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Introduction

The E-Z96.[®] 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 plate to efficiently remove cell debris and simultaneously homogenize the lysate. In combination with HiBind[®] RNA plate, 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 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
- Poly A+ RNA selection

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 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 well of HiBind[®] RNA plate 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 No.	R1027-01	R1027-02
HiBind™ RNA columns	2	8
2 ml deep well collection plate*	6	24
Homogenization Plate	2	8
Racked Microtubes (1.2ml)	2 x 96	8 x 96
Buffer RB	110 ml	440 ml
RNA Wash Buffer I	150 ml	600 ml
RNA Wash Buffer II, Concentrate	60 ml	2 x 100 ml
DEPC-treated water	25 ml	100 ml
User Manual	1	1

* 2ml Deep well plates are reusable; see Page 7 for instructions.



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

Before Starting

IMPORTANT	Wash Buffer II Concentrate must be diluted with absolute ethanol as follows:	
Once diluted, store RNA Wash Buffer II at room temp.	R1027-01	Add 240 ml 100 % ethanol
	R1027-02	Add 400 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 (β -mercaptoethanol) is key in denaturing endogenous RNases and must be added to an aliquot of Buffer RB and Buffer RPL before use. Add 20 μ l of 2-mercaptoethanol per 1 ml of Buffer RB or RPL. This mixture can be stored for 1 week at room temperature.

E-Z 96® Plant RNA Protocol

Materials to be provided by user:

- Centrifuge capable of 4,000 x g
- Adaptor for 96-well microplate.
- Multichannel pipet
- RNase-free filter pipette tips
- Racked RNase-Free 1.2 ml microtubes
- 2ml 96-well deep well plate
- Adhesive sealing film for microplate (5 per plate)
- 2-mercaptoethanol
- Absolute (96%-100%) ethanol
- β -Mercaptoethanol
- Isopropyl alcohol (isopropanol)
- Liquid nitrogen for freezing/disrupting samples
- Water bath or heat block preset at 55°C
- Preheat an aliquot (100 μ l 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 \leq 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) into each each well of 2.0ml deep well plate (Suppled) contains 500 μ l Buffer RB/2-mercaptoethanol.** We recommend starting with 50 mg tissue at first. If results obtained are satisfactory increase amount of starting material. Add 20 μ l 2-mercaptoethanol per 2ml of Buffer RB and then add 500 μ l of RB/2-mercaptoethanol 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 μ l 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.

2. Centrifuge the plate at 4000 x g for 10 minutes.
3. **Pipet the lysate directly into a Homogenization Spin Plate placed on top of a 2ml collection plate (supplied). Centrifuge at ≥ 3500 x g for 10 min at room temperature.**
3. **Carefully transfer the supernatant of the flow-through fraction to a new 2ml deep well 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 4.**

TIP: In most cases 450 μ l supernatant can easily be removed. This will require 225 μ l 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.

4. **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 film. Centrifuge at 4000 x g for 5 minutes at room temperature. Discard the flow-through liquid and place the **HiBind[®] RNA Plate** back on top the collecting plate.

Optional on-membrane DNase I digestion: This is the starting point to perform DNase I digestion. See Page 6 for detailed protocol.

5. **Apply 500 μ l RNA Wash Buffer I into each well of the HiBind[®] RNA Plate, seal the plate with sealing film, centrifuge at 4,000 x g for 5 minutes.** Discard both flow-through liquid.
 6. **Place HiBind[®] RNA Plate in a clean 2ml collection plate (not supplied), and add 700 μ l Wash Buffer II diluted with ethanol. Seal the plate with flim,** Centrifuge at 4000 x g for 5 minutes at room temperature and discard flow-through. Re-use the collection plate in step 7.
- Note:** Wash Buffer II Concentrate must be diluted with absolute ethanol before use. Refer to label on bottle for directions.
7. **Wash HiBind[®] RNA Plate with a second 700 μ l of Wash Buffer II by repeating step 6.** Centrifuge and discard flow-through. Then with the collection plate empty, centrifuge the **HiBind[®] RNA Plate** for **10 min at 4000 x g** to completely dry the HiBind[™] matrix.
 8. **Elution of RNA. Transfer the HiBind[®] RNA Plate on top a 1.2 ml microtube rack supplied) and elute the RNA with 100 μ l of DEPC-treated water (supplied with kit).** Make sure to add water directly onto center of matrix. Centrifuge 10 min at 4000 x g. A second elution into the same tube may be necessary if the expected yield of RNA >50 μ g.

