Contents

Introduction 2

Overview 2

Storage and Stability 2

Kit Contents 3

Before Starting 3

Tissue Homogenization Protocols 4

Plant miRNA Isolation Protocol. 5

Troubleshooting Tips 7

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Introduction

Majority of the current commercial products for isolating miRNA involve organic extraction (most commonly phenol-based) in the procedure. E.Z.N.A.? Plant miRNA Kit uses an innovative buffer system that completely eliminate the use of phenol. This product provides a rapid and easy method for the isolation of up to 50 µg of small and large-size RNA from plant samples. Typically, up to 100 mg plant tissue sample can be used .

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

Overview

The E.Z.N.A.? Plant miRNA Kit combines the reversible binding properties of HiBind? matrix, a new silica-based material, with the unique lysis and binding procedure to extract micro and large (>200 nt) RNA from a wide variety of starting materials. A specially formulated high-salt lysis and binding buffer system allows up to 50 µg of RNA to bind to the matrix. Fresh plant samples are first homogenized with Plant miRNA Lysis buffer that lyses the cells and inactivates RNases. After adjusting condition with XD Binding Buffer, the lysate is loaded to a gDNA Removal Column to remove cell debris and other contaminants. The flow-through liquid, which contains the miRNA, is mixed with ethanol and loaded onto a MicroElute? RNA Column to bind the miRNA. After a few quick wash steps, the miRNA can be eluted from the MicroElute? RNA Column with nuclease-free water.

Storage and Stability

All components in E.Z.N.A.? Plant miRNA Kits should be stored between 22-25°C.

Kit Contents

Product Number	R6727-00	R6727-01	R6727-02
Purification times	5 Preps	50 Preps	200 Preps
MicroElute? RNA Column	5	50	200
HiBind? gDNA Remove Column	5	50	200
2 ml Collection Tubes	10	100	400
MCL Lysis Buffer	4 ml	40 ml	160 ml
XD Binding Buffer	5 ml	50 ml	200 ml
RNA Wash Buffer II	2 ml	20 ml	2 X 40 ml
DEPC water	2 ml	20 ml	60 ml
Instruction Manual	1	1	1

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	
	RNA Wash Buffer II must be diluted with absolute ethanol (96-100%) before use and store at room temperature.
	R6727-00 Add 8 ml absolute ethanol
	R6727-01 Add 80 ml absolute ethanol to each bottle
	R6727-02 Add 160 ml absolute ethanol to each bottle

- 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.
- Although the binding capacity for the MicroElute? RNA Columns are approximately 50 μg, the maximum amount of starting material depends on the type of tissue being processed and its corresponding RNA content. It is essential to begin with the correct amount of tissue to get optimal RNA yield and purity with the E.Z.N.A.? Plant miRNA Kit. For the first time user, we recommend using less than 50 mg of tissue per sample. Depending on the yield and purity obtained, it may be possible to increase the starting material up to 100 mg.

Tissue Homogenization Protocols

A. Liquid Nitrogen Method-Recommened

- 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
- Pour the suspension into a pre-cooled 15 ml polypropylene tube.

  Note: Unless the tube is pre-cooled in liquid nitrogen, the suspension will boil vigorously possibly causing loss of tissue.
- Once the liquid nitrogen has completely evaporated, continue to Step 1 of the? Plant miRNA Isolation Protocol? on page 5.

B. Beads Beating Tissue Lyser

Beads beadting tissue lyser can effectively disrupt and homogenize most plant tissues. The process usually takes less than a minute depending on the tissue. Many beads-beating tissue lyser operate with differently sized adapts that allow processing of small volumes in microcentrifuge tubes or deep-well plate. We strongly recommand to use Geno/Grinder from SPEX SamplePrep Group (NJ, USA) for this protocol.

Plant miRNA Isolation protocol

Materials supplied by user

- Absolute ethanol (100%)
- 2-Mercaptoethanol
- RNase-free pipette tips and microcentrifuge tubes
- Centrifuge capable of 14,000 x g
- Ice Bucket

Prepare Lysis Mixture according to the following table (per sample):

Components	Volume
XD Binding Buffer	300 µL
MCL Lysis Buffer	650 µL
Absolute ethanol	50 µL
2- Mercaptoethanol	10 µL

1. Collect 50-100mg fresh plant samples and grind sample with liquid nitrogen. Add 700 µL Lysis Mixture.
2. Vortex at maximum speed for 30 seconds to mix the sample throughly.
3. Incubate at 55?C for 3 minutes.
4. Centrifuge at 12,000 x g for 5 minutes at room temperature.
5. Transfer the supernatant into a gDNA Removal Column placed in 2 ml collection tube. Centrifuge at 12,000 x g for 2 minutes at room temperature. Discard the HiBind gDNA Remove Column and transfer the flow-through lysate into a new 2ml tube.
6. Measure or estimate the volume of the filtrate , add 1.1 volumes of ethanol to the tube. Vortex 20 seconds to mix thoroughly.
7. Place a MicroElute RNA Column in 2 ml collection tube, transfer 750 µl of

the mixture from Step 6 to the MicroElute RNA Column.

8. Centrifuge at 12,000 x *g* for 1 minute. Discard the flowthrough and re-use the collection tube.
9. Repeat Steps 7-8 until all the remaining sample has been transferred to the MicroElute RNA Column.
10. Add 500 µL 96-100% Ethanol to the MicroElute RNA Column, centrifuge at 12,000xg for 1min, discard the flowthrough and re-use the collection tube.
11. Add 500 µL XD Binding Buffer to the the MicroElute RNA Column and centrifuge at 12,000 x *g* for 1 minute. Discard the filtrate and reuse the collection tube.
12. Add 750 µL RNA Wash Buffer II to the MicroElute RNA Column.
   Note: RNA Wash Buffer II must be diluted with ethanol before use.
13. Centrifuge at 12,000xg for 1min.
14. Repeat Steps 12-13 for a second RNA Wash buffer II wash step. Disacrd the flowthrough and re-use the collection tube.
15. Centrifuge at maximum speed (≥ 12,000 x *g)* for 2 minutes to completely dry the HiBind? matrix.
   Note: It is important to dry the HiBind? matrix before elution. Residual ethanol may interfere with downstream applications.
16. Place the MicroElute RNA Column in a new 1.5ml RNase-free microcentrifuge tube.
17. Add 30-50ul DEPC Water.
   Note: Make sure to add DEPC Water directly onto the center of HiBind matric.
18. Incubate at room temperature for 5 minutes.
19. Centrifuge at maximum speed (≥ 12,000 x *g)* for 1 minute. Store eluted RNA at -70?C.
   Note: Any combination of the following steps can be used to help increase RNA yield.
   - Preheat the DEPC Water to 70?C before elution.
   - Increase the incubation time to 5-10 minutes.
   - Increase the elution volume.
   - Repeat the elution step with fresh DEPC Water (this may increase the yield, but decrease the concentration).
   - Repeat the elution step using the eluate from the first elution (this may increase yield while maintaining elution volume).

Trouble shooting Tips

Problem	Cause	Suggestion
Little or no RNA eluted	RNA remains on the membrane	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 RNA Wash Buffer II Concentrate has been diluted with 4 volumes of 100% ethanol as indicated on bottle. 1 X RNA Wash Buffer II must be stored and used at room temperature. Repeat wash with RNA Wash Buffer II.
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