

Method 1696.1: Characterization of Human Fecal Pollution in Water by HF183/BacR287 TaqMan[®] Quantitative Polymerase Chain Reaction (qPCR) Assay

Acknowledgments

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Introduction

EPA has validated numerous quantitative polymerase chain reaction (qPCR) methods to identify fecal indicator bacteria. However, EPA did not have a validated method for microbial source tracking (MST) to characterize fecal pollution in recreational waters originating from a specific animal group, such as humans. Because EPA did not have a validated, approved method for MST, stakeholders could not use such a method in their National Pollutant Discharge Elimination System permits or for other instances where an approved method is required.

In response to stakeholders' needs for a validated method for MST to characterize human sources of fecal pollution in recreational waters, EPA conducted a multi-laboratory validation (MLV) study to assess method performance across 14 laboratories focusing on both fresh and marine recreational water matrices.

Advances in environmental molecular microbiology have identified Bacteroidales human-associated gene sequences commonly found in feces (primary source) and secondary sources (sewage and septage) of human waste. Although deoxyribonucleic acid (DNA) from these organisms is rarely present in other animal sources at low concentrations, their presence in environmental water is typically an indication of the presence of human fecal pollution.

Method 1696.1 is based on the isolation of Bacteroidales on membrane filters, extraction of total DNA, and detection of human-associated target sequences in purified DNA extracts by real time qPCR using TaqMan® Environmental master mix PCR reagent and the TaqMan® probe chemistry. TaqMan® chemistry signals the formation of PCR products by a process involving the enzymatic hydrolysis of a labeled fluorogenic probe that hybridizes to the target sequence. In addition, this method incorporates a series of quality control (QC) parameters to ensure proper implementation and generation of high quality data.

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Characterization of Human Fecal Pollution in Water by TaqMan® Quantitative Polymerase Chain Reaction (qPCR) Assay

1.0 Scope and Application

- 1.1 This method describes a quantitative polymerase chain reaction (qPCR) procedure for the measurement of human-associated gene sequences from *Bacteroides* isolated from environmental water samples. This method is based on the collection of *Bacteroides* on membrane filters, extraction of total deoxyribonucleic acid (DNA), and detection of human-associated target sequences in purified DNA extracts by qPCR using TaqMan® Environmental Master Mix PCR reagent and probe system.
- 1.2 *Bacteroides* human-associated gene sequences are commonly found in the feces of humans (primary source) and secondary sources of human fecal pollution (sewage and septage). Although DNA from human-associated *Bacteroides* is sometimes present in other animal sources, typically at lower concentrations, their presence in environmental waters is generally considered an indicator of human fecal pollution (References 15.1-15.4).
- 1.3 This method is recommended for the characterization of human fecal pollution in ambient marine and fresh waters.
- 1.4 This protocol relies on the accurate and reproducible application of all method steps. Successful implementation requires that a laboratory is set up properly, all equipment is functional and calibrated, and staff exhibit an adequate proficiency in required molecular techniques, as well as proper laboratory preparation and storage of all reagents and reference DNA materials. Ongoing demonstration of laboratory capability should be documented through successful performance of the method (e.g., meet method-specific acceptance/criteria) prior to processing environmental water samples. Guidance is provided in Section 9 of this document on how laboratories can monitor the initial and ongoing performance of the method and ensure the generation of high quality data. It is expected and has been observed that the performance of the method will improve as laboratories gain additional experience with the method through continued use.
- 1.5 It is recommended that users perform a specificity test (**Appendix C**) with reference pollution source materials collected from non-human fecal sources in the same geographic area as water quality testing prior to analyzing environmental water samples. The shedding of HF183/BacR287 DNA target sequences in non-human animals may vary from one geographical location to another and/or one animal population to another. These rare, but potential differences could confound data interpretations.
- 1.6 It is recommended that users perform a sensitivity test (**Appendix C**) with reference fecal pollution source materials collected from potential human waste sources (e.g., onsite systems, wastewater treatment plants/facilities-prior to disinfection) in the same geographic area as water quality testing prior to analyzing environmental samples. The shedding of HF183/BacR287 DNA target sequences in human populations could vary from one geographic location to another and/or by source. Sensitivity testing is important to confirm the suitability of the method for human fecal source identification.

- 1.7** This method assumes the use of a Thermo Fisher Scientific Real-Time PCR System as the default platform. The user should refer to **Appendix A** for instrument-specific instructions. Users should thoroughly read the method in its entirety before preparation of reagents and analysis of environmental water samples.

2.0 Summary of Method

The method is initiated by concentrating an environmental water sample through a membrane filter. Following filtration, the membrane with bacterial cells containing DNA is placed in a microcentrifuge tube with glass beads and buffer, and then agitated to release DNA into solution. The total DNA suspended in the supernatant is then isolated and purified. The purified total DNA is used for qPCR amplification and measurement of target sequences using the TaqMan® Environmental Master Mix PCR reagent and probe system. Results are reported as log₁₀ copies per reaction.

3.0 Acronyms, Abbreviations and Definitions

3.1 Acronyms and Abbreviations

ANOVA	Analysis of variance
BSA	Bovine serum albumin
°C	Degree Celsius
C _q	Quantification cycle
DNA	Deoxyribonucleic acid
<i>E</i>	Amplification efficiency
EPA	U. S. Environmental Protection Agency
FEB	Fecal extraction blank
g	Gram
IAC	Internal amplification control
LLOQ	Lower limit of quantification
MB	Method blank
MLV	Multi-laboratory validation
MST	Microbial source tracking
µg	Microgram
µL	Microliter
µm	Micrometer
mg	Milligram
mL	Milliliter
ng	Nanogram

NIST	National Institute of Standards and Technology
NTC	No template control
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
QA	Quality assurance
QC	Quality control
qPCR	Quantitative polymerase chain reaction
R ²	R squared
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
ROQ	Range of quantification
SEB	Secondary extraction blank
SPC	Sample processing control
UV	Ultraviolet radiation
X	Times

3.2 Definitions

- 3.2.1** Human-associated *Bacteroides*: A subpopulation of microorganisms that is closely associated with human fecal material.
- 3.2.2** Target sequence: A segment of a human-associated *Bacteroides* gene containing a nucleotide sequence that is complementary to the primers and probe used in this qPCR assay.
- 3.2.3** Sample processing control (SPC) sequence: A segment of the ribosomal ribonucleic acid (rRNA) gene operon, internal transcribed spacer region 2 of chum salmon, *Oncorhynchus keta* and other salmon species, containing a nucleotide sequence that is complimentary to the primers and probe used in the SPC qPCR assay (Sketa22). SPC sequences are added as part of a total salmon DNA solution in equal quantities to all environmental water and method blank (MB) sample filters prior to extracting DNA. The purpose of this control is to monitor the efficiency of DNA extraction, screen for substances that may interfere with DNA purification and/or amplification, as well as identify any potential laboratory errors.
- 3.2.4** Reference DNA material: A purified, RNA-free and quantified plasmid DNA preparation. In this method, two different plasmid constructs are used, DNA standard reference material and an internal amplification control (IAC) spike. These reference materials are used to generate calibration curves, determine performance characteristics of the qPCR assay, and monitor for amplification interference (Sections 9.4 and 9.6, respectively).

- 3.2.4.1** DNA standards: A type of reference DNA material consisting of purified, RNA-free, and quantified plasmid DNA dilutions used to generate calibration curves for estimating the concentration of human-associated target sequences in environmental water samples and evaluating various performance characteristics of this qPCR assay (Sections 12.3 and 9.4, respectively).
- 3.2.4.2** Internal amplification control (IAC): Reference DNA material consisting of a purified, RNA-free and quantified plasmid DNA preparation spiked into the qPCR assay mix to monitor for amplification interference. This process is described in Section 9.6.
- 3.2.5** No template control (NTC): These controls ensure that reagents and/or the laboratory environment did not introduce contaminants that could result in false positives. NTC results are used to verify the absence of contaminating target sequences that may be introduced during preparation of the reagents and/or reactions and to establish the amplification interference threshold for inhibition testing (Section 9.2).
- 3.2.6** Method blank (MB): These controls (100 mL PCR-grade water samples) are used to verify the absence of measurable levels of contaminating target sequences that may be introduced during filtration, DNA extraction and/or preparation of the reagents or reactions. These controls are also used to establish acceptance thresholds for the SPC quality assurance (QA) tests (Section 9.5).
- 3.2.7** Fecal extraction blank (FEB): These controls are recommended for evaluation of method specificity and sensitivity testing prior to analysis of environmental water samples. FEB results are used to verify the absence of measurable levels of contaminating target sequences that may be introduced during DNA extraction and/or preparation of the reagents or reactions. The absence of a fluorescence amplification growth curve (e.g., undetermined) for the HF183/BacR287 assay indicates the absence of contaminant target DNA. Refer to **Appendix C** for detailed specificity and sensitivity procedures.
- 3.2.8** Internal amplification control (IAC) proficiency criteria: The potential for amplification inhibition remains a significant challenge when using qPCR to quantify target sequences isolated from environmental water samples (Reference 15.5). Since the presence of inhibitors can decrease the accuracy and precision of qPCR measurements, and in extreme circumstances can lead to false-negative results (Reference 15.6), the inclusion of an inhibition control with each environmental water sample helps ensure the integrity of the findings and promote consistency between laboratories (Reference 15.6). There are many strategies available to test for inhibition ranging from dilution testing to kinetic outlier detection (Reference 15.7), and any approach may be appropriate. The IAC acceptance criteria (Section 9, **Table 2**) is used on an ongoing basis to evaluate the performance of the IAC test for a particular laboratory and instrument run.
- 3.2.9** Sample processing control (SPC) proficiency metric: Criteria used to evaluate the performance of the SPC test for a particular laboratory and sample batch. This procedure is described in Section 9.5.

- 3.2.10** Amplification efficiency (*E*): A measure of the efficiency at which calibration curve standards are measured. *E* values should range from 0.90 to 1.10 (Reference 15.8) and are calculated from the calibration curve slope as described in Section 9.4.
- 3.2.11** R^2 : A measure of the variability in the qPCR assay calibration curve. R^2 values should be ≥ 0.980 (Reference 15.9) Section 9, Table 2.
- 3.2.12** Lower limit of quantification (LLOQ): The lowest concentration of target sequence that can be quantified with the calibration curve. The LLOQ is calculated from the reference DNA calibration curve (Section 9.4).
- 3.2.13** Reference pollution source material: An individual fecal sample (primary source) or secondary source of fecal pollution (sewage or septage) from a known source used to estimate method specificity, sensitivity, and measure abundance of target sequences in a local area of interest. [Appendix C]
- 3.2.14** Specificity: The ability of this method to discriminate between human and other fecal sources and is expressed as $d/(a + d) \times 100$, where *a* represents false positives and *d* represents true negatives. The specificity of this method (HF183/BacR287 assay) is typically $> 90\%$ (References 15.3 and 15.10), but may vary from one geographic location to another. Specificity is determined by testing primary non-human reference pollution source materials only (fecal only, do not include secondary sources [agricultural lagoons]). [Appendix C]
- 3.2.15** Sensitivity: The ability of this method to detect human fecal pollution sources is expressed as $b/(b + c) \times 100$, where *c* represents false negatives and *b* represents true positives. The sensitivity of the HF183/BacR287 method may vary from one geographic location to another or by human fecal pollution source (e.g., individual human fecal samples, septage, sewage). Sensitivity is determined by testing primary and/or secondary reference pollution source materials. [Appendix C]
- 3.2.16** Instrument run: Refers to all data generated from a single run on a thermal cycle instrument.
- 3.2.17** Batch: Refers to all environmental and method blank (MB), fecal extraction blank (FEB) or secondary extraction blank (SEB) samples subjected to DNA purification at the same time.
- 3.2.18** Quantification cycle (*C_q*): Nomenclature describing the fractional PCR cycle used for quantification (Reference 15.6).
- 3.2.19** Secondary extraction blank (SEB): These controls are recommended for evaluation of method sensitivity testing prior to analysis of environmental water samples. SEB results are used to verify the absence of measurable levels of contaminating target sequences that may be introduced during DNA extraction and/or preparation of the reagents or reactions. The absence of a fluorescence amplification growth curve (e.g., undetermined) for the HF183/BacR287 assay indicates the absence of contaminant target DNA. Refer to Appendix C for detailed sensitivity procedures.

4.0 Interferences

Environmental water samples containing colloidal or suspended particulates may interfere with DNA recovery and subsequent qPCR analysis. Interferences can bias estimated concentrations of a DNA target sequence. To identify potential interferences, SPC and IAC procedures are recommended (Sections 9.5 and 9.6, respectively).

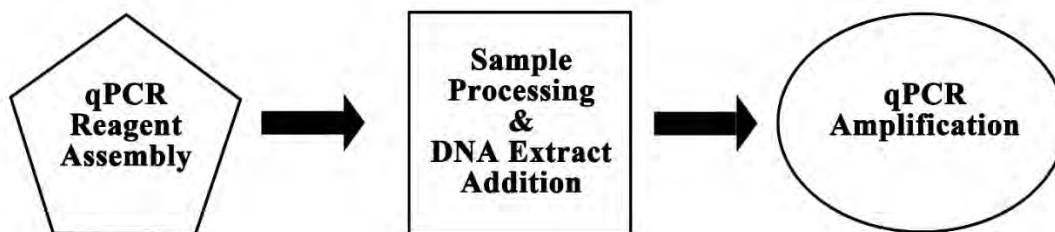
5.0 Safety

5.1 The analyst/technician must know and observe the normal safety procedures required in microbiology and molecular biology laboratories while preparing, using, and disposing of reagents/materials, and while operating sterilization equipment.

5.2 Mouth pipetting is prohibited.

6.0 Laboratory Organization, Equipment, and Supplies

6.1 Contamination from extraneous sources potentially introduced throughout a qPCR method can be problematic. DNA from equipment, other samples, and previously synthesized amplicons can contaminate qPCR amplifications leading to false positives and misinterpretation of results. Extraneous DNA from these sources can be limited through the use of physical barriers and dedicated equipment. It is recommended that qPCR reagent assembly, sample processing (filtering, DNA extraction, and addition of template), and qPCR amplifications occur in three separate laboratories or zones with dedicated equipment. In addition to physical barriers and dedicated equipment, qPCR analysis should progress in a single direction (**Figure 1**). Unidirectional progression prevents backtracking of purified DNA from environmental and reference samples, as well as qPCR amplicons generated from DNA amplification.



