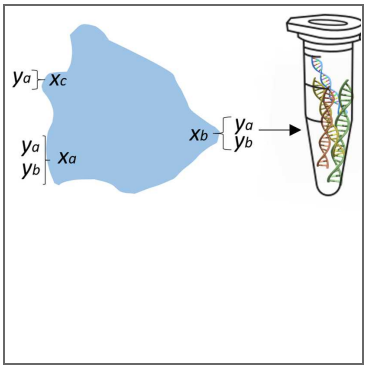

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Protocol status: Working
We use this protocol and it's working

Optimized Macherey-Nagel NucleoSpin Tissue Protocol for Environmental DNA Extraction

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ABSTRACT

This document aims at providing a transparent method and detailing mandatory steps to produce reproducible 1) preparation of an eDNA filter, and 2) environmental DNA extraction.

GUIDELINES

The extraction of environmental DNA should be processed in a dedicated room (not the same room as tissue DNA extraction) with filtered air and positive air pressure. All the samples, consumables and material entering the room should be cleaned with 0.5% sodium hypochlorite solution. Laboratory users should be trained to work in clean conditions (specific instructions about when to wear and change sterile gloves, coats, mobcaps, chirurgical masks, and overshoe protections). The extraction room should be decontaminated between projects or every week with a 0.5% sodium hypochlorite solution.

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MATERIALS

Equipment:

1. Tyvex lab coat (VWR #80200-600)
2. Disposable hair caps
3. Surgical mask
4. Scissors or scalpel
5. Tweezers or sterilized and disposable toothpicks
6. Weigh boats
7. Tube opener
8. -20 °C freezer
9. Pipettes 200 µL (P200)
10. Pipettes 1000 µL (P1000)
11. Racks
12. Vortexer
13. Thermomixer with 2 mL block adaptor
14. Microcentrifuge with rotor for 2 mL tubes
15. Safety wash bottle of ethanol 70%
16. Safety wash bottle of Milli-Q water
17. Safety wash bottle of sodium hypochlorite solution 0.5 %
18. Solid trash
19. Liquid trash

Reagents:

1. Macherey-Nagel NucleoSpin Tissue kit (Macherey-Nagel #740952.250)
2. T1 buffer (Macherey-Nagel #740940.100)
3. B3 Buffer (Macherey-Nagel #740920)
4. Proteinase K 14-22 mg/mL, > 50 U/mL (Macherey-Nagel #740396)
5. Ethanol 95-100%
6. Commercial sodium hypochlorite 6 or 12%
7. Alconox detergent

Consumables:

1. 2 mL microtubes (Ultident #87-B200-C)
2. 1.5 mL Eppendorf Safe-Lock microtubes (VWR #CA21008-959)
3. Lyse&Spin baskets with associated collection tubes (QIAGEN #19598)
4. Collection tubes (QIAGEN #19201)
5. Pipette tips with filter for P200 (VWR #CA89092-968) and P1000 (VWR #CA76416-026)
6. Kimwipes
7. Nitrile gloves
8. Filters: 1.2 or 1.5 glass fibers, 47 or 25 mm (Millipore, Cat no. 1822-047, 1822-025, 1827-047 and 1827-025)

SAFETY WARNINGS

From NucleoSpin handbook:
When working with the NucleoSpin Tissue kit wear suitable protective clothing (e.g., lab coat, disposable gloves, and protective goggles). For more information consult the appropriate Material Safety Data Sheets (MSDS available online at
http://www.mn-net.com/msds).
Caution: Guanidine hydrochloride in buffer B3 and buffer BW can form highly reactive compounds when combined with bleach! Thus, do not add bleach or acidic solutions directly to the sample preparation waste. The waste generated with the NucleoSpin Tissue kit has not been tested for residual infectious material. A contamination of the liquid waste with residual infectious material is highly unlikely due to strong denaturing lysis buffer and Proteinase K treatment but it
cannot be excluded completely. Therefore, liquid waste must be considered infectious and should be handled and discarded according to local safety regulations.

The following risk and safety phrases apply to components of MN NucleoSpin Tissue kit:
- Buffer B3: H302, H315, H319
- Buffer BW: H226, H302, H315, H319, H336
- Proteinase K: H315, H319, H334

H226: Flammable liquid and vapour
H302: Harmful if swallowed
H315: Causes skin irritation
H319: Causes serious eye irritation
H334: May cause allergy or asthma symptoms or breathing difficulties if inhaled
H336: May cause drowsiness or dizziness

From the safety data sheet of the Ethanol Solution 96% from ThermoFisher Scientific (version 24.12.2021):

- Ethanol solution 96%: flammable liquid, serious eye damage/irritation.

