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Introduction

The E.Z.N.A.® Insect DNA Kit is designed for efficient recovery of genomic DNA up to 60 kb in size from insects, arthropods, roundworms, flatworms, and some plant tissue samples rich in polysaccharides. The method is suitable for samples frozen or preserved in alcohol or DNE solution, and good results can be obtained with formalin preserved material. The 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. Following a rapid alcohol precipitation step, binding conditions are adjusted and DNA 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.™ Insect DNA Kit, except the Proteinase K and RNase A should be stored at 22°C-25°C. Once reconstituted in water, Proteinase K should be stored -20°C.Under at these conditions, DNA has successfully been purified and used for PCR after 24 months of storage. During shipment, or storage in cool ambient conditions, precipitates may form in some buffers. It is possible to dissolve such deposits by incubation the solution at 65°C. Store RNase A at -20°C.

Expiration Date: All E.Z.N.A.® Insect DNA Kit components are guaranteed for at least 24 months from the date of purchase when stored at 22-25°C.

Binding Capacity

Each HiBind™ DNA column can bind approximately 100 µg DNA. Using greater than 30 mg tissue is not recommended.

Kit Contents

Product	D0926-00	D0926-01	D0926-02
Purification times	5	50	200
HiBind® DNA Columns	5	50	200
2 ml Collecting tubes	10	100	400
Buffer CTL	2 ml	20 ml	80 ml
Buffer CBL	2.5 ml	25 ml	100 ml
Proteinase K	3 mg	30 mg	4 x 30 mg
RNase A	30 µl	270 µl	1.1 ml
Buffer HB	3 ml	30 ml	110 ml
DNA Wash Buffer	2 ml	20 ml	3 x 20 ml
Elution Buffer	1 ml	20 ml	50 ml
User Manual	1	1	1

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 60°C
- Absolute (96%-100%) ethanol
- Chloroform:isoamyl alcohol (24:1)

Before Starting

- Please read the entire booklet to become familiar with the E.Z.N.A.™ Insect DNA Kit protocol.
- **Dilute DNA Wash Buffer with absolute ethanol as follows** and store at room temperature.

D0926-00	Add 8 ml absolute (96%-100%) ethanol.
D0926-01	Add 80 ml (96%-100%) ethanol .
D0926-02	Add 80 ml (96%-100%) ethanol to each bottle.

- **Prepare proteinase K stock solution as following:**

D0926-00	Add 150 µl Elution Buffer to the vial
D0926-01	Add 1.5 ml Elution Buffer to the vial
D0926-02	Add 1.5 ml Elution Buffer to each vial

Vortex vial briefly prior to use. We recommend that you aliquot and store vials of reconstituted protease at -20°C

Insect DNA Isolation Protocol

Insect samples preserved in formalin should be rinsed in xylene and then ethanol before processing. Note that results obtained with formalin-fixed tissues generally depend on age and size of specimen. Purified material is usually adequate for PCR amplification, but fresh or frozen samples should be used for southern analysis.

Insects

1. **Pulverize no more than 50 mg of tissue in liquid nitrogen** with mortar and pestle and place the powder in a clean 1.5 ml microcentrifuge tube. If ceramic mortar and pestle are not available, homogenize the sample in the microfuge tube using a disposable microtube pestle (Omega Bio-Tek, Cat Cat# SSI-1015-39 & SSI-1014-39).**Proceed to Step 2 below.**

Arthropods (and other soft tissued invertebrates and plant samples)

1. **Grind no more than 30 mg tissue in liquid nitrogen** with mortar and pestle and place the powder in a clean 1.5 ml microcentrifuge tube. If ceramic mortar and pestle are not available, homogenize the sample in the microfuge tube using a disposable microtube pestle (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. **Proceed to Step 2 below.**

   Amount of starting material depends on sample and can be increased if acceptable results are obtained with the suggested 30 mg tissue. For easy to process specimens, the procedure may be scaled up and the volumes of all buffers used increased in proportion. In any event, use no more than 50 mg tissue per HiBind® spin-column as DNA binding capacity (100 µg) may be exceeded. *Meanwhile, difficult tissues may require starting with less than 30 mg tissue and doubling all volumes to ensure adequate lysis.*

2. **Add 350 µl Buffer CTL followed by 25 µl Proteinase K (20 mg/ml).** Vortex briefly to mix and incubate at 60°C for a minimum of 30 min or until entire sample is solubilized. Actual incubation times

vary and depend on elasticity of tissues. Most samples require no more than 4 hours. Alternatively an overnight incubation at 55°C will produce adequate results.

OPTIONAL: Certain tissues have high levels of RNA which will be co-purified with DNA using this kit. While it will not interfere with PCR, the RNA may be removed at this point. Add 5µl (assuming a sample size of 30 mg) RNase A (25 mg/ml) and incubate at room temperature for 10-15 minutes. Proceed with the protocol.

3. **To the lysate add 350 µl chloroform:isoamyl alcohol (24:1) and vortex to mix.** Centrifuge at 10,000 x g for 5 min at room temperature. Carefully transfer the **upper** aqueous phase to a clean 1.5 ml microfuge tube. Avoid the milky interface containing contaminants and inhibitors.

