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Full 18S metabarcoding of environmental samples of various substrats with Minion Nanopore

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18S Metabarcoding Minion

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We use this protocol and it's working

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Disclaimer

There are already a couple of metabarcoding protocols existing on this platform (e.g. **Aquatic eDNA sampling and plant community metabarcoding with portable Nanopore Flongle sequencing (v0.0.3) V.3**, **Metabarcoding using MinION: PCR, Multiplexing and Library Preparation V.2**, or **Nanopore amplicon sequencing V.4**) but because of the fast development of Nanopore technology, none of them used the most updated multiplex approach. In addition, some of them are too specific, and some focused only on sequencing, or library prep sections. Therefore, we estimated that this new exhaustive and global protocol was a valuable addition to this recent fast-evolving approach.

Abstract

We describe here a flexible protocol for eDNA metabarcoding with Oxford Nanopore's MinIon MK1C platform from sampling to sequencing.

The first section summarizes some key steps of sampling and sample preservation for both aquatic and terrestrial environments. The second one describes the DNA extraction protocol with the DNeasy PowerSoil Pro Kit of Qiagen for different types of samples (i.e., soil, liquid and dead plant materials). Our DNA amplification and bead purification protocols are characterized in the third section. Finally, the library prep with the Native amplicon Barcoding Kit 96 V14 (SQK-NBD114.96) and sequencing with the R10.4.1 flow cells (FLO-MIN114) and MinION Mk1C device are presented in the last sections.

This protocol has been optimized for protists (microbial eukaryotes) and 18S marker, but should be easily adjustable for other organisms, by modifying the sampling and DNA extraction sections, or other markers, even longer ones such as the full ribosomal operon, if need be.

Our goal is to teach/train researchers from different fields and different expertise on obtaining nanopore sequences from environmental samples by guiding them from protocol to protocol, focusing on the key steps, and informing them of the expected results based on our 3 runs performed so far.

A comprehensive bioinformatic pipeline to treat the data produced, as well as a methodological article discussing this method and the best ways to use it are in preparation.

Sampling

15m

1 Select a pristine area without anthropic disturbance and collect your sample of interest (e.g. ~ 0.5-1 kg of soil, ~ 50-200 g of dry leaves or bark, ~ 1-2 L of water) in a clean plastic bag or bottle. Register all valuable sample information such as the date, label of the sample, localization of the site, coordinates, type of material, and name of the sampler, and label your samples accordingly. It is often recommended to take as well pictures of the habitat and the sample. Avoid as much as possible collecting stones, roots and animals as this will diminish the amount of DNA of interest. If you are worried about potential contamination during the sample collection you can use gloves. Moreover, sterilize your shovel, spoon, or any tool you use with ethanol or bleach to avoid cross-contamination between samples.

10m

2 As soon as your sample is collected it will be impacted by your storage condition, which matters a lot for microbial organisms, in particular, as they have a fast turnover. Consequently, you should avoid as much as possible to delay the return to the laboratory. In the meantime, you can use a cooler with ice or at least avoid storing your sample in a hot vehicle. Alternatively, aliquot ~ 2-2.5 mL of nucleotide buffer, such as **LifeGuard Soil Preservation Solution (Qiagen)**, in 5 ml tubes before starting the sampling, and place ~ 1 g of soil to stabilize it until the return to the lab.

3 Once back in the laboratory, the best is to proceed to the DNA extraction as soon as possible. Until then, storage in the freezer (-20 °C) is adequate for a few days for soils, but -80 °C is better for long-term storage. For aquatic samples, storing in the fridge overnight should have a low impact on microbial communities, but we do not recommend extending storage at 4 °C much longer. In addition, freezing aquatic samples is not recommended either as this could break cells during thawing and lead to DNA loss during further filtrations.

Preparation of soils samples for DNA extraction

4h

4 Remove your soil samples from the fridge, if sampled a few hours ago, or the freezer, and let them thaw.

Note

We usually worked on a series of 20 samples, but no more than 24 because of room limitations on the device. We recommend working with an even number of samples to help balance the centrifuge and bead-beating device.

5 Ideally, the DNA extraction should be performed under a laminar flow cabinet to avoid airborne contamination of the samples and reagents, this is particularly important when working with prokaryotes or fungi that are often dispersed in the air. Clean the cabinet with ethanol and install a microscale, a kit of DNeasy PowerSoil Pro (not open on the bench), a rack for 2 ml tubes size, a box of gloves, a bag of 5ml tubes, cleaning paper, a Bunsen burner, a lighter, a pen and a benchtop centrifuge machine. All reagents, boxes of gloves and tubes should not be open on the bench to avoid contamination! Once everything is prepared, sterilize well with the ethanol. 5m

6 Weigh ~ 0.25 g of soil sample in a clean 5ml tube, with a metallic lab spoon, avoiding mineral particles as well as obvious animals and roots or plant materials. Transfer the sample to a labeled PowerBead Pro tube previously spun. Sterilize the lab spoon with the ethanol and the Bunsen burner, and weigh the next sample. 1h 30m

Note

1h is an estimation for a series of 20 samples. Count 3-5 min per sample

7 Remove the microscale from the laminar flow cabinet and add a vortex device. Change your gloves and sterilize again the cabinet with ethanol. 3m

Preparation of alternative sample types for DNA extraction 4h

8 Despite being optimized for soil samples, the DNeasy PowerSoil Pro kit (Qiagen) can also be used for liquid samples, such as water, in order to limit DNA extraction bias when comparing different types of samples.

