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Simple protocol for combined extraction of exocrine secretion and RNA in small arthropods

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David Fröhlich[1], Bodner Michaela[1], Dr. Guenther Raspotnig[1], Christoph Hahn[1]

[1]University of Graz

David Fröhlich
University of Graz

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Abstract

We here introduce a novel combination of different methods, namely gas chromatography-mass spectrometry and RNAseq. The described method can be used to extract exocrine chemical compounds and RNA from the same individual. Using this protocol, metobolites like defensive secretions, pheromones, surface protectans and others can be linked to RNA-profiling. The protocol should be applicable for the majority of arachnids, insects and other arthropods.

Materials

Equipment:
Microentrifuge
Homogenizer
Fume cupboard

Material:
Crushed ice
Methylene chloride
100% isopropanol
95% ethanol
Eppendorf tubes
GC-MS-vials
Tubes filled with beads (Beadbug™ Prefilled 2ml Tubes with 1mm Triple-Pure-High-Impact Zirconium Beads)
RNA extraction kit: Promega ReliaPrep™ RNA Miniprep System (other RNA extraction methods should work too)

Before start

This protocol has been tested for oribatid mites. Adaptations regarding your study organism might be necessary (e.g. accirding to organism size). Suitable modifications may include volume and types of solvent, duration of chemical extraction (adjustment recommended), additional preparation steps (e.g. using particular tissue only).

Preparation

1 To prevent any kind of contamination (chemical substances, RNase,...) follow good laboratory practice. Wear gloves all the time.

2 **Chemical extraction.**

2.1 Put some **crushed ice** into a box and place it at your fume cupboard for chemical extraction.

2.2 For each sample, **label two GC-MS vials with glass inlets** and **put them** for cooling **into the crushed ice.**

2.3 **Take** some **methylene chloride** as solvent (other solvents should also be used, but were not tested).

3 **Prepare** all chemicals and equipment for **RNA extraction.** We used Promega ReliaPrep™ RNA Miniprep System for RNA extraction. Steps according the manunfacturers protocol are marked with.*
We recommend to check the manufacturers protocol for more details before starting wth the extraction. Here you can find the manufacturers protocol:

reliaprep-rna-tissue-miniprep-system-… 540KB

3.1 **Before first use of the extraction kit** prepare:*
-**DNase I** by adding nuclease-free water. Mix gently, do not vortex. Store at -20°C. Make aliquots to reduce freeze-thaw cylces.
-**LBA + TG Buffer** by adding 1-Thioglycerol to LBA Buffer. Mark bottle that you have performed this step.
-**RNA Wash Solution** by adding 95% ethanol (Not included in the kit). Mark bottle that you have performed this step.
-**Column Wash Solution** by adding 95% ethanol (Not included in the kit). Mark bottle that you have performed this step.
95% ethanol not included in the kit. Use volumes according to your kit size as mentioned in the manufacturers protocol p. 7f.

-Take **100% isopropanol** for the extraction. (Not included in the kit)

3.2 Prepare **2 eppendorf-tubes** 1.5ml and **one Beadbug™** Prefilled 2ml **Tubes** with 1mm Triple-Pure-High-Impact Zirconium Beads **per sample**. **Label them** accordingly to your samples. Prepare **one additional eppendorf-tube** too. (Not included in the kit)

3.3 Make sure a **homogenizer** and a **centrifuge** are ready.

4 Make sure all **individuals of your organism of interest** are ready.

Chemical extraction

15m

5 Pipette 30 µL **of methylene chlorid** into one GC-MS-vial.

6 **Transfer the individual(s) into the GC-MS-vials.** Extract for 00:15:00 . The vials shall be placed on the crushed ice during chemical extraction. Please note that we emphasis to **shorten the extraction time**. The ideal **minimal extraction time** may be species specific. Modify due to **your knowledge on the study organism!**

15m

7 **Transfer** the secretion-loaded **solvent** into a new vial. The extract can be stored at -20°C

8 If remnants of the solvent are visible, let them evaporate. **Transfer the individual(s)** into the **bead-filled tubes** for RNA extraction. Last remnants will evaporate during transfer.

RNA extraction

4m

9 Pipette 250 µL **LBA + TG Buffer into the bead-filled tube**. We use the manufacturers protocol with the tissue input range of ≤5mg. If your organism is larger (>5mg to 20mg) double the volume. We note every step where you have to adjust the volume.

