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Introduction

The E.Z.N.A.™ High Performance (HP) Fungal DNA Midi Kit is designed for efficient recovery of genomic DNA up to 60 kb in size from fresh and dried Fungal tissue samples rich in polysaccharides or lower DNA contents. Up to 0.5g of wet tissue (or 150 mg dry tissue) can be processed in less than 1 hour. The system combines the reversible nucleic acid-binding properties of the 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.™ High Performance (HP) Fungal DNA Midi Kit for the first time, please read this booklet to become familiar with the procedures. This procedure relies on the well established properties of the cationic detergent, cetyltrimethyl ammonium bromide (CTAB), in conjunction with the selective DNA binding of Omega Bio-Tek's HiBind® matrix. Samples are homogenized and lysed in a high salt buffer containing CTAB and extracted with chloroform to remove polysaccharides and other components that interfere with many routine DNA isolations and downstream applications. Binding conditions are then adjusted and DNA is further purified using HiBind® DNA spin columns. In this way salts, proteins and other contaminants are removed to yield high quality genomic DNA suitable for downstream applications such as endonuclease digestion, thermal cycle amplification, and hybridization techniques.

Storage and Stability

All components of the E.Z.N.A.™ HP Fungal DNA Midi 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 CPL and Buffer CXD. 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.

Kit Contents

Product Number	D3196-00	D3196-01	D3196-02
HiBind® DNA Midi Columns	2	10	25
15 mL Collection Tubes	2	10	25
Buffer CPL	20 mL	80 mL	160 mL
Buffer CXD	10 mL	20 mL	50 mL
SPW Wash Buffer	10 mL	25 mL	2 x 25 mL
Elution Buffer (10mM Tris,pH8.5)	5 mL	20 mL	60 mL
Instruction Booklet	1	1	1

Before Starting

- Please read the entire booklet to become familiar with the E.Z.N.A.™ High Performance Fungal DNA Kit Midi procedure.
- Dilute SPW Wash Buffer Concentrate with ethanol as follows and **store at room temperature**.
 - D3196-00** Add 40 mL absolute (96%-100%) ethanol.
 - D3196-01** Add 100 mL absolute (96%-100%) ethanol to bottle
 - D3196-02** Add 100 mL absolute (96%-100%) ethanol per 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 ~150 mg powdered tissue. DNA Yields range from 25µg to more than 100µg/100mg dry tissue.
 - B. Fresh/Frozen Specimens** (Page 6) For processing ≤0.5g fresh (or frozen) tissue. Yield is similar to A.
 - C. Lower DNA content samples** (Page 9) For processing up to 0.3g dried or 1g fresh (or frozen) tissue. Yield is similar to A.

E.Z.N.A.™ High Performance Fungal DNA Midi Protocol

A. Dry Specimens

Materials to be provided by user:

- Swinging-bucket centrifuge capable of 3,000-5,000 x g
- Nuclease-free 15 mL microfuge tubes
- chloroform:isoamyl alcohol (24:1)
- Waterbath equilibrated to 65°C
- Absolute (96%-100%) ethanol
- RNase A stock solution at 50 mg/mL
- Optional: 2-mercaptoethanol

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 periods 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 ~300 mg of dried tissue into a 50 mL microfuge tube and grind using a pellet pestle. 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 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 75-150mg powdered dry tissue add 4 ml Buffer CPL in a 50 mL microfuge tube.** Optional: Add 40 µl 2-mercaptoethanol and vortex vigorously to mix. Make sure to disperse all clumps.

TIP: Process in sets of four to six tubes: grind, add Buffer CPL and 2-mercaptoethanol, and proceed to Step 2 before starting another set. Initially, do not exceed 150 mg dried tissue. Amount can be increased according to results.

2. **Incubate at 65°C for 30-60 min. Mix sample twice during incubation by inverting tube.** Optional: If necessary, add 60 µL of RNase into the lysate before incubation to remove the RNA.
3. **Add 4 mL chloroform/Isoamyl alcohol (24:1) and vortex to mix.** Centrifuge at 3,000-5,000 x g for 10 min.
4. **Carefully aspirate 3 ml of the supernatant to a new 15 mL microfuge tube making sure not to disturb the pellet or transfer any debris.**

5. **Adjust binding conditions of the sample by adding 1.5ml Buffer CXD followed by 3 ml absolute ethanol and vortex to obtain a homogeneous mixture.** A precipitate may form upon addition of ethanol; it will not interfere with DNA isolation.

NOTE: This the point to start the optional vacuum/spin protocol. (See Page 10 for details.)

