Contents

Revised February 2007

Introduction

E.Z.N.A.® Blood RNA Midiprep Kits are designed for isolation of total intracellular RNA from up to 10 ml of fresh, whole blood treated with any common anticoagulant such as heparin, EDTA, or acid-citrate-dextrose. 10 ml of blood typically yields 0.7- 1mg of total RNA. The procedure completely removes contaminants and enzyme inhibitors making total RNA isolation fast, convenient, and reliable. 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. The kit is also suitable for isolation of total RNA from cultures cells, tissues, and bacteria, and from RNA viruses.

RNA purified using the E.Z.N.A.® Blood RNA method is ready for applications such as RT-PCR*.

Principle

The E.Z.N.A.® Blood RNA Kits use the reversible binding properties of HiBind® matrix, a new silica-based material. This is combined with the speed of mini-column spin technology. Red blood cells are selectively lysed and white cells collected by centrifugation. After lysis of white blood cells under denaturing conditions that inactivate RNases, total RNA is purified on the HiBind® spin column. A specifically formulated high salt buffer system allows RNA molecules greater than 200 bases to bind to the matrix. Cellular debris and other contaminants (such as hemoglobin) are effectively washed away and high quality RNA is finally eluted in DEPC-treated sterile water.

Storage

E.Z.N.A.® Blood RNA Kits should be stored at room temperature. During shipment crystals may form in the TRK Lysis Buffer. Warm to 37ºC to dissolve. All components are guaranteed for at least 24 months from the date of purchase.

*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.® Blood RNA Kits	2 Preps	10 Preps	25 Preps
Product Number	R6615-00	R6615-01	R6615-02
Purifications	2	10	25
HiBind™ RNA Midi Columns	2	10	25
Midi Homogenizer Columns	2	10	25
15 ml Collection Tubes	4	20	50
Buffer ERL, 10 X Concentrate	20 ml	80 ml	2 x 100 ml
TRK Lysis Buffer	10ml	45 ml	110 ml
RNA Wash Buffer I	10 ml	40 ml	120 ml
RNA Wash Buffer II Concentrate	5 ml	25 ml	50 ml
DEPC-ddH_2O	1.5 ml	5 ml	20 ml
Instruction Manual	1	1	1

Important Notes

Harvesting and Storage of Blood

E.Z.N.A.® Blood RNA Kits are designed for purification of total RNA from up to 10 ml **fresh** human whole blood. Whole blood should be collected in the presence of anticoagulant such as EDTA or Heparin. For optimized result, the sample should be processed within a few hours of collection.

E.Z.N.A.® Blood RNA Kit can also be used for isolating total RNA from tissue and cultured cells. Tissue should either flash-frozen in liquid nitrogen and store at -70°C or processed immediately after excision. Frozen tissue can not be thawed during handling. Cultured cells should be collected as pellet and either be flash-frozen or processed immediately.

Alternately, sample can be stored at -70°C in the TRK Lysis Buffer after the disruption and homogenization.

Starting amount of sample:

Typically, 10 ml of health adult human blood (4-7 x 10^6 leukocytes per milliliter) can be processed in one HiBind® RNA Midi-column. For blood with elevated number of leukocytes, less than 10 ml blood must be used. The maximum number of leukocytes that can be processed is 1 x 10^8 per column. If more leukocytes are processed, they will not be fully lysed and contaminates will not completely removed. Also large number of leukocytes also could cause the

clogging of the column, thus significantly reduce the yield.

For tissue and cells, less than 300 mg tissue or 1 x 10^8 cells should be used per column.

Before Starting

IMPORTANT	
	Dilute **Buffer ERL** with sterile **deionized water** as follows. **R6615-00** Add 180ml deionized water **R6615-01** Add 720ml deionized water per bottle **R6615-02** Add 900 ml deionized water per bottle
	Dilute **Wash Buffer II** Concentrate with **absolute ethanol** before use. **R6615-00** Add 20ml ethanol **R6615-01** Add 100ml ethanol to each bottle **R6615-02** Add 200ml ethanol to each bottle

Please take a few minutes to read this booklet thoroughly and become familiar with the protocol. Prepare all materials required before starting to minimize RNA degradation.

