

Contents

Introduction.....	2
New In This Edition.	2
Storage and Stability.	2
Binding Capacity.....	2
Kit Contents.....	3
Before Starting.....	3
Working with RNA.	4
Materials to Be Provided by User.....	4
E.Z.N.A.® HP Plant RNA Protocol.	5
DNase I Digestion Protocol (Optional)	7
Quantization and Storage of RNA.	9
RNA Quality.	9
Troubleshooting Guide.....	10

Introduction

The E.Z.N.A.® HP Plant RNA Kit provides a convenient and rapid method for the isolation of total RNA from a variety of plant samples. The kit include detergent and gDNA Filter Column to efficiently remove cell debris, gDNA, polysaccharose and polyphenols. 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. E.Z.N.A.® HP 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.

New in this edition

- On-Membrane DNase I digestion protocol included. (Page 7)
- New capped spin column ensures the elimination of potential contamination during operation.

Storage and Stability

All components of the E.Z.N.A.® HP Plant RNA Kit except Buffer RA should be stored at 22°C-25°C. Buffer RA must be store at 4°C for a long time storage. 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 RC. In case of such an event, heat the bottle at 37°C to dissolve. Store Buffer RC 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	R6837-00	R6837-01	R6837-02
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 RA	5 ml	30 ml	110 ml
Buffer RC	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	10 ml	40 ml
User Manual	1	1	1

Buffer RA and Buffer RC 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.	
	R6837-00	Add 8 ml absolute ethanol to bottle
	R6837-01	Add 48 ml absolute ethanol to bottle
	R6837-02	Add 48 ml absolute ethanol to each bottle

- 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 RC. This is normal and the bottle may be warmed to redissolve the salt.

Materials to Be Provided by User

- Microcentrifuge capable of 14,000 x g
- 1.5ml and 2ml Nuclease-free microfuge tubes
- Absolute (96%-100%) ethanol
- Water-saturated phenol
- Chloroform
- Liquid nitrogen for freezing/disrupting samples
- Preheat an aliquot (100 µl per sample) of DEPC-treated water at 55°C.

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.

E.Z.N.A.™ HP Plant RNA Kit Protocol

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 RA.** 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 RA is added. 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. Process in sets of four to six tubes: fill all tubes with liquid nitrogen, grind, add Buffer RA, and continue to step 2 before starting another set. 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 150 μl water-saturated phenol and 100 μl Chloroform to the sample,** shake vigorously for 15 seconds.
3. Centrifuge at 14,000 x g for 5 min at room temperature.
4. **Transfer the supernatant to a new 2ml Nuclease-free microfuge tube, add equal volume Buffer RC,** mix by vortexing.
5. Transfer the mixture from step 4 into a *gDNA Filter Column* in 2 ml collection tube and centrifuge at 14,000 x g for 2 min at room temperature.
6. **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 500 μl of flow-through can easily be transferred. This will require 250 μl absolute ethanol. Note that depending on the sample, the volume of flow-through

may vary, measure the volume and add the correct amount of ethanol.

7. Apply the entire mixture from step 6, 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. If the sample volume exceeds the column capacity, load successively and repeat Step 7.

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.

(Optional): On-membrane DNase I digestion: This is the starting point to perform DNase I digestion. See Page 7 for detailed protocol.

8. **Add 400 μl RWC Wash Buffer and centrifuge as above.** Discard flow-through liquid and collection tube.
9. 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 10.

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

10. **Wash column with a second 500 μl RNA Wash Buffer II by repeating step 9.** 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.
11. 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.

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. (Polyphenols has an absorbance maximum at 275 nm and can interfere with spectrophotometric analysis of DNA or RNA. However, the E.Z.N.A.® HP Plant RNA Kit eliminates the use of polyphenols 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	<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.
	Column is overloaded	<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
Precipitated RNA will not dissolve.	High nucleic acid and polysaccharide content.	<ul style="list-style-type: none"> • 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	<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.
	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.