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Introduction

The E.Z.N.A.® Plant RNA Maxi Kit provides a convenient and rapid method for the isolation of total RNA from a variety of plant samples. The kit include shredding/ homogenizing units to efficiently remove cell debris and simultaneously homogenize the lysate. In combination with HiBind® RNA spin columns, this permits purification of high quality RNA from as much as 5 g fresh plant tissue. Typical yields are shown in Table 1. The E.Z.N.A.® Plant RNA Maxi Kit is ideal for processing multiple plant samples in less than one hour. The need for organic extractions is eliminated, making total RNA isolation fast, safe, and reliable. Purified RNA has Abs260/Abs280 ratios of 1.8-2.0 and is suitable for the following applications:

- RT-PCR
- Northern Analysis
- Differential display
- Poly A+ RNA selection

Table 1. Yields obtained with E.Z.N.A.® Plant RNA Maxi Kit

Arabidopsis sp	800µg
Tobacco leaves	2.50 mg
Mustard leaves	1.50 mg
Maize Fresh Seeds	600 µg

Storage and Stability

All components of the E.Z.N.A.® Plant RNA Maxi Kit should be stored at 22ºC-25ºC. Under these conditions, RNA has successfully been purified and used for RT-PCR after 24 months of storage. Under cool ambient conditions, a precipitate may form in the Buffer RB. In case of such an event, heat the bottle at 37ºC to dissolve. Store Buffer RB at room temperature.

Binding Capacity

Each HiBind® RNA maxi column can bind approximately 5 mg RNA. Using greater than 5 g plant tissue usually will not dramatically improve yields and sometimes has adverse effects.

Kit Contents

Product No.	R6629-00	R6629-01	R6629-02
Purification Times	2 Preps	5 Preps	20 Preps
HiBind™ RNA Maxi columns	2	5	20
50 ml Collection Tubes*	4	10	40
gDNA Filter Column	2	5	20
Buffer RB	50 ml	120ml	2 x 220 ml
Buffer RCL	50 ml	120 ml	2 x 220 ml
Buffer RCB	50 ml	120 ml	2 x 220 ml
RWC Wash Buffer	25 ml	60 ml	5 x 50 ml
RNA Wash Buffer II Concentrate	12 ml	3 x 12 ml	11 x 12 ml
DEPC-treated water	5 ml	20 ml	50 ml
User Manual	1	1	1

Note: 50 ml collection tubes have been inserted with HiBind™ RNA Maxi Column and Homogenization column.

Materials to be provided by user

- centrifuge capable of ≥4,000 x g
- Nuclease-free 50ml conical centrifuge tubes
- 2-mercaptoethanol
- Absolute (96%-100%) ethanol
- Isopropyl alcohol (isopropanol)
- Liquid nitrogen for freezing/disrupting samples
- Preheat an aliquot (3 ml per sample) of DEPC-treated water at 60°C.

Before Starting

IMPORTANT	**RNA Wash Buffer II Concentrate** must be diluted with absolute ethanol (96-100%) as follows:	
	R6629-00	Add 48 ml absolute ethanol to bottle
	R6629-01	Add 48 ml absolute ethanol/bottle
	R6629-02	Add 48 ml absolute ethanol/bottle
	Store diluted RNA Wash Buffer II at room temperature.	

Working with RNA

Please take a few minutes to read this booklet thoroughly to become familiar with the protocol. Prepare all materials required before starting to minimize RNA degradation.

- Whenever working with RNA, always wear latex gloves to minimize RNase contamination. Change gloves frequently. Use only clean RNase-free disposable plastic pipette tips when using the supplied reagents.
- During the procedure work carefully but quickly.
- Under cool ambient conditions, crystals may form in Buffer RB. This is normal and the bottle may be warmed to redissolve the salt.
- 2-mercaptoethanol (ß-mercaptoethanol) is key in denaturing endogenous RNases and must be added to an aliquot of Buffer RB and Buffer RPL before use. Add 20 µl of 2-mercaptoethanol per 1 ml of Buffer RB or RPL. This mixture can be stored for 1 week at room temperature.

