



Method 1611: Enterococci in Water by TaqMan[®] Quantitative Polymerase Chain Reaction (qPCR) Assay

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Introduction

Enterococci are commonly found in the feces of humans and other warm-blooded animals. Although these organisms can be persistent in the environment, the presence of enterococci in water is an indication of fecal pollution and the possible presence of enteric pathogens. Epidemiological studies have led to the development of criteria for promulgating recreational water standards based on established relationships between the measured density of enterococci colony forming units (CFU) in the water by culture based methods and the risk of gastrointestinal illness (GI) associated with swimming in the water (References 17.3, 17.6, 17.7, and 17.8).

Method 1611 describes a quantitative polymerase chain reaction (qPCR) procedure for the detection of DNA from enterococci bacteria in ambient water matrices based on the amplification and detection of a specific region of the large subunit ribosomal RNA gene (1srRNA, 23S rRNA) from these organisms. The advantage of this method over currently accepted culture methods that require 24 – 48 hours to obtain results is its relative rapidity. Results can be obtained by this method in 3 – 4 hours.

EPA conducted a single-laboratory validation (SLV) study of Method 1611 in marine and fresh water matrices, to assess potential matrix issues across multiple fresh and marine water matrices from different geographic regions across the U.S. During the SLV thirty-seven volunteer laboratories collected and shipped recreational water (marine and/or fresh) samples to the two analytical laboratories for Method 1611 analyses. For all 25 (each) marine and fresh water matrices, no matrix inhibition was observed for Method 1611. EPA then conducted multi-laboratory validation studies to assess method performance and reproducibility and develop quantitative quality control (QC) acceptance criteria.

Results of the multi-laboratory validation studies enabled performance characterization of Method 1611 in the reference matrix phosphate buffered saline (PBS) and in marine and fresh water matrices. Results also enabled the development of initial precision and recovery/ongoing precision and recovery (IPR/OPR) and matrix spike (MS) QC acceptance criteria. In addition, results from marine water and fresh water samples (5 different sources for each matrix type) spiked with commercially available target organism cells were used to assess method reproducibility.

The highly variable recoveries observed during these studies should be taken into consideration when analyzing results from Method 1611. It is recommended that analysts generate standard curves with each new lot of qPCR reagents or primers and probes to demonstrate comparable performance with these new reagents. In addition, weekly calibrator samples should be monitored to ensure that they are within the acceptable range of the methods; if calibrator samples are not within the acceptable range, new calibrator samples should be prepared and analyzed. Following these recommendations should increase analyst proficiency and minimize variability of recoveries. In addition, care should be placed to verify the accuracy of all reference materials used in the method (i.e. DNA standards, calibrator cells, control DNA's and spike materials).

In Method 1611, water samples are filtered to collect enterococci on polycarbonate membrane filters. Following filtration, total DNA is solubilized from the filter retentate using a bead beater. Enterococci target DNA sequences present in the clarified homogenate are detected by the real time polymerase chain reaction (PCR) technique using TaqMan[®] Universal master mix PCR reagent and the TaqMan[®] probe system. The TaqMan[®] system signals the formation of PCR products by a process involving enzymatic hydrolysis of a fluorogenically-labeled oligonucleotide probe when it hybridizes to the target sequence.

Method 1611 uses an arithmetic formula, referred to as the $\Delta\Delta C_T$ comparative cycle threshold calculation ($\Delta\Delta C_T$) method to calculate the ratio of *Enterococcus* 1srRNA gene target sequences (target sequences)

recovered in total DNA extracts from water samples relative to those in similarly-prepared extracts of calibrator samples containing a known quantity of *Enterococcus* cells. The $\Delta\Delta C_T$ relative quantitation method also uses measurements of a reference target sequence (from total salmon DNA, added in equal amounts to both test and calibrator samples) to normalize these ratios for potential differences in DNA recovery. The reference target sequence measurements are also used to signal inhibition or fluorescence quenching of the PCR analysis caused by sample matrix components or possible technical error. These ratios are converted to calibrator cell equivalents (CCEs) recovered from the test samples.

Table of Contents

1.0	Scope and Application	1
2.0	Summary of Method	2
3.0	Definitions	2
4.0	Interferences.....	3
5.0	Safety	3
6.0	Equipment and Supplies	3
7.0	Reagents and Standards	5
8.0	Sample Collection, Handling, and Storage	9
9.0	Quality Control	9
10.0	Calibration and Standardization of Method-Related Instruments.....	14
11.0	Procedure	14
12.0	Data Analysis and Calculations	25
13.0	Sample Spiking Procedure.....	27
14.0	Method Performance.....	33
15.0	Pollution Prevention.....	34
16.0	Waste Management.....	35
17.0	References.....	35
18.0	Acronyms.....	35

List of Appendices

Appendix A: ABI StepOnePlus™, ABI 7900, and ABI 7500 Real-Time PCR System Operation

Appendix B: Cepheid Smart Cycler® Operation

Method 1611: Enterococci in Water by TaqMan[®] Quantitative Polymerase Chain Reaction (qPCR) Assay

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1.0 Scope and Application

- 1.1** Method 1611 describes a quantitative polymerase chain reaction (qPCR) procedure for the measurement of large subunit ribosomal RNA (lsrRNA, 23S rRNA) target gene sequences (target sequences) from all known species of enterococci bacteria in water. This method is based on the collection of enterococci on membrane filters, extraction of total DNA using a bead beater, and detection of enterococci target sequences in the supernatant by real time polymerase chain reaction (qPCR) using TaqMan[®] Universal master mix PCR reagent and the TaqMan[®] probe system. The TaqMan[®] system signals the formation of PCR products by a process involving the enzymatic hydrolysis of a labeled fluorogenic probe that hybridizes to the target sequence.
- 1.2** The Method 1611 test is recommended as a measure of ambient marine and fresh recreational water quality. The significance of finding *Enterococcus* DNA target sequences in recreational water samples stems from the association between the density of these sequences and the reported occurrence of gastrointestinal (GI) illness associated with swimming observed in recent epidemiological studies (References 17.4 and 17.10).
- 1.3** The highly variable recoveries observed during the validation studies should be taken into consideration when analyzing results from Method 1611. Guidance is provided in section 9 of this document on how laboratories can monitor the initial and ongoing performance of the method in their hands. It is expected and has been observed that the performance of the method will improve as laboratories gain additional experience with it through continued practice.
- 1.4** This method assumes the use of an Applied Biosystems (ABI) Real-Time PCR System as the default platform. The Cepheid Smart Cycler[®] may also be used. The user should refer to the platform specific instructions for these instruments in the appendices. Users should thoroughly read the method in its entirety before preparation of reagents and commencement of the method to identify differences in protocols for different platforms.

2.0 Summary of Method

The method is initiated by filtering a water sample through a membrane filter. Following filtration, the membrane containing the bacterial cells and DNA is placed in a microcentrifuge tube with glass beads and buffer, and then agitated to extract the DNA into solution. The supernatant is used for PCR amplification and detection of target sequences using the TaqMan[®] Universal master mix PCR reagent and probe system.

3.0 Definitions

- 3.1** Enterococci: all species of the genus *Enterococcus* for which *lsrRNA* gene nucleotide sequences were reported in the GenBank database (<http://www.ncbi.nlm.nih.gov/Genbank>) at the time of method development.
- 3.2** Target sequence: A segment of the *Enterococcus* *lsrRNA* gene containing nucleotide sequences that are homologous to both the primers and probe used in the *Enterococcus* qPCR assay and that are common only to species within this genus.
- 3.3** Sample processing control (SPC) sequence (may also be referred to as reference sequence): A segment of the ribosomal RNA gene operon, internal transcribed spacer region 2 of chum salmon, *Oncorhynchus keta* (*O. keta*) and other salmon spp., containing nucleotide sequences that are homologous to the primers and probe used in the SPC qPCR assay. SPC sequences are added as part of a total salmon DNA solution in equal quantities to all water and calibrator sample filters prior to extracting DNA from the samples. The purpose of this control is to determine, and, if necessary, correct for different relative efficiencies of total DNA recovery from water and calibrator sample extracts using the $\Delta\Delta C_T$ comparative cycle threshold calculation method ($\Delta\Delta C_T$ method) as described in Section 12.3. This control is also used to signal potential co-extraction of water sample components that interfere with the PCR analysis as described in Section 9.12.
- 3.4** DNA standard: A purified, RNA-free and spectrophotometrically quantified and characterized *E. faecalis* strain ATCC[®] # 29212[™] genomic DNA preparation. DNA standards are used to generate standard curves for determination of performance characteristics of the qPCR assays and instrument with different preparations of master mixes containing TaqMan[®] reagent, primers and probe as described in Section 9.10. Also used for quantifying target sequences in calibrator sample extracts as described in Section 12.3.
- 3.5** Calibrator sample: Samples containing constant added quantities of *E. faecalis* strain ATCC[®] # 29212[™] cells and SPC sequences that are extracted and analyzed in the same manner as water sample filtrates. Calibrator sample analysis results are used as positive controls for the *Enterococcus* target sequence and SPC qPCR assays and as the basis for target sequence quantification in water sample filtrates using the $\Delta\Delta C_T$ comparative cycle threshold calculation method ($\Delta\Delta C_T$ method) as described in Section 12.3. Analysis results of these samples provide corrections for potential method-related variations in *Enterococcus* cell lysis, target sequence recovery and PCR efficiency.
- 3.6** $\Delta\Delta C_T$ method: A calculation method derived by ABI (Reference 17.1) for calculating the ratios of target sequences in two DNA samples (*e.g.*, a calibrator and water filtrate sample) that normalizes for differences in total DNA recovery from these samples using qPCR analysis C_T values for a reference (SPC) sequence that is initially present in equal quantities prior to DNA extraction.

- 3.7** Amplification factor (AF): A measure of the average efficiency at which target or SPC sequences are copied and detected by their respective primer and probe assays during each thermal cycle of the qPCR reaction that is used in the $\Delta\Delta C_T$ method. AF values can range from 1 (0% of sequences copied and detected) to 2 (100% of sequences copied and detected) and are calculated from a standard curve as described in Section 12.2. For an explanation of test inhibition, see Section 9.12.

4.0 Interferences

Water samples containing colloidal or suspended particulate materials can clog the membrane filter and prevent filtration. These materials can also interfere with DNA recovery and with the qPCR analysis by inhibiting the enzymatic activity of the Taq DNA polymerase, and/or inhibiting the annealing of the primer and probe oligonucleotides to sample target DNA, enzyme or quenching of hydrolyzed probe fluorescence.

5.0 Safety

- 5.1 The analyst / technician must know and observe the normal safety procedures required in a microbiology and/or molecular biology laboratory while preparing, using, and disposing of cultures, reagents, and materials, and while operating sterilization equipment.
- 5.2 Where possible, facial masks should be worn to prevent sample contamination.
- 5.3 Mouth-pipetting is prohibited.

6.0 Equipment and Supplies

- 6.1** Separated, and dedicated workstations for reagent preparation and for sample preparation, preferably with HEPA-filtered laminar flow hoods and an Ultraviolet (UV) light source, each having separate supplies (*e.g.*, pipettors, tips, gloves, etc.). *Note:* The same workstation may be used for the entire procedure provided that it has been cleaned with bleach and UV sterilized as specified in Section 11.6.1 between reagent and sample preparation. Under ideal conditions, two dedicated workstations are used for sample preparation: one for preparing samples with high target sequence DNA concentrations (*e.g.*, DNA standards and calibrator samples) and one for preparing samples with expected low target sequence DNA concentrations (*e.g.*, filter blanks and ambient water samples).
- 6.2** Balance capable of accuracy to 0.01 g
- 6.3** Extraction tubes: semi-conical, screw cap microcentrifuge tubes, 2.0 mL (PGC 506-636 or equivalent)
- 6.4** Glass beads, acid washed, 212 – 300 μm diameter (Sigma G-1277 or equivalent)
- 6.5** Autoclave, capable of achieving and maintaining 121°C [15 lb pressure per square inch (PSI)] for minimally 15 minutes (optional)
- 6.6** Workstation for water filtrations, preferably a HEPA-filtered laminar flow hood with a UV light source. This can be the same as used for sample preparation (Section 6.1).

- 6.7 Sterile bottles/containers for sample collection
- 6.8 Membrane filtration units (filter base and funnel) for 47 mm diameter filters, sterile glass, plastic (Pall Gelman 4242 or equivalent), stainless steel, or disposable plastic (Nalgene CN 130-4045 or CN 145-0045 or equivalent), cleaned and bleach treated (rinsed with 10% v/v bleach, then 3 rinses with reagent-grade water), covered with aluminum foil or Kraft paper, and autoclaved or UV-sterilized if non-disposable.
- 6.9 Line vacuum, electric vacuum pump, or aspirator for use as a vacuum source. In an emergency or in the field, a hand pump or a syringe equipped with a check valve to prevent the return flow of air can be used.
- 6.10 Flask, filter, vacuum, usually 1 L, with appropriate tubing
- 6.11 Filter manifold to hold a number of filter bases
- 6.12 Flask for safety trap placed between the filter flask and the vacuum source
- 6.13 Forceps, straight or curved, with smooth tips to handle filters without damage, 2 pairs
- 6.14 Polycarbonate membrane filters, sterile, white, 47 mm diameter, with 0.45 μm pore size (GE Osmonics Inc. K04CP04700 or equivalent)
- 6.15 Graduated cylinders, 100 - 1000 mL, cleaned and bleach treated (rinsed with 10% v/v bleach, then 3 rinses with reagent-grade water), covered with aluminum foil or Kraft paper and autoclaved or UV-sterilized.
- 6.16 Petri dishes, sterile, plastic or glass, 100 \times 15 mm with loose fitting lids
- 6.17 Disposable loops, 1 and 10 μL
- 6.18 Permanent ink marking pen for labeling tubes
- 6.19 Visible wavelength spectrophotometer capable of measuring at 595 nm
- 6.20 Single or multi-place bead beater (Biospec Products Inc. 3110BX or equivalent)
- 6.21 Microcentrifuge capable of 12,000 \times g
- 6.22 Micropipettors with 10, 20, 200 and 1000 μL capacity. Under ideal conditions, each workstation should have a dedicated set of micropipettors (one micropipettor set for pipetting reagents not containing cells or reference DNA and one set for reagents containing reference DNA and for test samples). Ideally, micropipettors should be calibrated monthly.
- 6.23 Micropipettor tips with aerosol barrier for 10, 20, 200 and 1000 μL capacity micropipettors.
Note: All micropipetting should be done with aerosol barrier tips. The tips used for reagents not containing DNA should be separate from those used for reagents containing DNA and test samples. Each workstation should have a dedicated supply of tips.
- 6.24 Microcentrifuge tubes, low retention, clear, 1.7 mL (GENE MATE C-3228-1 or equivalent)
- 6.25 Test tube rack for microcentrifuge tubes, use a separate rack for each set of tubes
- 6.26 Conical centrifuge tubes, sterile, screw cap, 50 mL
- 6.27 Test tubes, screw cap, borosilicate glass, 16 \times 125 mm
- 6.28 Pipet containers, stainless steel, aluminum or borosilicate glass, for glass pipets
- 6.29 Pipets, sterile, T.D. bacteriological or Mohr, disposable glass or plastic, of appropriate volume (disposable pipets preferable)
- 6.30 Vortex mixer (ideally one for each work station)

- 6.31 Dedicated lab coats for each work station
- 6.32 Disposable powder-free gloves for each work station
- 6.33 Refrigerator, 4°C (ideally one for reagents and one for DNA samples)
- 6.34 Freezer, -20°C or -80°C (ideally one for reagents and one for DNA samples)
- 6.35 Ice, crushed or cubes for temporary preservation of samples and reagents
- 6.36 Printer (optional)
- 6.37 Data archiving system (*e.g.*, flash drive or other data storage system)
- 6.38 UV spectrophotometer capable of measuring wavelengths of 260 and 280 nm using small volume capacity (*e.g.*, 0.1 mL) cuvettes or NanoDrop[®] (ND-2000 spectrophotometer or equivalent) capable of the same measurements at 2 µL sample volumes
- 6.39 ABI StepOnePlus[™], ABI 7900, or ABI 7500
 - 6.39.1 Optical 96 well PCR reaction tray (ABI N801-0560 or equivalent)
 - 6.39.2 Optical adhesive PCR reaction tray tape (ABI 4311971 or equivalent) or MicroAmp[™] caps (ABI N8010534 or equivalent)
 - 6.39.3 ABI StepOnePlus[™], ABI 7900, or ABI 7500 Real-Time PCR System
- 6.40 Cepheid (optional to ABI)
 - 6.40.1 Smart Cycler[®] 25 µL PCR reaction tubes (Cepheid 900-0085 or equivalent)
 - 6.40.2 Rack and microcentrifuge for Smart Cycler[®] PCR reaction tubes. *Note:* Racks and microcentrifuge are provided with the Smart Cycler[®] thermocycler
 - 6.40.3 Cepheid Smart Cycler[®] System Thermocycler

7.0 Reagents and Standards

Note: The *E. faecalis* stock culture (Section 7.8), Salmon DNA/extraction buffer (Section 7.12), and DNA extraction tubes (Section 7.18), may be prepared in advance.

