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Environmental DNA (eDNA) metabarcoding protocol for fish species

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Environmental DNA metabarcoding universal primers targeting the hypervariable region of the 12S rRNA gene

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Serial dilutions of mock community was prepared as a positive control

MATERIALS

Agencourt Ampure XP **Beckman Coulter Catalog #A63AA0**

UltraPure™ DNase/RNase-Free Distilled Water **Thermo Fisher Scientific Catalog #10977015**

10 mM dNTPs **Life Technologies Catalog #10297-018**

Q5 High-Fidelity DNA Polymerase - 500 units **New England Biolabs Catalog #M0491L**

The 1st part of the protocol is performed in the pre-PCR room.
The 2nd part in the post-PCR room.
Never bring back PCR products to the pre-PCR room.
Always add a negative control samples in each PCR run

Laboratory work space and equipment were sterilized by UV-light and DNase solution and 70% ethanol. Filter pipet tips were used in all steps of the laboratory work.

1 DNA extraction can be performed using Qiagen DNeasy power water sterivex kit. The quality of the extracted DNA was estimated using Nanodrop.

Qiagen DNeasy power water sterivex kit: https://www.qiagen.com/se/resources/resourcedetail?id=c5fe7d5f-070a-4ebe-ac04-4bbf05a13e91&lang=en

2 Perform the first PCR (triplicates/duplicates of each sample) using Illumina adaptor attached primers that target the gene of your choice.

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2.1 MiFish primers

A modified version of the universal primers targeting the hypervariable region of the 12S rRNA gene (163-185 bp) (Miya et al., 2015) was used. The sequence of the primer set is

MiFish-UF: 5'ACA CTC TTT CCC TAC ACG ACG CTC TTC CGA TCT NNN NNN GTC GGT AAA ACT CGT GCC AGC

MiFish-UmR: 5'AGA CGT GTG CTC TTC CGA TCT NNN NNN CAT AGT GGG GTA TCT AAT CCC AGT TTG.

2.2 Mock community

DNA extract of 10 fish species were pooled and used as a positive control

-The fish species are *Clupea harengus, Glypthocephalus cynoglossus, Scomber scombus, Thunnus alalunga, Pleuroneates platessa, Pollachius virens, Salmo salar, Gadus morhua, Reinhardtius hippoglossoides and Melanogrammus aeglefinus*.

First PCR reaction

3

First PCR reaction for fish amplification

3.1

Components	Working conc.	Final conc.	1 reaction (µl)
5xQ5 Reaction Buffer	5X	1X	5
MiFish_F	10 µM	0,3 µM	0,75
MiFish_R	10 µM	0,3 µM	0,75
dNTPs	2 mM	0,2 mM	2,5
Q5 HF DNA polymerase	2 U/µl	0.02 U/µl	0,25
Template DNA			5
Nuclease-Free water			10,75
Total			25

For environmental sample add 5 µl and for mock community add 1 µl as a template.

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A	B	C
Step	Temp	Time
Initial denaturation	98 C	30 sec
	98 C	20 sec
30 cycles	60 C	30 sec
	72 C	1 min
Final extension	72 C	7 min
Hold	4 C	

PCR visualization

4 Check PCR products with Agarose gel electrophoresis (1%) - optional

5 Pool PCR duplicate samples together and perform purification with magnetic beads (Agencourt AMPure or similar) 1h 30m

Second PCR

6 A second PCR is conducted for attaching standard illumina handles and index primers
Multiplex_fwd
AATGATACGGCGACCACCGAGA{TCTACAC}-[i5 index] ACACTCTTTCCCTACACGACG
Multiplex_rev
CAAGCAGAAGACGGCATACGAGAT-[i7 index]-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
We have in total 20 different forward index/barcode primers and 20 different reverse index/barcode primers.
By combining both primers (20X20), it is possible to generate 400 tags in one final pool for sequencing.

6.1

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Components	Working conc.	Final conc.	1 reaction (20 µl)
5xQ5 Reaction Buffer	5X	1X	4
Forward index (i5, illu-N501-N508)	5µM	0.25 µM	1
Reverse index (i7, illu-N701-N712)	5µM	0.25 µM	1
dNTPs	2mM	200 µM	2
Q5 HF DNA polymerase	2 U/µl	0.02 U/µl	0.2
Template from 1st PCR			2
Nuclease-Free water			9.8
Σ			20

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STEP	TEMP.	TIME
Initial Denaturation	98 C	30 sec
	98 C	10 sec
15 cycles	66 C	30 sec
	72 C	30 sec
Final Extension	72 C	2 min
Hold	6 C	∞

7 Check second PCR products with Agarose gel electrophoresis (1%)

8 Perform purification with magnetic beads (Agencourt AMPure)
https://research.fhcrc.org/content/dam/stripe/hahn/methods/mol_biol/Agencourt%20AMPure%20XP.pdf

9 Quantification of the concentration of second PCR product before pooling using PicoGreen assay
http://tools.thermofisher.com/content/sfs/manuals/PicoGreen-dsDNA-protocol.pdf

Calculate PCR samples concentration and volume before pooling

10 Pool the PCR samples in equal DNA amount (ng) or for unequal length amplicons, in equal molecule amount (mol). You will get one tube including a mix of all the samples.

To calculate the volume of each sample to be pooled (DNA amount mixing):
- Use the lowest concentration sample to define the minimum amount of DNA (ng) that you have available from a single sample:
DNA concentration (ng/µL) of the lowest concentration sample multiplied with its volume (µL).

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This will be your target DNA amount for each sample.
- Calculate how many µLs of each sample you need to achieve the target DNA amount: divide the target DNA amount with the concentration of each sample.
- Pipette into one tube the calculated volume of each sample.
Aim to use the same pipette for all samples (dilute or pipette multiple times) to avoid pipette calibration errors.

11 Gel purify the pool and requantify with PicoGreen before submitting to sequencing facility.

Sequencing

12 Sequencing was performed illumina paired end sequence strategy (150 bp).
Analysis was carried out by DADA2 pipeline https://benjjneb.github.io/dada2/tutorial.html

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