

Contents

Introduction.....	2
Overview.....	2
Storage and Stability.....	2
Kit Contents.....	3
Before Starting.....	3
Fungal RNA Protocol A (Standard Protocol).....	4
Fungal RNA Protocol B (For difficult Samples).....	6
On-Membrane DNase Digestion Protocol.....	8
RNA Isolation from Arthropods.....	10
Quantization of RNA.....	11
Troubleshooting.....	12

Revised September 2006

Introduction

E.Z.N.A.™ Fungal RNA Miniprep Kit provides a rapid and reliable method for isolation of total RNA from a wide variety of fungal samples. The kit does not require the use of cumbersome or expensive shredding/homogenizing accessories as an attempt to shear viscous fungal lysates. Rather, the method involves a simple and rapid precipitation step for removal of much of the polysachrides and phenolic compounds commonly found in fungal tissues. In combination with HiBind® RNA spin columns, this method permits purification of high quality RNA from as much as 200 mg tissue. The system is efficient enough to allow total RNA from as little as 10 mg of tissue or 100 cells. Typical yields are shown in Table 1. The procedure involves no organic extractions, thus reducing plastic waste and hands-on time. E.Z.N.A.™ Fungal RNA Kits are ideal for processing multiple fungal samples in parallel in 1 hour. Purified RNA has A260/A280 ratios of 1.8-2.0 and is suitable for the following applications:

- RT-PCR
- Northern Analysis
- Differential display
- Poly A+ RNA selection

Acremonium crysogenum	50 µg
Fusarium avenaceu	37 µg
Mushrooms	43 µg

Storage and Stability

All components of the E.Z.N.A.™ Fungal RNA Kit are stable for at least 24 months from date of purchase when stored at 22°C-25°C. During shipment or storage in cool ambient conditions, precipitates may form in RB Buffer. It is possible to dissolve such deposits by warming the solution at 37°C, though we have found that they do not interfere with overall performance.

Binding Capacity

Each HiBind® RNA column can bind approximately 100 µg RNA. Using greater than 250 mg fungal tissue in many cases will not dramatic improve yields and sometimes has adverse affects.

Kit Contents

Product Number	R6640-00 R6840-00	R6640-01 R6840-01	R6640-02 R6840-02
HiBind [®] RNA Columns	5	50	200
Homogenizer Columns	5	50	200
2 ml Collection Tubes	15	150	600
Buffer RFL	5 ml	40 ml	150 ml
Buffer SP	2 ml	10 ml	40 ml
Buffer RB	5 ml	30 ml	120 ml
RNA Wash Buffer II	5 ml	12 ml	50 ml
RNA wash Buffer I	5 ml	30 ml	120 ml
DEPC Water	1 ml	5ml	20 ml
Instruction Booklet	1	1	1

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

Before Starting

Please read the entire booklet to become familiar with the E.Z.N.A.™ Fungal RNA procedure.

- Dilute Wash Buffer Concentrate with ethanol as follows and **store at room temperature**.

R6640-00 R6840-00	Add 20 ml absolute (96%-100%) ethanol.
R6640-01 R6840-01	Add 48 ml absolute (96%-100%) ethanol to each bottle.
R6640-02 R6840-02	Add 200 ml absolute (96%-100%) ethanol to each bottle.

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

E.Z.N.A.™ Fungal RNA Protocol A (Standard Protocol)

Materials to be provided by user

- Microcentrifuge capable of 10,000 x g
- Nuclease-free microfuge tubes
- 2-mercaptoethanol
- Absolute (96%-100%) ethanol
- Isopropyl alcohol (isopropanol)
- Liquid nitrogen for freezing/disrupting samples
- 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 fungal sample, sample size should be limited to ≤ 100 mg. 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 2.0 ml microfuge tube and freeze by dipping in liquid nitrogen with a pair of tweezers to fill the tube. Grind the tissue using disposable homogenization pestles (available from Omega Bio-Tek, Cat# SSI-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 fungal tissue cannot be replaced with mechanical homogenizers.

Note: All centrifugation steps must be carried out at room temperature.

- Collect frozen ground fungal sample (up to 100 mg) in a microfuge tube and immediately add 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 600 µl 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 sample.

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

2. **Pipet the lysate directly into a Homogenization Spin Column (Green) placed in 2ml collection tube. Centrifuge at $\geq 13,000 \times g$ for 5 min at room temperature.**

3. **Carefully transfer the supernatant of the flow-through fraction to a new 1.5ml microfuge tube, making sure not to disturb the pellet or transfer any debris. Add 0.5 volume absolute ethanol and mix by vortexing.**

TIP: In most cases 450 µl supernatant can easily be removed. This will require 225µl 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 spin column (Red) assembled in a clean 2 ml collecting tube (supplied). Close the cap gently. Centrifuge at 10,000 x g for 30 sec 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 8 for detailed protocol.

5. **Add 500 µl RNA Wash Buffer I, close the tube gently. Centrifuge at 10,000 x g for 30 sec.** Discard both flow-through liquid and collecting tube.

6. **Place column in a clean 2ml collection tube (supplied), and add 700 µl Wash Buffer II diluted with ethanol. Close the column gently,** Centrifuge at 10,000 x g for 30 sec at room temperature and discard flow-through. Re-use the collection tube 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 column with a second 500 µl of Wash Buffer II by repeating step 6.** Centrifuge and discard flow-through. Then with the collection tube empty, centrifuge the spin cartridge for **1 min at full speed** to completely dry the HiBind™ matrix.