- 7.1 Purity of Reagents: Molecular-grade reagents and chemicals shall be used in all tests
- 7.2 Control Culture
 - *Enterococcus faecalis* (*E. faecalis*) ATCC[®] #29212[™] (ATCC, Manassas VA) or
 - *E. faecalis* ATCC[®] # 29212[™] (550 colony forming units [CFU]) BioBalls (bioMérieux Inc., Durham NC)
- 7.3 Sample Processing Control (SPC) DNA
 - Salmon testes DNA (Sigma D1626 or equivalent)

7.4 Phosphate Buffered Saline (PBS)**7.4.1** Composition:

Monosodium phosphate (NaH ₂ PO ₄)	0.58 g
Disodium phosphate (Na ₂ HPO ₄)	2.50 g
Sodium chloride	8.50 g
Reagent-grade water	1.0 L

7.4.2 Dissolve reagents in 1 L of reagent-grade water in a flask and dispense in appropriate amounts for dilutions in screw cap bottles or culture tubes, and/or into containers for use as rinse water. Autoclave after preparation at 121°C (15 lb PSI) for 15 minutes. Final pH should be 7.4 ± 0.2.

7.5 Brain heart infusion broth (BHIB)**7.5.1** Composition:

Calf brains, infusion from 200.0 g	7.7 g
Beef heart, infusion from 250.0 g	9.8 g
Proteose peptone	10.0 g
Sodium chloride	5.0 g
Disodium phosphate (Na ₂ HPO ₄)	2.5 g
Dextrose	2.0 g
Reagent-grade water	1.0 L

7.5.2 Add reagents to 1 L of reagent-grade water, mix thoroughly, and heat to dissolve completely. Dispense 10 mL volumes in screw cap 16 × 125 mm tubes and autoclave at 121°C (15 PSI) for 15 minutes. Final pH should be 7.4 ± 0.2.

7.6 Brain heart infusion agar (BHIA)**7.6.1** Composition:

BHI agar contains the same components as BHIB with the addition of 15.0 g agar per liter of BHI broth.

7.6.2 Add agar to formula for BHIB provided above. Prepare as in Section 7.5.2. After sterilization, dispense 12 – 15 mL into 100 × 15 mm petri dishes. Final pH should be 7.4 ± 0.2.

7.7 Sterile glycerol (used for preparation of *E. faecalis* stock culture as described in Section 7.8)**7.8** Preparation of *E. faecalis* (ATCC® # 29212™) stock culture

Rehydrate lyophilized *E. faecalis* per manufacturer's instructions (for ATCC® stocks, suspend in 5 - 6 mL of sterile BHIB and incubate at 37°C for 24 hours). Centrifuge to create pellet. Using a sterile pipet, discard supernatant. Resuspend pellet in 10 mL of fresh sterile BHIB containing 15% glycerol and dispense in 1.5 mL aliquots in microcentrifuge tubes. Freeze at -20°C (short term storage) or -80°C (long term storage). *Note:* Aliquots of suspension should be plated to determine CFU concentration as described in Section 11.1. It is also advisable to verify the *E. faecalis* culture as described, for example, in Section 15 of EPA Method 1600.

- 7.9** PCR-grade water (OmniPur water from VWR EM-9610 or equivalent). Water must be DNA/DNase free.
- 7.10** Isopropanol or ethanol, 95%, for flame-sterilization
- 7.11** AE Buffer, pH 9.0 (Qiagen 19077 or equivalent) *Note:* pH 8.0 is acceptable
Composition:
10 mM Tris-Cl (Tris-chloride)
0.5 mM EDTA (Ethylenediaminetetraacetic acid)
- 7.12** Salmon DNA/extraction buffer
- 7.12.1** Composition:
Stock Salmon testes DNA (10 µg/mL) (Section 7.3)
AE Buffer (Section 7.11)
- 7.12.2** Preparation of stock Salmon testes DNA: Using sterilized scissors and sterilized forceps, cut a piece of DNA to weigh approximately 20 mg, and place in a sterile 50 mL conical tube. After weighing, add 20 mL of PCR-grade water. Cap tightly, and resuspend by 2 – 4 hours of vigorous shaking (note: longer times may be required to fully suspend the DNA – check for even consistency by repeatedly drawing up the suspension and expelling with a sterile 25 ml pipet) . The concentration should be about 1 mg/mL. Dilute a 1ml aliquot of this 1 mg/mL stock with 99 ml PCR-grade water to make 10 µg/mL working stock. Check absorbance (OD₂₆₀) and calculate DNA concentration using the assumption 50 µg/mL DNA is equal to 1 OD₂₆₀, (i.e. 10 µg/mL should equal ~ 0.2 OD₂₆₀). If measured concentration is different than 10 µg/mL, adjust dilutions to make 0.2 µg/mL Salmon DNA/extraction buffer in 7.12.3 accordingly. Dispense 1 mg/mL stock and 10 µg/mL working stock suspensions into aliquots (e.g. 1 ml) and freeze at -20°C. Frozen aliquots of working stock should be thawed only once, but once thawed can be stored in a refrigerator for several months.
- 7.12.3** Dilute Salmon testes DNA working stock with AE buffer to make 0.2 µg/mL Salmon DNA/extraction buffer. Extraction buffer may be prepared in advance and stored at 4°C for a maximum of 1 week.
- Note:* Determine the total volume of Salmon DNA/extraction buffer required for each day or week by multiplying volume (600 µL) × total number of samples to be analyzed including controls, water samples, and calibrator samples. For example, for 18 samples, prepare enough Salmon/DNA extraction buffer for 24 extraction tubes: 3 tubes for water sample filtration blank (method blank) and 3 tubes for calibrator samples). *Note:* At least one method blank should be included with each sample batch. Additionally, prepare excess volume to allow for accurate dispensing of 600 µL per tube, generally enough for 1 extra tube. Thus, in this example, prepare sufficient Salmon/DNA extraction buffer for 24 tubes plus one extra. The total volume needed is 600 µL × 25 tubes = 15,000 µL. Dilute the Salmon testes DNA working stock 1:50, for a total volume needed (15,000 µL) ÷ 50 = 300 µL of 10 µg/mL Salmon testes DNA working stock. The AE buffer needed is the difference between the total volume and the Salmon testes DNA working stock. For this example, 15,000 µL – 300 µL = 14,700 µL AE buffer needed.
- 7.13** Bleach solution: 10% v/v bleach (or other reagent that hydrolyzes DNA), used for cleaning work surfaces
- 7.14** Sterile water (used as rinse water for work surface after bleaching)

- 7.15** TaqMan[®] Universal PCR master mix (ABI 4304437 or equivalent)
Composition:
AmpliTaq Gold[®] DNA Polymerase
AmpErase[®] UNG
dNTPs with dUTP
Passive Reference 1 (ROX[™] fluorescent dye)
Optimized buffer components (KCl, Tris, EDTA, MgCl₂)
- 7.16** Bovine serum albumin (BSA), fraction V powder (Sigma B-4287 or equivalent)
Dissolve in PCR-grade water to a concentration of 2 mg/mL.
- 7.17** Primer and probe sets: Primer and probe sets may be purchased from commercial sources. Primers should be desalted, probes should be HPLC purified.
- 7.17.1** *Enterococcus* primer and probe set (Reference 17.5):
Forward primer: 5'-GAGAAATTCCAAACGAACTTG
Reverse primer: 5'-CAGTGCTCTACCTCCATCATT
TaqMan[®] probe: [6-FAM]-5'-TGGTTCTCTCCGAAATAGCTTTAGGGCTA-TAMRA
- 7.17.2** Salmon DNA primer and probe set (Reference 17.4):
Forward primer: 5'-GGTTTCCGCAGCTGGG
Reverse primer (Sketa 22): 5'-CCGAGCCGTCCTGGTC
TaqMan[®] probe: [6-FAM]-5'-AGTCGCAGGCGGCCACCGT-TAMRA
- 7.17.3** Preparation of primer/probes: Using a micropipettor with aerosol barrier tips, add PCR grade water to the lyophilized primers and probe from the vendor to create stock solutions of 500 µM primer and 100 µM probe and dissolve by extensive vortexing. Pulse centrifuge to coalesce droplets. Store stock solutions at -20°C.
- 7.18** DNA extraction tubes
Note: It is recommended that tube preparation be performed in advance of water sampling and DNA extraction procedures.
Prepare 1 tube for each sample, and 1 extra tube for every 6 samples (*i.e.*, for method blank) and a minimum of 3 tubes per week for calibrator samples. Weigh 0.3 ± 0.01 g of glass beads (Section 6.4) and pour into extraction tube. Seal the tube tightly, checking to make sure there are no beads on the O-ring of the tube. Check the tube for proper O-ring seating after the tube has been closed. Autoclave tubes at 121°C (15 PSI) for 15 minutes. *Note:* Sterile extraction tubes containing glass beads are available from Generite LLC., (50205-50 or equivalent).
- 7.19** Purified, RNA-free quantified and characterized *E. faecalis* genomic DNA preparations for use as standards used to generate a standard curve (see Section 11.2 for preparation instructions).
- 7.20** RNase A 5 mg/mL (epicentre[®] MRNA092 or equivalent)
- 7.21** DNA extraction kit (Gene-Rite K102-02C-50 DNA-EZ[®] RW02 or equivalent)

8.0 Sample Collection, Handling, and Storage

8.1 Sampling procedures are briefly described below. Adherence to sample preservation procedures and holding time limits is critical to the production of valid data. Samples not collected according to these procedures should not be analyzed. *Note:* It is recommended that when possible that three samples be collected and analyzed from each sample location.

8.2 Sampling Techniques

Samples are collected by hand or with a sampling device if the sampling site has difficult access such as a dock, bridge or bank adjacent to a surface water. Composite samples should not be collected, since such samples do not display the range of values found in individual samples. The sampling depth for surface water samples should be 6 – 12 inches below the water surface. Sample containers should be positioned such that the mouth of the container is pointed away from the sampler or sample point. After removal of the container from the water, a small portion of the sample should be discarded to provide head space for proper mixing before analyses.

8.3 Storage Temperature and Handling Conditions

Ice or refrigerate water samples at a temperature of <10°C during transit to the laboratory. Do not freeze the samples. Use insulated containers to assure proper maintenance of storage temperature. Ensure that sample bottles are tightly closed and are not completely immersed in water during transit.

8.4 Holding Time Limitations

Examine samples as soon as possible after collection. Do not hold samples longer than 6 hours between collection and initiation of filtration.

9.0 Quality Control

9.1 Each laboratory that uses Method 1611 is required to operate a formal quality assurance (QA) program that addresses and documents instrument and equipment maintenance and performance, reagent quality and performance, analyst training and certification, and records storage and retrieval. Additional recommendations for QA and quality control (QC) procedures for microbiological laboratories are provided in Reference 17.2.

9.2 The minimum analytical QC requirements for the analysis of samples using Method 1611 include an initial demonstration of laboratory capability through performance of the initial precision and recovery (IPR) analyses (Section 9.3), ongoing demonstration of laboratory capability through performance of the ongoing precision and recovery (OPR) analysis (Section 9.4) and matrix spike (MS) analysis (Section 9.5), and the routine analysis of positive control (Section 9.6), no template controls (Section 9.7), method blanks (Section 9.8), media sterility checks (Section 9.9), DNA standard curves (9.10), calibrator samples (9.11), and sample processing controls (9.12). For the IPR, OPR and MS analyses, it is necessary to spike samples with either laboratory-prepared spiking suspensions or BioBalls as described in Section 13.

9.3 Initial precision and recovery (IPR) — The IPR analyses are used to demonstrate acceptable method performance (recovery and precision) and should be performed by each laboratory before the method is used for monitoring field samples. An IPR should be performed by each analyst. The IPR analyses are performed as follows:

9.3.1 Prepare four, 100 mL samples of PBS and spike each sample with the control culture, *E. faecalis* ATCC® # 29212™ according to the spiking procedure in Section 13. Spiking

with laboratory-prepared suspensions is described in Section 13.4 and spiking with a commercially prepared product (e.g., BioBalls) is described in Section 13.7. Filter and process each IPR sample according to the procedures in Section 11 and calculate the calibrator cell equivalents (CCEs) in the samples according to Section 12. *Note:* prepare calibrator samples from the same source of cells used to prepare the spike suspensions, i.e. for samples spiked with laboratory-prepared cell suspensions, use the same laboratory-prepared cell suspensions to prepare calibrator samples; for samples spiked with BioBalls, use the same lot of BioBalls to prepare calibrator samples.

- 9.3.2** Calculate the percent recovery (R) for each IPR sample according to the Method 1611 Calculation Excel file using the appropriate spreadsheets “Laboratory-prepared” or “BioBalls” for samples spiked with laboratory-prepared spiking suspensions or BioBalls, respectively.
- 9.3.3** Using the percent recoveries of the four analyses, calculate the mean percent recovery and the relative standard deviation (RSD) of the recoveries. The RSD is the standard deviation divided by the mean, multiplied by 100.
- 9.3.4** Compare the mean recovery and RSD with the corresponding IPR criteria in **Table 1**, below. If the mean and RSD for recovery of enterococci meet acceptance criteria, system performance is acceptable and analysis of field samples may begin. If the mean recovery or the RSD fall outside of the required range for recovery, system performance is unacceptable. In this event, identify the problem by evaluating each step of the analytical process, media, reagents, and controls, correct the problem and repeat the IPR analyses.

Table 1. Calculated Initial Precision and Recovery (IPR) Acceptance Criteria at 550 CFU

Spike Type	IPR Mean Recovery (%)	IPR RSD	OPR Recovery (%)
Lab-prepared	Detect – 2684%	132	Detect – 2846%
BioBall®	Detect – 286%	112	Detect – 325%

- 9.4** Ongoing precision and recovery (OPR) — To demonstrate ongoing control of the analytical system, the laboratory should routinely process and analyze spiked PBS samples. The laboratory should analyze one OPR sample after every 20 field and matrix spike samples or one per week that samples are analyzed, whichever occurs more frequently. The OPR analysis is performed as follows:
- 9.4.1** Prepare one, 100 mL sample of PBS and spike with *E. faecalis* ATCC® # 29212™ according to the spiking procedure in Section 13. Spiking with laboratory-prepared suspensions is described in Section 13.4 and spiking with BioBalls is described in Section 13.7. Filter and process the OPR sample according to the procedures in Section 11 and calculate the target sequences in the samples according to Section 12.
- 9.4.2** Calculate the percent recovery (R) for the OPR sample according to the Method 1611 Calculation Excel file using the appropriate spreadsheets “Laboratory-prepared” or “BioBalls” for samples spiked with laboratory-prepared spiking suspensions or BioBalls, respectively.
- 9.4.3** Compare the OPR result (percent recovery) with the corresponding OPR recovery criteria in Table 1, above. If the OPR result meets the acceptance criteria for recovery, method performance is acceptable and analysis of field samples may continue. If the OPR result falls outside of the acceptance criteria, system performance is unacceptable. In this

event, identify the problem by evaluating each step of the analytical process, reagents, and controls, correct the problem and repeat the OPR analysis.

