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Introduction

The E.Z.N.A.® Mollusc DNA Kit is designed for efficient recovery of genomic DNA up to 60 kb in size from molluscs, insects, arthropods, roundworms, flatworms, and other invertebrate tissue samples rich in mucopolysaccharides. The method is suitable for invertebrates 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 mucopolysaccharides. Following a rapid alcohol precipitation step, binding conditions are adjusted and DNA further purified using HiBind® DNA Mini 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.™ Mollusc 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 at -20°C. Store Rnase A at -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.® Mollusc 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	D3373-00	D3373-01	D3373-02
Purification times	5 Preps	50 Preps	200 Preps
HiBind® DNA Columns	5	50	200
2 ml Collecting tubes	10	100	400
Buffer ML1	2 ml	20 ml	80 ml
Buffer MBL	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 Concentrate	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 14,000 x g
- Nuclease-free 1.5 ml or 2 ml microfuge tubes
- Water bath equilibrated to 65°C
- Equilibrate sterile dH_2O or 10 mM Tris pH 9.0 at 65°C.
- Absolute (96%-100%) ethanol
- Chloroform

Before Starting

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

D3373-00	Add 8 ml absolute (96%-100%) ethanol.
D3373-01	Add 80 ml (96%-100%) ethanol to each bottle.
D3373-02	Add 80 ml (96%-100%) ethanol to each bottle.

- Prepare proteinase K stock solution as following:

D3373-00	Add 150 µl Elution Buffer to the vial
D3373-01	Add 1.5 ml Elution Buffer to the vial
D3373-02	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

E.Z.N.A.™ Mollusc / Arthropod DNA Protocol

Invertebrates 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 analyses.

Arthropods

1a. **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 No. SSI-1015-39; Eppendorf, Cat No. 0030 120.973; VWR, Cat No. KT 749520-0000).**Proceed to step 2 below.**

Molluscs (and other soft tissued invertebrates)

1b. **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 with 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™ DNA column as 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 ML1 followed by 25 µl Proteinase K.** Vortex to mix and incubate at 60°C for a minimum of 30 min or until entire sample is solubilized. Actual incubation time varies and depends on elasticity of tissue.

Most samples require no more than 4 hours. Alternatively an overnight incubation at 37°C will produce adequate results.

3. **To the lysate add 350 µl chloroform:isoamyl alcohol (24:1) and vortex to mix.** Centrifuge 10,000 x g for 2 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 another of 200 µl of ML1 Buffer and vortex to mix. Centrifuge as above and transfer the upper aqueous phase to tube.

4. **OPTIONAL:** Add 5 µl (assuming a sample size of 30 mg) RNase A and incubate at room temperature for 10-30 minutes. Proceed with the protocol.

5. **Measure the volume of supernatant from step 4 and add one volume of Buffer MBL and vortex at maxi speed for 15s.** Incubate at 70°C for 10 minutes.

6. **Add 0.5 volume of absolute ethanol (room temperature, 96-100%) and mix well by vortexing at maxi speed for 15s.**

Tips: 300 µl upper aqueous solution, add 300 µl Buffer MBL and 300 µl of absolute ethanol.

7. **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 collecting tube (supplied).** Centrifuge at 10,000 x g for 1 min at room temperature. Discard flow-through liquid and re-use collection tube.

8. 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.

9. **Place the column into a new 2 ml collection tube (supplied) and wash by adding 500 µl HB Buffer**. Centrifuge at 10,000 x g for 30 seconds. Discard the flow-through and collection tube.

10. **Place column into the same 2 ml collecting tube (supplied) and wash by adding 700 µl DNA Wash Buffer diluted with absolute ethanol.** Centrifuge 10,000 x g 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.

11. Repeat step 9 with a second 700 µl DNA Wash Buffer diluted with ethanol. Discard liquid and re-insert the column to the empty collecting tube, centrifuge the column at 15,000 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 suplied). To elute DNA add 50 -100 µl of Elution Buffer (or 10 mM Tris buffer, pH 9.0) preheated to 60°C-70°C directly onto the HiBind® matrix. Allow to soak for 2 min at room temperature. Centrifuge at 10,000 x gfor 1 min to Elute DNA.

13. Repeat elution step with a second 50 -100 µl Elution Buffer.

    Typically a total of 5-15 µg DNA with absorbance ratio (A_{260}/A_{280}) of 1.7-1.9 can be obtained from 1 gram soil 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:

$$\text{Concentration} = 50\ \mu g/ml \times \text{Absorbance}_{260} \times \{\text{Dilution Factor}\}$$

Troubleshooting Guide

Problem	Possible Cause	Suggestions
Clogged Column	Incomplete lysis	Increase incubation time with Buffer ML1 / Proteinase K. An overnight incubation may be necessary.
	Sample too large	Do not use greater than recommended amount of starting material. For larger samples, divide into multiple tubes.
	Incomplete homogenization	Pulverize 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. Incubate the column at 70°C for 5 min before spin
	Poor binding to column.	Follow protocol closely when adjusting binding conditions.
	Improper washing	DNA Wash Buffer Concentrate must be diluted with ethanol before use.
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 ML1. An overnight incubation may be necessary
	Trace protein contaminants remain.	Following step 8, wash column with a mixture of [300 µl Buffer ML2 + 300 µl ethanol] before proceeding to step 9.
No DNA eluted	Poor cell lysis.	Increase incubation time with Buffer ML1 . An overnight incubation may be necessary.

Problem	Possible Cause	Suggestions
	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 MBL and absolute ethanol.
	No ethanol added to DNA Wash Buffer Concentrate.	Dilute Wash Buffer with the indicated volume of absolute ethanol before first use.