For Research Use Only Not for Diagnostic Use

EZ-RNA 96 Kit

Catalog# Z7040005-1 2X96 Purifications
Catalog# Z7040005-2 6X96 Purifications (3XZ7040005-1)

INTRODUCTION

The EZ-RNA 96 Kit is designed for the high-throughput purification of total cellular RNA from cultured cells in a 96-well plate format. The kit can also be used to isolate total cellular RNA from plasma or serum, or to cleanup up to 96 RNA samples at a time. 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 final elution of the RNA product is performed using a table-top centrifuge.

Cells are lysed under denaturing conditions using a lysis 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-RNA 96 Kit 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. In fact, these plates are directly compatible with QIAGEN's RNeasy 96 kit, Invitrogen's PureLink 96 RNA Purification kit, Ambion's RNAqueous 96 kit, and Promega's SV 96 Total RNA Isolation Kit. All of these kits utilize a similar guanidine-based lysis buffer, followed by RNA binding to a silica membrane filter matrix, washing of the membrane and then sample elution.

The RNA extraction protocol will take 30-45 min. 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-RNA 96 Kit (and replacement plates), the linearity of RNA content with cell number and the interwell variation are superior to those found in all other comparable 96-well RNA purification kits.

Z7040005-1 KIT CONTENTS

Component	Contents per Kit
Filter plates (Z7040005-3)	2 X 96-well
Collection plates (Z7040005-4)	2 X 96-well
Lysis buffer (Z7040005-5)	1 X 20 ml
RNase-free water (Z7040005-6)	1 X 20 ml
Wash buffer 1 concentrate (Z7040005-7)	1 X 70 ml (for 140 ml)
Wash buffer 2 concentrate (Z7040005-8)	1 X 60 ml (for 300 ml)
RNase-free porous tape (Z7040005-9)	4 X 96-well plate covers
Instruction Manual	1

Z7040005-2 contains threeZ7040005-1 kits

STORAGE CONDITIONS

All contents of the EZ-RNA 96 Kit including the buffers should be stored at room temperature, and the kit is stable for one year under these conditions.

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-RNA 96 ASSAY

General Considerations

In general, the quality of the RNA isolated is a reflection of the metabolic state of the cells from which they were isolated. In general, and in particular with regards to confluent cultures, RNA quality will be increased when the cells can be fed fresh media 2-3 hrs before the RNA is extracted from them.

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-RNA 96 Kit can be used to isolate total cellular RNA from between 50 to $5X10^5$ cultured cells using a 96-well format. RNA can still be isolated quantitatively from fewer cells if required, however the well-to-well variation in the extraction efficiency will be increased slightly. Similarly, RNA can be isolated from more cells if required. A maximum of $5X10^5$ cultured cells were used during the testing of this product, which yields ~1 µg RNA, while other experiments performed by us indicate that each well of the plate can hold a maximum of >5 µg RNA. Thus, we anticipate that larger quantities of RNA could be purified using this system. Both adherent and suspension cultures can be utilized. Since confluent cultures of mammalian cells generally reach a maximum confluence of approximately $5X10^4$ cells/96-well, $1X10^5$ cells/48-well and $3X10^5$ cells/24-well plate, the kit can be used directly to isolate RNA from a variety of original plate formats other than 96-well plates.

RNA Yield

The final total RNA yield is directly proportional to the number of cells utilized for the extraction. The maximum number of cells tested during the development of the kit was $5X10^5$ which provides on the order of 1 µg total RNA product per well, depending on the cell type utilized.

Lysis and Wash Buffer

The EZ-RNA 96 Kit Lysis buffer requires the addition of β-mercaptoethanol just before use. EZ-RNA 96 Kit Wash Buffers 1 and 2 are provided as concentrates that require the addition of 100% ethanol to them before use.

Tissue Samples

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Tissue samples can be used with the kit, however their performance has not been validated. Based on our experience with the kit we anticipate that 1 mg samples can be processed using the kit. The use of greater amounts of starting materials may lead to filter clogging, reduced RNA qualities and poor recoveries.

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
- 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-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.

1. Suspension cells should be aliquoted into the wells of a 96-well plate (not included) and centrifuged for 5 min. at 500g. Remove the supernatant completely by pipetting before proceeding with step 2.

Adherent cells grown in a 96-well plates can be used directly in step 2 after the complete removal of the media. Rinsing of the cells with PBS before continuing with the protocol may also be desirable.

Serum and plasma samples need to be centrifuged briefly before use (12,000g X 1min) to spin out any cryoprecipitates that might otherwise clog the filters and slow sample processing.

2. Add 100 µl of Cell lysis buffer containing BME to each plate well. Shake the plate by sliding it back and forth across the bench five times.

Once the cell 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 -80ºC). Frozen plates must be thawed to room temperature before proceeding.

For RNA cleanup applications, add 100 µl of lysis buffer containing BME to 50 µl of sample.