- Whenever working with RNA, always wear latex gloves to minimize RNase contamination. Change gloves frequently. Use only clean RNase-free disposable plastic pipette tips when using the supplied reagents.
- During the procedure work carefully but quickly. Samples may be stored at -70ºC following lysis of white blood cells with TRK Lysis Buffer/2-mercaptoethanol.
- Under cool ambient conditions, crystals may form in TRK Lysis Buffer. The bottle should be warmed to redissolve the salt.
- **2-mercaptoethanol** (ß-mercaptoethanol) is key in denaturing RNases and must be added to an aliquot of **TRK Lysis Buffer** before use. Add 20 µl of 2-mercaptoethanol (commercial solutions are usually 14.5 M) per 1 ml of TRK Lysis Buffer. This mixture can be stored for 2 weeks at room temperature. Dispense 2-mercaptoethanol in a fume-hood.
- All the subsequent steps must be performed using acentrifuge

capable of at least 5000 x g. Ensure that an appropriate rotor adaptor is in place to prevent damage to the collecting tube.

E.Z.N.A.® Blood RNA Protocol

Materials supplied by user

- 2-mercaptoethanol
- 70% ethanol in DEPC-treated sterile distilled water
- Sterile RNase-free pipette tips and 15 ml centrifuge tubes (Polycabonate thick wall centrifuge tube preferred).
- Tubes for erythrocyte lysis (15 ml-15 ml depending on sample size)
- High speed centrifuge capable of 8,000Xg.
- Centrifuge with swinging-bucket rotor for 50 ml centrifuge tubes.
- Disposable latex gloves

Note: After red blood lysis and removal, all other steps must be carried out at room temperature. Work quickly, but carefully.

Procedure:

1. To 1 volume of whole fresh blood (start with 5 ml blood) add 5 volumes of 1 x Buffer ERL. For example, add 25 ml Buffer ERL to 5 ml blood in a 50 ml centrifuge tube. Mix by vortexing.

   Note: **Buffer ERL is supplied as a 10 x concentrate and must be diluted with sterile deionized water before use. Refer to page 4 or label on bottle for directions.**

2. Incubate for 15 min on ice, mixing by brief vortexing twice. Lysis of red blood cells is indicated when the solution becomes translucent. Blood samples from individuals with an elevated hematocrit or ECR, extend incubation time to 20 min.

3. Pellet leukocytes by centrifuging at 450 x g for 10 min at 4ºC. Completely remove and discard the supernatant containing lysed red blood cells.

4. Wash the white blood cell pellet with 2 volumes of Buffer ERL per volume of whole blood used in step 1. Thoroughly vortex to resuspend cells.

   Tip: If you used 5 ml of whole blood, wash with 10 ml of Buffer ERL.

5. Centrifuge at 450 x g for 10 min at 4ºC. Again, completely remove and discard the supernatant.

6. Loosen the leukocytes pellet by flicking the tube. Add an appropriate volume of TRK Lysis Buffer/2-mercaptoethanol to the pelleted leukocytes and vortex thoroughly to mix. For ≤5 ml healthy whole blood add 2 ml TRK Lysis Buffer. If 5 ml-10 ml health blood was used in step 1, add 4 ml TRK Lysis Buffer. Samples may safely be stored at -70ºC after addition of TRK Lysis Buffer.

   Note: 2-mercaptoethanol is crucial for inactivating endogenous RNases and must be added to an aliquot of TRK Lysis Buffer. Add 20 µl 2-mercaptoethanol per 1 ml of TRK Lysis Buffer. This mixture is stable at room temperature for 2 weeks.

7. **Homogenization: Vortex the sample for 30 seconds and transfer the lysate directly into a Midi Homogenizer Column assembled in a 15 ml centrifuge tube (Supplied). Centrifuge at 6000-8000 x g for 5 minutes to homogenize the sample. Discard the Homogenizer column.**

   Alternately, a conventional rotor-stator homogenizer can also be used at this step to obtain homogenized cell lysate. Generally homogenization with rotor-stator homogenizer results in higher RNA yield compared with other homogenization methods.

8. **Add an equal volume of 70% ethanol and vortex to mix throughly. A precipitate may form on addition of ethanol, resuspend the precipitate completely by vigorous shaking and immediately proceed to step 9.** Insufficiently resuspension of the precipitate will cause DNA contamination and lead to impurity of the RNA.

9. **Apply the entire sample (including any precipitate) to a HiBind® RNA Midi column assembled in a 15 ml collection tube (supplied).** The maximum capacity of the HiBind™ RNA spin column is 4 ml. (**Larger volumes can be loaded successively**.) Centrifuge at 3000-6000 x g for 5 minutes. Discard flow-through and re-use the tube. Load and repeat step 9 with the remaining of cell lysates.

10. **Wash column with Wash Buffer I by pipetting 4 ml directly into the spin column. Centrifuge at 3000-6000 x g for 5 minutes and discard the 15 ml collecting tube**. It is strongly recommended to discard the 15ml collection tube and change a new 15 ml RNase free centrifuge tube (not provided) to avoid RNase contamination before going to next step.
    Note: This the starting point if on-membrane DNase I digestion

(page 9) is desired.

