



Jul 26, 2021

Custom eDNA High Throughput Sequencing with MinION (Oxford Nanopore Technologies)

Luca Mirimin¹¹Galway-Mayo Institute of Technology

In Development



Share

This protocol is published without a DOI.

GMIT_eDNA

Luca Mirimin

DISCLAIMER

DISCLAIMER – FOR INFORMATIONAL PURPOSES ONLY; USE AT YOUR OWN RISK

The protocol content here is for informational purposes only and does not constitute legal, medical, clinical, or safety advice, or otherwise; content added to protocols.io is not peer reviewed and may not have undergone a formal approval of any kind. Information presented in this protocol should not substitute for independent professional judgment, advice, diagnosis, or treatment. Any action you take or refrain from taking using or relying upon the information presented here is strictly at your own risk. You agree that neither the Company nor any of the authors, contributors, administrators, or anyone else associated with protocols.io, can be held responsible for your use of the information contained in or linked to this protocol or any of our Sites/Apps and Services.

ABSTRACT

This protocol is a custom workflow to carry out High Throughput Sequencing (HTS) on environmental DNA samples using a MinION portable sequencer (Oxford Nanopore Technologies). The current protocol was tested to target fish-specific amplicons (340 and 1200 bp) in eDNA samples from both marine and freshwater environments.

PROTOCOL CITATION

Luca Mirimin 2021. Custom eDNA High Throughput Sequencing with MinION (Oxford Nanopore Technologies). **protocols.io**
<https://protocols.io/view/custom-edna-high-throughput-sequencing-with-minion-bws8pehw>



LICENSE

———— This is an open access protocol distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

CREATED

Jul 22, 2021

LAST MODIFIED

Jul 26, 2021

PROTOCOL INTEGER ID

51776

DISCLAIMER:

DISCLAIMER – FOR INFORMATIONAL PURPOSES ONLY; USE AT YOUR OWN RISK

The protocol content here is for informational purposes only and does not constitute legal, medical, clinical, or safety advice, or otherwise; content added to protocols.io is not peer reviewed and may not have undergone a formal

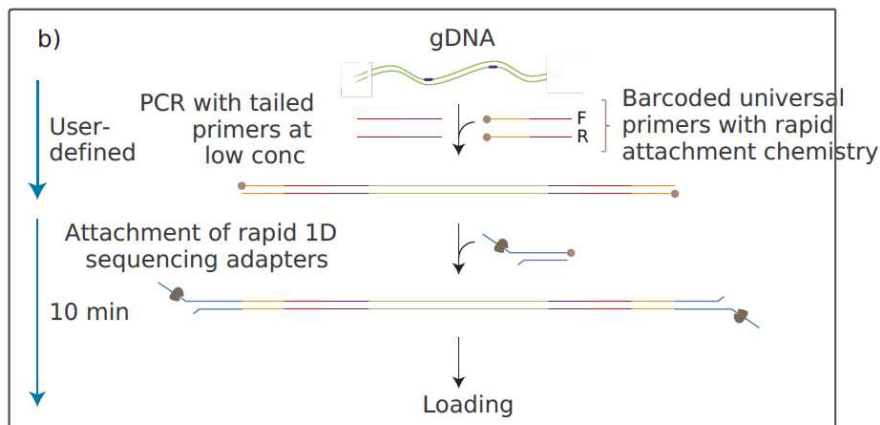
approval of any kind. Information presented in this protocol should not substitute for independent professional judgment, advice, diagnosis, or treatment. Any action you take or refrain from taking using or relying upon the information presented here is strictly at your own risk. You agree that neither the Company nor any of the authors, contributors, administrators, or anyone else associated with protocols.io, can be held responsible for your use of the information contained in or linked to this protocol or any of our Sites/Apps and Services.

INTRODUCTION

- 1 This document provides instructions on carrying out the "Four-primer PCR protocol" for amplicon sequencing from eDNA samples using the PCR barcoding kit SQK-PBK004.

The official version of this protocol is "Version: FFP_9038_v108_revP_14Aug2019" and can be found [here](#) (note that to access this protocol you will need to register for a Nanopore Community account).

The general workflow of this protocol is shown below:



© 2008 - 2021 Oxford Nanopore Technologies.

Plan your experiment/run based on the goals of the study, keeping in mind the following:

- This protocol is recommended (by the manufacturer) for 30ng of starting DNA per reaction, but it has worked for approx. 10ng. Thus, it can work with eDNA samples that contain low amounts of template DNA.
- The barcoding kit used below is for 12 distinct libraries, but there are strategies to increase the throughput (e.g. wash and re-run the flow cell; Incorporate custom barcodes in amplicons; "multiplexing" multiple genes per sample).
- Related to the above, keep in mind that reagents supplied by the manufacturer are meant for 12 samples/libraries with some excess), so this must be kept in mind when considering to increase throughput

MATERIALS REQUIRED

4h

2

The enzyme and PCR mix used can be changed as long as the enzyme used is efficient and/or recommended for environmental samples. In this case we used Platinum™ II Taq Hot-Start DNA Polymerase (Invitrogen™)

Hula mixer
Mixer
Invitrogen 15920D
Any rotator mixer

Mini Amp
Thermal Cycler
Applied Biosystems A37834 [↗](#)

