Environmental DNA (eDNA) 16S metabarcoding Illumina MiSeq NGS PCR Protocol Version 2

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Abstract

This protocol is aimed at amplifying the 16S rRNA hypervariable region 4 (16S V4) in prokaryotes. The primers (515fB and 806rB, 926R) used in this protocol are based on the primer utilized in Apprill et al 2015 and the Earth Microbiome Project (EMP).

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Citation
Apprill, A., McNally, S., Parsons, R. & Weber, L. Minor revision to V4 region SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton. Aquat Microb Ecol 75, 129–137 (2015). doi:10.3354/ame01753

Earth Microbiom Project
http://press.igsb.anl.gov/earthmicrobiome/protocols-and-standards/16s/

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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.

Before start

Disinfect work surfaces with 10% bleach, followed by 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.

Protocol

PCR

Step 1.

Both forward (515F-Y) and reverse (926R) primers for the 16S rRNA gene, targeting microorganisms, were tagged yielding dual-indexed reads (Apprill et al., 2015; Kozich et al., 2013; Parada et al., 2016).

PCR

Step 2.

PCR reactions were carried out in triplicate according to Apprill et al. (2015) with 1:10 dilutions on all extracts on a two-step fluidigm PCR protocol.

PCR

Step 3.

For general amplification (without barcodes) 1 µl DNA template was added to the master mix consisting of

- 3.9 µl sterile water
- 5.0 µl 2.0X Hot Master Mix (Qiagen)
- 0.05 µl of each primer

Final concentration of 5 µM

PCR

Step 4.

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

PCR

Step 5.

Cycling parameters:

- 95 °C for 3 minutes
- Thirty cycles of 95 °C for 45 seconds
- 50 °C for 45 seconds
- 68 °C for 90 seconds
- 68 °C for 5 minutes

PCR

Step 6.

For the secondary PCR step to attach barcodes to individual samples, 1 µl DNA template was added to the master mix consisting of

- 7.0 µl sterile water
- 6.0 µl 2.5X Hot Master Mix (Qiagen)
- 0.5 µl of each primer

A final concentration of 6 µM

PCR

Step 7.

Secondary PCR cycling parameters:

95 °C for 3 minutes

Fifteen cycles of 95 °C for 15 seconds

60 °C for 30 seconds

72 °C for 1 minite

72 °C for 3 minutes.

Quality control, PCR clean-up

Step 8.

After 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).

Quality control, PCR clean-up

Step 9.

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

Quality control, PCR clean-up

Step 10.

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

Quality control, PCR clean-up

Step 11.

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

Quality control, PCR clean-up

Step 12.

Equimolar pools were constructed and quantified with Qubit dsDNA HS kit to confirm pool concentration prior to library preparation.

Quality control, PCR clean-up

Step 13.

One library was constructed for each location sampled in using the KAPA HyperPrep and Library Quantification kits following manufacturer’s protocol.

Sequencing

Step 14.

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

Sequencing

Step 15.

The pooled product 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.

Sequencing

Step 17.

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

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.

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.