Contents

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 10–50 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 extraction, and time-consuming steps such as CsCl gradient ultracentrifugation, and precipitation with isopropanol or LiCl. The kit is also suitable for isolation of total RNA from culture cells, tissues, bacteria, and 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 are 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

All components except RNA-Solv Reagent in E.Z.N.A.® Total RNA Kits should be stored 22-25°C. RNA-Solv® Reagent should be store at 4°C for long term storage. All E.Z.N.A.™ Total RNA Kit components are guaranteed for at least 24 months from the date of purchase when stored as above.

*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 No	R6615-00	R6615-01	R6615-02
Purification times	2	10	25
HiBind™ RNA Midi Columns	2	10	25
15 ml Collection Tubes	4	20	50
Buffer ERL, 10 X Concentrate	20 ml	50 ml	120 ml
RNA-Solv Reagent	15 ml	45 ml	120 ml
RNA Wash Buffer I	10 ml	50 ml	2 x 50 ml
RNA Wash Buffer II	5 ml	12 ml	3 x 12 ml
DEPC-Water	1.5 ml	10 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 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 15 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.

Before Starting

IMPORTANT

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

R6615-00	Dilute with 180 ml deionized water.
R6615-01	Empty contents of the bottle supplied into an appropriately sized vessel and add 450 ml deionized water per bottle of Buffer ERL.
R6615-02	Empty contents of the bottle supplied into an appropriately sized vessel and add 1080 ml deionized water per bottle of Buffer ERL.

2 **RNA Wash Buffer II Concentrate** must be diluted with absolute ethanol before use.

R6615-00	Add 20 ml 100 % ethanol
R6615-01	Add 48 ml 100% ethanol to each bottle
R6615-02	Add 48 ml 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.
- During the procedure work carefully but quickly. Samples may be stored at -70°C following lysis of white blood cells with RNA-Solv Reagent.
- All the subsequent steps must be performed using a centrifugation capable of at least 5000 x g. Ensure that an appropriate rotor adaptor is in place to prevent damage to the collection tube.

E.Z.N.A.® Blood RNA Midi Protocol

Materials supplied by user:

- 70% ethanol
- Sterile RNase-free pipette tips and 15 ml centrifuge tubes (Polycarbonate thick wall centrifuge tube preferred).
- Tubes for erythrocyte lysis (15 ml-50 ml depending on sample size)
- High speed centrifuge capable of 3,000-5,000 x g.
- Centrifuge with swinging-bucket rotor for 15 and 50 ml centrifuge tubes.
- Disposable latex gloves

Procedure

1. **To 1 volume of whole fresh blood (maximum of 10 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 1 x Buffer ERL per volume of whole blood used in step 1.** Thoroughly vortex to resuspend cells.
   Tip: If you used 10 ml of whole blood, wash with 20 ml of Buffer ERL.

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

6. **Add RNA-Solv Reagent to the pelleted white blood cells and pipetting up and down to lyse the cells.** For ≤5 ml whole blood add 3 ml RNA-Solv Reagent. If 5 ml-10 ml blood was used in step 1, add 6 ml RNA-Solv Reagent. Samples may safely be stored at -70°C after addition of RNA-Solv Reagent.

7. **Homogenize the lysate according to step 2a or 2b.**
   7a. Homogenize the lysate for 30 seconds *using a rotor-stator homogenizer*. Proceed to step 8.

   7b. Pass the lysate at least 5 times through *a blunt 20-gauge needle* (0.9 mm diameter) fitted to an Rase-Free syringe. Proceed to Step 8.

8. **Add 0.2 volume of (600 ul or 1.2 ml) of chloroform into the lysate.** Cap sample tubes securely and shake vigorously for 15 seconds. Incubate at room temperature for 3 minutes.

9. **Centrifuge at 3,000-5,000 x g for 10 minutes at 4°C .** Transfer the supernatant into a new tube (Rnase-Free, Not supplied).

10. **Add equal volume of 70% ethanol to the lysate and vortex to mix.**
    TIP: In most cases 1.5 ml or 3 ml of supernatant can easily be transferred. This will require 1.5 ml or 3 ml 70% ethanol.

    Step 11 to 15 should be performed in swinging-bucket rotor for maximal RNA yields. All centrifugation steps must be carried out at room temperature.

11. **Apply the sample to a HiBind® RNA Midi column assembled in a 15 ml collection tube (supplied).** The maximum capacity of the HiBind™ RNA Midi column cartridge is 4 ml. (**Larger volumes can be loaded successively**.) Centrifuge at 2,000 x g for 3 minutes at room temperature. Discard flow-through and proceed to step 12.

12. **Wash column with RNA Wash Buffer I by pipetting 3.5 ml directly into the spin column. Centrifuge as above and discard the 15 ml collecting tube**. It is strongly recommended to discard the 15 ml collection tube and change a new 15 ml centrifuge tube (provided).

13. **Place column in a clean 15 ml collection tube (supplied)**, and add 3.5 ml RNA Wash Buffer II diluted with ethanol . Centrifuge and discard flow-through. Reuse the collection tube in step 14.
    Note: RNA Wash Buffer II Concentrate must be diluted with absolute ethanol before use. Refer to label on bottle for directions.

14. **(Option) Wash column with a second 2.5ml of Wash Buffer II as in step 13.** Centrifuge and discard flow-through. Then with the collection tube empty, centrifuge the column for **10 min at maxi speed (5,000 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.**

15. **Elution of RNA.** Transfer the column to a new 15ml centrifuge tube (**Rnase-Free,**

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 5000x 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 888-832-8896 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 centrifugation 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	• Mix thoroughly after addition of TRK Lysis Buffer. • Increase centrifugation time. • Reduce amount of starting material
Degraded RNA	Source	• Do not freeze blood • Do not store blood samples for more than 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 RNA Wash Buffer II has been diluted with 4 volumes of 100% ethanol as indicated on bottle. • RNA Wash Buffer II must be stored at room temperature. • Repeat wash with RNA 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.