NEON 16S/ITS qPCR Standard Operating Procedure v.8

Prepared for:
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I. Version History

Version 8	1. Sections IV.A.1 and A.2 clarified standard preparation. 2. Section IV.A.1 added Equation 1 and Equation 2 to show how the standard DNA concentration (GC/µL) is calculated to improve clarity and transparency. 3. Section IV.A.2 clarified to show how the standard DNA concentration (GC/µL) is calculated to improve clarity and transparency by referring to Equation 1 and 2. 4. Sections IV.A.1 and A.2 added language state that the 2020 genome size data obtained from ATCC Genome Portal is used as inputs for calculation of each standard concentration (GC/µL). 5. Section IV.A.1 added Table 2 to specify the mixing ratios of each prokaryotic standard DNA used to prepare the prokaryotic standard mixture, achieving a final concentration of 2×10^6 GC per 2 µL. 6. Section IV.A.2 added Table 3 to specify the mixing ratios of each fungal standard DNA used to prepare the fungal standard mixture, achieving a final concentration of 1×10^5 GC per 2 µL.
Version 7.3	1. An additional manufacturer was added as a source of bacterial standard. 2. A change was made for the software used for data analysis, the Applied Biosystems Design and Analysis 2 (DA2) version 2.8 was added to replace Applied Biosystems 7500 software, version 2.3 for data analysis only. Applied Biosystems 7500 software, version 2.3 will be used to run the instrument.
Version 7.2	1. Added option to manually adjust the baseline in Section IV.B. 2. Modified individual sample level QAQC criteria to allow for option of repeating samples that fall outside of standard curve range.
Version 7.1	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.
Version 7	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.
Version 6	1. Section III, Recommended Materials, was updated to allow the genomic DNA standards to vary from batch to batch. Exact standards in use are reported in the batch-level data; DNA extraction kit added. 2. Section IV.A.1 was changed to allow for direct isolation of bacterial gDNA from a 22-strain consortium.

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, Genomic DNA from *Lachancea thermotolerans* strain RRL Y-8284	ATCC	56472D-5™
Fungal gDNA Standard, Genomic DNA from *Aspergillus fumigatus* strain CBS 101355	ATCC	MYA-4609D-2™
Fungal gDNA Standard, Genomic DNA from *Cutaneotrichosporon dermatis* strain VITEK 303483	ATCC	204094D-5™
Archaeal gDNA Standard, Genomic DNA *Halobacterium salinarum* strain NRC-1	ATCC	700922D-5™
Archaeal gDNA Standard, Genomic DNA from *Thermococcus gorgonarius* strain DSM 10395	ATCC	700654D™
Bacterial Standard, Genomic DNA from Microbial Mock Community B	BEI Resources	HM-782D
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 bacteria 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. To determine concentration (Gene Copies (GC)/µL) of each prokaryotic standard gDNA, Equations 1 and 2 are used[1]:

$$Standard\ gDNA\ concentration\ (GC/\mu L) = \frac{Standard\ gDNA\ concentration\ (ng/\mu L)}{Standard\ DNA\ mass\ per\ GC\ (ng)} \qquad (1)$$

$$Standard\ DNA\ mass\ per\ GC\ (ng) = \frac{Genome\ size\ (bp) * 1.0\ x10^{9}\ \left(\frac{ng}{g}\right) * 660\ \left(\frac{g}{mol}\right)}{6.0221\ x10^{23}\ \left(\frac{molecules}{mol}\right)} \qquad (2)$$

Genome size data from the ATCC Genome Portal[2] (accessed in 2020, based on the corresponding prokaryotic standard DNA source strain) is used as inputs to calculate concentration (GC/µL) of each prokaryotic standard. To create a prokaryotic gDNA standard mixture with a final concentration of 2 x 10^{6} GC/2 µL, the bacterial gDNA standard stock and archaeal gDNA standards are combined in the proportions (ratios) specified in Table 2. 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} 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. To determine concentration (GC)/µL) of each fungal standard gDNA, the Equations 1 and 2 given in Section IV.A.1 are used. Genome size

[1] URL1: https://www.idtdna.com/page/support-and-education/decoded-plus/calculations-converting-from-nanograms-to-copy-number/, Access Date: 12/22/2025
[2] URL 2: https://genomes.atcc.org/genomes, Access Date: 01/2020

information from the ATCC Genome Portal[2] (accessed in 2020, based on the corresponding fungal standard DNA source strain) is used as inputs to calculate concentration (GC/µL) of each fungal standard.

