Contents

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 does not require the use of cumbersome or expensive shredding/homogenizing accessories as an attempt to shear DNA in viscous plant lysates. Rather, the method involves a simple and rapid precipitation step for removal of much of the polysaccharides and phenolic compounds commonly found in plant tissues. In combination with HiBind® RNA spin columns, this permits purification of high quality RNA from as much as 200 mg tissue. The system is efficient enough to allow isolation of total RNA from as little as 10 mg of tissue or 100 cells. 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. This Kit can also be used to purify RNA from enzymatic reaction mixtures and to desalt RNA. 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 Kits

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

New in this edition

- On-column Dnase I digestion protocol included. (Page 5)
- New capped V-Spin column (Kit# R6827) 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 approximately100 µg RNA. Using greater than 200 mg plant tissue usually will not dramatically improve yields and sometimes has adverse effects.

Kit Contents

Product	R6627-00 R6827-00 Trial Sample	R6627-01 R6827-01 50 Preps	R6627-02 R6827-02 200 Preps
Components			
HiBind™ RNA columns	5	50	200
2 ml Collection Tubes	10	100	400
Buffer RPL	5 ml	50 ml	120 ml
Buffer SP	2 ml	10 ml	40 ml
Buffer RB	5 ml	30 ml	100 ml
RNA Wash Buffer I	5 ml	45 ml	175 ml
RNA Wash Buffer II, Concentrate	5 ml	12 ml	45 ml
DEPC-treated water	1 ml	20 ml	50 ml
User Manual	1	1	1

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

Before Starting

IMPORTANT Once diluted, store RNA Wash Buffer II at room temp.	Wash Buffer II Concentrate must be diluted with absolute ethanol as follows: R6627-00/R6827-00 Add 20ml 100 % ethanol R6627-01/R6827-01 Add 48 ml 100 % ethanol R6627-02/R6827-02 Add 180 ml 100% ethanol

It is not necessary to DEPC-treat the absolute ethanol before adding to Wash Buffer II Concentrate.

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

Materials to be provided by user:
- **!** Microcentrifuge capable of 10,000 x g
- **!** Nuclease-free microfuge tubes
- **!** 2-mercaptoethanol
- **!** Absolute (96%-100%) ethanol
- **!** Isopropyl alcohol (isopropanol)
- **!** Liquid nitrogen for freezing/disrupting samples
- **!** Preheat an aliquot (100 µl per sample) of DEPC-treated water at 65ºC.

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 ≤200 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 a1.5-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 Kontes pellet pestles (available from VWR, Cat# KT749521-0500) or equivalent. Alternatively, one can allow liquid nitrogen to evaporate and then store samples at -70ºC 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 200 mg) in a microfuge tube and immediately add 600 µl Buffer RPL/2-mercaptoethanol.** We reccomend starting with 50 mg tissue at first. If results obtained are satisfactory inccrease amount of starting material. Add 20 µl 2-mercaptoethanol per 1ml of Buffer RPL and then add 600 µl to the sample. **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.

   Note: **Add 20 µl 2-mercaptoethanol per 1 ml of Buffer RPL 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 RPL/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. **Add 140 µl Buffer SP and vortex thoroughly to mix. Centrifuge at 10,000 x g for 10 min at room temperature.**

3. **Carefully aspirate cleared lysate to a new microfuge tube 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 600 µl supernatant can easily be removed. This will require 600 µl 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 10,000 x g for 2 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 microfuge tube on a paper towel for 1 min to allow residual liquid to drain. Drying the pellet is not necessary.

6. **Add 100 µl of sterile DEPC-treated water (supplied) or pre-heated to 65ºC and vortex to resuspend the pellet.** A brief incubation at 65ºC may be necessary to effectively dissolve the RNA.
Important: RB buffer also can be used to dissolve the RNA pellet at this step, especially when the degradation was found after the elution. RB buffer contains strong RNase inhibitor.

7. **Add 350 µl Buffer RB/2-mercaptoethanol followed by 250 µl absolute ethanol.** Vortex thoroughly to mix. This will adjust binding conditions prior to loading the HiBind® RNA column.

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.

8. **Apply the entire sample, including any precipitates that may form to an HiBind® RNA spin column** assembled in a clean 2 ml collecting tube (supplied). Centrifuge at 10,000 x g for 30 sec 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 6 for detail protocol.