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.

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: (see DNase I, Cat # E1091 for further information).

1. Follow the standard protocol until the samples **completely** pass through the HiBind[®] RNA Plate (Steps 1-4). Prepare the following:

- A. Add 300 μ l of RNA wash Buffer I to the column and centrifuge at $\geq 10,000$ x G for 1 min.
- B. For each HiBind[®] RNA column, prepare the DNase I digestion reaction mix as follows:

OBI DNase I Digestion Buffer	73.5 μ l
RNase-free DNase I (20 Kunitz unites/ μ l)	1.5 μ l
Total volume	75 μ l

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 μ l 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.
 - D. Incubate at room temperature(25-30°C) for 15 minutes
2. **Place HiBind[®] RNA Plate on top of a clean 2ml collection Plate,** and add 400 μ l RNA Wash Buffer I. **Incubate 5 minutes at room temperature.** Centrifuge at 4000 x g and discard flow-through. Reuse the collection plate.
 3. **Place HiBind[®] RNA Plate on top of the same 2ml collection plate,** and add 700 μ l RNA Wash Buffer II diluted with ethanol. Centrifuge at 4000 x g for 5 minutes and discard flow-through. Reuse the collection palte.
Note: Wash Buffer II Concentrate must be diluted with absolute ethanol before use. Refer to label on bottle for directions.

4. Wash **HiBind® RNA Plate** with a second 700 µl of Wash Buffer II by repeating step 3. Centrifuge at 4000 x g for 5 minutes and discard flow-through. Then with the collection plate empty, centrifuge the **HiBind® RNA Plate at 4000 x g for 10 min** at to completely dry the HiBind® matrix.
5. **Elution of RNA.** Transfer the **HiBind® RNA Plate** on top of 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 directly onto center of matrix. Centrifuge 10 min at 4000 x g to elute RNA.

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.

Clean the 2ml deep well plates:

Two 2ml deep well plate are supplied with each kit. If extra plates are needed, please call our customer service department for ordering information. To reuse the deep well plates, rinse them thoroughly 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 -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 membrane	<ul style="list-style-type: none"> ● Repeat elution. ● Pre-heat DEPC-water to 70° C prior to elution. ● Incubate column for 10 min with water prior to centrifugation.
	Plate is clogged	<ul style="list-style-type: none"> ● Reduce quantity of starting material.
Clogged column	Incomplete disruption or lysis of plant tissue.	<ul style="list-style-type: none"> ● Completely disrupt sample in liquid nitrogen. ● Increase centrifugation time. ● Reduce amount of starting material
Degraded RNA	Source	<ul style="list-style-type: none"> ● 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 B buffer as dissolvent instead of DEPC water.
	RNase contamination	<ul style="list-style-type: none"> ● Ensure not to introduce RNase during the procedure. ● Check buffers for RNase contamination.
Problem in downstream applications	Salt carry-over during elution	<ul style="list-style-type: none"> ● 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	<ul style="list-style-type: none"> ● Digest with RNase-free DNase and inactivate at 75°C for 5 min.
Low Abs ratios	RNA diluted in acidic buffer or water	<ul style="list-style-type: none"> ● DEPC-treated water is acidic and can dramatically lower Abs₂₆₀ values. Use TE buffer (pH 8) to dilute RNA prior to spec analysis.