6.1.1 Separate 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 equipment and supplies (e.g., pipettors, tips, gloves), is required.

6.1.2 A workstation for sample filtration with dedicated supplies that is physically separate from the reagent preparation and DNA extraction workstations

- 6.2 Sterile bottles/containers for sample collection
- 6.3 Disposable membrane filtration units (filter base, polycarbonate filter [0.4 μm] with 47 mm diameter, and 100 mL capacity funnel), individually bagged, and gamma-irradiated (Pall MicroFunnels™ filter funnels FMFNL1050 or equivalent)
- 6.4 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.
- 6.5 Flask, filter, vacuum, usually 1 L, with appropriate tubing
- 6.6 Filter manifold to hold a number of filter bases
- 6.7 Flask for safety trap placed between the filter flask and the vacuum source
- 6.8 Polycarbonate membrane filters, white, 47 mm diameter, with 0.45 μm pore size (Millipore HHTP04700 or equivalent). *Note: These filters will not be needed if Pall MicroFunnels™ filter funnels FMFNL1050 (See 6.3) are used because polycarbonate membrane filters are supplied with funnel assembly.*
- 6.9 Stainless steel forceps, straight or curved, with smooth tips to handle filters without damage, 2 pairs
- 6.10 Permanent ink marking pen for labeling tubes
- 6.11 Microcentrifuge capable of 12,000 $\times g$
- 6.12 Homogenizer (MP Biomedicals, LLC. MP FastPrep-24™ 5^G or equivalent)
- 6.13 Micropipettors with 10, 20, 200 and 1000 μL capacity. Each workstation should have a dedicated set of micropipettors. Micropipettors should be calibrated on a regular basis.
- 6.14 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 environmental water samples. Each workstation should have a dedicated supply of tips.*
- 6.15 Microcentrifuge tubes, low-retention, clear, 1.7 mL (GENE MATE C-3228-1 or equivalent)
- 6.16 5 mL round bottom tube with snap cap (BD Falcon™ 352003 or equivalent [optional])
- 6.17 50 mL conical tubes (GENE MATE C-3394-3 or equivalent)
- 6.18 Sterile DNA extraction tubes containing glass beads (Gene-Rite LLC S0205-50 or equivalent) *Note: Extraction tubes with glass beads are included in the Gene-Rite K102-02C-50 DNA-EZ® RW02 (see Section 7.15).*
- 6.19 Rack for microcentrifuge tubes, use a separate rack for each set of tubes
- 6.20 Vortex mixer
- 6.21 Dedicated lab coats for each work station
- 6.22 Sterile disposable powder-free gloves for each work station
- 6.23 Refrigerator, 4°C (ideally, one for reagents and one for DNA samples)
- 6.24 Freezer, -20°C (storage of reagents and reference DNA stock solutions)
- 6.25 Freezer, -80°C (storage of filters)
- 6.26 Ice, crushed or cubes for temporary preservation of environmental water samples and reagents

- 6.27** Data archiving system (flash drive or other data storage system)
- 6.28** UV spectrophotometer capable of using small volume capacity (e.g., 0.1 mL) cuvettes or NanoDrop® (ND2000 spectrophotometer or equivalent) capable of the same measurements at 2 µL sample volumes or other technology capable of estimating the concentration of DNA molecules.
- 6.29** Dry block heater (VWR 75838-270 or equivalent) and 2 mL tube blocks (VWR 12985-048 or equivalent) or microbiological incubator
- 6.30** Thermo Fisher Scientific StepOnePlus™, 7900, or QuantStudio™ 6
- 6.30.1** Vendor recommended optical 96 well PCR reaction tray (Fisher Scientific N8010560 or equivalent)
- 6.30.2** Optical adhesive PCR reaction tray tape (Fisher Scientific 4311971 or equivalent) or (MicroAmp™ caps N8010534 or equivalent)
- 6.30.3** Aluminum-foil sealing films for storage (AlumaSeal96™ F-96-100 or equivalent)

7.0 Reagents and Standards

- 7.1** Purity of Reagents: Molecular-grade reagents and chemicals shall be used in all tests
- 7.2** Sample Processing Control (SPC) DNA: Salmon testes DNA (Sigma D7656-1mL or equivalent)
- 7.3** PCR-grade water (OmniPur water [VWR EM-9610 or equivalent]). Water must be DNA/DNase free.
- 7.4** AE Buffer (Qiagen 19077 or equivalent)
- Composition:
- 7.4.1** 10 mM Tris-Cl (Tris-chloride)
- 7.4.2** 0.5 mM EDTA (Ethylenediaminetetraacetic acid)
- 7.5** 1X Phosphate Buffered Saline, (Thermo Fisher Scientific BP2438-4 or equivalent) [**Appendix C**]
- 7.5.1** Composition:
- | | |
|--|--------|
| Monosodium phosphate (NaH ₂ PO ₄) | 0.58 g |
| Disodium phosphate (Na ₂ HPO ₄) | 2.50 g |
| Sodium chloride | 8.50 g |
| PCR-grade water | 1.0 L |
- 7.5.2** Dissolve reagents in 1 L of PCR-grade water in a flask and dispense in appropriate amounts. Final pH should be 7.4 ± 0.2.
- 7.6** Bleach solution: 10% v/v bleach (or other reagent that hydrolyzes DNA), used for cleaning work surfaces
- 7.7** Sterile water (used as rinse water for work surface after bleaching)
- 7.8** TaqMan® Environmental PCR Master Mix 2.0 (Thermo Fisher Scientific 4396838 or equivalent)
- 7.9** Bovine serum albumin (BSA), fraction V (Gibco™ 15260037 or equivalent)
- Dilute in PCR-grade water to a concentration of 2 mg/mL.

7.10 Primer and probe sets: Primer and probe sets may be purchased from commercial sources. Primers should be desalted, probes should be HPLC purified.

7.10.1 HF183/BacR287 primer and probe set:

Forward primer (HF183): 5'-ATCATGAGTTCACATGTCCG-3'

Reverse primer (BacR287): 5'-CTTCCTCTCAGAACCCCTATCC-3'

TaqMan[®] probe (BacP234MGB): [6-FAM]-5'-CTAATGGAACGCATCCC-MGB

TaqMan[®] probe (Bac234IAC): [VIC]-5'-AACACGCCGTTGCTACA-MGB

7.10.2 Sketa22 primer and probe set:

Forward primer (SketaF2): 5'-GGTTTCCGCAGCTGGG-3'

Reverse primer (SketaR2): 5'-CCGAGCCGTCCTGGTC-3'

TaqMan[®] probe (SketaP2): [6-FAM]-5'-AGTCGCAGGCGGCCACCGT-TAMRA

7.10.3 Preparation of primer/probes: Using a micropipettor with aerosol barrier tips, add AE Buffer to the lyophilized primers from the vendor to create stock solutions of 500 µM and dissolve by gently vortexing. Pulse centrifuge to coalesce droplets. Probes arrive from vendor in liquid form at 100 µM concentration. Store stock solutions at -20°C.

7.11 Purified, RNA-free quantified and characterized DNA standard reference material (Section 7.16).

5'- ATCATGAGTTCACATGTCCGCATGATTAAGGTATTTTCCGGTAGACGATGGGGA
TGCGTTCCATTAGCTCGAGATAGTAGGCGGGGTAACGGCCCACCTAGTCAACGATG
GATAGGGGTTCTGAGAGGAAGG-3'

7.12 Purified, RNA-free quantified and characterized IAC plasmid (Section 7.17).

5'- ATCATGAGTTCACATGTCCGCATGATTAAGGTATTTTCCGGTAGACGATGTGTA
GCAACGGCGTGTTATAGTAGGCGGGGTAACGGCCCACCTAGTCAACGATGGATAGG
GGTTCTGAGAGGAAGG-3'

7.13 Not1-HF[™] RE-Mix[®] restriction endonuclease 10X master mix (New England BioLabs R3189 or equivalent). *Note: A different restriction endonuclease may be necessary if the plasmid vector does not contain a unique NotI restriction site.*

7.14 QIAGEN QIAquick PCR Purification Kit (28104 or equivalent)

7.15 DNA extraction kit (pre-loaded bead tubes, binding buffer, washing buffer, elution buffer, DNAsure columns, and collection tubes [Gene-Rite K102-02C-50 DNA-EZ[®] RW02 or equivalent])

7.16 Preparation of Plasmid-Derived DNA Standards

DNA standards should be prepared in a laboratory or work area (Section 6.1) separate from areas used for reagent mixing, sample filtration, and DNA purification, using dedicated supplies (e.g., pipet tips) and instruments (e.g., microcentrifuge) to prevent cross-contamination with environmental water samples.

Plasmid-derived DNA standard reference materials (Section 7.11) contain sequences corresponding to human-associated primer and hydrolysis probe sets and can be constructed in-house or ordered from companies specializing in custom gene synthesis. *Note: Plasmid reference materials should be sequenced to confirm that primer and probe sequences are correct prior to use.*

- 7.16.1** Follow plasmid resuspension instructions provided by vendor.
- 7.16.2** Digest $\geq 4 \mu\text{g}$ of plasmid with the NotI-HF™ RE-Mix® (Section 7.13) reagent according to manufacturer instructions. A successful endonuclease restriction digest should result in a single cut to linearize the plasmid. *Note: A different restriction endonuclease may be necessary if the plasmid vector does not contain a unique NotI restriction site.*
- 7.16.3** Clean digested product using the QIAGEN QIAquick PCR Purification kit or equivalent (Section 7.14) according to manufacturer directions. Final elution volume should be $50 \mu\text{L}$.
- 7.16.4** Measure spectrophotometric absorbance of cleaned product at 260 nm (A_{260}) in triplicate and average the readings. *Note: Other methods such as digital PCR or fluorescence-based DNA quantitation are also acceptable.*
- 7.16.5** Use plasmid length (bp) and measured average concentration of plasmid solution ($\text{ng}/\mu\text{L}$) to determine plasmid copies per μL of plasmid solution as follows:

$$\text{Number of copies (molecules)}/\mu\text{L} = \frac{X \text{ ng}/\mu\text{L} \times 6.0221 \times 10^{23} \text{ molecules/mole}}{(N \times 650 \text{ g/mole}) \times (1 \times 10^9 \text{ ng/g})}$$

Where X indicates the measured average mass of plasmid DNA ($\text{ng}/\mu\text{L}$), N represents the length of the plasmid, 6.0221×10^{23} denotes Avogadro's number (molecules/mole), and 650 g/mole is the average mass of 1 bp of double stranded DNA.

Example: Calculation for a $3,000 \text{ bp}$ plasmid solution with an average concentration of $5 \text{ ng}/\mu\text{L}$ is as follows:

$$\text{Number of copies (molecules)}/\mu\text{L} = \frac{5 \text{ ng}/\mu\text{L} \times 6.0221 \times 10^{23} \text{ molecules/mole}}{(3,000 \times 650 \text{ g/mole}) \times (1 \times 10^9 \text{ ng/g})}$$

$$\text{Number of copies (molecules)}/\mu\text{L} = 1.54 \times 10^9$$

- 7.16.6** Using the plasmid stock solution average number of copies/ μL values calculated in Section 7.16.5, prepare the following plasmid reference DNA material dilutions in AE buffer: 10^5 copies/ $2 \mu\text{L}$, 10^4 copies/ $2 \mu\text{L}$, 10^3 copies/ $2 \mu\text{L}$, 10^2 copies/ $2 \mu\text{L}$, and 10^1 copies/ $2 \mu\text{L}$. *Note: Dilutions are prepared for a $2 \mu\text{L}$ volume to increase pipetting accuracy.*

Example: Preparation of 10^5 copies/2 μL , 10^4 copies/2 μL , 10^3 copies/2 μL , 10^2 copies/2 μL , and 10^1 copies/2 μL dilutions using a plasmid stock solution with 1.54×10^9 copies/ μL is as follows:

Step 1: Prepare a 1:100 dilution of plasmid stock solution by adding 10 μL of the plasmid stock solution to 990 μL AE Buffer to yield 1.54×10^7 copies/ μL and mix well.

Step 2: Prepare a 10^6 copies/2 μL dilution in a final volume of 10 mL

$$\frac{(10^6 \text{ copies}/2 \mu\text{L}) \times 10,000 \mu\text{L}}{1.54 \times 10^7 \text{ copies}/\mu\text{L}} = 325 \mu\text{L plasmid stock solution}$$

Create a 10^6 copies/2 μL plasmid solution by adding 325 μL of 1.54×10^7 copies/ μL of the diluted plasmid stock solution to 9,675 μL AE Buffer and mix well.

Step 3: Prepare 10^5 copies/2 μL , 10^4 copies/2 μL , 10^3 copies/2 μL , 10^2 copies/2 μL , and 10^1 copies/2 μL dilutions by making serial dilutions (10-fold dilutions).

For example, to make the 10^5 copies/2 μL plasmid solution, add 1 mL of the 10^6 copies/2 μL plasmid solution to 9 mL of AE Buffer and mix well.

Note: An automated DNA copy number and dilution calculator can be found at <https://www.thermofisher.com/us/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/dna-copy-number-calculator.html>

- 7.16.7** Prepare aliquots of each dilution and store in low-retention plastic microcentrifuge tubes at -20°C . Aliquots should be discarded after three freeze/thaw cycles to minimize the effect of template degradation (**Table 1**).

7.17 Preparation of IAC

The IAC should be prepared in a laboratory separate from work areas (Section 6.1) used for reagent mixing, sample filtration, and DNA extraction and purification, using dedicated supplies and instruments to prevent cross-contamination with environmental water samples.

