Colorado State University's Next-Generation Sequencing Core
16S and ITS Amplicon Library Preparation Protocol

PCR is performed in duplicate on submitted DNA using Platinum II Hot Start Master Mix (Invitrogen) on a SimpliAmp thermocycler (ThermoFisher). No template controls are processed with the samples.

The 16S primers (515F/806R) and ITS primers (ITS1-F/ITS2) are described by the Earth Microbiome Project here.

ITS primers:
ITS1-F: Smith DP, Peay KG. Sequence depth, not PCR replication, improves ecological inference from next generation DNA sequencing. PLoS One. 2014 Feb 28;9(2):e90234. doi: 10.1371/journal.pone.0090234. PMID: 24587293; PMCID: PMC3938664.

ITS2: Bellemain, E., Carlsen, T., Brochmann, C. *et al.* ITS as an environmental DNA barcode for fungi: an *in silico* approach reveals potential PCR biases. *BMC Microbiol* **10**, 189 (2010). https://doi.org/10.1186/1471-2180-10-189

16S primers:
515F: Parada, A.E., Needham, D.M. and Fuhrman, J.A. (2016), Every base matters: assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. Environmental Microbiology, 18(5): 1403-1414. https://doi.org/10.1111/1462-2920.13023

806R primer: Apprill, A., McNally, S., Parsons, R., & Weber, L. (2015). Minor revision to V4 region SSU rRNA 806R gene primer greatly increases detection of SAR11 bacterioplankton. Aquatic Microbial Ecology, 75(2), 129–137. http://doi.org/10.3354/ame01753

Thermocycler Program

94°C	2 min	
94°C	15 sec	
60°C	15 sec	x35 cycles
68°C	60 sec	
72°C	10 min	
4°C	hold	

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After confirming amplification via gel electrophoresis, individual libraries were quantified using the Quant-iT PicoGreen dsDNA assay kit (Invitrogen). Libraries were pooled in equimolar amounts by plate and then cleaned using AMPure XP beads (Beckman Coulter). Pooled libraries by plate are then quantified using the Qubit 1X ds DNA HS Assay kit on a Qubit 4 fluorometer (Invitrogen). The pools are then combined in equimolar amounts into a final library.

The final pooled library goes through three quality and quantification steps:

1. Quantification on the Qubit 4 using the 1X ds DNA HS Assay kit
2. Checking the size distribution of the library using the High Sensitivity D1000 ScreenTape and Reagents (Agilent) on the Agilent 4150 TapeStation System
3. qPCR quantification using the Collibri Library Quantification Kit (Invitrogen) on an Applied Biosystems QuantStudio 3 Real-Time PCR System

After quality and quantification steps are performed and library is found to meet standards, the library is sequenced on an Illumina MiSeq at the Next-Generation Sequencing Core at Colorado State University. A 15% phiX spike is used for both 16S and ITS amplicon sequencing.

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