8. **Elution of RNA. Transfer the column to a clean 1.5 ml microfuge tube (not supplied with kit) and elute the RNA with 50-100 µl of DEPC-treated water (supplied with kit).** Make sure to add water directly onto column matrix. Centrifuge 1 min at maximum speed. 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 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. 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.

E.Z.N.A.™ Fungal RNA Protocol B (for difficult samples)

Certain fungal 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 fungal tissues. Use this protocol when standard protocol did not yield RNA or get lower yield.

1. **Grind fungal sample as described on page 4. Collect frozen ground fungal tissue (up to 100 mg) in a microfuge tube and immediately add 600 µl Buffer RFL/2-mercaptoethanol.** We recommend starting with 50 mg tissue at first. If results obtained are satisfactory increase amount of starting material. Add 20 µl 2-mercaptoethanol per 1ml of Buffer RFL and then add 600 µl to the sample. **Samples should not be allowed to thaw before Buffer RFL/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 RFL 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. Process in sets of four to six tubes: fill all tubes with liquid nitrogen, grind, add Buffer RFL/2-mercaptoethanol, and continue to step 2 before starting another set. Centrifuge all tubes simultaneously (step 2 below). 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 140 µl Buffer SP and vortex thoroughly to mix. Centrifuge at 10,000 x g for 10 min at room temperature.**
3. **Carefully aspirate cleared lysate to a new microfuge tube making sure not to disturb the pellet or transfer any debris. Add one volume 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 600 µl supernatant can easily be removed. This will require 600 µl 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 10,000 x g for 2 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 1 min to allow residual liquid to drain. Drying the pellet is not necessary.
6. **Add 100 µl of sterile DEPC-treated water (supplied) or 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.
Important: RB buffer is recommended to dissolve the RNA pellet at this step, especially when the degradation was found after the elution. RB buffer contains strong RNase inhibitor. In some case, it is difficult to dissolve the RNA pellet with RB Buffer, DEPC-treated water should be used in those case.
7. **If DEPC-treated water was used to dissolve RNA pellet in step 6, add 350 µl Buffer RB/2-mercaptoethanol followed by 250 µl absolute ethanol. If RB buffer was used in step 6, add 250 µl Buffer RB/2-mercaptoethanol followed by 350 µl of 70% ethanol.** Vortex thoroughly to mix. This will adjust binding conditions prior to loading the HiBind® RNA column.

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.

8. **Apply the entire sample, including any precipitates that may form to an HiBind® RNA spin column** assembled in a clean 2 ml collecting tube (supplied). Centrifuge at 10,000 x g for 30 sec at room temperature. Discard the flow-through liquid and place the column back into the collecting tube

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

9. **Add 500 µl RNA Wash Buffer I and centrifuge at 10,000 x g for 30 sec.** Discard both flow-through liquid and collecting tube.
10. **Place column in a clean 2ml collection tube (supplied), and add 500 µl Wash Buffer II diluted with ethanol.** Centrifuge at 10,000 x g for 30 sec at room temperature and discard flow-through. Reuse the collection tube in step 11.
11. **Wash column with a second 500 µl of Wash Buffer II as in step 10.** Centrifuge and discard flow-through. Then with the collection tube empty, centrifuge the spin cartridge for **1 min at full speed** to completely dry the HiBind™ matrix.
12. **Elution of RNA. Transfer the column to a clean 1.5 ml microfuge tube (not supplied with kit) and elute the RNA with 50-100 µl of DEPC-treated water (supplied with kit).** Make sure to add water directly onto column matrix. Centrifuge 1 min at maximum speed. A second elution into the same tube may be necessary if the expected yield of RNA >50 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 **completely** pass through the HiBind® RNA column (Steps 1-7). Prepare the following:

- a. For each HiBind® RNA 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.**
- b. 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 column.
- c. Incubate at room temperature(25-30°C) for 15 minutes
- 2. Place column in a clean 2ml collection tube**, and add 500 µl RNA Wash Buffer I. **(If on-membrane DNase digestion was performed in the previous step, wait at least 5 minutes before proceeding).** Centrifuge and discard flow-through. Reuse the collection tube in Step 7.
 - 3. Place column in the same 2ml collection tube**, and add 500 µl RNA Wash Buffer II diluted with ethanol. Centrifuge and discard flow-through. Reuse the collection tube in step 7.
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 500 µl of Wash Buffer II by repeating step 3.** Centrifuge and discard flow-through. Then with the collection tube empty, centrifuge the spin cartridge for **1 min at full speed** to completely dry the HiBind® matrix.
 - 5. Elution of RNA.** Transfer the column to a clean 1.5 ml microfuge tube (not supplied with kit) and elute the RNA with 50-100 µl of DEPC-treated 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.

RNA Isolation from Arthropods

The exoskeleton of arthropods poses the same problems as encountered with many plant and fungal specimens. Pigments and polysaccharides often co-purify with nucleic acids and interfere with downstream applications. The E.Z.N.A.™ Fungal RNA Kit includes a simple isopropanol precipitation step that improves RNA quality and column performance when difficult samples are processed.

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

1. Freeze and grind up to 100 mg arthropod tissue under liquid nitrogen. Grind tissue completely to obtain a fine homogenous powder.
2. **Immediately add 500 µl Buffer RB/2-mercaptoethanol.** Add 20 µl 2-mercaptoethanol per 1ml of Buffer RB and then add 500 µl 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 Fungal RNA Protocol from step 2 (page 4).

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.® Fungal 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	<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 amount of starting material.
Clogged column	Incomplete disruption or lysis of fungal 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, use Buffer RB to dissolve the RNA pellet.
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 RFL.
	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.