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Introduction

The E.Z.N.A.™ RNA family of products is an innovative system that radically simplifies the extraction and purification of RNA from a variety of sources. The key to this system is that it uses the reversible binding properties of the HiBind® Matrix (a new silica-based material) in combination with the speed of midi-column spin technology, thereby permitting single or multiple simultaneous processing of samples. There is no need for phenol/chloroform extractions, and time-consuming steps such as CsCl gradient ultracentrifugation, and precipitation with isopropanol or LiCl, are eliminated. RNA purified using the E.Z.N.A.™ RNA Purification System 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.™ Total RNA Midi Kit can purify up to 600 µg of Total RNA from cultured eukaryotic cells, tissue or bacteria. Normally, 5×10^6 - 1×10^8 eukaryotic cells, 5×10^8 - 1×10^{10} bacterial cells, or 25-200 mg of tissue, can be processed in a single experiment. Lysis of Cells or Tissue occurs under denaturing conditions by practically inactivating RNases. After the homogenization process, samples are applied to the HiBind® RNA Midi column to which Total RNA Binds. Cellular debris, and other contaminants are effectively washed away after a few quick wash steps. High quality RNA is finally eluted in Sterile DEPC-treated Water.

While this kit may be used for the isolation of RNA from whole blood, we recommend that you use the E.Z.N.A.™ Blood RNA Midi Kit (Product # R6615) as it is specifically designed for effective hemolysis, and hemoglobin removal, thereby giving higher RNA yields.

Storage and Stability

All E.Z.N.A.™ Total RNA Midi Kit components are guaranteed for at least 12 months from the date of purchase when stored at Room Temperature. During Shipment crystals may have formed in the TRK Lysis Buffer. Dissolve by warming Buffer to 37°C.

Binding Capacity

Each HiBind® RNA Midi column can bind approximately 1mg of RNA. Using greater than 200 mg of tissue or 10^8 cells is not recommended.

Kit Contents

Product Number	R6664-00	R6664-01	R6664-02
Purification Times	2 preps	10 preps	25 preps
HiBind® RNA Midi Columns	2	10	25
15 ml Collection Tubes	2	10	25
TRK Lysis Buffer	10 ml	40 ml	120 ml
RNA Wash Buffer I	10 ml	40 ml	110 ml
RNA Wash Buffer II	5 ml	20 ml	40 ml
DEPC Water	2 ml	5 ml	20 ml
Instruction Booklet	1	1	1

Before Starting

It is strongly advised that you familiarize yourself with the entire booklet before starting. E.Z.N.A.™ Kits are designed to be simple, fast, and reliable provided that all steps are followed diligently.

Materials and Reagents to be supplied by user

For All Protocols:

- Absolute (~96-100%) Ethanol
- Sterile RNase-free pipet tips and 15 ml centrifuge tubes
- 14.3M β-mercaptoethanol (β-ME, 2-mercaptoethanol)
- Microcentrifuge capable of at least 14,000 x g
- 70% ethanol in Sterile DEPC-treated Water.
- Disposable latex gloves

Specific to Protocol:

Sample Disruption and homogenization equipment.

One or more of the following are required, depending on the method chosen.

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

For Total RNA Isolation from Heart, Muscle, and Skin Tissue:

- RNase Free Proteinase K Solution, > 600mAU/ml
- Water Bath preheated to 55°C

For Total RNA Isolation from Bacteria

- Rnase-free Lysozyme
- TE Buffer (10 mM Tris-HCl, pH 7.6, 1mM EDTA)

DNase I Digestion Protocol

- RNase-free DNase Set (product no. E1091)

Key Notes:

- Dilute RNA Wash Buffer II Concentrate with absolute ethanol as follows:

R6664-00	Add 20 ml of absolute ethanol(96-100%) to bottle
R6664-01	Add 80 ml of absolute ethanol (96-100%) to bottle
R6664-02	Add 160 ml of absolute ethanol (96-100%) to bottle
- Add 20µl of 2-mercaptoethanol (β-mercaptoethanol) per 1 ml of TRK Lysis Buffer. This mixture can be stored for 1 week at room temperature.
NOTE: 2-mercaptoethanol is the key in denaturing RNases and must be added before use.
- Please Remember to always wear latex gloves whenever working with RNA. This will 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 TRK Lysis Buffer. This is normal; warm at 37°C to dissolve.
- **ALL CENTRIFUGATION STEPS MUST BE CARRIED OUT AT ROOM TEMPERATURE 22°C - 25°C.**

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 reduce RNA binding to the HiBind® RNA Midi Column, and occasionally may clog the column thus resulting in lower or no yields.