CITATION

Singer D, Seppey CVW, Lentendu G, Dunthorn M, Bass D, Belbahri L, Blandenier Q, Debroas D, de Groot GA, de Vargas C, Domaizon I, Duckert C, Izaguirre I, Koenig I, Mataloni G, Schiaffino MR, Mitchell EAD, Geisen S, Lara E (2021). Protist taxonomic and functional diversity in soil, freshwater and marine ecosystems..

LINK

https://doi.org/10.1016/j.envint.2020.106262

9 For water samples, filter directly the sample through a 250 µm to remove large particles, e.g. a **SE filter GP2-1100 1/100 inch size** from Amazon. Always sterilized the mesh with bleach before starting the next sample.

10 For leaves or bark samples start by weighing ~ 5 g of material in a clean bottle, add 250 mL of clean water (we used Deer Park) and shake vigorously for about 00:01:00. Then, filter the sample through a 250 µm mesh to remove large particles, e.g. a **SE filter GP2-1100 1/100 inch size** from Amazon. 1m

11 Then pour this filtrate into a Nalgene cellulose nitrate membrane with 0.2 µm pores and 50 mm diameter (**121-0020**) until saturation of the membrane or all sample filtration (cf. Fig 1). A vacuum pump can be used to speed up the process. 1h

Note

We worked on the bench for practical reasons and we used a Bunsen burner to avoid air contamination above the membrane or the sample.

Note

It is hard to estimate the time required for a series of samples as the duration depends on the speed of water filtration on the membrane. Some muddy samples took a half hour and few volumes, i.e. 0.125 L, were filtrated before saturation of the membrane.

12 Transfer the Nalgene filter to the laminar hood cabinet, cut the membrane loose and then in two parts with a sterilized scalpel. Finally, transfer these two pieces of membrane directly into the PowerBead Pro tube previously spun. Always sterilize the scalpel with ethanol before starting the next sample. 30m

Note

If working with a large number of samples, consider storing the filters in PowerBead overnight in the freezer before pursuing the protocol.

Note

In order to control if our approach was sterile, we added a blank composed of an unused membrane.

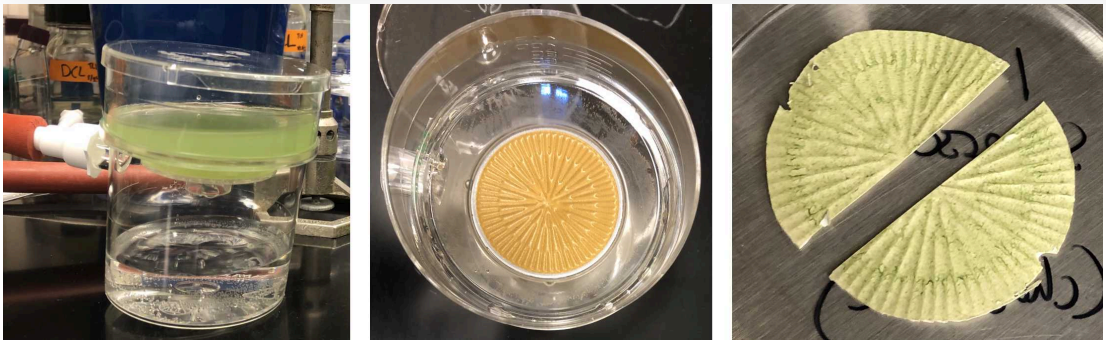


Fig 1 showing the filtration process described in steps 9 and 10. Note the variation of color displayed by these three different samples.

13 Ideally, the DNA extraction should be performed under a laminar flow cabinet to avoid airborne contamination of the samples and reagents, this is particularly important when working with prokaryotes or fungi. Clean the cabinet with ethanol and install a vortex device, a kit of DNeasy PowerSoil Pro (not open on the bench), a rack for 2 ml tubes size, a box of gloves, cleaning paper, a pen and a benchtop centrifuge machine. All reagents, boxes of gloves and tubes should not be open on the bench to avoid contamination! Once everything is prepared, sterilize well with the ethanol.

DNA extraction with DNeasy PowerSoil Pro kit (Qiagen)

4h

14 After the transfer of the samples or filters to the PowerBead Pro tubes, perform a bead beating step with a FastPrep24 homogenizer (MP Biomedicals, USA) with a program of 2 cycles composed of 00:00:20 at 4 m/s followed by 00:05:00 of break.

5m 20s

fastprep24_Manual.pdf

Note

Label the tubes both on the top AND on the side as this step often erases the label due to rubbing.

15 From now on, follow the protocol DNeasy PowerSoil Pro kit from Qiagen (corresponding here to steps 13-26). First centrifuge the PowerBead Pro Tube at 15,000 x g for 00:01:00 .

1m

Qiagen_DNeasyPowerSoilProKit.pdf

16 Transfer the supernatant to a clean 2 ml Microcentrifuge Tube (provided).

Note

Note: Expect 500-600 µL. The supernatant may still contain some soil or filter particles.

17 Add 200 µL of Solution CD2 and vortex for 00:00:05. 5s

18 Centrifuge at 15,000 x g for 00:01:00. Avoiding the pellet, transfer up to 700 µL of supernatant to a clean 2 ml Microcentrifuge Tube (provided). 1m

Note

Note: Expect 500-600 µL.