Qubit Fluorometer
Fluorometer
Invitrogen Q33238 [↗](#)

1-step PCR (amplicons and barcodes)

4h

4h

3 [🕒 04:00:00](#) Includes experimental design planning, sample prep and PCR

- 3.1 Once an experimental plan has been established, prepare the following PCR mix. NOTE that the "Mastermix" recommended is for 3 replicate PCR per sample/library.

| A | B | C | D |
|---|---|---|--------------------------------------|
| Reagent name and starting concentration | Amount (μL) in each individual reaction | Mastermix to enable 3 PCR replicates per sample (3.5 X) | Final concentration in each reaction |
| Water, nuclease-free | up to 31.6 | 110.6 | - |
| 5X Platinum™ II PCR Buffer | 10 | 35 | 1X |
| 10 mM dNTP mix | 1 | 3.5 | 0.2 mM each |
| 10 μM forward primer | 0.0625 | 0.21875 | 0.0125 nM |
| 10 μM reverse primer | 0.0625 | 0.21875 | 0.0125 nM |
| 10 μM forward primer with ONT1 adapter | 0.1875 | 0.65625 | 0.0375 nM |
| 10 μM reverse primer ONT2 adapter | 0.1875 | 0.65625 | 0.0375 nM |
| BPXX (SQK-PBK004) | 1.5 | 5.25 | |
| Platinum™ II Taq Hot-Start DNA Polymerase | 0.4 | 1.4 | 0.04 U/μL |
| Template DNA | up to 5 | | Up to 30 ng per reaction |
| | | | |
| Total volume | 50 | | |

3.2 THERMAL CYCLING CONDITIONS

13m

The following conditions were adopted when using

 Platinum™ Hot Start DNA Polymerase Invitrogen - Thermo

Fisher Catalog #14966

and

targeting 2 amplicon sizes (340bp and 1200bp).

1X

 **94 °C** -  **00:02:00**

7x

 **94 °C**  **00:01:00**

 **52 °C**  **00:00:30**

 **68 °C**  **00:01:30**

35x

 **94 °C**  **00:01:00**

 **62 °C**  **00:00:30**

 **68 °C**  **00:01:30**

1x

 **68 °C**  **00:05:00**

PCR check, clean up and quantification

7m

4

This section aims at verifying that amplicons + barcoding libraries have been successfully

generated and cleaned prior to the next section.

4.1 SUCCESSFUL AMPLICON/LIBRARY CHECK

Once the PCR has completed, load **10 µl** of PCR product on a **2 % (v/v)** Agarose Gel to confirm presence of bands in the expected size range. NOTE that the size of amplicons must take into account the addition of both ONT adapters and barcoding indexes, which are incorporated in the PCR step (e.g. add approx. 100bp to the original target size).

4.2 PCR CLEAN-UP

7m

Once amplicons of expected size have been confirmed, pool **33.3 µl** from each of the three PCR replicates (per library) and clean the mixture with

[Agencourt AMPure XP magnetic beads](#) **Beckman**

Coulter Catalog #A63880

as follows:

Transfer the **100 µl** pooled PCR into a *1.5 ml DNA LoBind Eppendorf* tube and add **180 µl** of resuspended AMPure XP beads to the reaction and mix by flicking the tube.

Incubate on a Hula mixer (rotator mixer) for **00:05:00** at room temperature.

Briefly spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant.

Keep the tube on the magnet and wash the beads with **300 µl** of freshly prepared 70% ethanol without disturbing the pellet. Remove the ethanol using a pipette and discard.

Repeat the previous step. Spin down and place the tube back on the magnet. Pipette off any residual 70% ethanol and briefly allow to dry.

Remove the tube from the magnetic rack and resuspend pellet in 10 µl of 10 mM Tris-HCl pH 8.0 with 50 mM NaCl.

Incubate for **00:02:00** at room temperature. Pellet beads on magnet until the eluate is clear and colourless.

Remove and retain **10 µl** of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.

4.3 QUANTIFICATION AND NORMALIZATION

Quantify 1 µL of purified PCR product using

[Qubit™ dsDNA BR Assay Kit](#) **Thermo Fisher**

Scientific Catalog #Q32853

in a Qubit



Fluorometer 3.0.

Using 10 mM Tris.HCl pH 8.0 with 50 mM NaCl as a solvent solution, dilute each PCR product to obtain a desired concentration before pooling all libraries in a clean 1.5 ml Eppendorf DNA LoBind tube and aiming for a final volume of 10 µL and a total concentration of 100 fmol of PCR product to **10 µl**.

At this stage purified PCR products can be stored at **4 °C** overnight prior to continuing with the next step.

4.4 ATTACHMENT OF RAPID 1D SEQUENCING ADAPTERS

This step must be done just prior to priming and loading the flow cell.

Add  **1 µl** RAP reagent to the  **10 µl** amplified DNA library. Mix gently by flicking the tube, and briefly spin down.

Incubate the reaction for 5 minutes at room temperature and keep on ice prior to flow cell loading.

4.5 Priming and loading the SpotON flow cell

Follow the steps as detailed from Page 11 in the Nanopore Protocol Four-primer PCR (SQK-PSK004 or SQK-PBK004; Version: FFP_9038_v108_revP_14Aug2019).

NOTE that it is recommended to run a flow cell check prior to carrying out the loading of the flow cell.