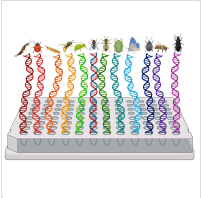

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High-throughput papain-based DNA extraction from whole invertebrates

Forked from High-throughput and cost effective pan trap DNA extraction

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ABSTRACT

This protocol is designed for extracting DNA from individual invertebrates in 96-well plate format for downstream barcoding or metabarcoding. The reagents and methods proposed offer a cost effective and high-throughput method for molecular identification, dietary analysis, parasitism diagnostics and more from individual invertebrate samples using standard lab equipment.

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FORK NOTE

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MATERIALS TEXT

For field collection and initial storage:

- Small collection tubes for storage of samples (e.g., 1.5 mL microcentifuge tubes)
- 100 % ethanol

For DNA extraction:

- Hardened 3 mm carbon steel ball bearings
- 1.2 mL 96-well plates (or larger) for initial lysis and protein denaturation
- 2.2 mL deep well plates for spin-column flow-through (these can be bleached and reused across sessions)
- Silica membrane 96-well spin-column plates
- 0.5 mL deep-well 96-well plates to collect eluted DNA
- Breathable plate seals
- Plate seals for long-term storage

Buffers and reagents:

- Sodium chloride
- 1 M Tris-HCl
- 0.5 M EDTA
- Nuclease-free water
- SDS
- Guanidine HCl
- 100 % ethanol
- Papain (100 mg/mL)

Equipment:

- -20 °C freezer
- Geno/Grinder 2010 or similar bead beater for homogenisation

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SAFETY WARNINGS

Check safety guidelines for individual reagents before commencing work. Some reagents will be toxic, corrosive or will otherwise present health and safety risks. Appropriate personal protective equipment should be used at all times, not only for personal safety but also reduction of contamination risk.

BEFORE STARTING

Consider steps that can be taken to limit cross-contamination throughout the process. Contamination between samples and from the environment can produce false positive data and inaccurate results to which downstream analyses like DNA metabarcoding can be incredibly sensitive.

Collection of samples

1 Consider how systematic the study needs to be and the various constraints imposed on the data by the study design.

2 Collect individual invertebrates from the field into a suitable preservative (e.g., 100 % ethanol).

Ensure that the collection equipment is clean and free of DNA contaminants by cleaning it with Chemgene or diluted bleach prior to and between each use.

Ensure appropriate permissions are in place for the site. If your site is publicly accessible or likely to be visited, consider using signage to reduce the risk of tampering (e.g., vandalism or benevolent liberation of the dead invertebrates).

Consider as well the ethical implications of your collections. Limit unnecessary collection and implement measures to limit bycatch of non-target organisms where applicable.

3 Store samples at -20 °C until ready to process.

Preparation and homogenisation of samples 1d 8h 28m

4 Add one 3 mm hardened carbon steel bead to each well of a 1.2 mL 96-well plate. 5m

Beads are usually shipped coated in manufacturing oil (especially the carbon steel beads). To remove this, place beads in a borosilicate glass beaker or Duran bottle with plastic pouring lip and lid removed then bake for at least 12 hours at 250 °C.

96-well bead dispensers make this step significantly quicker and less liable to

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contamination.

5 Add individual invertebrates to each well of the plate. 15m

Ensure appropriate sterilisation of tweezers between each transfer of samples by using bleach, ethanol and water to prevent cross contamination

6 Freeze the samples overnight at -20 °C. 16h

This will ensure the specimens are brittle and grind adequately; -80 °C will facilitate even better grinding, but it will make the plastic brittle too, so check the plasticware will survive without samples before proceeding.

7 Directly from the freezer, grind the bulk samples in a Geno/Grinder at full speed (1750 RPM) for 2 minutes. 2m

If the sample is not fully homogenised, repeat this step.

8 To each sample, add 140 µL of fresh **Lysis Buffer 1,** 60 µL of **Lysis Buffer 2** and 20 µL of 100 mg/mL papain: 2m

Borosilicate bottles are ideal for making up larger quantities of buffer.

To sterilise borosilicate bottles prior to making up the buffers, acid washing with ~100 mL 0.4 M hydrochloric acid followed by neutralisation with ~100 mL 0.4 M sodium bicarbonate is ideal for sterilisation without introducing bleach or other chemicals that might destroy or contaminate the DNA. Following neutralisation, wash twice with ~100 mL water to remove the reagents. Residual amounts of NaCl may remain, but this is inert and will be present in many of the buffers anyway.

For 0.4 M sodium bicarbonate add 33.604 g $NaHCO_3$ into 1000 mL water.

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Proteinase K can be used instead of papain, but is much more expensive. Given the low cost of papain, it is used at ten-fold concentration to ensure adequate lysis, but could be used as per the standard concentration of Proteinase K (10 mg/mL).

