Contents

Introduction. 2
Principle. 2
Storage and stability. 2
Kit Contents. 3
Important Notes. 3
Before Starting. 4
E.Z.N.A.? Blood RNA Mini Kit Protocol. 5
E.Z.N.A.? Protocol for Frozen Blood Samples. 8
Viral RNA From Acellular Body Fluids. 9
Quantitation and Storage of RNA. 10
RNA Quality. 10
Trouble Shooting Guide. 11

Introduction

E.Z.N.A.? Blood RNA Kit is designed for the 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. 1 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 the isolation of total RNA from cultures cells, tissues, 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 Kit uses 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 water. Trace of the DNA that may co-purified with RNA will be removed by DNase treatment on the RNA spin column. After two washing steps, purified total RNA will be eluted with RNase-free water.

Storage and stability

E.Z.N.A.? Blood RNA Kit should be stored at room temperature. During shipment crystals may form in the MRC Lysis Buffer. Warm to 37°C to dissolve. All kit 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

Product Number	R6814-00	R6814-01	R6814-02
Purification times	5	50	200
HiBind? RNA Mini column	5	50	200
2 ml Collection Tubes	10	100	400
Buffer ERL, 10 x Concentrate	5 ml	50 ml	3 x 50 ml
MRC Lysis Buffer	5 ml	40 ml	125 ml
RNA Wash Buffer I	5 ml	45 ml	2 x 90 ml
RNA Wash Buffer II	2 ml	12 ml	4 x 12 ml
DEPC Water	1.5 ml	10 ml	40 ml
Instruction Manual	1	1	1

Important Notes

Harvesting and Storage of Blood

E.Z.N.A.? Blood RNA Kit is 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 can not be reached.

Samples should be collected in the presence of an anticoagulant (preferably acid-citrate-dextrose) and processed within a few hours. Minimize storage prior 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 can not 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 Kit may also be used for the isolation of total RNA from cultured cells, tissues, bacteria and 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 I, **product # R6834**, is recommended for isolation of total RNA from cultured cells, tissues and bacteria.)

Before Starting

IMPORTANT

1. **Buffer ERL** is supplied as a 10 x concentrate and must be diluted with sterile deionized water as follows.

R6814-00	Add 45 ml deionized water.
R6814-01 R6814-02	Empty contents of each bottle supplied into an appropriately sized vessel and add 450 ml deionized water per bottle of Buffer ERL.

2. **RNA Wash Buffer II Concentrate** must be diluted with absolute ethanol(~96-100%) before use.

R6814-00	Add 8 ml absolute ethanol
R6814-01	Add 48 ml absolute ethanol
R6814-02	Add 48 ml absolute ethanol to each bottle

3. **Optional :** Prepare the DNase I digestion mixture: For each RNA isolation, add 1.5µl of DNase I with 73.5µl DNase I digestion buffer

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 -70°C following lysis of white blood cells with MRC Lysis Buffer/2-mercaptoethanol.
- Under cool ambient conditions, crystals may form in MRC Lysis Buffer. The bottle should be warmed to redissolve the salt.
- 2-mercaptoethanol (?-mercaptoethanol) is key in denaturing RNases and must be added to an aliquot of MRC Lysis Buffer before use. Add 20 µl of 2-mercaptoethanol (commercial solutions are usually 14.5 M) per 1 ml of MRC 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 Mini Kit Protocol

Materials supplied by user:

- 2-mercaptoethanol
- 70% ethanol in DEPC-treated sterile distilled water
- RNase-Free DNase I (optional)
- Sterile RNase-free pipette tips and microcentrifuge tubes
- Tubes for erythrocyte lysis (1 ml-15 ml depending on sample size)
- Table top microcentrifuge at room temperature.
- 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 1 x Buffer ERL**. 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. 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 4°C.** 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 1 ml of whole blood, wash with 2 ml of Buffer ERL.

5. **Centrifuge at 450 x g for 10 min at 4°C.** Completely remove and discard the supernatant.