For serum and plasma samples, add 50 µl of lysis buffer to 50 µl serum/plasma and pipette up and down three times to mix. Incubate at room termperature for 5 min.

3. 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.

For RNA cleanup applications add 150 µl of 100% ethanol (1 volume) to the samples. The rest of the RNA cleanup application protocol is identical to the RNA extraction protocol.

Plasma and serum samples are also processed using 200 µl of ethanol, as above. The rest of the protocol to isolate RNA from plasma/serum is the same as indicated below.

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

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

6. 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.

7. 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 avg/900g max 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.

8. 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 TrisHCl, pH 7.6, 2.5mM $MgCl_2$, 0.5mM $CaCl_2$ 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 5mM 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 Xpress EZ-RNA 96 Kit RNA cleanup protocol can be followed when absolutely all nucleotides, EDTA and salts must be removed from RNA samples.

Kit Performance

Figure 1 shows the results of two TaqMan real-time RT-PCR experiments that were undertaken in order to quantify the levels of a representative RNA polymerase I- (18S rRNA) and that of a RNA polymerase II-transcribed gene (GAPDH RNA) throughout the dynamic range of the kit and to quantify the well-to-well variation observed between samples. With 50-50,000 cells extracted, the GAPDH RNA levels were linear with an $r^2 = 0.9991$, while rRNA $r^2 = 0.9995$. Well-to-well variation as assessed by CV = mean/SD X

100% was between 0.84-2.94% for GAPDH and between 0.88-2.22% for rRNA.

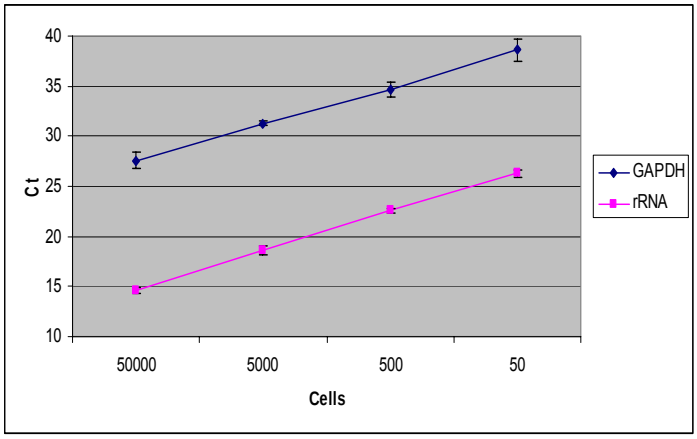

Figure 1. TaqMan RT-PCR analysis of threshold cycle (C_t) versus cell number for RNA isolated using the EZ-RNA 96 Kit. Hela cells were trypsinized, counted in a hemocytometer, and then four replicates each of 50,000, 5,000, 500 and 50 were placed into the wells of a 96-well plate. The plate was centrifuged and the supernatants were removed. The EZ-RNA 96 Kit protocol was then followed (with no DNase I treatment) and the resulting RNA product was analyzed in an ABI Prism 7000 using one-step RT-PCR master mix with either TaqMan rRNA control reagents or the human GAPDH endogenous control primer and probe set (Applied Biosystems, Inc.) under standard RT-PCR conditions with the RNA analyzed neat for GAPDH or diluted 50X in RNase-free water for the rRNA experiment. Shown are the mean +/- SD C_t values observed.

To assess the level of DNA contamination in RNA samples, we examined the absolute levels of GAPDH or rRNA DNA levels by TaqMan PCR, without the RT step (2X master mix, Applied Biosystems, Inc.), using the four replicates of RNA extracted from 50,000 Hela cells. The single copy gene GAPDH DNA could not be detected, while the multicopy rRNA DNA (~200 copies per haploid genome) was detectable. Using a specific rRNA quantitation standard in both the PCR and the RT-PCR we calculate that the rRNA DNA gave rise to 0.022% of the RNA signal. Thus the contribution of contaminating genomic DNA to the quantitation of the two RNAs examined was negligible.

RELATED PRODUCTS

Product	Catalogue Number
Filter plates	Z7040005-3
Collection plates	Z7040005-4
Lysis buffer	Z7040005-5
RNase-free water	Z7040005-6
Wash buffer 1 concentrate	Z7040005-7
Wash buffer 2 concentrate	Z7040005-8
RNase-free porous tape	Z7040005-9

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-RNA 96 KIT
EXPERIENCED USERS MINPROTOCOL

1. 100 µl cell lysis buffer containing 20 µl BME per ml per total RNA extraction sample or per 50 µl RNA sample for RNA cleanup.
2. Shake plate across bench- back and forth 5 times.
3. 100 µl of 100% ethanol per well to RNA extraction samples or 150 µl of 100% ethanol to RNA cleanup samples.
4. Pipette up and down 3X, 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/well, cover filter plate with porous tape, incubate 1 min., centrifuge 650--900g X 2 min.
10. Repeat the elution using 50 µl RNase-free water.

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