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

Luca Mirimin[1]

[1]Galway-Mayo Institute of Technology

In Development Share

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GMIT_eDNA

Luca Mirimin

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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

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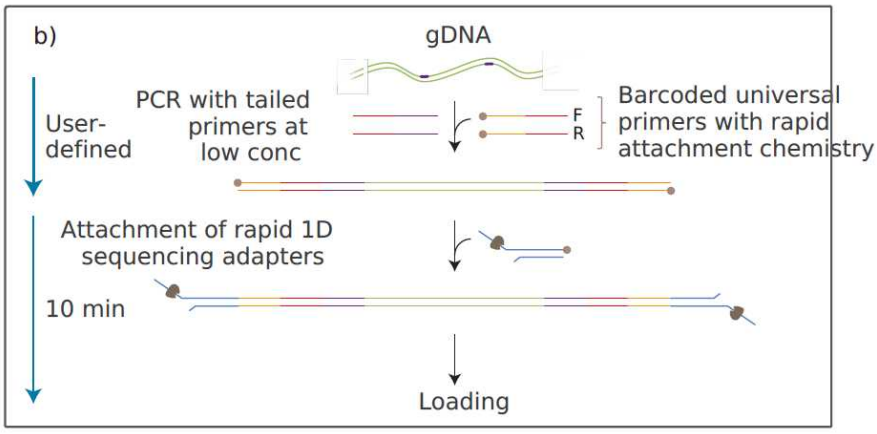
INTRODUCTION

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:



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**

4 PCR check, clean up and quantification 7m

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 **[M]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 1uL 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 10uL 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.