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Introduction

E.Z.N.A.™ Fungal DNA Miniprep Kits allows for the rapid and reliable isolation of high-quality total cellular DNA from a wide variety of fungal species and tissues. Up to 200 mg of wet tissue (or up to 50 mg dry tissue) can be processed in less than 1 hour. The system combines the reversible nucleic acid-binding properties of Omega's HiBind® matrix with the speed and versatility of spin column technology to eliminate polysaccharides, phenolic compounds, and enzyme inhibitors from fungal tissue lysates. Purified DNA is suitable for PCR, restriction digestion, and hybridization techniques. There are no organic extractions, thus reducing plastic waste and hands-on time to allow multiple samples to be processed in parallel.

Overview

If using the E.Z.N.A.™ Fungal DNA Miniprep Kit for the first time, please read this booklet to become familiar with the procedures. Dry or fresh fungal tissue is disrupted and then lysed in a specially formulated buffer containing detergent. Proteins, polysaccharides, and cellular debris are subsequently precipitated. Contaminants are further removed by isopropanol precipitation of DNA. Binding conditions are then adjusted and the sample is applied to an HiBind® DNA spin-column. Two rapid wash steps remove trace contaminants such as residual polysaccharides, and pure DNA is eluted in water or low ionic strength buffer. Purified DNA can be directly used in downstream applications without the need for further purification.

Storage and Stability

All components of the E.Z.N.A.™ Fungal Miniprep 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 Buffer FG3. It is possible to dissolve such deposits by warming the solution at 37°C.

Kit Contents

Product Number	D3390-00	D3390-01	D3390-02
HiBind® DNA Columns	5	50	200
2 ml Collection Tubes	10	100	400
Buffer FG1	5 ml	50 mL	180 mL
Buffer FG2	2 mL	10 mL	40 mL
Buffer FG3	2 mL	20 mL	80 mL
Equilibration Buffer	1.5 mL	7 mL	25 mL
RNase A	40 µL	250 µL	1 mL
DNA Wash Buffer Concentrate	12 mL	48 mL	2 x 60 mL
Elution Buffer	1.5 mL	15 mL	50 mL
Instruction Booklet	1	1	1

* Equilibration Buffer contains Sodium Hydroxide. Wear gloves and safety glasses when handling.

Before Starting

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

- Pre-heat Elution Buffer to 65° C.
- Dilute Wash Buffer Concentrate with ethanol as follows and **store at room temperature**.

D3390-00	Add 18 mL absolute (96%-100%) ethanol.
D3390-01	Add 72 mL absolute (96%-100%) ethanol to each bottle.
D3390-02	Add 90 mL absolute (96%-100%) ethanol to each bottle.

- Choose the most appropriate protocol to follow. Procedures are described for each of dried and fresh (or frozen) specimens. In addition, a short protocol is given for isolation of DNA for PCR reactions.

A. Dry Specimens (Page 4)	For processing ≤ 50 mg powdered tissue. Yield is sufficient for several tracks on Southern assay.
B. Fresh/Frozen Specimens (Page 6)	For processing ≤ 200 mg fresh (or frozen) tissue. Yield is similar to A.
C. Short protocol (Page 9)	Rapid protocol for dried or fresh samples. Yield is sufficient for PCR.

Fungal DNA Miniprep Protocol

A. Dry Specimens

Materials to be provided by user

- Microcentrifuge capable of at least 10,000 x g
- Nuclease-free 1.5 mL or 2 mL microfuge tubes
- Water bath equilibrated to 65° C
- Equilibrate sterile dH₂O water at 65° C
- Isopropyl alcohol (isopropanol)
- Absolute (96%-100%) ethanol
- Paper towels

This is the most robust method for isolation of total cellular (mitochondrial, chloroplast, and genomic) DNA. Yields are usually sufficient for several tracks on a Southern blot for RFLP mapping.

