

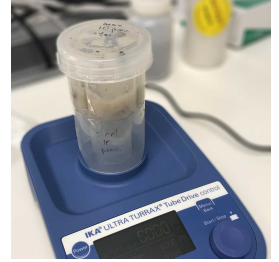


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Sample homogenization and DNA extraction for bulk insect catches

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Abstract

Here we describe a protocol to homogenize and extract DNA for bulk insect catches collected via Malaise traps. By following this protocol you will homogenize the entire catch into an insect soup followed by digestion of the entire insect soup with lysis buffer and proteinase K. After DNA lysis, an aliquot of the homogenate is purified using magnetic beads. The DNA obtained can then be used to amplify target genes for metabarcoding purposes. This protocol assumes you have bulk insect samples that have been drained of ethanol and for which you have recorded the wet biomass of each individual sample. You can obtain that by following steps 1 to 9 from FAVIS protocol: <https://www.protocols.io/view/favis-fast-and-versatile-protocol-for-metabarcodin-kqdg36261g25/v2>. We have used this protocol to extract DNA from 870 bulk insect samples from the Insect Biome Atlas project (www.insectbiomeatlas.org) that had first been subjected to DNA extraction following the mild lysis FAVIS protocol. To make sure we use all available DNA from each catch we combine the insect soup obtained in this protocol with the lysate obtained from the FAVIS protocol before proceeding with DNA purification with magnetic beads (optional section 4 of this protocol).

Guidelines

- Work in the trays on the bench to minimize contamination between samples.
- The highest risk for contamination is when opening the DT-50 rotor stator tube after homogenization. Change gloves between samples (or clean them with bleach).
- Spray the UltraTurrax instrument with 10% bleach after homogenizing each sample.

Materials

In this section we list consumables, chemicals and reagents that are needed to run the protocol. This is not an exhaustive list as we assume that you have access to common lab equipment (pipettes, centrifuges, vortex, etc) and certain common lab consumables (filtered pipette tips, petri dishes etc.). For less commonly used lab equipment and consumables that are used in the protocol we opt to reference them below.

LIST OF EQUIPMENT

A	B	C
Item	Brand	Reference
Large tweezers, 200 mm long	VWR®	232-2196
Shaking incubator - INCU-Line ILS 6	VWR®	444-0763
Small tweezers, 110 mm long	Sargenta	802-50
Tray	Buerkle™	4201-1318
Squirt bottle (for 10% bleach solution)		
Graduated cylinders (50 mL, 100 mL, 200 mL, 300 mL, 400 mL)		
Large glass petri dish		
Ultra Turrax Drive P control	IKA	0025005981
Silicon spatula (200 mm long)		
Silicon corner spatula (200 mm long)		
Small funnels		
Water bath	homemade	
Microscope		

LIST OF CONSUMABLES

A	B	C
Item	Brand	Reference
Nalgene 500 mL bottle (blank sample)	Fisher scientific	2105-0016



	A	B	C
	Homogenate collecting bottle 250 mL	Fisher scientific	2189-0008
	Homogenate collecting bottle 500 mL	Fisher scientific	2189-0016
	96-well microtube rack	VWR®	211-0213
	Microtubes (2 mL screw cap)		
	DT-50 rotor stator tubes (50 mL)	IKA	0003699600

CHEMICALS AND OTHER REAGENTS

- Sodium dodecyl sulphate (SDS) - CAS: 151-21-3
- Sodium Chloride (NaCl) - CAS: 7647-14-5
- Tris ultrapure 99,9% - CAS: 77-86-1
- Hydrochloric acid (HCl) - CAS: 7647-01-0
- EDTA disodium dihydrate - CAS: 6381-92-6
- DNase/RNase-Free Distilled Water (ddH₂O)
- Proteinase K Solution (20 mg/mL) - OMEGA, ref.PROK50

STOCK SOLUTIONS AND BUFFERS

Below we describe recipes for all stock solutions and buffers used in the protocol. These should be prepared before starting the protocol.

LYSIS BUFFER, 5 L

We use the buffer by Vesterinen et al. (2016), who modified it from Aljanabi and Martinez (1997).

- Add 4.5 L of DNase/RNase-Free Distilled Water (ddH₂O) to a 5 litre glass bottle.
- Add 116.9 g of Sodium Chloride (NaCl).
- Add 2.9 g EDTA disodium dihydrate.
- Add 50 mL 1M Tris-HCl, pH 8.0.
- Add 20 g of Sodium dodecyl sulphate (SDS).
- Dissolve the reagents by adding a magnetic flea stirrer into the glass bottle and placing the bottle on a magnetic stirrer at 60°C. Close the bottle with the lid but make sure to leave the lid a bit loose.
- After all ingredients have dissolved, remove the magnetic stirrer from the bottle with the help of a metal magnet.
- Top up the solution to 5 L with ddH₂O and firmly close the bottle with the lid.