8.1 **Lysis Buffer 1** should be pH 9 and comprised of the following reagents: 2m

A	B	C	D
Reagent	**Required concentration in buffer**	**Chemical molarity**	**Amount per 100 mL**
NaCl	120 mM	58.44	0.701 g
1 M Tris-Hcl	50 mM	-	5 mL
0.5 M EDTA	20 mM	-	4 mL
Water	-	-	91 mL

8.2 **Lysis Buffer 2** should be pH 9 and comprised of the following reagents: 2m

A	B	C	D
Reagent	**Required concentration in buffer**	**Chemical molarity**	**Amount per 100 mL**
Sodium chloride	120 mM	58.44	0.701 g
1 M Tris-Hcl	50 mM	-	5 mL
0.5 M EDTA	20 mM	-	4 mL
SDS	3 %	-	3 g
H2O	-	-	91 mL

9 Vortex each sample to mix and incubate at 37 °C overnight (12-16 hours). Alternatively, incubate at 56°C for three hours. 16h

Sample washes and DNA elution 1h 6m

10 Add 400 µL of master mix of **Protein Denaturation Buffer and ethanol** (detailed in the sub-step below) to each well of the 2.2 mL 96-well plate. 2m

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Create master mix of 1:1 ratio of protein denaturation buffer and ethanol.

10.1 Protein Denaturation Buffer should be comprised of the following reagents: 2m

A	B	C	D
Reagent	**Required concentration in buffer**	**Chemical molarity**	**Amount per 100 mL**
Guanidine HCl	5 M	95.53	47.7 g
H2O	-	-	100 mL

10.2 Per 96-well plate, the **Protein Denaturation Buffer** and ethanol master mix should comprise: 2m

A	B	C
Reagent	**Amount per sample**	**Amount per 96-well plate**
Protein Denaturation Buffer	220 µL	21.1 mL
Ethanol (100 %)	220 µL	21.1 µL

11 Vortex samples briefly to ensure thorough mixing 1m

12 Add all of the sample solution (~ 600 µL) to a well in a 96-well silica membrane spin-column plate and cover with a breathable seal. 5m

Ensure there is a suitable reservoir beneath into which the flow-through will go (e.g., 2.2 mL deep-well plate).

13 Centrifuge at ≥ 6,000 x *g* for 10 minutes and discard the flow-through. 10m

If the centrifuge cannot reach 6000 x *g*, a longer centrifugation will work.

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14 Add 500 µL **Wash Buffer 1** to each spin column and cover with a breathable seal. 2m

14.1 **Wash Buffer 1** should be comprised of the following reagents: 2m

A	B	C	D
Reagent	**Required concentration in buffer**	**Chemical molarity**	**Amount per 100 mL**
Guanidine HCl	7 M	95.53	29.4 g
Ethanol	56 %	-	56 mL
H2O	-	-	44 mL

15 Centrifuge at ≥ 6,000 x *g* for 5 minutes and discard the flow-through. 5m

If the centrifuge cannot reach 6000 x *g*, a longer centrifugation will work.

16 Add 500 µL **Wash Buffer 2** to each spin column and cover with a breathable seal. 2m

16.1 **Wash Buffer 2** should be pH ~7 and comprised of the following reagents: 2m

A	B	C	D
Reagent	**Required concentration in buffer**	**Chemical molarity**	**Amount per 100 mL**
Ethanol (100 %)	70 %	58.44	70 mL
1 M Tris-Hcl	10 mM	-	1 mL
H2O	-	-	29 mL

17 Centrifuge at ≥ 6,000 x *g* for 15 minutes and discard the flow-through. 15m

If the centrifuge cannot reach 6,000 x *g*, a longer centrifugation will work.

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18 Carefully move the spin column plate to a new 0.5 mL DNA collection plate. 1m

The liquid level following the final wash will be close to the base of the spin column, so take care not to let it touch to prevent ethanol transfer to the soon-to-be eluted DNA.

19 Add 100 µL **Elution Buffer** directly to the silica membrane and leave it at room temperature for 5 minutes, covering with a breathable seal. 6m

19.1 **Elution Buffer** should be pH ~7 and comprised of the following reagents: 2m

A	B	C	D
Reagent	**Required concentration in buffer**	**Chemical molarity**	**Amount per 100 mL**
1 M Tris-Hcl	10 mM	-	1 mL
H2O	-	-	99 mL

20 Centrifuge at ≥ 6000 x *g* for 2 minutes. The DNA is now in the collection plate and can be taken forward to molecular analysis. 2m

Steps 20-21 can be repeated for increased DNA yield but a lower overall concentration.

If the centrifuge cannot reach 6000 x *g*, a longer centrifugation (e.g., 5 minutes) will work, although should not be necessary for this step.

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