IAC plasmids contain a sequence corresponding to the human-associated primers and the Bac234IAC (HF183/BacR287) probe sequences and can be constructed in-house or ordered from companies specializing in custom gene synthesis. **Note:** Plasmid IAC reference materials should be sequenced to confirm that primer and probe sequences are correct.

- 7.17.1** Follow plasmid resuspension instructions provided by vendor.
- 7.17.2** Digest $\geq 4 \mu\text{g}$ of plasmid with the NotI-HF™ RE-Mix® (Section 7.13) reagent according to manufacturer instructions. A successful endonuclease restriction digest should result in a single cut to linearize plasmid. **Note:** A different restriction endonuclease may be necessary if the plasmid vector does not contain a unique NotI restriction site.
- 7.17.3** Clean digested product using the QIAGEN QIAquick PCR Purification kit (Section 7.14) according to manufacturer directions. Final elution volume should be 50 μL .

- 7.17.4** Measure spectrophotometric absorbance of cleaned product at 260 nm (A_{260}) in triplicate and average the readings. *Note: Other methods such as digital PCR or fluorescence-based DNA quantitation are also acceptable.*
- 7.17.5** Use plasmid size (base pairs) to determine plasmid copies per gram as described above in Section 7.16.5 and 7.16.6.
- 7.17.6** Use absorbance reading to calculate the concentration of the plasmid in copies per 2 μL . Use this value to make the following plasmid dilution in AE buffer (Section 7.4): 100 copies/2 μL . *Note: Dilutions are prepared for a 2 μL volume to increase pipetting accuracy.*
- 7.17.7** Prepare aliquots of the dilution and store in low-retention plastic microcentrifuge tubes at -20°C . Aliquots should be discarded after three freeze/thaw cycles to minimize the effect of template degradation on results. See Table 1 (below) for further information on recommended storage and handling.

7.18 Salmon DNA Extraction Buffer

Salmon DNA SPC preparations should be prepared in a laboratory or work area separate from areas used for reagent mixing, sample filtration, and DNA extraction and purification (Section 6.1) using dedicated supplies and instruments to prevent cross-contamination with environmental water samples.

- 7.18.1** Dilute an aliquot of the stock salmon testes DNA (10 mg/mL, Section 7.2) to a concentration of 10 $\mu\text{g}/\text{mL}$ (1:1000 dilution) by adding 50 μL of the 10 mg/mL stock to 49.95 mL AE buffer (Section 7.4).
- 7.18.2** Measure spectrophotometric absorbance at 260 nm (A_{260}) of the 10 $\mu\text{g}/\text{mL}$ solution. *Note: If a blank measurement is required to calibrate instrument, use AE buffer.*
- 7.18.3** Use absorbance reading (1 OD = 33 $\mu\text{g}/\text{mL}$) to calculate volume of AE buffer needed to dilute the 10 $\mu\text{g}/\text{mL}$ solution to a 0.2 $\mu\text{g}/\text{mL}$ salmon DNA working stock. *Note: Document the dilution factor used to prepare 0.2 $\mu\text{g}/\text{mL}$ working stock.*

$$[(A_{260} \text{ value}) \times (33 \mu\text{g}/\text{mL})]/[0.2 \mu\text{g}/\text{mL}] = \text{required dilution}$$

Example Calculation:

$$A_{260} = 0.303$$

$$\frac{[(0.303) \times (33 \mu\text{g}/\text{mL})]}{[0.2 \mu\text{g}/\text{mL}]} = 50 \text{ fold dilution}$$

- 7.18.4** Make 1 mL aliquots of 10 $\mu\text{g}/\text{mL}$ working stock solution and store in low-retention plastic microcentrifuge tubes at 4°C . Store 1 mL aliquots of salmon DNA 10 $\mu\text{g}/\text{mL}$ solution at 4°C for no longer than 12 months. See Table 1 (below) for further information on proper storage and handling. *Note: Sketa22 control mean Cq can vary from one salmon DNA 10 $\mu\text{g}/\text{mL}$ solution preparation to another. To avoid discontinuity within a study requiring more than 50 mL of salmon DNA 10 $\mu\text{g}/\text{mL}$ solution over a 12-month period, prepare a larger volume appropriate for respective study needs.*
- 7.18.5** Determine the total volume of Salmon DNA extraction buffer required for each day by multiplying volume (600 μL per tube) \times total number of samples to be analyzed including controls and environmental water samples. Extraction buffer may be

prepared in advance and stored at 4°C for a maximum of 24 hours. *Note: Discard excess 0.2 µg/mL working stock after DNA extraction on a daily basis.*

For example, for 7 environmental water samples (7 × 3 replicates = 21 filters), prepare enough Salmon DNA extraction buffer for 26 extraction tubes, (21 tubes for environmental water samples, 3 extra tubes for MBs (Sections 9.3 and 11.1), and 2 extra tubes to account for losses due to mixing and pipetting).

- 1) The total volume needed: 600 µL × 26 tubes = 15,600 µL
- 2) Dilute the Salmon testes DNA working stock 1:50 (Section 7.18.3)

$$15,600 \mu\text{L} \div 50 = 312 \mu\text{L of } 10 \mu\text{g/mL Salmon testes DNA working stock.}$$

The AE buffer needed is the difference between the total volume Salmon DNA extraction buffer and the Salmon testes DNA working stock.

For this example, 15,600 µL - 312 µL = 15,288 µL AE buffer needed

Proper storage of DNA preparations is important to ensure consistency of method performance. Most aliquots can be stored for an extended period of time. See Table 1 for recommended storage conditions. *Note: All reference DNA material preparations should be stored in low-retention plastic microcentrifuge tubes at all times (See Section 6.15).*

Table 1. Recommended Storage Conditions for Reference DNA Material Preparations

Type	Reference DNA Material	Storage Temp	Storage Duration
Storage Stock	Calibration curve DNA standard	-20°C	12 months
	Internal amplification control (IAC)		
	Method blank salmon DNA 10 mg/mL	4°C	
	Method blank salmon DNA 10 µg/mL		
Working Stock	Internal amplification control (IAC)	4°C	30 days
	Calibration curve DNA standard		24 hours
	Method blank salmon DNA 10 µg/mL		

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. Environmental water samples not collected and handled according to these procedures should not be analyzed.

8.2 Sampling Techniques – Environmental water 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. The sampling depth for surface water samples should be 6 - 12 inches below the water surface. Sterile ≥ 500 mL 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 environmental water samples at a temperature of <10°C during transit to the laboratory and prior to initiation of filtration. Do not freeze the samples. Use insulated containers to maintain proper storage

temperature. Ensure that sample bottles are tightly closed and are not completely immersed in ice water during transit.

- 8.4 Holding Time Limitations** – Filter environmental water samples as soon as possible after collection. Do not hold environmental water samples longer than 6 hours between sample collection and initiation of filtration.

9.0 Quality Control

- 9.1** Quality control (QC) parameters are necessary to generate reliable estimates of target sequence concentration in environmental test samples. Successful implementation of this method requires that the laboratory is setup properly (Section 6.1), all equipment is functional and calibrated (Sections 6 and 10), and staff are proficient with molecular techniques, as well as proper laboratory preparation and storage of all reagents and reference DNA materials (Section 7). Errors can arise from numerous sources in the qPCR method ranging from improper sample handling, degradation of DNA standards, and lack of laboratory technician proficiency, to interferences originating from the environmental sample itself. Each laboratory is required to operate a formal 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.

The minimum analytical QC requirements for the analysis of samples includes an initial demonstration of method proficiency prior to processing environmental samples. A detailed procedure for demonstrating initial method proficiency is provided in **Appendix B**. The analysis of environmental samples should not be conducted until the analyst is able to demonstrate proficiency with the method according to **Appendix B** and the criteria provided in Table 2. Ongoing demonstration of analyst capability should be documented through successful performance of the method (e.g., meeting method performance criteria, Table 2) when run with environmental samples. Failure to ‘PASS’ all acceptance criteria can result from a number of reasons such as poor laboratory technique, improper preparation/storage of reference DNA materials, and/or poor calibration of equipment (i.e., pipets, qPCR instrument).

The method performance criteria (Table 2) were derived from a multi-laboratory validation study (Reference 15.11), Thermo Fisher Scientific recommendations (Reference 15.8), and qPCR reports (References 15.9 and 15.6). These analyses (**Appendix B**) should be repeated for each new preparation of DNA reference materials (DNA standards [Section 7.16], IAC plasmid DNA [Section 7.17], and SPC salmon DNA [Section 7.18]).

Values for each metric are determined from a series of analyses that are designed to generate calibration curve, NTC, and MB control data (**Appendix B**).

Table 2. Method Performance Criteria

Metric	Acceptance Criteria	Data Source	Reference
R ²	≥ 0.98 ¹	Calibration curve data per instrument run	15.9
Amplification efficiency (E)	0.90 to 1.10 ¹		15.8
No-template controls (NTC)	Undetermined result in all HF183/BacR287 and Sketa22 test reactions ²	6 test reactions per instrument run	15.11
Method blank (MB)	Undetermined result in all HF183/BacR287 test reactions ²	3 MBs with triplicate reactions per extract	

Metric	Acceptance Criteria	Data Source	Reference
Internal amplification control (IAC) proficiency	Multiplex VIC Cq standard deviation ≤ 1.16 Cq for HF183/BacR287	6 NTC reactions	
Sample processing control (SPC) proficiency	Sketa22 assay standard deviation ≤ 0.62 Cq	3 method blanks with triplicate reactions per extract	

¹ Value calculated from single instrument run using calibration curve data generated from a minimum of five different standard concentrations with at least triplicate reactions at each dilution level.

² Although no reaction should yield a Cq value, Cq values greater than the LLOQ are acceptable for quantification applications only. However, the laboratory should report this practice including all control data results.

- 9.2 No Template Control (NTC)** – For the NTC 2 μ L of PCR-grade water (Section 7.3) is added to the NTC wells on a PCR plate in place of DNA reference material or sample DNA extract. The laboratory should set up six NTC reactions per plate. If any of the NTC reactions from an instrument run elicit true positive logarithmic amplification with quantification cycle (Cq) values below 40, the analyses should be repeated after cleaning work areas and instruments, and using new working stock preparations. *Note: Although NTC reactions should not yield a ≤ 40 Cq, values greater than the LLOQ are acceptable for quantification applications only. However, the laboratory should report this practice including all NTC results.*
- 9.3 Method Blank (MB)** – Filtration of 100 mL of PCR-grade water that is processed, and analyzed in the same manner as environmental samples. Refer to Section 11.2 for filter preparation and Section 11.3 for DNA extraction. The absence of a fluorescence amplification growth curve for HF183/BacR287 assay (e.g., undetermined) indicates the absence of contaminant target DNA. Prepare three MB filters for each batch of samples. If any of the MB reactions from an instrument run elicit true positive logarithmic amplification with Cq values below 40 for HF183/BacR287, the analyses should be repeated after cleaning work areas and instruments, and using new working stock preparations. *Note: MB extracts contain salmon DNA, but should not yield Cq values ≤ 40 with the HF183/BacR287 assay.*
- 9.4 DNA Standards and Calibration Curves** – A calibration curve should be generated from triplicate analyses of each dilution of DNA standard reference material and subjected to linear regression analysis. Calibration curve acceptance criteria are used to evaluate the suitability of a particular curve for estimating target DNA sequence concentrations in environmental water samples. Acceptable performance is assessed based on three metrics R^2 , amplification efficiency (E), and LLOQ. **Figure 2** provides an example calibration curve that meets the method acceptance criteria. In the event that values from a subsequent calibration curve are outside of these acceptance ranges, the DNA standards should be re-analyzed. If this difference persists, new working stocks of reference DNA standard material should be prepared and tested.

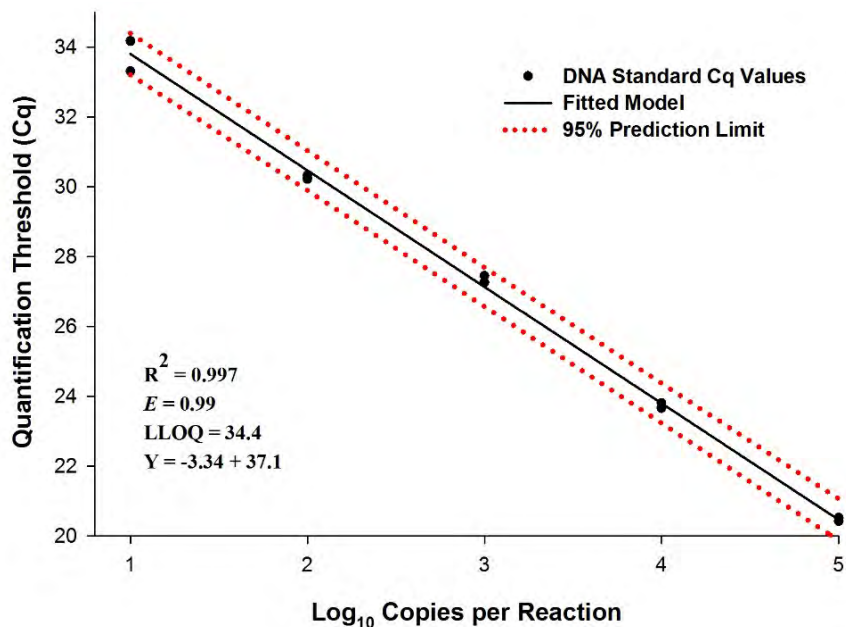


Figure 2. HF183/BacR287 Calibration Curve

The calibration curve is generated from a single instrument run including triplicate reactions at each calibration curve DNA standard dilution ranging from 1 to 5 log₁₀ copies per reaction. Shaded black circles indicate replicate DNA standard Cq values and the black line represents the simple linear regression fitted line, and the dotted red line denotes the 95% prediction limit. E indicates the amplification efficiency. LLOQ refers to the 95% prediction upper limit at the 1 log₁₀ copy DNA standard dilution.