   Note: This step will remove much of the polysaccharides and proteins from solution and improve spin-column performance downstream. If very few upper aqueous phase present after centrifugation, add 200 µl of Buffer CTL and vortex to mix. Centrifuge as above and transfer the upper aqueous phase to tube.

4. **Add one volume of Buffer CBL and vortex at maxi speed for 15s.** Incubate at 60°C for 10 minutes.

5. **Add one volume of absolute ethanol (room temperature, 96-100%) and mix well by vortexing at maxi speed for 15s.**

   Tips: 250 µl upper aqueous solution, add 250 µl Buffer CBL and 250 µl of absolute ethanol.

6. **Apply 750 µl of the mixture from step 5, including any precipitation that may have formed, to a HiBind® DNA column assembled in a 2 ml collection tube (supplied).** Centrifuge at 10,000 x g for 1 min at room temperature. Discard flow-through liquid and re-use collection tube.

7. **Place HiBind® DNA column back into the same collection tube, apply the remaining of mixture into the column and centrifuge as above.** Discard flow-through liquid and collection tube.

8. **Place the column into the same collection tube and wash by adding 500 µl Buffer HB**. Centrifuge at 10,000 x g for 1min. Discard the flow-through and collection tube.

9. **Place column into a new 2 ml collection tube (supplied) and wash by adding 700 µl DNA Wash Buffer diluted with absolute ethanol.** Centrifuge 10,000 x g for 1 min as above. Discard flow-through liquid and re-use collecting tube in next step.

   Note that DNA Wash Buffer is provided as a concentrate and must be diluted with absolute ethanol as indicated on the bottle and page 4. If refrigerated, the diluted DNA wash buffer must be brought to room temperature before use.

10. **Place column into a new 2 ml collection tube (supplied) and wash by another 700 µl DNA Wash Buffer diluted with ethanol.** Centrifuge as above and discard liquid.

11. **Place the empty column back into the same collection tube**. Centrifuge the column at 10,000 x g for 2 min at room temperature.

    This step is critical in removing traces of ethanol that will interfere with downstream applications.

12. **Place column into a clean 1.5 ml microfuge tube (not supplied). To elute DNA, add 50-100 µl of Elution Buffer preheated to 60°C directly onto the HiBind® matrix**. Allow to soak for 2 min at room temperature. Centrifuge at 10,000 x g for 1 min to Elute DNA.

13. **Optional: Repeat elution step with a second 50-100 µl Elution Buffer.**
    Typically a total of 5-50 µg DNA with absorbance ratio (A_{260}/A_{280}) of 1.7-1.9 can be obtained from 30 mg tissue sample. Yields vary depending on source and quantity of starting material used.
    TIP: To increase DNA Yield add Elution buffer and incubate the column at 60°C-70°C for 5 min before elution.

Determination of DNA Quality and Quantity

Dilute a portion of the eluted material approximately 10-20 fold in DNA Elution Buffer or 10 mM Tris, pH 8.0. Measure absorbance at 280 nm and at 260 nm to determine the A_{260}/A_{280} ratio. Values of 1.7-1.9 generally indicate 85%-90% purity. The concentration of DNA eluted can be determined as follows:

Concentration = 50 µg/ml x $Absorbance_{260}$ x {Dilution Factor}

Troubleshooting Guide

Problem	Possible Cause	Suggestions
Clogged Column	Incomplete lysis	Increase incubation time with Buffer CTL / Proteinase K. An overnight incubation may be necessary.
	Sample too large	Do not use more than recommended amount of starting material. For larger samples, divide into multiple tubes.
	Incomplete homogenization	Pulverize starting material as indicated in liquid nitrogen to obtain a fine powder.
Low DNA yield	Clogged column	See above
	Poor elution	Repeat elution or increase elution volume (see Page 7 for options).
	Poor binding to column.	Follow protocol closely when adjusting binding conditions.

Problem	Possible Cause	Suggestions
	Improper washing	DNA Wash Buffer Concentrate must be diluted with absolute (100%) ethanol.
Low A_{260}/A_{280} ratio	Extended centrifugation during elution step.	Resin from the column may be present in eluate. Avoid centrifugation at speeds higher than specified. The material can be removed from the eluate by centrifugation — it will not interfere with PCR or restriction digests.
	Poor cell lysis.	Increase incubation time with Buffer CTL / Proteinase K.
	Trace protein contaminants remain.	Following Step 8, wash column with 300 ul Buffer HB again before proceeding to Step 9.
	Incomplete homogenization	Pulverize starting material as indicated in liquid nitrogen to obtain a fine powder.
	Absolute ethanol not added before adding sample to column.	Before applying DNA sample to column, add Buffer CBL and absolute ethanol as indicated in Step 5, Page 6.
	No ethanol added to DNA Wash Buffer Concentrate.	Dilute Wash Buffer with the indicated volume of absolute ethanol before first use.