E.Z.N.A.™ Plant RNA Maxi Protocol I (Standard Protocol)

NOTE: Use extreme caution when handling liquid nitrogen.

This protocol is suitable for most fresh or frozen tissue samples allowing efficient recovery of RNA. However, due to the tremendous variation in water and polysaccharide content of plants, sample size should be limited to ≤5 g. Best results are obtained with young leaves or needles.

Note that all centrifugation steps must be carried out at room temperature.

1. **Weigh up to 5 g plant sample. Immediately place the weighed sample in liquid nitrogen, and grind throughly with a mortar and pestle.** Decant the powder and liquid nitrogen into an RNase-free, liquid nitrogen cooled, 50ml centrifuge tube (not supplied). Allow the liquid nitrogen to evaporate, but do not allow the sample to thaw.

2. **Immediately add 20 ml Buffer RB/2-mercaptoethanol.** We recommend starting with 2 g tissue at first. If results obtained are satisfactory increase amount of starting material. Add 20 µl 2-mercaptoethanol per 1 ml of Buffer RB. Samples should not be allowed to thaw before Buffer RB/2-mercaptoethanol is added. **Vortex vigorously to make sure that all clumps are dispersed. RNA cannot be effectively extracted from clumped tissue.**

3. Centrifuge the lysate at ≥4,000 x g for 10 min at room temperature. **Transfer the supernatant directly into a gDNA Filter Maxi-Spin Column placed in collection tube. Centrifuge at ≥4,000 x g for 5 min at room temperature.**

4. Discard the column and **add 0.5 volume absolute ethanol (room temperature, 96-100%) and mix by vortexing at maxi speed for 20 seconds.**

5. **Apply one half of the mixture to a HiBind® RNA Maxi column** assembled in a 50.0 ml collection tube (supplied). Close the cap gently. Centrifuge at ≥4,000 x g for 5 minutes at room temperature. Discard the flow-through liquid and place the column back into the collecting tube.

6. Apply the remaining of the mixture to the RNA Maxi Column. Centrifuge at ≥4,000 x g for 5 minutes at room temperature. Discard the flow-through liquid and place the column back into the collecting tube.I.

Optional on-membrane DNase I digestion: This is the starting point to perform DNase I digestion. See Page 8 for detailed protocol.

7. **Add 10 ml RWC Wash Buffer, close the tube gently and centrifuge as above.** Discard both flow-through liquid and collection tube.

8. **Place column in a clean 50ml collection tube, and add 15ml RNA Wash Buffer II diluted with absolute ethanol. Close the column gently,** Centrifuge as above and then discard flow-through. Re-use the collection tube in step 9.

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

9. **Wash column with a second 15 ml of RNA Wash Buffer II.** Centrifuge as above and discard flow-through. Then with the collection tube empty, centrifuge the Maxi column for **10 min at >4000 x g** to completely dry the HiBind™ matrix.

10. **Elution of RNA. Transfer the column to a new RNase-free 50 ml centrifuge tube (not supplied with kit) and elute the RNA with 1 ml of DEPC-treated water (supplied with kit).** Make sure to add water directly onto column matrix. Incubate at room temperature for 5 minutes. Centrifuge at 4000 x g for 5 minutes. A second elution into the same tube may be necessary if the expected yield of RNA >2mg.

    Note: 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.

Plant RNA Maxi Protocol II (for difficult samples)

Certain plant samples are very difficult for RNA isolation because of amount of material and type of secondary metabolites. This method involves a simple and rapid precipitation step for removal of much of the polysaccharides and phenolic compounds commonly found in plant tissues. **Use this protocol when standard protocol did not yield RNA or get lower yield.**

1. **Grind plant sample as described on page 4. Collect frozen ground plant tissue (up to 5g) in a microfuge tube and immediately add 20 ml Buffer RC L/2-mercaptoethanol.** We recommend starting with 2 g tissue at first. If results obtained are satisfactory increase amount of starting material. Add 20 µl 2-mercaptoethanol per 1ml of Buffer RCL. **Samples should not be allowed to thaw before Buffer RCL/2-mercaptoethanol is added**. Vortex vigorously to make sure that all clumps are dispersed. RNA cannot be effectively extracted from clumped tissue.

