NEON 16S v4v5 Marker Gene Sequencing Standard Operating Procedure, v.1.2

Prepared for:
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I. Version History

Version 1: The first version specific to 16S sequencing and will only be used to analyze samples collected in Award Year 2019 (AY19, samples collected November 1, 2018 – October 31, 2019).

Version 1.1: corrected error in the primer

Version 1.2: Amended sample-level acceptance criteria to include only a minimum raw read requirement.

II. Objective and Overview

Using the tailed primers described in Tables 1 and 2, 16S gene markers are targeted and amplified for sequencing. Field samples are prepared simultaneously with negative extraction controls (NEC, extraction blanks containing extraction kit reagents) as described in Section IV.A. After marker genes are amplified using polymerase chain reaction (PCR), the PCR products are purified by an AMPure XP bead clean-up and then individual index tags for each sample are attached using the Nextera XT index PCR primers to prepare the sequencing library. Each of the final per-sample libraries is purified by an AMPure XP bead clean-up to remove any leftover primers and enzymes, and then normalized using the Omega Bio-Tek Mag-Bind EquiPure Library Normalization Kit. The per-sample libraries from one extraction plate are pooled (resulting in 4 library pools per sequence run) and a Qubit HS DNA Assay is performed on each pool to quantify DNA concentration. Each pool is also run on the Agilent Bioanalyzer to ensure expected library fragment length (~520 bp, including insert and adapters), and if any small fragments (< 100 bp) are still present, an additional AMPure XP bead cleanup is performed to remove these small fragments. After a final Agilent run to ensure removal of small fragments, the four pooled libraries are combined into one final library pool and sequenced on an Illumina MiSeq. A schematic of this library prep workflow is shown in Figure 1.