11. **Place column in a clean 15 ml collection tube (Not supplied)**, and add 3 ml RNA Wash Buffer II diluted with ethanol . Centrifuge and discard flow-through. Reuse the collection tube in step 12.

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

12. **(Option) Wash the column with another 3.0 ml of RNA Wash Buffer II as in step 11.** Centrifuge and discard the flow-through.

13. Then with the collection tube empty, centrifuge the spin cartridge for **10 min at 3000-6000 x g** to completely dry the HiBind™ matrix. **Do not skip this step- it is critical for removing traces of ethanol that may otherwise interfere with downstream applications.**

14. **Elution of RNA.** Transfer the column to a new 15 ml centrifuge tube (**Not Supplied**) and elute the RNA with 250-500 µl of DEPC-treated water (supplied with kit). Make sure to add water directly onto column matrix. Centrifuge 10 min at 3000-6000 x g. A second elution may be necessary if >5 ml whole blood (>5x10^8 white blood cells) is used.

RNA Isolation from Tissue or Cultured cells

1. Disrupt cells or tissues with 2-4 ml of TRK Lysis Buffer. **Remember to add 20 µl of 2-mercaptoethanol per 1 ml of TRK Lysis Buffer before use.** Homogenize sample with a rotor-stator homogenizer at maximum speed for 1 minutes.

   2 ml of TRK Lysis Buffer is sufficient for 3 x 10^7 cells or approximately 100 mg disrupted tissue (~1.5 mm cube). For difficult tissues, more than 3 x 10^7 cells, or greater than 100 mg tissue, use 4 ml of TRK Lysis Buffer. However, use no more than 250 mg tissue.

   For culture cells grown in **monolayer** (fibroblasts, endothelial cells, etc.), lyse the cells directly in the culture vessel as follows. Aspirate culture medium completely and add TRK Lysis Buffer directly to the cells. Use 2 ml for ≤ 3 x10^7 cells and use 4 ml of TRK Lysis Buffer for >3 x10^7 cells . Pipette buffer over entire surface of vessel to ensure complete lysis. Transfer lysate from all flask to a clean 15 or 50 ml centrifuge tube and proceed to step 2 below. (This method is preferable to trypsinization followed by washing because it minimizes RNA degradation by nuclease contamination.)

   For cells grown in **suspension cultures**, pellet cells at no greater than 1,500 rpm (400 x g) for 5 min. Discard supernatant, add TRK

Lysis Buffer, lyse by pipetting up and down, and transfer to a clean 15 or 50 ml microfuge tube. Proceed to step 2.

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.

2. Transfer the cell lysate into a Midi-Homogenizer column inserted in a 15 ml tube. Centrifuge at 5000 x g for 5 minutes. Discard the Midi-Homogenizer column and transfer the pass through liquid into a new 15 ml tube.

3. Add an equal volume (2ml or 4ml) 70% Ethanol to the lysate and mix thoroughly by vortexing. precipitate may form after addition of ethanol. Vortex vigorously to resuspend the precipitate.

4. Apply sample onto HiBind® RNA Midi-spin column inserted in a 15 ml centrifuge tube (supplied). The maximum capacity of the Midispin column is 4.0 ml. (Larger volumes can be loaded successively.) Centrifuge at 3000-6000 x g for 5 minutes at room temperature. Discard the flow-through and re-use the tube. Repeat this step until all the sample from step 3 pass through the column.

5. Wash column with RNA Wash Buffer I by pipetting 4 ml directly into the spin column. Centrifuge as above and **discard the the flow-through and the 15 ml collecting tube**.

   Note: This the starting point if on-membrane DNase I digestion (page 9) is desired.

6. **Place column in a clean 15 ml collection tube (not supplied)** , and add 3 ml RNA Wash Buffer II diluted with ethanol. Centrifuge at 3000-6000 x g for 5 minutes at room temperature. Discard flow-through and 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.

7. Wash column with a second 3 ml of Wash Buffer II as in step 6. Centrifuge and discard flow-through. Then with the collection tube empty, centrifuge the spin cartridge for **10 min at 3000 - 6000 x g** to completely dry the HiBind® matrix.

8. **Elution of RNA.** Transfer the column to a clean 15 0r 20 ml centrifuge tube (Not supplied) and elute the RNA with 250-500 µl of DEPC-treated water (supplied with kit). Make sure to add water directly onto column matrix. Centrifuge 5 min at 5000 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 column 5 min at room temperature before centrifugation may increase yields.