- 9.5 Sample Processing Control (SPC)** – Variability in sample processing efficiency is measured for each environmental sample filter with a spike preparation consisting of a fixed concentration of salmon DNA. The resulting DNA eluate is tested with the Sketa22 qPCR assay. The demonstration of consistent spiked DNA recovery from one filter DNA extract to the next is achieved by establishing a SPC acceptance threshold based on repeated control experiments. Environmental test filter DNA extracts that fail the SPC acceptance threshold are either eligible for Cq adjustment or should be considered invalid (**Figure 3**).

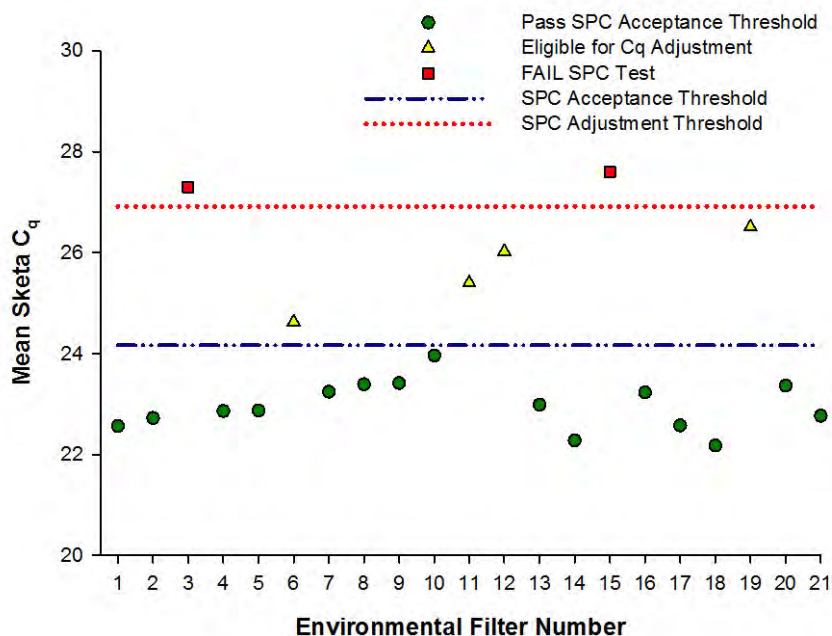


Figure 3. SPC Data from a Single Instrument Run of 21 Environmental Samples

The dash-dot line (blue) denotes the SPC acceptance threshold based on the Sketa22 MB mean C_q + (3 standard deviations). The dotted line (red) represents the SPC adjustment threshold (Sketa22 MB mean C_q + 3.3). Green circles represent environmental filters within the SPC acceptance threshold range. Environmental test filters that fail the SPC acceptance threshold may be eligible for C_q adjustment if the shift between the environmental sample mean Sketa22 C_q and SPC adjustment threshold is ≤ 3.3 C_q. Yellow triangles represent test filters that failed the SPC acceptance threshold, but are eligible for C_q adjustment (pass SPC adjustment threshold). Red boxes show filters that fail the SPC acceptance threshold and are not eligible for C_q adjustment. These data should be invalidated and removed from the data set prior to data interpretation.

9.5.1 SPC acceptance threshold determination. The SPC acceptance threshold is based on three MB filters spiked with salmon DNA (triplicate C_q measurements for each MB extract) per batch. A mean and standard deviation are then calculated from the resulting MB Sketa22 qPCR C_q data. The batch-specific SPC acceptance threshold equals the Sketa22 MB control mean C_q + (3 standard deviations). Test sample mean C_q values below the respective SPC acceptance threshold indicates acceptable sample processing efficiency.

9.5.2 Eligibility determination for C_q adjustment. Unacceptable test sample Sketa22 C_q values indicate that the HF183/BacR287 C_q measurements are not suitable for data interpretation without accounting for sample matrix interference. Test samples that fail the SPC acceptance threshold are eligible for HF183/BacR287 C_q adjustment if the difference between the respective test sample mean Sketa22 C_q and SPC acceptance threshold is less than 3.3 C_q [(test sample mean Sketa22 C_q – SPC acceptance threshold) ≤ 3.3 C_q]. Test samples that fail the SPC acceptance threshold and are not eligible for adjustment should be removed from the data set.

9.6 **Monitoring for Inhibition with the IAC** – The IAC strategy used in this method evaluates the suitability of purified DNA for qPCR amplification. This competitive amplification approach

utilizes a composite primer technique where the target and the IAC are simultaneously amplified with a common set of primers and under the same conditions in the same reaction. The only difference between the two constructs is the probe sequence and respective reporter molecules (FAM for the target sequence and VIC for the IAC). The criterion for establishing the presence or absence of inhibition in an environmental sample DNA extract relies on data from the calibration curve, NTC reactions, and environmental samples from the same instrument run.

9.6.1 Instrument run-specific amplification interference threshold. The instrument run-specific amplification interference threshold is calculated as follows: [mean VIC NTC Cq + (3 standard deviations)]. In each instrument run, the six VIC NTC results representing ideal conditions (PCR-grade water in the absence of other DNA target sequences or interfering substances) are used to establish the amplification interference threshold. A three-standard deviation threshold is used to establish a range inclusive of approximately 99.7% of measurements under control experimental conditions. Individual reactions from an environmental sample DNA extract can either “FAIL” (VIC Cq > interference threshold) or “PASS” (VIC Cq < interference threshold). If at least two of the three replicates “PASS”, then the environmental sample DNA extract shows no evidence of amplification interference. However, if two or all three replicates “FAIL”, then data suggests the presence of amplification interference.

9.6.2 Distinguishing between Inhibition and Competition. Amplification interference can result from either inhibition (interference from substances that persist in the environmental sample DNA extract after DNA extraction) or competition between the native human-associated target sequence and IAC spike. To discriminate between inhibition and competition, an IAC range of quantification (ROQ) and competition threshold are determined for each instrument run. An instrument run-specific IAC ROQ is derived using VIC Cq data from the 10^2 copy/reaction IAC spike associated with each DNA standard concentration (10 , 10^2 , 10^3 , 10^4 , 10^5 copies/reaction) in multiplex reactions. The range of standard concentrations where at least two or more of the three replicates “PASS” (VIC Cq < interference threshold) for each standard dilution indicate the respective instrument run-specific IAC ROQ. For example, **Figure 4** depicts an IAC ROQ range from 1 to $3 \log_{10}$ copies per reaction. The competition threshold is defined as the calibration model FAM Cq that intersects the upper bound of a respective instrument run-specific IAC ROQ. Any environmental sample DNA extract exhibiting amplification interference (determined from IAC assay VIC Cq measurements), where the sample mean FAM Cq from the native sequence target (calculated from environmental sample DNA extract triplicate FAM Cq measurements) is greater than the respective competition threshold indicates inhibition and should be invalidated and removed from the data set. Environmental sample DNA extracts indicating evidence of amplification interference with filter mean FAM Cq values less than the respective competition threshold are influenced by competition between the IAC and the sample DNA target sequences rather than inhibition.

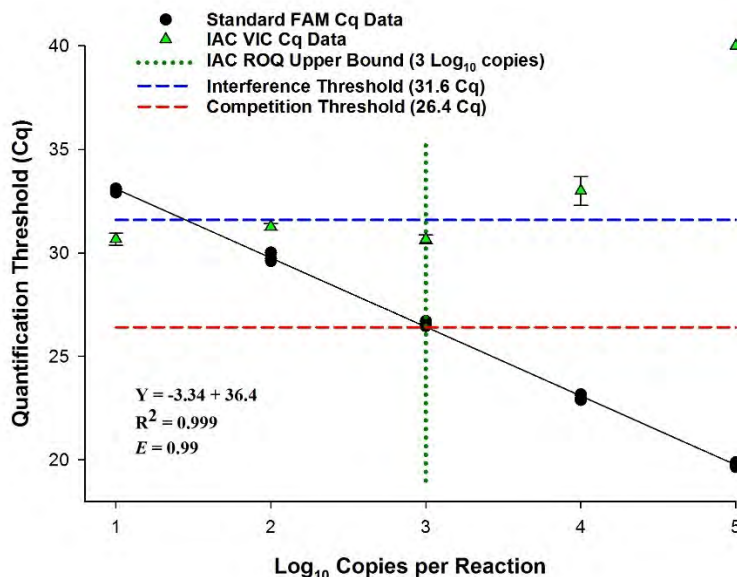


Figure 4. Plot of Human-Associated qPCR Assay HF183/BacR287 Calibration

The calibration curve is a curve from a single instrument run and includes the determination of internal amplification control range of quantification (IAC ROQ), and establishment of a competition threshold.

9.6.3 Options when Test Filter DNA Extract shows Evidence of Inhibition. Data associated with a sample DNA extract with evidence of amplification inhibition are both invalidated and removed from the data set or the DNA extract can be diluted and re-tested. *Note: If the DNA extract is diluted, then the log₁₀ copies per reaction determination must be adjusted based on the dilution factor.*

9.6.4 Options when Test Filter DNA Extract shows Evidence of Competition. Competition results when the quantity of the human-associated target sequence prevents normal amplification of the IAC spike. If sample DNA extracts are consistently showing evidence of competition, the DNA extract can be diluted and re-tested. *Note: If the DNA extract is diluted, then the log₁₀ copies per reaction determination must be adjusted based on the dilution factor.*

9.7 Specificity, Sensitivity, and Measuring Abundance of Target Sequences in Local Sampling Area (Appendix C) – Specificity refers to the ability of the human-associated method to discriminate between human and other fecal pollution sources. The HF183/BacR287 fecal source identification qPCR method is reported to be closely associated with human fecal pollution (References 15.1-15.3, 15.10). However, these methods do not always exclusively detect human pollution. For example, there are reports that HF183/BacR287 can cross-react with DNA targets in some dog, deer, and chicken populations, albeit typically at much lower concentrations compared to human sources (Reference 15.10). The shedding of HF183/BacR287 DNA target sequences in non-human animals may vary from one geographical location to another and/or one animal population to another. These rare, but potential differences could confound data interpretations. As a precaution, it is recommended that users perform a specificity test with reference pollution source materials collected from potential sources in the same geographic area as water quality testing prior to analyzing environmental water samples. If false positives are observed, it is then recommended that the abundance of target (human sources) and non-target (other animal sources) sequences is determined to aid in data interpretation. The prevalence and

concentration of HF183/BacR287 target sequences in human waste source types such as individual fecal samples, sewage, and septage may also vary from one population to another or source type. As a result, it is recommended that users perform a sensitivity test with human waste reference pollution source materials collected in the same geographic area as water quality testing prior to analyzing environmental samples. This information can be used to confirm the presence of human-associated DNA target sequences in human waste source types in the area of interest. Refer to **Appendix C** for detailed description of process.

- 9.8 Fecal Extraction Blank (FEB) (Appendix C)** – For a FEB, 300 µL of PCR-grade water (Section 7.3) is added to the extraction tube instead of fecal material prior to the bead beating process. These controls are recommended for evaluation of human-associated method specificity and sensitivity testing. Refer to **Appendix C** for preparation and DNA extraction. The absence of a fluorescence amplification growth curve from the human-associated assays during qPCR analysis of these controls (reported as “undetermined” on Thermo Fisher Scientific instruments) indicates the absence of contaminant target DNA. Prepare three FEB extractions for each batch of samples. *Note: Although FEB reactions should not yield a ≤ 40 Cq, values greater than the LLOQ are acceptable for quantification applications only. However, the laboratory should report this practice including all FEB results.*
- 9.9 Secondary Extraction Blank (SEB) (Appendix C)** – For a SEB, 250 µL of PCR-grade water (Section 7.3) is substituted for secondary source samples (sewage or septage) during sample filtration. These controls are recommended for evaluation of human-associated method sensitivity testing prior to analysis of environmental water samples. Refer to **Appendix C** for preparation and DNA extraction. The absence of a fluorescence amplification growth curve from the human-associated assays during qPCR analysis of these controls (reported as “undetermined” on Thermo Fisher Scientific instruments) indicates the absence of contaminant target DNA. Prepare three SEB extractions for each batch of samples. *Note: Although SEB reactions should not yield a ≤ 40 Cq, values greater than the LLOQ are acceptable for quantification applications only. However, the laboratory should report this practice including all SEB results.*

10.0 Calibration and Standardization of Method-Related Instruments

- 10.1** Check temperatures in refrigerators and freezers daily and record to ensure correct operation.
- 10.2** Check thermometers at least annually against a National Institute of Standards and Technology (NIST) certified thermometer or one that meets the requirements of NIST Monograph SP 250 - 23.
- 10.3** The spectrophotometer may need to be calibrated each day of use using optical density calibration standards between 0.01 – 0.5. Follow manufacturer instructions for calibration if needed.
- 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: Salmon DNA extraction buffer (Section 7.18) should be prepared each day of analyses. Prepare assay mixes each day before handling environmental water samples.

11.1 Environmental water sample filtration and method blank (MB) preparation

Note: Three replicates (100 mL) of each environmental water sample should be processed and analyzed. Three MB samples should also be filtered and analyzed with every batch of environmental water samples. They should be processed after environmental water samples for each of the steps described below.