Sample Disruption using a Mortar and Pestle followed by your choice 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 TRK lysis buffer. Continue the procedure as outlined. *This is the preferred method of disrupting tissue samples*

Using a Rotor-Stator Homogenizer for Sample Disruption and Homogenization

Can effectively simultaneously disrupt and homogenize most samples. The process usually takes less than a minute depending on your sample. Many Rotor-stator homogenizers operate with differently sized probes or generators that allow sample processing in 50ml tubes.

Using Bead Milling for 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.

Homogenization of lysate with the Syringe and Needle Method

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

Isolation of Total RNA from Animal Cells

1. **Determine the proper amount of starting material:** It is critical to use the correct number of starting cells in order to obtain optimal yield and purity with the HiBind® RNA Midi column. The maximum number of cells that can be processed on a HiBind® RNA Midi column is dependent on the specific RNA contents and type of cell line. The maximum binding capacity of the HiBind® RNA Midi column is 1mg. The maximum number of cells that TRK Lysis Buffer can use in the Total RNA Protocol is 1×10^8 .

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	4×10^7	850
Hela	7×10^7	1000
293HEK	6×10^7	850
HIN3T3	7×10^7	1000

2. **Harvest Cells** by choosing one of the following methods (do not use more than 1×10^8 cells)
 - 2a. For cells grown in suspension, **determine the number of cells. Pellet the appropriate number of cells by centrifuging at 500 x g for 5 minutes. Aspirate the supernatant** and continue with step 3 of this protocol.
 - 2b. For cells grown in a monolayer: These cells can either be lysed directly in the cell-culture dish or trypsinized and collected as a cell pellet prior to lysis. Cells grown in cell-culture flasks should always be trypsinized.

For direct cell lysis: **Determine cell number, and aspirate the cell-culture medium completely.** Immediately proceed to step 3.

NOTE: Not removing cell-culture medium completely will inhibit lysis and dilute the lysate. This will affect the conditions for binding of RNA to the HiBind® RNA Midi column, and may reduce RNA yield.

To trypsinize and collect cells:

Determine cell number. Aspirate the medium and wash cells with PBS. Aspirate the PBS, and add 0.1-25% trypsin in PBS. Add medium (containing

serum to inactivate the trypsin), after the cells detach from the dish or flask.

Transfer cells to an RNase-free glass or polypropylene centrifuge tube (not supplied), **and centrifuge at 500 x g for 5 minutes. Aspirate the supernatant completely.** Proceed to step 3.

NOTE: Not removing cell-culture medium completely will inhibit lysis and dilute the lysate. This will affect the conditions for binding of RNA to the HiBind® RNA Midi column, and may reduce RNA yield.

3. **Disrupt cells** (do not use more than 1×10^8 cells) **with TRK Lysis Buffer.**
NOTE: Remember to add 20µl of β-ME per 1ml of TRK Lysis Buffer before use.

For pelleted cells, loosen the cell pellet thoroughly by flicking the tube, and adding the appropriate amount of TRK Lysis Buffer based on the table below.

For direct lysis of cells grown in a monolayer, add the appropriate amount of TRK Lysis Buffer directly to the dish based on the table below as well.