1M TRIS-HCL, pH 8.0, 1 L



- Add 121.14 g Tris ultrapure 99.9% to a beaker.
- Adjust volume to 800 mL with ddH₂O.
- Adjust pH to 8.0 with hydrochloric acid (HCl).
- Adjust volume to 1 L with ddH₂O.
- Sterilize by filtering the solution with a 0.2 µM filter membrane and store at room temperature.

Before start

This protocol assumes you have bulk insect samples that have been drained of ethanol and for which you have recorded the wet biomass of each individual sample. You can obtain that by following steps 1 to 9 from FAVIS protocol: <https://www.protocols.io/view/favis-fast-and-versatile-protocol-for-metabarcodin-kqdg36261g25/v2>.

Prepare lysis buffer stock solution in advance as described in Materials section. Place the 1L bottles of lysis buffer into a large shaking incubator (we recommend INCU-Line ILS6; VWR) and set to 65-70°C, 90 rpm, to preheat. Invert the bottle every hour to help the salts fully dissolve - especially important during cold days as salts precipitate at low temperature.

SECTION 1: Set up working stations and prepare samples for processing

- 1 Start by creating individual working stations for each insect bottle that you will process by dividing the lab bench into 30 cm wide working stations. Number each station with a running number from 1 to $n+1$ (n is the total number of samples you will process) to account for one station to process your blank sample.
- 2 Use a squirt bottle with bleach 10% solution and a paper towel to wipe all lab benches.
- 3 At each working station place the following:
 - 1 tray
 - 1 bottle for temporary storage of homogenate (samples with ≥ 10 gr of insect biomass only)
 - 500 mL Nalgene HDPE bottle (for the blank sample)
 - small tweezers
 - large tweezers
 - large petri dish
 - microscope
 - silicon spatula (200 mm long - Fig. 1)
 - silicon corner spatula (200 mm long - Fig. 1)
 - 1 large glass petri dish
 - IKA DT-50 rotor stator tube (50 mL)
 - 1 screw cap microtube (2.0 mL)



Fig 1. Corner spatula in white and normal spatula in yellow.

- 4 Place one drained insect bottle in each working station and one empty bottle (Nalgene, 500 ml HDPE wide mouth) in the last working station ($n+1$) working station (blank sample).
- 5 Using a marker pen, label each bulk insect bottle and rotor stator tube with the respective working station number.

- 6 If you have a nylon circle mesh (used to decant ethanol from the bulk insect bottle during step 7.9 of the FAVIS protocol) and/or any archive label inside your bulk insect bottle, remove it with a pair of large tweezers and place it inside a large glass petri dish.
- 7 Put the petri dish under a microscope. Using the small tweezers, transfer the insects that are stuck to the round mesh and/or the archive label into its respective the bulk insect bottle.

SECTION 2: Sample lysis

- 8 Add the appropriate amount of lysis buffer to each insect bottle according to Table 1 using a graduated cylinder and a small funnel to get the right amount of buffer. The funnel is important so that the lysis buffer is poured slowly and accurately from the 1L bottle into the graduated cylinder, reducing foaming.

A	B	C
Sample weight (g)	Lysis buffer (mL)	Proteinase K (μL)
< 5.00	30	30
5.00 – 9.99	50	50
10.00 - 19.99	100	100
20.00 - 29.99	200	200
30.00 - 39.99	300	300
> 40.00	400	400
Blank sample	50	50

Table 1. Specification on amount of lysis buffer and proteinase K to be added to each insect bottle relative to the insect biomass of each sample.

NOTE: When adding the lysis buffer to each insect bottle, use the buffer to rinse the insect bottle walls so that all insect specimens that are attached to the walls fall at the bottom of the bottle and are soaked in the lysis buffer.

- 9 Add the correct amount of proteinase K as shown in Table 1 to each insect bottle.

NOTE: To speed up this step you can first add proteinase K to the lysis buffer before adding the mixed solution to the insect bottles. To minimize waste, first calculate how much buffer you will need for the samples you are processing that day (you will know



this after having the wet-weight of each sample). Add the correct amount of proteinase K to the total amount of buffer and then simply add the correct amount of "lysis buffer + proteinase K" solution to each insect bottle according to Table 12.

SECTION 3: Sample homogenization with Ultra Turrax Drive - IKA™

- 10 Gently pour the contents of the insect bulk sample into its respective DT-50 rotor stator tube. Make sure to scoop out any remaining insects from the bottle using the different spatulas. Close the rotor stator tube with the lid.

NOTE: For samples of up to 50 mL lysis buffer, all contents will fit in one rotor stator tube and homogenization can be performed in one go. However for larger samples (≥ 100 mL) you have to perform several rounds of homogenization of 50 mL batches depending on the size of the sample. You can use the same rotor stator tube for homogenizing several 50 mL batches of the same sample. After each homogenization round, transfer the homogenate of that round into an appropriate storage bottle that can accommodate the entire insect soup after all batches from that sample are processed.