- 11.1.1 Place a fresh membrane filtration funnel assembly (Section 6.3) on the filter base.
Note: If using a disposable filtration unit, confirm that the unit contains the correct filter type – replace if necessary. (Section 6.8)
- 11.1.2 Shake the environmental water sample bottle vigorously 25 times to distribute the bacteria uniformly, and measure 100 mL of the environmental water sample using the graduated markings on the funnel. Filter sample.
- 11.1.3 After filtering the sample, turn off the vacuum.
- 11.1.4 Label an extraction tube containing glass beads (Section 6.18) to identify environmental water sample. Remove the funnel from the filter base. Using sterile forceps, 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 environmental water sample. Insert the rolled filter into the labeled extraction tube with glass beads.
- 11.1.5 Filter the remaining environmental water samples and place filters in labeled extraction tubes with glass beads (Section 11.1.4). Filter MB samples (100 mL PCR-grade water, Section 7.3) and place filters in labeled extraction tubes with glass beads (Section 11.1.4).
- 11.1.6 Store tubes containing folded filters at -80°C until time of DNA extraction and purification. *Note: Filters should not be stored for more than 18-months.*

11.2 DNA extraction of environmental sample and method blank filters

The following are modified instructions for using the Gene-Rite DNA extraction kit (Section 7.15). *Note: Do not follow the manufacturer instructions.*

- 11.2.1 Warm elution buffer (Section 7.15) to 60°C using an incubator or heating block (Section 6.29).
- 11.2.2 Using a 1000 µL micropipettor, dispense 600 µL of the Salmon DNA extraction buffer (Section 7.18) into each labeled extraction tube with glass beads containing environmental water sample or MB filters from Section 11.1. Process the MB filters last.
- 11.2.3 Tightly close the tubes, making sure that the O-ring is seated properly.
- 11.2.4 Place the tubes in the homogenizer and shake for 30 seconds at a rate of 6 m/s or equivalent.
- 11.2.5 Remove the tubes from the homogenizer and centrifuge at 12,000 × g for 3 minutes to pellet the glass beads and debris. *Note: To minimize contamination, a new pair of gloves should be donned for this step.*

- 11.2.6 Using a 200 μL micropipettor, carefully transfer a minimum of 400 μL of the supernatant to a corresponding fresh, labeled low-retention 1.7 mL microcentrifuge tubes, taking care not to aspirate glass beads or sample debris (pellet). If unable to recover 400 μL of supernatant, centrifuge again, and repeat. Recover MB filter supernatants last.
 - 11.2.7 Centrifuge microcentrifuge tubes containing supernatant at $12,000 \times g$ for 1 minute.
 - 11.2.8 Prepare for DNA binding step by adding 760 μL of binding buffer (Section 7.15) to fresh, labeled low-retention 1.7 mL microcentrifuge tubes.
 - 11.2.9 Transfer 380 μL of the clarified supernatant, taking care not to disturb the pellet, to respective low-retention 1.7 mL microcentrifuge tubes containing binding buffer, and gently mix. *Note: It is important to pipet exactly 380 μL to yield reliable SPC data.* Recover the MB filter supernatants last.
 - 11.2.10 Label columns (Section 7.15) and place into collection tubes (Section 7.15).
 - 11.2.11 Transfer approximately 600 μL of each of the mixtures (clarified supernatant and binding buffer, Section 11.2.9) to the appropriately labelled columns and centrifuge at $12,000 \times g$ for 1 minute.
 - 11.2.12 Discard flow through and repeat Step 11.2.11 using the remaining mixture and the same column. Discard flow through. Repeat Step 11.2.12 for each of remaining mixtures (Section 11.2.9)
 - 11.2.13 Add 500 μL of washing buffer (Section 7.15) into each of the columns and centrifuge at $12,000 \times g$ for 1 minute. Discard flow through and repeat (total of two washing buffer rinses per column). Discard flow through.
 - 11.2.14 Transfer the columns to labelled, low-retention 1.7 mL microcentrifuge tubes
 - 11.2.15 Using a 200 μL micropipettor, add 50 μL of elution buffer (warmed to 60°C) directly to each column membrane and centrifuge at $12,000 \times g$ for 1 minute. *Note: Do not discard the eluates.*
 - 11.2.16 Repeat Step 11.2.15 for a total elution volume of 100 μL .
 - 11.2.17 Discard columns after final spin.
 - 11.2.18 Store purified DNA at 4°C . *Note: Purified DNA extracts should be stored at 4°C for no longer than 48 hours prior to testing.*
- 11.3 Preparation of qPCR assay mixes
- Initial qPCR reagent mixing should be performed in a separate workspace using dedicated supplies and equipment (Section 6.1). To minimize DNA contamination, routinely treat all work surfaces in the dedicated reagent preparation workstation 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.
- 11.3.1 Remove primers and probe stocks from the freezer and verify that they have been diluted to solutions of 500 μM primer and 100 μM probe. *Note: Primer stock should not undergo more than three freeze/thaw cycles.*
 - 11.3.2 Prepare working stocks of HF183/BacR287 and Sketa22 primer/probe mixes as outlined in **Table 3**. *Note: Prepare a sufficient quantity of primer/probe mix for the number of reactions, including reference DNA standards, NTCs, MBs and environmental water samples plus an additional 10% of anticipated total reaction*

volume (i.e., if 50 reactions expected, then prepare assay mix with additional 5 reactions [$50 \times 0.10 = 5$] for a total of 55 reactions).

Table 3. Preparation of Primer and Probe Mix for qPCR Assays

Reagent	Stock Solution	Volume Per Reaction	Primer/Probe Mix (200 reactions)	
			HF183/BacR287	Sketa22
Forward Primer	500 μ M	0.05 μ L	10 μ L	10 μ L
Reverse Primer	500 μ M	0.05 μ L	10 μ L	10 μ L
6FAM Probe	100 μ M	0.02 μ L	4 μ L	4 μ L
VIC Probe	100 μ M	0.02 μ L	4 μ L	---
PCR-grade water	---	2.86/2.88 ¹ μ L	572 μ L	576 μ L

¹ Volume of PCR-grade water per reaction for Sketa22 primer and probe mix preparation

- 11.3.3** Remove TaqMan[®] Environmental Master Mix (Section 7.8), and BSA (Section 7.9) reagents from storage. Gently mix each reagent microtube (primer/probe mix and BSA) and pulse microcentrifuge to coalesce any droplets.
- 11.3.4** Using dedicated micropipettors for reagent mixing, prepare an assay mix for each assay in separate, sterile, labeled 1.7 mL low-retention microcentrifuge tubes or 5 mL polystyrene tubes (Section 6.16) as described in **Table 4**. Prepare a sufficient quantity of each assay mix for the number of reactions (**Table 5**), reference DNA standards, NTCs, and MBs plus an additional 10% of total reactions (i.e., if 57 reactions expected, then prepare assay mix with additional 10% volume [$57 \times 0.10 = 5.7$] for a total of 63 reactions [$57 + 6 = 63$]). **Note:** For assay mix preparations containing more than 40 reactions total, use a 5 mL round bottom test tube (Section 6.16).

Table 4. Preparation of Assay Mix for Each qPCR Assay

Reagent	Final Concentration	Volume for one 25 μ L reaction	
		HF183/BacR287	Sketa22
TaqMan [®] Environmental Master Mix	1 X	12.5 μ L	12.5 μ L
BSA	0.2 mg/mL	2.5 μ L	2.5 μ L
Primer/probe working stock solution	1 μ M/80 nM	3.0 μ L	3.0 μ L
PCR-grade water	---	3.0 μ L	5.0 μ L
IAC plasmid ¹	---	2 μ L	---






¹ IAC plasmid should be added to assay mix in same work area as DNA template addition (Section 6.1)

Table 5. Example Instrument Run

Sample	Reactions
DNA standards – 5 dilutions \times 3 replicates per extract	15
3 Method blanks \times 3 replicates per extract	9
6 NTCs	6
3 Environmental water samples \times 3 filters per sample \times 3 replicates per extract	27
Total number of reactions	57

- 11.3.5** Place cap firmly on tube (either 1.7 mL microcentrifuge tube or 5 mL polystyrene tube) containing TaqMan® Environmental Master Mix, BSA, primer/probe working stock solution, and PCR-grade water and transfer to DNA template addition work area (Section 6.1). Store on ice until ready to add IAC plasmid (for HF183/BacR287 reactions only) and DNA templates.
- 11.3.6** Remove working stock DNA standards and IAC spike aliquots from freezer (-20°C). Place all materials on ice.
- 11.3.7** In DNA template addition work area (Section 6.1), pipet appropriate volume of IAC plasmid (2 µL × number of reactions [Table 4]) into HF183/BacR287 qPCR assay mix tube and mix gently and thoroughly.
- 11.3.8** Pipet 23 µL of qPCR assay mix into each well of a 96-well qPCR plate. *Note:* Procedure may use separate 96-well qPCR plates for HF183/BacR287 and Sketa22 amplifications.
- 11.3.9** Lightly cover plate with aluminum adhesive qPCR tape (do not seal tape onto plate).
- 11.3.10** Label plate and store on ice in dark for transport to dedicated Laminar flow hood in DNA purification workstation (Section 6.1) for the addition of DNA template.
- 11.3.11** In the laminar flow hood, add 2 µL of DNA template (environmental water sample extracts [Section 11.2.18]) into appropriate wells using a dedicated pipettor. Include a minimum of six NTC reactions (2 µL of PCR grade water substituted for DNA template), triplicate reactions for each MB filter (2 µL per reaction), and triplicate reactions for each environmental water sample DNA extract (2 µL per reaction). Refer to **Figure 5** for 96-well recommend format.
- 11.3.12** In addition, add 2 µL of each DNA standard of each concentration ranging from 10 to 1 × 10⁵ copies into appropriate wells in HF183/BacR287 96-well qPCR plate (**Figure 5, Panel A**).

	1	2	3	4	5	6	7	8	9	10	11	12
A.												
A	10 ⁵ copies	10 ⁴ copies	10 ³ copies	10 ² copies								
B	10 ⁵ copies	MB 1	MB 2	MB 3								
C	Filter 1	Filter 2	Filter 3	Filter 4								
D	Filter 5	Filter 6	Filter 7	Filter 8								
E	Filter 9	Filter 10	Filter 11	Filter 12								
F	Fecal 13	Filter 14	Filter 15	Filter 16								
G	Filter 17	Filter 18	Filter 19	Filter 20								
H	Filter 21											

	Calibration Curve Standards
	No Template Controls (NTC)
	Method Blank (MB)
	Water Test Sample (Filters 1-21)
	Empty Well

	1	2	3	4	5	6	7	8	9	10	11	12
B.												
A												
B				MB 1	MB 2	MB 3						
C	Filter 1	Filter 2	Filter 3	Filter 4								
D	Filter 5	Filter 6	Filter 7	Filter 8								
E	Filter 9	Filter 10	Filter 11	Filter 12								
F	Fecal 13	Filter 14	Filter 15	Filter 16								
G	Filter 17	Filter 18	Filter 19	Filter 20								
H	Filter 21											





	No Template Controls (NTC)
	Method Blank (MB)
	Water Test Sample (Filters 1-21)
	Empty Well

Figure 5. Recommended 96-Well Plate Format for Target Sequence Concentration Determination for Batch of 21 Environmental Water Samples

Panel A represents suggested format for instrument run for the HF183/BacR287 assay. Panel B shows format for Sketa22 qPCR assay.

- 11.3.13** Seal plate with optical adhesive PCR tape.
- 11.3.14** Place plate into real-time qPCR instrument according to manufacturer's instructions. Set amplification conditions to 95°C for 10 minutes and then forty cycles of 95°C for 15 seconds and 60°C for 1 minute. Analyze data with the Manual Cq Threshold set at **0.03** for HF183/BacR287 and Sketa22. Platform-specific instructions are provided in **Appendix A**.

12.0 Data Analysis and Calculations

- 12.1 Overview** – This method employs a 'single' set of DNA standard Cq measurements originating from a specific instrument run (Reference 15.12) to estimate \log_{10} DNA target sequence copies per reaction for environmental samples. This approach requires that environmental samples and corresponding calibration curve DNA standard measurements be from the same instrument run. The calibration curve is fitted with simple linear regression based on data from triplicate reactions at five standard concentrations. To identify outliers, individual Cq values are removed from the fitted model if the absolute value of the studentized residual is > 3 . Figure 2 (Section 9.4) shows a calibration curve model for the HF183/BacR287 qPCR method. To help ensure the integrity of results, promote consistency between laboratories, and enhance transparency of results, this protocol uses a standardized data procedure that includes a rigorous series of QA criteria in accordance with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines (Reference 15.6). Adherence to this recommended procedure will allow for more reliable and reproducible interpretation of qPCR results.
- 12.2 Data Acceptance Criteria** – Prior to estimating the concentration of the target DNA, the standard curve, SPC, IAC, MB and NTC results should be reviewed to verify that all data acceptance criteria are met. Data acceptance criteria include a broad range of conditions and are summarized in **Table 6** (Reference 15.11). Data that satisfies all QA conditions are appropriate for estimating the concentration of human-associated DNA target sequences. Refer to Section 9 if any of the data does not meet the acceptance criteria to determine appropriate steps to resolve the issue(s). Please refer to Section 12.3.2 for test sample(s) SPC results that are unacceptable, but are eligible for HF183/BacR287 Cq adjustment.

Table 6. Data Acceptance Criteria Summary (Reference 15.9)

Type	Metric	Acceptance Criteria
Calibration Curve	R ²	≥ 0.98 ¹
	Amplification efficiency (<i>E</i>)	0.90 to 1.10 ¹
	Lower limit of quantification (LLOQ)	95% prediction upper limit at 1 log ₁₀ copy DNA standard dilution
Extraneous DNA	No-template controls (NTC)	Undetermined result in all HF183/BacR287 test reactions per instrument run ²
	Method blank (MB)	Undetermined result in all Sketa22 test reactions per extraction batch ²
Matrix and Amplification Control Proficiency	Internal amplification control (IAC)	Instrument run-specific multiplex VIC Cq standard deviation ≤ 1.16 Cq for HF183/BacR287
	Sample processing control (SPC)	Batch-specific Sketa22 qPCR extraction blank standard deviation ≤ 0.62 Cq
Environmental Water Samples	Matrix interference with SPC	Refer to Section 9.5
	Inhibition screen with IAC	Refer to Section 9.6

¹ Value calculated from single instrument run using calibration curve data generated from a minimum of 5 different standard concentrations with at least triplicate reactions at each dilution level.

² Although no reaction should yield a Cq value < 40, Cq values greater than the lower limit of quantification (LLOQ) are acceptable for quantification applications only. However, laboratory should report this practice including all control data results.

12.3 Estimating Target Concentration in a Test Sample – Estimating the log₁₀ copies per reaction in an environmental sample is a multiple step process. Estimates are achieved by 1) verification that each test sample replicate Cq used to generate an estimate is within the range of quantification (< LLOQ); 2) determination of sample mean Cq; and 3) estimation of log₁₀ copies per reaction using the respective calibration curve. In this approach, estimates include a standard error term that incorporates variability from replicate reactions, replicate filters, as well as calibration curve intercept and slope parameters.