Drying allows storage of field specimens for prolonged period of time prior to processing. Samples can be dried overnight in a 45° C oven, powdered, and stored dry at room temperature. To prepare dried samples place ~50 mg of dried tissue into a microfuge (2 mL tubes are recommended for processing of >50 mg tissue) tube and grind using a pellet pestle. Disposable Kontes pestles work well and are available from Omega Bio-Tek (Cat# SSI1015-39). For critical work such as PCR and cloning, pestles are best used a single time then soaked in a dilute bleach solution immediately after use until cleaning. Disposable pestles may be autoclaved several times. For standard Southern analysis, the same pestle can be reused several times to grind multiple tissue samples by rinsing with ethanol and wiping the surface clean between samples. A fine powder will ensure optimal DNA extraction and yield. Process in sets of four to six tubes until Step 2 before starting another set.

1. **To 10-50 mg powdered dry tissue add 800 μ L Buffer FG1.** Vortex vigorously to mix. Make sure to disperse all clumps.
TIP: Process in sets of four to six tubes: grind, add Buffer FG1, then proceed to Step 2 before starting another set. Do not exceed 50 mg dried tissue.
2. **Incubate at 65°C for 10 min. Mix sample twice during incubation by inverting tube.**
3. **Add 180 μ L Buffer FG2 and vortex to mix. Incubate on ice for 5 minutes. Centrifuge at $\geq 10,000 \times g$ for 10 min.**
4. **Carefully aspirate supernatant to a new microfuge tube, making sure not to disturb the pellet or transfer any debris. Add 0.7 volume isopropanol and vortex to precipitate DNA.** This step will remove much of the polysaccharide content and improves spin-column performance by increasing DNA binding capacity (and hence yield) in the steps that follow. No incubation is required after addition of isopropanol.
TIP: In most cases 700 μ L supernatant can easily be removed. This will require 490 μ L isopropanol (i.e., 0.7 volume). 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.
5. **Immediately centrifuge at 10,000 $\times g$ for 2 min to pellet DNA.** Longer centrifugation does not improve yields.
6. **Carefully aspirate or decant the supernatant and discard, making sure not to dislodge the DNA pellet.** Invert the microfuge tube on a paper towel for 1 min to allow residual liquid to drain. It is not necessary to dry the DNA pellet.
7. **Add 300 μ L of sterile deionized water pre-heated to 65°C and vortex to resuspend the pellet.** A brief incubation at 65°C may be necessary to effectively dissolve the DNA. Add 4 μ L RNase and mix. No additional incubation is required for RNase treatment.
TIP: While incubating at 65°C to dissolve the DNA, label and place the required number of HiBind® DNA columns in 2 mL collection tubes.
8. Add 100 μ L of equilibration buffer to the HiBind DNA column and incubate for 4 minutes. Centrifuge at maximum speed for 30-60 seconds. Discard flowthrough.
9. **Adjust binding conditions of the sample by adding 150 μ L Buffer FG3**

followed by 300 μ L absolute ethanol and vortex to obtain a homogeneous mixture. A precipitate may form upon addition of ethanol; it will not interfere with DNA isolation.

- 10.. **Apply the entire sample (including any precipitate that may have formed) to a HiBind® DNA column placed in a 2 mL collection tube (supplied) .** Centrifuge the column at 10,000 $\times g$ for 1 min to bind DNA. Discard both the 2 mL collection tube and the flow-through liquid.
11. **Transfer column to a second collection tube and wash by adding 750 μ L Wash Buffer diluted with absolute (96%-100%) ethanol.** Centrifuge at 10,000 $\times g$ for 1 min and discard the flow-through liquid. Reuse the collection tube in Step 12 below.
NOTE: Wash Buffer Concentrate must be diluted with absolute (96%-100%) ethanol prior to use. Follow directions on label.
12. **Repeat wash step with an additional 750 μ L Wash Buffer.** Centrifuge at 10,000 $\times g$ for 1 min. Discard flow-through and reuse 2 mL collection tube in Step 13
- 13 **Centrifuge empty column 2 min at maximum speed to dry.** This step is **critical** for removing residual ethanol that may otherwise be eluted with DNA and interfere with downstream applications.
14. **Transfer column to a clean 1.5 mL tube. Apply 100 μ L Elution Buffer (or sterile deionized water) pre-warmed to 65°C and incubate at room temperature for 3 to 5 min. Centrifuge at 10,000 $\times g$ for 3 to 5 min to elute DNA.** Smaller volumes will significantly increase DNA concentration but give lower yields. Use of more than 200 μ L of buffer for elution is not recommended.
15. **Repeat Step 14 with an additional 100 μ L of Elution Buffer.** This may be performed using another 1.5 mL tube to maintain a higher DNA concentration in the first eluate.
TIP: To increase DNA concentration add Elution Buffer and incubate the column at 60°C-65°C for 5 min before elution

Total DNA yields vary depending on type and quantity of sample. Typically, 10-50 μ g DNA with a A_{260}/A_{280} ratio of 1.7-1.9 can be isolated using 50 mg dried tissue.

