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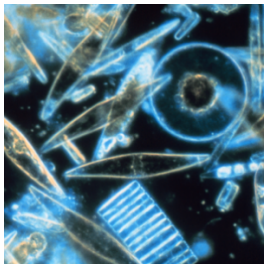
Environmental DNA (eDNA) 12S Metabarcoding Illumina MiSeq NGS PCR Protocol (Touchdown) V.1



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MBON eDNA

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Protocol status: Working
We use this protocol in our group and it is working.

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Abstract

The 12S protocol is aimed at amplifying the hypervariable region of the mitochondrial DNA 12S rRNA gene in eukaryotes. The primers (MiFish-U-F & MiFish-U-R) used in this protocol were developed by Miya et al., 2015 for metabarcoding environmental DNA (eDNA) from fishes.

Touchdown thermocycling protocols were adapted from the CALeDNA group.

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Citations
1) Miya M et al. 2015 MiFish, a set of universal PCR primers for metabarcoding environmental DNA from fishes: detection of more than 230 subtropical marine species. R.Soc.opensci. 2: 150088.
http://dx.doi.org/10.1098/rsos.150088
2) CALeDNA. 2019. University of California Conservation Genomics Consortium.

Guidelines

Local containment involves the employment of biological safety cabinets for initial specimen disinfection and DNA extraction. Such cabinets should not be used for PCR setup, as this procedure should be conducted apart from the aforementioned disinfection and extraction to minimize contamination.

1. Do not use any tube or plate that is not appropriate for the PCR machine you are using.
2. Make sure tubes and especially plates are well sealed before you begin run.
3. Clean up any spilled solutions and dispose of in appropriate biohazard boxes.
4. Be careful with PCR machine lids. These can be damaged if you slam or drop lids.
5. Make sure PCR heater block is clean before you start a run. Check each tube receptacle before you start.
6. Distribute tubes evenly across block so lid will seat flat against top of tubes for even heating and sealing.
7. Turn PCR machine off when you are done using.

Troubleshooting

Safety warnings

Always observe proper laboratory safety warning and precautions. Wear a lab coat, gloves, safety goggles and use UV-proof face shield when visualising gels with UV transilluminator. House the transilluminator in self-contained 'dark room.' All chemicals used as reagents in PCR reaction have Control of Substances Hazardous to Health Regulations (COSHH) storage form available along with procedure COSHH forms for PCR.

DNA visualization within the agarose gels requires the use of potentially hazardous ultraviolet light and ethidium bromide DNA intercollating dye. Personnel exposure to ultraviolet light will be minimized with the use of complete face shields designed to block UV ray transmission, as well as the use of long sleeved lab coats, gloves and the built in shield on the UV light box.

Before start

Disinfect work surfaces with 10% bleach or RNase Away followed by a MilliQ / DI water rinse and 70% ethanol wipe. Clean pipet surfaces with RNase Away and ethanol wipe.

UV pipets, molecular grade water, and tube racks for 30 minutes prior to starting protocol.

Setup and Primary PCR

1 **eDNA template & PCR processing were performed at the Monterey Bay Aquarium Research Institute (MBARI).**

PCR reactions for the 12S locus were performed with a two-step amplification protocol for each sample using the MiFish_U primers (Miya et al. 2015) with Fluidigm adapters CS1 & CS2.

All primers listed in the 5′ to 3′ direction. MiFish primers are in **bold**.

Fluidigm CS1 + **12S MiFish_U** (forward):
ACACTGACGACATGGTTCTACA**GTCGGTAAAACTCGTGCCAGC**

Fluidigm CS2 + 1**2S MiFish_U** (reverse):
TACGGTAGCAGAGACTTGGTCT**CATAGTGGGGTATCTAATCCCAGTTTG**

2 The primary PCR amplifications were carried out in triplicate 25 µl reactions using:

- 1 µL eDNA extract template
- 12.5 µL AmpliTaq Gold™ Fast PCR Master Mix
- 1 µL forward primer (10 µM)
- 1 µL reverse primer (10 µM)
- 9.5 µL molecular-grade, nuclease-free water

3 PCR reactions were performed in 96-well plates with a no-template control (NTC) for each PCR plate, for a total of 3 PCR negative controls. An artificial community was used as a positive control.

