NEON Metagenomics Standard Operating Procedure, v.4

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I. Version History

Version 4	Updated references to manufacturer manuals and catalog numbers, clarified statements regarding use of negative controls, clarified NextSeq Sample Sheet Preparation, combined DNA denaturation reagent tables for simplicity, added information for confirming presence of DNA libraries prior to sequencing using an automated electrophoresis system, added calculations used for determining sequencing library size.
Version 3	The number of samples prepared and sequenced on each run was increased to 60 from 40.

II. Objective and Overview

Using the KAPA HyperPrep kit from Kapa Biosystems, samples are prepared for metagenomic shotgun sequencing alongside a negative control (an extraction blank: wells not containing sample processed with the extraction kit reagents). The final libraries are quantified by qPCR, normalized, pooled into sets of 60 samples each, with each set including an extraction blank, and then sequenced on an Illumina NextSeq 550 (Manual: 15069765v01). Sequence data are uploaded to MG-RAST for analysis and sent to NEON.

III. Recommended Materials

Material	Manufacturer	Catalog #
KAPA HyperPrep Kit	KAPA Biosystems	KK8505
KAPA Pure Beads	KAPA Biosystems	KK8002
KAPA Unique Dual-Indexed Adapter	KAPA Biosystems	KK8727
MinElute PCR Purification Kit	Qiagen	28006
KAPA Library Quant (Illumina) Universal qPCR Mix Kit	KAPA Biosystems	KK4824
2X KAPA SYBR Fast qPCR Master Mix Universal Kit	KAPA Biosystems	KK4600
1M Tris-HCl, pH 7	Fisher	BP1756-100
2N NaOH	Fisher	SS264-1
High Output Sequencing Kit, including: **Flow Cell** **Reagent Cartridge** **Accessory Box (HT1)** **Buffer Cartridge**	Illumina	20024908 15065973 15057931 15058251 15057941

IV. Procedure

A. Kapa HyperPrep Library Preparation

The initial step in the NEON metagenomics procedure is to use the KAPA HyperPrep Library Preparation Kit (Manual: KR0961 – v9.20) to construct metagenomic libraries for Illumina sequencing. All work takes place in a decontaminated biosafety cabinet (BSC). Prior to work, the BSC is wiped down with 10% bleach, followed by 70% isopropyl alcohol. The germicidal light is turned on for a minimum of 10 minutes.

1. Covaris Fragmentation

Metagenomic libraries containing EDTA-free double-stranded DNA (dsDNA) are fragmented mechanically using a Covaris S220 according to the manufacturer's instructions (Manual: S220[x] User Manual). The Covaris shears DNA via an acoustic transducer that emits pulses of energy at user-specified frequencies.

2. End Repair and A-tailing

In the same plate containing the fragmentation reactions, the end repair and A-tailing reactions are assembled according to the manufacturer's instructions (KAPA, Manual: KR0961 – v9.20). This allows unique dual-index adapters to be ligated to fragmented samples.

3. Adapter Ligation

The adapter stocks (KAPA Unique Dual-Indexed Adapters) are diluted to the appropriate concentration as outlined in Table 1. In the same plate used to perform the end repair and A-tailing, the adapter ligation reactions are assembled according to the manufacturer's instructions (KAPA, Manual: KR0961 – v9.20).

Table 1 – Adapter stock concentrations

Input DNA	Adapter Stock Concentration	Adapter : Insert molar ratio
1 µg	15 µM	10:1
500 ng	15 µM	20:1
250 ng	15 µM	40:1
100 ng	15 µM	100:1
50 ng	15 µM	200:1
25 ng	7.5 µM	200:1
10 ng	3 µM	200:1
5 ng	1.5 µM	200:1
2.5 ng	750 nM	200:1
1 ng	300 nM	200:1

B. Post Ligation Cleanup

After adapter ligation, the Qiagen MinElute PCR Purification Kit is used to clean the post-ligation samples to eliminate any remaining reagents from the library preparation that may interfere with sequencing, according to the manufacturer's instructions (Qiagen, Manual: MinElute Handbook). Next, a double-size selection using KAPA Pure beads is performed to select fragments with a length of 150 – 350 bp (KAPA, Manual: KR0961 – v9.20).