19 Add 600 µL of Solution CD3 and vortex for 00:00:05. 5s

20 Load 650 µL of lysate to an MB Spin Column. Centrifuge at 15,000 x g for 00:01:00. 1m

21 Discard the flow-through and repeat the previous step to ensure that all of the lysate has passed through the MB Spin Column.

22 Carefully place the MB Spin Column into a clean 2 ml Collection Tube (provided). Avoid splashing any flow-through onto the MB Spin Column.

23 Add 500 µL of Solution EA to the MB Spin Column. Centrifuge at 15,000 x g for 00:01:00. 1m

24 Discard the flow-through and place the MB Spin Column back into the same 2 ml Collection Tube.

25 Add 500 µL of Solution C5 to the MB Spin Column. Centrifuge at 15,000 x g for 00:01:00 . 1m

26 Discard the flow-through and place the MB Spin Column into a new 2 ml Collection Tube (provided).

27 Centrifuge at up to 16,000 x g for 00:02:00 . Carefully place the MB Spin Column into a new 1.5 ml Elution Tube (provided). 2m

28 Add 50-100 µL of Solution C6 to the center of the white filter membrane.

Note

We used 100 µL of solution C6 to be able to quantify easily the DNA (cf. step 30).

29 Aliquot 20 µL of the resulting DNA for working on the bench, and keep the remaining 80 µL sterile by opening it only when necessary and below the laminar flow cabinet only. We labeled each tube by adding: DNA, the name of the project, the names of the samples, the dates, the person who performed the extraction, and either Aliquot or Stock, then stored both tubes at -20 °C .

30 Quantify 2 µL of all samples with Qubit High Sensitivity Double Strand (HS dsDNA) kit in order to assess the concentration of DNA. Qubit_dsDNA_HS_Assay_UG.pdf

Note

The values we obtained ranged from 4.6-114 (mean = 46.5, median = 48) ng/µl.

31 Evaluate the quality of each sample with 1 µL of DNA with Nanodrop.

nd-1000-v3.8-users-manual-8 5x11.pdf

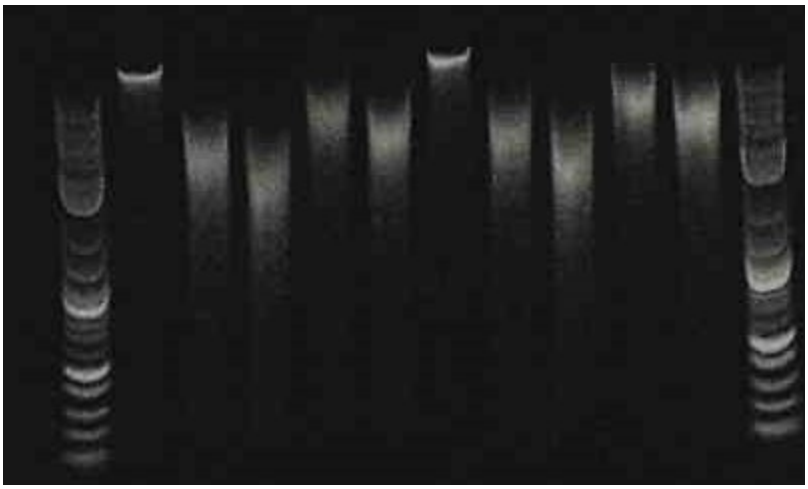
Note

The concentration values we obtained ranged from 6.3-184 (mean = 62.5, median = 57.7) ng/µl. The values for the ratio 260/280 ranged from 1.91-2.48 (mean = 1.93, median = 1.9) and those for the ratio 260/230 ranged from 0.01-0.75 (mean = 0.65, median = 0.5).

32 Control the integrity of the DNA, for instance by running a 1% TA gel at 70 volts for 03:00:00 with 3 µL DNA. Use a ladder with long fragments such as 1kb+ Biolabs indicating up to 10kb fragment size. 3h

Note

DNA fragmentation is particularly problematic for the amplification of long sequences. Based on our experience, fragments of 4-5 kb are long enough for the amplification of 2kb. With our described bead-beating program we obtained sequences of 5-8 kb. Reducing the intensity of the program decrease significantly the DNA yield and increase the risk that some resistant cells might not be properly broken, inducing a bias in the resulting community.



1% TA gel at 70 volts for 03:00:00 with 3 µL DNA and 1kb+ ladder Biolabs (both extremities) to evaluate the fragmentation in two soil samples with 5 different programs.
1st + 6th columns correspond to 2 cycles of vortex full speed for 2.5 min with 3 min of break.
2nd + 7th columns correspond to 2 cycles of bead-beating at 6.5 m/s for 45s with 5 min of break.
3rd + 8th columns correspond to 5 cycles of bead-beating at 6.5 m/s for 45s with 5 min of break.
4th + 9th columns correspond to 2 cycles of bead-beating at 4 m/s for 20s with 5 min of break.
5th + 10th columns correspond to 5 cycles of bead-beating at 4 m/s for 20s with 5 min of break.

Amplification of the full 18S

16h

33 Dilute e.g. 6 µL of all your DNA to 10 ng/µL with Elution Buffer. If some samples have a lower concentration dilute to 5 ng/µL instead.

34 Dilute your primers to 10 µM.

We used the primer forward S1 (5- AAC CTG GTT GAT CCT GCC -3) and reverse RibB (5- GAT CCT TCT GCA GGT TCA CC- 3) designed by Fiore-Donno et al. 2005 and 2008, based on Medlin primers, and which amplify a region of ~1800 bp corresponding to the almost entire 18S.

