Apr 01, 2025

Environmental DNA (eDNA) 12S metabarcoding Illumina MiSeq NGS (2-step) PCR Protocol

This protocol is a draft, published without a DOI.

Kathleen Pitz[1], Kristine Walz[2]

[1]MBARI; [2]Monterey Bay Aquarium Research Institute

Kristine Walz
Monterey Bay Aquarium Research Institute

Protocol Citation: Kathleen Pitz, Kristine Walz 2025. Environmental DNA (eDNA) 12S metabarcoding Illumina MiSeq NGS (2-step) PCR Protocol. **protocols.io https://protocols.io/view/environmental-dna-edna-12s-metabarcoding-illumina-wxxffpn**

Protocol status: Other
This protocol has been used historically but is no longer in use.

Created: January 10, 2019

Last Modified: April 01, 2025

Protocol Integer ID: 19159

Funders Acknowledgements:
NASA
Grant ID: NNX14AP62A

Abstract

This protocol is aimed at amplifying the 12S rRNA gene in eukaryotes. The primers (Mifish_U) utilized in this protocol are based on the primers utilized in Miya et al., 2015. This protocol was used to process data historically but is no longer in use.

12S MiFish_U (forward):
GTC GGT AAA ACT CGT GCC AGC

12S MiFish_U (reverse):
CAT AGT GGG GTA TCT AAT CCC AGT TTG

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

Primers used (at MBARI):
Fluidigm CS1+mifish_U (forward)
Fluidigm CS2+mifish_U (reverse)

Secondary 12S PCR primers (at MSU):
PE1-BC-CS1
PE2-BC-CS2

This work was supported by NASA grant NNX14AP62A 'National Marine Sanctuaries as Sentinel Sites for a Demonstration Marine Biodiversity Observation Network (MBON)' funded under the National Ocean Partnership Program (NOPP RFP NOAA-NOS-IOOS-2014-2003803 in partnership between NOAA, BOEM, and NASA), and the U.S. Integrated Ocean Observing System (IOOS) Program Office.

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.

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

Safety 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.

Before start

Disinfect work surfaces with 10% bleach, followed by 70% ethanol, then RNase Away, and clean pipets with RNase Away. UV pipets, molecular grade water, and tube racks for 20 minutes prior to starting protocol.

PCR

1 PCR reactions for 12S were run with Fluidigm two-step amplification protocol for each sample.

Primary PCR

2 Primary PCR amplifications were carried out in triplicate 25-µl reactions using:
- 1 µl DNA extract
- 12.5 µl AmpliTaq Gold Fast PCR master mix (Applied Biosystems)
- 1 µl each of forward and reverse primers (10 µM)
- 9.5 µl molecular-biology grade water

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

4 Primary 12S PCR cycling parameters:
- 95 °C for 10 minutes
- 40 cycles of the following three steps:
- 95 °C for 15 seconds
- 55 °C for 30 seconds
- 72 °C for 30 seconds
- A final elongation step of 72 °C for 10 minutes
- Hold at 4 °C

5 12S Primary PCR primers (primers listed in 5′ to 3′ direction)
Fluidigm CS1+**MiFish_U(forward)**:
ACACTGACGACATGGTTCTACA **GTCGGTAAAACTCGTGCCAGC**
Fluidigm CS2+**Mifish_U(reverse)**:
TACGGTAGCAGAGACTTGGTCT **CATAGTGGGGTATCTAATCCCAGTTTG**

Primary PCR clean-up

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).

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

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

Secondary PCR

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.

10 Secondary PCR amplifications were carried out as single 15-µl reactions using:
- 1 µl template of primary PCR product (1:75 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

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

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

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).

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

Sequencing

15 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.

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

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.

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

19 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

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