- 11 Processing one sample at a time, assemble the 50 mL rotor statue tube in the Ultra Turrax Drive and homogenize the insects + lysis buffer solution for 2 minutes at 6000 rpm. Spray the UltraTurrax instrument with 10% bleach after homogenizing each sample.

- 12 For the blank, pour 50 mL of the lysis buffer solution into a rotor stator tube and homogenize it as you did for the real samples.

- 13 Move the rotor stator tube to its respective working station. Let the sample sit for a while after homogenization for the foam to go down. Then open the rotor stator tube and transfer the homogenate back to the original insect sample bottle. For the blank sample use the 500 mL HDPE bottle to store the homogenized buffer. Use the spatula to transfer all the homogenate that is stuck to the rotor stator tube walls.

- 14 Close the rotor stator tube with the lid and discard it.

- 15 Proceed with sample pre-incubation in a water bath for 30-40 minutes depending on the biomass of the sample (see NOTE below) followed by incubation at 56°C for 2hr and 45 min in a dry shaking incubator at 90 rpm.

NOTE: It is important to preheat the insect bottles in a water bath before starting the incubation period as they take time to heat up to the desired incubation temperature of 56°C in a dry incubator. The water bath speeds up this process considerably and ensures that each sample is at 56°C when starting the incubation period in the dry





incubator. You can use a commercial shaking water bath or you can create your own one as we did in our lab (see details in Favis protocol, step 11: <https://www.protocols.io/view/favis-fast-and-versatile-protocol-for-metabarcoding-kqdg36261g25/v2>). For small size samples (30 mL, 50 mL, 100 mL) it takes 30 minutes of pre-heating in the water bath for the samples to reach the desired temperature of 56°C, for large samples (200 mL, 300 mL, 400 mL) it will take 40 minutes. These timings are specific to the bottles we use to collect insects and the temperature of the tap water that circulates in the water bath (60°C in our case) and they might need to be adjusted for your own project depending on the bottles and water bath you use.

- 16 Remove homogenized insect bottles from the dry incubator and move each bottle back to its respective working station.

SECTION 4: Combining homogenate with lysate (optional)

- 17 We have used this protocol to extract DNA from 870 samples from the Insect Biome Atlas project (www.insectbiomeatlas.org) that had first been subjected to mild lysis following the FAVIS protocol. If your original bulk insect sample has first been processed through the FAVIS protocol as ours, you should combine the insect soup (homogenate) obtained in step 16 above with the lysate obtained from the FAVIS protocol before proceeding with DNA purification to make sure you use all available DNA from each catch.
 - 17.1 Thoroughly thaw the corresponding lysate from the mild lysis protocol for each sample.
 - 17.2 Pour the lysate into the corresponding homogenate bottle and close with the lid.

NOTE: For samples with ≤ 200 mL lysate, this can be done in the same bottle but for samples with 300 and 400 mL lysate, a 1000 mL bottle is needed.
 - 17.3 Mix the combined homogenate+lysate solution thoroughly by inverting the bottle several times until you obtain a clear mixture.

SECTION 6: Taking homogenate aliquot for DNA purification

- 18 In the next steps we will transfer a 1.8 mL aliquot of the homogenate to a 2 mL microtube which will be used as a working-stock for DNA purification. If you proceed with DNA purification straight away you can save time by transferring the precise aliquot of homogenate (we use 225 μ L) directly from the homogenate bottle into a deep well plate and proceed with DNA purification.
 - 18.1 Gather a 1000 μ L micropipette, one box of 1000 μ L filtered tips and a 96 well microtube rack.



- 18.2 Gently shake the insect soup bottle to homogenize the liquid inside before taking the aliquots.
- 18.3 Cycle through each work station and transfer 2×900 µL aliquots of homogenate from each bottle into its corresponding microtube.
- 18.4 Fill in the microtube rack with the microtubes starting with A1 to A12, B1 to B12, and so on. Leave the last four wells (H9; H10; H11 and H12) empty as these will serve as negative (H9) and positive (H10) DNA purification controls and negative (H11) and positive (H12) library preparation controls. Label each 96-well microtube rack with a unique label.
- 18.5 The homogenate bottles and homogenate aliquots in the microtube racks should be individually labelled with a unique ID and stored at -20°C for long term storage if not used straightaway.
- 18.6 Proceed with DNA purification following step 16 of the FAVIS protocol (<https://www.protocols.io/view/favis-fast-and-versatile-protocol-for-metabarcoding-kqdg36261g25/v2>). In our project we purified 225 µL of homogenate using the KingFisher™ Cell and Tissue DNA Kit - THERMO SCIENTIFIC™ (discontinued) on a KingFisher Flex robot - THERMO SCIENTIFIC™ (ref. 5400620).

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