- 9.4.4** As part of the laboratory QA program, results for OPR and IPR samples should be charted and updated records maintained in order to monitor ongoing method performance. The laboratory should also develop a statement of accuracy for Method 1611 by calculating the average percent recovery (R) and the standard deviation of the percent recovery (s_r). Express the accuracy as a recovery interval from $R - 2s_r$ to $R + 2s_r$.
- 9.5** Matrix spikes (MS) — MS analysis are performed to determine the effect of a particular matrix on enterococci recoveries. The laboratory should analyze one MS sample when recreational water samples are first received from a source from which the laboratory has not previously analyzed samples. Subsequently, 5% of field samples (1 per 20) from a given source should include a MS sample. MS samples must be accompanied by the analysis of an unspiked field sample sequentially collected from the same sampling site. When possible, MS analyses should also be accompanied by an OPR sample (Section 9.4), using the same spiking procedure (laboratory-prepared spiking suspension or BioBalls). The MS analysis is performed as follows:
- 9.5.1** Prepare two, 100 mL field samples that were sequentially collected from the same site. One sample will remain unspiked and will be analyzed to determine the background or ambient concentration of enterococci for calculating MS recoveries (Section 9.5.3). The other sample will serve as the MS sample and will be spiked with *E. faecalis* ATCC® # 29212™ according to the spiking procedure in Section 13.
- 9.5.2** Spike the MS sample volume(s) with a laboratory-prepared suspension as described in Section 13.4 or with BioBalls as described in Section 13.7. Immediately filter and analyze both the unspiked and spiked field samples according to the procedures in Section 11.
- 9.5.3** For the MS sample, calculate the number of target sequences according to Section 12 and adjust based on any background enterococci observed in the unspiked matrix sample.
- 9.5.4** Calculate the percent recovery (R) for the MS sample (adjusted based on ambient enterococci in the unspiked sample) according to the Method 1611 Calculation Excel file using the appropriate spreadsheets “Laboratory-prepared” or “BioBalls” for samples spiked with laboratory-prepared spiking suspensions or BioBalls, respectively.
- 9.5.5** Compare the MS result (percent recovery) with the appropriate method performance criteria in **Table 2**, below. If the MS recovery meets the acceptance criteria, system performance is acceptable and analysis of field samples from this disinfected wastewater source may continue. If the MS recovery is unacceptable and the OPR sample result associated with this batch of samples is acceptable, a matrix interference may be causing the poor results. If the MS recovery is unacceptable, all associated field data should be flagged.
- 9.5.6** Laboratories should record and maintain a control chart comparing MS recoveries for all matrices to batch-specific and cumulative OPR sample results analyzed using Method 1611. These comparisons should help laboratories recognize matrix effects on method recovery and may also help to recognize inconsistent or sporadic matrix effects from a particular source.

Table 2. Calculated Matrix Spike Precision and Recovery Acceptance Criteria at 550 CFU

Matrix	Spike Type	MS Recovery (%)	MS RPD ¹ (%)
MW	Lab-prepared	Detect – 1198%	160
	BioBall [®]	Detect – 1123%	191
FW	Lab-prepared	Detect – 3473%	110
	BioBall [®]	Detect – 333%	354

¹ Relative percent difference

- 9.6 Positive controls** — The laboratory should analyze positive controls to ensure that the method is performing properly. Fluorescence amplification growth curve (PCR amplification trace) with an appropriate cycle threshold (C_T) value during PCR indicates proper method performance. On an ongoing basis, the laboratory should perform positive control analyses every day that samples are analyzed. In addition, controls should be analyzed when new lots of reagents or filters are used.
- 9.6.1** Calibrator samples will serve as the positive control. Analyze as described in Section 11.0. *Note:* Calibrator samples contain the same amount of extraction buffer and starting amount of Salmon DNA as the test samples; hence *E. faecalis* calibrator sample extracts (Section 11.3) will be used as a positive control for both *Enterococcus* and SPC qPCR assays.
- 9.6.2** If the positive control fails to exhibit the appropriate fluorescence growth curve response, check and/or replace the associated reagents, and reanalyze. If positive controls still fail to exhibit the appropriate fluorescence growth curve response, prepare new calibrator samples and reanalyze (see Section 9.11).
- 9.7 No template controls** — The laboratory should analyze “No Template Controls” (NTCs) to ensure that the PCR master mix reagents are not contaminated. On an ongoing basis, the laboratory should perform NTC analyses every day that samples are analyzed. If greater than one-third of the NTC reactions for a PCR master mix exhibit true positive logarithmic amplification traces with C_T values below 45 (not from chemical degradation of probe with linear kinetics that exhibit rising baseline) or if any one NTC reaction has a C_T value lower than 35, the analyses should be repeated with new master mix working stock preparations.
- 9.8 Method blank** (water sample filtration blank) — Filter a 50 mL volume of sterile PBS before beginning the sample filtrations. Remove the funnel from the filtration unit. Using two sterile or flame-sterilized forceps, fold the filter on the base of the filtration unit and place it in an extraction tube with glass beads as described in Section 7.18. Extract as in Section 11.5. The absence of a fluorescence amplification growth curve during PCR analysis of these samples (reported as “0” on Smart Cycler[®] and “undetermined” on ABI instruments) indicates the absence of contaminant target DNA (see Data Quality Acceptance, below). **Prepare at least one method blank filter for every PCR run (e.g., minimally one method blank per 96 well plate or Smart Cycler run).**
- 9.9 Media sterility check** — The laboratory should test media sterility by incubating one uninoculated unit (tube or plate) from each batch of medium (BHIA/BHIB) as appropriate and observing for growth. Absence of growth indicates media sterility. On an ongoing basis, the laboratory should perform media sterility checks when preparing new *E. faecalis* cell stocks.
- 9.10 DNA standards and standard curves** — Purified, RNA-free and spectrophotometrically quantified *E. faecalis* genomic DNA should be prepared as described in Section 11.2. Based on reported values for its size, the weight of a single *E. faecalis* genome can be estimated to be ~3.6 fg and there are four *lsrRNA* gene copies per genome in this species (see the rRNA operon

database at <http://rrmdb.cme.msu.edu>). The concentration of *lsrRNA* gene copies per μL in the standard *E. faecalis* genomic DNA preparation can be determined from this information and from its spectrophotometrically determined total DNA concentration by the formula:

$$\text{Concentration of } lsrRNA = \frac{\text{Total DNA concentration (fg}/\mu\text{L)}}{4 \text{ } lsrRNA \text{ gene copies}}$$

A composite standard curve should be generated from multiple analyses of serial dilutions of this DNA standard using the *Enterococcus* primer and probe assay and subjected to linear regression analysis as described in Section 12.2. From that point on, it is recommended that additional standard curves be generated from triplicate analyses of these same diluted standard samples with each new lot of TaqMan[®] master mix reagents or primers and probes to demonstrate comparable performance by these new reagents. The r^2 values from regressions of these curves should ideally be 0.99 or greater. Comparable performance is assessed by their slopes and y-intercepts which should be consistent with those from the initial composite analyses (e.g., within the 95% confidence range of the average values). *Note:* The 95% confidence ranges for these parameters in the initial composite standard curve can be generated using the Regression Analysis Tool which can be accessed from the “Data Analysis” selection under the “Tools” menu in Excel. Subsequent regressions can be performed by plotting the data using the Chart Wizard in Excel and using the “add trend line” selection in the Chart menu and “display equation on chart” selection under Options to obtain slope and y-intercept values as illustrated in Section 12.2.

In the event that the slope value from a subsequent standard curve regression is outside of the acceptance range, the diluted standards should be re-analyzed. If this difference persists, new working stocks of the reagents should be prepared and the same procedure repeated. If the differences still persist, the amplification factor values used for calculations of target cell numbers, as described in Section 12, should be modified based on the new slope values. If the slopes are within acceptance range but y-intercepts are not within acceptance range of this previous average, new aliquots of serial dilutions of the DNA standard should be analyzed or prepared and analyzed as described in Section 11.2 and above.

- 9.11 Calibrator samples** — The cell concentration of each cultured *E. faecalis* stock suspension used for the preparation of calibrator sample extracts should be determined as described in Section 11.1. A minimum of nine calibrator sample extracts should initially be prepared from three different freezer-stored aliquots of these stocks as described in Section 11.3. Dilutions of each of these calibrator sample extracts equivalent to the anticipated dilutions of the test samples used for analysis (e.g., 1-, 5- and/or 25-fold) should be analyzed with the *Enterococcus* primer and probe assay. The average and standard deviation of the C_T values from these composite analyses should be determined. From that point on, a minimum of three fresh calibrator sample extracts should be prepared from an additional frozen aliquot of the same stock cell suspension at least weekly and preferably daily before analyses of each batch of test samples. Dilutions of each new calibrator sample extract equivalent to the initial composite dilutions (e.g., 1-, 5- and/or 25-fold) should be analyzed using the *Enterococcus* primer and probe assay. The average C_T value from these analyses should not be significantly different from the laboratory’s average values from analyses of the initial calibrator sample extracts from the same stock cell suspension (i.e., not greater than three standard deviations). If these results are not within this acceptance range, new calibrator extracts should be prepared from another frozen aliquot of the same stock cell suspension and analyzed in the same manner as described above. If the results are still not within the acceptance range, the reagents should be checked by the generation of a standard curve as described in Section 9.10.

9.12 Salmon DNA SPC sequence analyses — The Salmon DNA SPC is used as a DNA isolation efficiency control. While not essential, it is good practice to routinely prepare and analyze standard curves from serial dilutions of Salmon DNA working stocks in a manner similar to that described for the *Enterococcus* DNA standards in Section 9.10. At this time rRNA gene operon copy numbers per genome have not been reported in the literature for the salmon species *O. keta*. Therefore log-transformed total DNA concentration values or dilution factor values can be substituted for target sequence copy numbers as the x-axis values in these plots and regression analyses.

In general, target DNA concentrations in test samples can be calculated as described in Section 12. However, the Salmon DNA PCR assay results for each test sample's 5-fold dilution should be within 3 C_T units of the mean of the 5-fold diluted calibrator (and/or method blank) Salmon DNA sample results. Higher C_T values may indicate significant PCR inhibition or poor DNA recovery possibly due to physical, chemical, or enzymatic degradation. Repeat the *Enterococcus* and Salmon DNA PCR assays of any samples whose 5-fold dilution exhibits a Salmon DNA PCR assay C_T value greater than 3 C_T units higher than the mean of the calibrator sample results using a 5-fold higher dilution (net dilution: 25-fold) of the extracts. The *Enterococcus* PCR result from assaying the original 5-fold dilution of the sample can be accepted if its Salmon DNA assay C_T value is lower than that of the corresponding 25-fold dilution of the sample. This pattern of results is indicative of poor recovery of total DNA in the extract rather than PCR inhibition. The poor DNA recovery is compensated for by the calculation method. Contrarily, if the Salmon PCR assay C_T value of the 25-fold dilution of the sample is lower than that of the 5-fold dilution of the sample, then the *Enterococcus* PCR assay result from the 25-fold dilution of the sample is considered more accurate. However the *Enterococcus* PCR results should be reported as questionable if the Salmon DNA assay's result is still not within 3 C_T units of the mean C_T result of the 25-fold dilution of the three calibrators.

10.0 Calibration and Standardization of Method-Related Instruments

- 10.1 Check temperatures in incubators twice daily with a minimum of 4 hours between each reading to ensure operation within stated limits.
- 10.2 Check thermometers at least annually against a NIST certified thermometer or one that meets the requirements of NIST Monograph SP 250 -23. Check columns for breaks.
- 10.3 The spectrophotometer should be calibrated each day of use using OD calibration standards between 0.01 – 0.5. Follow manufacturer instructions for calibration.
- 10.4 Micropipettors should be calibrated at a minimum annually, ideally monthly, and tested for accuracy on a weekly basis. Follow manufacturer instructions for calibration.
- 10.5 Follow manufacturer instructions for calibration of real-time PCR instruments.

11.0 Procedure

Note: *E. faecalis* cell suspensions (Section 11.1) and *E. faecalis* DNA standards (Section 11.2) may be prepared in advance. Calibrator samples (Section 11.3) should be prepared at least weekly.

11.1 Preparation of *E. faecalis* cell suspensions for DNA standards and calibrator samples

- 11.1.1** Thaw an *E. faecalis* (ATCC® # 29212™) stock culture (Section 7.8) and streak for isolation on BHIA plates. Incubate plates at $37^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ for 24 ± 2 hours.
- 11.1.2** Pick an isolated colony of *E. faecalis* from BHIA plates and suspend in 1 mL of sterile PBS and vortex.
- 11.1.3** Use 10 μL of the 1 mL suspension of *E. faecalis* to inoculate a 10 mL BHIB tube. Place the inoculated tube and one uninoculated tube (sterility check) on a shaker set at 250 rpm and incubate at $37^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ for 24 ± 2 hours. *Note:* It is advisable to verify that the selected colony is *Enterococcus* as described, for example, in Section 15 of EPA Method 1600.
- 11.1.4** After incubation, centrifuge the BHIB containing *E. faecalis* for 5 minutes at $6000 \times g$.
- 11.1.5** Aspirate the supernatant and resuspend the cell pellet in 10 mL PBS.
- 11.1.6** Repeat the two previous steps twice and suspend the final *E. faecalis* pellet in 5 mL of sterile PBS. Label the tube as *E. faecalis* undiluted stock cell suspension, noting cell concentration after determination with one of the following steps (11.1.7).
- 11.1.7** Determine calibrator sample cell concentrations based on one of two the options indicated below.

- **Option 1: Spectrophotometric absorbance** – Remove three 0.1 mL aliquots of undiluted cell suspension and dilute each with 0.9 mL of PBS (10^{-1} dilution). Read absorbance at 595 nm in spectrophotometer against PBS blank (readings should range from 0.05 to 0.3 OD). Calculate cells/mL (Y) in undiluted cell suspension using the formula from the standard curve shown below where X is the average 595 nm spectrophotometer reading. *Note:* Cell concentration (spectrophotometric determination) of undiluted *E. faecalis* cell suspension should be at least $\sim 10^9$ CFU/mL in order to obtain adequate DNA yield for accurate UV spectrophotometric reading.

$$Y = (1 \times 10^9 \text{ cells / mL} \times X) / 0.19$$

- **Option 2: plating** – *Note:* PBS and BHIA plates should be prepared in advance if this option is chosen. For enumeration of the *E. faecalis* undiluted cell suspension, dilute and inoculate according to the following.
 - A) Mix the *E. faecalis* undiluted cell suspension by vigorously shaking the 5 mL tube a minimum of 25 times. Use a sterile pipette to transfer 1.0 mL of the undiluted cell suspension to 99 mL of sterile PBS, cap, and mix by vigorously shaking the bottle a minimum of 25 times. This is cell suspension dilution “A”. A 1.0 mL volume of dilution “A” is 10^{-2} mL of the original undiluted cell suspension.
 - B) Use a sterile pipette to transfer 11.0 mL of cell suspension dilution “A” to 99 mL of sterile PBS, cap, and mix by vigorously shaking the bottle a minimum of 25 times. This is cell suspension dilution “B”. A 1.0 mL volume of dilution “B” is 10^{-3} mL of the original undiluted cell suspension.
 - C) Use a sterile pipette to transfer 11.0 mL of cell suspension dilution “B” to 99 mL of sterile PBS, cap, and mix by vigorously shaking the bottle a minimum of 25 times. This is cell suspension dilution “C”. A 1.0 mL volume of dilution “C” is 10^{-4} mL of the original undiluted cell suspension.

- D) Use a sterile pipette to transfer 11.0 mL of cell suspension dilution “C” to 99 mL of sterile PBS, cap, and mix by vigorously shaking the bottle a minimum of 25 times. This is cell suspension dilution “D”. A 1.0 mL volume of dilution “D” is 10^{-5} mL of the original undiluted cell suspension.
- E) Use a sterile pipette to transfer 11.0 mL of cell suspension dilution “D” to 99 mL of sterile PBS, cap, and mix by vigorously shaking the bottle a minimum of 25 times. This is cell suspension dilution “E”. A 1.0 mL volume of dilution “E” is 10^{-6} mL of the original undiluted cell suspension.
- F) Prepare BHIA (Section 7.6). Ensure that agar surface is dry. *Note:* To ensure that the agar surface is dry prior to use, plates should be made several days in advance and stored inverted at room temperature or dried using a laminar-flow hood.
- G) Each of the following should be conducted in triplicate, resulting in the evaluation of nine spread plates:
- Pipet 0.1 mL of dilution “C” onto surface of BHIA plate [10^{-5} mL (0.00001) of the original cell suspension].
 - Pipet 0.1 mL of dilution “D” onto surface of BHIA plate [10^{-6} mL (0.000001) of the original cell suspension].
 - Pipet 0.1 mL of dilution “E” onto surface of BHIA plate [10^{-7} mL (0.0000001) of the original cell suspension].
- H) For each spread plate, use a sterile bent glass rod or spreader to distribute inoculum over surface of medium by rotating the dish by hand or on a rotating turntable.
- I) Allow inoculum to absorb into the medium completely.
- J) Invert plates and incubate at $35^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ for 24 ± 4 hours.
- K) Count and record number of colonies per plate. Refer to the equation and **Table 3** below for calculation of undiluted cell suspension concentration.

$$\text{CFU/mL}_{\text{undiluted}} = \frac{\text{CFU}_1 + \text{CFU}_2 + \dots + \text{CFU}_n}{V_1 + V_2 + \dots + V_n}$$

Where:

$\text{CFU/mL}_{\text{undiluted}}$ = *E. faecalis* CFU/mL in undiluted cell suspension

CFU = number of colony forming units from BHIA plates yielding counts within the ideal range of 30 to 300 CFU per plate

V = volume of undiluted sample in each BHIA plate yielding counts within the ideal range of 30 to 300 CFU per plate

n = number of plates with counts within the ideal range

Table 3. Example Calculations of *E. faecalis* Undiluted Cell Suspension Concentration

Examples	CFU / plate (triplicate analyses) from BHIA plates			<i>E. faecalis</i> CFU / mL in undiluted suspension ^a
	10 ⁻⁵ mL plates	10 ⁻⁶ mL plates	10 ⁻⁷ mL plates	
Example 1	275, 250, 301	30, 10, 5	0, 0, 0	$(275+250+30) / (10^{-5}+10^{-5}+10^{-6}) = 555 / (2.1 \times 10^{-5}) = 26,428,571 = 2.6 \times 10^7 \text{ CFU / mL}$
Example 2	TNTC ^b , TNTC, TNTC	TNTC, 299, TNTC	12, 109, 32	$(299+109+32) / (10^{-6}+10^{-7}+10^{-7}) = 440 / (1.2 \times 10^{-6}) = 366,666,667 = 3.7 \times 10^8 \text{ CFU / mL}$

^a Cell concentration is calculated using all plates yielding counts within the ideal range of 30 to 300 CFU per plate

^b Too numerous to count

11.1.8 Divide remainder of undiluted cell suspension (approximately 5 mL) into 6 × 0.5 mL aliquots for DNA standard preparations and 100 – 200 × 0.01 mL (10 µL) aliquots for calibrator samples and freeze at -20°C. *Note:* Cell suspension should be stirred while aliquoting. It is also recommended that separate micropipettor tips be used for each 10 µL aliquot transfer and that the volumes in each tip be checked visually for consistency.

