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Introduction

E-Z 96® Fungal DNA Kits allow rapid and reliable isolation of high-quality total cellular DNA from a wide variety of fungal species in a 96 well plate. Up to 30 mg of wet sample (or 10 mg dry sample) can be processed in each well in less than 1 hour. The system combines the reversible nucleic acid-binding properties of HiBind® matrix with the speed and versatility of E-Z 96® DNA plate 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 up to 96 samples to be processed at one time.

Overview

If using the E- Z 96® Fungal DNA Kit for the first time, please read this booklet to become familiar with the procedure. Dry or fresh fungal sample is disrupted and then lysed in a specially formulated buffer containing a proprietary detergent mixture. 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 E-Z 96® DNA plate. 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 96® Fungal DNA Kit are stable for at least 24 months from date of purchase when stored at 22ºC-25ºC. 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 37ºC.

Kit Contents

Product Number	D1090-01	D1090-02
Preps	2 x 96	8 x 96
E-Z 96® DNA Plate	2	8
96 well Collection Plate (300 µL)	2	8
96 well Collection Plate (2 mL)	2	2
Buffer FG1	90 mL	350 mL
Buffer FG2	35 mL	125mL
Buffer FG3	60 mL	250 mL
DNA Wash Buffer Concentrate	120 mL	2 x 300 mL
Elution Buffer	100 mL	400 mL
Instruction Booklet	1	1

Before Starting

Please read the entire booklet to become familiar with the E-Z 96® Fungal DNA Kit procedures.

Important	Prepare an RNase stock solution at 20 mg/mL and aliquot into adequate portions. Store each aliquot at -20°C and thaw before use. Each sample will require 10 µL of this solution.
	Dilute **Wash Buffer** Concentrate with **ethanol** as follows and store at room temperature. **D1090-01** Add 180 mL absolute (96%-100%) ethanol to each bottle. **D1090-01** Add 450 mL absolute (96%-100%) ethanol to each bottle.

Sample Size

Choose the most appropriate protocol to follow. Procedures are described for 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 ≤10 mg powdered sample in each well. Yield can be up to 50 µg, depending on the species of Fungal.
B. Fresh/Frozen Specimens (page 6)	For processing ≤30 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.

E-Z 96 Fungal DNA Protocol

Materials to be provided by user:

- Laboratory centrifuge equipped with swinging-bucket rotor (for centrifugation protocol).
- Rotor-adapter for deep-well microplate (for centrifugation processing)
- Nuclease-free 1.5 mL or 2 mL microfuge tubes
- Waterbath equilibrated to 65°C
- Equilibrate sterile dH_2O water or 10 mM Tris pH 9.0 at 65°C.
- Isopropyl alcohol (isopropanol; absolute)
- Absolute (96%-100%) ethanol
- RNase A stock solution at 20 mg/mL
- Absorbent paper towels
- Liquid nitrogen for freezing/disrupting samples (For fresh/Frozen specimens)
- Vacuum manifold (for vacuum manifold processing only)

Section I: Sample Preparation

A. Dry Specimens.
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 ~10 mg of dried tissue into a microfuge tube and grind using a pellet pestle. Disposable Kontes pestles work well and are available (Cat# SSI-1015-39 & SSI-1014-39). Addition of a pinch of white quartz sand, -50 to 70 mesh (Sigma Chemical Co. Cat No. S9887) will help. For critical work such as PCR and cloning, pestles are best used a single time. Crucibles may be 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. The samples can also be processed in suitable 96 well blocks with a mechanical mixer.

1. **To 10 mg powdered dry sample add 400 µl 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, and 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 or shaking the plate.**

3. **Add 140 µL Buffer FG2 and vortex to mix.** Incubate the sample for 10 minutes at -20° C. This step helps to remove the proteins, polysacchrides and other inhibitors.

3. **Centrifuge at ≥10,000 x g for 10 min. (If 96-well block is used, centrifuge at 4000 x g for 20 minutes to pellet the precipitate.)**

   NOTE: *Optional* E-Z® 96 Lysate Clearance Plates (Product No. EZ1096C) are available for use with this kit at this step. Optionally, desired volume of supernatant following -20° C incubation in Step 5 can be transferred to the E-Z® 96 Filter Plate to be placed over a 1.2 mL 96-well plate (not supplied) and centrifuged at 3,000-5,000 x g for 5 min.

