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Introduction

E.Z.N.A.[®] Blood RNA Kits are designed for isolation of total intracellular RNA from up to 1 ml of fresh, whole blood treated with any common anticoagulant such as heparin, EDTA, or acid-citrate-dextrose. One ml of blood typically yields 1–5 µg 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 cultured 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 in the HiBind[®] spin column. A specially 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 37°C to dissolve. All kit components are guaranteed for at least 24 months from 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	Trial Kit 5 Preps	RNA Prep 50	RNA Prep 200
Product Number	R6614-00	R6614-01	R6614-02
Purifications	5	50	200
HiBind [®] Columns	5	50	200
Homogenizer columns	5	50	200
2 ml Collection Tubes	15	150	600
Buffer ERL, 10 X Concentrate	5 ml	50 ml	3 x 50 ml
TRK Lysis Buffer	5 ml	40 ml	150 ml
RNA Wash Buffer I	5 ml	40 ml	4 x 50 ml
RNA Wash Buffer II Concentrate	5 ml	12 ml	4 x 12 ml
DEPC-ddH ₂ O	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 1 ml **fresh** whole blood. The system is not limited by RNA binding capacity of HiBind[®] RNA columns (which can bind up to 100 µg RNA), but by lysis volume. Due to the abundance of erythrocytes and proteins, greater than 1 ml whole blood will significantly lower RNA quality. The relatively low RNA content of leukocytes means that the maximum binding capacity of HiBind[®] RNA columns cannot be reached.

Samples should be collected in the presence of an anticoagulant (preferably acid-citrate-dextrose) and processed within a few hours. Minimize storage time prior to RNA isolation as leukocyte transcripts generally have variable stabilities.

Avoid freezing blood samples at all costs. The E.Z.N.A.[®] Blood RNA procedure involves erythrocyte lysis and removal which cannot

be accomplished with frozen blood. For such samples we recommend the modified protocol (see Page 7). Note that only 150 µl frozen blood can be used with the modified procedure.

Modified Protocols

E.Z.N.A.[®] Blood RNA Kits may also be used for isolation of total RNA from cultured cells, tissues, bacteria and from acellular body fluids. In addition, RNA from enzymatic reactions, such as *in vitro* transcription, can be purified with the system. Please call our Technical Staff for these additional protocols. (The E.Z.N.A.[®] Total RNA Kit, **Product # R6634**, is recommended for isolation of total RNA from cultured cells, tissues and bacteria.)

Before Starting

IMPORTANT	1. Buffer ERL is supplied as a 10x concentrate and must be diluted with sterile deionized water as follows.			
	<table> <tbody> <tr> <td>Trial Sample (R6614-00)</td> <td>Add 45 ml deionized water.</td> </tr> <tr> <td>R6614-01 and R6614-02</td> <td>Empty contents of each bottle supplied into an appropriately sized vessel and add 450 ml deionized water per bottle of Buffer ERL.</td> </tr> </tbody> </table>	Trial Sample (R6614-00)	Add 45 ml deionized water.	R6614-01 and R6614-02
Trial Sample (R6614-00)	Add 45 ml deionized water.			
R6614-01 and R6614-02	Empty contents of each bottle supplied into an appropriately sized vessel and add 450 ml deionized water per bottle of Buffer ERL.			
	2. Wash Buffer II Concentrate must be diluted with absolute ethanol before use.			
	<table> <tbody> <tr> <td>R6614-00 (Trial Sample)</td> <td>Add 20 ml 100 % ethanol</td> </tr> <tr> <td>R6614-01 and R6614-02</td> <td>Add 48 ml 100% ethanol to each bottle</td> </tr> </tbody> </table>	R6614-00 (Trial Sample)	Add 20 ml 100 % ethanol	R6614-01 and R6614-02
R6614-00 (Trial Sample)	Add 20 ml 100 % ethanol			
R6614-01 and R6614-02	Add 48 ml 100% ethanol to each bottle			

Please take a few minutes to read this booklet thoroughly to 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 -70°C 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 (β -mercaptoethanol) is key in denaturing RNases and must be added to an aliquot of TRK Lysis Buffer before use. Add 20 μ l 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.

E.Z.N.A.® Blood RNA Protocol

Materials supplied by user:

- 2-mercaptoethanol
- 70% ethanol in DEPC-treated sterile distilled water
- RNase-free pipette tips and RNase-free microcentrifuge tubes
- Tubes for erythrocyte lysis (1 ml-15 ml depending on sample size)
- Table top microcentrifuge at 4°C (if available) and at room temperature.
- RNase-free 15 ml and 1.5 ml centrifuge tubes
- Centrifuge with swinging-bucket rotor for 15 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 (maximum of 1 ml) add 5 volumes of Buffer ERL (diluted). For example add 5 ml Buffer ERL to 1 ml blood. 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. For blood samples from individuals with an elevated hematocrit or ECR, extend incubation time to 20 min.
3. Pellet leukocytes by centrifuging at 600 x g for 10 min at 4°C. Completely remove and discard the supernatant containing lysed red blood cells. If a refrigerated centrifuge is not available, centrifuge at room temperature, but quickly complete Step 4 below.
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 1 ml of whole blood, wash with 2 ml of Buffer ERL.