12.3.1 Verification that replicate Cq measurements are within the range of quantification. The plasmid-derived DNA standard dilutions used to construct the calibration curve define the range of quantification (ROQ). The lowest concentration that is eligible for DNA target estimation is used to determine the LLOQ. LLOQ is defined as the 95% prediction upper limit at the 1 log₁₀ copy DNA standard dilution (Section 9.4). In this method, each environmental water sample results in three replicate filters. Amplification is performed in triplicate for each filter resulting in a maximum of nine Cq measurements per sample (1 sample × 3 filters × 3 replicates = 9 replicates). Each replicate is scored as a ‘PASS’ (Cq ≤ LLOQ) or ‘FAIL’ (Cq > LLOQ). All replicate Cq measurements that ‘FAIL’ the LLOQ requirement should not be included in the determination of sample mean Cq.

12.3.2 Conditional Cq measurement adjustment procedure. Before making DNA target concentration estimates, individual Cq measurements may be adjusted on a filter basis contingent on respective sample SPC results (Section 9.5.2). For eligible test samples,

each respective replicate HF183/BacR287 FAM Cq measurement may be adjusted as follows: HF183/BacR287 Cq - (test sample mean Sketa22 Cq – SPC acceptance threshold).

12.3.3 Estimation of human-associated DNA target concentration and associated error.

The mean \log_{10} copies per reaction is calculated as follows:

$$\frac{[\text{Sample mean Cq} - \text{intercept}]}{\text{slope}}$$

Where the calibration curve parameters originate from the same instrument run as the sample mean Cq data. Associated standard error is also determined and includes replicate reactions, replicate filters, as well as calibration model intercept and slope parameters. *Note: If the environmental sample DNA extract is diluted due to IAC inhibition, then the \log_{10} copies per reaction determination must be adjusted based on the dilution factor.*

- 12.4** Reporting Results: Uniformity and standardization in the report of findings is essential for users and reviewers to evaluate the quality of data. However, procedures for reporting MST qPCR data are typically overlooked. In order to effectively communicate the relevant information needed to properly assess the quality of human-associated qPCR results, it is highly recommended that users report the information listed in **Table 7** [checklist adapted from MIQE guidelines (Reference 15.6)].

Table 7. Checklist for Reporting Findings

<i>Sample Information</i>	<i>Reference DNA Optimization</i>
Description	Calibration standard dilution Cq and variation
Sample holding time prior to filtration	Salmon DNA SPC spike Cq mean and variation
Volume/mass processed	IAC spike mean Cq and variation from NTC data
Processing procedure	IAC range of quantification
If frozen, how?	<i>qPCR Validation</i>
Sample storage conditions and duration	Evidence of specificity and sensitivity
<i>DNA Extraction</i>	Calibration curve (slope and intercept)
Procedure	Amplification efficiency (<i>E</i>)
Kit name and any modifications	R ² of calibration curve
Source of all reagents	Range of quantification
Elution volume	LLOQ
<i>qPCR Oligonucleotides</i>	<i>Data Analysis</i>
Primer sequences	Calibration model with error
Probe sequences	Outlier identification and disposition
Details of any modifications	Evidence for no contamination (NTC, MB and FEB)
Oligonucleotide manufacturer	Evidence of no inhibition
<i>qPCR Protocol</i>	IAC acceptance threshold
Complete reaction conditions	IAC competition threshold
Reaction volume	Evidence of acceptable sample processing
Volume of DNA extract amplified	SPC acceptance threshold
Complete thermal cycling parameters	SPC adjustment threshold
Instrument manufacturer	Software (source, version)
Manual Cq threshold settings	
Number of cycles	

12.5 Automated data analysis support tool: HF183/BacR287 qPCR data analysis can be time consuming, repetitive, and prone to error. To help simplify this procedure, reduce manual errors, and promote standardization in calculations, a companion Microsoft Excel workbook tool is available online at <https://www.epa.gov/cwa-methods/other-clean-water-act-test-methods-microbiological>.

12.5.1 Tool functions. The automated data analysis support tool is designed to quickly and accurately apply all data acceptance criteria and generate HF183/BacR287 target sequence concentration estimates in surface water samples (reported as log₁₀ copies per reaction ± error). For a step-by-step description of data acceptance and human-associated qPCR concentration estimate calculations from water samples, please refer to Sections 12.2 and 12.3, respectively.

12.5.2 Tool organization. The automated data analysis support tool is organized into five tabs including (1) Instructions, (2) Standards Data, (3) Sketa22_Data, (4) Samples Data, and (5) Final Report. The Instruction Tab provides directions for tool use. The Standards Data, Sketa22_Data, and Samples Data tabs contain Tables for entry of data necessary to apply all data acceptance criteria and generate HF183/BacR287 target sequence concentrations in surface water samples. Results are organized into easy-to-read Tables and Figures for quick and accurate access to all QC and water sample concentration information (refer to Section 12.5.4 for additional information).

- 12.5.3 Data entry.** Data is entered into the automated data analysis tool by cutting and pasting Cq values from the thermal cycle instrument export file (see Appendix A for platform-specific information on data exporting). HF183/BacR287 standard curve and NTC measurements are entered into Table 1 (Standards Data tab), while MB results are pasted into Table 2. Sketa22 Cq measurements for MB and NTC tests are entered into Tables 3 and 4, respectively (Sketa22_Data tab). Water sample data including HF183/BacR287 and Sketa22 Cq measurements are entered in Table 5 (Samples Data tab).
- 12.5.4 Final report features.** The Final Report tab automates the presentation of all data acceptance metrics, calibration model, and water sample estimated HF183/BacR287 DNA target sequence concentrations. Table 1 lists all calibration model performance metrics including slope \pm standard deviation, intercept \pm standard deviation, amplification efficiency (E), R^2 , lower limit of quantification (Cq value), lower limit of quantification in \log_{10} copies per reaction, number of outliers, range of quantification (minimum and maximum reported in \log_{10} copies per reaction), and internal amplification control range of quantification (minimum and maximum reported in \log_{10} copies per reaction). Figure 1 shows the HF183/BacR287 calibration model with 95% prediction interval. Table 2 shows quality control and proficiency metric criteria results for calibration curve model (R^2 and E), extraneous DNA (NTC and MB), and matrix and amplification control proficiency (IAC proficiency and SPC proficiency). Table 3 displays amplification and matrix interference thresholds for HF183/BacR287 IAC interference and IAC competition thresholds, as well as Sketa22 SPC acceptance and SPC adjustment values. Figure 2 provides an illustration of SPC water filter testing results plotting the SPC adjustment threshold and SPC acceptance threshold followed by color-coded mean Sketa22 Cq values for each water sample filter (red = filter fails the SPC test; Yellow = filter is eligible for Cq adjustment; Black = filter passes SPC acceptance threshold; Brown = filter mean Sketa22 Cq values is > 30). Table 4 reports water test sample estimated concentrations as \log_{10} copies per reaction \pm standard deviation, along with the number individual reactions used to generate the estimate (n), and the final mean Cq value used to generate the concentration estimate (*Note: The original Cq value may have been adjusted depending on results of SPC test*).

13.0 Pollution Prevention

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

14.0 Waste Management

- 14.1** The laboratory is responsible for complying with all Federal, State, and local regulations governing waste management, particularly hazardous waste identification rules and land disposal restrictions, and for protecting 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. An overview of requirements can be found in Environmental Management Guide for Small Laboratories (EPA 233-B-98-001).
- 14.2** Samples, reference materials, and equipment known or suspected to have environmental fecal material attached or contained must be sterilized prior to disposal.
- 14.3** 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 N.W., Washington D.C. 20036.

15.0 References

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- 15.3** Layton B.A., *et al.*, Performance of human fecal anaerobe-associated PCR-based assays in a multi-laboratory method evaluation study. *Water Research*, 2013. 47(18):6897-6908.
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- 15.5** Wilson, I.G., Inhibition and facilitation of nucleic acid amplification. *Applied and Environmental Microbiology*, 1997. 63:3741-3751.
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- 15.10** Shanks, O.C., *et al.*, Performance of PCR-based assays targeting Bacteroidales genetic markers of human fecal pollution in sewage and fecal samples. *Environmental Science and Technology*, 2010. 44(16):6281-6288.

- 15.11** Shanks, O.C., et al., Data acceptance criteria for standardized human-associated fecal source identification quantitative real-time PCR methods. *Applied and Environmental Microbiology* 2016, 82:2773-2782.
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Appendix A:
Thermo Fisher Scientific StepOnePlus™, 7900, and QuantStudio™ 6 Real-
Time PCR Systems Operations

StepOnePlus™, 7900, and QuantStudio™ 6 Real-Time PCR Systems Operations

1.0 StepOnePlus™

- 1.1 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.
- 1.2 On the StepOnePlus™ home screen select **Advanced Set Up**.
- 1.3 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). Next, go through the following fields:
 - 1.3.1 Which instrument is going to be used to run the experiment: **StepOnePlus™ Instrument** (96 wells) is default (highlighted).
 - 1.3.2 What experiment do you want to set up: **Quantitation-Standard Curve** is default (highlighted).
 - 1.3.3 Which reagents will be used to detect the target: **TaqMan® Reagents** is default (highlighted).
 - 1.3.4 Which ramp speed do you want to use in the instrument run: Click on **Standard-2 hours** (Not Default)
- 1.4 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 1.8).
- 1.5 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.
- 1.6 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. The selected targets will then be added on to the define target and sample screen.
- 1.7 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 appropriate reporter for a particular assay (**Table A-1**). From the **Quencher** dropdown menu, select appropriate quencher for a particular assay (Table A-1). Leave the default in the color field.

Table A-1. Reporter and Quencher Settings for qPCR Assays for the StepOnePlus™

Assay	Detector	Reporter	Quencher
HF183/BacR287 ¹	HF183 FAM	FAM	Non-fluorescent
	HF183 VIC	VIC	Non-fluorescent
Sketa22	Sketa22 FAM	FAM	TAMRA

¹ Each multiplex assay is assigned two detectors: one for the target sequence, which uses FAM as the reporter, and one for the IAC, which uses VIC as the reporter.

- 1.8 Click on the tab **Assign Targets and Samples** to see the screen view of the plate layout with 96 wells.
 - 1.8.1 Select the wells, based on the plate set up, by highlighting, one target at a time.

- 1.8.2 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.
 - 1.8.3 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.
 - 1.8.4 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
- 1.9 Run the Method
- 1.9.1 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.
 - 1.9.2 Make sure the reaction volume per well field displays 25 μ L, this is not the default setting.
 - 1.9.3 Set the thermal profile to the following holding and cycling stages: Holding Stage 1: 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.
 - 1.9.4 *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.*
- 1.10 Load the plate into the instrument.
- 1.11 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.
- 1.11.1 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.
 - 1.11.2 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.*
- 1.12 Once the run is completed remove the reaction plate and discard.
- 1.13 Analyze the run
- 1.13.1 Click on the tab in the top right corner of screen to **Analyze the Run**.
 - 1.13.2 Highlight all of the sample wells and click on **Analysis Settings** to get to Cq settings (default Cq settings).

- 1.13.3** Highlight all the targets running in the plate; uncheck the default setting for automatic threshold and **set the threshold to 0.03 for HF183/BacR287 and Sketa22**. 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.
- 1.14** 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**.

2.0 7900 Real-Time PCR System Operation

- 2.1** Turn on the Thermo Fisher Scientific Model 7900 and then the computer. Launch the **SDS** 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.
- 2.2** Under File menu, select **New**.
- 2.3** The **New Document** window that appears, change container selection from **384 well clear plate** to **96 well clear plate** using the drop-down menu. Accept default selections of **Absolute Quantification** and **Blank Template**. Click **OK** to display a new plate document.
- 2.4** 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.
- 2.5** Above the right-hand window, click on **Setup** tab.
- 2.6** 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 (Section 2.7).*
- 2.7** 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. Under **Group** select Default. Under **Reporter** select appropriate terms based on assay (**Table A-2**). Under **Quencher** select appropriate quencher for a particular assay (Table A-2). 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 2.8).

Table A-2. Reporter and Quencher Settings for qPCR Assays for the 7900 System

Assay	Detector	Reporter	Quencher
HF183/BacR287 ¹	HF183 FAM	FAM	Non-fluorescent
	HF183 VIC	VIC	Non-fluorescent
Sketa22	Sketa22 FAM	FAM	TAMRA

¹ Each multiplex assay is assigned two detectors: one for the target sequence, which uses FAM as the reporter, and one for the IAC, which uses VIC as the reporter.

- 2.8** In pop-up window that was opened in Step 2.7, select the desired detector under Names menu and click on **Copy to Plate Document** button. Click on **Done** button to return to setup screen.

- 2.9 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. Repeat for VIC detector depending on assay (Table A-2).
- 2.10 Click on **Instrument** tab right hand window.
- 2.11 In instrument screen, change **Sample Volume** to 25 μ L and choose **9600 Emulation**.
- 2.12 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.
- 2.13 Insert PCR reaction tray with prepared reactions in holder.
- 2.14 Click on **Open/Close** button to close PCR reaction tray holder door on instrument.
- 2.15 Click on **Start** button in lower right hand “Real Time” window to start thermal cycling in instrument.
- 2.16 Name run file at prompt.
- 2.17 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.
- 2.18 At termination of the run success complete, choose **Analysis Settings** from the toolbar. In that box enter a value for the **Manual Cq Threshold (0.03 for HF183/BacR287 and Sketa22)**. Click on **OK**. Click on **Analyze** from the toolbar. You should see Cq values in the Results Table.
- 2.19 Calculated Cq 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.
- 2.20 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**.