4. **Carefully aspirate supernatant to a 96-well plate (not supplied) making sure not to disturb the pellet or transfer any debris. Add 0.7 volume isopropanol and vortex to mix the sample and precipitate DNA.** This step will remove much of the polysaccharides 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 350 µL supernatant can easily be removed. This will require 245 µL isopropanol (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 5 min to pellet DNA.** Longer centrifugation does not improve yields. **(If a 96-well plate is used, centrifuge at 4000 x g for 25 minutes to pellet the precipitate.)**

6. **Carefully aspirate or decant the supernatant and discard, making sure not to dislodge the DNA pellet.** Invert the plate on a paper towel for 1 min to allow residual liquid to drain. It is not necessary to dry the DNA pellet.

7. **Add 150 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 10 µL RNase A (20 mg/mL) and mix. No additional incubation is required for RNase A treatment.

8. **Adjust binding conditions of the sample by adding 75 µL Buffer FG3, followed by 150 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. **Apply the entire sample (including any precipitate that may have formed) to an E-Z 96® DNA plate by following the procedure described in section II (Vacuum Manifold Processing) or section III (Centrifugation Protocol).**

B. Fresh/Frozen Specimens

NOTE: Use extreme caution when handling liquid nitrogen.

This protocol is suitable for most fresh or frozen samples allowing more efficient recovery of DNA. However, due to the tremendous variation in water and polysaccharide content of fungi, sample size should be limited to ≤30 mg.

To prepare samples, collect sample 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# SSI-1014-39 & SSI-1015-39). Alternatively, one can allow liquid nitrogen to evaporate and then store samples at -70ºC for later use. For critical work such as PCR and cloning, pestles are best used a single time. Crucibles may be 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 sample in a microfuge tube (or a 2mL 96-well plate) and immediately add 300 µL Buffer FG1.** Mix throughly by vortexing. Make sure to disperse all clumps. DNA cannot be effectively extracted from clumped tissue.

2. **Incubate at 65°C for 10 min. Mix sample twice during incubation by inverting tube or shaking the plate.**

3. **Add 100 µL Buffer FG2 and vortex to mix.** Incubate the sample for 10 minutes at -20°C. This step helps to remove the proteins, polysacchrides and other inhibitors.

3. **Centrifuge at ≥10,000 x g for 10 min. (If 2 mL 96-well plate is used, centrifuge at 4000 x g for 20 minutes to pellet the precipitate.)**

   NOTE: ***Optional*** E-Z® 96 Lysate Clearance Plates (Product No. EZ1096C) are available for use with this kit at this step. Optionally, desired volume of supernatant following -20°C incubation in Step 5 can be transferred to the E-Z® 96 Filter Plate to be placed over a 1.2 mL 96-well plate (not supplied) and centrifuged at 3,000-5,000 x g for 5 min.

4. **Carefully aspirate cleared lysate to a new microfuge tube (or 96-well plate) 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 polysaccharides 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 300 µL supernatant can easily be removed. This will require 210 µL isopropanol (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 5 min to pellet DNA.** Longer centrifugation does not improve yields. **(If a 96-well plate is used, centrifuge at 4000 x g for 25 minutes to pellet the precipitate.)**

6. **Carefully aspirate or decant the supernatant and discard making sure not to dislodge the DNA pellet.** Invert the microfuge tube or the 96-well plate on a paper towel for 1 min to allow residual liquid to drain. It is not necessary to dry the DNA pellet.

7. **Add 150 µL of sterile deionized water, pre-heated to 65°C, and vortex to resuspend the pellet.**. A brief incubation at 65ºC may be necessary to effectively dissolve the DNA. Add 10 µL RNase A (20 mg/mL) and mix. No additional incubation is required for RNase A treatment.

8. **Adjust binding conditions of the sample by adding 75 µL Buffer FG3, followed by 150 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. **Apply the entire sample (including any precipitate that may have formed) to an E-Z 96® DNA plate by following the procedure described in Section II (Vacuum Manifold Processing) or Section III (Centrifugation Protocol).**

Section II: Vacuum Manifold Processing

Note: The following protocol is based on using OBI's vacuum manifold (Product No. VAC-03).