To create a fungal gDNA standard mixture with a final concentration of 1 x 10^5 /2 µL, fungal gDNA standards are combined according to the ratios (proportions) shown in Table 3. 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

Samples are run by quantitative PCR (qPCR) using the Applied Biosystems 7500 Fast PCR platform (software version 2.3) 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 4 and 5.

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 Design and Data Analysis 2 (DA2) software, version 2.8, according to the manufacturer's instructions with automatic or manual baseline and 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 Cycle Threshold (Ct).

For each assay, the standard curve is generated only using standard concentrations that have three positive replicates. A replicate is considered positive if its Ct value is lower than the lowest Ct 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 considered the lower limit of quantification (LLOQ) for the assay, and the highest concentration standard that has three positive replicates is considered the upper limit of quantification (ULOQ) for the assay.

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[3]
	Reverse	5′-GACTACNVGGGTATCTAATCC-3′	
ITS-1	Forward	5′-CTTGGTCATTTAGAGGAAGTAA-3′	ITS1f and ITS2[4]
	Reverse	5′-GCTGCGTTCTTCATCGATGC-3′	

Table 2 - Prokaryotic gDNA Standard Preparation

Prokaryotic Standard gDNA	gDNA Standards Mixture Proportions	Standards Mixture Percentages
Microbial Mock Community B gDNA	0.923	42%
***H. salinarum* strain NRC-1 gDNA**	1.000	45%
***T. gorgonarius* strain DSM 10395 gDNA**	0.294	13%

Table 3 – Fungal gDNA Standard Preparation

Fungal Standard gDNA	gDNA Standards Mixture Proportions	Standards Mixture Percentages
***C. dermatis* strain VITEK 303483 gDNA**	0.886	44%
***A. fumigatus* strain CBS 101355 gDNA**	0.133	7.0%
***L. thermotolerans* strain RRL Y-8284 gDNA**	1.000	50%

Table 4 - 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	

[3] 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.

[4] 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.

Melt Curve Dissociation Step	1%	95 °C	15 seconds		
Melt Curve - Step 3	100%	60 °C	15 seconds		

Table 5 - 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		
Anneal	100%	50 °C	1 minute		**35**
Extend	100%	72 °C	1 minute	On Hold	
Hold	100%	72 °C	10 min		Hold
Melt Curve - Step 1	100%	95 °C	15 seconds		
Melt Curve - Step 2	100%	60 °C	1 minute	On Ramp	
Melt Curve Dissociation Step	1%	95 °C	15 seconds		**1**
Melt Curve - Step 3	100%	60 °C	15 seconds		

C. Acceptance Criteria

1. Batch-Level Acceptance Criteria

For each qPCR assay, data is 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 this 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 two of three replicates have amplification which falls between that of the LLOQ and the ULOQ.

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 flagged as failing QA/QC and may be repeated if a change to the sample dilution would likely result in a Cq value between the ULOQ and LLOQ. NEON approval must be obtained prior to performing any repeat analysis.

Individual samples will be analyzed no more than two times. If a sample's repeat analysis has two replicates with Cq values that fall outside the range of Cq values between the ULOQ and LLOQ, the sample will be reported and flagged as failing QA/QC.

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 less than the LLOQ, greater than the ULOQ, or that have Ct values with a standard deviation ≥2 among replicates receive quality flags. These quality flags are reported in the data returned for each sample.