CITATION

Fiore-Donno, A.-M., Berney, C., Pawlowski, J., Baldauf, S.L. (2005). Higher-order phylogeny of plasmodial slime molds (Myxogastria) based on EF1A and SSU rRNA sequences. J. Eukaryot. Microbiol.

LINK

DOI: 10.1111/j.1550-7408.2005.00032.x

CITATION

Fiore-Donno AM, Meyer M, Baldauf SL, Pawlowski J (2008). Evolution of dark-spored Myxomycetes (slime-molds): molecules versus morphology..

LINK

https://doi.org/10.1016/j.ympev.2007.12.011

35 Prepare a PCR mix with:

- 12.5 µL GoTaq 2x Master Mix
- 0.5 µL primer S1 10 µM
- 0.5 µL primer RibB 10 µM
- 10.5 µL Nuclease-free water

to obtain a total of 24 µL per sample. Multiply this ratio of reagent by the number of samples (+1 extraction blank, +1 PCR negative control, and +1 additional volume) and mix it well by pipetting.

36 Distribute 24 µL of PCR mix in as many tubes as samples, or use 96-well plate. Add 10 ng of DNA sample in a volume of 1 µL, to reach a final volume of 25 µL and mix well by pipetting.

37 Amplify the 18S gene from your samples, in a thermal cycler (e.g. Dual 48 Well DNA Engine PTC0200, BioRad, USA), using the following cycling conditions: 3h

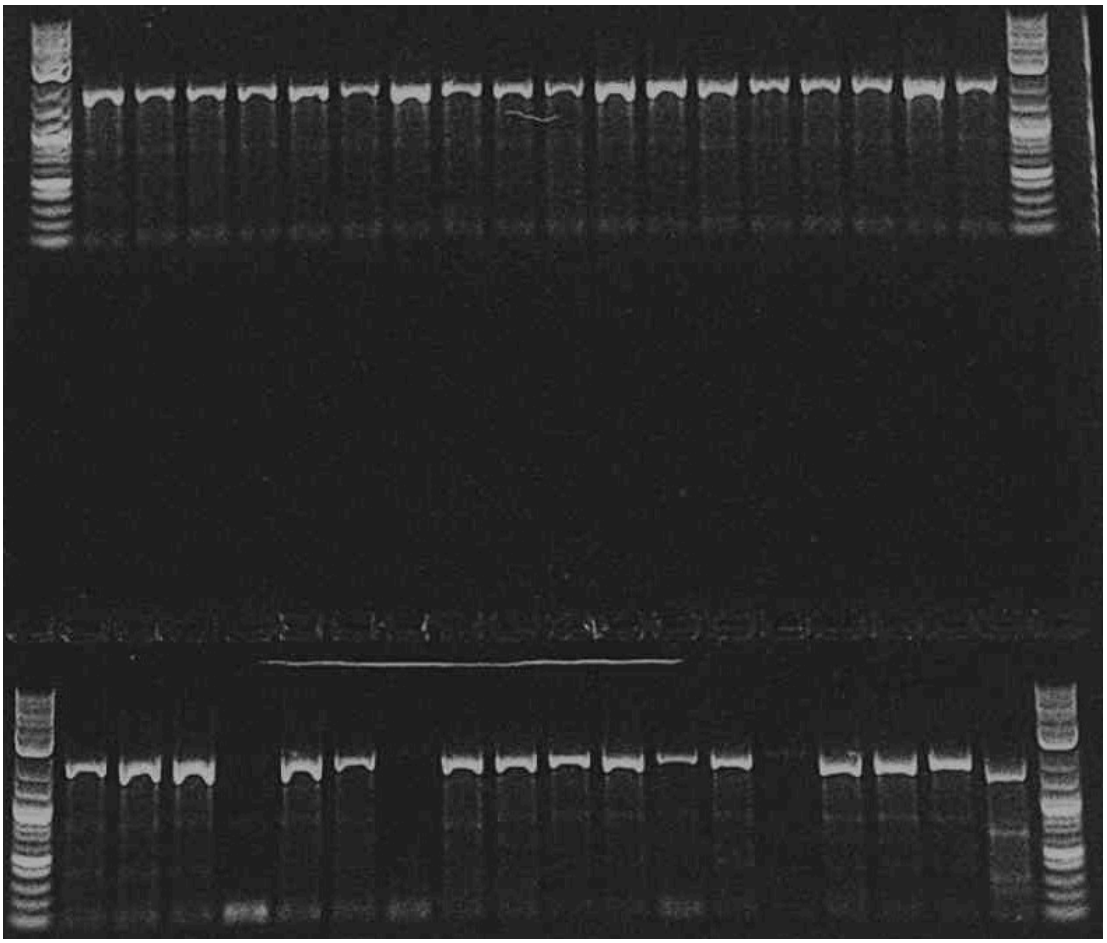
1. Initial denaturation 00:02:00 at 95 °C (1 cycle),
2. Denaturation 00:00:30 at 95 °C (33 cycles),
3. Annealing 00:00:30 at 48 °C (33 cycles),
4. Extension 00:04:00 at 72 °C (33 cycles),
5. Final extension 00:05:00 at 72 °C (1 cycle),
6. Hold at 10 °C (1 cycle)

38 In order to avoid PCR bias and to obtain enough DNA for the sequencing, perform all sample amplification in three independent replicates.

