FISH DNA extraction:

EcoALpsWater protocol applied for fish DNA extraction from VigiDNA® filtration cartridge using an adaptation of the NucleoSpin® Soil kit (MACHEREY-NAGEL)

Interreg Alpine Space Eco-AlpsWater project – WP1

WPT1 Innovative monitoring approaches (environmental DNA and metabarcoding protocols)
Deliverable D.T1.1.2 Identification and formalization of protocols for eDNA analysis

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I- CONTEXT & GENERAL DESCRIPTION

This protocol is part of the DNA workflow applied in the Eco-AlpsWater Project.

The methodological step described here is the extraction of DNA, this is a critical step for obtaining relevant results since molecular inventories might be influenced by the DNA extraction method used.

The choice of the methodology for fish DNA extraction is based on previous studies and in particular on the study of Pont et al (2018), with some adaptations for the Eco-AlpsWater project.

For the Eco-AlpsWater project, water sampled for eDNA Fish is collected with filtration cartridge adapted to treat large volume of water (VigiDNA® 0.45-µm capsule OR alternatively Envirochek HV 1µm). A preservation buffer is added to the cartridge that is tired at RT until extraction (the extraction should be done within 3 months after the sampling has been done).

The DNA extraction protocol presented below is based on a protocol adapted from the NucleoSpin® Soil kit (MACHEREY-NAGEL) with specific modifications according to the detailed protocol presented by (Pont et al. 2018) and the tests done in the context of the Eco-AlpsWater project.

II. DNA EXTRACTION MATERIAL LIST

- Samples (Cartridge filled with preservation buffer)
- NucleoSpin® Soil kit (MACHEREY-NAGEL)
- ATL buffer from the DNeasy Blood & Tissue Extraction Kit (Qiagen) (720 µL per sample)

- Absolute ethanol: 33 ml per sample
- Proteinase K: 20 µL per sample
- Sodium acetate solution (3 M): 1.50 ml per sample
- Flask shaker (e.g. S50 shaker (cat Ingenieurbüro™))
- A microcentrifuge for 1.5 to 2 mL tubes (relative centrifugal force needed: 11,000 to 18,000 x g)
- A refrigerant centrifuge for 50 mL tubes (relative centrifugal force needed: 15,000 x g at a temperature of 6°C)
- Freezer (-20°C)
- Vortex
- Incubator (56°C)
- Gloves
- 5/10 mL pipette + tips 5/10 mL
- 1000 µL pipette + tips 1000 µL
- 200 µL pipette + tips 200 µL
- 50 mL sterile tube : 1 per sample
- 2 mL sterile microcentrifuge tubes
- 1.5 or 2 mL sterile microcentrifuge tubes to collect DNA at the end of extraction : 1 per sample

III. STEPWISE PROTOCOL

III-1 Precautions before sampling

The following precautions must be applied:

- Wear gloves throughout the extraction process
- Clean the bench with DNA off or 10% commercial bleach before and after manipulation
- Use tips with filters to avoid contaminations
- **All steps have to be performed under a specific DNA-work station (sterile area equipped with air filtration and UV systems)**
- **If possible work in a room equipped specifically for rare/degraded DNA** (i.e. room equipped with positive air pressure, UV treatment, frequent air renewal, and accessible only with full protective clothing and mask). In any case the extraction can be done in a room were PCR products are amplified

III-2 FISH DNA extraction procedure

1. Prepare the sample
    - Agitate the filtration capsule containing the preservation buffer for 15 min on a flask shaker at 800 rpm

- Collect 50 mL of buffer in a 50 mL tube, and preserve the rest of the buffer (about 30 mL) in case of problems during extraction
- Centrifuge at 15,000 x g for 15 min
- Remove the supernatant with sterile 5/10 mL pipette, leaving 15 mL of liquid at the bottom of the 50 mL tube
- Add 33 ml of absolute ethanol and 1.5 mL of 3M sodium acetate to the 50 mL tube
- Store the tube at least one night at -20°C
- Centrifuge the tube at 15,000 x g for 15 min at 6°C
- Discard the supernatant

