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## Introduction

The E.Z.N.A.® Plant RNA Midiprep Kit provides a convenient and rapid method for the isolation of total RNA from a variety of plant samples. The kit includes 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 500 mg of tissue. Typical yields are shown in Table 1. E.Z.N.A.® 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

| Yields obtained with E.Z.N.A.® Plant RNA Kits | |
|---|---|
| Arabidopsis sp | 150 µg |
| Tobacco leaves | 200 µg |
| Mustard leaves | 150 µg |
| Maize | 140 µg |

### Storage and Stability

All components of the E.Z.N.A.® 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 HiBind® RNA column can bind approximately100µg of RNA. Using greater than 200 mg of plant tissue usually will not dramatically improve yields and sometimes has adverse effects.

**Kit Contents**

| Product No. | R6628-00 | R6628-01 | R6628-02 |
|---|---|---|---|
| HiBind™ RNA columns | 2 | 10 | 25 |
| 15 ml Collection Tubes | 4 | 20 | 50 |
| Homogenizer Midi Columns | 2 | 10 | 25 |
| Buffer RPL | 10 ml | 40ml | 100 ml |
| Buffer SP | 2 ml | 10 ml | 20 ml |
| Buffer RB | 6 ml | 30 ml | 70 ml |
| RNA Wash Buffer I | 10 ml | 40 ml | 90 ml |
| RNA Wash Buffer II, Concentrate | 5 ml | 12 ml | 50 ml |
| DEPC-treated water | 1.5 ml | 20 ml | 30 ml |
| User Manual | 1 | 1 | 1 |

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

**Before Starting**
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.

| IMPORTANT | Dilute **Wash Buffer II** with **absolute ethanol** as follows<br><br>**R6628-00** Add 20ml 100 % ethanol<br>**R6628-01** Add 48 ml 100 % ethanol<br>**R6628-02** Add 200 ml 100% ethanol |
|---|---|

## Working with RNA

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

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

**Materials to be provided by user**

- Swing Bucket centrifuge capable of 3,000-5,000 x g
- Nuclease-free 15ml conical centrifuge tubes
- 2-mercaptoethanol
- Absolute (96%-100%) ethanol
- Isopropyl alcohol (isopropanol)
- Liquid nitrogen for freezing/disrupting samples
- Preheat an aliquot (500 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 ≤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 500mg of plant sample. Immediately place the weighed sample in liquid nitrogen, and grind thoroughly with a mortar and pestle. Decant the powder and liquid nitrogen into an Rnase-free, liquid nitrogen cooled, 50ml centrifuge tube (not supplied). Allow the liquid nitrogen to evaporate, but do not allow the sample to thaw.**

2. **Immediately add 2.5ml of Buffer RB/2-mercaptoethanol.** We recommend starting with 250 mg of tissue at first. If results obtained are satisfactory increase amount of starting material. Add 25µl of 2-mercaptoethanol per 2.5 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 of the 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. **Transfer the lysate directly into a Homogenization Midi-Spin Column placed in the collection tube. Centrifuge at** 3,000-5,000 **x g for 15 minutes at room temperature.**

4. **Carefully transfer the supernatant of the flow-through fraction to a new 15 ml centrifuge tube, making sure not to disturb the pellet**

**or transfer any debris. Add 0.5 volume of absolute ethanol and mix by vortexing.**

**TIP:** In most cases 2.0 ml supernatant can easily be removed. This will require 1.0 ml 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.

5. **Apply the entire sample, including any precipitates that may form to a HiBind® RNA Midi-Spin column** assembled in a 15.0 ml collecting tube (supplied). Centrifuge at 3,000-5,000 x g for 5 minutes at room temperature. Discard the flow-through liquid and place the column back into the collection tube.

   **Optional on-membrane DNase I digestion: This is the starting point to perform DNase I digestion. See Page 8 for detailed protocol.**

6. **Add 3.5 ml RNA Wash Buffer I. Centrifuge at** 3,000-5,000 **x g for 5 minutes.** Discard the flow-through liquid and place the column back into the collection tube.

7. **Add 3.5ml Wash Buffer II diluted with ethanol.** Centrifuge at 3,000-5,000 x g for 2 minutes at room temperature. Then discard the flow-through and place the column back into the collection tube.

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

8. **Wash column with a second 3.5ml of Wash Buffer II by repeating previous step.** Centrifuge and discard the flow-through.

9. Then with the collection tube empty, centrifuge the Midi-spin column for **10 min at** 3,000-5,000 **x g** to completely dry the HiBind™ matrix.