4

Primary 12S cycling parameters, using the CALeDNA Touchdown method:

1. 95°C 15 minutes
∗13 cycles of the following 3 steps (*step 3 changes -1.5°C each cycle; "touchdown"*)
2. 94°C 30 seconds*
3. 69.5°C 30 seconds*
4. 72°C 90 seconds*
∗25 cycles of the following 3 steps
5. 94°C 30 seconds*
6. 50°C 30 seconds*
7. 72°C 45 seconds*
8. 72°C 10 minutes
9. 4°C HOLD

Quality Control and Product Cleanup

5 After primary PCR amplification of the marker region, the PCR products were pooled (75 µL total per unique environmental sample) and run through a 2% agarose gel to confirm the presence of target bands and absense of non-specific amplification across environmental samples.

6 Primary PCR products were purified and size selected using the Agencourt AMPure XP bead system (Beckman Coulter, USA) at 1.2x volume beads to product.

7 A second agarose gel was run to confirm primer removal and retention of target amplicons after purification.

NTCs were also tested using a Qubit dsDNA 1x high sensitivity kit to ensure no amplification.

Secondary Amplification

8 **Secondary amplification and NGS were performed at Michigan State University's Research Technology Support Facility (RTSF).**

An aliquot of 20 µL from each purified primary PCR product was sent to RTSF Genomics Core at MSU for secondary PCR amplification with primers which targeted the CS1/CS2 ends of the primary PCR products and added dual indexed, Illumina compatible adapters with barcodes.

PE1-BC-CS1 (forward):
AATGATACGGCGACCACCGAGATCT-[i5-BC(index 2)]-ACACTGACGACATGGTTCTACA

PE2-BC-CS2 (reverse):
CAAGCAGAAGACGGCATACGAGAT-[i7-BC(index 1)]-TACGGTAGCAGAGACTTGGTCT

9 The secondary PCR amplifications were carried out in 15 µL reactions, using 1 µL original pooled PCR product triplicates.

- 6 µl 2.5X HotMaster Mix
- 7 µl DI water
- 1 µl Primer Mix (6uM)
- 1 µl original eDNA PCR product

10 Secondary 12S cycling parameters:

1. 95°C 3 minutes
2. 95°C 15 seconds

3. 60°C 30 seconds
4. 72°C 1 minute
5. **Repeat steps 2-4 15 times**
6. 72°C 3 minutes
7. 25°C Hold

11 An agarose gel was run after secondary PCR to confirm the presence of target bands and absence of non-specific amplification across environmental samples as well as the absence of amplification in NTCs.

12 After secondary PCR, products were run through Invitrogen SequalPrep Normalization Plate (ThermoFisher Scientific) using manufacturer's protocol to create pooled library.

Sequencing

13 The pooled product for the genetic locus was loaded on a standard MiSeq v2 flow cell and sequenced in a 2×250bp paired end format using a v2 500-cycle MiSeq reagent cartridge.

14 The MiSeq run was performed with a 10% PhiX spike added.

15 Primers complementary to the Fluidigm CS1 & CS2 oligomers were added to appropriate wells of the reagent cartridge to server as sequencing and index read primers.

16 Base calling was done by Illumina Real Time Analysis (RTA) v1.18.54 and output of RTA was demultiplexed and converted to FastQ format with Illumina Bcl2fastq v2.18.0

17 12S Sequencing primers (5′ to 3′ direction):

FL1-CS1(read1)	A+CA+CTG+ACGACATGGTTCTACA
FL1-CS2(read2)	T+AC+GGT+AGCAGAGACTTGGTCT
FL2-CS1rc	T+GT+AG+AACCATGTCGTCAGTGT
FL2-CS2rc(index)	A+GAC+CA+AGTCTCTGCTACCGTA