2. Addition of Lysis buffer
    - Add 720 µL of ATL buffer from the DNeasy Blood & Tissue Extraction Kit (Qiagen)
    - Vortex at full speed for 5 min.
    - Transfer the mixture to a 2 mL tube containing 20 µL of Proteinase K
    - Mix by pipetting
    - Incubate 2 hours at 56°C
    - Centrifuge 2 min at 11,000 x g
    - Transfer the clear supernatant to a new 2 mL tube

3. Adjust binding conditions (step 6 from the NucleoSpin® Soil (MACHEREY-NAGEL GmbH & Co., Düren Germany)
    - Pool the lysate from step 4 in a new 2mL collection tube
    - Add 250 µL of SB Buffer
    - Close the lid and vortex 5 seconds

4. Bind DNA
    - Place a NucleoSpin® Soil Column (green ring) in a Collection tube (2 mL)
    - Load 550 µL of sample onto the column
    - Centrifuge 1 min at 11,000 x g
    - Discard the flow through and place the column back in a collection tube
    - Load the remaining sample onto the column
    - Centrifuge for 1 min at 11.000 x g
    - Discard the flow through and place the column back into the collection tube

    Note: If a large pellet accumulates in the column and blocks the passage of the sample, resuspend the pellet in SB buffer and recentrifuge the column.

5. Wash and dry silica membrane

    Note: the same collection tube is used throughout the entire washing procedure to reduce plastic waste
    - 1st wash
        - Add 500 µL of Buffer SB to the NucleoSpin® Soil Column

- Centrifuge for 30 seconds at 11.000 x g
- Discard the flow through and place the column back into the collection tube

- 2nd Wash
  - Add 550 µL of Buffer SW1 to the NucleoSpin® Soil Column
  - Centrifuge for 30 seconds at 11.000 x g
  - Discard the flow through and place the column back into the collection tube
- 3rd Wash
  - Add 650 µL of Buffer SW2 to the NucleoSpin® Soil Column
  - Close the lid and vortex for 2 seconds.
  - Centrifuge for 30 seconds at 11.000 x g
  - Discard the flow through and place the column back into the collection tube
- 4th Wash
  - Add 650 µL of Buffer SW2 to the NucleoSpin® Soil Column
  - Close the lid and vortex for 2 seconds
  - Centrifuge for 30 seconds at 11.000 x g
  - Discard the flow through and place the column back into the collection tube

6. Dry silica membrane
   - Centrifuge for 2 min at 11.000 x g

   Note: if for any reason, the liquid in the collection tube has touched the NucleoSpin® Soil Column after the drying step, discard flow through and centrifuge again

7. Elute DNA
   - Place the NucleoSpin® Soil Column into a new microcentrifuge tube (not provided in the kit)
   - Add 50 µL of Buffer SE previously heated to 37°C to the column
   - Do not close the lid and incubate for 1 min 30 seconds at room temperature (18-25°C)
   - Close the lid and centrifuge for 30 s at 11.000 x g
   - Repeat the same steps with the same column and the same collection tube to get a final extract of 100 µl: add 50 µL of Buffer SE previously heated to 37°C to the column
   - Do not close the lid and incubate for 1 min 30 seconds at room temperature (18-25°C)
   - Close the lid and centrifuge for 30 seconds at 11.000 x g
   - Throw the column and keep the tube containing the 100 µL of DNA

III-3 Inhibition test and DNA Quantification

After the DNA extraction the samples should be tested for inhibition by qPCR. If the sample is considered inhibited, it should be diluted (5-fold to 10 fold) before the amplification.

Different protocols/equipment can be used to quantify the total amount of DNA extracted

-Qubit ®Fluorometer system
- Nanodrop
- Spectrofluorimetry

III-4 Storage of DNA working stocks

We recommend storing DNA frozen at -20°C until preparation of DNA library for HTS (or at - 40°C to -80°C for longer storage).

IV References:

NucleoSpin® Soil kit (MACHEREY-NAGEL) - User Manual
https://www.mn-net.com/Portals/8/attachments/Redakteure_Bio/Protocols/Genomic%20DNA/UM_gDNASoil.pdf

Pont, D., M. Rocle, A. Valentini, R. Civade, P. Jean, A. Maire, N. Roset, M. Schabuss, H. Zornig, and T. Dejean. 2018. "Environmental DNA reveals quantitative patterns of fish biodiversity in large rivers despite its downstream transportation." *Scientific Reports* 8. doi: 10.1038/s41598-018-28424-8.