11.2 Preparation of *E. faecalis* genomic DNA standards

11.2.1 Remove two 0.5 mL undiluted *E. faecalis* cell suspensions (Section 11.1.8) from freezer and thaw completely.

11.2.2 Transfer cell suspensions to extraction tubes with glass beads.

11.2.3 Tightly close the tubes, making sure that the O-rings are seated properly.

11.2.4 Place the tubes in bead beater and shake for 60 seconds at the maximum rate (5000 rpm).

11.2.5 Remove the tubes from the mini bead beater and centrifuge at 12,000 × g for one minute to pellet the glass beads and debris.

11.2.6 Using a 200 µL micropipettor, transfer 350 µL of the supernatants to sterile 1.7 mL microcentrifuge tubes. Recover supernatants without disrupting the glass beads at the tube bottom.

11.2.7 Centrifuge crude supernatants at 12,000 × g for 5 minutes and transfer 300 µL of clarified supernatant to clean, labeled 1.7 mL low retention microcentrifuge tubes, taking care not to disturb the pellet.

11.2.8 Add 1 µL of 5 µg/µL RNase A solution to each clarified supernatant, mix by vortexing and incubate at 37°C for 1 hour.

Note: Steps 11.2.9 - 11.2.15 may be substituted with an optional method if a DNA purification procedure is chosen other than the DNA-EZ purification kit. In such a case, manufacturer instructions should be followed rather than these steps. Continue onward from step 11.2.16.

11.2.9 Add 0.6 mL of binding buffer solution from a DNA-EZ purification kit to each of the RNase A-treated extracts and mix by vortexing. *Note:* In general, a minimum concentration of 5 × 10⁸ cells is required for this step.

- 11.2.10** If using the DNA-EZ purification kit, perform the following steps. Insert one DNAsure™ column from the DNA-EZ purification kit into a collection tube (provided with kit) for each of the two extracts. Transfer the extract and binding buffer mixtures from Section 11.2.9 to a DNAsure™ column and collection tube assembly and centrifuge for 1 minute at $12,000 \times g$.
- 11.2.11** Transfer each of the DNAsure™ columns to new collection tubes. Discard previous collection tubes and collected liquid.
- 11.2.12** Add 500 μL EZ-Wash Buffer from the DNA-EZ purification kit to each of the DNAsure™ columns and centrifuge at $12,000 \times g$ for 1 minute. Discard the liquid in the collection tube.
- 11.2.13** Repeat step 11.2.12.
- 11.2.14** Transfer each of the DNAsure™ columns to a clean, labeled 1.7 mL low retention microcentrifuge tube and add 50 μL of DNA elution buffer to each column. Centrifuge for 30 seconds at $12,000 \times g$. Repeat this procedure again to obtain a total DNA eluate volume of $\sim 100 \mu\text{L}$ from each column.
- 11.2.15** Pool the two eluates to make a total volume of approximately 200 μL .
- 11.2.16** Transfer the entire purified DNA eluate volume from each column to a clean and sterile microcuvette for UV spectrophotometer and read absorbance at 260 and 280 nM. (*Note:* The cuvette should be blanked with DNA elution buffer before reading sample). If necessary, the sample may be diluted with elution buffer to reach the minimum volume that can be accurately read by the spectrophotometer (see manufacturer's recommendation), however, this may reduce the DNA concentration to a level that cannot be accurately read by the spectrophotometer. If available, readings can be taken of 2 μL aliquots of the sample with a NanoDrop™ Spectrophotometer.
- 11.2.17** Sample is acceptable as a standard if ratio of $\text{OD}_{260}/\text{OD}_{280}$ readings is ≥ 1.75 .
- 11.2.18** Record UV spectrophotometer results.
- 11.2.19** Calculate total DNA concentration in sample by formula:
- $$\text{OD}_{260} \text{ reading} \times 50 \text{ ng}/\mu\text{L DNA}/\text{OD}_{260}$$
- 11.2.20** Transfer sample back to labeled 1.7 mL non-retentive microcentrifuge tube and store at -20°C .
- 11.2.21** Using total DNA concentration (Section 11.2.19), calculate *E. faecalis* PCR target sequence concentration (TSC) in purified extract (Section 9.10).

Example:

$$\begin{aligned} \text{OD}_{260} = 0.7271 \quad \text{Total DNA concentration} &= \text{OD}_{260} \text{ Reading} \times 50 \text{ ng}/\mu\text{L} \\ 0.7271 \times 50 = 36.36 \text{ ng}/\mu\text{L} &= 36.36 \times 10^6 \text{ fg}/\mu\text{L} = 3.636 \times 10^7 \text{ fg}/\mu\text{L} \\ \frac{3.636 \times 10^7 \text{ fg}/\mu\text{L}}{3.6 \text{ fg/genome}} \times \frac{4 \text{ lsrRNA gene copies}}{\text{genome}} &= 4.04 \times 10^7 \text{ gene copies}/\mu\text{L} \end{aligned}$$

- 11.2.22** Record UV spectrophotometer results and all calculations.

- 11.2.23** In appropriately labeled 1.7 mL low retention microcentrifuge tubes, perform serial dilutions using AE buffer to prepare DNA standards of 4×10^3 TSC/5 μ L, 4×10^2 TSC/5 μ L, 100 TSC/5 μ L and 40 TSC/5 μ L (see below).
- Note:* Perform initial dilution of purified and quantified DNA as required to achieve a concentration of 2×10^8 TSC/5 μ L or 2×10^7 TSC/5 μ L (as appropriate) prior to initiating serial dilutions.
- 11.2.23.1** Perform 10-fold serial dilutions of the 2×10^8 or 10^7 TSC/5 μ L stock (10 μ L : 90 μ L) with AE buffer to achieve 2×10^7 TSC/5 μ L; 2×10^6 TSC/5 μ L; and 2×10^5 TSC/5 μ L
- 11.2.23.2** Perform 5-fold dilution of 2×10^5 TSC/5 μ L stock (100 μ L : 400 μ L) with AE buffer to achieve 4×10^4 TSC/5 μ L stock
- 11.2.23.3** Perform successive 10-fold serial dilutions of the 4×10^4 TSC/5 μ L stock (100 μ L : 900 μ L) to achieve 4×10^3 TSC/5 μ L; 4×10^2 TSC/5 μ L stocks
- 11.2.23.4** Perform a 4-fold dilution of 4×10^2 TSC/5 μ L stock (100 μ L : 300 μ L) to achieve 100 TSC/5 μ L stock
- 11.2.23.5** Perform a final 2.5-fold dilution of the 100 TSC/5 μ L stock (100 μ L : 150 μ L) to achieve 40 TSC/5 μ L stock
- Note:* It is recommended that DNA standards be stored and manipulated in a different room than that used for routine sample processing in order to minimize the potential for laboratory contamination.
- 11.2.24** Store DNA standards in refrigerator at 4°C. For long term storage, freeze aliquots at -20°C or -80°C
- 11.2.25** Generate DNA standard curves using the 40, 100, 400, and 4000 TSC/5 μ L dilutions (3 replicates each) according to Section 11.7 (ABI) or 11.8 (Cepheid), as appropriate.
- 11.2.26** qPCR analyses of these diluted standards using the *Enterococcus* primer and probe assay should initially be performed at least six separate times in triplicate. From that point on, it is recommended that additional standard curves be generated from triplicate analyses of these same diluted standard samples with each new lot of TaqMan® master mix reagents or primers and probes to demonstrate comparable performance by these new reagents.

11.3 Extraction of *E. faecalis* calibrator samples

- 11.3.1** A minimum of three calibrator extracts should be prepared during each week of analysis.
- Note:* To prevent contamination of water sample filtrates and filter blanks, this procedure should be performed at a different time, and, if possible, in a different work station than the procedures in Sections 11.1 and 11.2 above and Section 11.5 below.
- 11.3.2** Remove one tube containing a 10 μ L aliquot of *E. faecalis* undiluted stock cell suspension (Section 11.1.8) from the freezer and allow to thaw completely on ice.
- Note:* If using BioBalls for calibrators add a single BioBall® to each of 3, 1.0 mL sterile PBS blanks, filter (Section 11.4), and extract according to Section 11.5.
- 11.3.3** While cell stock is thawing, using sterile (or flame-sterilized) forceps, place one polycarbonate filter (Section 6.14) in an extraction tube with glass beads. Prepare one filter for each sample to be extracted in this manner.

- 11.3.4** Dispense 590 μL of Salmon DNA/extraction buffer (Section 7.12) into three extraction tubes with glass beads and filters. Prepare one tube for each of the three calibrator samples to be extracted in this manner. Label tubes appropriately.
- 11.3.5** When *E. faecalis* suspension has thawed, transfer 990 μL AE buffer (Section 7.11) to the 10 μL *E. faecalis* stock cell tube and mix thoroughly by vortexing. Pulse microcentrifuge tube briefly (1 – 2 sec.) to coalesce droplets in tube.
- 11.3.6** Immediately after vortexing the *E. faecalis* suspension, spot 10 μL onto the polycarbonate filter in the extraction tube from step 11.3.4.
Note: For the blank extraction tube, spot 10 μL AE buffer onto the polycarbonate filter
- 11.3.7** Tightly close the tube, making sure that the O-ring is seated properly and label tubes appropriately.
- 11.3.8** Repeat Sections 11.3.6 and 11.3.7 for the other two filters to prepare three calibrator samples with *E. faecalis*.
- 11.3.9** Place the tubes in the mini bead beater and shake for 60 seconds at the maximum rate (5000 rpm).
- 11.3.10** Remove the tubes from the mini bead beater and centrifuge at $12,000 \times g$ for one minute to pellet the glass beads and debris.
- 11.3.11** Using a 200 μL micropipettor, transfer the crude supernatant to the corresponding labeled sterile 1.7 mL microcentrifuge tube. Transfer 400 μL of supernatant without disrupting the debris pellet or glass beads at the tube bottom. *Note:* The filter will normally remain intact during the bead beating and centrifugation process. Generally, 400 μL of supernatant can be easily collected. Collect an absolute minimum of 100 μL of supernatant. Record volume of supernatant collected.
- 11.3.12** Centrifuge at $12,000 \times g$ for 5 minutes and transfer clarified supernatant to a clean, labeled 1.7 mL tube, taking care not to disturb the pellet. *Note:* Cell pellet may not be visible in calibrator samples.
- 11.3.13** Label the tubes as undiluted or 1X *E. faecalis* calibrator extracts. Label additional 1.7 mL tubes for 5- and 25-fold dilutions. In appropriately labeled tubes, using a micropipettor, add a 50 μL aliquot of each 1X *E. faecalis* calibrator extracts and dilute each with 200 μL AE buffer (Section 7.11) to make 5-fold dilutions. In appropriately labeled tubes using a micropipettor, add a 50 μL aliquot of each 5-fold dilution and dilute each with 200 μL AE buffer to make 25-fold dilutions. Store all diluted and undiluted extracts in refrigerator.
- 11.3.14** If the extracts are not analyzed immediately, refrigerate.
- 11.4** Water sample filtration
- Note:* It is required that minimally one water sample filtration blank (method blank) be analyzed for every qPCR run of water samples (Section 9.8)
- 11.4.1** Place a polycarbonate filter (Section 6.14) on the filter base (shiny side "up" facing you), and attach the funnel to the base so that the membrane filter is now held between the funnel and the base.
- 11.4.2** Shake the sample bottle vigorously 25 times to distribute the bacteria uniformly, and measure 100 mL of sample into the funnel.
- 11.4.3** Filter 100 mL of water sample. After filtering the sample, rinse the sides of the funnel with 20 - 30 mL of sterile PBS (Section 7.4) and continue filtration until all liquid has

been pulled through the filter. Turn off the vacuum and remove the funnel from the filter base.

11.4.4 Label an extraction tube containing glass beads (Section 6.4) to identify water sample. Leaving the filter on the filtration unit base, fold filter into a cylinder with the sample side facing inward, being careful to handle the filter only on the edges, where the filter has not been exposed to the sample. Insert the rolled filter into the labeled extraction tube with glass beads. Prepare one filter for each sample filtered in this manner.

11.4.5 Cap the extraction tube. Refrigerate.

11.5 DNA extraction of water sample filter extracts and method blanks

11.5.1 Using a 1000 μL micropipettor, dispense 590 μL of the Salmon DNA/extraction buffer (Section 7.12) into each labeled extraction tube with glass beads containing water sample or method blank filters from Section 11.3.4. Extract the method blank last.

11.5.2 Tightly close the tubes, making sure that the O-ring is seated properly.

11.5.3 Place the tubes in the mini bead beater and shake for 60 seconds at the maximum rate (5000 rpm).

11.5.4 Remove the tubes from the mini bead beater and centrifuge at $12,000 \times g$ for 1 minute to pellet the glass beads and debris. *Note:* To help prevent contamination, a new pair of gloves may be donned for this step, and steps 11.5.6 and 11.5.7.

11.5.5 Using the 200 μL micropipettor, transfer 400 μL of the supernatant to a corresponding labeled sterile 1.7 mL microcentrifuge tube, taking care not to aspirate glass beads or sample debris (pellet). *Note:* The filter will normally remain intact during the bead beating and centrifugation process. Generally, 400 μL of supernatant can easily be collected. Collect an absolute minimum of 100 μL of supernatant. Recover the method blank supernatant last.

11.5.6 Centrifuge crude supernatant from Section 11.5.5 for 5 minutes at $12,000 \times g$. Transfer 350 μL of the clarified supernatant to another 1.7 mL tube, taking care not to disturb the pellet. *Note:* Pellet may not be visible in water samples. Recover the method blank supernatant last.

11.5.7 Label the tubes from Section 11.5.6 as undiluted or 1X water sample extracts with sample identification. These are the water sample filter extracts. Also label tubes for method blanks. Label additional 1.7 mL tubes for 5-fold dilutions. In appropriately labeled tubes, using a micropipettor, add a 50 μL aliquot of each 1X water sample extract and dilute each with 200 μL AE buffer (Section 7.11) to make 5-fold dilutions. Dilute the method blank supernatant last.

11.5.8 Optional: For samples where the concentration of target DNA is anticipated to be high, an additional 25-fold dilution can be done. In appropriately labeled tubes using a micropipettor, add a 50 μL aliquot of each 5-fold dilution and dilute each with 200 μL of AE buffer to make 25-fold dilutions. Dilute the method blank supernatant last.

11.5.9 Store all diluted and undiluted extracts in refrigerator.

11.5.10 *Note:* Use of 5-fold diluted samples for analysis is currently recommended if only one dilution can be analyzed. Analyses of undiluted water sample extracts have been observed to cause a significantly higher incidence of PCR inhibition while analyses of 25-fold dilutions may unnecessarily sacrifice sensitivity.

11.5.11 Refrigerate the extracts if they are not analyzed immediately. Water sample filter extracts should be analyzed the same day that they are prepared.

