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Introduction

E.Z.N.A.® miRNA Kit is designed for isolating total RNA, including miRNA and other small RNA molecules with size less than 200 nucleotides, from culture cells and variety of animal tissues. a rapid and easy method for the isolation of up to 50 µg of small and large sizeRNA from cultured eukaryotic cells, tissues, bacteria, plant or fungal. The kit allows single or multiple, simultaneous processing of samples in less than 30 min. Normally, up to 1 x 10^6 eukaryotic cells, up to 1 x 10^9 bacterial cells, 50 mg tissue or 100 mg plant samples can be used in a single experiment.

RNA purified using the E.Z.N.A.® miRNA method is ready for applications such as RT-PCR*, Northern blotting, nuclease protection.

Principle

E.Z.N.A.® miRNA Kit combines the reversible binding properties of HiBind® matrix, a new silica-based, time saving spin technology material, with the efficiency of the time proved phenol/Guanidine-based monophasic solution to purify total RNA from variety of samples. The sample is lysed and homogenized first under highly denaturing buffer contains phenol and Guanidine Thiocyanate (RNA-Solv Reagent) to protect RNA from degradation. After addition of Chloroform, the homogenate is separated into aqueous and organic phases by centrifugation. The upper aqueous phase which contains RNA is extracted and mixed with ethanol to adjust binding condition for large RNA. The sample is loaded into a HiBind® RNA column to which the large RNA molecules including mRNA are bound. The flow-through sample is mixed with ethanol again to create proper binding condition for smaller RNA and loaded into a MicroElute® RNA column to bind smaller RNA moleculaes. After two brief wash steps, the enriched miRNA and other smaller RNA molecules are eluted with RNase-free water.

Storage and Stability

All components in E.Z.N.A.® miRNA Kits should be stored 22-25°C. RNA-Solv® Reagent should be store at 4°C after addition of water-saturated-phenol for long term storage.

Kit Contents

Product Number	R6842-00	R6842-01	R6842-02
Purification times	5 Preps	50 Preps	200 Preps
MicroElute® RNA Column	5	50	200
HiBind® RNA Mini Column	5	50	200
2 ml Collection Tubes	15	150	600
RNA-Solv® Reagent	6 ml	60 ml	220 ml
RWC Wash Buffer	5 ml	40 ml	160 ml
RWB Wash Buffer	4 ml	2 x 12 ml	2 x 50 ml
DEPC water	5 ml	20 ml	60 ml
Instruction Manual	1	1	1

Note: RNA-Solv® Reagent contains Guanidine Thiocyanate and Phenol,handle those reagents with extra care.Safety and risk phase:R20-24/25-32-34, S13-26-36/37/39-45.

Before Starting?

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.

IMPORTANT	**RWB Wash Buffer** must be diluted with absolute ethanol (96-100%) before use and store at room temperature. R6842-00 Add 16 ml absolute ethanol R6842-01 Add 48 ml absolute ethanol R6842-02 Add 200 ml absolute ethanol

- Whenever working with RNA, always wear latex gloves to minimize RNase contamination. Use only clean RNase-free disposable plastic pipette tips when using the supplied reagents.
- During the procedure work carefully but quickly.

- **It is very important to determine the correct amount of starting material before the experiment**. If the maximum amount of starting material is 100mg. The capacity of the HiBind® RNA column is 100µg. For samples contains high amount of RNA, we suggest to use 30mg tissue to start. For samples contains lower level RNA, the maximum amount of starting material (100mg) can be used.

Homogenization of Tissues

A. Liquid Nitrogen Method

Wear gloves and take great care when working with liquid nitrogen. Excise tissue and promptly freeze in a small volume of liquid nitrogen. Grind tissue with a ceramic mortar and pestle under approximately 10 ml of liquid nitrogen and pour the suspension into a pre-cooled 15 ml polypropylene tube. Unless the tube is pre-cooled (in liquid nitrogen), the suspension will boil vigorously possibly causing loss of tissue. When the liquid nitrogen has completely evaporated, add RNA-Solv® Reagent and continue with the procedure as outlined below. This is the preferred method of disrupting tissue samples.

B. Rotor-Stator Homogenizers

Rotor-stator homogenizers effectively homogenize most tissues. The process usually takes less than a minute depending on the tissue. Many Rotor-stator homogenizers operate with differently sized probes or generators that allow processing of small volumes in microfuge tubes. Such homogenizers are available from:

- Tekmar Inc., Cincinnati, OH (Tissuemizers ®)
- BIOSPEC Products, Bartlesville, OK (Tissue-Tearor ®)
- Craven Laboratories, Austin, TX.

