

Enzymax LLC

Tel: 859-219-8482 Fax: 859-219-0653 Web: www.enzymax.net

PRODUCT INFORMATION

miRNA micro Kit for small amount Cultured Cells, Tissues and LCM (laser Captured Microdissection) sample (Cat# 701)

Kit Contents:

Components	Cat#701	Storage
RL Buffer*	12.5ml	18 months at room temperature (except, RL Solution should be kept at 2-8°C). For longer storage, keep all contents at 4°C.
WASH 1 Solution** (concentrated)	9ml	
WASH 2 Solution** (concentrated)	3ml	
DEPC-Water (RNase-free)	2.5ml	
RNA Tini Spin Column (with collection tubes)***	50	

(*) RL Solution should be kept at 2-8°C. It may form a precipitate upon storage. If necessary, dissolve the precipitate by warming the solution at 37°C.

(**) WASH 1 & 2 Solutions: Before use add **6ml** 100% ethanol to 9 ml WASH1 and **12ml** 100% ethanol to 3ml WASH2.

(***) RNA Tini Spin columns are sold separately for leftover solutions.

Reagents and equipment supplied by the user

- RNase-free Ethanol (100%)
- Centrifuge for micro-centrifuge tubes
- RNase-free micro-centrifuge tubes
- Manual micro-pipettors and sterile, RNase-free tips
- Equipment for sample disruption

Principle:

This kit is designed for fast isolation of total RNA (including miRNA, 5S rRNA, and tRNA) from **small amount** of samples from human and animal cell culture (eg: LCM sample, 10^1 - 10^5 cells), animal tissues, and also for RNA clean up or concentration. The reagent contains disruptive and protective properties of guanidine isothiocyanate and β -mercaptoethanol to inactivate the ribonucleases present in cell extracts. The kit contains a membrane embedded spin column for binding up to 20 μ g of RNA. Nucleotides, proteins, salts, and other impurities do not bind to the Tini Spin Column. The recommended sample size is $<5 \times 10^5$ cell and <5 mg tissue. For sample $>5 \times 10^5$ up to 5×10^6 cells and 30mg tissue, please use our MiRNA Kit (cat# EZC301).

Features:

- ✓ Fast (20 min procedure) and High quality (OD_{260}/OD_{280} ratio >1.9) using RNA spin column format.
- ✓ No phenol / chloroform extraction or ethanol precipitation needed ✓ Columns (Cat#EZCR101) are sold separately for leftover solutions

Note: Care must be taken when working with RNA. It is important to maintain an RNase-free environment starting with RNA sample preparation and continue through purification and analysis. Use RNase free tubes, tips, gels. Wear gloves at all times. Change gloves frequently to avoid contaminating samples with RNases.

RNA is exposed to RNA-degrading enzymatic activity until the sample is frozen or disrupted using RNase-inhibiting agents. Plant and animal tissue samples should be flash frozen in liquid N2 immediately and stored at -80°C or processed as soon as possible.

Procedures for Isolation of Total RNA (including miRNA) from Small Amount of Animal and Human Cells

Important Notes:

In order to obtain optimal RNA yield and purity, it is essential to use the correct amount of starting material and the amount of lysis solution (RL) for efficient cell lysis. Here is the recommendation for different sample types:

RL Solution	Number of Cells	Fresh Tissue	Tissue stored in RNAlater	Difficult to lyse issue samples
175 μ l	$<1 \times 10^5$ (48 well plate)	<5 mg		
350 μ l	$<5 \times 10^6$ (<60 mm dish)	<20 mg	<20 mg	
600 μ l	5×10^6 - 1×10^7 (60-100mm dish)	20-30mg	20-30mg	<30 mg

*If 600 μ l RL Buffer and ethanol are used, sample must be loaded onto the column in two successive centrifugation steps.

Cell numbers in Different Sizes of Multiwell Culture Plates and Dishes

Multiwell plates	Number of Cells	Culture Dishes	Number of Cells
96-well	$4-5 \times 10^4$	35mm dish	1×10^6
48-well	1×10^5	60mm dish	2.5×10^6
24-well	2.5×10^5	100mm dish	7×10^6 to 1×10^7
12-well	5×10^5	145-150mm	2×10^7
6-well	$>1 \times 10^6$	40-50ml Flask	3×10^6
		250-300 ml Flask	1×10^7
		650-750ml Flask	2×10^7

1. Samples Preparation ($<5 \times 10^5$ Cells)

- a) Count cells; pellet up to 5×10^5 cells by centrifugation, and thoroughly remove supernatant by aspiration.

b) The cell pellet can be washed with 1X phosphate buffered saline (PBS) prior disruption, but this is not essential. Wash cells in PBS, resuspend in 1 mL PBS, and pellet the cells, and thoroughly remove the fluid.

c) Proceed immediately to the next step, sample disruption.