11.6 Preparation of qPCR assay mix

- 11.6.1** To minimize environmental DNA contamination, routinely treat all work surfaces with a 10% bleach solution, allowing the bleach to contact the work surface for a minimum of 15 minutes prior to rinsing with sterile water. If available, turn on UV light for 15 minutes. After decontamination, discard gloves and replace with a new pair.
- 11.6.2** Remove primers and probe stocks from the freezer and verify that they have been diluted to solutions of 500 μ M primer and 100 μ M probe.
- 11.6.3** Prepare working stocks of *Enterococcus*, and Salmon DNA (Sketa 22) primer/probe mixes by adding 10 μ L of each *Enterococcus* or Salmon DNA (Sketa 22) primer stock and 4 μ L of respective probe stock to 676 μ L of PCR-grade water, and vortex. Pulse centrifuge to create a pellet. Use a micropipettor with aerosol barrier tips for all liquid transfers. Transfer aliquots of working stocks for single day use to separate tubes and store at 4°C.
- 11.6.4** Using a micropipettor, prepare assay mix of the *Enterococcus* and Salmon DNA (Sketa 22) reactions in separate, sterile, labeled 1.7 mL microcentrifuge tubes as described in **Table 4**.

Table 4. PCR Assay Mix Composition

Reagent	Volume/Sample (multiply by # samples to be analyzed per day)
Sterile H ₂ O	1.5 μ L
BSA	2.5 μ L
TaqMan [®] master mix	12.5 μ L
Primer/probe working stock solution	3.5 μ L

Note: This will give a final concentration of 1 μ M of each primer and 80 nM of probe in the reactions. Prepare sufficient quantity of assay mix for the number of samples to be analyzed per day including calibrators and negative controls plus at least two extra samples. Prepare assay mixes each day before handling DNA samples.

- 11.6.5** Vortex the assay mix working stocks; then pulse microcentrifuge to coalesce droplets. Return the primer/probe working stocks and other reagents to the refrigerator.

11.7 ABI StepOnePlus™, ABI 7900, and ABI 7500 (all non-Fast mode) qPCR assay preparation (Reference 17.1)

Transfer 20 μ L of either the master mix containing *Enterococcus* primers and probe or the master mix containing the Salmon DNA primers and probe to the wells of a 96 well PCR reaction tray equal to number of samples to be analyzed including calibrator and negative control samples. (*Note:* The same tip can be used for pipetting multiple aliquots of the same assay mix as long as no contact is made with anything else).

Example: For the analysis of 18 recreational water samples, 47 wells will require the addition of assay mix with *Enterococcus* primers and probe as follows: 18 samples, two replicates each (36), 3 method blanks, one replicate each (3), 3 calibrators, 2 replicates each (6) and 1 no template control (NTC), 2 replicates (2) = 47 wells. In addition, 47 wells will require the addition of assay mix with Salmon DNA assay primers and probe as follows: 18 samples, two replicates each (36),

3 method blanks, one replicate each (3), 3 calibrators, 2 replicates each (6) and 1 NTC, 2 replicates (2) = 47 wells. Thus a total of 94 wells are used for 18 recreational water samples.

- 11.7.1** Transfer 20 μL of either the master mix containing *Enterococcus* primers and probe or the master mix containing the Salmon DNA primers and probe to wells of a 96 well PCR reaction tray equal to number of samples to be analyzed including calibrator and negative control samples. Pipet into the center of the wells, taking care to not touch the well walls with the pipet tip. (*Note:* The same tip can be used for pipetting multiple aliquots of the same assay mix as long as it doesn't make contact with anything else).
- 11.7.2** When all wells are loaded transfer to the PCR preparation station used for handling DNA samples (Section 6.1). *Note:* All aliquoting of assay mixes to reaction trays must be performed each day before handling of DNA samples.
- 11.7.3** DNA standards – Transfer 5 μL each concentration of the undiluted DNA extracts of (Section 11.2.24) to separate wells of the PCR reaction tray containing *Enterococcus* assay mix. *Note:* Record positions of each concentration. See Section 11.2.26 regarding the frequency of DNA standard analyses.
- 11.7.4** Water samples – Transfer 5 μL each of the diluted (or undiluted) DNA extracts of method blanks and water samples (Section 11.5.7), and corresponding dilutions of calibrator samples (Section 11.3.13), to separate wells of the PCR reaction tray containing *Enterococcus* assay mix. *Note:* Record positions of each sample.
- 11.7.5** Transfer 5 μL each of the diluted (or undiluted) DNA extracts of method blanks and water samples (Section 11.5.7), and corresponding diluted calibrator samples (Section 11.3.13) to separate wells of the PCR reaction tray containing Salmon DNA assay mix. Record positions of each sample.
- 11.7.6** Transfer 5 μL aliquots of AE buffer to wells of PCR reaction tray containing *Enterococcus* master mix that are designated as NTCs. Record positions of these samples.
- 11.7.7** Tightly cap wells of PCR reaction tray containing samples or cover tray and seal tightly with optical adhesive. *Note:* 96 well plates should be freshly prepared just prior to analyses. Do not prepare and refrigerate multiple plates prior to analyses, as signal degradation may occur.
- 11.7.8** Run reactions in ABI StepOnePlus™, ABI 7900, or ABI 7500 (all in non-Fast mode) Real-Time PCR Systems. For platform-specific operation see Appendix A. *Note:* The ABI 7500 Fast may be used but it should be run in the 9600 emulation mode.
- 11.8** Smart Cycler® qPCR assay preparation
- 11.8.1** Label 25 μL Smart Cycler® tubes with sample identifiers and assay mix type (see Section 11.8.8 for examples) or order tubes in rack by sample number and label rack with assay mix type. It is recommended that the unloaded open Smart Cycler® tubes be irradiated under UV light in a PCR cabinet for 15 minutes. Using a micropipettor, add 20 μL of the *Enterococcus* assay mix (Section 11.6.4) to labeled tubes. Avoid generating air bubbles, as they may interfere with subsequent movement of the liquid into the lower reaction chamber. The same tip can be used for pipetting multiple aliquots of the same assay mix as long as it doesn't make contact with anything else. Repeat procedure for Salmon DNA (Sketa 22) assay mix.
- 11.8.2** Add 5 μL of AE buffer to NTC tubes and close tubes tightly.

- 11.8.3** Close the other PCR tubes loosely and transfer to refrigerator or directly to the PCR preparation station used for handling DNA samples (Section 6.1). *Note:* All aliquoting of assay mixes to reaction tubes must be performed each day before handling of DNA samples.
- 11.8.4** DNA standards – Transfer 5 µL of each concentration of the undiluted DNA extracts (Section 11.2.24), to tubes containing *Enterococcus* mix. Close each tube tightly after adding sample. Label the tube tops as appropriate. See Section 11.2.26 regarding the frequency of DNA standard analyses.
- 11.8.5** Water samples – Transfer 5 µL each of the diluted (or undiluted) DNA extracts of method blanks and water samples (Section 11.5.7), and then corresponding dilutions of calibrator samples (Section 11.3.13) to tubes containing *Enterococcus* and Salmon DNA (Sketa 22) mixes. Close each tube tightly after adding sample. Load the method blank last. Label the tube tops as appropriate.
- 11.8.6** When all Smart Cycler® tubes have been loaded, place them in a Smart Cycler® centrifuge, and spin for 2 – 4 seconds.
- 11.8.7** Inspect each tube to verify that the sample has properly filled the lower reaction chamber. A small concave meniscus may be visible at the top of the lower chamber, but no air bubbles should be present. (If the lower chamber has not been properly filled, carefully open and reclose the tube, and re-centrifuge). Transfer the tubes to the thermocycler.
- 11.8.8** For platform-specific operation see Appendix B.
- 11.8.9** Suggested sample analysis sequence for Smart Cycler®
- Example:* For analyses on a single 16 position Smart Cycler®, calibrator samples and water samples will need to be analyzed in separate runs and a maximum of 6 water samples (or 2 replicates of 3 samples) can be analyzed per run, as described in **Tables 5 and 6**, below.

Table 5. Calibrator PCR Run - 14 Samples

Sample Description ¹	Quantity	PCR Assay Master Mix
3 Calibrators x 2 replicates (1-, 5-, or 25-fold dilution)	6	<i>Enterococcus</i>
3 Calibrators x 2 replicates (1-, 5-, or 25-fold dilution)	6	Salmon DNA
No template controls (reagent blanks)	2	<i>Enterococcus</i>

¹ Diluted equivalently to the water samples

Table 6. Water Sample PCR Run - 14 Samples

Sample Description ¹	Quantity	PCR Assay Master Mix
Water samples (1-, 5-, or 25-fold dilution)	6	<i>Enterococcus</i>
Method blank (1-, 5-, or 25-fold dilution)	1	<i>Enterococcus</i>
Water samples (1-, 5-, or 25-fold dilution)	6	Salmon DNA
Method blank (1- 5-, or 25-fold dilution)	1	Salmon DNA

¹ Use of 5-fold diluted samples for analysis is currently recommended if only one dilution can be analyzed. Analyses of undiluted water sample extracts have been observed to cause a significantly higher incidence of PCR inhibition while 25-fold dilutions analyses may unnecessarily sacrifice sensitivity.

12.0 Data Analysis and Calculations

- 12.1 **Overview:** This section describes a method for determining the ratio of the target sequence quantities recovered from a test (water filtrate) sample compared to those recovered from identically extracted calibrator samples using an arithmetic formula, referred to as the $\Delta\Delta C_T$ method. The $\Delta\Delta C_T$ relative quantitation method also normalizes these ratios for differences in total DNA recovery from the test and calibrator samples using qPCR analysis C_T values for a reference sequence provided by the SPC DNA. These ratios are converted to absolute measurements of total target sequence quantities recovered from the test and calibrator sample extracts and these ratios are converted to CCEs recovered from the test samples. The complete procedure for determining target sequence quantities and CCEs in water samples is detailed below.
- 12.2 **Generation of C_T value vs. target sequence number standard curve:** Three replicate serial dilutions of a DNA standard, prepared as described in Section 11.2 should be prepared to give concentrations of 4×10^3 , 4×10^2 , 1×10^2 , and 4×10^1 lsrRNA gene sequences per 5 μL (the standard sample volume added to the PCR reactions) and the replicates of each dilution pooled. *Note:* A procedure for the determinations of target sequence concentrations in the DNA standard is also provided in Section 9.10.

Aliquots of each of these dilutions should be frozen in low retention microcentrifuge tubes. Thawed aliquots should be stored at 4° C and can be reused for repeated qPCR analyses. qPCR analyses of these diluted standards using the *Enterococcus* primer and probe assay should initially be performed at least six separate times in triplicate. C_T values from these composite analyses should be subjected to regression analysis against the log₁₀-transformed target sequence numbers per reaction as described in Section 9.10 with example results illustrated in **Figure 1**.

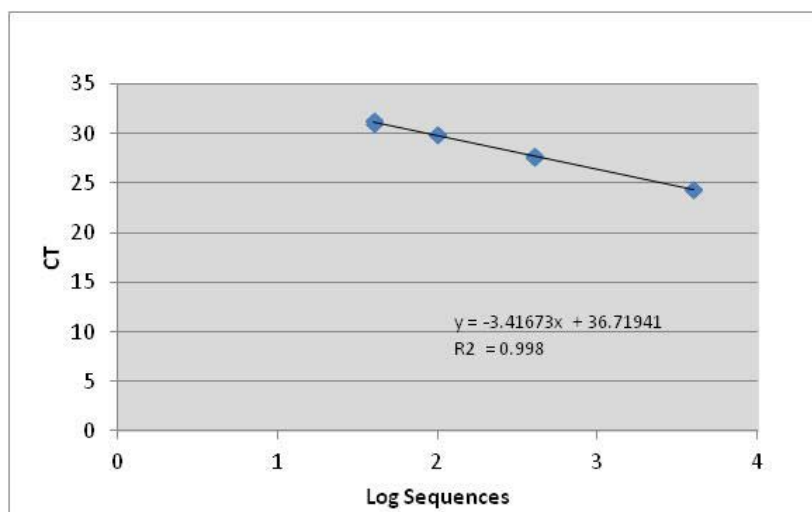


Figure 1: Example plot and regression analysis of qPCR analysis C_T values vs. log target sequences per reaction.

AF used for subsequent comparative C_T calculations (Section 12.4) can be calculated from the slope value of this curve by the formula $AF = 10^{(1 / (-)\text{slope value})}$. An example calculation using the slope value from the example regression is shown below:

$$AF = 10^{(1 / 3.41673)} = 1.96$$

- 12.3 **Calculation of average target sequence recovery in calibrator sample extracts:** A minimum of nine calibrator sample extracts should initially be prepared from at least three different freezer-

stored aliquots of each cultured *E. faecalis* stock suspension that is prepared as described in Section 11.1. Dilutions of each of these calibrator sample extracts equivalent to the anticipated dilutions of the test samples used for analysis (e.g., 1-, 5- and/or 25-fold) should be analyzed with the *Enterococcus* primer and probe assay. The average C_T value from these analyses should be interpolated on the standard curve generated from the DNA standard (Section 12.2) to determine the average number of target sequences per 5 μL of extract used in the reactions. An example calculation using an average calibrator extract C_T value of 24.31 and standard curve from Figure 1 is shown below:

$$\text{Average calibrator target sequences}/5 \mu\text{L extract} = 10^{[(24.31-36.7194) / -3.4167]} = 4285.0744$$

Note: Four places should be kept from this calculation for the following calculation (i.e., 4285.0744). Dividing this value by 5 gives the average calibrator target sequences μL extract which can be multiplied by the total volume of the extract at the applicable dilution level (e.g., 600 μL of original extract volume \times 5 = 3000 μL for a 5-fold diluted sample) to determine the average total quantity of target sequences recovered in the calibrator sample extracts. An example of this calculation using the average calibrator target sequences/reaction value determined immediately above is shown below:

$$(4285.0744/5) \times 3000 = 2571045 \text{ target sequences} / \text{total extract}$$

12.3.1 Calculation of average target sequence recovery per *Enterococcus* cell in calibrator sample extracts (optional): In previous studies, measurements of recreational water quality by the qPCR method have been reported as *Enterococcus* calibrator cell equivalents (Reference 17.4).

12.3.2 A prerequisite for making such comparisons is to determine that the ratio of the numbers of target sequences recovered in calibrator sample extracts to the numbers of *Enterococcus* cells added to these samples is consistent in different studies. *Example:* The quantity of lab prepared cells added to each calibrator sample is ~100,000. Dividing the average target sequences recovered per calibrator extract (determined as described in Section 12.3) by this number provides the ratio of target sequence numbers to cell numbers. An example of this calculation using the average target sequences/calibrator extract value determined in Section 12.3 is shown below:

$$\text{Ratio of target sequence numbers to cell numbers} = 2571045/100,000 = 25.71$$

12.4 Calculation of CCEs in test samples: A minimum of three fresh calibrator samples should be extracted and analyzed at least on a weekly basis and preferably on a daily basis in association with each batch of water sample filtrates. QC analysis of the results from these calibrator extracts should be performed as described in Section 9.11. C_T values from the *Enterococcus* and salmon DNA SPC qPCR assays for both the calibrator and test samples are used in the $\Delta\Delta C_T$ method to determine the ratios of target sequences in the test and calibrator sample extracts and these ratios are converted to CCEs recovered from the test samples as specified below and illustrated in **Table 5**.

12.4.1 Subtract the SPC assay C_T value (C_T , SPC) from the target assay C_T value (C_T , target) for each calibrator sample extract to obtain ΔC_T value and calculate the average ΔC_T value for these calibrator samples.

12.4.2 Subtract the SPC assay C_T value (C_T , SPC) from the target assay C_T value (C_T , target) for each water sample filtrate extract to obtain ΔC_T values for each of these test samples. *Note:* If multiple analyses are performed on these samples, calculate the average ΔC_T value.

- 12.4.3** Subtract the average ΔC_T value for the calibrator samples from the ΔC_T value (or average ΔC_T value) for each of the test samples to obtain $\Delta\Delta C_T$ values.
- 12.4.4** Calculate the ratio of the target sequences in the test and calibrator samples using the formula: $AF^{(-\Delta\Delta C_T)}$, where AF = amplification factor of the target organism qPCR assay, determined as described in Section 12.2.
- 12.4.5** Multiply the ratio of the target sequences in the test and calibrator samples by the estimated count (e.g., CFU) determined in Section 11.1.7 to determine the CCEs for each of the test samples. *Note:* This calculation can be applied without modification to the analyses of diluted extracts if both the test sample and calibrator extracts are equally diluted and equal volumes of diluted extracts are analyzed.

