Contents

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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 includes a homogenization plate to efficiently remove cell debris and simultaneously homogenize the lysate. In combination with the HiBind® RNA plate, this kit permits purification of high quality RNA from as much as 40 mg seed tissue or as much as 100 mg plant tissue. The system is efficient enough to allow isolation of total RNA from as little as 0.5 mg 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
- qPCR
- 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

Storage and Stability

All components of the E-Z 96® 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. The crystals may be dissolved by heating the buffer at 37ºC and gently shaking its container. Store Buffer RB and all other components 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 often has adverse effects.

Kit Contents

Product No.	R1027-00	R1027-01	R1027-02
HiBind® RNA Plate	1	2	8
96-Well Collection Plate (2ml)*	3*	6*	12*
E-Z 96® **Homogenizer Plate**	**1**	**2**	**8**
Racked Microtubes (1.2ml)	**1 x 96**	**2 x 96**	**8 x 96**
Aera Seal Film	**5**	**10**	**40**
8-Strip Microtube Caps	**12 x 8**	**25 x 8**	**100 x 8**
Buffer RB	**55 ml**	**110 ml**	**2 x 220**
RNA Wash Buffer I	**75 ml**	**150 ml**	**2 x 300**
RNA Wash Buffer II	**35 ml**	**2 x 35 ml**	**5 x 50 ml**
DEPC-treated water	**15 ml**	**25 ml**	**100 ml**
User Manual	**1**	**1**	**1**

*** 96-Well Collection Plates (2ml) are reusable; see Page 7 for instructions.**

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

Before Starting

Important	Dilute **Wash Buffer II** Concentrate with **absolute ethanol** (96%-100%) as follows: **R1027-00** Add 140 ml absolute ethanol per bottle **R1027-01** Add 140 ml absolute ethanol per bottle **R1027-02** Add 200 ml absolute ethanol per 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. 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 before use. Add 10 µl of 2-mercaptoethanol per 1 ml of Buffer RB. This mixture can be stored for 1 month at room temperature. If RB Buffer is stored for more than 1 month, 2-mercaptoethanol should be added again.

E-Z 96® Plant RNA Protocol

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
- Racked RNase-free 1.2 ml microtubes
- 2ml 96-well deep well plate
- 2-mercaptoethanol
- Absolute (96%-100%) ethanol
- Isopropyl alcohol (isopropanol)
- 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 65°C.

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 plant tissue and ≤40 mg seed tissue. (Less starting material often results in better quality yields.) Best results are obtained with young leaves or needles. This 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 -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. Add 500 µl Buffer RB/2-mercaptoethanol per sample to the wells of a 2 ml deep-well plate.

   Note: Add 10 µl 2-mercaptoethanol 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-mercaptoethanol. 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. Centrifuge the plate at 4,000 x g for 10 minutes.

4. Pipet the lysate directly into a E-Z 96-Well Homogenizer Plate placed on top of a new 2 ml collection plate (supplied). Centrifuge at ≥3500 x g for 10 min at room temperature.

5. Carefully transfer the supernatant of the flow-through fraction to a new 2ml collection plate (supplied), making sure not to disturb the pellet or transfer any debris. Add 0.5 volume absolute ethanol and mix by vortexing. Keep and re-use the 2 ml collection plate for Step 6.

   Tip: In most cases 450 µl supernatant can easily be removed. This will require 225µl ethanol. The volume of supernatant 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 the 2ml collection plate from Step 5. Seal the plate with 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 RNA Wash Buffer I into each well of the HiBind® RNA Plate. Seal the plate with aera seal film. Then centrifuge at 4,000 x g for 5 minutes. Discard the flow-through liquid. Use a clean collection plate in the next step.

8. Place the HiBind® RNA Plate onto a clean 2ml collection plate, and add 700 µl RNA Wash Buffer II diluted with ethanol. Seal the plate with aera seal film and centrifuge at 4,000 x g for 5 minutes at room temperature. Discard the flow-through and re-use the collection plate 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 and re-use the collection plate. 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 atop a 1.2 ml microtube rack (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. 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.

    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.

DNase I Digestion Protocol (Optional)

Since the HiBind® RNA technology eliminates most of the DNA, DNase I digestion is not necessary for most downstream applications. However, certain sensitive RNA applications might require further DNA removal. Follow the steps below for on-membrane DNase I digestion (see DNase I Digestion Set, Cat # E1091 for further information).

1. Follow the standard protocol until the samples completely pass through the HiBind® RNA Plate (Steps 1-6). Then complete the procedure using the steps following steps:

   A. Add 300µl RNA Wash Buffer I to each well of the HiBind® RNA Plate and centrifuge at ≥4,000 x g for 1 min.

   B. For each RNA sample, prepare the DNase I digestion mixture as follows:

OBI DNase I Digestion Buffer	73.5 µl
RNase-free DNase I (20 Kunitz units/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 beginning the RNA isolation procedure.

2. Standard DNase buffers may not be compatible with Omega Bio-Tek's DNase I Digestion Set.

C. Pipet 75 µl DNase I digestion mixture directly onto the surface of the membrane in each well of the HiBind® RNA Plate. Be certain to pipet the mixture directly onto each membrane, as DNA digestion might not be complete if some of the mixture is retained on the walls or the O-rings of the HiBind® RNA Plate.

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

2. Place the HiBind® RNA Plate on top of a clean 2ml collection plate and add 400 µl RNA Wash Buffer I. Incubate 3 minutes at room temperature. Centrifuge at 4,000 x g and discard flow-through. Re-use the collection plate.

3. Place the HiBind® RNA Plate on top of the same 2ml collection plate and add 700 µl RNA Wash Buffer II diluted with ethanol. Centrifuge at 4,000 x g for 3 minutes and discard flow-through. Re-use the collection plate.

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

4. Wash the HiBind® RNA Plate with a second 700 µl RNA Wash Buffer II by repeating Step 3. Centrifuge at 4,000 x g for 3 minutes. Discard flow-through and re-use the collection plate. Then centrifuge the empty HiBind® RNA Plate at 4,000 x g for 10 min to *completely* dry the HiBind® matrix. (Complete drying is critical.)

5. Elution of RNA: Place the HiBind® RNA Plate on top of a 1.2 ml microtube rack (supplied). Add 100 µl of DEPC-treated water (supplied with kit) to each well. Make sure to add DEPC-water directly to the center of each membrane for optimal elution results. Incubate at room temperature for 1 minute. Centrifuge for 5 min at 4,000 x g to elute RNA.

Alternatively, 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. Pre-heating the DEPC-water to 65°C before adding to the HiBind® RNA Plate and incubating the plate for 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} for 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 Total 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 to the HiBind® matrix. The kit optimally binds molecules from 200 bp to 40 kb.

Troubleshooting Guide

Problem	Cause	Suggestion
Little or no RNA eluted	RNA remains on the membrane	• Repeat elution. • Pre-heat DEPC-water to 65° C prior to elution. • Incubate HiBind® plate containing DEPC-water for 10 min prior to elution.
	Plate is clogged	• Reduce amount of starting material.
Clogged HiBind® RNA Plate membrane	Incomplete disruption or lysis of plant tissue	• Completely disrupt sample in liquid nitrogen. • Increase centrifugation time. • Reduce amount of starting material.
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 RNA Wash Buffer II has been diluted with absolute ethanol as indicated on bottle. • Diluted RNA Wash Buffer II must be stored at room temperature. • Repeat wash with RNA 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.