6. Add 100µl of DEPC Water and vortex to re-suspend the white blood cells.

7. **Add MRC Lysis Buffer/2-mercaptoethanol and vortex thoroughly to mix.** For ≤500 µl whole blood add 350 µl MRC Lysis Buffer. If 0.5 ml-1.0 ml blood was used in step 1, add 600 µl MRC Lysis Buffer. Samples may safely be stored at -70°C after addition of MRC Lysis Buffer.

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

8. Homogenize the lysate by passing it at least 5 times through a blunt 20-gauge needle (0.9 mm) fitted to an Rnase-Free syringe or by pipetting up and down for 5-10 times. **Add an equal volume of 70% ethanol and vortex well by pipetting.**

9. **Apply the entire sample (including any precipitate) to a HiBind? RNA mini**

column assembled in a 2 ml collection tube (supplied). The maximum capacity of the HiBind? RNA spin cartridge is 800µl. (Larger volumes can be loaded successively.) Centrifuge at 10,000 x g for 30 seconds. Discard flow-through and proceed to step 10.

10. **Pipet 300µl RNA wash Buffer I into the column. Centrifuge as above and** Discards the flow-through. Re-use the collection tube in step 11. If the On-Membrane DNase treatment is not desired, simply increase the volume of RNA wash Buffer I to 700µl, centrifuge at 10,000 x g for 30 seconds to wash the column and discards the flow-through and collection tube. Continue the protocol with step 12.

11. A. (**Optional): Prepare the DNase I mixture:** In a 1.5 ml microtube, add 1.5µl DNase I to 73.5µl DNase I Digestion Buffer. Mix gently by inverting the tube. Do not vortex, DNase I is especially sensitive with physical denaturation.

    B. Pipet the DNase incubation mixture directly onto the center of HiBind? RNA column silica membrane. Incubate at room temperature for 15 minutes.

    C. **Pipet 500µl RNA wash Buffer I into the column and incubate at room temperature for 5 min**. Centrifuge at 10,000 x g for 30 seconds. Discard the flow-through and collection tube.

12. **Place column in a clean 2ml collection tube (supplied)**, and add 500 µl RNA Wash Buffer II diluted with ethanol. Centrifuge and discard flow-through. Reuse the collection tube in step 13.

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

13. **Wash column with a second 500 µl of RNA Wash Buffer II as in step 12.** Centrifuge and discard flow-through. Then with the collection tube empty, centrifuge the spin cartridge for **2 min at full speed** to completely dry the HiBind? matrix.

14. **Elution of RNA.** Transfer the column to a clean 1.5 ml microfuge tube (not supplied with kit) and elute the RNA with 30-50 µl of DEPC 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 (>$2x10^6$ white blood cells) is used.

No RNA extraction procedure can completely remove genomic DNA. For sensitive (such as RT-PCR or differential display) 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 800-832-8896 for assistance. We can help design primers suited to your needs.

E.Z.N.A.? X-Press Protocol for Blood Samples

This protocol is designed for fast isolation of Total RNA from 1-150ul fresh or frozen Blood.

1. **Pipette 150 µl of blood into a sterile microcentrifuge tube**.

2. **Add 350 µl of MRC Lysis Buffer/?-mercaptoethanol and vortex for 30 seconds to thoroughly mix**.

3. **Incubate at 65°C for 10 minutes.** Mix the sample twice by inversion during the incubation.

4. **Centrifuge sample at 13,000 x g for 3 min and transfer 450 µl supernatant to a sterile microfuge tube.**

5. **Add 250 µl of absolute ethanol to the mixture**, vortex for 10 seconds, and proceed to step 9, (page 6) of main protocol (addition of sample to RNA HiBind? column/collection tube assembly).

   On 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 dH_2O 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 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 MRC 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 MRC Lysis Buffer.

5. Follow the main Blood RNA protocol from step 8 (page 6). Use 30-50 µl DEPC Water for elution to obtain a high 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.

Trouble Shooting Guide

Problem	Cause	Suggestion
Little or no RNA eluted	RNA remains on the column	• 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 lysis	• Mix thoroughly after addition of MRC Lysis Buffer. • Increase centrifugation time. • Reduce amount of starting material.
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 contaminati on	• 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		• Digest with RNase-free DNase and inactivate at 75°C for 5 min.