Contents

Revised November 2012

Introduction

The E-Z 96® 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 plate, this kit permits purification of high quality RNA from as much as40mg seed tissue or as much as 100 mg tissue. The system is efficient enough to allow isolation of total RNA from as little as 0.5 mg of plant tissue. Typical yields are shown in Table 1. E-Z 96® 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 10)
- New capped spin column ensures the elimination of potential contamination during operation.

Storage and Stability

All components of the E-Z 96® Plant RNA 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/Buffer RCL/Buffer RCB. In case of such an event, heat the bottle at 37°C to dissolve. Store Buffer RB at room temperature.

Binding Capacity

Each well of the HiBind® RNA plate can bind approximately 100 µg RNA. Using more than the recommended maximum amount of plant tissue usually will not improve yields significantly and ofte has adverse effects.

Kit Contents

Product Number	R1027-00	R1027-01	R1027-02
Purification Times	1x96	2x96	8x96
HiBind® RNA Plate	1	2	8
96-Well gDNA Remove Plate	1	2	8
96-Well Collection Plate(2ml) *	3 *	6 *	12 *
Elution Plate(300 µl)	1	2	8
Aera Seal Film	5	10	40
8-Strip Microtube Caps	12x8	25x8	100x8
Buffer RCL	55 ml	110 ml	2x220 ml
Buffer RCB	55 ml	110 ml	2x220 ml
Buffer RB	55 ml	110 ml	2x220 ml
RWC Wash Buffer	75 ml	150 ml	3 x 200 ml
RNA Wash Buffer II	35 ml	2x35 ml	5 x50 ml
DEPC water	15 ml	25 ml	100 ml
User Manual	1	1	1

** 96-Well Collection Plate(2ml) are reusable; see Page 7 for instructions.*
** Buffer RB/Buffer RCL/Buffer RCB contains 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.	
	R1027-00	Add 140 ml absolute ethanol to each bottle
	R1027-01	Add 140 ml absolute ethanol to each bottle
	R1027-02	Add 200 ml absolute ethanol to each bottle

Note: 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/Buffer RCL /Buffer RCB. 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 RCL before use. Add 20 µl of 2-mercaptoethanol per 1 ml of Buffer RB or RCL. This mixture can be stored for 1 week at room temperature.

Materials to Be Provided by User

- Centrifuge capable of 4,000 x g
- Centrifuge rotor adaptor for 96-well microplates
- Multichannel pipet
- RNase-free filter pipette tips
- 2-Mercaptoethanol
- Absolute (96%-100%) ethanol
- Liquid nitrogen for freezing/disrupting samples
- Water bath or heat block preset at 55°C.
- Preheat an aliquot (100 µl per sample) of DEPC-treated water at 55°C.

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 plant tissue and ≤40 mg seed tissue.(Less starting material ofter results in better quality yields). Best results are obtained with young leaves or needles. The method isolates sufficient RNA for a few tracks on a standard Northern assay, depending on the type and quality of the sample.

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 -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. **Add 500 µl Buffer RB/2-mercaptoethanol per sample to the wells of a 2ml deep-well plate.**

   Note: Add 10 µl 2-Me. per 1 ml of Buffer RB before use. This mixture can be made and stored at room temperature for 1 month. 2-mercaptoethanol should be added again(estimate same proportion) if Buffer RB is stored for more than 1 month.

2. **Collect frozen ground plant tissue (up to 100 mg) or seed tissue (up to 30 mg) and add to a well containing Buffer RB / 2-mercaptoethanol.** Samples should not be allowed to thaw before adding to Buffer RB / 2-Me. We recommend starting with 30 to 50 mg plant tissue or 12 to 20 mg seed tissue. If results obtained are satisfactory, increase amount of starting material up to maximum limits. Vortex vigorously to make sure that all clumps are dispersed. RNA cannot be effectively extracted from clumped tissue.

   Tip: As a guide, a 2 cm diameter leaf square weighs approximately 100 mg.

3. Seal the plate with aera seal film. Centrifuge the plate at 4,000 x g for 10 minutes.

4. **Pipet the lysate directly into a 96-Well gDNA Remove Plate placed on top of a new 2 ml collection plate (supplied)**. Seal the plate with a new aera seal film. Centrifuge at 3,500 x g for 10 min at room temperature, reuse the film in the next step.

5. **Add 0.5 volume absolute ethanol ,** seal the plate with aera seal film and mix by vortexing.

   Tip: In most cases 450 µl flowthrough can easily be removed. This will require 225 µl ethanol. The volume of flowthrough may vary. For convenience, a fixed volume may be used. Measure the volume and add the correct amount of ethanol.

6. Apply the entire sample, including any precipitates that may form to a HiBind® RNA Plate placed on top of a new 2ml collection plate. Seal the plate with a new aera seal film, centrifuge at 4,000 x g for 5 minutes at room temperature. Discard the flow-through liquid and place the HiBind® RNA Plate back on top of the collection plate. If the sample volume exceeds the well capacity, load successively and repeat Step 6.

   Note: Be sure that the lysate has passed completely through each well. If any lysate remains, repeat centrifugation for an additional 3 to 5 minutes.

DNase I Digestion (OPTIONAL): This is the point to begin optional DNase I digestion. If DNase I digestion is necessary for downstream applications, go to Page 6 to complete the procedure using the DNase I Digestion Protocol; otherwise continue with Step 7.

7. **Apply 600 µl RWC Wash Buffer into each well of the HiBind® RNA Plate.** Seal the plate with a new aera seal film. Then centrifuge at 4,000 x g for 5 minutes. Discard the flow-through liquid. Reuse the collection plate and film in next step.