9. **Add 750 µl RNA Wash Buffer I and centrifuge at 10,000 x g for 30 sec.** Discard both flow-through liquid and collecting tube.

10. **Place column in a clean 2ml collection tube (supplied), and add 500 µl 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.

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

11. **Wash column with a second 500 µl of Wash Buffer II as in step 10.** Centrifuge and discard flow-through. Then with the collection tube empty, centrifuge the spin cartridge for **1 min at full speed** to completely dry the HiBind™ matrix.

12. **Elution of RNA. Transfer the column to a clean 1.5 ml microfuge tube (not supplied with kit) and elute the RNA with 50-100 µl 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 into the same tube may be necessary if the expected yield of RNA >50 µg.

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. 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 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. Following steps provide on-membrane DNase I digestion:(see DNase I cat.# E1091for detail information)

1. Follow the standard protocol until the samples **completely** pass through the HiBind RNA column (step1-7). Prepare the following:

a. 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

Note:
1. **DNase I is very sensitive for physical denaturaion, so do not vortex this 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 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.

c. Incubate at room temperature(25-30^{N}C) for 15 minutes

6. **Place column in a clean 2ml collection tube**, and add 500 µl RNA Wash Buffer I. **(If on-membrane DNase digestion was performed in the previous step, wait at least 5 minutes before proceeding).** Centrifuge and discard flow-through. Reuse the collection tube in step 7.

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

8. Wash column with a second 500 µl of Wash Buffer II as in step 5. Centrifuge and discard flow-through. Then with the collection tube empty, centrifuge the spin cartridge for **1 min at full speed** to completely dry the HiBind® matrix.

9. **Elution of RNA.** Transfer the column to a clean 1.5 ml microfuge tube

(not supplied with kit) and elute the RNA with 50-100 µl 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 >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 70ºC 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. The E.Z.N.A.® Plant RNA Kit includes a simple isopropanol precipitation step that improves RNA quality and column performance when difficult samples are processed.

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 600 µl Buffer RPL/2-mercaptoethanol.** Add 20 µl 2-mercaptoethanol per 1ml of Buffer RPL and then add 600 µl of this mixture to the sample. **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.

   Note: **Add 20 µl 2-mercaptoethanol per 1 ml of Buffer RPL 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 4).

RNA Isolation from Fungal

E.Z.N.A.® Plant RNA Kit can also be used for fungal RNA isolation since fungal sample poses similar problem as encountered with many plant specimens. It is recommended to use E.Z.N.A.® Fungal RNA Kit for fungal RNA isolation because its buffers are specially modified for Fungal samples.

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 600 µl Buffer RPL/2-mercaptoethanol.** Add 20 µl 2-mercaptoethanol per 1ml of Buffer RPL and then add 600 µl of this mixture to the sample. **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.

3. Proceed with the Plant RNA Protocol from step 2 (page 4).

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 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

Product No.	Product Name	Description
E.Z.N.A.™ Blood DNA Kits		
D3481-01/02 D3491-01/02	Blood DNA Kit I (Proteinase included)	Isolation of total cellular DNA from fresh and dry blood
D3482-01/02 D3492-01/02	Blood DNA Kit II (OB Protease included)	Isolation of total cellular DNA from fresh and dry blood
D3493-01/02	Blood DNA Midiprep Kit (Proteinase not included)	Isolation of total cellular DNA from up to 10ml blood.
D3494-01/02	Blood DNA Midiprep Kit (OB Proteinase included)	Isolation of total cellular DNA from up to 10ml blood.
D2492-01/02	Blood DNA Maxiprep Kit (OB Proteinase included)	Isolation of total cellular DNA from up to 30ml blood.
D1192-01/02	EZ 96™ Blood DNA Kit	Isolation of total cellular DNA from fresh and dry blood with 96 well plate
E.Z.N.A.™ Tissue DNA Kits		
D3395-01/02 D3495-01/02	Tissue DNA Kit I (Proteinase not included)	Isolation of total cellular DNA from tissue samples.
D3396-01/02 D3496-01/02	Tissue DNA Kit II (OB Protease included)	Isolation of total cellular DNA from tissue samples.
D1196-01/02	EZ 96™ Tissue DNA Kit	Isolation of total cellular DNA from tissue samples with 96 well plate
D3592-01/02	Forensic DNA Kit	Isolation of genomic DNA from forensic samples. OB collection paper included.