Number of Cells	Amount of TRK Lysis Buffer (ml)
$5 \times 10^6 - 5 \times 10^7$	2
$5 \times 10^7 - 1 \times 10^8$	4

4. **Homogenize cells with a rotor-stator homogenizer or until the sample is uniformly homogenized.** Alternatively, sample can be homogenized by using the syringe and needle method as described on page 5.
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 produces better yields.
5. **Add an equal volume (2ml or 4ml) of 70% Ethanol to the lysate and mix thoroughly by vortexing. DO NOT CENTRIFUGE.** If any sample has lost its volume during homogenization, adjust the volume of ethanol accordingly.
NOTE: During RNA purification from certain cell lines, a precipitate may form after the addition of ethanol. This does not affect the procedure.
6. **Apply the sample** (including any precipitate that may have formed) **to a HiBind® RNA Midi column placed into a 15ml collection tube** (supplied). **Centrifuge at 4,000-5,000 x g for 5 minutes at room temperature. Discard**

flow-through and proceed to the next step.

NOTE: The maximum capacity of the HiBind® RNA Midi column is 4 ml. Larger volumes can be loaded on to the column successively in the same HiBind® RNA Midi column. Discard flow-through after each centrifugation.

- **OPTIONAL:** This is the starting point if performing the optional on-column DNase I digestion . Follow protocol as outlined on page 15, after completing step 6 of this protocol.
7. **Wash the HiBind® RNA Midi column with RNA Wash Buffer I by pipetting 3.5ml directly onto the spin column. Centrifuge at 10,000 x g for 1 minute, and discard the 15ml collecting tube.**
8. **Place the HiBind® RNA Midi column into a clean 15 ml centrifuge tube. Add 3 ml of RNA Wash Buffer II diluted with absolute ethanol. Centrifuge at 4,000-5,000 x g for 5 minutes at room temperature. Discard flow-through, and reuse the collection tube in the next step.**
9. **Wash the column as before, and use 3.5 ml of RNA Wash Buffer II diluted with absolute ethanol. Centrifuge at 4,000-5,000 x g for 5 minutes, and discard flow-through.**
10. **With the empty 15 ml collection tube, centrifuge the HiBind® RNA Midi column for 10 minutes at 5,000 x g to completely dry the HiBind® matrix.**
11. **Elution of RNA: Transfer the column into a clean 15 or 20 ml centrifuge tube** (not supplied), **and elute the RNA with 250-500µl of DEPC-treated water** (supplied). **Make sure that you add the water directly onto the column matrix. Centrifuge for 5 minutes at 5,000 x g.** A second elution may be necessary if the expected yield of RNA is > 50 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. Pre-heating the water to 70°C before adding it to the column, and incubating the column for 5 min at room temperature before centrifugation may increase yields.

Isolation of Total RNA from Animal Tissue

1. **Determine the proper amount of starting material:** It is critical to use the

correct number of starting cells in order 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 is dependent on the specific RNA contents and type of tissue. The maximum binding capacity of the HiBind® RNA Midi column is 1mg. The maximum amount of tissue that TRK Lysis Buffer can use in the Total RNA Protocol is 250 mg.

Use the following table as a guideline to select the correct starting material. If you have no information regarding your starting material, use 100 mg as a starting amount. Given the yield and quality of RNA obtained from 100 mg, adjust the starting amount in the next purification.

Average Yield of Total Cellular RNA

Source	Amount of Tissue	RNA Yield (µg)
Mouse		
Brain	100	100
Kidney	150	450
Liver	150	650
Heart	200	100
Spleen	150	500
Lung	100	120
Pancreas	100	400
Thymus	100	200

2. **Disrupt tissue** (do not use more than 250mg of tissue) with TRK Lysis Buffer.

NOTE: Remember to add 20µl of β-ME per 1ml of TRK Lysis Buffer before use. Add the appropriate amount of TRK Lysis Buffer based on the table below.

Amount of Tissue (mg)	Amount of TRK Lysis Buffer (µl)
20-120	2
120-250	4

3. **Homogenize cells with a rotor-stator homogenizer or until the sample is**

uniformly homogenized. Alternatively, sample can be homogenized by using the syringe and needle method as described on page 5.

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 produces better yields.

