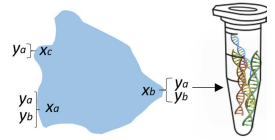




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Optimized Macherey-Nagel NucleoSpin Tissue Protocol for Environmental DNA Extraction



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We use this protocol and it's working

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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, chirurgial masks, and overshoe protections). The extraction room should be decontaminated between projects or every week with a 0.5% sodium hypochlorite solution.

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

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 extraction, pre-identified 2 mL Eppendorf Safe-Lock for back up, tweezers, scissors, weigh boats, waste beaker, microtube opener, and gloves. 1m
- 3 Above a clean weigh boat, take a filter with clean tweezers, unfold it, and cut it in two equal halves with 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. 1m
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. 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 of 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 immersed in the solution.





- 16 Place microtubes in the thermomixer. Incubate  Overnight at  56 °C with shaking at 900 rpm to 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 . 
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 . 

- 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. 
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 $^{\circ}\text{C}$) 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 $^{\circ}\text{C}$ for short-term storage, at  -20 $^{\circ}\text{C}$ for medium-term storage, and at  -80 $^{\circ}\text{C}$ 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. 

Protocol references

[Instruction-NucleoSpin-Tissue.pdf \(mn-net.com\)](#)

[msds \(fishersci.ca\)](#)

[msds \(fishersci.com\)](#)