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PRODUCT INFORMATIOIN

RNA micro Kit for small amount Cultured Cells, Tissues and LCM (laser Captured Microdissection) samples *(Cat#700)*

Kit Contents:

Components	*Cat#700*	Storage
RL Buffer*	12.5ml	18 months at room temperature (except, RL Solution should be kept at 2-8^{0}C). For longer storage, keep all contents at 4^{0}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^{0}C. It may form a precipitate upon storage. If necessary, dissolve the precipitate by warming the solution at 37^{0}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 (>200) 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 µg of RNA. Nucleotides, proteins, salts, and other impurities do not bind to the Tini Spin Column. The recommended sample size is <5x10^5 cell and <5mg tissue. For sample >5x10^5 up to 5x10^6 cells and 30mg tissue, please use our RNA Kit (cat# EZCR300).

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#EZC107) 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 –70°C or processed as soon as possible.

Procedures for Isolation of Total RNA from 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 µl	<1x10^5 (48 well plate)	<5mg		
350 µl	<5x10^6 (<60mm dish)	<20mg	<20mg	
600 µl	5x10^6-1x10^7(60-100mm dish)	20-30mg	20-30mg	<30mg

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

Cell numbers in Different Sizes of Multiwall Culture Plates and Dishes

Multiwell plates	Number of Cells	Culture Dishes	Number of Cells
96-well	4-5x10^4	35mm dish	1x10^6
48-well	1x10^5	60mm dish	2.5x10^6
24-well	2.5x10^5	100mm dish	7x10^6 to 1x10^7
12-well	5x10^5	145-150mm	2x10^7
6-well	>1x10^6	40-50ml Flask	3x10^6

1. Samples Preparation (**<5x10^5** Cells)
   a) Count cells; pellet up to 5x10^5 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. To 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 <5x10^5).
   b) Vortex vigorously for cell homogenization. No cell clumps should be visible before proceeding the next step.

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

3. RNA isolation: Add 88µl 100% ethanol (1/2 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. Discard the flow-through or save the flow-through if you want to recover small RNA (includes miRNA, 5S rRNA, and tRNA), for details, see Option 2 below.

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 x 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 x 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 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^{0}C for long term storage.

Procedure for Isolation of Total RNA from Animal Tissue:

Important:

A. 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.

B. 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.

1. Samples Preparation: It is essential to use correct amount of tissue. Excess sample may increase the risk of RNA degradation.
   a. 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.
   b. 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

1. Microdissected sample preparation
   Usually frozen sections (rather than paraformaldehyde or formalin fixed, paraffin-embedded sections) are used to extract RNA.
2. 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.
3. Incubate the sample at 42^{0}C for 30min.
4. Vortex briefly and quick spin the tube to collect the fluid at the bottom of the tube.
5. Pre-wet the Tini spin column with30µl RL solution. Quick spin the column to remove the solution before applying the lysate to the column (step 7).
6. 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^{0}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^{0}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 H_2O, 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:** skit 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 H_2O 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^{0}C.

Option 1:

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^{0}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 2–8ºC 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!

Option 2: Recover small RNA from flow-though **(includes miRNA, 5S rRNA, and tRNA)**

This protocol is designed to recover small RNA by using Tini spin column (EZC107)

1. Add 0.64 volumes of 100% ethanol to flow-through from step 4 (first protocol).
2. Apply to RNA Tini spin column.
3. Add 180 ul of WASH 2 solution to the Tini Spin Column, spin at 13,000rpm (>11,000xg) for 1 min. Discard the flow-through and spin once more to remove residue of RPE solution.
4. Add 180ul of 100% ethanol and wash the column, spin at 13,000rpm (>11,000xg) for 1 min. Discard the flow-through and spin once more to remove residue of ethanol.
5. Transfer the Tini spin columne to a clean RNase-free 1.5ml micro-centrifuge tube and add 5-10ul of RNase-free water to the center 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^{0}C.