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Revised May 2006

Introduction

The E.Z.N.A.® Plant RNA Maxiprep 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 10 gram tissue. Typical yields are shown in Table 1. E.Z.N.A.® Plant RNA Kits are 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

Yields obtained with E.Z.N.A.® Plant RNA Kits	
Arabidopsis sp	1.5 mg
Tobacco leaves	2.5 mg
Mustard leaves	1.5 mg
Maize	1.5 mg

Storage and Stability

All components of the E.Z.N.A.® Plant RNA Maxiprep Kit should be stored at 22°C-25°C. 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 37°C to dissolve. Store Buffer RB at room temperature.

Binding Capacity

Each HiBind® RNA Maxi column can bind approximately 3 mg RNA. Using greater than 15 gram plant tissue usually will not dramatically improve yields and sometimes has adverse effects.

Kit Contents

Product No.	R6629-00	R6629-01	R6629-02
HiBind™ RNA Maxi-columns	2	5	20
50 ml Collection Tubes	4	10	40
Homogenization Maxi Columns	2	5	20
Buffer RPL	25 mL	60 ml	250 ml
Buffer SP	5 mL	15 ml	60 ml
Buffer RB	25 mL	60 ml	250 ml
RNA Wash Buffer I	25 mL	60 ml	250 ml
RNA Wash Buffer II, Concentrate	12 mL	25 ml	100 ml
DEPC-treated water	10 mL	30 ml	110 ml
User Manual	1	1	1

Before Starting

IMPORTANT	Dilute **Wash Buffer II** with **absolute ethanol** as follows **R6629-00** Add 48 mL of 96-100% ethanol **R6629-01** Add 100 mL of 96-100% ethanol **R6629-02** Add 400 mL of 96-100% ethanol

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 Protocol I (Standard Protocol)

Materials to be provided by user

- centrifuge capable of ≥15,000 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 (500 µl per sample) of DEPC-treated water at 65°C.
- 30ml or 50 ml High speed centrifuge tubes (polycarbonate or Corex®)

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 ≤10 gram. 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 10 gram 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, 30 or 50ml polycarbonate or Corex® centrifuge tube (not supplied). Allow the liquid nitrogen to evaporate, but do not allow the sample to thaw.**

2. **Immediately add 10ml Buffer RB/2-mercaptoethanol.** We recommend starting with 3-5 gram plant tissue at first. If results obtained are satisfactory increase amount of starting material. Add 20 µl 2-mercaptoethanol per 1ml 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 at 15,000 x g for 5 minutes at room temperature.

4. **Transfer the lysate directly into a Homogenization Maxi-Spin Homogenizer Column placed in collection tube. Centrifuge at ≥4,000 x g for 15 min at room temperature.**

5. **Carefully transfer the supernatant of the flow-through fraction to a new 50 ml centrifuge tube (not spplied), making sure not to**

disturb the pellet or transfer any debris. Add 0.5 volume absolute ethanol and mix by vortexing.

6. **Apply the entire sample, including any precipitates that may form to a HiBind® RNA Maxi-Spin column** assembled in a 50 ml collecting 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.

   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 RNA Wash Buffer I, close the tube gently. Centrifuge at ≥4,000 x g for 5 minutes.**

8. Discard both flow-through liquid and collecting tube.

9. **Place column in a clean 50ml collection tube (Not supplied), and add 10ml Wash Buffer II diluted with ethanol. Close the column gently,** Centrifuge at 6,000 x g for minutes at room temperature and discard flow-through. Re-use the collection tube in step 7.

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

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

8. **Elution of RNA: Transfer the column to a new RNase-free 50ml centrifuge tube (not supplied with kit) and elute the RNA with 3-4ml of DEPC-treated water (supplied with kit).** Make sure to add water directly onto column matrix. Centrifuge at 4000 x g for 5 minutes. A second elution into the same tube may be necessary if the expected yield of RNA >2 mg.

   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.

No RNA extraction procedure can completely remove genomic DNA. For sensitive work (such as RT-PCR or differential display) we suggest that you treat the eluted RNA with RNase-free DNase. Also for RT-PCR, use intron-spanning primers that allow easy identification of DNA-contamination.

