NEON 16S/ITS qPCR Standard Operating Procedure v.7.1

Prepared for:
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I. Version History

1. Section IV.A.1 and A.2 clarified standard preparation and the relation to the acceptance criteria. Added Section IV.A.3 on assay controls.
2. Section IV.B clarified extract dilution and dilution incorporation into calculations; moved acceptance criteria into Section IV.C.
3. Section IV.C combined all acceptance criteria into this section and added details.
4. Added Section IV.D Reporting.
5. Clarification of sample level acceptance criteria and minor wording changes in Section IV.A.1 that do not impact work already completed under SOP v7.
6. the PCR primer sequence in Table 1 for primer Pro805R, which is used for the 16S marker gene, is now corrected compared to earlier versions of the SOP.

II. Objective and Overview

To assess microbial abundance in aquatic samples, quantitative polymerase chain reaction (qPCR) is performed using primers adapted for hypervariable regions V3 and V4 from bacterial and archaeal 16S ribosomal DNA (rDNA) and fungal-specific primers for the ITS-1 region of fungal rDNA (Table 1).

III. Recommended Materials

Material	Manufacturer	Catalog #
Fungal gDNA Standard	ATCC	varies
Archaeal gDNA Standard	ATCC	varies
Bacterial standard	ATCC	varies
Quantus ONE dsDNA Assay Kit	Promega	E4871
Wizard® Genomic Purification Kit	Promega	A1120
10 mg/mL Lysozyme	Sigma	L6876
10 mg/mL Lysostaphin	Sigma	L7386
Kapa SYBR Green Master Mix	Kapa Biosystems	KK4602
Genomic-tip 100/G Kit	Qiagen	10243

IV. Procedure

A. Preparation of Standards and Controls

Bacterial, archaeal, and fungal genomic DNA standards are used for generating standard curves and calculating gene copy numbers. The exact standard in use may vary from batch to batch based on standard availability and is tracked at the batch level for each data set.

1. Prokaryotic Standards

Archaeal and bacterial genomic DNA (gDNA) standards are rehydrated according to the manufacturer's instructions, as needed.

If archaeal and/or bacterial genomic DNA is prepared from a whole cell microbial consortium, the entire contents of the vials are extracted directly using a commercial genomic DNA extraction kit, such as the Wizard® Genomic Purification Kit, according to the manufacturer's instructions. This ensures that all organisms present in the consortium are represented in the gDNA.

Bacterial and archaeal gDNA concentrations are determined using the Quantus Fluorometer with the Quantus ONE dsDNA assay kit. The bacterial gDNA stock and archaeal gDNA are combined to create a gDNA prokaryotic standard, normalized by calculated DNA content based on the mass of the genome and concentration of extracted DNA. A ten-fold dilution series is prepared for the prokaryotic gDNA standard curve ranging from approximately 1 x 10^1 to 1 x 10^6 Gene Copies (GC)/2 µL. Exact standard curve concentrations may vary, as long as initially there is a six-point standard curve prepared. Refer to Section C for acceptance criteria of standards.

2. Fungal Standards

Commercially available fungal genomic DNA (gDNA) standards are rehydrated according to the manufacturer's instructions. Genomic DNA concentrations are determined using the Quantus Fluorometer with the Quantus ONE dsDNA assay kit. Fungal gDNA standards are combined, and a ten-fold dilution series is prepared for the fungal gDNA standard curve ranging from approximately 1 to 1 x 10^5 GC/2 µL. Exact standard curve concentrations may vary, as long as initially there is a six point standard curve prepared. Refer to Section C for acceptance criteria of standards.

3. Assay Controls

3.1 Negative Controls: Each qPCR analysis will include No Template Controls (NTCs) as the assay negative control. The NTCs are prepared in the same manner as the samples, using the same primers and reagents, but with DNA-free water added in lieu of sample.

3.2 Positive Controls: The prokaryotic and fungal standard curves will serve as the assay positive control for the 16S and ITS abundances, respectively.

B. qPCR Analysis

Quantitative PCR (qPCR) is performed using the Applied Biosystems 7500 Fast PCR platform with Kapa SYBR Green Master Mix. The master mix is prepared according to the manufacturer's instructions with the primers at a concentration of 0.2 µM. Each DNA extract is analyzed for 16S

(archaeal and bacterial) and ITS (fungal) abundances using the appropriate primers as described in Table 1, and the appropriate cycling conditions as described in Tables 2 and 3.

Sample DNA extracts will undergo a 10-fold or greater dilution prior to analysis and will be run in triplicate. Each qPCR assay is conducted using a 96 well plate that contains the sample DNA extracts to be analyzed, a six-point standard curve run in triplicate, and five NTCs.

The data are analyzed using the Applied Biosystems 7500 software, version 2.3, according to the manufacturer's instructions with automatic baseline setting and automatic or manual threshold setting, with no more than two significant digits. The baseline is the fluorescence background of everything in the early stages of PCR, and the threshold is the point where fluorescence is significantly past the baseline, assigning a Quantification Cycle (Cq).

For each assay, the standard curve is generated only using standard concentrations that have three positive replicates. A replicate is considered positive if its Cq value is lower than the lowest Cq value among the NTCs within that assay that pass the NTC acceptance criteria of having fewer than 10^3 gene copies/2 µL. The lowest concentration standard that has three positive replicates is identified as the lower limit of quantification (LLOQ) for the assay and the highest concentration standard that has three positive replicates is identified as the upper limit of quantification (ULOQ) for the assay. The Cq values representing the LLOQ and the ULOQ are the average Cq values of the three positive replicates that constitute the LLOQ and the ULOQ, respectively.

