Environmental DNA (eDNA) 18S metabarcoding Illumina MiSeq NGS PCR Protocol

Forked from Environmental DNA (eDNA) 18S metabarcoding Illumina MiSeq NGS PCR Protocol

Monika Hassan[1]

[1]protocols.io

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Working

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private for test

Monika Hassan
protocols.io

ABSTRACT

This protocol is aimed at amplifying the 18S rRNA hypervariable region 9 (18S V9) in eukaryotes with a focus on microbial eukaryotes. Amplicons generated using this protocol can then be sequenced using the Illumina platform. The primers (1391F, EukBr) utilized in this protocol are based on the primer utilized in Amaral-Zettler et al 2009 and the Earth Microbiome Project (EMP).

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Citations

Amaral-Zettler LA, McCliment EA, Ducklow HW, Huse SM (2009) A Method for Studying Protistan Diversity Using Massively Parallel Sequencing of V9 Hypervariable Regions of Small-Subunit Ribosomal RNA Genes. PLoS ONE 4(7): e6372. doi:10.1371/journal.pone.0006372

Earth Microbiome Project - http://www.earthmicrobiome.org/emp-standard-protocols/18s/

TAGS

dna

PCR

Show tags

PROTOCOL STATUS

Working

We use this protocol in our group and it is working

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 bgdfe fif 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.

MATERIALS TEXT

fdsfdfdsfsdd

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 refdsfdfdfdsuires 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 STARTING

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

PCR

1 PCR reactions were run in triplicate 25-µl reactions for each sample using 12-basepair Golay barcoded reverse primers (Amaral-Zettler et al., 2009).

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2 PCR reactions for 18S rDNA were carried out using
- 1 µl DNA extract (1:10 dilution)
- 12.5 µl Amplitaq Gold Fast PCR mastermix (Applied Biosystems)
- 1 µl each of forward and reverse primers (5 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 18S thermal cycling parameters:
- 95° C for 10 minutes
- 35 cycles of the following three steps:
- 94° C for 45 seconds
- 57° C for 30 seconds
- 68 °C for 90 seconds
- Final elongation step of 72° C for 10 minutes
- Hold at 4 °C

5 18S Forward PCR sequence (**Euk1391F**):
*AATGATACGGCGACCACCGAGATCTACAC*TATCGCCGTT *CG***GTACACACCGCCCGTC**
18S Reverse PCR sequence (**EukBr**):
*CAAGCAGAAGACGGCATACGAGAT XXXXXXXXXXXX*AGTCAGTCAG *CA***TGATCCTTCTGCAGGTTCACCTAC**
(where *XXXXXXXXXXXX* is unique 12-bp barcode location, all primers listed in 5' to 3' direction)

Quality control, PCR clean-up and sequencing parameters

6 After PCR amplification of the marker region, PCR products were pooled by sample (75 ul) and 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 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.

9 Purified products were then quantified using Quant-It Picogreen dsDNA Assay (Life Technologies) on an fmax Molecular Devices Fluorometer with SoftMaxPro v1.3.1

10 Library pooling was done by combining equimolar volumes from each sample.

Sequencing

11 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.

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

13 Custom sequencing primers were added to appropriate wells of the reagent cartridge.

14 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

15 18S rRNA Sequencing primers (5' to 3' direction):

Read 1 (pad+linker+1391f): TATCGCCGTT+CG+**GTA CAC ACC GCC CGT C**
Read 2 (pad+linker+EukBr): AGTCAGTCAG+CA+**TGA TCC TTC TGC AGG TTC ACC TAC**
Index read (rcEukBr+rcPad+rcLinker): **GTA GGT GAA CCT GCA GAA GGA TCA**+TG+CTGA CTGACT

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