Table 5. Example Calculations (AF = 1.96)

Estimated Cell Count ¹	Sample Type	$C_{T,target}$	$C_{T,SPC}$	ΔC_T	$\Delta\Delta C_T$	Ratio calib/test $1.96^{-\Delta\Delta C_T}$	CCEs in Test Sample Extract ($1.96^{-\Delta\Delta C_T} \times$ Est cell count)
1200000	Calibrator	25.71	34.13	-8.42	----		----
	Test	33.74	25.94	7.8	7.59	0.006	$0.006 \times 1200000 = 7264.74$

¹ Estimated cell count is based on plating results taking into account the dilution factor (100) and the volume of the dilution (0.1 mL) added to the calibrator sample

- 12.4.6** The geometric mean (optional) of the CCEs and associated coefficients of variation in multiple water samples can be determined from individual sample C_T values using the following procedure:
- 12.4.6.1** Use ΔC_T value for each individual water sample extract and the mean calibrator ΔC_T value to calculate the CCEs in each water sample extract, as described above.
 - 12.4.6.2** Calculate the log₁₀ of the CCEs in each water sample (log N)
 - 12.4.6.3** Calculate the mean (M) and standard deviation (S) from the values of log N obtained in the previous step for all of the water sample extracts.
 - 12.4.6.4** Calculate the geometric mean as 10^M .
 - 12.4.6.5** The implied coefficient of variation (CV) is calculated, based on the log normal distribution, as the square root of $10^{V/0.434} - 1$, where $V = S^2$.
- 12.5 Reporting Results:** Where possible, duplicate analyses should be performed on each sample. Report the results as *Enterococcus* CCEs per volume of water sample filtered.

13.0 Sample Spiking Procedure

- 13.1** Method 1611 QC requirements (Section 9) include the preparation and analysis of spiked reference (PBS) and matrix samples in order to monitor initial and ongoing method performance. For the IPR (Section 9.3), OPR (Section 9.4), and MS (Section 9.5) analyses it is necessary to spike samples with either laboratory-prepared spiking suspensions (Section 13.3) or BioBalls (Section 13.7).
- 13.2** Preparation of Laboratory-Prepared Undiluted Spiking Suspension. *Note:* when using laboratory-prepared cultures, it is recommended to prepare calibrator samples and spike suspensions from the same culture using the same cell enumeration results (see section 11.1).

13.2.1 Stock Culture (*E. faecalis*). Prepare a stock culture by inoculating a BHIA slant with *E. faecalis* ATCC® #29212™ (e.g., KWIK-STIK™) and incubate at 35°C ± 0.5°C for 21 ± 3 hours. This stock culture may be stored in the dark at room temperature for up to 30 days.

13.2.2 *E. faecalis* Spiking Suspension (Undiluted). From the stock culture of *E. faecalis* ATCC® #29212™, transfer a small loopful of growth to the BHIB and vigorously shake a minimum of 25 times. Incubate at 35°C ± 0.5°C for 21 ± 3 hours. The resulting spiking suspension contains approximately 1.0×10^9 enterococci CFU per mL. This is referred to as the “undiluted spiking suspension.”

13.2.3 Proceed to Section 13.3 for sample spiking and enumeration of spiking suspension (Section 13.2.2).

13.3 Laboratory-Prepared Sample Spiking and Spiking Suspension Enumeration

Since the objective of spiking the sample is to establish percent recovery, it is necessary to determine the concentration of *E. faecalis* in the laboratory-prepared undiluted spiking suspension (Section 13.2.2). This section provides instructions for sample spiking (Section 13.4) and spiking suspension enumeration (Section 13.5). If you are using BioBalls instead of a laboratory preparation, go to Section 13.7.

13.4 Sample Spiking

Please be sure to vigorously shake the spiking suspensions in the steps below to ensure accurate sample spiking and spiking suspension enumeration.

13.4.1 Dilute spiking suspension

Perform steps 13.4.1.1 through 13.4.1.5 using the laboratory-prepared undiluted spiking suspension (Section 13.2.2).

13.4.1.1 Mix the undiluted spiking suspension by vigorously shaking the bottle a minimum of 25 times. Use a sterile pipette to transfer 1.0 mL of the undiluted spiking suspension to 99 mL of sterile PBS; cap and mix by vigorously shaking the bottle a minimum of 25 times. This is spiking suspension dilution “A.” A 1.0 mL volume of dilution “A” is equivalent to a concentration of 1×10^7 CFU per mL (10^{-2} mL of the original undiluted spiking suspension).

13.4.1.2 Mix spiking suspension “A” (Section 13.4.1.1) by vigorously shaking the bottle a minimum of 25 times. Use a sterile pipette to transfer 1.0 mL to 99 mL of sterile PBS; cap and mix by vigorously shaking the bottle a minimum of 25 times. This is spiking suspension dilution “B.” A 1.0 mL volume of dilution “B” is equivalent to a concentration of 1×10^5 CFU per mL (10^{-4} mL of the original undiluted spiking suspension).

13.4.1.3 Mix spiking suspension “B” (Section 13.4.1.2) by vigorously shaking the bottle a minimum of 25 times. Use a sterile pipette to transfer 1.0 mL to 99 mL of sterile PBS; cap and mix by vigorously shaking the bottle a minimum of 25 times. This is spiking suspension dilution “C.” A 1.0 mL volume of dilution “C” is equivalent to a concentration of 1×10^4 CFU per mL (10^{-5} mL of the original undiluted spiking suspension).

13.4.1.4 Mix spiking suspension “C” (Section 13.4.1.3) by vigorously shaking the bottle a minimum of 25 times. Use a sterile pipette to transfer 1.0 mL to 99 mL of sterile PBS; cap and mix by vigorously shaking the bottle a minimum of 25 times. This is spiking suspension dilution “D.” A 1.0 mL volume of

dilution “D” is equivalent to 1×10^3 CFU per mL (10^{-6} mL of the original undiluted spiking suspension).

13.4.1.5 Mix spiking suspension “D” (Section 13.4.1.4) by vigorously shaking the bottle a minimum of 25 times. Use a sterile pipette to transfer 11.0 mL to 99 mL of sterile PBS; cap and mix by vigorously shaking the bottle a minimum of 25 times. This is spiking suspension dilution “E.” A 1.0 mL volume of dilution “E” is equivalent to 1×10^2 CFU per mL (10^{-7} mL of the original undiluted spiking suspension).

13.4.2 Spike samples

13.4.2.1 550 CFU Spike

To spike the sample, add 5.5 mL of spiking suspension dilution “E” (Section 13.4.1.5) to 100 mL of unspiked sample and mix by vigorously shaking the bottle a minimum of 25 times. This is the “spiked” sample with a concentration of 550 CFU per 100 mL. The volume (mL) of undiluted spiking suspension added is 5.5×10^{-7} mL/100 mL. This is referred to as V_{spiked} per 100 mL sample in Section 13.6.2.

13.5 Enumeration of Spiking Suspensions (Prepared in Section 13.3.1)

13.5.1 Prepare BHIA according to manufacturer’s instructions; add 12 – 15 mL of BHIA per 100×15 mm petri dish and solidify. Ensure the agar surfaces are dry. *Note:* Agar plates must be dry and free from condensation prior to use. To ensure the agar surfaces are dry, plates should be made several days in advance and stored inverted at room temperature or dried using a laminar-flow hood.

13.5.2 Each of the following should be conducted in triplicate, resulting in the evaluation of nine spread plates:

- Pipet 0.1 mL of dilution “C” (Section 13.4.1.3) onto surface of pre-dried BHIA plate (10^{-6} mL of the original spiking suspension).
- Pipet 0.1 mL of dilution “D” (Section 13.4.1.4) onto surface of pre-dried BHIA plate (10^{-7} mL of the original spiking suspension).
- Pipet 0.1 mL of dilution “E” (Section 13.4.1.5) onto surface of pre-dried BHIA plate (10^{-8} mL of the original spiking suspension).

13.5.3 After pipetting the 3 spread plates for each dilution, use a sterile bent glass rod or spreader to distribute inoculum over the surface of the medium by rotating the dish by hand or on a turntable. Repeat for next 2 dilutions. *Note:* Please ensure that inoculum is evenly distributed over entire surface of the plate.

13.5.4 Allow inoculum to absorb into the medium completely.

13.5.5 Invert plates and incubate at $35^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ for 21 ± 3 hours.

13.5.6 Count and record number of colonies per plate. Refer to Section 13.6 for calculation of spiking suspension concentration.

13.6 Calculation of Spiked *E. faecalis* Percent Recovery (Laboratory-Prepared Undiluted Spiking Suspension)

The spiked *E. faecalis* percent recovery will be calculated in three steps as indicated in Sections 13.6.1 through 13.6.3 below. *Note:* The calculated numbers in the table examples provided below have been rounded at the end of each step. If your laboratory recalculates the examples using a

spreadsheet and rounds only after the final calculation (Section 13.6.3), the percent recovery may be slightly different.

13.6.1 Calculate Concentration of *E. faecalis* (CFU/mL) in Undiluted Spiking Suspensions

- 13.6.1.1** The number of *E. faecalis* (CFU/mL) in the undiluted spiking suspensions will be calculated using all BHIA plates from Section 13.5.2 yielding counts within the ideal range of 30 to 300 CFU per plate.
- 13.6.1.2** If the number of colonies exceeds the upper range (i.e., >300) or if the colonies are not discrete, results should be recorded as “too numerous to count” (TNTC).
- 13.6.1.3** Calculate the concentration of *E. faecalis* (CFU/mL) in the undiluted spiking suspensions according to the following equation. An example calculation is provided in **Table 6**.

$$E. faecalis_{\text{undiluted spike}} = (CFU_1 + CFU_2 + \dots + CFU_n) / (V_1 + V_2 + \dots + V_n)$$

Where,

- $E. faecalis_{\text{undiluted spike}}$ = *E. faecalis* (CFU/mL) in undiluted spiking suspension
- CFU = Number of colony forming units from BHIA plates yielding counts within the ideal range of 30 to 300 CFU per plate
- V = Volume of undiluted sample on each BHIA plate yielding counts within the ideal range of 30 to 300 CFU per plate
- n = Number of plates with counts within the ideal range

Table 6. Example Calculation of *E. faecalis* Spiking Suspension Concentration

CFU / plate (triplicate analyses) from BHIA plates			<i>E. faecalis</i> CFU / mL in undiluted spiking suspension ($E. faecalis_{\text{undiluted spike}}$)*
10 ⁻⁶ mL plates	10 ⁻⁷ mL plates	10 ⁻⁸ mL plates	
TNTC, TNTC, TNTC	94, 106, 89	9, 11, 28	$(94+106+89) / (10^{-7}+10^{-7}+10^{-7}) =$ $289 / (3.0 \times 10^{-7}) =$ $9.6 \times 10^8 \text{ CFU/mL}$

* $E. faecalis_{\text{undiluted spike}}$ is calculated using all plates yielding counts within the ideal range of 30 to 300 CFU per plate

13.6.2 Calculate “True” Spiked *E. faecalis* (CFU/100 mL)

Calculate the true concentration of spiked *E. faecalis* (CFU/100 mL) according to the following equation. Example calculation is provided in **Table 7**.

$$T_{\text{Spiked } E. faecalis} = (E. faecalis_{\text{undiluted spike}}) \times (V_{\text{spiked per 100 mL sample}})$$

Where,

- $T_{\text{Spiked } E. faecalis}$ = Number of spiked *E. faecalis* (CFU/100 mL)
- $E. faecalis_{\text{undiluted spike}}$ = *E. faecalis* (CFU/mL) in undiluted spiking suspension
- $V_{\text{spiked per 100 mL sample}}$ = mL of undiluted spiking suspension per 100 mL sample

Table 7. Example Calculation of Spiked *E. faecalis*

<i>E. faecalis</i> undiluted spike	V_{spiked} per mL sample	$T_{\text{Spiked } E. faecalis}$
9.6×10^8 CFU/mL	5.5×10^{-7} mL/100 mL of sample	$(9.6 \times 10^8 \text{ CFU/mL}) \times (5.5 \times 10^{-7} \text{ mL} / 100 \text{ mL}) = 528 \text{ CFU} / 100 \text{ mL}$

13.6.3 Calculate Percent Recovery

13.6.3.1 Calculate percent recovery (R) using the following equation.

$$R = 100 \times \frac{(N_s - N_u)}{T}$$

Where,

R = Percent recovery

N_s = *E. faecalis* (CFU/100 mL) in the spiked sample

N_u = *E. faecalis* (CFU/100 mL) in the unspiked sample

$T_{\text{Spiked } E. faecalis}$ = True spiked *E. faecalis* (CFU/100 mL) in spiked sample

Example percent recovery calculation is provided in **Table 8**.

Table 8. Example Percent Recovery Calculation

N_s (CFU / 100 mL)	N_u (CFU / 100 mL)	$T_{\text{Spiked } E. faecalis}$ (CFU / 100 mL)	Percent recovery (R)
521	3 ^a	528	$100 \times (521 - 3)/528 = 98\%$

^a The result of 3 CFU is acceptable if the sample was a recreational water; for PBS N_u should be <1 CFU/100 mL.

13.7 BioBall® Sample Spiking and Enumeration

This section provides instructions for sample spiking (Section 13.7.1) and spiking suspension enumeration (Section 13.7.2) using BioBalls instead of a laboratory preparation of *E. faecalis*.

Upon receipt, BioBalls should be stored at -20°C. Preparation of BioBalls prior to spiking is not necessary, as they can be spiked directly into the sample once the vial is opened.

13.7.1 Sample Spiking

13.7.1.1 Open BioBall® vial by removing the cap. Aseptically add one 550 CFU *E. faecalis* BioBall® to 100 mL of sample and mix by vigorously shaking the bottles a minimum of 25 times.

13.7.1.2 This is the “spiked” sample with a concentration of ~550 CFU per 100 mL. Note: BTF provides mean and standard deviation of CFU concentrations in their certificates of analysis for each lot of BioBalls. These values can be accessed at <http://www.btfbio.com/cofa.php?nav=BioBall>.

13.7.2 Enumeration of BioBall®

13.7.2.1 Prepare BHIA according to manufacturer's instructions. Add 12 – 15 mL of BHIA per 100 × 15 mm petri dish and let solidify. Ensure the agar surfaces are dry. *Note:* Agar plates must be dry and free from condensation prior to use. To ensure agar surfaces are dry, plates should be made several days in advance and stored inverted at room temperature or dried using a laminar-flow hood.

13.7.2.2 The following should be conducted in triplicate, resulting in the evaluation of three spread plates.

- Open BioBall® vial by removing the cap and aseptically add one BioBall® to 1.0 mL of sterile PBS. Allow BioBall® to dissolve completely.
- Mix suspension to homogenize. Pipet 0.1 mL of the BioBall® suspension onto a 100 × 15 mm BHIA plate.

13.7.2.3 For each spread plate, use a sterile bent glass rod or spreader to distribute BioBall® inoculum over the medium surface by rotating the dish by hand or on a turntable. *Note:* Please ensure that inoculum is evenly distributed over entire surface of plate.

13.7.2.4 Allow inoculum to absorb into medium completely.

13.7.2.5 Invert plates and incubate at 35°C ± 0.5°C for 21 ± 3 hours.

13.7.2.6 For each dilution, count and record the sum of the number of colonies (CFU) on the triplicate plates.

13.8 Calculation of BioBall® Spike Percent Recovery

Spiked BioBall® percent recovery will be calculated using the lot mean value provided by manufacturer according to Section 13.8.2. Recovery may also be calculated following the two steps as indicated below in Sections 13.8.1 and 13.8.2. *Note:* The calculated numbers in the example provided in the tables below have been rounded at the end of each step. If your laboratory recalculates the examples using a spreadsheet and rounds only after the final calculation (Step 2), the percent recovery may be slightly different.

13.8.1 Calculate Mean *E. faecalis* per BioBall® and “True” Spiked *E. faecalis* (CFU/100 mL)

The mean concentration of *E. faecalis* (CFU) in the BioBalls will be calculated using all three BHIA plates (from Section 13.7.2.6). Since one BioBall® is spiked per 100 mL sample, use the mean number of *E. faecalis* per BioBall® as the “true” spiked *E. faecalis*. For example, if the triplicate results from Section 13.7.2.6. are 57, 52, and 49, then the triplicate plate counts for the 550 CFU BioBall® concentration are as follows:

$$T_{\text{Spiked } E. faecalis} \text{ (CFU/100 mL)} = [(54 + 52 + 49) \times 10] / 3 = 517 \text{ per BioBall}^{\circledR}$$

Where,

$$T_{\text{Spiked } E. faecalis} = \text{True spiked } E. faecalis \text{ (CFU/100 mL) in spiked sample}$$

Thus, 517 is the “true” spiked *E. faecalis* (CFU/100 mL)

13.8.2 Calculate Percent Recovery

Calculate percent recovery (R) using the following equation:

$$R = \frac{100 \times (N_s - N_u)}{T}$$

Where,

R = Percent recovery

N_s = *E. faecalis* (CFU/100 mL) in the spiked sample (or CFU per BioBall®)

N_u = *E. faecalis* (CFU/100 mL) in the unspiked sample

$T_{\text{Spiked } E. faecalis}$ = True spiked *E. faecalis* or CFU/100 mL in spiked sample

Percent recovery example calculation is provided in **Table 9**.