10 **Homogenize** the samples (4m/s for 20 second, repeated after 20 seconds).
(After lysis in LBA + TG Buffer samples may be stored at −20°C to −70°C for up to three months.*)

11 Add 250 µL **RNA Dilution buffer (RDB)** (double for larger organisms; see step 9). **Vortex** for 10 seconds. **Incubate** for 00:01:00 . **Transfer** the lysate into **a new eppendorf-tube**.

1m

12 Clear homogenates by **centrifugation** 10000 x g, Room temperature, 00:03:00 to pellet insoluble debris. **Transfer the cleared lysates to clean tubes**, taking care to **avoid** any pelleted **debris**.* 3m

13 **Add** 170 µL **100% isopropanol**. For large samples (see step 9) use 340 µL .*

14 Wear clean gloves and open the packs of tubes and minicolumns carefully. Remove **one ReliaPrep™ Minicolumn, two Collection Tubes and one Elution Tube for each sample** to be processed. Place the Collection Tubes in a microcentrifuge tube rack, and place the ReliaPrep ™ Minicolumn into a Collection Tube. Be sure to **label** all your tubes and minicolumns to maintain sample identity. Always wear gloves when handling the tubes and minicolumns.*

15 **Transfer** up to 700 µL of **lysate to** a ReliaPrep™ **Minicolumn** and **centrifuge** 12000-14000 x g, 20-25°C, 00:01:00 . If your original homogenate LBA + TG volume was 500 µL , a second load step will be required. Remove the ReliaPrep™ Minicolumn and discard the liquid in the Collection Tube. Place the ReliaPrep™ Minicolumn back into the Collection tube. Repeat the centrifugation step.* 1m

16 Remove the ReliaPrep™ Minicolumn, and **discard the liquid in the Collection Tube**. Place the ReliaPrep™ Minicolumn back into the Collection Tube. Verify that the RNA Wash Solution has been diluted with ethanol. **Add** 500 µL **of RNA Wash Solution** to the ReliaPrep™ Minicolumn. **Centrifuge** at 12000-14000 x g, 00:00:30 .* 30s

17 Empty the Collection Tube as before and place it in the microcentrifuge rack. In a sterile tube, **prepare the DNase I incubation mix** by combining (in this order) the following amount of each reagent per sample: 15m

24 µL of **Yellow Core Buffer**

3 µL **0.09M $MnCl_2$**

3 µL of **DNase I enzyme**.

Mix by gentle pipetting; do not vortex. Prepare only the amount of DNase I incubation mix needed. Store the DNase I mix on ice while it is thawed. **Apply** 30 µL of this freshly prepared **DNase I incubation mix directly to the membrane inside the column.** Make sure that the solution is in direct contact with and thoroughly covering the membrane. The incubation solution is yellow to make this easier to see.

Note: Do not mix the Yellow Core Buffer and 0.09M MnCl2 prior to this step. The Yellow Core Buffer and 0.09M $MnCl_2$ should be stored separately and mixed immediately prior to each set of RNA preparations.

Incubate for 00:15:00 **at room temperature (+20 to +25°C).***

18 After this incubation, **add** 200 µL **of Column Wash Solution** (verify that ethanol has been added) to the ReliaPrep™ Minicolumn. **Centrifuge at** 12000-14000 x g, 00:00:15 . There is no need to empty the Collection Tube before the next step.* 15s

19 **Add** 500 µL **of RNA Wash Solution** (with ethanol added) and **centrifuge** at 12000-14000 x g, 00:00:30 . Empty wash solutions and discard the Collection Tube.* 30s

20 Place the ReliaPrep™ Minicolumn into **a new Collection Tube. Add** 300 µL **of RNA Wash Solution** (with ethanol added). **Centrifuge at high speed for** , 00:02:00 .* 2m

21 For each sample, remove one capped 1.5ml Elution Tube. **Transfer the ReliaPrep™ Minicolumn** from the Collection Tube **to the Elution Tube**, and **add** 15 µL **Nuclease-Free Water** to the membrane (double the volume for large samles [see step 9]). Be sure to completely cover the surface of the membrane with the water.* 1m

Incubate for 00:01:00 **.**

22 Place the ReliaPrep™ Minicolumn in the centrifuge with the lids of the Elution Tubes facing out. **Centrifuge at** 12000-14000 x g, 00:01:00 . Remove the column and discard. Cap the Elution Tube containing the purified RNA and store at −70°C.* 3m

Optional: You can repeat the elution process by again adding 15µl Nuclease-Free Water to the membrane, incubate for 00:01:00 and centrifuge at 12000-14000 x g, 00:01:00 again. (This has not been done with the samples in the original paper)