GLOBAL ARMS PROGRAM

Smithsonian
National Museum of Natural History

16S Metabarcoding

Materials:

- 12596-4 Qiagen UltraClean 96 PCR Cleanup Kit (384 samples)
- Q32854 (Thermo Fisher) Invitrogen Qubit dsDNA HS Assay Kit (500 assays)
- MS-102-2002 (Illumina) MiSeq Reagent Kit v2 (300-cycle)
- AM9932 (Thermo Fisher) Ambion Nuclease-free water (not DEPC-treated)
    - ph 8.0, decanted in falcon tube under fume hood
- 13000013 (Thermo Fisher) Platinum Hot Start PCR Master Mix (2X), 200 reactions
- 16S customized primer plates (see below)
- 1.7 ml tubes
- 0.5 ml Qubit tubes
- 96-well PCR plates
- Filter tips
- Loading dye
- Gel red dye
- SeaKem Agarose
- Hi-Lo DNA Marker

Earth Microbiome Procedure:

This protocol is based on the 16S Earth Microbiome Protocol, which is available on the Earth Microbiome website: http://www.earthmicrobiome.org

<u>16S Amplification Primers</u>

1. Use this protocol for the amplification of prokaryotes using paired-end 16S sequencing with primers 515f-806r, which target the V4 region of the 16S SSU rRNA (see Caporaso et al. 2012, *ISME*; Walters et al. 2016, *mSystems*).
2. Modified 515f-806r primer pair:
    a. Barcodes are on the forward primer of the 515f-806rB primer pair.
    b. Degeneracy was added to forward and reverse primers to remove biases (see Parada et al. 2014, *Environ Microbiol*; Apprill et al. 2015, *Aquat Microb Ecol*).

<u>Primer Order</u>

1. Order primers in the 5′ -> 3′ orientation from IDT.
2. Barcoded primers are ordered in 96-well plates at 100 nmol.
3. Check the option to receive same amount per well (8 nmol).

Resuspension of Primers

1. Resuspend primers with ultra-pure water under sterile conditions in a hood.
2. Stock plate are 100 uM and should be aliquoted and diluted to 10 uM.
3. Use the standard desalting cleanup option.
4. If using a robot, plate volume should be 15 uL per well or greater.

515fB Forward Primer

1. 5′ Illumina adapter (index sequencing primer)
2. Golay barcode
3. Forward primer pad
4. Forward primer linker
5. Forward primer (515fB)

AATGATACGGCGACCACCGAGATCTACACGCT XXXXXXXXXXXX
TATGGTAATT GT GTGYCAGCMGCCGCGGTAA

806rB Reverse Primer

1. Reverse complement of 3′ Illumina adapter
2. Reverse primer pad
3. Reverse primer linker
4. Reverse primer (806rB)

CAAGCAGAAGACGGCATACGAGAT AGTCAGCCAG CC
GGACTACNVGGGTWTCTAAT

16S Amplification in Triplicate

1. Retrieve genomic material and put in fridge to thaw.
2. Get reagents for master mix from freezer.
3. Use 16S V4 515f-806rB primers in resuspended and aliquoted 96-well plates.
4. Let everything thaw on bench. Put primers back on ice after they have thawed.
5. Label 96-well plates. Samples are run in triplicate. Make plate maps of where each sample is placed in 96-well PCR plate.
6. Vortex & spin down everything. Put all components back on ice.
7. Set up PCR reaction:

PCR Reaction	Concentration	Volume
ddH_2O		13 ul
16S V4 515f	0.2 uM	0.5 ul
16S V4 806rB	0.2 uM	0.5 ul
Platinum HotStart PCR Master Mix	0.8x	5 ul
DNA template		1 ul
Total		25 ul

8. Briefly centrifuge PCR plates.
9. Place PCR plates in Thermal Cycler. PCR should run for about two hours.

Cycle	1	35			1	1
Temp	94.0	94.0	50.0	72.0	72.0	4.0
Time	3:00	0:45	1:00	1:30	10:00	∞

Pool PCR Triplicates

1. Pool PCR triplicates of each sample into a single volume (75 ul)
2. Create pool in fresh 96-well plate.

Run Gel

1. Prepare 1.2% agarose gel and gel dye red.
2. Protocol: 40 minutes at 100V with DNA Hi-Lo Ladder.
3. Should yield 300-350 bp PCR product.
4. Bioanalyzer is an optional alternative method.

Qubit & Pool Samples

1. Use Qubit protocol to record new concentrations of the samples.
2. Based on Qubit concentrations, combine an equimolar amount (240 ng) of amplicon from each sample in a single tube.
3. If spurious bands were present on gel, one-half of the final pool can be run on a gel and then gel extracted to select only the target bands.

Clean Samples

1. Clean the amplicon pool following the instructions from Qiagen UltraClean PCR Cleanup Kit.
2. Qubit the cleaned sample before evaporation/dilution.

MiSeq

1. Sample must be 2.0 nM to run on the MiSeq platform.
2. Use a V2 300 cycle kit.
3. 20% PhiX to increase diversity.