2. Disrupt cells and Homogenization of lysate:

a) Add 175µl RL Solution (see Note) to the cell pellet (cell $<5 \times 10^5$).

b) Vortex vigorously for cell homogenization. No cell clumps should be visible before proceeding with the next step.

Note: Incomplete loosening of the cell pellet may lead to inefficient lysis and reduced RNA yields.

3. RNA isolation: Add 275µl of 100% ethanol (or ~1.5 volume) to the homogenized lysate (flow-through from step 2), and mix by inverting the tube. Do not centrifuge. Proceed immediately to the next step.

Note: precipitate may form after adding ethanol, but this will not affect the procedure. Load all of the precipitate on the column as described in step 4.

4. Transfer above ethanol mixture to RNA Tini spin column, centrifuge at 13,000rpm (~11,000 x g) for 1 minute.

Note: Maximal loading capacity of the RNA Tini spin column is 350µl. Repeat the step 4 if more than 350µl is processed.

5a. Discard the flow-through. Add 200 µl of WASH 1 Solution to the Spin Column and spin at 13,000 rpm ($>11,000 \times g$) for 1 minute. Discard flow-through and place the column back to the same Collection Tube.

5b. On-column rDNase treatment if needed.

Option: Base on your application, On-column rDNase 1 (EZrDNase1 set sold separately) treatment will eliminate genomic DNA contamination (See procedure from **Option**).

6. Add 250 µl of WASH 2 Solution to the spin column, spin at 13,000 rpm ($>11,000 \times g$) for 1 minute. Discard the flow-through

7. Cut off the lid of the spin column and centrifuge for 2 min at full speed to remove residue of WASH 2 Solution. This step is very important to remove the residual ethanol in WASH 2 thoroughly.

8. Transfer the spin column to a clean RNase-free 1.5 ml microtube. Add 5-30 µl of RNase-free water to the center part of the column; incubate at room temperature for 2 minutes. Spin to elute the RNA at full speed for 1 minute. RNA is ready for use or kept at -80°C for long term storage.

Procedure for Isolation of Total RNA (including miRNA) from Small Amount of Animal Tissues

Important:

- In order to obtain high quality RNA, minimize the time between tissue collection and RNase inactivation. Immediately disrupt fresh tissue in RL Solution or freeze the tissue in liquid nitrogen, and store at -80°C .
- In order to obtain optimal RNA yield and purity, it is essential to use the correct amount of starting material and the amount of lysis solution (RL) for efficient cell lysis. Estimate the mass of small tissue samples:

RL Solution used for tissue sample	Tissue size (LxW) (mm)	Thickness (mm)	Estimated weight (mg)
175 µl	1.5mmx1.5mm	1-2mm	1mg
175 µl	2.0mmx2.0mm	1-2mm	3mg
175 µl	2.5mmx2.5mm	1-2mm	5mg
175 µl	3.0mmx3.0mm	1-2mm	7mg
175 µl	3.5mmx3.5mm	1-2mm	10mg
175 µl	4.0mmx4.0mm	1-2mm	15mg

Note: excess sample may increase the risk of RNA degradation.

- Samples Preparation: It is essential to use correct amount of tissue. Excess sample may increase the risk of RNA degradation.
 - Immediately place the weighted tissue (5-10mg) to liquid nitrogen and grind thoroughly with mortar and pestle. Transfer the tissue power with liquid nitrogen to a dry ice pre-chilled RNase-free microcentrifuge tube. Allow the liquid nitrogen to evaporate, but do not allow the tissue to thaw.
 - Add 175µl RL solution to the tissue sample and vortex to mix.

2. Follow the step 3-8 from the protocol above.

Note:

Fatty tissue (brain+adipose tissue): Lysis Additive (cat# LA101) is required during the lysis step.

Fibrous tissues (skin, skeletal muscle, heart): Proteinase K treatment is recommended during the lysis step.

RNA (including miRNA) isolation from LCM (Laser Capture Microdissection) Sample

- Microdissected sample preparation
Usually frozen sections (rather than paraformaldehyde or formalin fixed, paraffin-embedded sections) are used to extract RNA.
- In a 1.5ml RNase-free microcentrifuge tube, add 100µl of RL buffer, and then drop the captured cells into the tube. Make sure the sample is completely immersed in the RL solution.
- Incubate the sample at 42°C for 30min.
- Vortex briefly and quick spin the tube to collect the fluid at the bottom of the tube.
- Pre-wet the Tini spin column with 30µl RL solution. Quick spin the column to remove the solution before applying the lysate to the column (step 7).
- Add the ethanol to the lysate:

