NEON 16S/ITS PCR Amplification Standard Operating Procedure v.1

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I. Objective and Overview

Using the tailed primers described in Table 1, 16S and ITS gene markers are targeted and amplified for sequencing. Samples are prepared simultaneously with both positive and negative controls, where the positive controls are mixtures of genomic DNA (gDNA) from archaea, bacteria, and fungi and negative controls are extraction blanks (containing extraction kit reagents). After marker genes are amplified using polymerase chain reaction (PCR), the PCR products are purified by an AmpureXP bead clean-up and then amplified using the Nextera XT index PCR primers to attach individual index tags to complete the library molecules. The final libraries are purified by an AmpureXP bead clean-up to remove any leftover primers and enzymes, and then normalized using the Omega Bio-Tek Mag-Bind Equipure Library Normalization Kit. Eleven samples from each plate are quantified using the Agilent High Sensitivity DNA Kit to ensure that the average library size is near the expected value for each target (16S [~600bp] or ITS [~500bp]). An additional Qubit HS DNA Assay is performed on the library pool. The library products are then sequenced on an Illumina MiSeq.

II. Recommended Materials

Material	Manufacturer	Catalog #
KAPA HiFi Hot Start Ready Mix 2X	KAPA Biosystems	KK2601
AMPure XP Beads	Agencourt	A63880
Mag-Bind Library Normalization	Omega Bio-Tek	
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

III. Procedure

A. First Stage Amplification and Purification

The first stage PCR amplifies the target region (16S or ITS) of gDNA using Kapa HiFi HotStart ReadyMix 2X (#KK2601, Manual: KR0370 – v8.17). All PCR prep work is conducted at the Battelle King Avenue location in the 20-0-10 biosafety cabinets (BSC). Prior to work, the BSC is

decontaminated by wiping with 10% bleach and 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 1. The thermocycler is run using the conditions in Table 2 (16S) or Table 3 (ITS).

Table 1 – Primers to be used in First Stage PCR. Bold indicates PCR primer region and underlined region indicates Nextera XT adapter tail

Target gene	Primer	Oligonucleotide Sequence (5'-3')	Tm (C)	Reference
16S	Pro341F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**CCTACGGGNBGCASCAG**	49.8	Takahashi S et al. PLoS ONE. 2014, 9(8): e105592.
	Pro805R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**GACTACNVGGGTATCTAATCC**	47.0	
ITS	ITSF	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**CTTGGTCATTTAGAGGAAGTAA**	51.7	Walters et al. mSystems. 2015, 1(1): e00009-15.
	ITSR	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**GCTGCGTTCTTCATCGATGC**	62.7	

Table 2 – Thermocycler conditions for 16S first stage PCR

Temperature	Duration	Cycles
95°C	3 minutes	1
95°C	30 seconds	25
55°C	30 seconds	
72°C	30 seconds	
72°C	5 minutes	1
4°C	∞	Hold

Table 3 – Thermocycler conditions for ITS first stage PCR

Temperature	Duration	Cycles
95°C	3 minutes	1
95°C	30 seconds	35
55°C	1 minute	
72°C	1 minute	
72°C	5 minutes	1
4°C	∞	Hold

After completion of the first stage PCR, amplification products are purified 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.

B. 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). This is done 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 1. The stage 2 thermocycler conditions are listed in Table 4 (same for 16S and ITS).

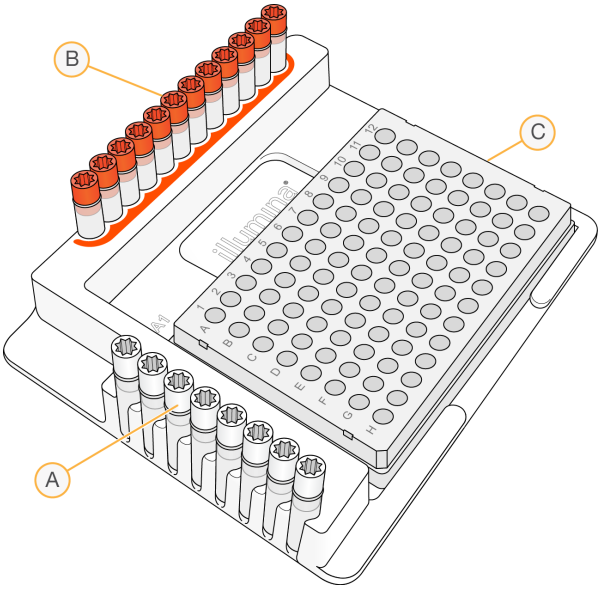

Figure 1 – Set up for dual indices on TruSeq Index Plate Fixture during second stage PCR. A: rows A-H Index 2 adapters; B: Columns 1-12 Index adapters; C: Indexed Amplification Plate.

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 using AMPure XP beads (#A63880) to purify the 16S or ITS libraries.

C. Normalization of Libraries

A Mag-Bind EquiPure Library Normalization Kit is used to create equimolar concentrations of 16S and ITS libraries after the second stage PCR clean-up according to the manufacturer's instructions.

D. DNA Size and Quantity Investigation using Agilent Bioanalyzer

An Agilent 2100 Bioanalyzer is used to evaluate the size and quantity of the DNA libraries (Manual: G2946-90004) according to the manufacturer's instructions.

E. Qubit dsDNA HS Assay

After completion of the Agilent analysis, the libraries are quantified using a Quibit 2.0 (Manual: MAN0002326v.B.0). The samples are then either prepared for sequencing or stored at -20°C.

IV. Quality Review

Samples with a positive amplicon concentration as determined via a Qubit 2.0 pass QAQC review.