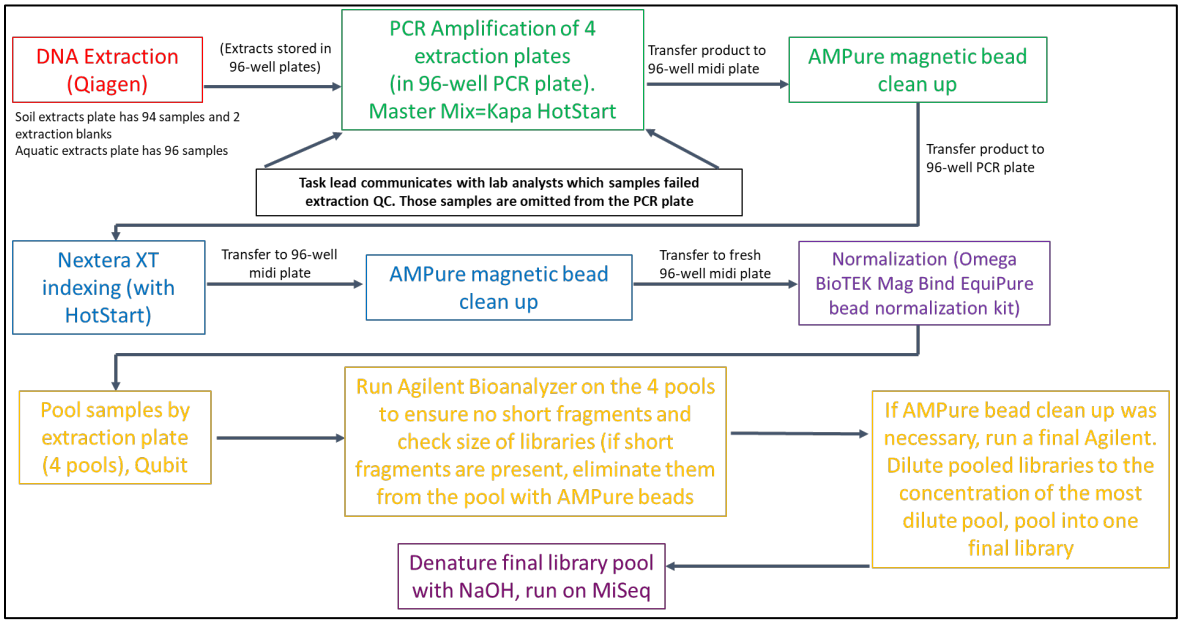

Figure 1: NEON marker gene sequencing workflow diagram

III. Recommended Materials

Table 1. Recommended Materials and their source

Material	Manufacturer	Catalog #
KAPA HiFi Hot Start Ready Mix 2X	KAPA Biosystems	KK2601
AMPure XP Beads	Agencourt	A63880
Qubit dsDNA HS Assay Kit	ThermoFisher	Q32851
Agilent High Sensitivity DNA Kit	Agilent Technologies	5067-4627
Agilent Chip	Agilent Technologies	5067-4626
Nextera XT Index Kit	Illumina	FC-131-1001 or FC-131-1002
Mag-Bind EquiPure Library Normalization Kit	Omega	M6445-00
PhiX v3	Illumina	15017397
HT1 (Hybridization Buffer)	Illumina	15027041
MiSeq v3 Reagent Kit	Illumina	15043894
MiSeq v3 Cartridge	Illumina	15043895
Flow Cell	Illumina	15028382
16S 515F Primer	Integrated DNA Technologies	Custom
16S 926R Primer	Integrated DNA Technologies	Custom

IV. Procedure

A. Sample Requirements

For AY19 samples, a marker gene sequencing run consists of 376 field samples and 8 NECs if only soil samples are analyzed, or 384 field samples with no NECs if only aquatic samples are analyzed. A combination of soil and aquatic samples may be analyzed in the same sequencing run by combining full 96-well PCR plates if possible. If a sequencing run requires less than an increment of a full 96-well plate of either soil or aquatic samples, then any NECs for those samples will also be included (example: 350 aquatic samples are to be supplemented with soil samples to fill the sequencing run; 32 DNA extracts from soil samples and the 2 NECs from the same soil DNA extraction plate will be added to fill the sequencing run [384 total wells]). Any samples with DNA concentrations below the minimum acceptance criteria specified in the current BMI_dnaExtractionSOP will be replaced with samples that meet the acceptance criteria for DNA extraction.

B. First Stage PCR and Purification

PCR processing follows the conditions described in Parada et al., 2016 (Table 3) using primers described by the Earth Microbiome Project (Table 2). The first stage PCR amplifies the 16S v4v5 target region of gDNA using Kapa HiFi HotStart ReadyMix 2X (#KK2601, Manual: KR0370 – v8.17) in a 96-well plate format. All PCR prep work is conducted in Class II biological safety cabinets (BSC). Prior to work, the BSC is decontaminated by wiping all surfaces with 10% bleach followed by 70% isopropyl alcohol. The germicidal UV light is turned on for a minimum of 10 minutes. The PCR master mix is prepared according to the manufacturer's instructions using the primers in Table 2. The thermocycler is run using the conditions in Table 3.

Table 2. Primers to be used in first stage PCR amplification. Bold indicates PCR primer region and underline text indicates Nextera XT adapter tail. *Source*: Parada et al., 2016.

Target gene	Primer	Oligonucleotide Sequence (5'-3')	Tm (C)
16S	515F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**GTG YCAGCMGCCGCGGTAA**	73.0
	926R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**CC GYCAATTYMTTTRAGTTT**	68.2

Table 3. Thermocycler conditions for 16S first stage PCR. *Source*: Parada et al., 2016.

Temperature	Duration	Cycles
95°C	3 minutes	1
95°C	45 seconds	25
50°C	45 seconds	
68°C	90 seconds	
68°C	5 minutes	1
4°C	∞	Hold

After completion of the first stage PCR, amplification products of each sample are purified separately using AMPure XP beads (#A63380, Manual: 000387v001) according to the manufacturer's instructions. If not proceeding immediately to the next step, the samples are stored at -20°C for a maximum storage time of one week.

C. Second Stage PCR and Purification

The purpose of the second stage PCR is to attach dual indices and Illumina sequencing adapters using the Nextera XT Index Kit (#FC-131-1001 and FC-131-1002, Manual: 15031942v02) and Kapa HiFi HotStart ReadyMix 2X (#KK2601, Manual: KR0370 – v8.17). The indexing is performed according to the manufacturer's instructions with the primers for Index 1 and Index 2 arranged in a TruSeq Index Plate Fixture as displayed in Figure 2. The second stage PCR thermocycler conditions are listed in Table 4.

