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Introduction

The E.Z.N.A.® Plant RNA Midi 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 spin columns, this permits purification of high quality RNA from as much as 5 g fresh plant tissue. Typical yields are shown in Table 1.

The E.Z.N.A.® Plant RNA Midi Kit is 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

Table 1. Yields obtained with E.Z.N.A.® Plant RNA Midi Kit

Arabidopsis sp	150 µg
Tobacco leaves	250 µg
Mustard leaves	150 µg
Maize Fresh Seeds	140 µg

Storage and Stability

All components of the E.Z.N.A.® Plant RNA Midi 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 HiBind® RNA midi column can bind approximately 500 µg RNA. Using greater than 1 g plant tissue usually will not dramatically improve yields and sometimes has adverse effects.

Kit Contents

Product No.	R6628-00	R6628-01	R6628-02
Purification Times	2 Preps	10 Preps	25 Preps
HiBind™ RNA Midi columns	2	10	25
15 ml Collection Tubes*	4	20	50
gDNA Filter Midi Column	2	10	25
Buffer RCL	10 ml	40 ml	90 ml
Buffer RCB	10 ml	40 ml	90 ml
Buffer RB	10 ml	40 ml	90 ml
RWC Wash Buffer	10 ml	50 ml	3 x 50ml
RNA Wash Buffer II	12 ml	2 x 12 ml	3 x 12ml
DEPC water	3 ml	20 ml	40 ml
User Manual	1	1	1

Note: 15 ml collection tubes have been inserted with HiBind™ RNA Midi Column and Homogenization Midi column.

Materials to be provided by user

- centrifuge capable of ≥4,000 x g
- Nuclease-free 15ml conical centrifuge tubes
- 2-mercaptoethanol
- Absolute (96%-100%) ethanol
- Isopropyl alcohol (isopropanol, optional for difficult samples)
- Liquid nitrogen for freezing/disrupting samples
- Preheat an aliquot (0.5 ml per sample) of DEPC water at 60°C.

Before Starting

IMPORTANT	1. RNA Wash Buffer II must be diluted with absolute ethanol (96-100%) as follows: **R6628-00** Add 48 ml absolute ethanol/bottle **R6628-01** Add 48 ml absolute ethanol/bottle **R6628-02** Add 48 ml absolute ethanol/bottle ***Store diluted RNA Wash Buffer II at room temperatur.*** 2. 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.

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 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.

E.Z.N.A.™ Plant RNA Midi Protocol I (Standard Protocol)

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 ≤500 mg. Best results are obtained with young leaves or needles.

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

1. Weigh up to 500 mg plant sample. Immediately place the weighed sample in liquid nitrogen, and grind throughly with a mortar and pestle. Decant the powder and liquid nitrogen into an RNase-free, liquid nitrogen cooled, 50 ml centrifuge tube (not supplied). Allow the liquid nitrogen to evaporate, but do not allow the sample to thaw.

2. **Immediately add 2.5ml Buffer RB/2-mercaptoethanol.** We recommend starting with 250 mg tissue at first. If results obtained are satisfactory increase amount of starting material. Add 20 µl 2-mercaptoethanol per 1 ml of Buffer RB. **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.

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

3. **Centrifuge the lysate at ≥4,000 x g for 10 min at room temperature. Transfer the supernatant directly into a gDNA filter Midi Column placed in collection tube.** Centrifuge at ≥4,000 x g for 5 min at room temperature.

4. **Discard the gDNA filter Midi Column and add equal volume of 70% ethanol (room temperature) to the flow-through. Mix by vortexing at maxi speed for 30 seconds.** If precipitation can be seen at this point, break the precipitation by passing through a needle using a syringe.

5. **Apply the entire mixture, including any precipitates that may form to a HiBind® RNA Midi column** assembled in a 15.0 ml collection tube (supplied). Close the cap gently. Centrifuge at ≥4,000 x g for 5 minutes 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 9 for detailed protocol.

6. **Add 3.5 ml RWC Wash Buffer, close the tube gently. Centrifuge at ≥4,000 x g for 2 minutes.** Discard both flow-through liquid and collection tube.

7. **Place column in a clean 15 ml collection tube (Not supplied), and add 3.5 ml RNA Wash Buffer II diluted with absolute ethanol. Close the column gently,** Centrifuge at 4,000 x g for 2 minutes at room temperature and discard flow-through. Re-use the collection tube in step 8.

   Note: RNA 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 3.5 ml of RNA Wash Buffer II by repeating step 7.** Centrifuge as above and discard flow-through. Then with the collection tube empty, centrifuge the Midi column for **10 min at >4000 x g** to completely dry the HiBind™ matrix.

9. **Elution of RNA. Transfer the column to a new RNase-free 15 ml centrifuge tube (not supplied with kit) and elute the RNA with 0.3-0.5 ml of DEPC water (supplied with kit).** Make sure to add water directly onto column matrix. Centrifuge at 4,000 x g for 5 minutes. A second elution into the same tube may be necessary if the expected yield of RNA >0.5mg.

   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.

E.Z.N.A.™ Plant RNA Midi 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.**

1. Weigh up to 500 mg plant sample. Immediately place the weighed sample in liquid nitrogen, and grind throughly with a mortar and pestle. Decant the powder and liquid nitrogen into an RNase-free, liquid nitrogen cooled, 50 ml centrifuge tube (not supplied). Allow the liquid nitrogen to evaporate, but do not allow the sample to thaw.