39 Control all PCR products by electrophoresis, e.g. with a 1% TBE or TA gel at 100 volts for 01:00:00 with 3 µL DNA. Use a ladder with long fragments such as 1kb+ Biolabs indicating up to 10kb fragment size. 1h

Note

Some light smires were sometimes observed but this did not impact the quality of the sequencing.



Example of a gel obtained with the described protocol. Light smires can be observed below the bands.

Design and creation of a Mock community

4h

40 In order to assess the quality of Nanopore sequencing, we selected 8 cDNA of diverse amoebae cultures for which we previously obtained transcriptomic data including the full 18S sequences.

41 We amplified independently these 8 cDNA with the same protocol described above.

42 We controlled 3 µL of each PCR product by electrophoresis as described earlier.

43 We quantified 1 µL of each of them with qubit HS as described earlier.

44 We merged equivolume, i.e. 20 µL, of all PCR products samples in a single tube.

Triplicates preparation, purification and quantification

8h

45 Once good bands are obtained for all sample triplicates, merge each triplicate independently together in a 0.2 ml tube (i.e. one tube per sample!).

> **Note**
>
> It is recommended to perform a quick centrifugation of the triplicates before merging in order to avoid losing DNA.

46 Merge all the PCR negative control PCR products in the same tube of adapted size.

47 Remove Mag-Bind® Total Pure NGS from the fridge about 00:30:00 before starting the purification. Then add a 1:1 ratio of temperate and well-vortexed Mag-Bind beads into each triplicate and leave 00:10:00 at room temperature in a Hula mixer at 160 speed. 40m

Omega_Mag-Bind_TotalPureNGS_Cl…

> **Note**
>
> In order to be safer and more precise, we worked with individual tubes instead of a plate, and performed several series of 6-8 tubes at a time in order to respect the duration and avoid losing DNA.

> **Note**
>
> It is extremely important that the beads are well-vortexed for at least 00:00:10 just before adding them to the DNA. Otherwise, it will bias the ratio of beads.

Note

An alternative to the Hula mixer is to pipette up and down a dozen time or to vortex it gently for 00:00:30 .

48 Perform a quick centrifugation and pellet the DNA in a magnet for 00:02:00 , or until the liquid is fully transparent, and then discard the supernatant without disturbing the beads. 2m

49 Without removing the tubes from the magnet, add 200 µL of freshly prepared 70% ethanol, let it stand for 00:00:30 , and remove the supernatant without disturbing or collecting the beads. 30s

50 Repeat the previous step for an additional washing step. 30s

51 Perform a quick centrifugation, return the tubes to the magnet, wait for 00:00:10 , remove leftover ethanol and let dry for 00:00:30 . 40s

Note

It is crucial that no ethanol remains in the tubes, but do not let the beads dry, otherwise you will lose DNA!

52 Add 31 µL of Elution Buffer in the samples and incubate for 00:10:00 at 37 °C with Hula mixer at 160 speed. For the PCR negative control add only 15 µL . 10m

Note

Warm conditions favor the DNA release from the beads.

53 Perform a quick centrifugation and pellet the beads on the magnet for 00:02:00, or until the liquid is fully transparent, and remove 30 µL of DNA without beads. 2m

54 Quantify all purified DNA samples, mock community and the two negative controls (i.e. the extraction of the clean membrane and the PCR without DNA, both should have an undetectable amount of DNA).

Note

We obtained values ranging from 45-116 (mean = 79.5, median =79.5) ng/µl, except for the negative controls that were undetectable.

Nanopore libraries planification 8h

55 *One of the great advantages of the Nanopore sequencing approach is its flexibility. Indeed, it is possible to sequence various samples with diverse fragment sizes simultaneously. In the present case, in addition to the 24 environmental samples, associated with the mock community and the two negative controls, we also added 9 samples of a single organism, with a different gene, with different sizes ranging from 630-750 bp. The results we obtained were adequate for all of them.*
Based on our 3 sequencing following this protocol with the same Nanopore chemistry, we observed that a number of samples ranging from 36 to 57 seems adequate but can certainly be extended toward fewer or more sequences. Keep in mind that the number of sequences per sample will decrease to a certain point with the addition of more samples and vice versa. But if too few samples are used there is the risk of having too little DNA for an optimal run of the flow cell.

56 In order to obtain the 200 fmol of DNA required for the library, it is necessary to calculate the mass it represents and this is directly related to the size of fragments obtained.

Some online calculators exist to help you, e.g.
https://www.bioline.com/media/calculator/01_07.html

With our primers combination, we know that the size of our fragments should be around 1.8 kb, which is confirmed by the electrophoresis gels. But, because we know that some taxa possess up to 2 kb sequences and that we prefer to obtain rather a bit more than too little DNA at this step, we chose to target 2 kb instead of 1.8 kb. Based on the calculator, 200 fmol of dsDNA

fragments correspond to a mass total of ~ 260 ng . As the initial DNA samples required input is 11.5 µL , the concentration needed is therefore 260/11.5 = 22.6 ng/µl.

Note

It is possible to combine different projects with different sequence sizes and different sequencing depths (e.g. for barcoding). If so, the optimal concentration has to be calculated for the different sizes.

57 Dilute all your samples to a final concentration of ~22.6 ng/µl. It is best to target a final volume greater than 11.5 µL as you will need at least 1 µL for a final quantification. Depending on your concentrations, we recommend a final volume of ideally ~ 15-30 µL .

Note

As it is hard to dilute DNA to a precise concentration and that total DNA is limited, we decided to accept a range of concentration between 20-25 ng/µl as an input. In the case of one sample below this threshold, we suggest taking it anyway, but it might yield fewer sequences than the others.