Hazard statements:
Highly flammable liquid and vapor
Causes serious eye irritation

Precautionary statements:
Prevention:
Use personal protective equipment as required

Wash face, hands and any exposed skin thoroughly after handling
Wear eye/face protection
Do not breathe dust/fume/gas/mist/vapors/spray
Use only outdoors or in a well-ventilated area
Keep away from heat/sparks/open flames/hot surfaces. - No smoking
Keep container tightly closed
Ground/bond container and receiving equipment
Use explosion-proof electrical/ventilating/lighting equipment
Use only non-sparking tools
Take precautionary measures against static discharge
Keep cool

Response
IF exposed or concerned: Get medical attention/advice
IF INHALED: Remove victim to fresh air and keep at rest in a position comfortable for breathing
IF ON SKIN (or hair): Take off immediately all contaminated clothing. Rinse skin with water/shower
IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing
If eye irritation persists: Get medical advice/attention
In case of fire: Use CO2, dry chemical, or foam for extinction

From the safety data sheet of the sodium hypochlorite solution (10-15%) from ThermoFisher Scientific (version 13.10.2023):

- Sodium hypochlorite: corrosive to metals, respiratory irritation, skin burns and eye damage, toxic gas when in contact with acids.

Hazard statements:
May be corrosive to metals
Causes severe skin burns and eye damage
May cause respiratory irritation
Contact with acids liberates toxic gas

Precautionary statements:
Prevention
Take any precaution to avoid mixing with acids
Do not breathe dust/fumes/gas/mist/vapours/spray
Wear respiratory protection
Wash face, hands and any exposed skin thoroughly after handling
Keep only in original container
Use only outdoors or in a well-ventilated area

Wear protective gloves/protective clothing/eye protection/face protection

Response
IF INHALED: Remove person to fresh air and keep comfortable for breathing.
Immediately call a POISON CENTER/doctor (Québec: 1-800-463-5060)
IF SWALLOWED: Rinse mouth. Do NOT induce vomiting
IF ON SKIN (or hair): Take off immediately all contaminated clothing. Rinse skin with water/ shower
IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing
Wash contaminated clothing before reuse
Absorb spillage to prevent material damage

BEFORE START INSTRUCTIONS

Wash Buffer B5 is supplied as concentrated solution. Before using for the first time, add the appropriate amount of ethanol (95–100%) as indicated on the bottle to obtain a working solution. Wash Buffer B5 can be stored at 15 – 25 °C for at least one year.

If proteinase K is solid, add the indicated volume of proteinase Buffer PB to dissolve lyophilized proteinase K. Proteinase K solution is stable at -20 °C for at least 6 months.

Preheat a thermomixer at 56°C and an another one at 70°C .

Filter preparation

6m

1 Clean bench before use with water (i.e., Milli-Q water, hereafter "water" for cleaning procedures, to remove Macherey-Nagel solutions with guanidine salts prior bleaching; see warnings for details), 0.5% sodium hypochlorite solution (i.e., to degrade DNA), and water (i.e. to rinse traces of sodium hypochlorite). Alternatively, clean bench before use with Alconox, 0.5% sodium hypochlorite solution and ethanol 70% (hereafter referred to ethanol for cleaning procedures).

2 Install all the material on the benchtop including 2 mL microtubes with Lyse&Spin baskets for extracti 1m pre-identified 2 mL Eppendorf Safe-Lock for back up, tweezers, scissors, weigh boats, waste beaker, microtube opener, and gloves.

3 Above a clean weigh boat, take a filter with clean tweezers, unfold it, and cut it in two equal halfs with 1m clean scissors. Alternatively, use sterilized and disposable toothpicks and scalpels to transfer and cut

filters in a weigh boat. Because filters were preserved with ethanol 95-100%, leave one half to air dry on the weigh boat.
Note: Try to divide the filtrate equally on each half.

4 Once air dried, roll the filter half (filtrate inside), and put it in the extraction microtube (2 mL microtube with a Lyse&Spin basket) using clean tweezers (or toothpicks and scalpel). If DNA extraction will occur on a subsequent day, put the filter half into a 2 mL microtube, and store it at -20 °C. If DNA extraction will occur on the same day, keep it at room temperature on the bench. 30s

5 Roll the second half of the filter with tweezers, put it into the Eppendorf Safe-Lock 2 mL previously identified, and store it as back up at -80 °C. Alternatively, store the second half filter in its original tubes containing ethanol 95-100% at -20 °C. 30s

6 If not using disposable equipment, rinse tweezers, scissors, and weigh boats with water, 0.5% sodium hypochlorite solution, and water for final rinse during at least 00:00:30, and ethanol (i.e., to dry equipment and avoid rust). Change gloves. Repeat steps 3 to 6 for each filter. 30s

7 Prepare an extraction negative control for each extraction day. Humidify the filter with 250 µL of Milli-Q water, add 250 µL of ethanol 95-100%, and proceed with steps 3 and 4. Alternatively, the extraction negative control could be prepared at step 14 by adding T1 solution only.
Note 1: Use a filter of material and porosity identical to those from the project.

8 Clean bench with water, 0.5% sodium hypochlorite solution, and water or with water, Alconox, 0.5% sodium hypochlorite solution, and ethanol.