1. **Set up vacuum manifold by following manufacturer's instructions.**

2. **Place waste collection tray inside the vacuum manifold, then place the E-Z 96® DNA plate on top part of the manifold.**

3. **Apply the entire sample (including any precipitate that may have formed) to an E-Z 96® DNA plate**.

   Note: It is always good idea to mark the E-Z 96® DNA plate and collection plate at this stage so that they can be easily identified throughout the protocol.

   ***Do not touch the rim of the wells with pipet tips to avoid cross-contamination.**

4. **Turn on the vacuum manifold and filter through the sample mixture by vacuum. Turn off the vacuum.**

5. **Add 700 µL DNA wash buffer into each well of the E-Z 96™ DNA plate by using multichannel pipet. Turning on vacuum until all the liquid through the plate. (Dilute the DNA wash buffer with ETOH before use.)**

6. **Wash the plate with another 700 µL DNA wash buffer by repeating step 5.**

7. **Repeat Step 6 by washing the plate with 400 µL 100% ethanol. Continue vacuum until the E-Z 96® DNA plate is completely dried.**

8. **Remove the E-Z 96® DNA plate from manifold and tap hard on a stack of paper towels to remove any residue ethanol. Discard the flow-through and collection plate.**

   Note: It is very important to completely dry the E-Z 96® DNA plate before elution. If a swing bucket centrifuge and 96-well plate adaptor are available, centrifuge at 4,000 x g for 10 minutes to dry the plate.

9. **Assemble the manifold by placing a new 300 µL collection plate (supplied) inside the vacuum manifold**. If an Omega VAC-03 is used, one 800 µL plate should be place under the 300 µL plate to give proper height for elution.

10. **Place the E-Z 96® DNA plate atop the vacuum manifold.**

11. **To elute the DNA, add 100 ul of preheated (65°C) Elution Buffer to each well using a multichannel pipet. Incubate for 5 min at room temperature. Apply the vacuum to elute the DNA into collection plate.**

    TIP: 100 ul water or TE buffer is sufficient to elute up to 85% of the DNA from each well of the E-Z 96® DNA plate. A second elution step with same 100 ul elutate containing DNA, reheated to 65°C, will increase yield by up to 10-15%.

    Total DNA yields vary depending on type and amount of sample. Typically, 5-10 µg DNA with a A_{260}/A_{280} ratio of 1.7-1.9 can be isolated using 10 mg dried tissue.

Section III: Centrifugation Processing

1. **Transfer the entire sample from step 8 of Section I into the E-Z 96® DNA plate. Seal the top of the DNA plate with adhesive plate film. Use a register chart to identify the positions of the samples.**

   Note: Do not touch the rims of the wells with pipet tips to avoid cross-contamination.

2. **Place the E-Z 96® DNA plate atop a 2 mL 96 well collection plate (supplied). Connect the two plates by using plastic tape if necessary.**

3. **Place the plates into the centrifuge and spin at 4,000 x g for 5 min.**

4. **Separate the plates and discard the flow-through liquid.** Re-use the collection plate.

5. Remove the adhesive plate film and carefully add 700 ul of DNA wash Buffer to each well of the E-Z 96® DNA plate.

6. **Seal the E-Z 96® DNA plate with new adhesive plate film.**

7. Reassemble the E-Z 96® DNA plate with the collection plate. Centrifuge at 4,000 x g for 5 min.

8. **Separate the plates and discard the flow-through liquid. Wash the DNA plate again with another 700 µL DNA wash Buffer by repeating step 5-7.**

9. Remove the adhesive film, discard the flow-through and place the E-Z 96® DNA plate atop the 300 µL collection plate (supplied). Centrifuge at 4,000 x g for 10 minutes.

   Note: Drying the membrane at this step is very important for DNA elution in next step. The residue of the DNA wash buffer contains ethanol which will inhibit PCR and cause low yield of DNA.

10. **To elute the DNA, add 100 ul of preheated (65°C) Elution Buffer to each well using a mutichannel pipet. Seal the E-Z 96® DNA plate with new adhesive film and incubate for 5 min at room temperature. Centrifuge at 4,000 x g for 5 min.**

    100 ul Elution Buffer is sufficient to elute up to 85% of the DNA from each well of the E-Z 96® DNA plate. A second elution step with same 100 ul elute containing DNA, reheated to 65°C, will increase yield by up to 10-15%

Section IV: Short Protocol

This simplified method allows rapid isolation of DNA from fresh, frozen, or dried specimens for use in PCR reactions. This procedure limits the amount of starting material, so DNA yields will generally be lower than those obtained with Protocols A and B in Section I.