Note

This step was certainly one of the most difficult and frustrating steps of the whole pipeline. Do not underestimate the time required to obtain the correct concentration, especially for a large number of samples. Fortunately, the proportion of sequences we obtained from the different samples was satisfactory at the end.

58 If your samples are too diluted to obtain 200fmol in 11.5 µL , it is possible to use a speed vacuum concentrator in order to reconcentrate your samples and dilute them to the correct dilution. Manual_SpeedVac_VacuumConcentr…

59 For the blank sample of extraction and the PCR negative control use 11.5 µL even if the concentration is extremely weak.

60 Download and follow the most recent version of the Nanopore protocol, in our case, it was the revision H from the protocol version from 15th September 2022.

ligation-sequencing-amplicons-native…

Note

Before preparing the library be sure to possess a mk1c nanopore device, a valid flow cell, the adapted kit, and all third-party reagents required. In our case, we used:
-Native Barcoding Kit 96 V14 (SQK-NBD114.96)
-R10.4.1 flow cells (FLO-MIN114)
-Flow Cell Wash Kit (EXP-WSH004)
-NEB Blunt/TA Ligase Master Mix (NEB, cat # M0367)
-NEBNext Ultra II End repair/dA-tailing Module (NEB, cat # E7546)
-NEBNext Quick Ligation Module (NEB, cat # E6056)

Note

The ONT protocol includes 4 steps summarised and commented in the next 4 sections of this protocol.
1) The **End-Prep** step to prepare the DNA ends for adapter attachment
2) The **Native Barcode Ligation** step to ligate a unique adapter to each sequence of a sample for multiplexing purposes.
3) The **Adapter ligation and clean-up** step to ligate Nanopore sequencing adapters to the sequences.
4) The **Priming and loading the SpotON flow cell** step to prepare the flow cell and start the run.

End-prep step, summary from the original protocol

1h 30m

61 Thaw the DNA Control Sample (DCS) at room temperature, mix by vortexing, and place on ice. Prepare the NEBNext Ultra II End Repair / dA-tailing Module reagents in accordance with manufacturer's instructions, and place on ice.

Note

Do not vortex the NEBNext Ultra II End Prep Enzyme Mix, but vortex well the buffer.

62 Dilute your DNA Control Sample (DCS) by adding 105 µL Elution Buffer (EB) directly to one DCS tube. Mix gently by pipetting and spin down.

Note

Using the DNA Control Sample is not mandatory, but recommended by ONT. We used it for all samples except the negative controls.

63 Prepare a mix composed of 1 µL Diluted DNA Control Sample (DCS), 1.75 µL Ultra II End-prep Reaction Buffer and 0.75 µL Ultra II End-prep Enzyme Mix (total 3.5 µL) by multiplying your sample number +1. Between each addition, pipette mix 10-20 times.

Note

To test for potential tag-jumping issues, we always prepared a library negative control by replacing DNA template by Elution Buffer (EB).

Note

For all negative controls, i.e., the DNA extraction Blank, the PCR negative controls and the library control, we created a different mix by replacing the 1 µL Diluted DNA Control Sample (DCS) by 1 µL of Elution Buffer (EB).

64 Distribute 3.5 µL of the freshly prepared mix to a 96-well plate, or 8-stripes tubes.

Note

To avoid wasting costly reagents, we used a single-channel pipette to distribute the mix to the tubes.

65 Distribute 11.5 µL of the 200 fmol amplicon DNA to the corresponding tubes and pipette mix 10-20 times (volume total 15 µL).

Note

To save time we used a multi-channel pipette to distribute the DNA.

66 Perform a quick centrifugation to spin down the volume and incubate at 20 °C for 00:05:00 and 65 °C for 00:05:00 using a thermal cycler. 10m

Note

The End-prep library can be stored overnight providing some additional steps, cf. Nanopore protocol. We always proceed directly with the Native barcode ligation step.

Native barcode ligation step, summary from the original protocol 3h

67 Thaw the AMPure XP Beads (AXP), the EDTA and the Native Barcodes (NB01-96) at room temperature. Keep the beads at room temperature at all times, but place the two others on ice. Vortex the EDTA and spin it down. The Native barcodes plate and the NEB Blunt/TA Ligase Master Mix have to be spun down and then mixed by performing several pipette mixes.

68 In a new 96-well plate or 8-stripes tubes, add the reagents in the following order per well: Nuclease-free water 3 µL, End-prepped DNA 0.75 µL, Native Barcode (NB01-96) 1.25 µL and Blunt/TA Ligase Master Mix 5 µL for a total of 10 µL. Always pipette mix 10 - 20 times between each addition.

Note

Select a unique barcode for every sample to be run together on the same flow cell. Up to 96 samples can be barcoded and combined in one experiment.

Note

We used a multi-channel pipette to distribute the Nuclease-free water, the End-prepped DNA and the Native Barcode, but used a single-channel pipette for the Blunt/TA Ligase to avoid any loss of this enzyme.

69 Ensure the reaction is thoroughly mixed by gently pipetting, then spin down briefly and incubate for 00:20:00 at room temperature. 20m

70 Add 1 µL of EDTA to each well to stop the ligation of the barcodes, mix thoroughly by pipetting and spin down briefly.

