NEON 16S and ITS Sequencing Standard Operating Procedure, v.1

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Business Sensitive

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I. Overview

Indexed libraries are combined to create analytical pools containing indexes. These pools are quantified by Qubit HS DNA Assay to ensure equimolarity of each pool before they are further combined for sequencing. Each sequencing run contains A-D indexes. For each sequencing run, a positive and a negative control from 16S and ITS library preparation are sequenced on the same flow cell along with 190x16S libraries and 190xITS libraries, for a total of 384 samples per run. Sequencing is performed as 300 bp paired end reads on each MiSeq v3 600 cycles cartridge (Manual: 15044983vB).

II. Materials

Material	Manufacturer	Catalog #
PhiX v3	Illumina	15017397
HT1 (Hybridization Buffer)	Illumina	15027041
MiSeq v3 Reagent Kit	Illumina	15043894
MiSeq v3 Cartridge	Illumina	15043895
Flow Cell	Illumina	15028382
Qubit dsDNA HS Assay Kit	ThermoFisher	Q32851

III. Procedure

A. Pooling of 16S and ITS Libraries

The 384 libraries are pooled prior to sequencing on the Illumina MiSeq according to the manufacturer's instructions. Briefly, the concentrations of PCR products from each of 4, 96-well plates are quantified with a Qubit High Sensitivity Kit and recorded (Manual: MAN0002326vB.0). Equal volumes of equimolar index sets A, B, C, and D are added to each plate, and the concentration of DNA across each plate is normalized. Equal volumes of sample from each plate are combined into a single pool containing 384 samples. The concentration of the "Library Pool" is determined via a Qubit High Sensitivity DNA protocol. An aliquot of the "Library Pool" is used for high-throughput sequencing.

B. Sample Sheet Setup and Preparation

The sample sheet is created on the MiSeq using the Illumina Experiment Manager. The option "Create Sample Sheet" option is selected, as well as the appropriate options for the sequencing run (e.g., "MiSeq," "FASTQ Only," enter reagent cartridge barcode, "Nextera XT", and "Paired End Read").

C. Sequencing

1. Preparation

A heat block suitable for 1.7 mL tubes is set to 96°C and a MiSeq reagent cartridge is set at room temperature to thaw. An ice-water bath is prepared by combining 3 parts ice and 1 part water.

2. DNA Denaturation

Final pooled DNA library is denatured by adding 0.2 N NaOH in a microcentrifuge tube according to Table 1. 200 nM Tris-HCl (pH7) is added to the denatured DNA according to Table 2. Pre-chilled hybridization buffer (HT1) is added to the tube according to Table 3, resulting in a denatured library of 20 pM in 1 mM NaOH. The tube is placed on ice until proceeding to the final dilution step.

Table 1 – Library and 0.2 N NaOH volume according to library concentration

Library concentration	Library volume	0.2 N NaOH volume
4 nM	5 µL	5 µL
2 nM	10 µL	10 µL
1 nM	20 µL	20 µL
0.5 nM	40 µL	40 µL

Table 2 – Library and 200 mM Tris-HCl volume according to library concentration

Library concentration	Library volume	200 mM Tris-HCl (pH 7) volume
4 nM	5 µL	5 µL
2 nM	10 µL	10 µL
1 nM	20 µL	20 µL
0.5 nM	40 µL	40 µL

Table 3 – Volume of HT1 according to library concentration

Library concentration	Prechilled HT1 volume
4 nM	985 µL
2 nM	970 µL

1 nM	940 µL
0.5 nM	880 µL

3. DNA Dilution

The library of denatured DNA is diluted with pre-chilled HT1 from the MiSeq Kit according to Table 4 (Note: Illumina suggests starting with 4 pM and then adjusting runs from there). The tube is inverted several times to mix and then placed on ice.

Table 4 – Final sample dilutions according to library concentration

Final Concentration	2 pM	4 pM	6 pM	8 pM	10 pM
20 pM denatured library	60 µL	120 µL	180 µL	240 µL	300 µL
Pre-chilled HT1	540 µL	480 µL	420 µL	360 µL	300 µL

4. Denaturing and Dilution of PhX Control

The PhiX library is diluted according to the manufacturer's instructions. The PhiX library is brought to the same concentration as the DNA library according to Table 4. The tube is inverted several times, centrifuged, and then placed on ice.

5. Combing Amplicon Library and PhiX Control

PhiX library (30 µL) is combined to 570 µL of amplicon library in a microcentrifuge tube and incubated on a heat block at 96°C for 2 minutes. The tube is inverted 1-2 times and immediately placed in the ice water bath for 5 minutes.

6. Loading the MiSeq Cartridge and running the MisSeq

The MiSeq cartridge is prepared according to the manufacturer's instructions, and the MiSeq is run following the prompts on the instrument.

IV. Quality Review

Samples must have a minimum of 3000 reads after quality filtering in order to pass QA/QC. The quality filtering criteria for each read are:

- Minimum mean quality score = 20, after trimming
- Maximum 1 ambiguous base call