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Introduction

The EZgene™ Mollusc gDNA Kit is designed to extract 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.

Samples are homogenized and lysed in a high salt buffer and extracted with chloroform to remove mucopolysaccharides. Following a rapid alcohol precipitation step, binding conditions are adjusted and DNA further purified using ezBind DNA spin columns. While proteins and other contaminants are removed by wash buffer, high quality genomic DNA is eluted with elution buffer or sterile water. The purified genomic DNA is suitable for downstream applications such as Southern Blot, restriction digestion, and PCR.

Storage and Stability

All components of the EZgene™ Mollusc gDNA Kit, except the Proteinase K and RNase A should be stored at 22^{o}C -25^{o}C. Once reconstituted in water, Proteinase K should be stored -20^{o}C.Under 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 ^{o}C. Store RNase A at -20 ^{o}C. All EZgene™ Mollusc gDNA Kit components are guaranteed for at least 12 months from the date of purchase when stored at 22 ^{o}C -25 ^{o}C.

Binding Capacity

Each ezBind DNA column can bind approximately 100 μg DNA. Use less than 30 mg of sample per column.

Kit content

Product	GD2414-00	GD2414-01	GD2414-02
ezBind DNA Columns	4	50	250
2 mL collecting tubes	8	100	500
Buffer MTL	2 mL	20 mL	100 mL
Buffer MBL	2 mL	20 mL	100 mL
Buffer KB	2.8 mL	28 mL	135 mL
Proteinase K	3 mg	30 mg	5 x 30 mg
RNase A（20 mg/mL）	55 μL	270 μL	1350 μL
DNA Wash Buffer	2 mL	15 mL	3 x 24 mL
Elution Buffer	1 mL	15 mL	70 mL
User Manual	1	1	1

Before Starting

Please read the entire booklet to become familiar with the EZgene protocol.

Dilute DNA Wash Buffer with 100% ethanol as follows:

GD2414-00	Add 8 mL absolute (96%-100%) ethanol.
GD2414-01	Add 60 mL (96%-100%) ethanol to each bottle.
GD2414-02	Add 96 mL (96%-100%) ethanol to each bottle.

Reconstitute ***Proteinase K*** stock solution. Vortex vial briefly prior to use. We recommend that you aliquot and store vials of reconstituted protease at -20 ℃.

GD2414-00	Add 110 μL Elution Buffer to the vial
GD2414-01	Add 1.3 mL Elution Buffer to the vial
GD2414-02	Add 5 x 1.3 mL Elution Buffer to each vial

EZgene™ Mollusc / Arthropod DNA Protocol

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 ddH_2O or 10 mM Tris pH 8.5 at 65 °C
- Absolute (96%-100%) ethanol
- Chloroform: isoamyl alcohol (24:1)

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.

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 ezBind DNA column TM 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.

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# S1015-39 & 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.

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. **Proceed to step 2 below.**

2. Add **350 µL Buffer MTL** 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 200 µL of MTL Buffer and vortex to mix. Centrifuge as above and transfer the upper aqueous phase to tube**.**

4. Add **one volume** of **Buffer MBL** followed by **5 µL RNase A,** vortex at maxi speed for 15 s. Incubate at 70 °C for 10 minutes.

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

   Note: 500 µL upper aqueous solution, add 500 µL Buffer MBL and 500 µL of absolute ethanol.

6. Apply **750 µL** of **the mixture** from step 5, including any precipitation that may have formed, to an ezBind DNA column Centrifuge at 10,000 x g for 1 min at room temperature. Discard flow-through liquid and re-use collection tube.

7. Place ezBind 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 another a new 2 mL collection tube (supplied) and wash by adding **500 µL Buffer KB.** Centrifuge at 10,000 x g for 30 s. Discard the flow-through and re-use collection tube.

9. Place column into collection tube from previous step and add **650 µL DNA Wash Buffer** diluted with 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.

10. Repeat step 9 with a second **650 µL DNA Wash Buffer** diluted with **ethanol.** Discard liquid and collection tube. And insert the column into a new 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.

11. Place column into a clean 1.5 mL microfuge tube (not supplied). To elute DNA add **50 µL-100 µL** of **Elution Buffer** (or 10 mM Tris buffer, pH 9.0) preheated to 60 ^{o}C -70 ^{o}C directly onto the ezBind matrix. Allow soaking for 2 min at room temperature. Centrifuge at 10,000 x g for 1 min to Elute DNA.

12. Repeat elution step with a second **50 µL-100 µL Elution Buffer.**

    Tip: To increase DNA Yield add Elution buffer and incubate the column at 60^{o}C -70 ^{o}C for 5 min before elution.

Determination of DNA Quality and Quantity

Dilute a portion of the eluted material approximately 10-20 fold in ddH_2O Measure absorbance at 280 nm and at 260 nm to determine the A260/A280 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 Absorbance260 x {Dilution Factor}

Trouble Shooting Guide

Problem	Possible Cause	Suggestions
Clogged Column	Incomplete lysis	Increase incubation time with Buffer MTL / 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 MTL. An overnight incubation may be necessary
	Trace protein contaminants remain.	Following step 8, wash column with a mixture of [300 µL Buffer MBL + 300 µL ethanol] before proceeding to step 9.
No DNA eluted	Poor cell lysis.	Increase incubation time with Buffer MTL. 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 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