B. Fresh or Frozen Specimens

Materials to be provided by user

Microcentrifuge capable of 10,000 x g

- Nuclease-free microfuge tubes
- Waterbath equilibrated to 65°C
- Equilibrate sterile dH₂O water at 65°C
- Absolute (96%-100%) ethanol
- Isopropyl alcohol (isopropanol)
- Liquid nitrogen for freezing/disrupting samples
- Paper towels

NOTE: Use extreme caution when handling liquid nitrogen.

This protocol is suitable for most fresh or frozen tissue samples, allowing efficient recovery of DNA. However, due to the tremendous variation in water and polysaccharide content of various fungi, sample size should be limited to ≤200 mg. Best results are obtained with young leaves or needles. The method isolates sufficient DNA for several tracks on a standard Southern assay.

To prepare samples, 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 Kontes pellet pestles, which are available from Omega Bio-Tek (Cat# SSI1015-39). Alternatively, one can allow liquid nitrogen to evaporate and then store samples at -70°C for later use. For critical work such as PCR and cloning, pestles are best used a single time then soaked in a dilute bleach solution immediately after use until clean. Disposable pestles may be autoclaved several times. For standard Southern analysis, the same pestle can be reused several times to grind multiple tissue samples by rinsing with ethanol and carefully wiping the surfaces clean between samples.

1. **Collect ground fungal tissue (start with 100 mg) in a microfuge tube and immediately add 600 µL Buffer FG1.** Vortex vigorously. Make sure to disperse all clumps. DNA cannot be effectively extracted from clumped tissue.

TIP: Process in sets of four to six tubes: fill all tubes with liquid nitrogen, grind, add Buffer FG1, then proceed to Step 2 before starting another set. As a starting point, use 100 mg tissue per tube; if yield and purity are satisfactory increase to 200 mg.

2. **Incubate at 65°C for 10 min. Mix sample twice during incubation by inverting tube.**
3. **Add 140 µL Buffer FG2 and vortex to mix. Incubate on ice for 5 minutes. Centrifuge at ≥10,000 x g for 10 min.**
4. **Carefully aspirate cleared lysate to a new microfuge tube making sure not to disturb the pellet or transfer any debris. Add 0.7 volume isopropanol and vortex to precipitate DNA.** This step will remove much of the polysaccharide content and improves spin-column performance by increasing DNA binding capacity (and hence 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 420 µL isopropanol (i.e., 0.7 volume). 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.
5. **Immediately centrifuge at 10,000 x g for 2 min to pellet DNA.** Longer centrifugation does not improve yields.
6. **Carefully aspirate or decant the supernatant and discard making sure not to dislodge the DNA pellet.** Invert the microfuge tube on a paper towel for 1 min to allow residual liquid to drain. It is not necessary to dry the DNA pellet.
7. **Add 300 µL of sterile deionized water pre-heated to 65°C and vortex to resuspend the pellet.** A brief incubation at 65°C may be necessary to effectively dissolve the DNA. Add 4 µL RNase and mix. No additional incubation is required for RNase treatment.

TIP: While incubating at 65°C to dissolve the DNA, label and place the required number of HiBind® DNA columns in 2 mL collection tubes.
8. **Adjust binding conditions of the sample by adding 150 µL Buffer FG3 followed by 300 µL absolute ethanol and vortex to obtain a homogeneous mixture.** A precipitate may form upon addition of ethanol; it will not interfere with DNA isolation.