Table 9. Percent Recovery Example Calculation

Concentration	N_s (CFU / 100 mL)	N_u (CFU / 100 mL)	T (CFU / 100 mL)	Percent recovery (R)
550	505	10	517	$100 \times (505 - 10)/517 = 96\%$

14.0 Method Performance

14.1 Multi-laboratory Validation (MLV) of Method 1611 in Marine and Fresh Water

14.1.1 Marine Water

14.1.1.1 Eleven volunteer laboratories participated in the U.S. Environmental Protection Agency's (EPA's) MLV study of EPA Method 1611. The purposes of the study were to characterize method performance across multiple laboratories and multiple marine water matrices, assess method reproducibility, and to develop QC acceptance criteria. A detailed description of the study and results are provided in the validation study report (Reference 17.9). Results submitted by laboratories were validated using a standardized data review process to confirm that results were generated in accordance with study-specific instructions and the April 2010 draft version of EPA Method A (1611).

14.1.1.2 Recovery — Method 1611 was characterized by mean laboratory-specific recoveries, of enterococci from marine water samples spiked with BioBalls ranging from 68% to 841% (StepOnePlus™) and 89% to 632% (ABI 7500), with an overall mean recovery of 401% (StepOnePlus™) and 274% (ABI 7500). Mean laboratory-specific recoveries of enterococci from PBS samples spiked with BioBalls ranged from 37% to 606% (StepOnePlus™) and 89% to 627% (ABI 7500), with an overall mean recovery of 326% (StepOnePlus™) and 297% (ABI 7500).

14.1.1.3 Precision — Method 1611 was characterized by laboratory-specific relative standard deviations (RSDs) from marine water samples spiked with BioBalls ranging from 35% to 91% (StepOnePlus™) and 43% to 71% (ABI 7500), with an overall pooled, within-laboratory RSD of 67% (StepOnePlus™) and

58% (ABI 7500). For PBS samples spiked with BioBalls, laboratory-specific RSDs ranged from 29% to 114% (StepOnePlus™) and 48% to 74% (ABI 7500), with an overall pooled, within-laboratory RSD of 81% (StepOnePlus™) and 58% (ABI 7500).

14.1.1.4 Reproducibility — Each laboratory analyzed a total of five matrix samples received during a single week. Each sample was split into eight replicates (4 unspiked and 4 spiked). Samples were spiked with *E. faecalis* (550 CFU) BioBalls. Overall mean recoveries were 127%, 133%, 82%, 111%, and 228% for matrices 1 – 5, respectively.

14.1.2 Fresh Water

14.1.2.1 Ten volunteer laboratories participated in the U.S. Environmental Protection Agency's (EPA's) MLV study of EPA Method 1611. The purposes of the study were to characterize method performance across multiple laboratories and multiple fresh water matrices, assess method reproducibility, and to develop quantitative QC acceptance criteria. A detailed description of the study and results are provided in the validation study report (Reference 17.9). Results submitted by laboratories were validated using a standardized data review process to confirm that results were generated in accordance with study-specific instructions and the April 2010 draft version of EPA Method A (1611).

14.1.2.2 Recovery — Method 1611 was characterized by mean laboratory-specific recoveries, of enterococci from fresh water samples spiked with BioBalls ranging from 7% to 156%, with an overall mean recovery of 83%. Mean laboratory-specific recoveries of enterococci from PBS samples spiked with BioBalls ranged from 35% to 103%, with an overall mean recovery of 69%.

14.1.2.3 Precision — Method 1611 was characterized by laboratory-specific relative standard deviations (RSDs) from fresh water samples spiked with BioBalls ranging from 35% to 1554%, with an overall pooled, within-laboratory RSD of 554%. For PBS samples spiked with BioBalls, laboratory-specific RSDs ranged from 20% to 104%, with an overall pooled, within-laboratory RSD of 58%.

14.1.2.4 Reproducibility — Each laboratory analyzed a total of five matrix samples received during a single week. Each sample was split into eight replicates (4 unspiked and 4 spiked). Samples were spiked with *E. faecalis* (550 CFU) BioBalls. Overall mean recoveries were 13%, 151%, 87%, 35%, and -97% for matrices 1 – 5, respectively. It should be noted that for Matrix #5 the mean background enterococci concentration was 37,329 CCEs/100 mL; approximately 68 times greater than the spike level of 550 cells added to each sample.

15.0 Pollution Prevention

- 15.1 The solutions and reagents used in this method pose little threat to the environment when recycled and managed properly.
- 15.2 Solutions and reagents should be prepared in volumes consistent with laboratory use to minimize the volume of expired materials to be disposed.

16.0 Waste Management

- 16.1 It is the laboratory's responsibility to comply with all federal, state, and local regulations governing waste management, particularly the biohazard and hazardous waste identification rules and land disposal restrictions, and to protect the air, water, and land by minimizing and controlling all releases from fume hoods and bench operations. Compliance with all sewage discharge permits and regulations is also required.
- 16.2 Samples, reference materials, and equipment known or suspected to have viable enterococci attached or contained must be sterilized prior to disposal.
- 16.3 For further information on waste management, consult "The Waste Management Manual for Laboratory Personnel" and "Less Is Better: Laboratory Chemical Management for Waste Reduction," both available from the American Chemical Society's Department of Government Relations and Science Policy, 1155 16th Street NW, Washington, DC 20036.

17.0 References

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18.0 Acronyms

$\Delta\Delta C_T$	comparative cycle threshold calculation method
AF	amplification factor
BHIA	brain heart infusion agar
BHIB	brain heart infusion broth
BSA	bovine serum albumin
CCE	calibrator cell equivalent
CFU	colony forming units
C_T	cycle threshold
CV	coefficient of variation
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
GI	gastrointestinal illness
IPR	initial precision and recovery
lsrRNA	large subunit ribosomal RNA (i.e., 23S rRNA)
MS	matrix spike
MLV	multi-laboratory validation
NTC	no template control
OD	optical density
OPR	ongoing precision and recovery
PBS	phosphate buffered saline
PCR	polymerase chain reaction
QA	quality assurance
QC	quality control
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
RPD	relative percent difference
RSD	relative standard deviation
SLV	single-laboratory validation
SPC	sample processing control
TNTC	too numerous to count
TSC	target sequence concentration
UV	ultraviolet (light)

**Appendix A:
ABI StepOnePlus™, ABI 7900, and ABI 7500 Real-Time PCR System
Operation**

StepOnePlus™, ABI 7900, and ABI 7500 Real-Time PCR System Operation

1.0 ABI StepOnePlus™

1.0.1 Turn on the StepOnePlus™ and then the computer. Launch the **StepOnePlus™** software program by double clicking on its icon on the computer desktop or from the Computer Programs menu.

1.0.2 On the StepOnePlus™ home screen select **Advanced Set Up**.

1.0.3 On the right side of the main screen click in the box **Experiment Name**, enter identifying information for the experiment (Name/Date etc such that experiment can be identified). Then go through the following fields:

- Which instrument is going to be used to run the experiment: **StepOnePlus™ Instrument** (96 wells) is default (highlighted).
- What experiment do you want to set up: **Quantitation-Standard Curve** is default (highlighted).
- Which reagents will be used to detect the target: **TaqMan® Reagents** is default (highlighted).
- Which ramp speed do you want to use in the instrument run: Click on **Standard-2 hours** (Not Default)

1.0.4 Click on **Plate Set Up** from the navigational pane of the present screen to define the targets, and then assign them to wells in the reaction plate (Step 1.0.8).

1.0.5 Define target – You can add a new target or use a saved target. By clicking on **Add Saved Target** the window with the target library will open.

1.0.6 Select the target(s) for your assay(s) and click on **Add Selected Targets**. All of the targets may be selected simultaneously by holding the Ctrl key and highlighting the desired targets (e.g., Entero, Sketa). The selected targets will then be added on to the define target and sample screen.

1.0.7 Optional: If a new target is to be added click the **Enter Target Name** cell and type the name. From the **Reporter** dropdown menu, select FAM (default). From the **Quencher** dropdown menu, select Tamra (NFQ-MGB) (default). Leave the default in the color field.

1.0.8 Click on the tab **Assign Targets and Samples** to see the screen view of the plate layout with 96 wells.

- Select the wells, based on the plate set up, by highlighting, one target at a time.
- Select the wells for the first target by checking the box for the desired target in **Assign Target** to the selected wells and it will automatically populate the wells for that target. If more than one target is being used, repeat the process above for each target.
- The wells can be selected individually or by rows by clicking in the left corner of the row. In addition, the whole plate may be selected by clicking in the left corner of the plate.
- To deselect a row or well press Ctrl & click the selected portion one more time and it will deselect the row or well. Selected wells/rows will be highlighted grey, while unselected wells/rows will remain white.

1.0.9 Run the Method

- On the run method screen, review the reaction volume and the thermal profile for the default run. If needed: The default run method can be edited or replaced with one from the run method library. Click either the **Graphical view** (default) or **Tabular View** tab.
- Make sure the reaction volume per well field displays 25 μL , this is not the default setting.
- Set the thermal profile to the following holding and cycling stages: Holding Stage 1: 50.0°C for 2:00 minutes, Holding Stage 2: 95.0°C for 10:00 minutes, Cycling Stage: 95.0°C for 0:15 seconds. The second step of the Cycling Stage is defaulted at 60.0°C for 1 minute.
- *Note:* When using a run from the library click on the tab **Open Run Method** in the graphical view. Select **Run Method** and click ok. It will replace the default run with the saved run.

1.0.10 Load the plate into the instrument.

1.0.11 Click on the **Plate Set Up** tab and start the run by clicking the green button in the upper right hand corner of the screen.

- Save Experiment dialogue box – Click **Save** to accept default file name and location (the name assigned when setting up the experiment). The experiment is saved by default to the <drive>:\ applied Biosystems\<software name>\experiment folder.
- Run progress can be viewed from the touch screen of the instrument. *At the beginning of a run do not leave the instrument or computer until you verify that the run has started.*

1.0.12 Once the run is completed remove the reaction plate and discard.

1.0.13 Analyze the run

- Click on the tab in the top right corner of screen to **Analyze the Run**.
- Highlight all of the sample wells and click on **Analysis Settings** to get to C_T settings (default C_T settings).
- Highlight all the targets running in the plate; uncheck the default setting for automatic threshold and **set the threshold to 0.03**. Keep the Automatic Baseline. Click on **Apply Analysis Settings** to save changes. The new settings can be confirmed by viewing the threshold line on the amplification plot.

1.0.14 Press the **Export** tab on the left corner of the view plate layout screen. Click on the browse button to find the correct place to **export the data** and click Start Exporting. After the export is done **close export tool**.

1.1 ABI 7900 Real-Time PCR System Operation

- ### 1.1.1
- Turn on the ABI Model 7900 and then the computer. Launch the **SDS 2.2.2** software program by double clicking on its icon on the computer desktop or from the Computer Programs menu. The computer will establish communication with the 7900 instrument and if the connection is successful, the software will display the Connected icon in the status bar when a plate document is opened.

- 1.1.2 Under File menu, select **New**.
- 1.1.3 In resulting **New Document** window that appears, change container selection from **384 well clear plate** to **96 well clear plate** using drop down menu. Accept default selections of **Absolute Quantification** and **Blank Template**. Click **OK** to display a new plate document.
- 1.1.4 Click, hold and drag mouse over all PCR reaction tray wells containing samples in upper left window. Selected wells will be outlined with a bold line and their position numbers should appear in the results table in the lower left window. To unselect wells, repeat above process while holding down control key.
- 1.1.5 Above right hand window, click on **Setup** tab.
- 1.1.6 Click on **Add Detector** button at the bottom of the setup screen. *Note:* Before any analyses are performed, a specific detector for the method must be created. To do this, go to step 1.1.7. Once the detector has been created, go to step 1.1.8.
- 1.1.7 Click on **New** in the pop-up window that appears. Another pop-up window will appear. Under **Name**, type in a name for the detector that will be used by this method (*e.g.*, "Method 1611"). Under **Group** select Default. Under **Reporter** select FAM. Under **Quencher** select TAMRA. Click on **OK** to close second pop-up window. This step only needs to be performed before the initial analysis run of the method. The detector that is named is selected in all subsequent analysis runs as indicated in step 1.1.8)
- 1.1.8 In pop-up window that was opened in step 1.1.6, select the desired detector under Names menu (*e.g.*, Method 1611) and click on **Copy to Plate Document** button. Click on **Done** button to return to setup screen.
- 1.1.9 Click on **Use** box next to FAM detector in right hand window. This box should become marked with an X. Name and color code for FAM detector should appear in each of the selected well positions in the upper left window and a data column for this detector should be created for each of the selected well positions in the results table in the lower left window.
- 1.1.10 Click on **Instrument** tab right hand window.
- 1.1.11 In instrument screen, change **sample volume** to 25 μ L and choose **9600 emulation**.
- 1.1.12 Still in instrument screen, click on **Connect**, then click on **Open/Close** button in lower right hand "Real Time" window to open PCR reaction tray holder door on instrument.
- 1.1.13 Insert PCR reaction tray with prepared reactions in holder.
- 1.1.14 Click on **Open/Close** button to close PCR reaction tray holder door on instrument.
- 1.1.15 Click on **Start** button in lower right hand "Real Time" window to start thermal cycling in instrument.
- 1.1.16 Name run file at prompt.
- 1.1.17 At termination of the run, instrument-calculated cycle threshold values should automatically appear for each well position and detector entry in the lower left hand results table window.
- 1.1.18 At termination of the run success complete, choose **Analysis Settings** from the toolbar. In that box enter a value for the **Manual Ct Threshold** (see 1.1.19). Click on **OK**. Click on **Analyze** from the toolbar. You should see C_T values in the Results Table.

- 1.1.19** The instrument-selected threshold line is indicated by the bold red line on the plot and the value is listed below the window. This can be manually adjusted by either click-hold and dragging the line up or down or entering new values below. *Note:* Based on results thus far with the instrument, the threshold value can be adjusted from the default value of 0.2 to 0.03, however, this should be done only if the threshold line remains above the background values (seen before the growth curves) for all of the samples.
- 1.1.20** Calculated C_T values for each of the sample tray positions in the lower left hand “Results Table” will automatically be updated following adjustments of the threshold line. Once the threshold is adjusted to the desired level, select “Print Report” under the “File” menu. Check or uncheck desired report items by clicking on their associated boxes and the click on “Print” button. *Note:* Minimum report should have both detector boxes, *i.e.*, “FAM” checked which will show C_T values for all selected tray positions for this detector.
- 1.1.21** Export data by clicking on **File** from the toolbar. From the drop down menu choose **Export**. In the box you will see **Look in:** and here you choose a directory to send the exported file too. Click on **Export**. **Save changes** to document? will appear, click on **Yes**. Click **OK**.
- 1.2 ABI 7500 (non-Fast) Sequence Detector Operation
- 1.2.1** Turn on the ABI Model 7500 sequencer and then the computer. Launch the software program by double clicking on its icon on the computer desktop or from the Computer Programs menu. The computer will establish communication with the 7500 instrument. See “How to Set Up a New Experiment Using the ABI 7500” for screen shots.
- 1.2.2** Click on either the **New Experiment** or **Advanced Setup** button to create a new experiment, which will pull up the Experiment Menu.
- 1.2.3** From the **Setup** menu, select **Experimental Properties** to select the experiment type and give the experiment a name. Enter the experiment name in the asterisked box.
- Click on **7500 (96 Wells)** to select the instrument type. *Note:* This protocol is not designed for the 7500 Fast.
 - Scroll down to access more experiment properties options. Click on **Quantitation – Standard Curve** to select the experiment type
 - Click on **TaqMan® Reagents** to select the reagents used.
- 1.2.4** From the **Setup** menu, select **Plate Setup**. Click on the **Define Targets and Samples** tab to define the reporter-quencher dye for each target and also to enter the sample identifications (*e.g.*, Ent 5X).
- Click on **Add New Target** and enter the name of the target. Under the Reporter heading, click on the drop down menu to select the reporter dye **FAM**. Under the Quencher heading, click on the drop down menu to select **TAMRA** as the quencher.
 - Repeat the process above to add more than one target, *e.g.*, Sketa 5x.
 - In the **Define Samples** section, click on **Add New Sample** and enter the sample name in the Sample 1 box.
 - Click on the **Assign Targets and Sample** tab to assign the Target, sample ID and sample type to the wells.
 - Drag the mouse over the desired cells to assign the Target, Task and Sample.

