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Introduction

The E.Z.N.A.® Plant RNA 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 100 mg tissue. The system is efficient enough to allow isolation of total RNA from as little as 10 mg of plant 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 and Poly A+ RNA selection.

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

Arabidopsis sp	30 µg
Tobacco leaves	65 µg
Mustard leaves	34 µg
Maize	28 µg

New in this edition

- On-Membrane DNase I digestion protocol included. (Page 8)
- New capped spin column ensures the elimination of potential contamination during operation.

Storage and Stability

All components of the E.Z.N.A.® Plant RNA 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 column can bind approximately 100 µg RNA. Using greater than 200 mg plant tissue usually will not dramatically improve yields and sometimes has adverse effects.

Kit Contents

Product Number	R6827-00	R6827-01	R6827-02
Purification Times	5 Preps	50 Preps	200 Preps
HiBind™ RNA Mini column	5	50	200
2 ml Collection Tubes	15	150	600
gDNA Filter Column	5	50	200
Buffer RCL	5 ml	30 ml	110 ml
Buffer RCB	5 ml	30 ml	110 ml
Buffer RB	5 ml	30 ml	110 ml
RWC Wash Buffer	5 ml	50 ml	2 x 90 ml
RNA Wash Buffer II	2 ml	12 ml	4 x 12 ml
DEPC water	1 ml	15 ml	40 ml
User Manual	1	1	1

Buffer RB contains a chaotropic salt. Use gloves and protective eyeware when handling this solution.

Before Starting

IMPORTANT	**RNA Wash Buffer II** must be diluted with absolute ethanol (96-100%) as follows at store the diluted RNA Wash Buffer II at room temperature.	
	R6827-00	Add 8 ml absolute ethanol to bottle
	R6827-01	Add 48 ml absolute ethanol to bottle
	R6827-02	Add 48 ml absolute ethanol to each bottle

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.

Materials to Be Provided by User

- Microcentrifuge capable of 14,000 x g
- Nuclease-free microfuge tubes
- 2-mercaptoethanol
- Absolute (96%-100%) ethanol
- Liquid nitrogen for freezing/disrupting samples
- Preheat an aliquot (100 µl per sample) of DEPC-treated water at 55°C.

E.Z.N.A.™ Plant RNA Kit 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 ≤100 mg. Best results are obtained with young leaves or needles. The method isolates sufficient RNA for a few tracks on a standard Northern assay. Wearing latex disposable gloves, collect tissue in a 1.5-ml or 2-ml microfuge tube and freeze by dipping in liquid nitrogen with a pair of tweezers to fill the tube. Grind the tissue using disposable pestles (available from OBI Cat# SS-1014-39 &1015-39) or equivalent. Alternatively, one can allow liquid nitrogen to evaporate and then store samples at -70°C for later use. **Do not allow samples to thaw**. Use disposable pestles only once. Alternatively, a small clean mortar and pestle can be used. The above methods for disrupting plant tissue **cannot** be replaced with mechanical homogenizers.

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

1. **Collect frozen ground plant tissue (up to 100 mg) in a microfuge tube and immediately add 500 µl Buffer RB/2-mercaptoethanol.** We recommend starting with 50 mg tissue at first. If results obtained are satisfactory increase amount of starting material. **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.

   TIP: As a guide, a 2-cm diameter leaf square weighs approximately 100 mg. Process in sets of four to six tubes: fill all tubes with liquid nitrogen, grind, add Buffer RB/2-mercaptoethanol, and continue to step 2 before starting another set. Centrifuge all tubes simultaneously (step 2 below). We recommend starting with 50-100 mg tissue at first to ensure optimal results. Adjust amount of starting material according to results obtained.

2. Centrifuge at 14,000 x g for 5 min at room temperature.

3. **Transfer the supernatant directly into a *gDNA Filter Column* in 2 ml collection tube** and centrifuge at 14,000 x g for 2 min at room temperature.

4. **Add 0.5 volume absolute ethanol (room temperature) to the flow-through and mix well by pipetting up and down 5-10 times.**

   TIP: In most cases 450 µl of supernatant can easily be transferred. This will require 225µl absolute ethanol. 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 ethanol.

5. **Apply the entire mixture from step 4, including any precipitates that may form to a HiBind® RNA Mini column** assembled in a clean 2 ml collection tube (supplied). Centrifuge at 10,000 x g for 1 minute at room temperature. Discard the flow-through liquid and place the column back into the collection tube.

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

6. **Add 400 µl RWC Wash Buffer and centrifuge as above.** Discard both flow-through liquid and collection tube.

7. **Place column in a clean 2 ml collection tube (supplied), and add 500 µl RNA Wash Buffer II diluted with ethanol.** Centrifuge as above and discard flow-through. Re-use the collection tube in step 7.

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

8. **Wash column with a second 500 µl RNA Wash Buffer II by repeating step 6.** Centrifuge as above and discard flow-through. Then with the collection tube empty, centrifuge the column for 2 min at 10,000 x g to completely dry the column matrix.

