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Introduction

E.Z.N.A.® Blood RNA Maxiprep Kits are 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. 50ml of blood typically yields 50–150 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 Kits combine the reversible binding properties of HiBind® matrix with the speed of maxi-column spin technology to provide fast, easy method for the isolation of total cellular RNA from whole human blood. The red blood cells are selectively lysed and white cells are collected by centrifugation. White blood cells are lysed under denaturing conditions that inactivate RNases, then the lysate is homogenized with Midi-Homogenizer column with a quick centrifugation step. After the adjust of binding condition by addition of ethanol, the lysate is loaded to the HiBind® RNA Midi-column on which the RNA is bound to the matrix. Cellular debris and other contaminants (such as hemoglobin) are effectively washed away. Purified 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 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 Maxi Kits	2 Preps	5 Preps	20 Preps
Product Number	R6616-00	R6616-01	R6616-02
Purifications	2	5	20
HiBind™ RNA Maxi-Columns	2	5	20
Maxi Homogenizer Columns	2	5	20
50 ml Collection Tubes	4	10	40
Buffer ERL, 10 X Concentrate	50 ml	200 ml	3 x 250 ml
TRK Lysis Buffer	25 ml	75 ml	350ml
RNA Wash Buffer I	25 ml	60 ml	210 ml
RNA Wash Buffer II Concentrate	10 ml	50 ml	2x50 ml
DEPC-ddH_2O	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 50 ml **fresh** whole blood. The system is not limited by RNA binding capacity of HiBind® RNA columns (which can bind up to 1.5 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® maxi RNA columns can not be reached. **We recommend to use 10 ml blood for starting point. User can scale up the sample volume proportionally after get used to this protocol**.

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.

Modified Protocols

E.Z.N.A.® Blood RNA Maxiprep 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 # R6693**, is recommended for isolation of total RNA from cultured cells, tissues and bacteria.)

Before Starting

IMPORTANT	
	Dilute **Buffer ERL** with sterile deionized water as follows. **R6616-00** Add 450ml deionized water per bottle **R6616-01** Add 1800ml deionized water per bottle **R6616-02** Add 2250ml deionized water per bottle.
	Dilute **Wash Buffer II Concentrate** with **absolute ethanol** as follows **R6616-00** Add 40ml of ethanol **R6616-01** Add 200ml of ethanol **R6616-02** 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 -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** (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.

E.Z.N.A.® Blood RNA Maxiprep Protocol

Materials supplied by user

- 2-mercaptoethanol
- 70% ethanol in DEPC-treated sterile distilled water
- Sterile RNase-free pipette tips and 50 ml centrifuge tubes
- Tubes for erythrocyte lysis (50ml-250 ml depending on sample size)
- High speed centrifuge capable of 5,000Xg.
- Centrifuge with swinging-bucket rotor for 50 ml or 250 ml centrifuge bottles.

- 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 10 ml blood in 50 ml tubes) add 4 volumes of 1 x Buffer ERL. For example add 40 ml Buffer ERL to 10 ml blood in each tube. .

   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 3 volumes of Buffer ERL per volume of whole blood used in step 1. Thoroughly vortex to resuspend cells.

   Tip: If you used 20 ml of whole blood, wash each tube with 30 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 TRK Lysis Buffer/2-mercaptoethanol to the pelleted white blood cells and vortex thoroughly to mix.

Amount of Blood	Amount of TRK Lysis Buffer (ml)
10-25 ml	7.5
25-50 ml	15

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. Transfer the lysate directly into a Maxi Homogenizer Column setting in a 50 ml centrifuge tube (Supplied). Centrifuge at 5000 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.

8. **Add an equal volume of 70% ethanol to the homogenized cell lysate 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 Maxicolumn assembled in a 50 ml collection tube (supplied). The maximum capacity of the HiBind™ RNA spin cartridge is 25 ml. Cap the tube and centrifuge at 3500-5000 x g for 15 minutes. Discard flow-through and proceed to step.

10. Wash column with Wash Buffer I by pipetting 10 ml directly into the spin column. Centrifuge as above and discard the 50 ml collecting tube.. It is strongly recommended to discard the 50 ml collection tube and change a new 50 ml RNase free centrifuge tube (provided) to avoid RNase contamination before go to next step.

11. Place column in a clean 50 ml collection tube (supplied), and add 10 ml Wash Buffer II diluted with ethanol . Centrifuge and discard flow-through. Reuse 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. (Option) Wash column with a second 10 ml of RNA Wash Buffer II as in step 10. Centrifuge and discard flow-through. Then with the collection tube empty, centrifuge the spin cartridge for **15 min at 4000x 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**

13. **Drying the column**: choose either of the methods below to further dry the column before eluting DNA.

12.a. Remove the column and place into a vacuum chamber at room temperature. Any device connected to a vacuum source may be used. Seal the chamber and apply vacuum for 15 min. Remove the column and proceed to step 13.

12.b. Place the column into a incubator or oven and incubate at 65C for 10 minutes. If no vacuum chamber is available perform elution (step 13) now.

14. Elution of RNA. Transfer the column to a new 10 ml microfuge tube (**Supplied with kit**) and elute the RNA with 1ml 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 >20 ml whole blood (>$5x10^8$ white blood cells) is used.

If the column was NOT dried (step 12.a.) prior to elution, precipitate the RNA for final clean-up.
Note: The edge of the ring of the maxi column may retain some drops of RNA wash buffer after centrifugation, completely remove these buffer by using a pepet or pieces of absorbing paper.

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.

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. • Incubate the column at 70° C in a vacuum oven. • 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 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.