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User's Manual and Instructions

EZ-BLOOD RNA 96 KIT

Catalog# Z7040007-1 2 X 96 Purifications
Catalog# Z7040007-2 6 X 96 Purifications

INTRODUCTION

The EZ-Blood RNA 96 kit is designed for the high-throughput purification of total cellular RNA from whole blood in a 96-well plate format. No phenol-chloroform extraction or precipitation steps are involved. The sample addition and washing steps are performed using any 96-well compatible vacuum manifold, while the lysis of red blood cells (RBCs) and the final elution of the RNA product are performed using a table-top centrifuge.

The red blood cells (RBCs) in whole blood are first selectively lysed and the white blood cells are pelleted by centrifugation. The white blood cells are then lysed under denaturing conditions using a buffer containing the denaturant guanidine isothiocyanate. Ethanol is next added to the samples, which are then added to the filter plates. This step facilitates the binding of RNA to the filter plate's silica membrane matrix. Under these conditions the RNA binds to the membrane while other contaminants are washed through. The plate is then washed to further remove protein, buffer components and other contaminants using two ethanol-containing wash buffers and the final RNA product is eluted in RNase-free water. The final RNA product is high quality total cellular RNA that can be used directly for quantitative RT-PCR and other downstream applications. A protocol to treat the final RNA product with DNase I is also included, but most applications will not require its use since very little DNA contaminants are found in the final RNA product. Small RNAs (<200 nucleotides) such as tRNA are not efficiently isolated using this kit.

The EZ-Blood RNA 96 kit's 96-well silica membrane plates have a standard Society for Biomolecular Screening (SBS) footprint and are compatible with a variety of automated liquid handling workstations, all 96-well-compatible vacuum manifolds and most, if not all, 96-well plate-compatible rotors.

The RNA extraction protocol will take approximately 1.5-2 hr. to perform, however two vacuum manifolds in parallel can be used to process two 96-well plates in nearly the same amount of time.

In our experience, the quality and quantity of cellular RNA isolated from the EZ-Blood RNA 96 Kit, the linearity of RNA content with blood volume and the interwell variation are superior to those found in all other comparable 96-well RNA purification kits.

KIT CONTENTS

Component	Contents per Kit
Filter plates (Z7040007-3)	2 X 96-well
Collection plates (Z7040007-4)	2 X 96-well
Lysis buffer (Z7040007-5)	1 X 20 ml
RNase-free water (Z7040007-6)	1 X 20 ml
Wash buffer 1 concentrate (Z7040007-7)	1 X 70 ml (for 140 ml)
Wash buffer 2 concentrate (Z7040007-8)	1 X 60 ml (for 300 ml)
RNase-free porous tape (Z7040007-9)	4 X 96-well plate covers
96-well deep well plates (Z7040007-10)	2
Plate sealers (Z7040007-11)	4
10X RBC lysis buffer (Z7040007-12)	1 X 80 ml
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STORAGE CONDITIONS

All contents of the EZ-Blood RNA 96 Kit including the buffers should be stored at room temperature, and the kit is stable for one year under these conditions. Once the 10X RBC lysis buffer has been diluted with water it is stable for one week at room temperature.

SAFETY INFORMATION

The MSDS for this kit is available online at www.biochain.com.

TECHNICAL ASSISTANCE

Please refer any technical questions to TechSupport@biochain.com.

IMPORTANT NOTES BEFORE USING THE EZ-BLOOD RNA 96 KIT

General Considerations

Great care should be taken to not introduce ribonucleases (RNases) into the experiment. Hands and dust particles are the most common sources of RNase. Therefore, disposable gloves should be worn at all times and disposable plasticware should be used. Similarly, RT-PCR reactions should be set up in a biological safety hood or PCR workstation.

Sample Size and Type

The EZ-Blood RNA 96 Kit can be used to isolate total cellular RNA from up to 200 µl of whole blood using a 96-well format. The use of greater volumes of blood may be associated with insufficient lysis of RBCs and clogging of the filter plates. RNA can be isolated quantitatively from less blood if required.