E.Z.N.A.™ 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 10 gram) in a 30 or 50ml polycarbonate or Corex® centrifuge tube (not supplied) and immediately add 10.5 ml Buffer RPL/2-mercaptoethanol.** We recommend starting with 5g plant tissue at first. If results obtained are satisfactory increase amount of starting material. Add 20 µl 2-mercaptoethanol per 1ml of Buffer RPL. **Samples should not be allowed to thaw before Buffer RPL/2-mercaptoethanol is added**. Vortex vigorously to make sure that all clumps are dispersed. RNA cannot be effectively extracted from clumped tissue.

2. **Add 2.1 ml Buffer SP and vortex thoroughly to mix. Centrifuge at 15,000 x g for 20 min at room temperature.**

3. **Carefully aspirate cleared lysate to a new 30 or 50ml polycarbonate or Corex® centrifuge tube (not supplied) making sure not to disturb the pellet or transfer any debris. Add one volume isopropanol and vortex to precipitate RNA.** This step removes much of the polysaccharide content and improves spin-column performance by increasing RNA binding capacity (and therefore yield) in the steps that follow. No incubation is required after addition of isopropanol.

   TIP: In most cases 3.5 ml supernatant can easily be removed. This will require 3.5 ml isopropanol. Note that depending on the sample, the volume of supernatant may vary. After transferring to a fresh tube, measure the volume and add the correct amount of isopropanol.

4. **Immediately centrifuge at ≥15,000 x g for 10 min at room temperature to pellet RNA.** A longer centrifugation does not improve yields.

5. **Carefully aspirate or decant the supernatant and discard making sure not to dislodge the RNA pellet.** Invert the tube on a paper towel for 5 min to allow residual liquid to drain. Drying the pellet is not necessary.

6. **Add 5ml RB pre-heated to 65°C and vortex to resuspend the pellet.** A brief incubation at 65°C may be necessary to effectively dissolve the RNA.

7. **Add 5ml Buffer RB/2-mercaptoethanol followed by 10ml of 70% ethanol.** Vortex thoroughly to mix. This will adjust binding conditions prior to loading the HiBind® RNA Maxi-column.

8. **Apply the entire sample, including any precipitates that may form to an**

HiBind® RNA Maxi-spin column assembled in a clean 50 ml collecting tube (supplied). 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

Note: This is the starting point to perform DNase I digestion. See page 8 for detail protocol.

9. **Add 10ml RNA Wash Buffer I and centrifuge at ≥4,000 x g for 5 minutes.** Discard both flow-through liquid and collecting tube.

10. **Place column in a clean 50ml collection tube (Not supplied), and add 10 ml Wash Buffer II diluted with ethanol.** Centrifuge at 10,000 x g for 30 sec at room temperature and discard flow-through. Reuse the collection tube in step 11.

11. **Wash column with a second 10 ml of Wash Buffer II as in step 10.** Centrifuge and discard flow-through. Then with the collection tube empty, centrifuge the Midi-spin column **at 4000 x G for 10 minutes** to completely dry the HiBind™ matrix.

12. **Elution of RNA. Transfer the column to a RNase-free 50 ml microfuge tube (not supplied with kit) and elute the RNA with 3 ml of DEPC-treated water (supplied with kit).** Make sure to add water directly onto column matrix. Centrifuge at 6000 x G for 5 minutes at room temperature. A second elution into the same tube may be necessary if the expected yield of RNA >1.5 mg.

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:(Order product # E1091-02 for DNase I digestion set).

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

A. Pipet 1.5ml RNA Wash Buffer I into the HiBind® RNA Midi-spin column, and centrifuge at 6000 x G for 5 minutes to wash the column. Discard the flow through. For each HiBind® RNA Midi-spin column, prepare the DNase I digestion reaction mix as follows:

OBI DNase I Digestion Buffer	3.75ml
RNase-free DNase I (20 Kunitz unites/µl)	75 µl
Total volume	3.75 ml

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 3.75ml I 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 50ml collection tube**, and add 3 ml RNA Wash Buffer I. **Incubate 5 minutes at room temperature.** Centrifuge at 4000 x g for 5 minutes and discard flow-through. Reuse the collection tube.

3. **Place column in the same 50 ml collection tube**, and add 10ml RNA Wash Buffer II diluted with ethanol. Centrifuge at 6000 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 Wash Buffer II by repeating step 3. Centrifuge and discard flow-through. Then with the collection tube empty, centrifuge the Midi-spin 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 15 ml microfuge tube (not supplied with kit) and elute the RNA with 3-5 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 >1.5 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. Pre-heating the water to 70°C before adding to column and incubating column 5 min at room temperature before centrifugation may increase yields.