Resulting data from the software are in units of gene copies per µL DNA (GC/µL) which are adjusted for dilution as indicated in Section D2 before reporting.

Table 1 - qPCR Primer Sequences

Target	Primer	Sequence	Reference
16S v3-4	Forward	5'-CCTACGGGNBGCASCAG-3'	Pro341F and Pro805R[1]
	Reverse	5'-GACTACNVGGGTATCTAATCC-3'	
ITS-1	Forward	5'-CTTGGTCATTTAGAGGAAGTAA-3'	ITS1f and ITS2[2]
	Reverse	5'-GCTGCGTTCTTCATCGATGC-3'	

[1] Takahashi, S., J. Tomita, K. Nishioka, T. Hisada, and M. Nishijima. 2014. *Development of a Prokaryotic Universal Primer for Simultaneous Analysis of Bacteria and Archaea Using Next-Generation Sequencing.* PloS one 9.8 (2014): e105592.

[2] Walters, W., E. R. Hyde, D. B.-L., G. Ackermann, G. Humphrey, A. Parada, J. A. Gilbert, J. K. Jansson, J. G. Caporaso, J. A. Fuhrman, A. Apprill, and R. Knight. 2015. Improved Bacterial 16S rRNA gene (V4 and V4-5) and Fungal Internal Transcribed Spacer Marker Gene Primers for Microbial Community Surveys." *mSystems Methods and Protocols* 1.1 (2015): e00009-15.

Table 2 - 16S qPCR Cycling Conditions

Step	Ramp Rate	Temperature	Duration	Collect Data	Cycles
Polymerase Activation	100%	95 °C	3 minutes		Hold
Denature	100%	95 °C	30 seconds		**35**
Anneal	100%	50 °C	30 seconds		
Extend	100%	72 °C	30 seconds	On Hold	
Hold	100%	72 °C	5 min		Hold
Melt Curve - Step 1	100%	95 °C	15 seconds		**1**
Melt Curve - Step 2	100%	60 °C	1 minute	On Ramp	
Melt Curve Dissociation Step	1%	95 °C	15 seconds		
Melt Curve - Step 3	100%	60 °C	15 seconds		

Table 3 - ITS qPCR Cycling Conditions

Step	Ramp Rate	Temperature	Duration	Collect Data	Cycles
Polymerase Activation	100%	95 °C	3 minutes		Hold
Denature	100%	95 °C	30 seconds		**35**
Anneal	100%	50 °C	1 minute		
Extend	100%	72 °C	1 minute	On Hold	
Hold	100%	72 °C	10 min		Hold
Melt Curve - Step 1	100%	95 °C	15 seconds		**1**
Melt Curve - Step 2	100%	60 °C	1 minute	On Ramp	
Melt Curve Dissociation Step	1%	95 °C	15 seconds		
Melt Curve - Step 3	100%	60 °C	15 seconds		

C. Acceptance Criteria

1. Batch-Level Acceptance Criteria

For each qPCR assay, data are reported if the following acceptance criteria are satisfied:

1. The standard curve must have at least three of the six concentrations with three positive replicates, as described in Section B
2. The R^2 value for the standard curve is greater than or equal to 0.95
3. For each run, a minimum qPCR efficiency must be met: 70% for ITS and 60% for 16S
4. The majority of NTCs must be below 10^3 GC/2 µL. In the batch-level data table, NTCs that meet these acceptance criteria are reported as 'Pass'; those not meeting these criteria are reported as 'Fail'.

The efficiency thresholds defined here are based on efficiency data from all previous NEON qPCR assays performed at BMI. The acceptance criteria described above are reported with the batch-level data in the ingest file. If the assay does not meet these criteria, the assay is deemed a failure and all samples in the assay are repeated once. The data from the first assay that fails due to not meeting the acceptance criteria are not reported. Assays that fail a second time should be discussed with NEON and will be reported in the returned data using the appropriate quality flag.

2. Sample-Level Acceptance Criteria

For individual samples tested by qPCR, data are reported as passing QA/QC if at least two of the three replicates have Cq values that fall within the range of Cq values between the ULOQ and the LLOQ.

Individual samples with at least two replicates having Cq values that fall outside the range of Cq values between the ULOQ and LLOQ will be reported and flagged as failing QA/QC. Individual samples that fail QA/QC will not be repeated.

D. Reporting

1. Nucleic acid concentration

The reported nucleic acid concentration reflects the final concentration of nucleic acids in a sample used for the qPCR assay, which accounts for sample dilutions.

2. Gene copy calculations

The number of gene copies per nanogram of DNA (GC/ng) in the sample is calculated using the sample nucleic acid concentration, reported as nanograms per microliter (ng/µL), the volume of sample added to the qPCR reaction (2 µL), and the measured number of gene copies per microliter (GC/µL). The gene copy calculation is as follows:

*GC/ng = (GC/µL)/[2 µL * nucleic acid concentration (ng/µL)]*

Prior to reporting, this concentration is adjusted for the sample dilution:

*Reported Gene Copies per nanogram of DNA = [GC/ng]*dilution factor*

3. Quality flag reporting

All samples that have a calculated GC/µL quantity that is less than that of the LLOQ, greater than that of the ULOQ, or that have Cq values with a standard deviation ≥2 among replicates receive quality flags. These quality flags are reported in the data returned for each sample.