   Note: **Add 20 µl 2-mercaptoethanol per 1 ml of Buffer RCL before use**. This mixture can be made and stored at room temperature for 1 week.

2. **Incubate at 55oC for 1-3 minutes. Centrifuge at 4,000 x g for 10 min at room temperature.**

3. **Transfer the supernatant directly into a gDNA Filter Maxi-Spin Column placed in collection tube.** Centrifuge at ≥4,000 x g for 5 min at room temperature.

4. **Discard the column and add equal volume of Buffer RCB to the flow-through. Mix by vortexing at maxi speed for 20 seconds.**

5. **Apply one half of the mixture to a HiBind® RNA Maxi column** assembled in a 50.0 ml collection tube (supplied). Close the cap gently. Centrifuge at ≥4,000 x g for 5 minutes at room temperature. Discard the flow-through liquid and place the column back into the collecting tube.

6. Apply the remaining of the mixture to the RNA Maxi Column. Centrifuge at ≥4,000 x g for 5 minutes at room temperature. Discard the flow-through liquid and place the column back into the collecting tube.

   Optional on-membrane DNase I digestion: This is the starting point to perform DNase I digestion. See Page 8 for detailed protocol.

7. **Add 10 ml RWC Wash Buffer, close the tube gently and centrifuge as above.** Discard both flow-through liquid and collection tube.

8. **Place column in a clean 50ml collection tube, and add 15ml RNA Wash Buffer II diluted with absolute ethanol. Close the column gently,** Centrifuge as above and then discard flow-through. Re-use the collection tube in step 13.

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

9. **Wash column with a second 15 ml of RNA Wash Buffer II.** Centrifuge as above and discard flow-through. Then with the collection tube empty, centrifuge the Maxi column for **10 min at >4000 x g** to completely dry the HiBind™ matrix.

10. **Elution of RNA. Transfer the column to a new RNase-free 50 ml centrifuge tube (not supplied with kit) and elute the RNA with 1 ml of DEPC-treated water (supplied with kit).** Make sure to add water directly onto column matrix. Incubate at room temperature for 5 minutes. Centrifuge at 4000 x g for 5 minutes. A second elution into the same tube may be necessary if the expected yield of RNA >2mg.

    Note: 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.

DNase digestion Protocol (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. The following steps provide on-membrane DNase I digestion:(see DNase I, Cat # E1091 for further information).

1. Follow the standard protocol until the samples **completely** pass through the HiBind® RNA column. Prepare the following:

A. Pipet 5ml RNA Wash Buffer I into the HiBind® RNA Maxi column, and centrifuge at 4000 x g for 5 minutes to wash the column. Discard the flow through. For each HiBind® RNA Maxi column, prepare the DNase I digestion reaction mix as follows:

OBI DNase I Digestion Buffer	367.5 µl
RNase-free DNase I (20 Kunitz unites/µl)	7.5 µl
Total volume	375 µl

Note:

1. **DNase I is very sensitive and prone to physical denaturing; so do not vortex the DNase I mixture. Mix gently by inverting the tube. Prepare the fresh DNase I digestion mixture before RNA isolation.**

2. **OBI DNase I digestion buffer is supplied with OBI RNase-free Dnase set.**

3. **Standard DNase buffers are not compatible with on-membrane Dnase digestion.**

B. Pipet 375 µl of the DNase I digestion reaction mix directly onto the surface of the 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 sticks to the wall or the O-ring of the HiBind® RNA column.

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

2. **Place column in a clean 50.0ml collection tube**, and add 5 ml RWC Wash Buffer I. **Incubate 5 minutes at room temperature.** Centrifuge at 4000 x g for 2 minutes and discard flow-through. Reuse the collection tube.