RNA Isolation from 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 10 gram of arthropod tissue under liquid nitrogen. Grind tissue completely to obtain a fine homogenous powder.

2. **Immediately add 10ml 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 Midiprep Protocol from step 3 (page 5).

RNA Isolation from Fungi

E.Z.N.A.® Plant RNA 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 10 gram of fungal sample under liquid nitrogen. Grind tissue completely to obtain a fine homogenous powder.

2. **Immediately add 10 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).

Quantitation and Storage of RNA

To determine the concentration and purity of RNA, measure absorbance at 260 nm and 280 nm in a spectrophotometer. 1 O.D. unit measured at 260 nm corresponds to 40 µg of RNA per ml. The ratio of A_{260}/A_{280} of pure nucleic acids is 2.0, while for pure protein it is approximately 0.6. A ratio of 1.8-2.0 corresponds to 90%-100% pure nucleic acid. (Phenol has an absorbance maximum at 275 nm and can interfere with spectrophotometric analysis of DNA or RNA. However, the E.Z.N.A.® Plant RNA Kit eliminates the use of phenol and avoids this problem.) Store RNA samples at -70°C in water. Under such conditions RNA prepared with the E.Z.N.A. system is stable for more than a year.

RNA Quality

It is highly recommended that RNA quality be determined prior to all analyses. The quality of RNA can be assessed by denaturing agarose gel electrophoresis and ethidium bromide staining. Several sharp bands should appear on the gel. These are the 28S and 18S ribosomal RNA bands as well as certain populations of mRNA and possibly viral RNA bands. If these bands smear towards lower molecular weight RNAs, then the RNA has undergone major degradation during preparation, handling, or storage. RNA molecules less than 200 bases in length do not efficiently bind the HiBind matrix, thus the method enriches high quality RNA. Since no RNA extraction procedure can completely remove genomic DNA.

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 50-100 mg 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. • Follow protocol closely, and work quickly. • 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 as indicated on bottle. • 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 inactivate 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.

Ordering Information

Product Number	Product Name	Description
E.Z.N.A.™ Total RNA Miniprep Kits		
R6634-01/02 R6834-01/02	E.Z.N.A.™ Total RNA Kit	Total RNA isolation from animal cells or tissues.
R6614-01/02 R6814-01/02	E.Z.N.A.™ Blood RNA Kit	Total RNA Isolation from blood samples
R6627-01/02 R6827-01/02	E.Z.N.A.™ Plant RNA Kit	Total RNA Isolation from plant samples
R6640-01/02 R6840-01/02	E.Z.N.A.™ Fungal RNA Kit	Total RNA Isolation from fungal samples
R6670-01/02 R6870-01/02	E.Z.N.A.™ Yeast RNA Kit	Total RNA Isolation from yeast samples
R6850-01/02 R6950-01/02	E.Z.N.A.™ Bacterial RNA Kit	Total RNA Isolation from yeast samples
R6675-01/02 R6875-01/02	E.Z.N.A.™ Mollusc RNA Kit	Total RNA Isolation from mollusc, invertebrates samples.
E.Z.N.A.™ Total RNA Midi/maxi Kits		
R6664-01/02	E.Z.N.A.™ Total RNA Midi Kit	Total RNA isolation from animal cells or tissues
R6693-01/02	E.Z.N.A.™ Total RNA Maxi Kit	Total RNA isolation from animal cells or tissues
R6615-01/02	E.Z.N.A.™ Blood RNA Midi Kit	Total RNA isolation from blood samples
R6616-01/02	E.Z.N.A.™ Blood RNA Maxi Kit	Total RNA isolation from blood samples
R6628-01/02	E.Z.N.A.™ Plant RNA Midi Kit	Total RNA isolation from plant samples
Other RNA isolation kit, Reagent and supplies		
R6511-01/02	mRNA Enrichment kit	mRNA isolation
R6830-01/02	RNA-Solv™ reagent	Single reagent for total RNA isolation
R6248-01/02 R6249-01/02	E.Z.N.A.™ RNA Probe purification kit	RNA Probe purification
R6376-01/02	E.Z.N.A.™ Poly-Gel RNA Isolation Kit	Isolate RNA from poly-acrylamide gel
R6500-01/02	E.Z.N.A.™ Oligo (dT) Cellulose	High capacity oligo(dT) cellulose
E1091	RNase-free DNase I set	DNase I set for on-column DNase digestion