RNA Yield

The final total RNA yield is directly proportional to the number of white blood cells extracted. Typical yields from 200 µl blood are 1-3 µg RNA.

Buffer Concentrates

The lysis buffer requires the addition of β-mercaptoethanol just before use. Wash buffers 1 and 2 are provided as concentrates that require the addition of 100% ethanol to them before use. The 10X RBC lysis buffer needs to be diluted 10-fold with distilled delonized water (1 part 10X buffer concentrate and 9 parts water) before use. The diluted RBC lysis buffer is stable for one week at room temperature.

Increasing Throughput

The protocol can be expedited considerably when the wash buffers are applied to the wells of the filter plate using a wash bottle to deliver the buffer rather than by pipetting.

Reagents and Equipment to be Supplied by the User

- Pipetteman (multichannel pipettors desirable) with sterile RNase-free tips
- Disposable gloves
- 100% ethanol
- 14.5M β-mercaptoethanol
- Laboratory-grade adhesive tape
- Paper towels
- Distilled deionized water
- Sterile tubes or bottles for dilution of RBC lysis buffer concentrate
- Any 96-well plate-compatible vacuum manifold
- A vacuum source with a capacity of 18 liters/min. The use of a weak vacuum may reduce the RNA yield and purity
- A table-top centrifuge capable of providing 650g with rotors that can accommodate 96-well plates (including GH3.8, GH3.8A and J34.3)

EZ-Blood RNA 96 KIT PROTOCOL

Before starting: The Lysis buffer requires the addition of 20 µl 14.5M β-mercaptoethanol (BME) per ml just before use. If crystals appear in the lysis buffer then it should be warmed briefly at 37°C to solubalize it. The wash buffer 1 concentrate requires the addition of 70 ml of 100% ethanol before it can be used, while the wash buffer 2 concentrate requires that 240 ml of 100% ethanol is added to it before use. Both of the wash buffers are stable for one year after the addition of ethanol. The 10X RBC lysis buffer needs to be diluted 10-fold with distilled deionized water (1 part 10X buffer concentrate and 9 parts water) into a sterile tube or bottle (not provided with kit) before use. The diluted RBC lysis buffer is stable for one week at room temperature.

1. Transfer (up to) 200 µl of whole blood into the wells of the 96-well deep well plates. Add 1.8 ml of RBC lysis buffer (that has been diluted appropriately with water) per well. Pipette up and down twice to mix, seal the plate with a plate sealer and incubate at room temperature for 5 min. Spin the plate in a table top centrifuge for 5 min. at 400 g.

This kit is designed for the isolation of total cellular RNA from fresh whole blood. Whole blood should be collected in the presence of an anticoagulant. EDTA is the preferred anticoagulant, but other anticoagulants such as citrate or heparin may be employed. For optimal results, blood samples should be processed within 4 hr. of collection. Some RNA may degrade over time, so blood samples should be processed as quickly as possible for optimal results.

2. Pour off the supernatant and resuspend the pellets into 2 ml RBC lysis buffer per well. Incubate and centrifuge as above.

The pellet is best resuspended by adding 1 ml of RBC lysis buffer, adjusting the pipetman to a volume of 0.9 ml, and pipetting up and down 10 times. Next another 1 ml of RBC lysis buffer should be added per well and the mixture pipetted up and down twice.

3. Pour off the supernatant and resuspend the pellets into 100 µl of lysis buffer containing BME. Pipette each well up and down 5-10 times to resuspend.

Once the lysis buffer has been added to the cells, the RNA contained in it is stable. The protocol can be stopped briefly now at room temperature (~1 hr.). Samples that will be processed later in the day should be stored at 2-8°C, while samples that will be processed much later should be stored frozen (preferably at -80°C). These preparations of white blood cell lysate can be stored at -80°C for up to one month. Frozen plates must be thawed to room temperature before proceeding.