9. Add 100 µl of equilibration buffer to the HiBind DNA column and incubate for 4 minutes. Centrifuge at maximum speed for 30-60 seconds. Discard flowthrough.
10. **Apply the entire sample (including any precipitate that may have formed) to a HiBind® DNA column placed in a 2 mL collection tube (supplied) .** Centrifuge the column at 10,000 x g for 1 min to bind DNA. Discard both the 2 mL collection tube and the flow-through liquid.
11. **Transfer column to a second collection tube and wash by adding 750 µL Wash Buffer diluted with absolute (96%-100%) ethanol.** Centrifuge at 10,000 x g for 1 min and discard the flow-through liquid. Reuse the collection tube in Step 11 below.
NOTE: Wash Buffer Concentrate must be diluted with absolute (96%-100%) ethanol prior to use. Follow directions on label.
12. **Repeat wash step with an additional 750 µL Wash Buffer.** Centrifuge at 10,000 x g for 1 min. Discard flow-through and reuse 2 mL collection tube in Step 13.
13. Centrifuge empty column 2 min at maximum speed to dry. This step is critical for removing residual ethanol that may otherwise be eluted with DNA and interfere with downstream applications.
14. Transfer column to a clean 1.5 mL tube. Apply 100 µL Elution Buffer (or sterile deionized water) pre-warmed to 65°C and incubate at room temperature for 3 to 5 min. Centrifuge at 10,000 x g for 3 to 5 min to elute DNA. Smaller volumes will significantly increase DNA concentration but give lower yields. Use of more than 200 µL of buffer for elution is not recommended.
15. Repeat Step 14 with an additional 100 µL of Elution Buffer. This may be performed using another 1.5 mL tube to maintain a higher DNA concentration in the first eluate.
TIP: To increase DNA concentration add Elution Buffer and incubate the column at 60°C-65°C for 5 min before elution

Total DNA yields vary depending on type and quantity of sample. Typically, 20-50 µg DNA with a A_{260}/A_{280} ratio of 1.7-1.9 can be isolated using 200 mg fresh leaf tissue.

C. Short Protocol

This simplified method allows rapid isolation of DNA from fresh, frozen, or dried specimens for use in PCR reactions. The procedure limits the amount of starting material, so that DNA yields will generally be lower than those obtained with Protocols A and B. Thus, this short protocol is not recommended for Southern analysis or cloning work, as in most cases there will not be sufficient material.

Materials to be provided by user

- Microcentrifuge capable of 10,000 x g
- Nuclease-free microfuge tubes
- Waterbath equilibrated to 65°C
- Sterile dH₂O water equilibrated at 65°C
- Absolute (96%-100%) ethanol
- Liquid nitrogen for freezing/disrupting fresh samples
- Paper towels

Follow suggestions for preparation of dried or fresh samples as outlined in Sections A and B (Pages 4 and 6 respectively). Note the following limitations on sample size:

- **Dry Samples - Use a maximum of 10 mg ground tissue**
 - **Fresh Samples - Use a maximum of 40 mg fresh/frozen ground tissue**
1. **Collect ground sample in a microfuge tube and add 600 µL Buffer FG1 and 5 µL RNase (20 mg/mL).** Vortex vigorously to mix and incubate at room temperature for 1 min. Add 10 µL 2-mercaptoethanol and vortex to mix.
 2. **Incubate at 65°C for at least 5 min.** Mix sample once during incubation by inverting tube.
 3. **Add 140 µL Buffer FG2 and vortex to mix. Incubate on ice for 5 minutes. Centrifuge at $\geq 10,000$ x g for 10 min.**
 4. **Carefully aspirate 600 µL supernatant to a new microfuge tube making sure not to disturb the pellet or transfer any debris. Add ½ volume of**

Buffer FG3 and one volume of absolute ethanol. Vortex thoroughly to obtain a homogeneous mixture. A precipitate may form but will not affect the procedure.

TIP: Volume of supernatant will vary, and is usually lower with dried samples. For 600 µL of supernatant add 300 µL Buffer FG3 followed by 600 µL absolute ethanol.

