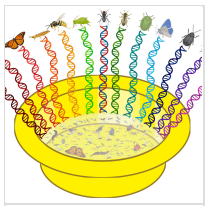

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High-throughput and cost effective pan trap DNA extraction

Tissue extraction from whole caterpillars

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This protocol is designed for extracting DNA from pan trap-collected invertebrates for biomonitoring and community metabarcoding. The reagents and methods proposed offer a cost effective and high-throughput method for molecular diversity analyses of bulk samples using standard lab equipment.

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For field collection and initial storage:

- Pan traps
- Detergent diluted in water for collection of invertebrates
- Small collection pots for storage of samples
- Sieves for isolating invertebrates
- 100 % ethanol
- Chemgene/diluted bleach for sterilisation of sieves
- 50 mL falcon tubes
- 125 mL sample pots (for full sample lysis)

For DNA extraction:

- Hardened carbon steel ball bearings
- 2.2 mL deep well plates (or up to 1.5 mL) for initial protein denaturation
- 2.2 mL deep well plates for archiving
- 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
- Proteinase K (10 mg/mL) OR papain (10 mg/mL)

Equipment:

- -20 °C freezer
- -80 °C freezer
- Geno/Grinder 2010 or similar bead beater for homogenisation

Check safety guidelines for individual reagents before commencing work. Some reagents will be toxic, corrosive or 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.

Collection of pan trap samples 3d 0h 30m

1 Select suitable sites and locations for pan traps. Consider how systematic the study needs to be and the various constraints imposed on the data by the study design.

2 Set a pan trap at approximately the same height as the surrounding vegetation. 15m

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Ideally, use a pan trap design that prevents overflow if the site is likely to experience heavy rainfall.

Ensure the trap is clean and free of DNA contaminants by cleaning it with Chemgene or diluted bleach prior to use.

3 Fill the traps with water containing detergent to reduce surface tension. Leave the traps out for 72 hours. 3d

Standardise the volume of soapy water across traps but ensure it is sufficient to avoid evaporation yet not so much as to make overflow likely.

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 traps. Limit unnecessary collection and implement measures to limit bycatch of non-target organisms where applicable.

4 Pour the trap contents through a sterilised funnel into a suitably sized pot labelled with all necessary sample information for transport to the laboratory. Store samples at -20 °C until ready to process. 15m

Preparation and homogenisation of samples 1d 9h

5 Wash samples with water in a fine mesh sieve to remove external contaminants, and transfer to a 50 mL falcon tube. 5m

6 Add three 3 mm hardened carbon steel beads to the tube. 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.

7 Freeze the samples overnight at -80 °C. 16h

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8 Directly from the freezer, grind the bulk samples in a Geno/Grinder at full speed (1750 RPM) for 4 minutes. 4m

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

9 Two lysis options are presented below. One standard protocol using Proteinase K to lyse a subsample of each trap (this may reduce detectable diversity but increases efficiency), and the other uses papain (a cheap alternative to Proteinase K) to lyse whole samples.
Step 9 includes a Step case.
Proteinase K
Papain

step case

Proteinase K

10 Add an appropriate volume of fresh **Lysis Buffer 1** (detailed in the sub-step below) to sufficiently cover the specimens. 1m

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, into 1000 mL water, add 33.604 g $NaHCO_3$.

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

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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

11 Grind the samples in a Geno/Grinder at full speed (1750 RPM) for 2 minutes. 2m

12 Centrifuge at 4,000 x *g* for 2 minutes. 2m

13 Take a subsample of 1 mL lysate from the supernatant and transfer it to a 2.2 mL 96-well plate. Store the remaining lysate and sample from the falcon tube at -20 °C. 1m

> It is possible to freeze the plate at this stage or proceed with overnight digestion depending on the desired completion time.

14 To the 1 mL lysate, add 500 µL of freshly-prepared **Proteinase Buffer**, a master mix of **Lysis Buffer 2** and proteinase K (detailed in the sub-steps below) and vortex to mix. 1m

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

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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

14.2 Per plate, the **Proteinase Buffer** master mix should comprise: 1m

A	B
Reagent	**Amount per 96-well plate**
Lysis solution 2	51.2 mL
Proteinase K (10 mg/mL)	1584 µL

15 Vortex each sample to mix and incubate at 37 °C overnight (12-16 hours). 16h

16 Centrifuge at 4,000 x *g* for 4 minutes. 1m

17 Transfer 1 mL of the supernatant to a clean 2.2 mL deep-well 96-well plate for archiving/backup. 1m

18 Transfer 200 µL of the supernatant to a clean 2.2 mL deep-well 96-well plate. 1m

The remaining lysate can now be stored at -20 °C for backup/future work.

DNA extraction and purification 22m

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19 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. 1m

19.1 **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

19.2 Per 96-well plate, the **Protein Denaturation Buffer and ethanol** master mix should comprise: 1m

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

20 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. 1m

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

21 Centrifuge at ≥ 6,000 x *g* for 10 minutes and discard the flow-through. 1m

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

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

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22.1 **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

23 Centrifuge at ≥ 6,000 x *g* for 5 minutes and discard the flow-through. 1m

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

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

24.1 **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

25 Centrifuge at ≥ 6,000 x *g* for 15 minutes and discard the flow-through. 3m

If the centrifuge cannot reach 20,000 x *g*, a longer centrifugation will work.

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26 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.

27 Add 200 µL **Elution Buffer** directly to the silica membrane and leave it at room temperature for 5 minutes, covering with a breathable seal. 1m

27.1 **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

28 Centrifuge at ≥ 6000 x *g* for 2 minutes. The DNA is now in the collection plate and can be taken forward to molecular analysis. 1m

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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