4. Place the filter plate onto the vacuum manifold. Add 100 µl of 100% ethanol per well to the samples containing lysis buffer, pipette the plate well contents up and down three times to mix it well and add the contents to the corresponding well of the filter plate. Turn on the vacuum pump for 30 seconds or until all of the sample material has been drawn through the wells of the filter plate. Then release the vacuum pressure from the plate assembly before turning off the vacuum.

If some of the plate wells will not receive any samples, first cover the wells that will not be used with ordinary laboratory adhesive tape (not supplied with the kit). This helps to increase the vacuum pressure to the wells that are employed and these covered wells can be used another day.

As with all 96- and 384-well applications, some small differences in efficiency may be experienced when the outer wells of the plate, and in particular the plate corner wells, are used. The inner wells of the plate should be employed first whenever possible when experiments are designed.

5. Wash the wells by adding 700 µl wash buffer 1 (which contains the added ethanol) per well and apply the vacuum as above.

6. Wash the wells twice by adding 700 µl wash buffer 2 (which contains the added ethanol) and apply the vacuum as above.

7. Remove the filter plate from the vacuum manifold and pat it down firmly on a stack of paper towels until no further liquid is released onto the paper towels. Return the filter plate to the vacuum manifold assembly and turn on the vacuum for 5 min. to completely dry the membrane.

The plate wells need to be dry in order to prevent alcohol carryover to the RNA product in the final elution step. Some vacuum manifolds may accomplish this in 3 min., while others may require 10 min. The use of a 5 min. filter plate drying step is sufficient for most users.

8. Place the filter plate into the bottom half of the 96-well collection plate, add 50 µl of RNase-free water per well, preferably using a multichannel pipettor. Cover the plate with the RNase-free porous tape, wait 1 min., and then centrifuge the filter plate/collection plate assembly (650g X 2 min.) to elute the final RNA product. Covering the plate helps keep dust (and RNase) out of the plate. Repeat the elution using 50 µl RNase-free water as above.

The use of one 50 µl elution rather than two elution steps will provide you with a slightly more concentrated RNA product, however the absolute yield of RNA will be reduced. The dead volume of the filter plate wells is approximately 20 µl, so one can expect to recover approximately 30 µl or 80 µl of final RNA product when one or two elution steps are used, respectively. Whenever possible, it is best to use the RNA directly after isolation. If this is not possible then freeze the RNA to -80°C and later thaw it to room temperature before use.

9. DNase digestion (optional). Almost all of the DNA is removed from the final RNA product with no DNase treatment. DNase treatment of the wells of the filter plate directly is not efficient and is not recommended. In those instances when even trace amounts of DNA in the final RNA product cannot be tolerated, make the RNA solution 10 mM Tris HCl, pH 7.6, 2.5mM $MgCl_2$, 0.5 mM $CaCl_2$ by using a 10X DNase I buffer concentrate (not supplied), add 1-2 U of RNase-free DNase I (not supplied), incubate for 15 min. at 37°C and inactivate the DNase I by heating the sample to 75°C for 10 min. If EDTA will not affect your downstream application or will be removed from the samples by using the RNA cleanup protocol it is recommended to add EDTA to 5 mM before the heat inactivation step. This will help protect the RNA during the enzyme inactivation. For most applications, this provides a sufficiently clean DNase-free RNA. However, the EZ-Blood RNA 96 Kit RNA cleanup protocol

(Catalogue number Z7040007) can be followed when absolutely all nucleotides, EDTA and salts must be removed from RNA samples.

Kit Performance

Figure 1 shows the result of TaqMan real-time RT-PCR analysis of 18S rRNA levels in RNA isolated from various volumes of blood from a healthy male volunteer using this kit. With 2-200 µl whole blood, rRNA levels were linear with an r^2 = 0.9840. Well-to-well variation as assessed by CV = mean/SD X 100% was between 5.0% (200 µl) and 7.2% (2 µl).

