NEON 16S/ITS qPCR Standard Operating Procedure v.1

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I. Version History

None. This is the first version of this SOP.

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 and ITS-2 regions of fungal rDNA (Table 1).

III. Recommended Materials

Material	Manufacturer	Catalog #
Fungal gDNA Standard	ATCC	204094D-5 56472D-5 MYA-4609D-2
Archaeal gDNA Standard	ATCC	700922D-5 700653D
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
PowerUp SYBR Green Master Mix	Fisher	A25777

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 x 10^6 to 1 Gene Copies (GC)/µL. Exact standard curve concentrations may vary, as long as there is a six point standard curve.

2. Fungal Standards

Fungal gDNA standards are purchased from ATCC (see Recommended Materials) and rehydrated according to the manufacturer's instructions. gDNA 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 x 10^6 to 1 (GC)/µL. 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 PowerUp 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.

Table 1 - qPCR Primer Sequences

Target	Primer	Sequence	Reference
16S v3-4	Forward	5′- CCTACGGGNBGCASCAG-3′	Pro341F and Pro805R[1]
	Reverse	5′- GGACTACNVGGGTATCTAATCC-3′	
ITS 1-2	Forward	5′- TCCTCCGCTTATTGATATGC-3′	White et al. 1990[2]
	Reverse	5′- GCTGCGTTCTTCATCGATGC-3′	Walters et al. (from Gardes and Bruns et al. Mol Ecol. 1993)[3]

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[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] White, T. J., T. Bruns, S. Lee, and J. Taylor. 1990. Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics. *PCR Protocols: A Guide to Methods and Applications.* Academic Press, Inc.

[3] 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
UDG Activation	100%	50 °C	2 minutes		Hold
Dual-Lock DNA Polymerase	100%	95 °C	2 minutes		Hold
Denature	100%	95 °C	3 seconds		40
Anneal	100%	55 °C	15 seconds		
Extend	100%	60 °C	1 minute	On 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	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
UDG Activation	100%	50 °C	2 minutes		Hold
Dual-Lock DNA Polymerase	100%	95 °C	2 minutes		Hold
Denature	100%	95 °C	3 seconds		40
Anneal/Extend	100%	60 °C	30 seconds	On 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	1%	95 °C	15 seconds		
Melt Curve Step 3	100%	60 °C	15 seconds		

C. Analysis Criteria

Data analyses are performed using the Applied Biosystems 7500 software, version 2.3, according to the manufacturer's instructions, with automatic baseline and threshold settings.

D. Acceptance Criteria

1. Assay Acceptance Criteria

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

1. The acceptance criteria in section D.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, may be re-tested.