- a. To recover only the large RNA (>75nt), add 0.5 volumes (~54µl) of 100% ethanol to the lysate and mix by pipetting up and down or by gently vortexing.
 - b. To recover both large RNA and small RNA (miRNA, 5S rRNA, and tRNA), add 1.25 volumes (~130µl) of 100% ethanol to the lysate, and mix by pipetting up and down or by gently vortexing.
7. Apply the entire lysate/ethanol mixture onto prepared Tini spin column and close the cap. Centrifuge for 1min at 11,000xg (13,000rpm). Discard flow-through.
 8. Add 180µl of WASH 1 Solution to the Tini Spin Column, spin at 13,000 rpm (>11,000 x g) for 1 minute. Discard the flow-through
 9. Add 180µl WASH 2 Solution to the Tini Spin column, spin at 13,000 rpm (>11,000 x g) for 1 minute. Discard the flow-through and open the lid, spin once more at full speed for 1 minute to remove residual fluid and dry the column.
 10. Transfer Tini Spin Column to a clean RNase-free 1.5 ml microtube. Add 5-10µl of RNase-free water (preheated at 80°C) to the center part of the column; incubate at room temperature for 2 minutes. Spin to elute the RNA at full speed for 1 minute. RNA is ready for use or kept at – 80°C.

Procedure for RNA Cleanup and concentration:

This procedure is to clean up or concentrate RNA that isolated by different methods or after enzymatic reactions.

1. Adjust sample to a volume of 50 µl with RNase-free H₂O, add 175µl of RL Solution, and mix well.
2. Add ethanol:
 - For recovering only the large RNA: Add 125 µl of 100% ethanol to the diluted RNA and mix gently. A precipitate may form by adding ethanol, do not centrifuge, and proceed immediately to the next step.
 - For recovering both large and small RNA: Add 300µl of 100% ethanol (>60% ethanol in final) the diluted RNA and mix gently. A precipitate may form by adding ethanol, do not centrifuge, and proceed immediately to the next step.
3. Place the Tini Spin Column in 2.0ml Collection Tube and transfer the mixture to the column and spin at >13,000 rpm (11,000 x g) for 1 minute, discard flow-through.
4. Add 400µl of WASH 1 Solution to the Spin Column and spin at 13,000 rpm (>11,000 x g) for 1 minute. Discard flow-through. **Note:** skip this wash step if you want to recover micro RNA. Small RNA will be washed off the column in 40% ethanol.
5. Add 400µl of WASH 2 Solution to the column and spin at 13,000 rpm (11,000 x g) for 1 minute, discard the flow-through.
6. Cut off the lid of the spin column and centrifuge for 2 min at full speed to remove residue of ethanol in WASH 2. This step is very important to remove the residual ethanol thoroughly.
7. Add 5-50 µl of RNase-free H₂O onto the center part of the membrane of the column and centrifuge at 13,000 rpm (11,000 x g) for 1 minute. Keep RNA sample at -80°C.

Option:

On column DNase (RNase free) treatment: Cat# EZrDNase1 set. In most case, this step is not necessary. However, for certain application that is sensitive to very small amount of DNA (e.g., TagMan RT-PCR analysis with a low-abundant target), and then DNase (RNase free) treatment can efficiently remove the DNA contamination.

Protocol:

Prepare rDNase1 stock solution: Inject or add 570ul of RNase-Free Water into rDNase vial, and mix by swirling (Do not vortex which will dramatically decrease the DNase 1 activity)—Aliquot the stock and keep at -80°C for up to 1-2 years. Avoid freeze thaw!

Prepare on column rDNase I cocktails:

- Tini Spin Column: Mix 3ul of rDNase1 stock with 21ul rDNase 1 reaction buffer gently by inverting the tube.
 - Mini Spin Column: Mix 6ul of rDNase1 stock with 42ul rDNase 1 reaction buffer gently by inverting the tube.
1. (After step 5a on the first protocol) Add 24 or 48µl of the cocktail to the center of the Tini or Mini Spin Column and close the cap. Centrifuge for 1 minute at 200xg or spin for 30 second pulse at full speed. Reload the flow-through on the center of the column and incubate at 25-37°C for 15 minutes. This is to ensure that the entire DNase I solution passes through the column. Repeat the step if needed.
 2. Add 120µl WASH 2 solution to the column and incubate for additional 5 minutes and then centrifuge ≥12,000 x g for 30 seconds. Discard the flow-through.
 3. Add 400µl WASH 2 solution to wash the column one more time.
 4. Elute the RNA with 10-40ul of RNase-free water.

Storage:

- The RNase-Free DNase Set is shipped at room temperature and should be stored at 2–8°C immediately upon receipt, which will be stable for at least 9 months.
- Aliquot the stock solution and keep at -80°C for up to 1-2 years. Avoid freeze thaw!

**PRODUCTS ARE INTENDED FOR BASIC SCIENTIFIC RESEARCH ONLY!
NOT INTENDED FOR HUMAN OR ANIMAL USE!**