

Contents

Introduction	2
Storage and Stability	2
Kit Contents	3
Before Starting	3
Supplied By User	3
Mollusc / Arthropod DNA Protocol	4
Arthropods	4
Molluscs	4
Determination of DNA Quality and Quantity	6
Troubleshooting Guide	7
References	8

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 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.

New in this edition

New capped S-Spin column (D3395 & D3396) ensures the elimination of potential contamination during the process.

Storage and Stability

All components of the Mollusc DNA Kit are stable for at least 24 months from the date of purchase when stored at 22°C-25°C. During shipment or storage in cool ambient conditions, salts may precipitate in certain buffers. Simply warm to 37°C and mix to dissolve. Contents of the kit should not be refrigerated at any time.

Kit Contents

Product	D3473-00 D3373-00	D3473-01 D3373-01	D3473-02 D3373-02
Preps	5	50	200
HiBind® DNA Columns	5	50	200
2 ml Collecting tubes	10	100	400
Buffer ML1	2 ml	20 ml	80 ml
Buffer ML2	2 ml	10 ml	40 ml
Proteinase K	3 mg	30 mg	4 x 30 mg
RNase A	30 µl	270 µl	1.1 ml
DNA Wash Buffer Concentrate	12 ml	40 ml	3 x 40 ml
DNA Elution Buffer	1 ml	20 ml	50 ml
User Manual	1	1	1

Before Starting

- DNA Wash Buffer is supplied as a concentrate and must be diluted with absolute ethanol before first use. Add 18 ml (*D3473-00, 5 prep*); 60 ml (*D3473-01, 50 prep*); or 60 ml (*D3473-02, 200 prep*) ethanol to each bottle of DNA Wash Buffer Concentrate as indicated on the label. Once diluted with ethanol, DNA Wash Buffer must be stored at room temperature.
- Proteinase K - prepare a stock solution of 20 mg/ml water or TE and store in aliquots at -20°C

D3473-00 & D3373-00	Dilute with 150µl water or TE Buffer
D3473-01 & D3373-01	Dilute with 1.5ml water or TE Buffer
D3473-02 & D3373-02	Dilute with 1.5ml water or TE Buffer

- Buffer ML2 contains a chaotropic agent. Handle with care, always wearing disposable latex gloves and appropriate protective eye-ware.

Supplied By User

- Microcentrifuge capable of 10,000 x g
- Sterile 1.5 ml microfuge tubes
- Absolute ethanol and 70% ethanol
- Water bath equilibrated to 60°C
- Sterile deionized water
- Chloroform - prepare Chloroform:isoamyl alcohol (24:1)
- Isopropanol (2-propanol)

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

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 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 tissue invertebrates)

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.

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 (30 µg) may be exceeded. Meanwhile, difficult tissues may require starting with less than 30 mg tissue and doubling all volumes to ensure adequate lysis.

Proceed with step 2 below.

2. Add 350 μ l Buffer ML1 followed by 25 μ l Proteinase K (20 mg/ml water). Vortex briefly 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 μ l 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.

4. Add 0.7 x vol isopropanol and mix to precipitate DNA. Incubate at room temperature 2 min and centrifuge 10,000 x g for 10 min. Carefully discard as much supernatant as possible without disturbing pellet. Air dry 2 min at room temperature.

Tip: For example if 600 μ l of aqueous phase is recovered in step 3, add 420 μ l isopropanol (i.e., 0.7 volume).

5. Resuspend DNA pellet in 300 μ l sterile deionized water. This may require incubation at 60°C-70°C for 20 minutes or more. Add 4 μ l RNase A and mix.
6. Adjust binding conditions by adding 200 μ l Buffer ML2 followed by 300 μ l absolute ethanol and vortex to mix. Apply entire mixture, including any precipitation that may have formed, to an HiBind® DNA column assembled in a 2 ml collecting tube (supplied). Centrifuge 8,000 x g for 1 min at room temperature. Discard flow-through liquid and collecting tube.

7. Place column into a clean 2 ml collecting tube and wash by adding 750 μ l DNA Wash Buffer diluted with absolute ethanol. Centrifuge 8,000 x g 1 min as above. Discard flow-through liquid and reuse collecting tube in next step.

Note: DNA Wash Buffer is supplied as a concentrate and must be diluted with absolute ethanol as indicated on page 3 of this booklet.

8. Repeat step 7 with a second 750 μ l DNA Wash Buffer. Discard liquid and using the empty collecting 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 (such as agarose gel electrophoresis of high molecular weight DNA).

9. Place column into a clean 1.5 ml microfuge tube. To elute DNA add 50 μ l-100 μ l of DNA Elution Buffer (or 10 mM Tris buffer, pH 8.0) preheated to 60°C-70°C directly onto the HiBind matrix. Allow to soak for 2 min at room temperature. Centrifuge at 8,000 x g for 1 min to collect DNA.

10. Repeat elution step with a second aliquot of DNA Elution Buffer. Typically a total of 15 μ g DNA with absorbance ratio (A_{260}/A_{280}) of 1.7-1.9 can be obtained from 30 mg buccal glands of a common garden snail. Yields vary depending on source and quantity of starting material used. However the product should be sufficient for multiple PCR reactions or 2-4 lanes of a Southern procedure.

Note: Eluting DNA with 200 μ l of Elution Buffer yield the same result as two elutions of 100 μ l. To increase DNA concentration in final product, elute with 50 μ l Elution Buffer. For high yields and concentration, elute once with 100 μ l and repeat with the first eluate. Also, incubation of the column at 70°C following addition of Elution Buffer may increase yields.

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:

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

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 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 for options). Incubation of column at 70°C for 5 min with dH ₂ O or Tris buffer prior to centrifugation may increase yields.
	Poor binding to column.	Follow protocol closely when adjusting binding conditions. If more than 300 µl water is required to dissolve DNA pellet, adjust volumes of Buffer ML2 and ethanol in proportion.
	Improper washing	DNA Wash Buffer Concentrate must be diluted with absolute (100%) ethanol as specified on page 3 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 / Proteinase K. An overnight incubation may be necessary

Problem	Possible Cause	Suggestions
	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 / Proteinase K. An overnight incubation may be necessary.
	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 ML2 and absolute ethanol as indicated in step 6, page 6.
	No ethanol added to DNA Wash Buffer Concentrate.	Dilute Wash Buffer with the indicated volume of absolute ethanol before first use.

References

- Doyle, J.J. & Doyle, J.L. (1987) *Phytochemical Bulletin* 19: 11-15
- Gustincich et al. (1991) *BioTechniques* 11: 298-302.
- Hempstead et al. (1990) *DNA and Cell Biology* 9: 57-61.
- Maki et al (1991) *Biochem Biophys Res Comm* 175: 768-774.
- Rogstad, S.H. (1992) *Taxon* 41: 701-708.