9. **Elution of RNA: Transfer the column to a clean 1.5 ml microfuge tube (not supplied) and elute the RNA with 30-50 µl of DEPC water (supplied).** Make sure to add water directly onto column matrix. Incubate at room temperature for 2 minutes. Centrifuge for 1 min at 10,000 x g. A second elution into the same tube may be necessary if the expected yield of RNA >30 µg.

   Note: RNA may be eluted with a greater volume of water. While additional elution 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. A control PCR reaction containing the RNA as template will also allow detection of DNA contamination. For designing intron-spanning primers, call our technical staff at 800-832-8896 for assistance. We can help design primers suited to your needs.

E.Z.N.A.™ Plant RNA 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. **Collect frozen ground plant tissue (up to 100 mg) in a microfuge tube and immediately add 500 µl Buffer RCL/2-mercaptoethanol.** We recommend starting with 50 mg tissue at first. If results obtained are satisfactory increase amount of starting material. 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 maxi speed (>14,000 x g) for 5 min at room temperature.

3. **Transfer the supernatant directly into a *gDNA Filter Column* in 2 ml collection tube** and centrifuge at 14,000 x g for 2 min at room temperature.

4. **Add eqaul volume Buffer RCB to the flow-through and mix well by pipetting up and down 5-10 times.**

   TIP: In most cases 450 µl of supernatant can easily be transferred. This will require 450µl Buffer RCB. 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 ethanol.

5. **Apply one half of the mixture from step 4 to a HiBind® RNA Mini column** assembled in a clean 2 ml collection tube (supplied). Centrifuge at 10,000 x g for 1 minute at room temperature. Discard the flow-through liquid and place the column back into the collection tube.

6. **Apply the regaining of the mixture from step 4 to the column.** Centrifuge at 10,000 x g for 1 minute at room temperature. Discard the flow-through liquid and place the column back into the collection 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 400 µl RWC Wash Buffer and centrifuge as above.** Discard both flow-through liquid and collection tube.

8. **Place column in a clean 2 ml collection tube (supplied), and add 500 µl RNA Wash Buffer II diluted with ethanol.** Centrifuge as above and 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 500 µl RNA Wash Buffer II by repeating step 8.** Centrifuge as above and discard flow-through. Then with the collection tube empty, centrifuge the column for 2 min at 10,000 x g to completely dry the column matrix.

10. **Elution of RNA: Transfer the column to a clean 1.5 ml microfuge tube (not supplied) and elute the RNA with 30-50 µl of DEPC water (supplied).** Make sure to add water directly onto column matrix. Incubate at room temperature for 2 minutes. Centrifuge for 1 min at 10,000 x g. A second elution into the same tube may be necessary if the expected yield of RNA >30 µg.

    Note: RNA may be eluted with a greater volume of water. While additional elution 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. A control PCR reaction containing the RNA as template will also allow detection of DNA contamination. For designing intron-spanning primers, call our technical staff at 800-832-8896 for assistance. We can help design primers suited to your needs.

DNase I 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 protocol until the samples **completely** pass through the HiBind® RNA Mini column. Prepare the following:

   A. **Add 300µl of RWC wash Buffer to the column** and centrifuge at 10,000 x g for 1 min.

   B. For each HiBind® RNA Mini 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

   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.

   C. Pipet 75 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 Mini column.

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

2. **Place column in a clean 2ml collection tube**, and add 400 µl RWC Wash Buffer. **Incubate 5 minutes at room temperature.** Centrifuge at 10,000 x g for 1 min at room temperature. Discard flow-through and reuse the collection tube.

3. **Place column in the same 2ml collection tube**, and add 500 µl RNA Wash Buffer II diluted with ethanol. Centrifuge as above and discard flow-through.

   Reuse the collection tube.

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

4. **Wash column with a second 500 µl RNA Wash Buffer II by repeating step 3.** Centrifuge as above and discard flow-through. Then with the collection tube empty, centrifuge the spin cartridge for 2 min at 10,000 x g to completely dry the HiBind® matrix.

5. **Elution of RNA. Transfer the column to a clean 1.5 ml microfuge tube (not supplied) and elute the RNA with 30-50 µl of DEPC water** (supplied). Make sure to add water directly onto column matrix. Centrifuge for 1 min at 10,000 x g. A second elution may be necessary if the expected yield of RNA >30 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.

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 100 mg arthropod tissue under liquid nitrogen. Grind tissue completely to obtain a fine homogenous powder.

2. **Immediately add 500 µl Buffer RB/2-mercaptoethanol.** Add 20 µl 2-mercaptoethanol per 1ml of Buffer RB and then add 500 µl 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 Protocol from step 2 (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 30 mg fungal sample under liquid nitrogen. Grind tissue completely to obtain a fine homogenous powder.

2. **Immediately add 500 µl Buffer RB/2-mercaptoethanol.** Add 10 µl 2-mercaptoethanol per 1ml of Buffer RB and then add 500 µl 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 2 (page 5).

Quantization 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. For sensitive work (such as RT-PCR or differential display) either on-membrane DNase I digestion treatment or after elution DNase I digestion will be needed. For modified protocols for DNase I digestion, call our technical staff at 800.832.8896 for assistance.

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 RCL. • 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.