C. Syringe Method

High molecular weight DNA is responsible for the viscosity of cell lysates and can be shredded by passing the sample several times through a narrow needle (19-21 gauge).

Protocol A: Isolation of miRNA from Cells and Tissue.

Materials supplied by user

- Absolute ethanol (100%)
- RNase-free filter pipette tips
- Centrifuge capable of 14,000 x g

1. **Lyse cells or tissues with 1 ml of RNA-Solv® Reagent.** 1ml of RNA-Solv® Reagent is sufficient for 1x 10^7 cultured cells, 30-50 mg animal tissue or 50-100 mg plant tissue.

   For culture cells grown in **monolayer** (fibroblasts, endothelial cells, etc.), lyse the cells directly in the culture vessel as follows. Aspirate culture medium completely and add RNA-Solv® Reagent directly to the cells. Pipette buffer over entire surface of vessel to ensure complete lysis. Transfer lysate to a clean 1.5 ml microfuge tube and proceed to step 2 below. (This method is preferable to trypsinization followed by washing because it minimizes RNA degradation by nuclease contamination.)

   For cells grown in **suspension cultures**, pellet cells at no greater than 1,500 rpm (400 x g) for 5 min. Discard supernatant, add RNA-Solv® Reagent, lyse by vortex or pipetting up and down, and transfer to a clean 1.5 ml microfuge tube. Proceed to step 2.

   For **tissue** samples, determine the size of the samples and homogenize by using one of the methods discussed on page 4. Unless using liquid nitrogen, homogenize samples directly in RNA-Solv® Reagent and proceed to step 2.

2. Incubate the tube contains homogenate at room temperature for 2-3 minutes.

3. **Add 0.2 mL of chloroform per 1 mL of RNA-*Solv* Reagent.** Cap sample tubes securely and shake vigorously for 15 seconds. Incubate on ice for 10 minutes.

4. **Centrifuge at 12,000 x g for 15 minutes at 4°C.** The mixture separates into a lower phenol-chloroform phase, an inter phase, and an upper aqueous phase. RNA remains entirely in the aqueous phase.

5. **Transfer no more than 80% of the aqueous phase to a new 2.0 ml tube and add 1/2 volume of absolute ethanol. Vortex to mix well.** (When process liver and spleen, 1/3 volume of absolute ethanol should be added.)
   Removal of Large RNAs (>200 nt)

6. Apply no more than 700 µl of the mixture from step 5 onto HiBind® RNA Mini

column. (Larger volumes can be loaded successively.) Vortex and add the entire mixture to the column. With the spin column inside a 2 ml collecting tube (supplied with kit), ***centrifuge at 10,000 x g for 30-60 seconds at room temperature.*** Transfer the flow-through into a new 2 ml tube for small RNA Isolation.

7. Repeat step 6 by loading the remaining sample to the column. Centrifuge as above and transfer the flow-through into the same 2 ml tube used at step 6 for small RNA isolation.

   Note: the HiBind RNA Mini Column can be used to isolate of large Size of Total RNA (>200 nt). See Protocol B.

   Purification of miRNAs

8. Measure the volume of the flow through from step 6-7. Add 0.9 volume of absolute ethanol to flow through and vortex to mix well. Take a MicroElute® RNA column and **place column in a clean 2 ml collection tube.**

9. **Apply the mixture from step 8 into the column, centrifuge at 10,000 x g for 30-60 seconds.** Discard the liquid.

10. **Repeat step 9 by loading the remaining the sample to the column and centrifuge as above.** Discard the liquid.

11. **Place column in the same 2 ml collection tube**, and add 500 µl RWB Wash Buffer. Centrifuge as above and discard flow-through. Reuse the collection tube in step 12.

    Note: RWB Wash Buffer must be diluted with absolute ethanol before use. Refer to label on bottle for directions.

12. **Wash column with a second 500 µl of RWB Wash Buffer as in step 11**. Centrifuge and discard flow-through. Then with the collection tube empty, centrifuge the spin cartridge for **2 min at full speed** (≥13,000 x g) to completely dry the HiBind® matrix.

13. **Elution of miRNA.** Transfer the column to a clean 1.5 ml microfuge tube (not supplied with kit) and elute the RNA with 15-30 µl of DEPC Water (supplied with kit). Make sure to add water directly onto column matrix. Let the column sit at room temperature for 2 minutes and centrifuge for 1 min at full speed. A second elution may be necessary if the expected yield of RNA >20 µg.

    Alternatively, RNA may be eluted with a greater volume of water. While additional elutions increase total RNA yield, the concentration will be lowered since more than 80% of RNA is recovered with the first elution. Pre-heating the

water to 70°C before adding to column and incubating column 5 min at room temperature before centrifugation may increase yields.

