Contents

Revised March 2004

Introduction

The E.Z.N.A.® Blood RNA Maxi Kit is designed for isolation of total intracellular RNA from up to 50 ml of fresh, whole blood treated with any common anticoagulant such as heparin, EDTA, or acid-citrate-dextrose. 50 ml of health blood typically yields 50–250 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 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 Kit uses the reversible binding properties of HiBind® matrix, a new silica-based material. This is combined with the speed of Spin-column 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 and Stability

The E.Z.N.A.® Blood RNA Kit should be stored at room temperature. During shipment crystals may form in the TRK Lysis Buffer. Warm to 37°C 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

Product Number	R6616-00	R6616-01	R6616-02
Purification Times	2 Preps	5 Preps	20 Preps
HiBind™ RNA Maxi Columns	2	5	20
50 ml Collection Tubes	2	5	20
Buffer ERL, 10 X Concentrate	80 ml	200 ml	4 x 200 ml
RNA-Solv Reagent	50 ml	120 ml	2 x 220 ml
RNA Wash Buffer I	35 ml	2 x 40 ml	8 x 40 ml
RNA Wash Buffer II Concentrate	12 ml	3 x 12 ml	11 x 12 ml
DEPC-ddH_2O	5 ml	10 ml	30 ml
Instruction Manual	1	1	1

Important Notes

Harvesting and Storage of Blood

The E.Z.N.A.® Blood RNA Maxi Kit is designed for purification of total RNA from up to 50 ml **fresh** whole blood. The system is not limited by RNA binding capacity of HiBind® RNA columns (which can bind up to 1 mg RNA), but by lysis volume. Due to the abundance of erythrocytes and proteins, greater than 50 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 may not be accomplished with frozen blood.

Before Starting

IMPORTANT

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

R6616-00	Dilute with 720 ml deionized water.
R6616-01	Empty contents of the bottle supplied into an appropriately sized vessel and add 1800 ml deionized water per bottle of Buffer ERL.
R6616-02	Empty contents of the bottle sipplied into an appropriately sized vessel and add 1800 ml deionized water per bottle of Buffer ERL.

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

R6616-00	Add 48 ml 96-100 % ethanol
R6616-01	Add 48 ml 96-100% ethanol to each bottle
R6616-02	Add 48 ml 96-100% 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.

.

- 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 Maxi Protocol

Materials supplied by user:

- Chloroform
- Rotor-stator homogenizer or syringe with 19-21 gauge needle
- Sterile RNase-free pipette tips
- 50 ml centrifuge Tubes capable of 5000 x G
- Tubes for erythrocyte lysis (50 ml-250 ml depending on sample size)
- centrifuge capable of 5,000 X G.
- 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. Estimate the volume of ERL Buffer to be used for lysis of red blood cells: To 1 volume of whole fresh blood add 3 volumes of 1 x Buffer ERL.

2. **Base on total volume of blood sample, prepare several 50ml tubes contains 30ml of 1 x ERL Buffer.** For example: for 50ml blood sample, prepare 5 of 50ml tubes contains 30ml 1 x ERL Buffer.
   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.**

3. **Add 10ml whole blood to each 50ml tube contains 30ml ERL Buffer.** Invert to mix and incubate for 10-15 minutes on ice. Mixing by inverting at least once during incubation. 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.

4. **Pellet leukocytes by centrifuging at 5,000 x g for 5 min at 4°C.** Completely remove and discard the supernatant containing lysed red blood cells.

5. **Wash the white blood cell pellet with 30ml of 1 x Buffer ERL per 10ml whole blood.** Thoroughly vortex to resuspend cells. centrifuging at 5000 x g for 5 min at 4°C. Completely remove and discard the supernatant.

6. **Add 4 ml RNA-Solv Reagent to the pelleted white blood cells and vortex thoroughly to mix.** Samples may safely be stored at -70°C after addition of RNA Solv Reagent. Combine samples by pool all samples into one 50ml tube (~20ml).

7. Homogenize the sample: Homogenize the cell lysate using a conventional rotor-stator homogenizer for 1 minute. Alternatively, cell lysate can be homogenized by using a syringe and needle. High molecular-weight DNA can be sheared with by passing the lysate through a narrow needle (19-21 gauge)fitted to an syringe for 5- 10 times or until the sample is uniformly homogeneous.

8. **Incubate the tube contains homogenate at room temperature for 2-3 minutes.**

9. **Add 4 mL of chloroform per 20 mL of RNA-*Solv* Reagent.** Cap sample tubes securely and shake vigorously for 15 seconds. Incubate on ice for 10 minutes.

10. **Centrifuge at 12,000 x g for 15 minutes at 4°C.** The mixture separates into a lower phenol-chloroform phase, an interphase, and an upper aqueous phase. RNA remains entirely in the aqueous phase.

11. **Transfer no more than 80% of the aqueous phase to a fresh tube and add 1/2 volume of absolute ethanol (~96-100%, room temperature)**. Vortex at maximum speed for 30 seconds.
    ***Note: All of the centrifugation step below should be carried out at room temperature*.**

12. **Apply the entire sample to a HiBind® RNA Maxi column assembled in a 50 ml collection tube (supplied).** The maximum capacity of the HiBind™ RNA Maxi column is 25 ml. (**Larger volumes can be loaded successively**.) Centrifuge at 2000 x g for 3 minutes. Discard flow-through and proceed to step 12.

13. **Wash column with Wash Buffer I by pipetting 15 ml directly into the RNA maxi spin column. Centrifuge at 5,000 x g for 3 minutes and discard the 50ml collecting tube**. It is strongly recommended to discard the 50 ml collection tube and change a new 50ml RNase free centrifuge tube (provided) to avoid RNase contamination before go to next step.

14. **Wash column by pipetting 15 ml RNA Wash Buffer II diluted with ethanol**. Centrifuge and discard flow-through. Reuse the collection tube in step 15.

15. **Wash column with a second 15ml RNA Wash Buffer II as in step 14.** Centrifuge and discard flow-through. Then with the collection tube empty, centrifuge the spin cartridge for **10 min at 5000x 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.

16. **Elution of RNA. Transfer the column to a new 50ml centrifuge tube (Not Supplied) and elute the RNA with 0.7-1ml of DEPC-treated water (supplied with kit).** Make sure to add water directly onto column matrix. Centrifuge 3 min at 4000 x g. A second elution may be necessary if >5 ml whole blood (>$5x10^8$ 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 888-OMEGA-88 for assistance. We can help design primers suited to your needs.

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. 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 lysis or incomplete homogenization	• Mix thoroughly after addition of RNA Solv Reagent • use a rotor-stator to homogenize the lysate • 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 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		• Digest with RNase-free DNase and inactivate at 75°C for 5 min.