3.0 QuantStudio™ 6

- 3.1 Turn on the QuantStudio™ 6 and then the computer. Launch the QuantStudio™ 6 Software by double clicking on its icon on the computer desktop or from the Computer Programs menu and click **Experiment Setup**. Click **Experiment Properties** to access the Experiment Properties screen.
- 3.2 Enter a unique experiment name in the Experiment Name field (Name/Date etc. such that experiment can be identified). Then go through the following fields:
 - 3.2.1 Which instrument is going to be used to run the experiment: **QuantStudio™ 6**
 - 3.2.2 Select the block type you are using to run the experiment: **96-Well Block (0.2 mL)**
 - 3.2.3 What experiment do you want to set up: **Quantitation-Standard Curve**
 - 3.2.4 Which reagents will be used to detect the target: **TaqMan®**
 - 3.2.5 Which ramp speed do you want to use in the instrument run: Click on **Standard-2 hours**

- 3.3** Click **Define** to access the Define screen. Define the targets, and then assign them to wells in the reaction plate
- 3.4** 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.
- 3.5** 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.
- 3.3** 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 appropriate reporter for a particular assay (**Table A-3**). From the **Quencher** dropdown menu, select appropriate quencher for a particular assay (Table A-3). Leave the default in the color field.

Table A-3. Reporter and Quencher Settings for qPCR Assays for the StepOnePlus™

Assay	Detector	Reporter	Quencher
HF183/BacR287 ¹	HF183 FAM	FAM	Non-fluorescent
	HF183 VIC	VIC	Non-fluorescent
Sketa22	Sketa22 FAM	FAM	TAMRA

¹ Each multiplex assay is assigned two detectors: one for the target sequence, which uses FAM as the reporter, and one for the IAC, which uses VIC as the reporter.

- 3.4** Click on the tab **Assign Targets and Samples** to see the screen view of the plate layout with 96 wells.
- 3.4.1** Select the wells, based on the plate set up, by highlighting, one target at a time.
- 3.4.2** 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
- 3.7.3** 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.
- 3.7.4** 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.
- 3.7.5** 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.
- 3.5** Define samples
- 3.5.1** Click **New** to add samples and name them.
- 3.5.2** In the samples table, click a cell in the **Sample Name** column for the sample to define and enter your sample name. The default sample name is Sample 1.
- 3.9** Assign Samples: Click **Assign** to access the Assign screen.
- 3.9.1** Select wells using the plate layout or the well table on the Assign screen.
- 3.9.2** Select the check box next to the sample to assign to the selected wells.

- 3.10** Run the Method
- 3.10.1** 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.
- 3.10.1** Make sure the reaction volume per well field displays 25 μ L, this is not the default setting.
- 3.10.2** Set the thermal profile to the following holding and cycling stages: Holding Stage 1: 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.*
- 3.11** Load the plate into the instrument.
- 3.12** 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.
- 3.12.1** 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.
- 3.12.2** 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.*
- 3.13** Once the run is completed remove the reaction plate and discard.
- 3.14** Analyze the run
- 3.14.1** Click on the tab **Analyze** to analyze the Run.
- 3.14.2** Highlight all of the sample wells and click on **Analysis Settings** to get to Cq settings (default Cq settings).
- 3.14.3** Highlight all the targets running in the plate; uncheck the default setting for automatic threshold and **set the threshold to 0.03 for HF183/BacR287 and Sketa22**. 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.
- 3.15** 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**.

Appendix B:
Method Proficiency Test Procedure

Method Proficiency Test Procedure

Each analyst should demonstrate initial proficiency with the method according to Appendix B and the criteria provided in **Table 2** (EPA Method 1696.1, Section 9.1) prior to processing environmental samples. This procedure should be repeated for each new preparation of DNA reference materials (DNA standards, IAC, and salmon DNA [SPC] stock solutions). Ongoing demonstration of analyst capability should be documented through successful performance of the method (e.g., meeting method performance criteria when run with environmental samples). Initial and ongoing proficiency is based on six metrics (**Table 2**, EPA Method 1696.1, Section 9.1). Values for each metric are determined from a series of analyses designed to generate calibration curve, NTC, and MB data. It is recommended that the procedure below should be repeated until the user can meet all acceptance criteria three times in a row.

1.0 Day One

- 1.1 Prepare three MB filters as described in EPA Method 1696.1, Sections 9.3 and 11.1.
- 1.2 Prepare fresh salmon DNA extraction buffer (EPA Method 1696.1, Section 7.18).
- 1.3 Perform DNA extraction on MB filters as described in EPA Method 1696.1, Section 11.2. Store purified DNA at 4°C. qPCR analysis should be performed within 48 hours of extraction.

2.0 Day Two

- 2.1 Prepare qPCR assay mixes as described in EPA Method 1696.1, Section 11.3. *Note: It is important to generate data as shown in **Figure B-1** for three independent instrument runs. Thus, three separate assay mixes must be prepared for each qPCR assay.*
- 2.2 Place cap firmly on 1.7 mL microcentrifuge tube containing TaqMan® Environmental Master Mix, BSA, primer/probe working stock solution, and PCR-grade water and transfer to DNA template addition work area (EPA Method 1696.1, Section 6.1). Store on ice until ready to add IAC plasmid and DNA templates.
- 2.3 Remove working stock DNA standards and IAC spike aliquots from freezer (-20°C). Remove MB DNA extracts from 4°C storage. Place all materials on ice.
- 2.4 In DNA template addition work area (EPA Method 1696.1, Section 6.1), pipet appropriate volume of IAC plasmid (2 µL × number of reactions) into HF183/BacR287 qPCR assay mix tube and mix gently and thoroughly.
- 2.5 Pipet 23 µL of qPCR assay mix into appropriate well of a 96-well qPCR reaction plate.
- 2.6 Lightly cover plate with aluminum adhesive qPCR tape (do not seal tape onto plate).
- 2.7 Label plate and store on ice in dark for transport to dedicated Laminar flow hood in DNA purification workstation (EPA Method 1696.1, Section 6.1) for the addition of DNA template.

	1	2	3	4	5	6	7	8	9	10	11	12	
A	10 ⁵ copies			10 ³ copies			10 ¹ copies			10 ⁰ copies			HF183/BacR287
B	10 ⁵ copies			MB 1			MB 2			MB 3			
C	NTC			NTC			NTC			NTC			
D	NTC			NTC			NTC			NTC			
E	NTC			NTC			NTC			NTC			Sketa22
F	NTC			NTC			NTC			NTC			
G	MB 1			MB 2			MB 3			NTC			
H	NTC			NTC			NTC			NTC			

Calibration Curve Standards
 No Template Controls (NTC)
 Method Blanks (MB)

Figure B-1. Recommended method proficiency test format for HF183/BacR287 and Sketa22 96-well plate set-up

- 2.8** In the laminar flow hood, add 2 μ L of DNA standards in triplicate for each concentration ranging from 10 to 1×10^5 copies into HF183/BacR287 assay wells and MB DNA extracts into appropriate wells using a dedicated pipette. For NTC reactions, add 2 μ L of PCR-grade water into appropriate wells.
- 2.9** Seal plate with optical adhesive PCR tape.
- 2.10** Place plate into real-time qPCR instrument according to manufacturer's instructions. Set amplification conditions to 95°C for 10 minutes and then forty cycles of 95°C for 15 seconds and 60°C for 1 minute. *Note: For platform-specific operation see Appendix A.* Analyze data with the Manual Cq Threshold set at **0.03** for **HF183/BacR287** and **Sketa22**.

3.0 Method Proficiency Data Analysis

- 3.1** Method proficiency calculations are completed with data generated from the qPCR instrument run described in Section 2 Day Two. It is recommended that the user repeats the proficiency test at least three times prior to processing environmental samples. In addition, with three iterations of data, expected Cq ranges for working stocks of reference DNA materials such as plasmid calibration curve, IAC plasmid spike, and salmon DNA preparations can be established.
- 3.2** **Table B-1** can be used to document and compare results with acceptance thresholds. Procedures to determine calibration curve R^2 and amplification efficiency (E) performance metrics, as well as the absence of contamination in reagents and laboratory environment with NTC and MB controls for each instrument run are provided in EPA Method 1696.1, Sections 9.2 and 9.3, respectively. IAC proficiency acceptance thresholds are evaluated by calculating the human-associated qPCR Cq standard deviation in VIC Cq values from NTC reactions for each instrument run. SPC proficiency is measured with the Sketa22 Cq standard deviation in MB filters for each instrument run. For each metric with observed values within the acceptance criteria is scored as 'PASS'. A proficient laboratory should meet all acceptance criteria (PASS) for all metrics and all three instrument runs. Failure to 'PASS' all acceptance criteria can result from a number of reasons such as poor laboratory technique, improper preparation/storage of reference DNA materials, and/or poor calibration of equipment (*i.e.*, pipets, qPCR instrument,). Issues such as poor laboratory technique, improper preparation/storage of reference DNA materials, and/or poor

calibration of equipment (i.e., pipets, qPCR instrument,) can result in the laboratory failing to meet the acceptance criteria.

Table B-1. Method Proficiency Metric Results Worksheet

Metric	Acceptance Threshold	Run 1		Run 2		Run 3	
		Observed	Pass	Observed	Pass	Observed	Pass
R ²	≥ 0.98 ¹						
Amplification efficiency (E)	0.90 to 1.10 ¹						
No-template controls (NTC)	≥ 40 Cq result ²						
Method blank (MB)	≥ 40 Cq result ²						
	Sketa22 ≥ 40 Cq result ²						
Internal amplification control (IAC) proficiency	Multiplex VIC Cq standard deviation ≤ 1.16 Cq for HF183/BacR287						
Sample processing control (SPC) proficiency	Sketa22 assay standard deviation ≤ 0.62 Cq						

¹ Value calculated from single instrument run using calibration curve data generated from a minimum of 5 different standard concentrations with at least triplicate reactions at each dilution level.

² Although no reaction should yield a Cq value < 40, Cq values greater than the lower limit of quantification (LLOQ) are acceptable for quantification applications only. However, laboratory should report this practice including all control data results.

4.0 Reference DNA Material Working Stock Worksheet

Currently there is no standardized source of reference DNA materials for this method. Until standardized reference DNA materials are commercially available, laboratories will need to prepare their own stocks. It is normal and expected that DNA reference material stock concentrations will vary from one preparation to another due to source material variation; technician variability (e.g., dilution preparation); variability in methodology used to measure initial concentration in stock preparations; and/or various other factors. As a result, it is recommended that each reference DNA material preparation (e.g., DNA standards, IAC plasmid spike, salmon DNA [SPC]) are documented prior to the analysis of environmental water samples. These values provide a laboratory with preparation-specific benchmarks for each reference DNA material. If expected Cq values change by more than two standard deviations between observed values from laboratory proficiency data in a future instrument run using the same working stock preparations, then it is likely that reference materials have degraded and should be discarded. **Table B-2** can be used to document observed mean Cq ± standard deviation for each reference material used in this method. Mean Cq and standard deviations should be calculated using data from all three instrument runs with filter and run-to-run variability included.

Table B-2. Observed Reference DNA Material Working Stocks Worksheet

Reference DNA Material Type	Test Concentration	Observed Mean Cq ± Standard
DNA standards	10 copies/reaction	
	10 ² copies/reaction	
	10 ³ copies/reaction	
	10 ⁴ copies/reaction	
	10 ⁵ copies/reaction	
Internal amplification control (IAC)	10 ² copies/reaction	
Method blank (SPC)	0.2 µg/mL	

Appendix C:
Specificity, Sensitivity, and Target Abundance in Reference Fecal Source
Materials Procedure

Procedure to Assess HF183/BacR287 Method Specificity, Sensitivity, and Target Abundance in Reference Fecal Source Materials

The HF183/BacR287 fecal source identification qPCR method does not always exclusively detect human pollution. The shedding of HF183/BacR287 DNA target sequences in non-human animals may vary from one geographical location to another and/or one animal population to another. These rare, but potential differences could confound data interpretations. As a precaution, it is recommended that users perform a specificity test with reference pollution source materials collected from potential sources in the same geographic area as water quality testing prior to analyzing environmental water samples. If false positives are observed, it is then recommended that the abundance of target (human sources) and non-target (other animal sources) sequences is determined to aid in data interpretation. The prevalence and concentration of HF183/BacR287 target sequences in human waste source types such as individual fecal samples, sewage, and septage may also vary from one population to another or by source type. As a result, it is recommended that users perform a sensitivity test with human waste reference pollution source materials collected in the same geographic area as water quality testing prior to analyzing environmental samples. This information can be used to confirm the presence of human-associated DNA target sequences in human waste source types in the area of interest.

Specificity and sensitivity testing should be conducted at least once during the water quality collection time frame, preferably before analysis of environmental water samples.

1.0 Reference Pollution Source Sample Collection

- 1.1 Fecal reference pollution source materials are samples collected from a known animal group and location. Ten individual samples should be collected from each animal source group that could potentially impact water quality in the area of interest (e.g., watershed).
- 1.2 For secondary fecal pollution sources such as sewage and agricultural lagoon wastes, it is recommended that samples are collected from at least one facility or location.
- 1.3 Fecal and secondary source materials should be collected using sterilized collection tools (e.g., scoops) and sample containers and gloves paying special attention to keep individual samples separate. For fecal samples (e.g., feces), collect ~2 g. For secondary sources (e.g., wastewater), collect a minimum of 100 mL.
- 1.4 During transit to the laboratory, samples should be stored on ice. In the laboratory, fecal samples can be stored at -80°C for up to 12 months prior to analysis. Secondary source samples, may be stored at 4°C for up to 8 hours from time of collection to initiation of filtration.
- 1.5 Samples should be labeled with date of collection, pollution source group, and location.

2.0 Reference Secondary Fecal Pollution Source Sample Filtration and Secondary Extraction Blank (SEB) Preparation

Note: Three SEB samples (25 mL) should be analyzed with each batch of secondary fecal samples. A single replicate (25 mL) of each secondary fecal sample should also be processed and analyzed for sensitivity testing. SEB controls should be processed after secondary fecal samples for each of the steps described below.