Materials to be provided by user:

- 96-well tube rack (Part# SSI-1760-00, sold separately) or a deep well plate.
- Microcentrifuge capable of at least 10,000 x rpm
- Laboratory centrifuge equipped with swinging-bucket rotor (for centrifugation protocol)
- Rotor adapter for deep-well plate (for centrifugation processing)
- Nuclease-free 1.5 mL or 2 mL microfuge tubes
- Waterbath equilibrated to 65°C
- Equilibrate sterile dH_2O water or 10 mM Tris pH 9.0 at 65°C.
- Isopropyl alcohol (isopropanol; absolute)
- Absolute (96%-100%) ethanol
- RNase A stock solution at 20 mg/mL
- Liquid nitrogen for freezing/disrupting samples (For fresh/Frozen specimens)

Follow suggestions for preparation of dried or fresh samples as outlined in sections I. Note the following limitations on sample size:

- **Dry Samples - use a maximum of 3 mg ground tissue**
- **Fresh Samples - use a maximum of 10 mg fresh/frozen ground tissue**

1. **Follow the protocols in Section I to prepare the dried or fresh samples.**

2. **Add 3 mg ground dried sample or 10 mg fresh/frozen samples to each microtube or to each well of the plate.**

   Tip: it is always good idea to mark the tube rack and use a register (96 well) to identify the positions of the samples

3. **Add 400 µL Buffer FG1 to each well of the 96 well plate.**

4. **Add 140 µL Buffer P2 to each well of the plate. Seal the plate with tape and shake to mix.**

5. **Incubate at 65°C for 10 min. Mix sample twice during incubation by shaking the plate.**

6. **Place the tube rack or 96-well plate into a centrifuge and spin at 4,000 x g for 15 min.**

7. **Carefully transfer the clear supernatant to a new microtube rack or deep well plate using multiple channel transfer pipet**.

   Note: Mark the plate to identify the positions of samples.

8. **Add ½ volume of Buffer FG3 into each well followed by addition of one volume of ethanol (96-100%). Seal the plate with adhesive sealing film. Mix sample by shaking the plate.**

   Tip: If the volume of cleared supernatant from step 7 is 300 µL, add 150 µL of FG3 followed by 300 µL absolute ethanol.

9. **Place the E-Z 96® DNA plate onto top of a 2 mL 96 well collection plate (supplied).**

10. **Load the sample from Step 8 into each well of the E-Z 96® DNA plate. Centrifuge at 4,000 x g for 5 min.**

11. Remove the adhesive plate film and discard the flow-through.

12. Carefully add 700 ul of DNA wash Buffer to each well of the E-Z 96® DNA plate.

13. **Seal the E-Z 96® DNA plate with new adhesive plate film.**

14. Reassemble the E-Z 96® DNA plate with the collection plate. Centrifuge at 4,000 x g for 5 min.

15. **Separate the plates and discard the flow-through liquid. Wash the DNA plate again with another 700 µL DNA wash Buffer by repeating step 12-14.**

16. Remove the adhesive film, discard the flow-through and place the E-Z 96® DNA plate atop the **300 µL** collection plate (supplied). Centrifuge at 4,000 x g for 10 minutes.
    Note: Drying the membrane at this step is very important for DNA elution in next step. The residue of the DNA wash buffer contains ethanol which will inhibit PCR and cause low yield of DNA.

17. **To elute the DNA, add 100 ul of preheated (65°C) Elution Buffer to each well using a mutichannel pipet. Seal the E-Z 96® DNA plate with new adhesive film and incubate for 5 min at room temperature. Centrifuge at 4,000 x g for 5 min.**

Troubleshooting Guide

Problem	Cause	Suggestions
Clogged well	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.	Do not exceed suggested amount of starting material. Alternatively, increase amounts of Buffers FG1 and FG2 proportionally.
	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 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 step, ensure that the plate is dried by centrifuging 10 min at 4000 x g.