11. **Elution of RNA. Transfer the column to a new RNase-free 15 ml centrifuge tube (not supplied) and elute the RNA with 500µl of DEPC-treated water (supplied).** Make sure to add water directly onto column matrix. Centrifuge at 3,000-5,000 x g for 5 minutes. A second elution into the same tube may be necessary if the expected yield of RNA >300 µg.

    **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 got lower yield.**

1. **Grind plant sample as described on page 4. Collect frozen ground plant tissue (up to 500 mg) in a microfuge tube and immediately add 3.5ml of Buffer RPL/2-mercaptoethanol.** We recommend starting with 250 mg of tissue at first. If results obtained are satisfactory increase amount of starting material. Add 20µl of 2-mercaptoethanol per 1ml of Buffer RPL. **Samples should not be allowed to thaw before Buffer RPL/2-mercaptoethanol is added**. Vortex vigorously to make sure that all of the clumps are dispersed. RNA cannot be effectively extracted from clumped tissue.

   **Note**: **Add 20µl of 2-mercaptoethanol per 1 ml of Buffer RPL before use**. This mixture can be made and stored at room temperature for 1 week.

2. **Add 700µl of Buffer SP and vortex thoroughly to mix. Centrifuge at** 3,000-5,000 **x g for 20 minutes at room temperature.**

3. **Carefully aspirate cleared lysate to a RNase-free 15ml centrifuge tube making sure not to disturb the pellet or transfer any debris. Add one volume of isopropanol and vortex to precipitate RNA.** This step removes much of the polysaccharide content and improves spin-column performance by increasing RNA binding capacity (and therefore yield) in the steps that follow. No incubation is required after addition of isopropanol.

   **TIP:** In most cases 3.5 ml supernatant can easily be removed. This will require 3.5 ml isopropanol. 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 isopropanol.

4. **Immediately centrifuge at** 3,000-5,000 **x g for 20 min at room temperature to pellet RNA.** A longer centrifugation does not improve yields.

5. **Carefully aspirate or decant the supernatant and discard making sure not to dislodge the RNA pellet.** Invert the microfuge tube on a paper towel for 5 min to allow residual liquid to drain. Drying the pellet is not necessary.

6. **Add 500µl of RB buffer pre-heated to 65°C and vortex to resuspend the pellet.** A brief incubation at 65°C may be necessary to effectively dissolve the RNA.

7. **Add 1.25ml of Buffer RB/2-mercaptoethanol followed by 1.75ml of 70% ethanol.** Vortex thoroughly to mix. This will adjust binding conditions prior to loading the HiBind® RNA column.

8. **Apply the entire sample, including any precipitates that may form to an HiBind® RNA Midi-spin column** assembled in a clean 15 ml collection tube (supplied). Centrifuge at 3,000-5,000 x g for 5 minutes at room temperature. Discard the flow-through liquid and place the column back into the collection tube

   **Note:** This is the starting point to perform DNase I digestion. See page 8 for detail protocol.

9. **Add 3.5ml of RNA Wash Buffer I and centrifuge at** 3,000-5,000 **x g for 5 minutes.** Discard the flow-through liquid and place the column back into the collection tube.

10. **Add 3.5 ml of Wash Buffer II diluted with ethanol.** Centrifuge at 3,000-5,000 x g for 30 seconds at room temperature. Discard the flow-through and place the column back into the collection tube.

11. **Wash the column with a second 3.5 ml of Wash Buffer II as in previous step.** Centrifuge and discard the flow-through.

12. Then with the collection tube empty, centrifuge the Midi-spin column **at** 3,000-5,000 **x g for 10 minutes** to completely dry the HiBind™ matrix.

13. **Elution of RNA. Transfer the column to a RNase-free 15 ml microfuge tube (not supplied) and elute the RNA with 500µl of DEPC-treated water (supplied).** Make sure to add water directly onto column matrix. Centrifuge at 6000 x g for 5 minutes at room temperature. A second elution into the same tube may be necessary if the expected yield of RNA >300 µg.

## 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 have **completely** passed through the HiBind® RNA column. Prepare the following:

   A. Pipet 1.5ml of RNA Wash Buffer I into the HiBind® RNA Midi-spin column, and centrifuge at 3,000-5,000 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 matrix 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 minutes

2. **Place column into a clean 15.0ml collection tube**, and add 3ml of RNA Wash Buffer I. **Incubate for 5 minutes at room temperature.** Centrifuge at 6000 x g for 2 minutes and discard the flow-through. Reuse the collection tube.

3. **Place the column into the same 15ml collection tube**, and add 3.5ml of RNA Wash Buffer II diluted with ethanol. Centrifuge at 3,000-5,000 x g for 5 minutes and discard the flow-through. Reuse the collection tube.
   **Note:** 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 Wash Buffer II by repeating step 3. Centrifuge and discard the flow-through.

