, or increase homogenization time. • Column overloaded. Reduce amount of starting material. • Precipitation occurs in lysate due to drop in centrifuge temperature. Ensure that centrifugation temperature is above 22°C. • Debris carried over to column from first centrifugation step. If necessary, sacrifice 2-5 ml of lysate following centrifugation. Adjust volume of ethanol to add accordingly before applying to column.