C. Library Quantification

Quantitative polymerase chain reaction (qPCR) is performed to quantify samples prior to sequencing using the KAPA Biosystems Universal qPCR kit, according to the manufacturer's instructions. The qPCR assay is run on a 7500 Real-Time system (Thermo Fisher) according to the conditions described Table 2.

Table 2 – qPCR conditions on 7500 Real-Time System

Step	Temperature	Time	Cycles
Initial Denaturation	95°C	5 minutes	1
Denaturation	95°C	30 seconds	35
Annealing/Extension/Data acquisition	60°C	45 seconds	

D. Metagenomics Shotgun Sequencing

1. NextSeq Sample Sheet Preparation

Initiating the sequencing run on the NextSeq does not require the use of a sample sheet. However, a sample sheet is required to demultiplex the run data.

The sample sheet required to demultiplex the NextSeq run data is created by using a ".csv" file containing both the sample ID associated with its corresponding unique dual-indexed adapter sequence. The adapter sequences are provided in the KAPA Unique Dual-Indexed Adapter kit manual for Illumina Platforms (KAPA; KR1736 – v3.20).

2. Sample Normalization

Prior to normalization, a negative control and ten randomly selected PCR-free libraries are loaded onto a High Sensitivity DNA Chip and analyzed with an automated electrophoresis system, such as the Agilent Bioanalyzer, to confirm the presence of DNA libraries. Due to the "forked" adapters in PCR-free workflows, the DNA libraries will appear to have a longer than expected size and or may display a broad or bimodal size distribution. Due to this configuration a base pair size of 373 bp, which represents the expected size after Covaris fragmentation, insert size, adaptor and index, will be used instead of the size provided by the Bioanalyzer traces.

The sample libraries concentrations are calculated using the results of the qPCR and the expected library size. Sample concentrations are determined based on the following formula:

$$\frac{(Average\ Conc.\ [pM])(452\ bp\ standards)}{373\ bp\ average\ amplicon} = W$$

W X D = Library Conc in pM

W = Library concentration in pM, D = dilution of samples input into Kapa Quant, i.e. if samples are diluted 1:10,000, then D=10,000

The library concentration is converted from picomolar (pM) to nanomolar (nM) using the conversion factor below:

$$\frac{pM\ Library\ conc}{1{,}000} = nM\ library\ conc.$$

The libraries are then normalized with sample dilution buffer to the closest target concentration of either 4 nM, 2 nM, 1 nM, or 0.5 nM.

3. DNA Denaturation

Based on the chosen normalized library concentrations, freshly diluted 0.2 N NaOH is combined with a volume of pooled DNA library in a microcentrifuge tube according to Table 3. Then, 200 nM Tris-HCl (pH 7) is added to the sample tube according to Table 3. The libraries are then diluted to 20 pM with pre-chilled HT1 buffer according to Table 3. The libraries are diluted to a final concentration of 1.8 pM by adding 117 µL of diluted library solution with 1,183 µL of pre-chilled HT1 in a new tube. This tube is inverted to mix.

Table 3 – Library and Reagent volumes according to library concentration

Library concentration	Library volume	0.2 N NaOH volume	200 mM Tris-HCl (pH 7) volume	Prechilled HT1 volume
4 nM	5 µL	5 µL	5 µL	985 µL
2 nM	10 µL	10 µL	10 µL	970 µL
1 nM	20 µL	20 µL	20 µL	940 µL
0.5 nM	40 µL	40 µL	40 µL	880 µL

4. Loading and Running the NextSeq

The NextSeq High Output 300 Cycle cartridge is prepared according to the Illumina instructions. The sequencing run is set up by following the appropriate prompts on the instrument. During run set up, the “paired-end” option is selected, as well as “151” for both Cycle Read 1 and Cycle Read 2 and “8 bp” for both Index 1 and Index 2.

V. Quality Review

Individual samples with fewer than 100,000 reads are reanalyzed by the lab. Samples should have 5-10 million reads, a minimum Q-score of 20, and a maximum of 1 ambiguous base per read.