- 2.1 Secondary fecal pollution sources should be filtered within 8 hours of collection. Triplicate filters should be prepared for each sample.
- 2.2 Place a fresh membrane filter funnel assembly (EPA Method 1696.1, Section 6.3) on the filter base. *Note: If using a disposable filtration unit, confirm that the unit contains the correct filter type – replace filter if necessary (Method 1696.1, Section 6.8).*
- 2.3 Shake the sample bottle vigorously 25 times to distribute the bacteria uniformly, and measure 25 mL of secondary fecal sample into the funnel using the graduated markings on the funnel.
- 2.4 After filtering the sample, turn off the vacuum.
- 2.5 Label an extraction tube containing glass beads (EPA Method 1696.1, Section 6.18) to identify secondary fecal sample. Remove the funnel from the filter base leaving the filter on the filtration unit base. Using sterile forceps, 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 and cap the tube.
- 2.6 Filter the remaining secondary fecal samples. Filter SEB samples (25 mL PCR-grade water, Section 7.3) after secondary fecal samples. *Note: A 25 mL volume is used for SEB filter preparation to coincide with the 25 mL of sewage sample.*
- 2.7 Store tubes containing folded filters at -80°C until time of DNA extraction. *Note: Filters should not be stored for more than 18-months.*

3.0 Secondary Pollution Source Nucleic Acid Extraction

The following are modified instructions for using the Gene-Rite DNA extraction kit (EPA Method 1696.1, Section 7.15). *Note: Do not follow the manufacturer instructions.*

- 3.1 Warm elution buffer (EPA Method 1696.1, Section 7.15) to 60°C using an incubator or heating block (EPA Method 1696.1, Section 6.29).
- 3.2 Using a 1000 µL micropipettor, dispense 600 µL of AE buffer (EPA Method 1696.1, Section 7.4) into each labeled extraction tube containing glass beads and sample or MB filters. Process the SEB (secondary extraction blank) filters last.
- 3.3 Tightly close the tubes, making sure that the O-ring is seated properly.
- 3.4 Place the tubes in the homogenizer (EPA Method 1696.1, Section 6.12) and bead beat for 30 seconds at a rate of 6 m/s or equivalent.
- 3.5 Remove the tubes from the homogenizer and centrifuge at 12,000 × g for 3 minutes to pellet the glass beads and debris. *Note: To minimize contamination, a new pair of gloves should be donned for this step.*
- 3.6 Using a 200 µL micropipettor, carefully transfer a minimum of 400 µL of the supernatant to a corresponding fresh, labeled low-retention 1.7 mL microcentrifuge tube, taking care not to aspirate glass beads or sample debris (pellet). If unable to recover 400 µL of supernatant, centrifuge again, and repeat. Recover SEB filter supernatants last.
- 3.7 Centrifuge microcentrifuge tubes containing supernatant at 12,000 × g for 1 minute.
- 3.8 Prepare for DNA binding step by adding 760 µL of binding buffer (EPA Method 1696.1, Section 7.15) to fresh, labeled low-retention 1.7 mL microcentrifuge tubes.

- 3.9 Transfer 380 μL of the clarified supernatants to the respective low-retention 1.7 mL microcentrifuge tubes taking care not to disturb the pellet and gently mix. *Note: Pellet may not be visible.* Recover the SEB supernatants last.
- 3.10 Label DNAsure columns (EPA Method 1696.1, Section 7.15) and insert into a collection tubes.
- 3.11 Transfer approximately 600 μL of the mixture (clarified supernatant + binding buffer) to the columns and centrifuge at $12000 \times g$ for 1 minute.
- 3.12 Discard flow through and repeat Step 3.11 using the remaining mixture and the same columns. Discard flow through.
- 3.13 Add 500 μL of washing buffer (EPA Method 1696.1, Section 7.15) into the columns and centrifuge at $12000 \times g$ for 1 minute. Discard flow through and repeat (total of two wash buffer rinses per column).
- 3.14 Transfer columns to a labeled low-retention 1.7 mL microcentrifuge tube and add 50 μL elution buffer (warmed to 60°C) using a 200 μL micropipettor directly to each column membrane and centrifuge at $12,000 \times g$ for 1 minute. *Note: Do not discard the eluates.*
- 3.15 Add another 50 μL elution buffer (heated to 60°C) using a 200 μL micropipettor directly to each column membrane. Centrifuge at $12,000 \times g$ for 1 minute. Discard column after final spin.
- 3.16 Stored purified nucleic acid at 4°C for up to 24 hours prior to measuring concentration.

4.0 Fecal Sample Nucleic Acid Extraction and Fecal Extraction Blank (FEB) Preparation

The following are modified instructions for using the Gene-Rite DNA extraction kit (EPA Method 1696.1, Section 7.15). *Note: Do not follow the manufacturer instructions.*

- 4.1 Warm elution buffer (EPA Method 1696.1, Section 7.15) to 60°C using an incubator or heating block (EPA Method 1696.1, Section 6.29).
- 4.2 Remove fecal sample from -80°C and thaw on ice.
- 4.3 Make fecal slurry for each individual sample by mixing ~ 0.25 g feces with 1 mL of phosphate buffered saline (PBS [EPA Method 1696.1, Section 7.5]). *Note: Slurry can be stored at -80°C for up to 3 months.*
- 4.4 Using a 1000 μL micropipettor, dispense 400 μL AE Buffer to labeled extraction tubes containing glass beads.
- 4.5 Using a 1000 μL micropipettor, add ~ 300 μL fecal slurry to labeled extraction tubes containing glass beads and AE Buffer (prepared in step 4.4).
- 4.6 Prepare triplicate FEB controls by adding 300 μL PCR-grade water to labeled extraction tubes containing glass beads and AE Buffer (prepared in step 4.4).
- 4.7 Tightly close the tubes, making sure that the O-ring is seated properly.
- 4.8 Place the tubes in homogenizer (EPA Method 1696.1, Section 6.12) and bead beat for 30 seconds at a rate of 6 m/s or equivalent.
- 4.9 Centrifuge bead mill tube at $12,000 \times g$ for 3 minutes.
- 4.10 Using a 1000 μL micropipettor, transfer supernatants to a 1.7 mL low-retention microtube and centrifuge at $12,000 \times g$ for 1 minute.

- 4.11 Prepare for DNA binding step by adding 760 μL of binding buffer (EPA Method 1696.1, Section 7.15) to fresh, labeled low-retention 1.7 mL microcentrifuge tubes.
- 4.12 Transfer 380 μL of the clarified supernatants to the respective low-retention 1.7 mL microcentrifuge tubes taking care not to disturb the pellet and gently mix.
- 4.13 Label a DNAsure columns (Method 1696.1, Section 7.15) and insert into a collection tubes.
- 4.14 Transfer 600 μL of the mixture (clarified supernatant + binding buffer) to columns. Centrifuge at $12,000 \times g$ for 1 minute. Discard the flow through.
- 4.15 Repeat Step 4.14 for remaining mixture using the same columns.
- 4.16 Using a 1000 μL micropipettor, transfer 500 μL wash buffer (EPA Method 1696.1, Section 7.15) to the columns and centrifuge at $12,000 \times g$ for 1 minute. Discard flow through.
- 4.17 Repeat Step 4.16.
- 4.18 Transfer columns to a labeled low-retention 1.7 mL microcentrifuge tube.
- 4.19 Using a 200 μL micropipettor, add 50 μL elution buffer (warmed to 60°C) directly to each column membrane and centrifuge at $12,000 \times g$ for 1 minute. *Note: Do not discard the eluate.*
- 4.20 Repeat step 4.19 for a total volume of 100 μL .
- 4.21 Discard column after final spin.
- 4.22 Stored purified nucleic acid at 4°C for up to 24 hours prior to measuring concentration.

5.0 Measurement of Total Nucleic Acid in Reference Pollution Source Material Extracts

- 5.1 Measure spectrophotometric absorbance of DNA extract at 260 nm (A_{260}) in triplicate and average the readings. Measurement of total nucleic acid concentration in each DNA extract preparation should be performed within 24 hours. Use averaged concentration value to make the following dilution: 0.5 ng of total nucleic acid per μL . *Note: Other methods such as fluorescence based DNA quantitation are also acceptable, but could lead to different results.*
- 5.2 Prepare aliquots of dilution and store in low-retention plastic microtubes at -20°C . Aliquots should be discarded after three freeze/thaw cycles to minimize the effect of template degradation on results.

6.0 Preparation of qPCR Mixes

- 6.1 Prepare qPCR assay mixes as described in EPA Method 1696.1, Section 11.3. Refer to **Figure C-1** for recommended 96-well format for each instrument run.

A.

	1	2	3	4	5	6	7	8	9	10	11	12
A	10 ⁸ copies	10 ⁷ copies	10 ⁶ copies	10 ⁵ copies								
B	10 ⁸ copies	FEB 1	FEB 2	FEB 3								
C	Fecal 1	Fecal 2	Fecal 3	Fecal 4								
D	Fecal 5	Fecal 6	Fecal 7	Fecal 8								
E	Fecal 9	Fecal 10	Fecal 11	Fecal 12								
F	Fecal 13	Fecal 14	Fecal 15	Fecal 16								
G	Fecal 17	Fecal 18	Fecal 19	Fecal 20								
H	Fecal 21	Fecal 22										

Calibration Curve Standards
 No Template Controls (NTC)
 Fecal Extraction Blanks (FEB)
 Fecal Samples

B.

	1	2	3	4	5	6	7	8	9	10	11	12
A	10 ⁸ copies	10 ⁷ copies	10 ⁶ copies	10 ⁵ copies								
B	10 ⁸ copies	SEB 1	SEB 2	SEB 3								
C	Filter 1	Filter 2	Filter 3	Filter 4								
D	Filter 5	Filter 6	Filter 7	Filter 8								
E	Filter 9	Filter 10	Filter 11	Filter 12								
F	Filter 13	Filter 14	Filter 15	Filter 16								
G	Filter 17	Filter 18	Filter 19	Filter 20								
H	Filter 21	Filter 22										

Calibration Curve Standards
 No Template Controls (NTC)
 Secondary Extraction Blank (SEB)
 Fecal Samples

Figure C-1. Recommended 96-well plate format

- 6.2** Place cap firmly on 1.7 mL microcentrifuge tube containing TaqMan[®] Environmental Master Mix, BSA, primer/probe working stock solution, and PCR-grade water and transfer to DNA template addition work area (Section 6.1). Store on ice until ready to add IAC plasmid and DNA templates.
- 6.3** Remove working stock DNA standards and IAC spike aliquots from freezer (-20°C). Remove DNA extracts and SEB or FEB from 4°C storage. Place all materials on ice.
- 6.4** In DNA template addition work area (Section 6.1), pipet appropriate volume of IAC plasmid (2 µL × number of reactions) into qPCR assay mix tube and mix gently and thoroughly.
- 6.5** Pipet 23 µL of qPCR assay mix into appropriate well of a 96-well qPCR reaction plate.
- 6.6** Lightly cover plate with aluminum adhesive qPCR tape (do not seal tape onto plate).
- 6.7** Label plate and store on ice in dark for transport to dedicated Laminar flow hood in DNA purification workstation (Section 6.1) for the addition of DNA template.
- 6.8** In the laminar flow hood, add 2 µL of 0.5 ng of total nucleic acid template dilution (Section 5.1) (reference source sample extracts) into appropriate wells using a dedicated pipettor. Include a minimum of six NTC reactions (2 µL of PCR grade water substituted for nucleic acid template), triplicate reactions for each fecal source sample extract on each plate, and triplicate SEB or FEB on each plate.
- 6.9** In addition, add 2 µL of each DNA standard in triplicate for each concentration ranging from 10 to 1 × 10⁵ copies.
- 6.10** Seal plate with optical adhesive PCR tape.
- 6.11** Place plate into real-time qPCR instrument according to manufacturer's instructions. Set amplification conditions to 95°C for 10 minutes and then forty cycles of 95°C for 15 seconds and 60°C for 1 minute. Analyze data with the Manual Cq Threshold set at **0.03 (HF183/BacR287)**. For platform-specific operation see **Appendix A**.

7.0 Data Analysis and Calculations

7.1 Estimating specificity

The specificity of a method is described as the proportion of reference fecal samples that are not human and test negative. Specificity is expressed as $d/(a + d) \times 100$, where a represents false positives and d represents true negatives (**Table C-1**). Specificity determinations interpret qPCR data in a qualitative manner (i.e., presence or absence of target sequence). Positive detections of the respective human-associated DNA target sequence are defined as any C_q value \leq LLOQ. Data is tallied as either 'PRESENT' ($C_q \leq$ LLOQ) or 'ABSENT' ($C_q >$ LLOQ). A specificity value can be calculated for each primary pollution source or by combining data from all non-human animal reference pollution source materials. There is currently no consensus among experts on what is an acceptable specificity. It is suggested that a specificity of 80% or more is adequate (EPA Method 1696.1, Section 15.13). For HF183/BacR287, reported specificities are typically $> 90\%$ although it is dependent on the reference pollution source material collection. *Note: Only use fecal reference material samples, not secondary pollution sources (sewage or septage).*

7.2 Estimating sensitivity

Method sensitivity is described as the proportion of reference samples that contain human waste and test positive. Sensitivity is expressed as $b/(b + c) \times 100$, where c represents false negatives and b represents true positives (**Table C-1**). Sensitivity determinations interpret qPCR data in a qualitative manner (i.e., presence or absence of target sequence). Positive detections of the respective human-associated DNA target sequence are defined as any C_q value \leq LLOQ. Data is tallied as either 'PRESENT' ($C_q \leq$ LLOQ) or 'ABSENT' ($C_q >$ LLOQ). A sensitivity value can be tabulated for each source type (e.g., fecal, septage, sewage) or by combining data from all human reference pollution source materials.

7.3 Estimating target sequence abundance in reference pollution sources

Estimation of target sequence concentrations in fecal or sewage samples uses the same mathematical and QA approaches used for water quality testing (see EPA Method 1696.1, Section 12.3). Exceptions include the unit of measure (\log_{10} copies per total nucleic acid mass) and the omission of the Sketa22 SPC. A SPC is not required because the test quantity of purified DNA from reference pollution source material samples is fixed to a test quantity of 1 ng of total nucleic acid per reaction.

Table C-1. Specificity and Sensitivity

		Result		
		+	-	All
True	+	B (TP) ¹	C (FP) ²	B+C
	-	A (FN) ³	D (TN) ⁴	A+D
	All	A+B	C+D	A+B+C+D

¹ True positive

² False positive

³ False negative

⁴ True negative