5. Centrifuge at 600 x g for 10 min at 4°C. Again, completely remove and discard the supernatant. If a refrigerated centrifuge is not available, centrifuge at room temperature, but quickly complete Step 6 below.
6. Add TRK Lysis Buffer/2-mercaptoethanol to the pelleted white blood cells and vortex thoroughly to mix. For ≤ 500 μ l whole blood add 400 μ l TRK Lysis Buffer. If 0.5 ml-1.0 ml blood was used in Step 1, add 700 μ l TRK Lysis Buffer. Samples may be safely stored at -70°C after addition of TRK Lysis Buffer. Vortex or pipet to remove any clumps.

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

7. Transfer the cell lysate directly into a Homogenizer Column setting in a 2 ml collection tube (supplied) and centrifuge at maximum speed for 2 minutes. Discard Homogenizer Column and save the homogenized lysate. Note: if too many cells have been used, the cell lysate will be too viscous to pipet. In this case, divide the

sample into two aliquots and adjust the volume of each aliquot to 700 µl with TRK Lysis Buffer. Continue the protocol from the step 7 with two Homogenizer Columns and two HiBind® RNA Columns.

8. Add an equal volume of 70% ethanol and vortex to mix. A precipitate may form on addition of ethanol, but will not interfere with RNA isolation.
9. Apply the entire sample (including any precipitate) to a HiBind® RNA column assembled in a 2 ml collection tube (supplied). The maximum capacity of the HiBind® RNA spin cartridge is 750 µl. (Larger volumes can be loaded successively.) Centrifuge at 10,000 x g for 15 seconds. Discard flow-through and proceed to Step 10.
10. Wash column with Wash Buffer I by pipetting 750 µl directly into the spin column. Centrifuge at 10,000 x g for 15 seconds and discard the 2 ml collection tube.
11. Place column in a new 2ml collection tube (supplied), and add 500 µl Wash Buffer II diluted with ethanol. Centrifuge at 10,000 x g for 15 seconds and discard flow-through. Re-use the collection tube in Step 11.

Note: Wash Buffer II Concentrate must be diluted with absolute ethanol before use. Refer to label on bottle for directions.
12. Wash column with a second 500 µl of Wash Buffer II as in Step 10. Centrifuge and discard flow-through. Then with the collection tube empty, centrifuge the spin cartridge for **1 min at full speed** to *completely* dry the HiBind® matrix.
13. **ELUTION OF RNA:** Transfer the column to a new 1.5 ml microfuge tube (not supplied with kit) and elute the RNA with 50-100 µl of DEPC-treated water (supplied with kit). Make sure to add water directly onto column matrix. Centrifuge 1 min at maximum speed. A second elution may be necessary if >0.5 ml whole blood (>2x10⁶ white blood cells) is used.

Note: No RNA extraction procedure can completely remove genomic DNA. For very sensitive work we suggest that you treat the eluted RNA with RNase-free DNase. Also for RT-PCR, use intron-spanning primers that allow easy identification of DNA-contamination. A control PCR reaction containing the RNA as template will also allow detection of DNA contamination. For designing intron-spanning primers, call our technical staff at **800-932-8896** for assistance. We can help design primers suited to your needs.

E.Z.N.A.® Protocol for Frozen Blood Samples

We recommend that blood be harvested and processed immediately using the main protocol on Page 5. However, if absolutely necessary, blood may be frozen at -70°C, but no more than 150 µl should be processed using the following modified method. RNA quality and yield is usually adequate for RT-PCR of transcripts ≤500 nt.

Additional materials required by user

- RNase-free Proteinase K
- Water bath preheated to 70°C.

Prepare a Proteinase K solution by preparing 450 µl TRK Lysis Buffer/2-mercaptoethanol containing 4 mg/ml Proteinase K. **Thaw out the frozen blood sample and work quickly to minimize RNA degradation.**

1. **Pipette 150 µl of blood into a RNase-free microcentrifuge tube.**
2. **Add 350 µl of TRK Lysis Buffer/β-mercaptoethanol containing 4 mg/ml Proteinase K and vortex for 30 seconds to thoroughly mix.**
3. **Incubate at 70°C for 10 minutes.** Mix the sample twice by inversion during the incubation.
4. **Centrifuge sample at 10,000 x g for 3 min and transfer 450 µl supernatant to an RNase-free microfuge tube.**
5. **Add 225 µl of absolute ethanol to the mixture, vortex for 10 seconds, and proceed to Step 8 (Page 6) of main protocol** (addition of sample to RNA HiBind® column/collection tube assembly step).

