Environmental DNA (eDNA) COI metabarcoding Illumina MiSeq NGS PCR Protocol

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Abstract

This protocol is aimed at amplifying the cytochrome c oxidase subunit I (COI) mitochondrial gene in eukaryotes. The primers (forward: mlCOIintF, reverse: HCO2198) utilized in this protocol are based on the primers utilized in Leray et al. 2013 (forward) and Folmer et al. 1994 (reverse).

Amplicons generated using this protocol can then be sequenced using the Illumina platform.

Primers used:

Fluidigm CS1+mlCOIintF

Fluidigm CS2+HCO2198

Secondary COI PCR primers

PE1-BC-CS1

PE2-BC-CS2

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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.

Protocol

PCR

Step 1.

PCR reactions for COI were run with Fluidigm two-step amplification protocol for each sample.

Primary PCR

Step 2.

Primary PCR amplifications were carried out in triplicate 25-µl reactions using

- 1 µl DNA extract (1:10 dilution)
- 12.5 µl AmpliTaq Gold Fast PCR master mix (Applied Biosystems)
- 1 µl each of forward and reverse primers (5 µM)
- 9.5 µl molecular-biology grade water.

Primary PCR

Step 3.

PCR reactions were run in 96-well plates with a NTC run in triplicate for each plate.

Primary PCR

Step 4.

Primary COI cycling parameters:

- 95 °C for 10 minutes
- 16 cycles of the following three steps:
- 94 °C for 10 seconds
- 62 °C for 30 seconds (this changes -1°C for each subsequent cycle)
- 68 °C for 60 seconds
- Then 25 cycles of the following three steps:
- 94 °C for 10 seconds
- 46 °C for 30 seconds
- 68 °C for 60 seconds
- A final elongation step of 72 °C for 10 minutes
- Hold at 4 °C

Primary PCR

Step 5.

COI Primary PCR primers (primers listed in 5’ to 3’ direction)

Fluidigm CS1+**mlCOIintF** (forward):

ACACTGACGACATGGTTCTACA **GGWACWGGWTGAACWGTWTAYCCYCC**

Fluidigm CS2+**HCO2198** (reverse):

TACGGTAGCAGAGACTTGGTCT **TAAACTTCAGGGTGACCAAAAAATCA**

Primary PCR clean-up

Step 6.

After primary PCR amplification of the marker region, the pooled PCR products were run through an agarose gel to confirm the presence of target bands and absense of non-specific amplification across environmental samples as well as the absence of amplification in no-template controls (NTCs).

Primary PCR clean-up

Step 7.

Primary PCR products were purified and size selected using the Agencourt AMPure XP bead system (Beckman Coulter, USA).

Primary PCR clean-up

Step 8.

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

Secondary PCR

Step 9.

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.

Secondary PCR

Step 10.

Secondary PCR amplifications were carried out as single 15-µl reactions using:

- 1 µl template of primary PCR product (no dilution)
- 6 µl OneTaq Hot Start 2X master mix with standard buffer (NEB)
- 1 µl of forward and reverse primer mix (6 µM)
- 7 µl molecular-biology grade water

Secondary PCR

Step 11.

Secondary COI cycling parameters:

- 95 °C for 3 minutes
- 15 cycles of the following three steps:
- 95 °C for 15 seconds
- 60 °C for 30 seconds
- 72 °C for 60 seconds
- Then a final elongation step of 72 °C for 3 minutes
- Hold at 25 °C

Secondary PCR

Step 12.

Secondary Fluidigm PCR primers (primers listed in 5’ to 3’ direction)

PE1-BC-CS1 (forward):

AATGATACGGCGACCACCGAGATCT-[i5-BC(index 2)]-ACACTGACGACATGGTTCTACA

PE2-BC-CS2 (reverse):

CAAGCAGAAGACGGCATACGAGAT-[i7-BC(index 1)]-TACGGTAGCAGAGACTTGGTCT

Quality control, PCR clean-up and sequencing parameters

Step 13.

An agarose gel was run after secondary PCR to confirm the presence of target bands and absense of non-specific amplification across environmental samples as well as the absence of amplification in no-template controls (NTCs).

Quality control, PCR clean-up and sequencing parameters

Step 14.

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

Sequencing

Step 15.

The pooled product for the genetic locus was loaded on a standard MiSeq v2 flow cell and sequenced in a 2x250bp paired end format using a v2 500-cycle MiSeq reagent cartridge.

Sequencing

Step 16.

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

Sequencing

Step 17.

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.

Sequencing

Step 18.

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

Sequencing

Step 19.

COI 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

Sequencing

Step 20.

Sequencing is performed at the Research Technology Support Facility (RTSF) Genomics Core at Michigan State University (MSU).

Warnings

Always observe proper laboratory safety warning and precautions. Wear 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.