6. **Apply 4 ml of the sample (including any precipitate that may have formed) to a HiBind® DNA Midi column placed in a 15 mL collection tube (supplied).** Centrifuge the column in at 3,000-5,000 x g for 5 min to bind DNA. Discard the flow-through liquid and reuse the collection tube in the next step.
7. Repeat Step 6 by transferring the remaining of the mixture into the column. Discard flow-through and reuse collection tube.
8. **Wash the column by adding 3mL SPW Wash Buffer diluted with absolute (96%-100%) ethanol.** Centrifuge at 3,000-5,000 x g for 5 min and discard the flow-through liquid. Reuse the collection tube in next step below.
NOTE: SPW Wash Buffer Concentrate must be diluted with absolute (96%-100%) ethanol prior to use. Follow directions on label.
9. **Repeat wash step with an additional 3 mL SPW Wash Buffer.** Centrifuge at 3,000-5,000 x g for 5 min. Discard flow-through and reuse 2.0 mL collection tube in next step.
10. **Place the column into collection tube and centrifuge empty column 15 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 15 mL tube. Apply 0.3-0.5 mL Elution Buffer (or sterile deionized water) pre-warmed to 65°C and immediately centrifuge at maximum speed for 5 min to elute DNA.** Smaller volumes will significantly increase DNA concentration but give lower yields.
12. **Repeat Step 11 with an additional 0.3-0.5 mL of Elution buffer.** This may be performed using another 15 mL tube to maintain a higher DNA concentration in the first eluate.

B. Fresh/Frozen Specimens

Materials to be provided by user:

- Swinging-bucket centrifuge capable of 3,000-5,000 x g
- Nuclease-free 15 mL microfuge tubes
- Water bath equilibrated to 65°C
- Equilibrate sterile dH₂O water or 10 mM Tris, pH 9.0 or 8.5 at 65°C.
- Absolute (96%-100%) ethanol
- chloroform:isoamyl alcohol (24:1)
- Liquid nitrogen for freezing/disrupting samples
- RNase A stock solution at 50 mg/mL
- Optional: 2-mercaptoethanol

NOTE: Use extreme caution when handling liquid nitrogen.

This protocol is suitable for most fresh or frozen tissue samples allowing more efficient recovery of DNA. However, due to the tremendous variation in water and polysaccharide content of Fungals, sample size should be limited to ≤1g. 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 50 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 VWR (Cat# KT749521-0500). 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 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 carefully wiping the surfaces clean between samples.

1. **Collect ground Fungal tissue (start with 0.25-0.5 g) in a 15 mL microfuge tube and immediately add 4 ml Buffer CPL.** Add 40 µL 2-mercaptoethanol and 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 CPL and 2-mercaptoethanol; proceed to Step 2 before starting another set.
2. Incubate at 65°C for 15-30 min. Mix sample twice during incubation by inverting tube. Optional: If necessary, add 100 µL of RNase into the lysate and incubate at 37°C for 30-60 min.
3. **Add 4ml chloroform/Isoamyl alcohol (24:1) and vortex to mix.** Centrifuge at ≥3,000-5,000 x g for 10 min.

4. Carefully aspirate 3ml supernatant to a new 15 mL microfuge tube making sure not to disturb the pellet or transfer any debris.
5. **Adjust binding conditions of the sample by adding 1.5 mL Buffer CXD followed by 3 ml absolute ethanol and vortex to obtain a homogeneous mixture.** A precipitate may form upon addition of ethanol; it will not interfere with DNA isolation.
6. **Apply 4 ml of the sample (including any precipitate that may have formed) to a HiBind® DNA Midi column placed in a 15 mL collection tube (supplied) .** Centrifuge the column at 3,000-5,000 x g for 5 min to bind DNA. Discard the flow-through liquid and repeat by transferring the remaining of the mixture into the column. Centrifuge as above. Discard flow-through and reuse collection tube.
NOTE: This the point to start the optional vacuum/spin protocol. (See Page 10 for details.)
7. **Wash the column by adding 3 mL SPW Wash Buffer diluted with absolute (96%-100%) ethanol.** Centrifuge at 3,000-5,000 x g for 5 min and discard the flow-through liquid. Reuse the collection tube in next step below.

NOTE: SPW Wash Buffer Concentrate must be diluted with absolute (96%-100%) ethanol prior to use. Follow directions on label.
8. **Repeat wash step with an additional 3 mL SPW Wash Buffer.** Centrifuge at 3,000-5,000 x g for 5 min. Discard flow-through and reuse 50 mL collection tube in next step below.
9. **Place the column into collection tube and centrifuge empty column 15 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.
10. **Transfer column to a clean 15 mL tube. Apply 0.3-0.5 mL 10 mM Tris buffer, pH 9.0 or 8.5 (or sterile deionized water), pre-warmed to 65°C and incubate at room temperature for 5 min.** Centrifuge at maxi speed for 5 min to elute DNA.
11. **Repeat Step 10 with an additional 0.3-0.5ml Elution buffer.** This may be performed using another 15 mL tube to maintain a higher DNA concentration in the first eluate.

C. Protocol For Samples With Lower DNA Contents

This modified method allows rapid isolation of DNA from fresh, frozen, or dried specimens for sample types with lower DNA content or when larger yields are essential. The procedure increases the amount of starting material so that DNA yields will generally be higher than those obtained with Protocols A and B.