3. **Place column in the same 50 ml collection tube**, and add 15ml RNA Wash Buffer II diluted with ethanol. Centrifuge at 4000 x g for 5 minutes and discard flow-through. Reuse the collection tube.

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

4. Wash column with a second 10 ml of RNA Wash Buffer II by repeating step 3. Centrifuge and discard flow-through. Then with the collection tube empty, centrifuge the column at 4000 x g for **10 min at full speed** to completely dry the HiBind® matrix.

5. **Elution of RNA.** Transfer the column to a clean 50 ml microfuge tube (not supplied with kit) and elute the RNA with 1 ml of DEPC-treated water (supplied with kit). Make sure to add water directly onto column matrix. Centrifuge 1 min at maximum speed. A second elution may be necessary if the expected yield of RNA >2 mg.

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.

Plant RNA Maxi Protocol for Arthropods

The exoskeleton of arthropods poses the same problems as encountered with

many plant specimens. Pigments and polysaccharides often co-purify with nucleic acids and interfere with downstream applications. Prepare all necessary materials and reagents (listed on page 4) and follow the procedure below:

1. **Freeze and grind up to 5 gram arthropod tissue under liquid nitrogen**. Grind tissue completely to obtain a fine homogenous powder.

2. **Immediately add 15ml Buffer RB/2-mercaptoethanol.** Add 20 µl 2-mercaptoethanol per 1ml of Buffer RB and then add 2.5 ml of this mixture to the sample. **Samples should not be allowed to thaw before Buffer RB/2-mercaptoethanol is added**. Vortex vigorously to make sure that all clumps are dispersed. RNA cannot be effectively extracted from clumped tissue.

   Note: **Add 20 µl 2-mercaptoethanol per 1 ml of Buffer RB before use**. This mixture can be made and stored at room temperature for 1 week.

3. Proceed with the Plant RNA Maxiprep Protocol from step 3 (page 5).

Plant RNA Maxi Protocol for Fungi

E.Z.N.A.® Plant RNA Maxi Kit can also be used for fungal RNA isolation since many fungal samples possess similar cellular attributes as many plant specimens.

1. **Freeze and grind up to 5 gram fungal sample under liquid nitrogen.** Grind tissue completely to obtain a fine homogenous powder.

2. **Immediately add 15 ml Buffer RB/2-mercaptoethanol.** Add 10 µl 2-mercaptoethanol per 1ml of Buffer RB and then add 2.5ml of this mixture to the sample. **Samples should not be allowed to thaw before Buffer RB/2-mercaptoethanol is added**. Vortex vigorously to make sure that all clumps are dispersed. RNA cannot be effectively extracted from clumped tissue.

3. Proceed with the Plant RNA Protocol from step 3 (page 5).

Troubleshooting Guide

Problem	Cause	Suggestion
Little or no RNA eluted	RNA remains on the column	• Repeat elution. • Pre-heat DEPC-water to 70° 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 disruption or lysis of plant tissue.	• Completely disrupt sample in liquid nitrogen. • Increase centrifugation time. • Reduce amount of starting material
Precipitated RNA will not dissolve.	High nucleic acid and polysaccharide content.	• Reduce amount of starting material. Generally it is best to start with 2-3gram at first. • To avoid RNA degradation, do not increase incubation time for resuspension.
Degraded RNA	Source	• Freeze starting material quickly in liquid nitrogen and store at -70°C without thawing. • Make sure that 2-mercaptoethanol is added to Buffer RPL. • Use RB Buffer as dissolvent instead of DEPC water.
	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 Wash Buffer II has been diluted with 100% ethanol • Diluted Wash Buffer II must be stored at room temperature. • Repeat wash with Wash Buffer II.
DNA contamination	Co-purification of DNA	• Digest with RNase-free DNase and nactivate at 75°C for 5 min.
Low Abs ratios	RNA diluted in acidic buffer or water	• DEPC-treated water is acidic and can dramatically lower Abs260 values. Use TE buffer (pH 8) to dilute RNA prior to spec analysis.