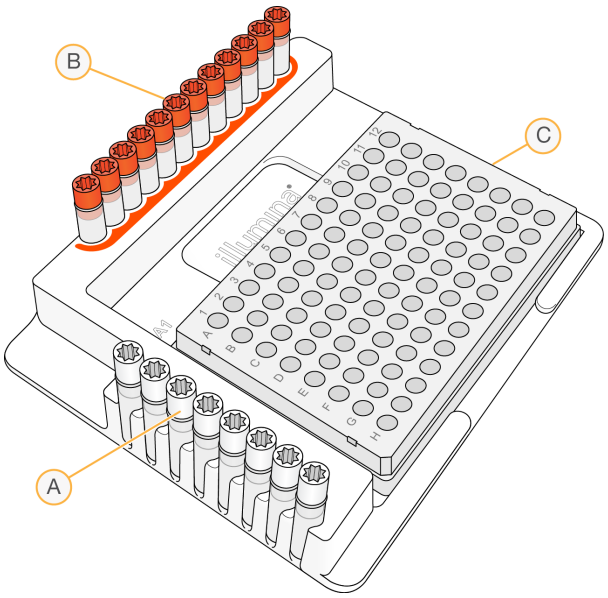

Figure 2. Set up for dual indices on TruSeq Index Plate Fixture during second stage PCR. A: rows A-H Index 2 adapters; B: Columns 1-12 Index adapters; C: Indexed Amplification Plate.

Table 4. Thermocycler conditions for second stage PCR

Temperature	Duration	Cycles
95°C	3 minutes	1
95°C	30 seconds	8
55°C	30 seconds	
72°C	30 seconds	
72°C	5 minutes	1
4°C	∞	Hold

After the second stage PCR, a clean-up is performed on each individual sample using AMPure XP beads (#A63880) to remove excess adapters and PCR reagents that may interfere with sequencing from the 16S libraries.

D. Normalization of Libraries

A Mag-Bind EquiPure Library Normalization Kit is used to create equimolar concentrations of 16S libraries after the second stage PCR clean-up. Following the manufacturer's instructions (#M6445-01, Manual: M6445-01 v.3.0), samples are incubated with the Mag-Bind® Particles LRQ and binding buffer to allow the DNA to bind to the particles. Following the incubation steps, unbound DNA is removed from the reaction and the particles bound with DNA are washed. Lastly, the normalized DNA is eluted.

E. Sample Pooling and Quality Assessment

The libraries from each PCR plate are combined into one pool, resulting in four pools; The DNA concentration of each of the four pooled libraries is quantified using a Qubit 2.0 (Manual: MAN0002326v.B.0). If a library pool has a concentration that is too low to detect on the Qubit, the samples for that plate are prepared again starting with PCR amplification.

The lengths of the DNA fragments in the pooled libraries are measured using an Agilent Bioanalyzer, with the expected length of the PCR products being ~520 bp. The Agilent Bioanalyzer will be set to detect faint peaks to allow for identification of small fragments. If small fragments (< 100 bp) are detected by the Bioanalyzer software, or when visually inspecting the Bioanalyzer trace of fragment distributions, the affected plate(s) will undergo an additional AMPure bead cleanup to remove them. Each pooled library that received additional cleanup is run on the Agilent Bioanalyzer again to ensure successful removal of small fragments. If the fragments are successfully removed, the pools are diluted with 10 mM Tris-HCl to match the DNA concentration of the pool with the lowest concentration, resulting in four equimolar pools. The pools may be stored for a maximum duration of one day at -20°C before processing continues. If small fragments are not successfully removed, or if no DNA fragment peak of ~520 bp is detected on the Bioanalyzer, then the library preparation is repeated starting at the PCR amplification step.

F. Final Library Preparation and DNA Sequencing

The four pooled libraries are combined into one final library. Following the MiSeq manufacturer's instructions, the MiSeq cartridge is thawed and gently mixed. The final pooled library is then denatured with fresh 0.2N NaOH and diluted to a final DNA concentration of 12 pM. Next, a 15% PhiX spike is added to the diluted, denatured library and this mixture is loaded on the MiSeq cartridge. The sequencing run is set up following the prompts on the instrument. The run setting is a 2 x 300 bp paired-end run with 15% PhiX spiked in.

V. Sequence Run Quality Review

Run-level data acceptance criteria: three quality metrics are monitored during the run (with their associated acceptance criteria in parentheses) and will be reported to NEON:

1) Q30, the percentage of sequenced bases with Phred-equivalent quality scores of at least 30 (greater than 70%)
2) Cluster density (700-900 K/mm^2), and
3) Percent PhiX aligned (+/- 5% of the PhiX spike-in, i.e., 10%-20%).

Deviations from these metrics will cause the run to be flagged and reported to NEON and may require reprocessing the sequencing run.

Sample-level data acceptance criteria: Each sample must produce at least 10,000 raw reads. Any samples that do not meet this criterion are flagged in the qaqcStatus field of the 16S sequencing data table as 'Fail'.

VI. REFERENCES

Parada, Alma E., David M. Needham, and Jed A. Fuhrman. 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): 1403–14. https://doi.org/10.1111/1462-2920.13023.