4. **Centrifuge at 4,000-5,000 x g for 10 minutes. Carefully transfer the cleared supernatant by pipetting to a clean 15 ml centrifuge tube** (not supplied). Use only this supernatant (lysate) in subsequent steps.
NOTE: In some preparations, a fatty upper layer will form after centrifugation. Transferring any pellet of fatty layer may reduce RNA yields, or clog the column.
5. **Add an equal volume (2ml or 4ml) of 70% Ethanol to the lysate and mix thoroughly by vortexing. Do not centrifuge.** If any sample has lost its volume during homogenization, adjust the volume of ethanol accordingly.
NOTE: A precipitate may form after the addition of ethanol in certain preparations. This does not affect the procedure.
6. **Apply the sample** (including any precipitate that may have formed) **to a HiBind® RNA Midi column placed into a 15ml collection tube** (supplied). **Centrifuge at 4,000-5,000 x g for 5 minutes at room temperature. Discard flow-through and proceed to the next step.**
NOTE: The maximum capacity of the HiBind® RNA Midi column is 800µl. Larger volumes can be loaded on to the column successively in the same HiBind® RNA Midi column. Discard flow-through after each centrifugation.
- **OPTIONAL:** This is the starting point if performing the optional on-column DNase I digestion . Follow protocol as outlined on page 15, after completing step 6 of this protocol.
7. **Wash the HiBind® RNA Midi column with RNA Wash Buffer I by pipetting 3 ml directly onto the spin column. Centrifuge as above, and discard the 15ml collecting tube.**
8. **Place the HiBind® RNA Midi column into a clean 15 ml centrifuge tube. Add 3ml of RNA Wash Buffer II diluted with absolute ethanol. Centrifuge at**

4,000-5,000 x g for 5 minutes at room temperature. Discard flow-through, and reuse the collection tube in the next step.

9. **Wash the column as before except this time use 3.5ml of RNA Wash Buffer II diluted with absolute ethanol. Centrifuge at 4,000-5,000 x g for 5 minutes, and discard flow-through.**
10. **With the empty 15 ml collection tube, centrifuge the HiBind® RNA Midi column for 10 minutes at 5,000 x g to completely dry the HiBind® matrix.**
11. **Elution of RNA: Transfer the column into a clean 15 ml or 20 ml centrifuge tube (not supplied), and elute the RNA with 250-500µl of DEPC-treated water (supplied). Make sure that you add the water directly onto the column matrix. Centrifuge for 5 minutes at 5,000 x g. A second elution may be necessary if the expected yield of RNA is > 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. Pre-heating the water to 70°C before adding it to the column, and incubating the column for 5 min at room temperature before centrifugation may increase yields.

Isolation of Total RNA from Heart, Muscle, and Skin Tissue.

Due to the rich contents of contractile proteins, connective tissue, and collagen, it is normally difficult to isolate RNA from heart, muscle, and skin tissue using the standard E.Z.N.A.™ Total RNA Midi Protocol. The following protocol is a modified version that has added Proteinase K digestion, which enables removal of the proteins described above.

1. **Determine the proper amount of starting material:** It is critical to use the correct number of starting cells in order 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 is dependent on the specific RNA contents and type of tissue. The maximum binding capacity of the HiBind® RNA Midi column is 1mg. The maximum amount of tissue that TRK Lysis Buffer can use in the Total RNA Protocol is 250mg. If you have no information regarding your starting material, use 150 mg as a starting amount. Given the yield and quality of RNA obtained from 150 mg, adjust the starting amount in the next purification.
2. **Disrupt tissue (do not use more than 250 mg of tissue) with 2ml of TRK Lysis Buffer.**

NOTE: Remember to add 20µl of β-ME per 1ml of TRK Lysis Buffer before use.