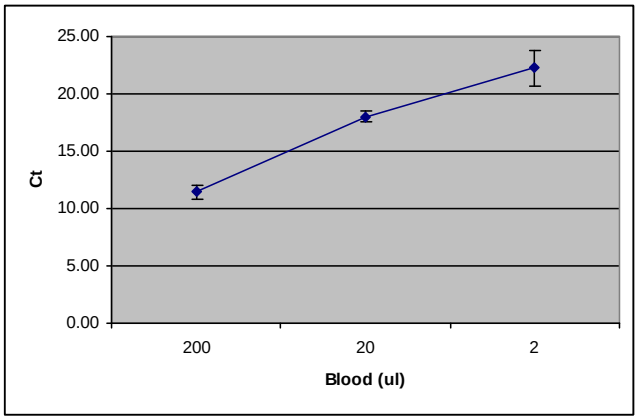

Figure 1. TaqMan RT-PCR analysis of threshold cycle (C_t) versus blood volume extracted for RNA isolated using the EZ Blood-RNA 96 kit. The resulting RNA product was analyzed in an ABI Prism 7000 using one-step RT-PCR master mix with TaqMan rRNA control reagents (Applied Biosystems, Inc.) under standard RT-PCR conditions. Shown are the mean +/- SD C_t values observed for four replicates each when the indicated blood volumes were extracted.

To assess the level of DNA contamination in RNA samples, we also examined the absolute levels of 18S rRNA DNA levels by TaqMan PCR, without the RT step (2X master mix, Applied Biosystems, Inc.). Using a specific rRNA quantitation standard in both the PCR and the RT-PCR we calculate that the rRNA DNA gave rise to <1.0% of the RNA signal. Thus the contribution of contaminating genomic DNA to the quantitation of RNA is negligible.

RELATED PRODUCTS

Product	Catalogue Number
Filter plates	Z7040007-3
Collection plates	Z7040007-4
Lysis buffer	Z7040007-5
RNase-free water	Z7040007-6
Wash buffer 1 concentrate	Z7040007-7
Wash buffer 2 concentrate	Z7040007-8
RNase-free porous tape	Z7040007-9
96-well deep well plates	Z7040007-10
Plate sealers	Z7040007-11
10X RBC lysis buffer	Z7040007-12

TROUBLESHOOTING

Problem	Comments and Suggestions
Little or no RNA eluted	Remove all traces of supernatant before beginning. All buffers must be at room temperature. Ensure that vacuum draws all liquid through filter membrane at each step. Perform RNA extraction with no interruptions. Measure final elution volume- ensure adequate final elution from final centrifugation steps.
Filters clog	Too much RNA/cells used. Reduce sample size.
Filters tear/plates break	Reduce centrifugation speed.
Degraded RNA/High Interwell variation	Ensure that cells have been well cared for- feed cells shortly before RNA extraction if possible. Consider finding sources of RNase contamination that might have been introduced. Inadequate vacuum during washing.
RNA performs poorly	Ensure that that plate is completely dry and that remaining traces of ethanol have been removed before final elution step (increase drying time to 10 min).

EZ-BLOOD RNA 96 KIT EXPERIENCED USERS MINIPROTOCOL

1. 200 µl whole blood & 1.8 ml RBC lysis buffer, mix, 5 min @ RT, 5 min @ 400 g.
2. Pellet to 2 ml RBC lysis buffer, mix, 5min @ RT, 5 min @ 400 g.
3. 100 µl lysis buffer, mix.
4. 100 µl of 100% ethanol, mix, add to filter plate, vacuum.
5. 700 µl wash buffer 1, vacuum.
6. 700 µl wash buffer 2, vacuum.
7. Repeat 700 µl wash buffer 2.
8. Pat filter plate on paper towels. Vacuum dry 5 min.
9. Place filter plate into collection plate, 50 µl of RNase-free water, incubate 1 min., centrifuge 2 min. @ 650 g.

Repeat the elution using 50 µl RNase-free water.