5. Then with the collection tube empty, centrifuge the Midi-spin column at 3,000-5,000 x g for **10 minutes at full speed** to completely dry the HiBind® matrix.

6..**Elution of RNA.** Transfer the column to a clean 15 ml microfuge tube (not supplied) and elute the RNA with 50-100 µl of DEPC-treated water (supplied). Make sure to add water directly onto the column matrix. Centrifuge for 1 minute 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 for 5 minutes 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 of arthropod tissue under liquid nitrogen. Grind tissue completely to obtain a fine homogenous powder.

2. **Immediately add 2.5 ml of Buffer RB/2-mercaptoethanol.** Add 20µl of 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 20µl of 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 prep Protocol from step 3 (page 4).

## RNA Isolation from Fungi

E.Z.N.A.® Plant RNA 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 of 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 10µl of 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 of the clumps are dispersed. RNA cannot be effectively extracted from clumped tissue.

3. Proceed with the Plant RNA Protocol from step 3 (page 4).

## Quantization and Storage of RNA

To determine the concentration and purity of RNA, measure absorbancy 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 absorbancy 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. 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.<br>• Pre-heat DEPC-water to 70° C prior to elution.<br>• Incubate column for 10 min with water prior to centrifugation. |
| | Column is overloaded | • Reduce the quantity of starting material. |
| **Clogged column** | Incomplete disruption or lysis of plant tissue. | • Completely disrupt sample in liquid nitrogen.<br>• Increase centrifugation time.<br>• Reduce amount of starting material |
| **Precipitated RNA will not dissolve.** | High nucleic acid and polysaccharide content. | • Reduce the amount of starting material. Generally it is best to start with 50-100 mg at first.<br>• 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.<br>• Follow protocol closely, and work quickly.<br>• Make sure that 2-mercaptoethanol is added to Buffer RPL.<br>• Use RB Buffer as dissolvent instead of DEPC water. |
| | RNase contamination | • Ensure not to introduce RNase during the procedure.<br>• 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.<br>• Diluted Wash Buffer II must be stored at room temperature.<br>• 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. |

## Ordering Information

| Product Number | Product Name | Description |
|---|---|---|
| **E.Z.N.A.™ Total RNA Miniprep Kits** | | |
| R6634-01/02<br>R6834-01/02 | E.Z.N.A.™ Total RNA Kit | Total RNA isolation from animal cells or tissues. |
| R6614-01/02<br>R6814-01/02 | E.Z.N.A.™ Blood RNA Kit | Total RNA Isolation from blood samples |
| R6627-01/02<br>R6827-01/02 | E.Z.N.A.™ Plant RNA Kit | Total RNA Isolation from plant samples |
| R6640-01/02<br>R6840-01/02 | E.Z.N.A.™ Fungal RNA Kit | Total RNA Isolation from fungal samples |
| R6670-01/02<br>R6870-01/02 | E.Z.N.A.™ Yeast RNA Kit | Total RNA Isolation from yeast samples |
| R6850-01/02<br>R6950-01/02 | E.Z.N.A.™ Bacterial RNA Kit | Total RNA Isolation from yeast samples |
| R6675-01/02<br>R6875-01/02 | E.Z.N.A.™ Mollusc RNA Kit | Total RNA Isolation from mollusc, invertebrates samples. |
| **E.Z.N.A.™ Total RNA Midi/maxi Kits** | | |
| R6664-01/02 | E.Z.N.A.™ Total RNA Midi Kit | Total RNA isolation from animal cells or tissues |
| R6693-01/02 | E.Z.N.A.™ Total RNA Maxi Kit | Total RNA isolation from animal cells or tissues |
| R6615-01/02 | E.Z.N.A.™ Blood RNA Midi Kit | Total RNA isolation from blood samples |
| R6616-01/02 | E.Z.N.A.™ Blood RNA Maxi Kit | Total RNA isolation from blood samples |
| R6628-01/02 | E.Z.N.A.™ Plant RNA Midi Kit | Total RNA isolation from plant samples |
| **Other RNA isolation kit, Reagent and supplies** | | |
| R6511-01/02 | mRNA Enrichment kit | mRNA isolation |
| R6830-01/02 | RNA-Solv™ reagent | Single reagent for total RNA isolation |
| R6248-01/02<br>R6249-01/02 | E.Z.N.A.™ RNA Probe purification kit | RNA Probe purification |
| R6376-01/02 | E.Z.N.A.™ Poly-Gel RNA Isolation Kit | Isolate RNA from poly-acrylamide gel |
| R6500-01/02 | E.Z.N.A.™ Oligo (dT) Cellulose | High capacity oligo(dT) cellulose |
| E1091 | RNase-free DNase I set | DNase I set for on-column DNase digestion |