71 Once the EDTA has been added to each library, pool the barcoded samples in a 1.5 ml Eppendorf DNA LoBind tube.

72 Resuspend the AMPure XP Beads (AXP) by vortexing and add to the reaction a multiple of sample number and 0.44 µL of beads, i.e. a 0.4x ratio. Mix well by pipetting.

Note

It is really important that the beads are well-homogenized initially, so vortex at least 00:00:30 just before pipetting.

73 Incubate on a Hula mixer (rotator mixer) for 00:10:00 at room temperature. During that time, prepare 2 mL of fresh 80% ethanol in nuclease-free water. 10m

74 Spin down the sample and pellet on a magnet for 5 minutes. Keep the tube on the magnetic rack until the eluate is clear and colourless, and pipette off the supernatant without disturbing the beads.

75 Keep the tube on the magnetic rack and wash the beads with 700 µL of freshly prepared 80% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.

76 Repeat the previous step for a second washing. Then, spin down and place the tube back on the magnetic rack. Pipette off any residual ethanol. Allow the pellet to dry for ~ 00:00:30 , 30s

but do not dry the pellet to the point of cracking.

77 Remove the tube from the magnetic rack and resuspend the pellet in 35 µL nuclease-free water by gently flicking.

78 Incubate for 00:10:00 at 37 °C. Every 00:02:00, agitate the sample by gently flicking for 00:00:10 to encourage DNA elution. 12m 10s

Note

We used a Hula mixer to maintain a constant agitation.

79 Pellet the beads on a magnetic rack until the eluate is clear and colourless. Then remove and retain 35 µL of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.

Note

The resulting barcoded DNA library can then be used directly for the next step or left overnight at 4C. Do not freeze it! We always paused overnight and resumed the library process the next day.

Adapter ligation and clean-up step, summary from the original protocol 1h

80 Spin down the Native Adapter (NA) and Quick T4 DNA Ligase, pipette mix and place on ice. Thaw the Elution Buffer (EB), Short Fragment Buffer (SFB) and NEBNext Quick Ligation Reaction Buffer (5x) at room temperature, mix by vortexing, spin down and place on ice.

Note

Do not vortex the Quick T4 DNA Ligase!

Note

Depending on the wash buffer (LFB or SFB) used, the clean-up step after adapter ligation is designed to either enrich for DNA fragments of >3 kb, or purify all fragments equally.
-To enrich for DNA fragments of 3 kb or longer, use Long Fragment Buffer (LFB)
-To retain DNA fragments of all sizes, use Short Fragment Buffer (SFB)

81 In a new 1.5 ml Eppendorf LoBind tube, mix in the following order: Pooled barcoded sample 30 µL, Native Adapter (NA) 5 µL, NEBNext Quick Ligation Reaction Buffer (5X) 10 µL and Quick T4 DNA Ligase 5 µL for a total of 50 µL. Between each addition, pipette mix 10 - 20 times.

82 Ensure the reaction is thoroughly mixed by gently pipetting, then spin down briefly and incubate the reaction for 00:20:00 at room temperature. 20m

83 Resuspend the AMPure XP Beads (AXP) by vortexing. Then add 20 µL of resuspended AMPure XP Beads (AXP) to the reaction and mix by pipetting.

Note

It is really important that the beads are well-homogenized initially, so vortex at least 00:00:30 just before pipetting.

84 Incubate on a Hula mixer (rotator mixer) for 00:10:00 at room temperature. 10m

85 Spin down the sample and pellet on the magnetic rack. Keep the tube on the magnet and pipette off the supernatant.

86 Wash the beads by adding 125 µL of Short Fragment Buffer (SFB). Flick the beads to resuspend, spin down, then return the tube to the magnetic rack and allow the beads to pellet. Remove the supernatant using a pipette and discard.

87 Repeat the previous step. Then spin down and place the tube back on the magnet. Pipette off any residual supernatant.

88 Remove the tube from the magnetic rack and resuspend pellet in 15 µL Elution Buffer (EB).

89 Spin down and incubate for 00:10:00 at 37 °C. Every 00:02:00, agitate the sample by gently flicking for 00:00:10 to encourage DNA elution. 12m 10s

> Note
>
> We used a Hula mixer to maintain a constant agitation.

90 Pellet the beads on a magnet until the eluate is clear and colourless, for at least 00:01:00 1m
.
Remove and retain 15 µL of eluate containing the DNA library into a clean 1.5 ml Eppendorf DNA LoBind tube.

91 Quantify 1 µL of the resulting eluted sample using a Qubit fluorometer (High Sensitivity dsDNA). Optionally, it is also possible to quantify 1 µL of the resulting library after the Native Barcode Ligation step for more control on the DNA loss during both purification.

> Note
>
> The quantification is highly dependent on the initial number of samples selected. The higher the number of samples, the higher the mass of DNA, the higher the DNA concentration.

92 ONT recommends to make up the library to 12 µl at 10-20 fmol. Too few fmol will leave the pores inactive which will reduce the yield of sequences but also damage the pores. Whereas, too much fmol will be detrimental as it could block the pores or perturbate the electrical signal. We always targeted just below 20 fmol. As 12 µL is the volume required for the loading step, we recommend preparing a higher volume of diluted library to compensate for the volume left during quantification(s) but also to be able to load several runs if required.

Similarly to the initial DNA input calculation for the library, we now have to calculate the mass of DNA to use for the loading step. Optionally, use the calculators if needed, e.g.

https://www.bioline.com/media/calculator/01_07.html. Based on a fragment size of 2kb (we considered the ligated barcode and Nanopore adapters size as negligible) we then need to load less than 26 ng in the flow cell. As the loaded volume will be 12 µL, the concentration maximal needed is therefore 26/12 = 2.17 ng/µl, whereas the minimal concentration will be 1.1 ng/µl.

