NEON Metagenomics Standard Operating Procedure, v.3

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I. Version History

The number of samples prepared and sequenced on each run was increased to 60 from 40.

II. Objective and Overview

Using the KAPA Hyper Plus 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, and then sequenced on an Illumina NextSeq 550 (Manual: 15069765v01). The negative control from the library preparation is sequenced simultaneously with the samples on the same flow cell. Sequence data are uploaded to MG-RAST for analysis and sent to NEON.

III. Recommended Materials

Material	Manufacturer	Catalog #
KAPA HyperPlus Kit	KAPA Biosystems	KK8514
KAPA Pure Beads	KAPA Biosystems	KK8002
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
High Output Flow Cell	Illumina	15065973
Reagent Cartridge v2	Illumina	15057931
Accessory Box v2	Illumina	15058251
Buffer Cartridge v2	Illumina	15057941

IV. Procedure

A. Kapa Hyper Plus Library Preparation

The initial step in the NEON metagenomics procedure is to use the KAPA Hyper Plus Library Preparation Kit (Manual: KR1145 – v.3.16) 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 KR1145 – v.3.16). This allows unique dual-index adapters to be ligated to fragmented samples.

3. Adapter Ligation

The adapter stocks 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 KR1145 – v.3.16).

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 a 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). After this a double-size selection using KAPA Pure beads is performed to select fragments with a length of 150 – 350 bp (KAPA, Manual: KR1245 – v.3.16).

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

The sample sheet for the NexSeq run is created using Illumina Experiment Manager. During set up the "paired-end" option is selected, as well as "151" for both Cycle Read 1 and Cycle Read 2.

2. Sample Normalization

Based on the results of the qPCR, the sample libraries are normalized to equal concentrations of either 4 nM, 2 nM, 1 nM, or 0.5 nM with sample dilution buffer.

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 4. The libraries are then diluted to 20 pM with pre-chilled HT1 buffer according to Table 5. 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 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 4 – 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 5 – Final NextSeq sample dilutions 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

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.

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.