5. Add 100 µL of equilibration buffer to the HiBind DNA column and incubate for 4 minutes. Centrifuge at maximum speed for 30-60 seconds. Discard flowthrough.
6. **Apply 800 µL of the mixture to a HiBind® DNA column assembled in a 2mL collection tube (supplied).** Centrifuge at 10,000 x g for 1 min to bind DNA. Discard flow-through liquid and reuse collection tube in the next step.
7. **Add the remainder of the sample (including any precipitate that may have formed) to the column.** Centrifuge at 10,000 x g for 1 min and discard both the 2mL collection tube and the flow-through liquid.
8. Place the column in a second 2mL tube and add 750 µL Wash Buffer diluted with absolute ethanol. Centrifuge at 10, 000 x g for 1 min and discard flow-through liquid. Keep 2 mL tube.
NOTE: Wash Buffer Concentrate must be diluted with absolute ethanol before use. Follow directions on bottle.
9. **Repeat wash step with an additional 750 µL Wash Buffer.** Centrifuge at 10,000 x g for 1 min. Discard flow-through and reuse 2 mL collection tube in Step 10.
10. **Centrifuge empty column 2 min at maximum speed to dry.** This step is critical for removing residual ethanol that may otherwise be eluted with DNA and interfere with downstream applications.
11. **Transfer column to a clean 1.5 mL tube. Add 100 µL DNA Elution Buffer. (or sterile deionized water) pre-warmed to 65°C and incubate at room temperature for 1 min. Centrifuge at 10,000 x g for 1 min to elute DNA.** Smaller volumes will significantly increase DNA concentration but give lower yields. Use of more than 200 µL of buffer for elution is not recommended.
12. **Repeat Step 10 with an additional 100 µL of Elution Buffer.** This may be

performed using another 1.5 mL tube to maintain a higher DNA concentration in the first eluate.

TIP: To increase DNA concentration add buffer and incubate the column at 60°C-70°C for 5 min before elution.

Yields vary according to sample size and whether dried or fresh. Between 2 µg-10 µg restrictable DNA can usually be obtained with this method.

Vacuum/Spin Protocol

Note: Please read through previous sections of this manual before using this protocol.

1. **Prepare wet or dry samples by following the standard Protocol in previous sections until loading DNA/FG3/Ethanol mixture to HiBind® DNA column.**
2. Prepare the vacuum manifold according to manufacturer's instructions and connect the V-Spin column to the manifold.
3. Load the DNA/FG3/Ethanol solution to the column.
4. Switch on vacuum source to draw the sample through the column and turn off the vacuum.
5. Wash the column by adding 750 µL DNA wash buffer. Draw the wash buffer through the column by turning on the vacuum source. Repeat this step with another 750 µL DNA wash buffer.
6. **Assemble the column into a 2 mL collection tube and transfer the column to a micro centrifuge. Spin 1 minute to dry the column.**
7. Place the column in a clean 1.5 mL microcentrifuge tube and add 100 µL DNA Elution Buffer or water. Stand for 1-2 minute and centrifuge 1 minute to elute DNA.

Troubleshooting Guide

Problem	Cause	Suggestions
Clogged column	Carry-over of debris.	Following precipitation with Buffer FG2, make sure no particulate material is transferred.
	DNA pellet not completely dissolved before applying sample to column.	In protocols A and B, ensure that DNA is dissolved in water before adding Buffer FG3 and ethanol. This may need repeated incubation at 65°C and vortexing.
	Sample too viscous.	In protocol C, do not exceed suggested amount of starting material. Alternatively, increase amounts of Buffers FG1 and FG2 and use two or more columns per sample.
	Incomplete precipitation following addition of FG2.	Increase RCF or time of centrifugation after addition of buffer FG2.
Low DNA yield	Incomplete disruption of starting material.	For both dry and fresh samples, obtain a fine homogeneous powder before adding Buffer FG1.
	Poor lysis of tissue.	Decrease amount of starting material or increase amount of Buffers FG1 and FG2.
	DNA remains bound to column.	Increase elution volume to 200 µL and incubate on column at 65°C for 5 min before centrifugation.
	DNA washed off.	Dilute Wash Buffer Concentrate by adding appropriate volume of absolute ethanol prior to use (page 3).
Problems in downstream applications	Salt carry-over.	Wash Buffer must be at room temperature.
	Ethanol carry-over	Following the second wash spin, ensure that the column is dried by centrifuging 2 min at maximum speed.