Targeting a final volume of for instance 30 µL, the volume of diluted DNA will be: (Final Concentration (i.e. <2.17) * Final Volume (i.e. 30)) / Measured concentration of diluted DNA. The volume of Elution Buffer (EB) needed will be: 30 - volume of diluted DNA.

Perform the dilution based on the described calculation, and confirm that the values are correct with 2-3 independent quantifications with 1 µL of HS dsDNA Qubit. In case of doubt regarding the library's purity, check another 1 µL with the Nanodrop.

Note

Store the library on ice until ready to load! The leftover library can be stored at 4 °C for a few days allowing performing multiple runs.

Note

We always obtained enough mass to load 5-10< runs with respectively 35 and 50 initial samples.

Priming and loading the SpotON flow cell step, summary from the original protocol

10m

93 Thaw the Sequencing Buffer (SB), Library Beads (LIB), Flow Cell Tether (FCT) and one tube of Flow Cell Flush (FCF) at room temperature. Mix by vortexing and spin down.

94 Prepare the flow cell priming mix in a clean 1.7ml tube by combining Flow Cell Flush (FCF) 1170 µL and Flow Cell Tether (FCT) 30 µL. Once combined, mix by inverting the tube and pipette mix at room temperature.

Note

The priming mix must be temperate before loading it in the flow cell. A cool mix could release detrimental gas within the flow cell due to redox chemistry during the run.

95 When the priming mix is done, open the MinION device lid and slide the flow cell under the clip. Press down firmly on the flow cell to ensure correct thermal and electrical contact. Complete a flow cell check to ensure the number of pores available is sufficient for the run before loading the library.

Additionally, take a moment to think of the parameters of the run you plan to use. Ideally, you could already select and prepare everything before loading the flow cell to reduce stress and avoid leaving the loaded flow cell waiting too long before running it.

96 Slide the flow cell priming port cover clockwise to open the priming port. After opening the priming port, check for a small air bubble under the cover. Draw back a small volume to remove any bubbles:
1. Set a P1000 pipette to 200 µl
2. Insert the tip into the priming port
3. Turn the wheel until the dial shows 220-230 µl, to draw back 10-30 µL, or until you can see a small volume of buffer entering the pipette tip
Note: Visually check that there is a continuous buffer from the priming port across the sensor array.

Note

This step is extremely critical and sensitive. Please watch tutorial videos before manipulating the flow cell. In addition, training with an old/already used flow cell could be beneficial and limit mistakes. Be aware that introducing air bubbles into the array can/will irreversibly damage pores.

97 Load 800 µL of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles. Wait for 00:05:00 During this time, prepare the library for loading by following the steps below.

5m

Note

Be careful to load the priming mix into the priming port and not into the SpotON sample port!

Note

This step is extremely critical and sensitive. Please watch tutorial videos before manipulating the flow cell. In addition, training with an old/already used flow cell could be beneficial and limit mistakes. Be aware that introducing air bubbles into the array can/will irreversibly damage pores.

98 Thoroughly mix the contents of the Library Beads (LIB) by vortexing immediately before pipetting it. In a new tube, prepare the library for loading as follows: Sequencing Buffer (SB) 37.5 µL, Library Beads (LIB) 25.5 µL, DNA library 12 µL for a total of 75 µL.

99 Complete the flow cell priming:
1. Gently lift the SpotON sample port cover to make the SpotON sample port accessible.
2. Load 200 µL of the priming mix into the flow cell priming port (not the SpotON sample port), avoiding the introduction of air bubbles.

Note

Be careful to load the priming mix into the priming port and not into the SpotON sample port!

Note

This step is extremely critical and sensitive. Please watch tutorial videos before manipulating the flow cell. In addition, training with an old/already used flow cell could be beneficial and limit mistakes. Be aware that introducing air bubbles into the array can/will irreversibly damage pores.

100 Mix the prepared library gently by pipetting up and down just prior to loading. Add 75 µL of the prepared library to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each
drop flows into the port before adding the next.

Note

Be careful to load the prepared library into the SpotON sample port and not into the priming port!

Note

This step is extremely critical and sensitive. Please watch tutorial videos before manipulating the flow cell. In addition, training with an old/already used flow cell could be beneficial and limit mistakes. Be aware that introducing air bubbles into the array can/will irreversibly damage pores.

101 Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port and replace the MinION or GridION device lid.

102 We selected the following settings: SQK-NBD114.96, 400 bs, 100 hours, min read length 200 bp, Fast basecalling on, Barcoding on. Start the run.

103 The activity of the pores should be extremely high in the first hours, but it starts to drop significantly and regularly after 1 day. ONT recommends pausing the run, washing the library, refueling the flow cell and loading a new library to maintain a high activity of the pores and optimize the sequences yield. From our experience, we did not observe a significant improvement while doing so. We therefore suggest running the flow cell as long as possible until it dies, i.e., around 72 hours, before stopping the run.

104 Download the sequencing run report. Optionally wash the flow cell following ONT protocol, this is particularly important if you plan to use it again.

Citations

Step 34

Fiore-Donno, A.-M., Berney, C., Pawlowski, J., Baldauf, S.L.. Higher-order phylogeny of plasmodial slime molds (Myxogastria) based on EF1A and SSU rRNA sequences
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