Protocol B: Isolation of Large RNA from HiBind RNA Mini Column

Materials supplied by user

- Absolute ethanol (100%)
- RNase-Free DNase I (optional)
- β-Mercaptoethanol
- RNase-free filter pipette tips
- Centrifuge capable of 14000 x g

Note: Reagents and collection tube supplied with this kit is sufficient for 50 or 200 miRNA isolations. To perform both large and miRNA isolation protocol, additional reagents and collection tubes are available to purchase separately.

1. Process step 1-7 from Protocol A.

2. Place the HiBind® RNA Mini column into a new 2 ml collection tube.

3. Add 500µl **RWC Wash Buffer** to the column. Spin at 10,000 x g for 30 seconds. Discard the flow-through and re-use the collection tube. **Skip this step if performing optional DNase I digestion step and go to step 5.**

   Note: If DNase-free RNA is desired, On-membrane DNase I digestion can be start at this point.

4. **DNase I digestion (Optional)**
   Since HiBind® RNA resin and spin-column technology actually removes most of DNA without the DNase treatment, it is not necessary to do DNase digestion for most downstream applications. However, certain sensitive RNA applications might require further DNA removal. Following steps provide on-membrane DNase I digestion:(see DNase I cat.# E1091for detail information)

   a. **Add 300 µl RWC Wash Buffer into the column**. Centrifuge as above and discard flow-through. Re-used the collection tube.

   b. For each HiBind® RNA column, prepare the DNase I digestion reaction mix as follows:

OBI DNase I Digestion Buffer	73.5 µl
RNase-free DNase I (20 Kunitz unites /µl)	1.5 µl
Total volume	75 µl

   c. Pipet 75 µl of the DNase I digestion reaction mix directly onto the surface of HiBind® RNA resin in each column. Make sure to pipet the DNase I digestion mixture directly onto the membrane. DNase I digestion will not be complete if some of the mix stick to the wall or the O-ring of the HiBind® RNA column.

   d. Incubate at room temperature(25-30°C) for 15 minutes**.**

   e. **Place column in the collection tube** and add 400 µl **RWC Wash Buffer**. Incubate at room temperature for 5 minutes before centrifugation). Centrifuge as above and discard flow-through. Place column in the same collection tube.

5. **Place column in the same 2 ml collection tube**, and add 500 µl RWB Wash Buffer diluted with absolute ethanol. Centrifuge as above and discard flow-through. Reuse the collection tube in step 4.

   Note: RWB Wash Buffer must be diluted with absolute ethanol before use. Refer to label on bottle for directions.

6. **Wash column with a second 500 µl of RWB Wash Buffer as in step 5**. Centrifuge and discard flow-through. Then with the collection tube empty, centrifuge the spin cartridge for **2 min at full speed** (≥13,000 x g) to completely dry the HiBind® matrix.

7. **Elution of RNA.** Transfer the column to a clean 1.5 ml microfuge tube (not supplied with kit) and elute the RNA with 30-50 µl of DEPC Water (supplied with kit). Make sure to add water directly onto column matrix. Let the column sit at room temperature for 2 minutes and centrifuge for 1 min at full speed. A second elution may be necessary if the expected yield of RNA >50 µg.

   Alternatively, RNA may be eluted with a greater volume of water. While additional elutions increase total RNA yield, the concentration will be lowered since more than 80% of RNA is recovered with the first elution. Pre-heating the water to 70°C before adding to column and incubating column 5 min at room temperature before centrifugation may increase yields.

Protocol C: Isolation of Total RNA, Including small RNA from Cells and Tissue.

Materials supplied by user

- Absolute ethanol (100%)
- RNase-Free DNase I (optional)
- β-Mercaptoethanol
- RNase-free filter pipette tips
- Centrifuge capable of 14000 x g
- Water bath or heat block preset at 55°C

1. **Lyse cells or tissues with 1 ml of RNA-Solv® Reagent.** 1ml of RNA-Solv® Reagent is sufficient for 1x 10^7 cultured cells, 30-50 mg animal tissue or 50-100 mg plant tissue.

   For culture cells grown in **monolayer** (fibroblasts, endothelial cells, etc.), lyse the cells directly in the culture vessel as follows. Aspirate culture medium completely and add RNA-Solv® Reagent directly to the cells. Pipette buffer over entire surface of vessel to ensure complete lysis. Transfer lysate to a clean 1.5 ml microfuge tube and proceed to step 2 below. (This method is preferable to trypsinization followed by washing because it minimizes RNA degradation by nuclease contamination.)