8. Place the HiBind® RNA Plate onto 2ml collection plate, **and add 700 µl RNA Wash Buffer II diluted with ethanol.** Seal the plate with aera seal film of step 7 and centrifuge at 4,000 x g for 5 minutes at room temperature. Discard the flowthrough, reuse the collection plate and film in next step.

   Note: RNA Wash Buffer II Concentrate must be diluted with absolute ethanol before use. Refer to Page 3 or to the bottle label for directions.

9. **Wash the HiBind® RNA Plate with a second 700 µl RNA Wash Buffer II as in the prior step. Discard flow-through, reuse the collection plate and film. Centrifuge the HiBind® RNA Plate for 10 min at 4,000 x g to completely dry the HiBind® matrix.**

10. Elution of RNA. Place the HiBind® RNA Plate onto a Elution Plate (supplied) **and elute the RNA with 100 µl of DEPC-treated water (supplied with kit).** Make sure to add water to the center of each membrane. Incubate at room

temperature for 1 minute. Seal the plate with a new aera seal film, centrifuge 5 min at 4,000 x g. A second elution into the same tube may be necessary if the expected yield of RNA >50 µg. Seal the Elution Plate with a new aera seal film.

Note: RNA may be eluted with a greater (or lesser) 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.

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.

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

1. **Add 500 µl Buffer RCL/ 2-mercaptoethanol per sample to the wells of a 2ml deep-well plate.**

   Note: Add 10 µl 2-Me. per 1 ml of Buffer RCL before use. This mixture can be made and stored at room temperature for 1 month. 2-Me should be added again(estimate same proportion) if Buffer RCL is stored for more than 1 month.

2. Collect frozen ground plant tissue (up to 100 mg) or seed tissue (up to 30 mg) and **add to a well containing Buffer RCL / 2-mercaptoethanol.** Samples should not be allowed to thaw before adding to Buffer RCL / 2-Me. We recommend starting with 30 to 50 mg plant tissue or 12 to 20 mg seed tissue. If results obtained are satisfactory, increase amount of starting material up to maximum limits. Vortex vigorously to make sure that all clumps are dispersed. RNA cannot be effectively extracted from clumped tissue.

   Tip: As a guide, a 2 cm diameter leaf square weighs approximately 100 mg.

3. **Incubate at 55°C for 3 minutes.** Seal the plate with aera seal film. Centrifuge the plate at 4,000 x g for 10 minutes.

4. Pipet the lysate directly into a 96-Well gDNA Remove Plate placed on top of a new 2 ml collection plate (supplied). Seal the plate with a new aera seal film.

Centrifuge at 3,500 x g for 10 min at room temperature, reuse the film in the next step.

5. **Add equal volume Buffer RCB**, seal the plate with aera seal film and mix by vortexing.

   Tip: In most cases 450 µl flowthrough can easily be removed. This will require 450 µl Buffer RCB. The volume of flowthrough may vary. For convenience, a fixed volume may be used. Measure the volume and add the correct amount of Buffer RCB.

6. Apply the entire sample, including any precipitates that may form to a HiBind® RNA Plate placed on top of a new 2ml collection plate. Seal the plate with a new aera seal film, centrifuge at 4,000 x g for 5 minutes at room temperature. Discard the flow-through liquid and place the HiBind® RNA Plate back on top of the collection plate. If the sample volume exceeds the well capacity, load successively and repeat Step 6.

   Note: Be sure that the lysate has passed completely through each well. If any lysate remains, repeat centrifugation for an additional 3 to 5 minutes.

DNase I Digestion (OPTIONAL): This is the point to begin optional DNase I digestion. If DNase I digestion is necessary for downstream applications, go to Page 6 to complete the procedure using the DNase I Digestion Protocol; otherwise continue with Step 7.

7. **Apply 600 µl RWC Wash Buffer into each well of the HiBind® RNA Plate.** Seal the plate with a new aera seal film. Then centrifuge at 4,000 x g for 5 minutes. Discard the flow-through liquid. Reuse the collection plate and film in next step.

8. Place the HiBind® RNA Plate onto 2ml collection plate, **and add 700 µl RNA Wash Buffer II diluted with ethanol.** Seal the plate with aera seal film of step 7 and centrifuge at 4,000 x g for 5 minutes at room temperature. Discard the flowthrough, reuse the collection plate and film in next step.

   Note: RNA Wash Buffer II Concentrate must be diluted with absolute ethanol before use. Refer to Page 3 or to the bottle label for directions.

9. **Wash the HiBind® RNA Plate with a second 700 µl RNA Wash Buffer II as in the prior step.** Discard flow-through, reuse the collection plate and film. Centrifuge the HiBind® RNA Plate for 10 min at 4,000 x g to completely dry the HiBind® matrix.

10. Elution of RNA. Place the HiBind® RNA Plate onto a Elution Plate (supplied) and elute the RNA with 100 µl of DEPC-treated water (supplied with kit). Make sure to add water to the center of each membrane. Incubate at room temperature for 1 minute. Seal the plate with a new aera seal film, centrifuge 5 min at 4,000 x g. A second elution into the same tube may be necessary if the expected yield of RNA >50 µg. Seal the Elution Plate with a new aera seal film.

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 70ºC before adding to column and incubating column 5 min at room temperature before centrifugation may increase yields.

Clean the 2ml collection plates

Two 2ml collection plates are supplied with each kit. If extra plates are needed, please call our customer service department for ordering information. To re-use the collection plates, rinse them throughly with tap water, incubate overnight in 0.2M NaOH/1mM EDTA, rinse with distilled water and dry by air.

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 -70ºC 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.