3. **Homogenize cells with a rotor-stator homogenizer or until the sample is uniformly homogenized.** Alternatively, sample can be homogenized by using the syringe and needle method as described on page 5.
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 produces better yields.
4. **Add 4ml of double distilled water to the homogenized lysate, and follow by adding 250µl of Proteinase K solution. Mix thoroughly by pipetting. Incubate at 55°C for 25 minutes.**
5. **Centrifuge at 4,000-5,000 x g for 10 minutes. Carefully transfer the cleared supernatant by pipetting to a clean 15 ml centrifuge tube (not supplied). Use only this supernatant (lysate) in subsequent steps.**
NOTE: In some preparations, a fatty upper layer will form after centrifugation. Transferring any pellet of fatty layer may reduce RNA yields, or clog the column.
6. **Add a .5 volume of absolute ethanol to the lysate and mix thoroughly by vortexing. DO NOT CENTRIFUGE** If any sample has lost its volume during homogenization, adjust the volume of ethanol accordingly.
NOTE: A precipitate may form after the addition of ethanol in certain preparations. This does not affect the procedure.
7. **Apply the sample (including any precipitate that may have formed) to a HiBind® RNA Midi column placed into a 15ml collection tube (supplied). Centrifuge at 4,000-5,000 x g for 5 minutes at room temperature. Discard flow-through and proceed to the next step.**
NOTE: The maximum capacity of the HiBind® RNA Midi column is 4 ml. Larger volumes can be loaded on to the column successively in the same HiBind® RNA Midi column. Discard flow-through after each centrifugation.
→ **OPTIONAL:** This is the starting point if performing the optional on-column DNase I digestion. Follow protocol as outlined on page 15, after completing step 7 of this protocol.
8. **Wash the HiBind® RNA Midi column with RNA Wash Buffer I by pipetting 3 ml directly onto the spin column. Centrifuge at 4,000-5,000 x g for 5 minutes , and discard the 15ml collecting tube.**
9. **Place the HiBind® RNA Midi column into a clean 15 ml centrifuge tube. Add 3ml of RNA Wash Buffer II diluted with absolute ethanol. Centrifuge at 4,000-5,000 x g for 5 minutes at room temperature. Discard flow-through,**

and reuse the collection tube in the next step.

10. **Wash the column with an additional 3 ml of RNA Wash Buffer II diluted with absolute ethanol. Centrifuge at 4,000-5,000 x g for 5 minutes , and discard flow-through.**
11. **With the empty 15 ml collection tube, centrifuge the HiBind® RNA Midi column for 10 minutes at 5,000 x g to completely dry the HiBind® matrix.**
12. Elution of RNA: **Transfer the column into a clean 15 ml centrifuge tube** (not supplied), **and elute the RNA with 250-500µl of DEPC-treated water** (supplied). Make sure that you add the water directly onto the column matrix. **Centrifuge for 5 minutes at 5,000 x g.** A second elution may be necessary if the expected yield of RNA is > 400 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. Pre-heating the water to 70°C before adding it to the column, and incubating the column for 5 min at room temperature before centrifugation may increase yields.

Isolating Total RNA from Bacteria

The E.Z.N.A.™ Total RNA Kit can be modified for the isolation of RNA from bacterial cultures. Only cells growing at log phase should be used. Measured at 600 nm an OD of 0.5-1.0 corresponds to ~10¹⁰ cells per ml. This method is suitable for no more than 10¹⁰ cells.

1. **Centrifuge 5 x 10⁹ cells at 4,000 x g for 5 min. Discard supernatant and add 500µl of TE buffer containing lysozyme** (1mg/ml for gram negative and 4 mg/ml for gram-positive bacteria) **Resuspend cells completely and incubate at room temperature for 7-10 min.**
2. **Add 2 ml of TRK Lysis Buffer and mix by pipetting several times.** Remember to add 20µl of β-mercaptoethanol per 1 ml of TRK Lysis Buffer.
3. **Centrifuge at 4000-5000 x g for 10 minutes. Transfer the cleared supernatant to a new 15ml tube.**
4. **Add 1.4 ml of absolute ethanol to the lysate and mix by vortexing.** A precipitate may form at this point. This will not interfere with RNA purification.
5. **Apply sample (~4ml) to a HiBind® RNA Midi column mounted in a clean 15ml collection tube** (supplied). **Centrifuge at 5,000 x g for 5 minutes (at room temperature). Discard flow-through and proceed to step 6.**

→ **OPTIONAL:** This is the starting point if performing the optional on-column DNase I digestion. Follow protocol as outlined on page 15, after completing step 5 of this protocol.