   For cells grown in **suspension cultures**, pellet cells at no greater than 1,500 rpm (400 x g) for 5 min. Discard supernatant, add RNA-Solv® Reagent, lyse by vortex or pipetting up and down, and transfer to a clean 1.5 ml microfuge tube. Proceed to step 2.

   For **tissue** samples, determine the size of the samples and homogenize by using one of the methods discussed on page 4. Unless using liquid nitrogen, homogenize samples directly in RNA-Solv® Reagent and proceed to step 2.

2. Incubate the tube contains homogenate at room temperature for 2-3 minutes.

3. **Add 0.2 mL of chloroform per 1 mL of RNA-*Solv* Reagent.** Cap sample tubes securely and shake vigorously for 15 seconds. Incubate on ice for 10 minutes.

4. **Centrifuge at 12,000 x g for 15 minutes at 4°C.** The mixture separates into a lower phenol-chloroform phase, an interphase, and an upper aqueous phase. RNA remains entirely in the aqueous phase.

5. **Transfer no more than 80% of the aqueous phase to a new 2.0 ml tube.**

6. **Add 1.5 volume of absolute ethanol to the sample. Mix throughly by vortexing. For example, if 350µl of aqueous is transferred, use 525µl absolute ethanol.**

7. Apply no more than 700 µl of the mixture from step 6 onto HiBind® RNA Mini column. (Larger volumes can be loaded successively.) A precipitate may form on addition of ethanol in step 6. Vortex and add the entire mixture to the column. With the spin column inside a 2 ml collecting tube (supplied with kit), ***centrifuge at 10,000 x g for 30-60 seconds at room temperature.*** Discard the Folw-through and re-use the collection tube for next step.

8. Add 500 µl RWB Wash Buffer diluted with absolute ethanol. Centrifuge as above and discard flow-through. Reuse for next step.

9. Add another 500 µl RWB Wash Buffer to the column and spin at 10,000 x g for 30 seconds. Discard the flow-through and re-use the collection tube for next step.

10. Place the column into the collection tube from last step and spin the empty column at maximum speed for 2 minutes to dry the HiBind® RNA Mini column.

11. **Elution of total RNA.** Transfer the column to a clean 1.5 ml microfuge tube (not supplied with kit) and elute the RNA with 40-50 µl of DEPC water (supplied with kit). Make sure to add water directly onto column matrix. let the column sit at room temperature for 2 minutes and centrifuge for 1 min at full speed. A second elution may be necessary if the expected yield of RNA >20 µg.

    Alternatively, RNA may be eluted with a greater volume of water. While additional elutions increase total RNA yield, the concentration will be lowered since more than 80% of RNA is recovered with the first elution. Pre-heating the water to 70°C before adding to column and incubating column 5 min at room temperature before centrifugation may increase yields.

Trouble shooting Tips

Problem	Cause	Suggestion
Little or no RNA eluted	RNA remains on the column	Repeat elution. Pre-heat DEPC-water to 65°C prior to elution. Incubate column for 10 min with water prior to centrifugation.
	Column is overloaded	Reduce quantity of starting material.
Clogged column	Incomplete homogenization	Completely homogenize sample. Increase centrifugation time. Reduce amount of starting material
	Too much starting material	Reduce the amount of starting material
	Centrifugation temperature is too low	Perform the centrifugation at room temperature
Degraded RNA	Source	Freeze starting material quickly in liquid nitrogen. Do not store tissue culture cells prior to extraction unless they are lysed first. 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 RWB Wash Buffer Concentrate has been diluted with 4 volumes of 100% ethanol as indicated on bottle. 1 X RWB Wash Buffer must be stored and used at room temperature. Repeat wash with RWB Wash Buffer.
Phase does not separate completely	No chloroform added or Chloroform is not pure	Make sure to add the chloroform use Chloroform without any additives such as isoamyl alcohol
	Sample is not throughly mixed after addition of chloroform	Mix sample throughly by votexing before centrifugation
	Sample contains organic solvents	Make sure the sample does not contain organic solvents (such as DMSO or alkaline reagents)
DNA contamination		Digest with RNase-free DNase and inactivate at 75°C for 5 min.
Low Abs ratios	RNA diluted in acidic buffer or water	DEPC Water is acidic and can dramatically lower Abs260 values. Use TE buffer to dilute RNA prior to spectrophotometric analysis.
miRNA concentration is low	Ethanol concentration is not correct	Make sure to use 200 proof ethanol (100%) Use correct ethanol volume as described in the protocol
poor performance of miRNA in downstream applications	Large RNA interference	Make sure to use protocol A to remove the large RNA