NOTE: The buffer supplies with this kit is designed for standard protocols (Protocol A & B). Additional buffer will be required by using this protocol. Buffers can be purchase separately from OBI, please contact OBI or its distributors for order information.

Materials to be provided by user:

- Swinging-bucket centrifuge capable of 3,000 x g
- Nuclease-free microfuge tubes
- Nuclease-free 15 mL tubes
- Waterbath equilibrated to 65°C
- Sterile dH₂O water or 10 mM Tris, pH 9.0 or 8.5 equilibrated at 65°C
- chloroform:isoamyl alcohol (24:1)
- Absolute (96%-100%) ethanol
- Liquid nitrogen for freezing/disrupting fresh samples
- RNase A stock solution at 20 mg/mL
- Optional: 2-mercaptoethanol

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 300 mg ground tissue**
 - **Fresh Samples - use a maximum of 1g fresh/frozen ground tissue**
1. **Collect ground sample in a 15 mL polypropylene tube and add 6 mL Buffer CPL.** Add 10 µL 2-mercaptoethanol per mL Buffer CPL and vortex to vigorously to mix.
 2. **Incubate at 65°C for 30-60 min.** Mix sample twice during incubation by inverting tube.
 3. **Add 6.0mL chloroform/Isoamyl alcohol (24:1) and vortex to mix.** Centrifuge at 3,000-5,000 x g for 10 min.
 4. **Carefully aspirate top aqueous phase to a new 15 mL tube making sure not to disturb the pellet or transfer any debris.**
 5. **Add 0.7 volume isopropanol and vortex to precipitate DNA.**

6. **Immediately centrifuge at 3,000-5,000 x g for 20 min to pellet DNA.** Longer centrifugation does not improve yields.
7. **Carefully aspirate or decant the supernatant and discard making sure not to dislodge the DNA pellet.** Place inverted microfuge tube on a paper towel for 1 min to allow residual liquid to drain. It is not necessary to dry the DNA pellet.
8. **Add 1 mL of sterile deionized water, pre-heated to 65°C, to each tube and vortex to resuspend the pellet.** Add 20 µL RNase (20 mg/mL) and mix. Incubate at 65°C for 30-60 minutes to effectively dissolve the DNA.
9. **Adjust binding conditions of the sample by adding 500 µL Buffer CXD followed by 1mL 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 mixture to a HiBind® DNA Midi column assembled in a 15 mL collection tube (supplied).** Centrifuge at 3,000-5,000 x g for 5 min to bind DNA. Discard flow-through liquid and reuse collection tube in the next step.
11. Wash the column by adding 3ml SPW Wash Buffer diluted with absolute ethanol. Centrifuge at 3,000-5,000 x g for 5 min and discard flow-through liquid.

NOTE: SPW Wash Buffer Concentrate must be diluted with absolute ethanol before use. Follow directions on bottle.

12. **Repeat wash step with an additional 3 mL Wash Buffer.** Centrifuge at 3,000-5,000 x g for 5 min. Discard flow-through and reuse 15 mL collection tube.
13. **Centrifuge empty column 15 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 15 mL tube. Add 0.3-0.5mL Elution Buffer (or sterile deionized water) pre-warmed to 65°C and incubate at room temperature for 5 min. Centrifuge at Maximum speed for 5 min to elute DNA.**
15. **Repeat Step 14 with an additional 0.3-0.5 mL Elution buffer.** This may be performed using another 50 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 for Fungal DNA Isolation (V-Spin column only)

Note: Please read through previous section of this book before using this protocol.

1. Prepare wet or dry samples by following the standard Protocol in previous sections until loading DNA/CDX/Ethanol mixture to HiBind[®] DNA Midi column.
2. Prepare the vacuum manifold according to manufacturer's instruction and connect the midi column to the manifold.
3. Load the DNA/CXD/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 3mL SPW wash buffer, draw the wash buffer through the column by turn on the vacuum source. Repeat this step with another 3ml SPW wash buffer.
6. Assemble the column into a 15 mL collection tube and transfer the column to a micro centrifuge. Spin 15 minute to dry the column.
7. Place the column in a clean 15 mL microcentrifuge tube and add 0.5-1mL elution Buffer or water. Centrifuge at maximum speed for 5 minute to elute DNA.

Troubleshooting Guide

Problem	Cause	Suggestions
Clogged column	Carry-over of debris.	Following extraction with chloro:isoamyl alcohol, make sure no particulate material is transferred.
	DNA pellet not completely dissolved before applying sample to column.	In Protocols C, ensure that DNA is dissolved in water before adding Buffer CXD and ethanol. This may need repeated incubation at 65°C and vortexing.
	Sample too viscous.	Do not exceed suggested amount of starting material. Alternatively, increase amounts of Buffers CPLand CXD and use two or more columns per sample.
Low DNA yield	Incomplete disruption of starting material.	For both dry and fresh samples, obtain a fine homogeneous powder before adding Buffer CPL
	Poor lysis of sample.	Decrease amount of starting material or increase amount of Buffers CPL, chlorosoamyl alcohol,and CXD.
	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.	DNA 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.