

# NEON 16S/ITS qPCR Standard Operating Procedure v.3

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## I. Version History

1. Section C: Analysis criteria were changed from automatic to manual baseline and threshold settings.

## II. Objective and Overview

To assess microbial abundance in soil and 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 #
<b>Fungal gDNA Standard</b>	ATCC	204094D-5 56472D-5 MYA-4609D-2
<b>Archaeal gDNA Standard</b>	ATCC	700922D-5 700653D
<b>Quantus ONE dsDNA Assay Kit</b>	Promega	E4871
<b>Wizard® Genomic Purification Kit</b>	Promega	A1120
<b>10 mg/mL Lysozyme</b>	Sigma	L6876
<b>10 mg/mL Lysostaphin</b>	Sigma	L7386
<b>Kapa SYBR Green Master Mix</b>	Kapa Biosystems	KK4602

## IV. Procedure

### A. Standard Preparation

#### 1. Prokaryotic Standards

Archaeal genomic DNA (gDNA) standards are rehydrated according to the manufacturer's instructions, as needed. Cultures used for preparing bacterial gDNA are grown from glycerol stocks (BEI # HM-280) overnight at 30 °C in Nutrient Broth (NB) and at 37 °C in Trypticase Soy Broth (TSB). Seven bacterial strains are included in the bacterial gDNA stock representing a variety of cellular morphologies. Each culture is plated on both Nutrient Agar (NA) and Trypticase Soy Agar (TSA) plates using a three-phase streak with an inoculation loop, and plates are incubated overnight at 30 °C and 37 °C, respectively. Individual colonies are harvested using a sterile swab, swirling the swab in 10 mL sterile water to dislodge the bacteria. The bacteria are pelleted by centrifugation, washed three times and resuspended in 10 mL sterile water. One milliliter aliquots of the washed bacteria are purified using the Wizard® Genomic DNA Purification Kit according to the manufacturer's instructions. 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 in equal proportions to

create a gDNA prokaryotic standard, normalized by calculated gene copy 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  $1 \times 10^6$  to 1 Gene Copies (GC)/ $\mu\text{L}$ . Exact standard curve concentrations may vary, as long as there is a six point standard curve.

## 2. Fungal Standards

Fungal genomic DNA (gDNA) standards are purchased from ATCC (see Recommended Materials) and 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  $1 \times 10^6$  to 1 GC/ $\mu\text{L}$ . Exact standard curve concentrations may vary, as long as there is a six point standard curve.

## B. qPCR Analysis

Quantitative PCR (qPCR) is performed using the Applied Biosystems 7500 Fast PCR platform with Kapa SYBR Green Master Mix according to the manufacturer's instructions. 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. The data are analyzed using the Applied Biosystems 7500 software, version 2.3, according to the manufacturer's instructions.

Samples are run in batches of up to 24 samples in triplicate and include 5 samples of water-only no-template controls.

**Table 1 - qPCR Primer Sequences**

Target	Primer	Sequence	Reference
<b>16S v3-4</b>	Forward	5'-CCTACGGGNBGCASCAG-3'	Pro341F and Pro805R <sup>1</sup>
	Reverse	5'-GGACTACNVGGGTATCTAATCC-3'	
<b>ITS 1</b>	Forward	5'-CTTGGTCATTTAGAGGAAGTAA-3'	Walters et al. (from Gardes and Bruns et al. Mol Ecol. 1993) <sup>2</sup>
	Reverse	5'-GCTGCGTTCTTCATCGATGC-3'	

<sup>1</sup> 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.

<sup>2</sup> 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
<b>Polymerase Activation</b>	100%	95 °C	3 minutes		Hold
<b>Denature</b>	100%	95 °C	30 seconds		<b>35</b>
<b>Anneal</b>	100%	50 °C	30 seconds		
<b>Extend</b>	100%	72 °C	30 seconds	On Hold	
<b>Hold</b>	100%	72 °C	5 min		Hold
<b>Melt Curve Step 1</b>	100%	95 °C	15 seconds		1
<b>Melt Curve Step 2</b>	100%	60 °C	1 minute	On Ramp	
<b>Melt Curve Dissociation Step</b>	1%	95 °C	15 seconds		
<b>Melt Curve Step 3</b>	100%	60 °C	15 seconds		

**Table 3 - ITS qPCR Cycling Conditions**

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

## C. Analysis Criteria

Data analyses will be performed using the Applied Biosystems 7500 software, version 2.3, according to the manufacturer's instructions with manual baseline and threshold setting, rounding threshold to no more than 2 significant digits.

## D. Acceptance Criteria

### 1. Assay Acceptance Criteria

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

1. The results are negative for each no-template control.
2. The standard curve contains at least three concentrations of standard for which there are two or more positive replicates.
3. The  $R^2$  value for the standard curve is greater than or equal to 0.95.

If any of these criteria are not met in an assay, all samples in the assay are repeated.

### 2. Sample Acceptance Criteria

For individual samples tested by qPCR, data are reported to the client if the following acceptance criteria are satisfied:

1. The acceptance criteria in section C. 1. were satisfied for the assay in which the sample was tested.
2. The sample was detected as positive for at least two of three replicates.

Samples not passing the above criteria, and samples passing the above criteria whose average quantity is calculated to be above the range of the standard curve, are re-tested at the discretion of the client.