D. DNase I Digestion Protocol

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. **Place column in a clean 15ml collection tube**, and add 2 ml RNA Wash Buffer I. Centrifuge and discard flow-through. Reuse the collection tube.

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

OBI DNase I Digestion Buffer	**175 µl**
RNase-free DNase I (20 Kunitz unites/µl)	**5µl**
Total volume	**180 µl**

a. Note: 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. OBI DNase I digestion buffer is supplied with OBI RNase-free DNase set.

c. Standard Dnase buffers are not compatible with on-membrane Dnase digestion

3. Pipet 180µl 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.

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

5. Add 2 ml of RNA Wash Buffer I to the column, centrifuge at 3000 -6000 x g for 5 minutes, discard the flow-through. Continue with first RNA Wash Buffer II washing step (Step 11 for standard protocol)

E.Z.N.A.™ Total RNA Kit Vacuum/Spin Protocol

Carry out lysis, homogenization, and sample loading onto the HiBind® RNA column steps as indicated in previous protocols(steps 1-9 for Blood RNA) . Instead of continuing with centrifugation, follow the steps below.

NOTE: Please read through previous section of this manual before proceeding with this protocol.

1. **Prepare the vacuum manifold according to manufacturer's instruction and connect the HiBind® RNA Column to the manifold**.

2. **Load the homogenized sample onto the HiBInd RNA Column.**

3. **Switch on the vacuum source to draw the sample through the column.**

➜ **OPTIONAL:** This is the starting point if performing the optional on-column DNase I digestion . Follow protocol as outlined on page 9 after completing step 3 of this protocol.

4. **Wash the column by adding 4 mL of RNA Wash Buffer I, draw the wash buffer through the column by turning on the vacuum source**.

5. **Wash the column by adding 3 ml of RNA Wash Buffer II, draw the wash buffer through the column by turning on the vacuum source.**

6. **Wash the column by adding 3 ml of RNA Wash Buffer II, draw the wash buffer through the column by turning on the vacuum source.**

7. **Assemble the column into a 15 ml centrifuge tube and transfer the column to a centrifuge. Centrifuge for 3-6 minutes at 3,000-6,000 g to dry the column matrix.** Try to Avoid carrying over the RNA Wash Buffer II when removing the spin column from the collection tube.

8. **Place the column in a clean 1.5 ml microcentrifuge tube** (not supplied), **and add 250-500µl of DEPC-treated water** (supplied). **Let it sit for 1-2 minutes, and then centrifuge for 1 minute to elute the RNA**

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. 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 spectrophotometric analysis of DNA or RNA. However, the

E.Z.N.A. Total RNA Kit eliminates the use of phenol and avoids 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

It is highly recommended that RNA quality be determined prior to all analyses. The quality of RNA can 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	• Increase the centrifuge speed or time to dry the column completely. • Repeat elution. • Pre-heat DEPC-water to 70° C prior to elution. • Incubate column for 5 min with water prior to centrifugation.
	Column is overloaded	• Reduce quantity of starting material.
Clogged column	Incomplete disruption and homogenization	• Mix thoroughly after addition of TRK Lysis Buffer.. • Increase centrifugation time. • Reduce amount of starting material • choose appropriate method for homogenization if tissue or cultured cells are used
	Centrifuge temperature too low . Low temperature caused precipitate and cause clooging	• The centrifuge temperature should be set at 20-25° C. Some centrifuge may cool to below 20°C even it is set at 20°C. Check the temperature of the actual temperature.
Degraded RNA	Source	• Do not freeze blood • Do not store blood samples for more that a few hours • Follow protocol closely, and work quickly.
	RNase contamination	• Ensure not to introduce RNase during the procedure. • Check buffers for RNase contamination.
Problem in downstream applications	Salt carry-over during elution	• Ensure Wash Buffer II has been diluted with 4 volumes of 100% ethanol as indicated on bottle. • 1 X Wash Buffer II must be stored at room temperature. • Repeat wash with Wash Buffer II.
	Inhibitors of PCR	• Use less starting material • Prolong incubation with Buffer ERL to completely lyse erythrocytes
DNA contamination	Insufficiently resuspend the precipitate before loading the column will cause the DNA contamination lead to impurity of the RNA	• Digest with RNase-free DNase and inactivate at 75ºC for 5 min. • Make sure to resuspend the precipitate by vortexing or vigorously shaking and immediately lodd to the column
Low A260/280 value	pH of the solution interfere the OD reading	use the Tris-HCl (pH 7.5), not DEPC water to dilute sample before measure the purity.