Critical Notes for DNA Extraction

9 *DO NOT TOUCH microtube edges with gloves while opening. Always use a microtube opener. In case doubt for contamination, change gloves before touching another sample.* 1m

DO NOT OPEN more than one microtube at a time to limit contamination. Alternatively, if using a multidispense pipette, leave space between opened tubes.

ALWAYS do a quick spin before microtube opening to limit aerosols. In case of doubt for contamination, rinse the microtube opener with water, 0.5% sodium hypochlorite solution, and water.

Optimally, change the pipette tip between microtubes when adding a solution, even if the same solution is added. Alternatively, use a multidispense pipette with sterile tips, not changing pipette tips while adding the same solution.

DNA Extraction

3h 17m

10 Before use, clean bench with water, 0.5% sodium hypochlorite solution, and water or with water, Alconox, 0.5% sodium hypochlorite solution, and ethanol.

11 Clean pipettes and centrifuges by wiping them down with water, 0.5% sodium hypochlorite solution, water, and ethanol.

12 Install all the material on the benchtop including microtubes, reagents, collection tubes from the Macherey-Nagel NucleoSpin Tissue kit, and racks.

13 Change gloves and work under extractor hood or arm for all subsequent steps because of proteinase K (i.e., may cause breathing difficulties if inhaled).

14 Prepare a mastermix of 360 µL of T1 solution and 50 µL of proteinase K to dispense to each microtube.
NOTE: Work as quickly as possible once proteinase K is mixed to T1 to avoid self digestion.

15 Mix microtubes by inversion for few seconds. Make sure that the filter stays immerged in the solution.

16 Place microtubes in the thermomixer. Incubate Overnight at 56 °C with shaking at 900 rpm 3h digest the filtrate.
NOTE: GF filters should not digest.
NOTE: If not overnight, incubation should last at least 03:00:00.

17 Centrifuge microtubes at 18,000 g during 00:01:00. 1m
NOTE: If lysis solution is still present in the column after centrifugation, redo step 17 until all the solution

went through the column. Make note in an excel sheet.

18 To avoid leaking issues, transfer the eluate to a new labeled 2 mL microtube.

19 Add 400 µL of B3 buffer to each microtube. Change pipette tip between each microtube or use a multidispense pipette with sterile tips, not changing pipette tips. Mix microtubes by inversion for few seconds or vortex microtubes for few seconds.

20 Incubate in the thermomixer for 00:10:00 at 70 °C. 10m

21 Quick spin.

22 Add 420 µL of ethanol 95-100% to each microtube. Change pipette tip between each microtube or use a multidispense pipette with sterile tips, not changing pipette tips.

23 Mix microtubes by inversion for few seconds or vortex microtubes for few seconds.

24 Quick spin.

25 Pipet up to 650 µL of the mix into a MN column placed in a 2 mL collection tube.

26 Centrifuge 00:01:00 at 11,000 g. Discard flow-through. 1m

NOTE: When flow-through is discarded in the trash, be careful not to contaminate gloves and bench top.

27 Repeat steps 25 to 26 until all the solution has passed through the column. Change the collection tube.

NOTE: DNA is on the column.

28 Add 500 µL of BW buffer. Change pipette tip between each microtube or use a multidispense pipette with sterile tips, not changing pipette tips.

29 Centrifuge 00:01:00 at 11,000 g. Discard flow-through and collection tube. 1m

30 Add 600 µL of B5 buffer. Change pipette tip between each microtube or use a multidispense pipette with sterile tips, not changing pipette tips.

31 Centrifuge 00:01:00 at 11,000 g. Discard flow-through. 1m

32 Centrifuge again at 11,000 g for 00:01:00 to dry the column. 1m

33 Place the column into a 2 mL labeled Eppendorf Safe-Lock microtube.

34 Add 50 µL of BE buffer (previously heated at 70 °C) on the center of the membrane.

35 Incubate at Room temperature for 00:01:00. 1m

36 Centrifuge 00:01:00 at 11,000 g. 1m

37 Repeat steps 34 to 36 for a second round of elution.

38 Discard the column and store the 2 mL labeled Eppendorf Safe-Lock microtube with the eluate at 4 °C for short-term storage, at -20 °C for medium-term storage, and at -80 °C for long-term storage.
NOTE: The storage protocol of DNA extracts maximizes chances of eDNA detections since freeze-thaw cycles limit detection of rare molecules (i.e., most likely due to deterioration of DNA's integrity). We favour the storage of DNA extracts within the ultraclean laboratory at -20 °C to limit contamination. We favour the long term storage of DNA extracts at -80 °C to maximize the preservation of DNA integrity, when no detections are planned.

39 Throw liquid bench top trash into the liquid trash of the laboratory.

40 Rinse the liquid bench top trash with water, discard this water in the liquid trash of the laboratory, then clean it with water, 0.5% sodium hypochlorite solution, and water.

41 Clean bench with water, 0.5% sodium hypochlorite solution, and water or with water, Alconox, 0.5% sodium hypochlorite solution, and ethanol.