Upon freezing whole blood, both red and white blood cells are lysed. Due to the abundance of contaminants such as hemoglobin, greater than 150 µl frozen blood can not be processed without adversely affecting RNA quality. As leukocytes have a relatively low RNA content, the maximum yield with this protocol is typically < 1 µg. For RT-PCR, a single elution of RNA should be carried out with 30 µl of water to maximize final concentration.

Viral RNA From Acellular Body Fluids (Plasma, Serum, Urine, Etc.)

The following modification of the main Blood RNA protocol is required for optimal binding to the RNA HiBind[®] matrix. You will require a stock solution of yeast tRNA to use as carrier.

Prepare a stock solution of yeast tRNA in DEPC-treated dH₂O at 5 mg/ml. Aliquot and freeze at -70°C until required.

1. Centrifuge no more than 5 ml sample for 20 min at 5,000 x g.
2. Filter sterilize by passing through a sterile 0.22 µm filter. This will remove any cells, thus avoiding cellular nucleic acid co-purification.
3. **Optional.** Some specimens may contain very few virions. It may be necessary to concentrate the filtered sample using a centrifugal micro-concentrator. Suitable devices include Centricon[™]-100 (Amicon, 2 ml, Cat# 4211), Ultrafree[™]-CL (Millipore, 2 ml, Cat# UFC4 THK 25), and equivalents. Centrifuge 3-5 ml of sample according to the manufacturer's protocol to obtain a ten- to twenty-fold concentration (final volume 200-300 µl).
4. Pipet 150 µl sample into a 1.5 ml microcentrifuge tube and add 750 µl Buffer TRK Lysis Buffer followed by 5 µl of yeast tRNA. Then add 600 µl absolute ethanol and vortex thoroughly. Immediately proceed to next step.

Note: Add 20 µl 2-mercaptoethanol per 1 ml TRK Lysis Buffer.

5. Follow the main Blood RNA protocol from Step 7 (Page 7). Use 30-50 µl DEPC-dH₂O for elution to obtain a higher RNA concentration.

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	<ul style="list-style-type: none"> 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	<ul style="list-style-type: none"> Reduce quantity of starting material.
Clogged column	Incomplete lysis	<ul style="list-style-type: none"> Mix thoroughly after addition of TRK Lysis Buffer.. Increase centrifugation time. Reduce amount of starting material
Degraded RNA	Source	<ul style="list-style-type: none"> Do not freeze blood Do not store blood samples for more that a few hours 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 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	<ul style="list-style-type: none"> Use less starting material Prolong incubation with Buffer ERL to completely lyse erythrocytes
DNA contamination		<ul style="list-style-type: none"> Digest with RNase-free DNase and inactivate at 75°C for 5 min.

Other Product Information

Product Number	Product Name	Description
E.Z.N.A.® Total RNA Miniprep Kits		
R6634-01/02 R6834-01/02	E.Z.N.A.® Total RNA Kit	Total RNA isolation from animal cells or tissues.
R6614-01/02 R6814-01/02	E.Z.N.A.® Blood RNA Kit	Total RNA Isolation from blood samples
R6627-01/02 R6827-01/02	E.Z.N.A.® Plant RNA Kit	Total RNA Isolation from plant samples
R6640-01/02 R6840-01/02	E.Z.N.A.® Fungal RNA Kit	Total RNA Isolation from fungal samples
R6670-01/02 R6870-01/02	E.Z.N.A.® Yeast RNA Kit	Total RNA Isolation from yeast samples
R6850-01/02 R6950-01/02	E.Z.N.A.® Bacterial RNA Kit	Total RNA Isolation from yeast samples
R6675-01/02 R6875-01/02	E.Z.N.A.® Mollusc RNA Kit	Total RNA Isolation from mollusc, invertebrates samples.
E.Z.N.A.® Total RNA Midi/maxi Kits		
R6664-01/02	E.Z.N.A.® Total RNA Midi Kit	Total RNA isolation from animal cells or tissues
R6693-01/02	E.Z.N.A.® Total RNA Maxi Kit	Total RNA isolation from animal cells or tissues
R6615-01/02	E.Z.N.A.® Blood RNA Midi Kit	Total RNA isolation from blood samples
R6616-01/02	E.Z.N.A.® Blood RNA Maxi Kit	Total RNA isolation from blood samples
R6628-01/02	E.Z.N.A.® Plant RNA Midi Kit	Total RNA isolation from plant samples
Other RNA isolation kit, Reagent and supplies		
R6511-01/02	mRNA Enrichment kit	mRNA isolation
R6830-01/02	RNA-Solv® reagent	Single reagent for total RNA isolation
R6248-01/02 R6249-01/02	E.Z.N.A.® RNA Probe purification kit	RNA Probe purification
R6376-01/02	E.Z.N.A.® Poly-Gel RNA Isolation Kit	Isolate RNA from poly-acrylamide gel
R6500-01/02	E.Z.N.A.® Oligo (dT) Cellulose	High capacity oligo(dT) cellulose
E1091	RNase-free DNase I set	DNase I set for on-column DNase digestion