- While the cells are highlighted, in the **Assign target(s) to the selected wells** section, check the box under **Assign** for the appropriate Target, then under **Task**, select **U** for unknown, **S** for standard or **N** for negative control.
 - In the **Assign sample(s) to the selected wells** section, check the box under **Assign** to label the highlighted cells as Sample 1.
 - Repeat the process above, by dragging the mouse over the appropriate cells, and then assigning them as **Unknowns**, **Standards** or **Negative Controls**, as appropriate. *Note:* For Standards, in the **Assign target(s) to the selected wells** section, if there are 4 different values, each cell will need to be highlighted separately, and a quantity (*e.g.* 40000) typed in the **Quantity** box.
 - Click on **Print Report** at the top of the screen to print a plate layout for loading master mix and sample extracts.
 - Select **Plate Layout** option by clicking on the box next to it.
 - Click **Print Report** to print the plate layout.
- 1.2.5** From the **Setup** menu, select **Run Method** to set up the thermo cycling profile.
- In the **Graphical View** tab, change the **Reaction Volume Per Well** from the default 50 μL to 25 μL by typing in 25.
 - Under **Cycling Stage** section, change the **Number of Cycles** to 45.
 - Check that the default settings of Temperature and Time for the two Holding Stages and the Cycling Stage are correct. Specifically, these should be: Holding Stage 1: 50.0°C for 2:00 minutes, Holding Stage 2: 95.0°C for 10:00 minutes, and Cycling Stage: 95.0°C for 0:15 seconds. *Note:* The purpose of Holding Stage 1 is to eliminate potential PCR carryover products in the reactions using the Amp-Erase[®] UNG enzyme provided in the TaqMan[®] Universal PCR master mix. The purpose of Holding Stage 2 is to inactivate the Amp-Erase[®] UNG enzyme.
 - The second step of the Cycling Stage is defaulted at 60.0°C for 1 minute – change the time to 2:00 minutes. *Note:* This sets the detection of the fluorescence signal to occur at the end of the second step in each cycle.
 - Click on **Save** at the top of the screen to save the run before actually running the experiment.
 - The Save screen will open so that you may select the folder to save the Run Data File. Type in the Experiment Name (if you did not already do so at the beginning of the Experiment Setup) and click on **Save**. If you had previously typed in an Experiment Name and there is no change, click on **Save**. *Note:* You must save to the hard drive (rather than a flash drive).
- 1.2.6** From the **Experiment** menu, select **Run** to monitor the run in real time.
- The Run Status screen will open - click on **Start Run**. When the run has successfully started, the **Start Run** button will turn change from green to red.
- 1.2.7** When the run has completed, from the **Setup** menu, select **Analysis** to export your results. See “How to Export Results from the ABI 7500 Software” for screen shots.
- The **Analysis Settings for Experiment name** screen will open.

- Under the **C_T Settings** tab, click on the Target desired to be set from Default to Manual Threshold setting (generally, this would be the Ent).
- In the **C_T Settings for ENT** section, uncheck the boxes next to **Use Default Settings**, and **Automatic Threshold**. Enter the desired Threshold (*e.g.*, 0.025). If more than one target is present, highlight the next target (*e.g.*, Sketa) and repeat this step.
- Click on **Apply Analysis Settings** to save the changes.

1.2.8 Click on **Export** at the top of the screen to save the experiment results data and to save the selected plots required in the report. The threshold value will have changed on the Amplification plot curve graphic.

- The Export Data screen will open. In the **Select data to export** section, check all of the boxes (*i.e.*, Sample Setup, Raw Data, Amplification Data, Results, and Multicomponent Data).
- For **Select one file or separate files**, select **One file** to export all the data into one file with multiple tabs.
- For **Export File Name**, enter the name of the Experimental data file to be exported, and for **File Type**, choose *.xls.
- For **Export File Location**, click on **Browse** to select the folder in which you wish to export your file. Always save your file to the hard drive.
- Click on **Start Export**. When this is completed, an Export Completed screen will open – click on **Close Export Tool** to complete this task.

1.2.9 How to interpret your results. See “How to Interpret Results from the ABI 7500” for screen shots.

- After exporting your data, from the **Experiment** menu, select **Analysis**.
 - From the **Analysis** menu, select **Amplification Plot** to view the results of the selected samples at different plot settings.
 - Drag the mouse over the desired cells to select the samples to be plotted in the amplification plot.
 - In the **Amplification Plot** section, in the **Plot Settings** tab, select the **Plot Type** (from the drop down menu) and the **Graph Type** (from the drop down menu).
 - If the plot requires the display of the Threshold and Baseline, in the **Options** tab, check the boxes next to **Threshold** and **Baseline**. If you want to see the results in tabular format, click on **View Well Table**, and scroll down or sideways for more samples and analysis parameters.
 - The toolbar above the graph can be used to make hard copy prints, saving the file as *.jpg, zooming in and out, and changing the plot properties.
- For the Standard Curve, select **Standard Curve** from the **Analysis** menu to view the results of the same sample (assuming they have the same target) and standards in the plot.
 - In the **Plot Settings** tab, click the drop down menu arrow beside **Target** to select a different target (if the standards were analyzed in more than

-
- one target), and click the drop down menu arrow beside **Plot Color** to change the plot color.
 - The standard curve parameter display below the plot (*i.e.* Target, Slope, Y-Intercept, R^2 , and Eff% (percent efficiency)) is useful for data analysis.
 - For Multicomponent plots, click on **Multicomponent Plot** from the **Analysis** menu to view the variation in the fluorescence of the dyes used in the experiment over the ascending repeat of the 45 thermo cycles based on well, target, or dye.
 - Click on the drop down menu arrow next to **Plot Color** in the **Plot Settings** tab to select the parameter for the basis of the graph (*e.g.*, well, target, or dye).
 - For raw data plots, select **Raw Data Plot** from the **Analysis** menu to view the variation in the emission of fluorescence of the dyes in different filters present over the ascending repeat of the 45 thermo cycles.
 - In the **Options** tab, drag the pointer on the cycle scale to the desired cycle number to view the fluorescence variation through filters of the samples at that cycle.
 - For quality controls, click on **QC Summary** from the **Analysis** menu to view the analysis summary of the samples.
 - To view all the plots, click on **Multiple Plots View** from the **Analysis** menu to see all of the plots for the samples in a single window. Each plot will have its own drop down menu as in Section 1.2.9.5.
- 1.2.10** To print the report, click on **Print Report** at the top of the screen.
- Check all of the boxes (*i.e.*, Experimental Summary, Results Summary, Plate Layout, Amplification Plot (3 boxes), Standard Curves, Results Table, and QC Summary).
 - Click on **Print Report** at the bottom of the screen.

**Appendix B:
Cepheid Smart Cycler[®] Operation**

Appendix B - Cepheid Smart Cycler[®] Operation

1.0 Smart Cycler[®] Operation

- 1.1 This protocol is intended to provide only information about critical instrument settings required to perform EPA Method 1611. Further details concerning the operation of the instrument can be obtained from the Smart Cycler[®] Operation Manual, Cepheid Part #D0190, Rev. D.
- 1.2 Turn on the Smart Cycler[®]; then the computer.
- 1.3 Double-click on the **Smart Cycler[®]** icon on the computer desktop.
- 1.4 The following steps for defining a protocol are only required before the initial run of the instrument. The protocol that is defined in these steps is used in all subsequent runs of the instrument. See "How to Set Up a New Experiment Using the Smart Cycler[®] (Software Version 2.0)" for screen shots.
 - 1.4.1 Click on the **Define Protocols** icon to go to **Define Protocols** screen.
 - 1.4.2 Click on the **New Protocol** button to open the **Protocol Name?** dialog. Enter "EPA Enterococcus Method 1611" for the new protocol name, and click **OK**. The protocol stages are defined in the series of boxes at the bottom of the **Define Protocol** screen. Make sure the new protocol is highlighted and begin to choose state settings. To define **Stage 1**, click on its drop-down box to display the menu of stage types; then select **Hold**. In the **Temp** column, enter 50.0, and in **Secs** column, enter 120, leaving the **Optics** setting as the default **Off** setting. (*Note:* This stage is performed to eliminate potential PCR carryover products in the reactions using the Amp-Erase[®] UNG enzyme provided in the TaqMan[®] Universal PCR master mix. The optical reading cannot be programmed during a Hold stage.).
 - 1.4.3 To define **Stage 2**, click on its drop-down box to display the menu of stage types, and again select **Hold**. In the **Temp** column, enter 95.0, and in **Secs** column enter 600. Again, leave the **Optics** setting on **Off**. (*Note:* This stage is performed to inactivate the Amp-Erase[®] UNG enzyme).
 - 1.4.4 To define **Stage 3**, select **2-Temperature Cycle** from its drop down menu. For the first step, enter 95.0 in the first row of the **Temp** column and 15 in the **Secs** column, **Optics** column **Off**. For the second step, enter 60.0 in the second row of the **Temp** column, 120 in the **Secs** column, and click on the **Optics** cell to select **On** from the drop-down menu. (*Note:* This sets the detection of the fluorescence signal to occur at the end of the second step in each cycle.) Enter 45 in the **Repeat** field at the top of the **Stage 3** box to specify that it should be repeated for 45 cycles. Click the **Save Protocol** button.
 - 1.4.5 To display primary curve graphs, click **Define Graphs**. Highlight **FAM** in **Graph** column. Check the box for **Automatically add to new runs**. Under **Graph Type** choose **Optics** from the pull down menu. Under **Channels** check the box for **Ch 1 (FAM)**. Under **Show** check the boxes for **Primary Curve**, **Threshold (Vertical)** and **Threshold (Horizontal)**. Under **Axes** check the box for **Fluorescence vs. Cycle**. At the bottom of the screen click on **Save Graph**.
- 1.5 Click on the **Create Run** icon to open the **Create Run** screen. For each new run, enter a unique name in the **Run Name** field. (*Note:* The software does not allow duplicate run names).
- 1.6 Enter any additional information about the run in the **Notes** field. Click the arrow in the **Dye Set** box to display a drop-down menu of the possible selections. Select **FTTC25**. (*Note:* This selects the dye set: FAM, TET, Tex Red, Cy5, and a 25 μ L reaction).

- 1.7 Click the **Add/Remove Sites** button. The **Select Protocols and Sites...** dialog will appear. Highlight (click on) the "EPA Method 1611" protocol developed prior to the first run (Section 1.4.2) in the **Protocols** list. In the **Sites** list, highlight the sites on the instrument to be used with this protocol in the current run by clicking on them with the shift key held down (*Note*: Sites refer to the I-core modules in the Smart Cyclers[®] processing block in which reaction tubes will be placed; a total of 16 are possible per block. When using multiple blocks, the site numbers will be preceded by the block letters, *e.g.*, A, B, C); then click the right pointing arrow to transfer the selected sites and protocol to the **Selections** table.
- 1.8 Click on the **OK** button to save the selections, and return to the **Create Run** screen.
- 1.9 Place the loaded Smart Cyclers[®] reaction tubes in the I-core module slots, selected above for current run. The tubes should snap into place. Either the front or back of the caps can face the front of the processing block.
- 1.10 In one of the **View** menus that is shown, select **Analysis Settings**. The displayed table includes one row for each of the four possible dye channels defined in the dye set. Click on the cell in the **FAM** row under the **Usage** column heading, and select **Assay** from the drop down menu. Set the **Usage** cells for all other dyes to **Unused** in the same manner. (*Note*: All assays in this protocol use FAM as the reporter dye). All other cells in this table should be left at default settings (See Smart Cyclers[®] Operation Manual).
- 1.11 In the other **View** menu that is shown, select the **Results Table**. Enter the sample identification information for each site in the **Sample ID** column (additional information can be entered into the **Notes** column.). Leave the other columns as default settings (see Smart Cyclers[®] Operation Manual.)
- 1.12 Click on the **Start Run** button. The orange LEDs on the Smart Cyclers[®] processing block should turn on, and the software will automatically switch to the **View Results** screen.
- 1.13 To display the real time temperature profiles for all sites, click **Temperature** in either of the **View** menus. To display real time growth curves for all samples (*i.e.*, the fluorescence signal vs. cycle), click **FAM** in the other **View** menu.
- 1.14 At the end of the run, it is recommended to check the cycle threshold values calculated by the instrument for each sample by opening the **Results Table** window by clicking on this selection in the upper **View** menu. It is also recommended to inspect the growth curves in the **FAM** window which can be opened in the same manner from the lower **View** menu. The default threshold fluorescence value is shown in this window as a single horizontal red line and the cycle thresholds for each site are shown as vertical red lines. To view the data for individual sites in this window, click on that site number in the table to the right of the graph. If the default threshold fluorescence line is well above all of the growth curve lines prior to visible amplification, the threshold fluorescence value can be changed to a lower value. This is done by reopening the **Analysis Settings** window from the upper **View** menu and entering a new value in the **Manual Thresh Fluor Units** cell in the **FAM** row. Conversely if the default threshold fluorescence line is below any of the growth curve lines prior to visible amplification, the threshold fluorescence value should be changed to a higher value in the same manner. Previous studies have indicated that a threshold value of 8 works well for most analyses. Click on the **Update Analysis** button to view the new threshold line in the **FAM** window. The cycle threshold values will be automatically updated in the **Results Table**.
- 1.15 Once the threshold fluorescence value is adjusted to an optimal value, click the **Save Run** button. (*Note*: The Smart Cyclers[®] Software does not give a prompt to save changes before printing or exporting. Therefore, it is possible to make changes to the Results Table or Analysis Settings, and immediately print or export the data, then close the run without saving the changes. In this case,

- the data saved in the Smart Cycler[®] database will not match the printed or exported data. If no changes are made in the threshold fluorescence value, the run data is automatically saved as it is when the program is closed or a new run is created.)
- 1.16 To set up automatic export of raw data, (see “How To Set Up Automatic Export of Raw Data” for screen shots) from the main menu, click on **Setup**, then **System Defaults**, then **Export Settings**. From the Export Settings dialog box, check the box beside Results Table and Analysis Settings. Click the **Browse** button to select the folder where you want your raw data saved. Click on the radio button next to Automatic export on run completion, then click the **OK** button.
 - 1.17 To manually save the **Results Table and Analysis setting** containing the instrument-calculated cycle threshold values for each sample (see “How to Manually Export Raw Data” for screen shots), click the **Export** button to display the **Export Data** dialog box. Check the box next to the heading **Export Results Table and Analysis Settings** by clicking on it. Leave all other boxes unchecked. Click on **Export**. A box labeled **Export Data** will appear with the run name in the file name box. Click on the drop down menu to the right of Save In: at the top left of the screen to change the directory where you want your data saved. Click on **Save**. Data are exported as comma-delimited text (*.cvs) files in MS Excel-compatible files to the Export folder in the Smart Cycler[®] folder: C:\Smart Cycler[®]\Export. Always save to the hard drive.
 - 1.18 To archive a run in the Smart Cycler[®] program, click on **Tools**. From the drop down menu click on **Data Management** and then **Archive Runs**. Click **Proceed**. Select the run to be archived by clicking on its name in the database list. Click **OK** and then **Proceed**. A box labeled **Archive Run** will appear on the screen. There will be a line labeled **Save in:**. Input which directory you would like the run to be saved in. There will be a line **File Name:**. Enter a file name. Click **Save**. Click **OK**.
 - 1.19 To print run data (see “How to Print Run Data” for screen shots), after the run is completed, right click on the graph area, and select **Print**, then **Print Graph with Results Table**. An Optics Graph print preview screen will pop up, click **Print**. The screen will switch back to the post run screen. To print the generated report, click **Report**. A Run Report print preview screen will pop up, click **Print**.
 - 1.20 To set up a new analysis (see “How to Set Up a New Run” for screen shots), from the post run screen, click **Create Run** at the top left. The results of your just completed will disappear and a **Add/Remove Sites** button will appear in the middle of the screen – click it. A Select Protocols and Sites screen will pop up, select **EPA Enterococcus Method 1611** in the Protocols box, and highlight the amount of sites needed for the assay (A1-A16) in the Sites box. Click the right arrow to the right of the sites box to move the sights to the Selections box. After double checking that the correct protocol is highlighted, click **OK**. Add the Run Name in the Run Name box. The Site ID, Protocol, Sample ID, Sample Type, Notes (etc.) box will pop up. Enter the Sample ID for each site. Under this box, find the Usage column and click on **Assay** to select **Unused** for Cy3, TxR and Cy5 (leave Assay for FAM). In the same box, find the Manual Thresh Fluor Units, and click on the units for FAM to change it from 30.0 to 8.0 (leave the units at 30.0 for Cy3, TxR and Cy5). After loading the Smart Cycler with tubes, click **Start Run**. A red light should appear on sites that are in use.