2. **Immediately add 2.5ml Buffer RCL/2-mercaptoethanol.** We recommend starting with 250 mg tissue at first. If results obtained are satisfactory increase amount of starting material. Add 20 µl 2-mercaptoethanol per 1 ml of Buffer RCL. **Samples should not be allowed to thaw before Buffer RCL/2-mercaptoethanol 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.

3. **Incubate at 55°C for 1-3 minutes.**

4. **Centrifuge the lysate at ≥4,000 x g for 10 min at room temperature. Transfer the supernatant directly into a gDNA filter Midi Column placed in collection tube.** Centrifuge at ≥4,000 x g for 5 min at room temperature.

5. **Discard the gDNA filter Midi Column and add equal volume of Buffer RCB to the flow-through. Mix by vortexing at maxi speed for 30 s.** If precipitation can be seen at this point, break the precipitation by passing through a needle using a syringe.

6. **Apply the entire mixture, including any precipitates that may form to a HiBind® RNA Midi column** assembled in a 15.0 ml collection tube (supplied). Close the cap gently. Centrifuge at ≥4,000 x g for 5 minutes 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 9 for detailed protocol.

7. **Add 3.5 ml RWC Wash Buffer, close the tube gently. Centrifuge at ≥4,000 x g for 2 minutes.** Discard both flow-through liquid and collection tube.

8. **Place column in a clean 15 ml collection tube (Not supplied), and add 3.5 ml RNA Wash Buffer II diluted with absolute ethanol. Close the column gently,** Centrifuge at 4,000 x g for 2 minutes at room temperature and discard flow-through. Re-use the collection tube in step 8.

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

9. **Wash column with a second 3.5 ml of RNA Wash Buffer II by repeating step 7.** Centrifuge as above and discard flow-through. Then with the collection tube empty, centrifuge the Midi column for **10 min at >4000 x g** to completely dry the HiBind™ matrix.

10. **Elution of RNA. Transfer the column to a new RNase-free 15 ml centrifuge tube (not supplied with kit) and elute the RNA with 0.3-0.5 ml of DEPC water (supplied with kit).** Make sure to add water directly onto column matrix. Centrifuge at 4,000 x g for 5 minutes. A second elution into the same tube may be necessary if the expected yield of RNA >0.5mg.

    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.

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 the standard protocol until the samples **completely** pass through the HiBind® RNA column. Prepare the following:

A. **Pipet 1.5 ml RWC Wash Buffer into the HiBind® RNA Midi-spin column, and centrifuge at 4000 x g for 5 minutes to wash the column.** Discard the flow through. For each HiBind® RNA Midi-spin column, prepare the DNase I digestion reaction mix as follows:

OBI DNase I Digestion Buffer	367.5 µl
RNase-free DNase I (20 Kunitz unites/µl)	7.5 µl
Total volume	375 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.**

B. Pipet 375 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 column.

C. Incubate at room temperature(25-30°C) for 15-30 minutes

2. **Place column in a clean 15.0ml collection tube**, and add 3 ml RWC Wash Buffer. **Incubate 5 minutes at room temperature.** Centrifuge at 4000 x g for 2 minutes and discard flow-through. Reuse the collection tube.

3. **Place column in the same 15 ml collection tube**, and add 3.5ml RNA Wash Buffer II diluted with ethanol. Centrifuge at 4000 x g for 5 minutes and discard flow-through. Reuse the collection tube.

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

4. Wash column with a second 3.5 ml of RNA Wash Buffer II by repeating step 3. Centrifuge and discard flow-through. Then with the collection tube empty, centrifuge the Midi-spin column at 4000 x g for **10 min at full speed** to completely dry the HiBind® matrix.

5. **Elution of RNA.** Transfer the column to a clean 15 ml microfuge tube (not supplied with kit) and elute the RNA with 500 µl of DEPC 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 70°C 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.

Prepare all necessary materials and reagents (listed on page 4) and follow the procedure below:

1. Freeze and grind up to 500 mg arthropod tissue under liquid nitrogen. Grind tissue completely to obtain a fine homogenous powder.

2. **Immediately add 2.5 ml Buffer RB/2-mercaptoethanol.** Add 20 µl 2-mercaptoethanol per 1ml of Buffer RB and then add 2.5 ml of this 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 µl 2-mercaptoethanol per 1 ml of Buffer RB before use. This mixture can be made and stored at room temperature for 1 week.

3. Proceed with the Plant RNA Midi Protocol I from step 3 (page 5).

RNA Isolation from Fungi

E.Z.N.A.® Plant RNA Midi Kit can also be used for fungal RNA isolation since many fungal samples possess similar cellular attributes as many plant specimens.

1. Freeze and grind up to 500 mg fungal sample under liquid nitrogen. Grind tissue completely to obtain a fine homogenous powder.

2. **Immediately add 2.5 ml Buffer RB/2-mercaptoethanol.** Add 10 µl 2-mercaptoethanol per 1ml of Buffer RB and then add 2.5ml of this 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.

3. Proceed with the Plant RNA Midi Protocol I from step 3 (page 5).

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 250mg 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 RNA 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 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 water is acidic and can dramatically lower Abs260 values. Use TE buffer (pH 8) to dilute RNA prior to spec analysis.