6. **Wash the column with 3ml of RNA Wash Buffer I. Centrifuge for 5 minutes at maximum speed and discard both flow-through and collection tube.**
7. **Place the spin column into a clean 15ml collection tube** (supplied), **and add 3 ml of RNA Wash Buffer II diluted with absolute ethanol. Centrifuge and discard flow-through as above.** Reuse the collection tube in the next step.
8. **Repeat wash step 7 with a second 3 ml Wash Buffer II. Centrifuge at 4,000-5000 x g for 5 minutes and discard flow-through. Then empty the collection tube and centrifuge the spin cartridge for 10 min at 4000-5000 x g to completely dry the HiBind® matrix.**
9. Elution of RNA: **Transfer the column into a clean 15 ml centrifuge tube** (not supplied), **and elute the RNA with 250-500µl of DEPC-treated water** (supplied). Make sure that you add the water directly onto the column matrix. **Centrifuge for 5 minutes at 5,000 x g.** A second elution may be necessary if the expected yield of RNA is > 500 µg.

Optional: DNase I Digestion Protocol

Omega Bio-tek, Inc.'s RNase-Free DNase Set (product no. E1091), provides efficient on-column digestion of DNA during RNA Isolation.

Important to Note:

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 1-6 of the standard protocol (making sure that all of your samples have completely passed through the HiBind® RNA Midi column), proceed with the following steps.

1. **Wash the HiBind® RNA Midi column by pipetting 1.5 ml directly into the spin column. Centrifuge at 4,000-5,000 x g for 5 minutes and discard the 15ml collection tube.**

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

E.Z.N.A.™ DNase I Digestion Buffer	176 µl
RNase-free DNase I (20 Kunitz/µl)	4 µl
Total Volume	180 µl

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 your 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.
3. **Pipet 180µl of the DNase I stock solution directly onto the surface of the HiBind® RNA resin in each column** (each column should be placed into a 15ml centrifuge tube). Make sure to pipet the stock solution directly onto the 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. **Place the HiBind® RNA Midi column into a new 15 ml centrifuge tube, and add 1.5 ml of RNA Wash Buffer I. Place the column on a bench top for 5 minutes. Centrifuge at 5,000-8,000 x g for 5 minutes and discard flow-through.** Reuse the collection tube in step 6.
6. **Place the HiBind® RNA Midi column in the same 15 ml centrifuge tube, and add 3 ml of RNA Wash Buffer II diluted with absolute ethanol. Centrifuge at 5,000-8,000 x g for 5 minutes and discard flow-through.** Reuse the collection tube in step 7.
7. **Wash the column with a second 3 ml of RNA Wash Buffer II diluted with absolute ethanol. Centrifuge and discard flow-through. Then, with the**

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

8. **Place the column in a clean 15 ml microcentrifuge tube** (not supplied), and **add 250-500 µl of DEPC-treated water** (supplied). Make sure to add water directly onto the HiBind® matrix. **Let it sit for 1 minute, and then centrifuge for 3 minutes at 8,000 x g to elute the RNA.** A second elution may be necessary if the expected yield of RNA > 500µg.

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. Pre-heating the water to 70°C before adding it to the column, and incubating the column for 5 min at room temperature before centrifugation may increase yields.

Storage of RNA

Purified RNA can be stored at -20° C or -70° C (RNase-free water). Under such conditions RNA prepared with the E.Z.N.A.™ Total RNA 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. However, the E.Z.N.A.™ Total RNA Midi Kit eliminates the use of phenol and avoids this problem.*]

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 your 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.

Troubleshooting Guide

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 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 RNA 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.

The following can be purchased separately

Product	Size	Product No.
TRK Lysis Buffer	100ml	PR021
RNA Wash Buffer I	100ml	PR030
RNA Wash Buffer II	25 ml Concentrate (add 100 mL before use)	PR031
DEPC Water	100ml	PR032
2ml capless collection tubes	500/BAG	SS1-1370-00
1.5ml DNase/RNase Free Centrifuge Tubes	500/BAG	SS1-1210-00
RNase-free DNase Set	50 preps	E1091
Proteinase K Solution	2ml/10ml	AC115/AC116

Please Call, Fax , or e-mail us to place an order.

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