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Introduction

E.Z.N.A.? Fungal RNA 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 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. E.Z.N.A.? Fungal RNA Kits are ideal for processing multiple fungal samples parallel in 1 hour. There are no organic extractions thus reducing plastic waste and hands-on time to allow multiple samples to be processed in parallel. Purified RNA has A260/A280 ratios of 1.8-20 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.? Fungal RNA Kits	
Acremonium crysogenum	50 mg
Fusarium avenaceu	37 mg
Mushrooms	43 mg

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. 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 ug RNA. Using greater than 250 mg fungal tissue in many cases will not dramatical improve yields and sometimes has adverse affects.

Kit Contents

Product No.	R6840-00	R6840-01	R6840-02
Purification times	5 Preps	50 Preps	200 Preps
HiBind? RNA Mini columns	5	50	200
2 ml Collection Tubes	15	100	400
RB Buffer	5 ml	30 ml	100 ml
RNA Wash Buffer I	5 ml	45 ml	180 ml
RNA Wash Buffer II Concentrate	2 ml	12 ml	50 ml
DEPC water	5 ml	20 ml	50 ml
User Manual	1	1	1

Before Starting

IMPORTANT	RNA Wash Buffer II Concentrate must be diluted with absolute ethanol as follows.
	R6840-00 Add 8 ml 96-100 % ethanol
	R6840-01 Add 48 ml 96-100 % ethanol
	R6840-02 Add 20 ml 96-100% ethanol
	Once diluted, store RNA Wash Buffer II at room temp

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

Materials to be provided by user

- Microcentrifuge capable of 10,000 x g
- Nuclease-free microfuge tubes
- 2-mercaptoethanol
- Absolute (96%-100%) ethanol
- 70% ethanol
- Liquid nitrogen for freezing/disrupting samples
- Preheat an aliquot (100 µl per sample) of DEPC-treated water at 65°C.
- Chloroform
- Water saturated phenol
- 2M NaAc (pH 4.0)

E.Z.N.A.® Fungal RNA Protocol

Note: Use extreme caution when handling liquid nitrogen.

This protocol is suitable for most fresh or frozen fungal tissue samples allowing efficient recovery of RNA. However, due to the tremendous variation in water and polysaccharide content of fungal, 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 a1.5-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 Kontes pellet 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 fungal tissue **cannot** be replaced with mechanical homogenizers.

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

1. **Collect frozen ground fungal tissue (up to 100 mg) in a microfuge tube and immediately add 500 µl 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 µl 2-mercaptoethanol per 1ml 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 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.

   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 RB/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 500 µl of water-saturated phenol and 100 µl 2M NaAc (pH 4.0)to the sample**. Vortex at maxi speed for 15 seconds.

3. **Add 0.2 mL of chloroform to the sample**. Cap sample tubes securely and shake vigorously for 15 seconds. Incubate on ice for 10 minutes.

4. **Centrifuge at 12,000 x g for 15 minutes at 4°C.** The mixture separates into a lower phenol-chloroform phase, an interphase, and an upper aqueous phase. RNA remains entirely in the aqueous phase.

5. Carefully transfer the upper aqueous phase to a new tube. **Add equal Volume of 70% ethanol to the sample**.Vortex at maxi speed for 10 seconds

6. **Apply the entire sample, including any precipitates that may form to a HiBind® RNA Mini column 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.

7. **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

8. **Place column in a clean 2 ml collection tube (supplied), and add 700 µl RNA 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: 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 500 µl of RNA Wash Buffer II by repeating step 8.** 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.

10. **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 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 intronspanning primers, call our technical staff at 800-832-8896 for assistance. We can help design primers suited to your needs.

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 Minicolumn (Steps 1-6). Prepare the following:

   A. Add 300 µl of RNA wash Buffer I to the column and centrifuge at ≥10,000 x g for 1 minute.

   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**

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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 500 µl RNA Wash Buffer I. **Incubate 5 minutes at room temperature.** Centrifuge at 10,000 x g for 30 sec and discard flow-through. 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 at 10,000 x g for 30 sec and discard 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 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 **2 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 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.

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	• 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 Fungal 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 50-100 mg 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 RB • 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 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	• 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.