Horse-Associated *Bacteroidales* Hof597 Marker in Water by Quantitative Polymerase Chain Reaction (qPCR) Assay

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1. SCOPE AND APPLICATION

1.1. This method describes a quantitative Polymerase Chain Reaction (qPCR) procedure that amplifies a gene sequence in the 16S rRNA gene found in multiple *Bacteroides* species which are associated with horse feces.

1.2. Tests covered by this SOP are PCR-HoF597, PCR-HoF597B, PCR-HoF597S, and PUR-HoF597.

2. SUMMARY OF THE METHOD

2.1. Bacteria present in a water sample are collected by filtration. The filter is processed by a bead beater in the presence of zirconium silica beads to extract the DNA from bacterial cells into a buffer solution. The clarified extract may also be purified using a purification kit. The extract is analyzed with qPCR amplification and detection in a thermocycler system using a primers/probe assay specific to horse associated *Bacteroidales*. Solid samples are weighed and purified prior to qPCR amplification and detection.

2.2. Interferences: Colloidal or suspended particulate materials in water can clog the filter and prevent filtration of enough sample volume to detect the target sequence. Some substances, including humic and tannic acids, can act as inhibitors and reduce efficiency by either binding to the DNA polymerase or interfering with the annealing and extension cycles. Sample purification of dilution can help alleviate the effects of inhibitors.

2.3. Definitions:

2.3.1. qPCR: Quantitative Polymerase Chain Reaction is a procedure in which the amount of DNA amplified in each PCR cycle is measured using fluorescent dyes to determine the original amount of DNA in the sample.

2.3.2. Target sequence: The DNA sequence in the gene of interest (GOI) that is amplified by the primers in each PCR cycle. The primers/probe set is designed to attach specifically to this system.

2.3.3. *Bacteroidales*: An order of anaerobic, Gram-negative rod bacteria notably includes genera *Prevotella* and *Bacteroides*, that are typically found in the gastrointestinal tract of humans and animals.

2.3.4. Standard Curve: A dilution series of standards with known DNA concentrations is analyzed and plotted on a graph with the log of the known concentrations on the x-axis and the quantification cycle (C_q) value of each dilution on the y-axis to create a standard curve. This is used to determine the concentration of unknown samples and the performance of the reaction.

3. EQUIPMENT AND SUPPLIES

3.1. Instrumentation –

Instrument Name	Serial Number	Manufacturer

qPCRQuantStudio3_1 Thermocycler	272311740	Applied Biosystems
qPCR QuantStudio3_2 Thermocycler	272314393	Applied Biosystems

3.2. Equipment –

Equipment	Notes
Autoclave	S/N 031761851
Mettler Toledo MS403TS/00	S/N B815673069
Tube Racks	N/A
96-Well Plate Holders and Stands	N/A
Mettler Toledo MS403TS/00	S/N B815673069
CBS Optimizer PCR Workstation #1	S/N 007631
CBS Optimizer PCR Workstation #2	S/N 007632
Micropipettes	N/A
Fisher Mini Vortex	N/A
Fisher Microcentrifuges	N/A
Megafuge ST4F Plus Centrifuge	S/N 43085295
PCR#2, Thermo Freezer	S/N 0121174101121218; Maintained at -20 ± 10°C, Room B246A
PCR#4, Thermo Freezer	S/N 0121174201121218; Maintained at -20 ± 10°C, Room B246B
PCR#5, Thermo Fridge/Freezer	S/N 0121174701121218; Maintained at 4 ± 2°C and -20 ± 10 °C, Room B246
PCR#9, Thermo Fridge	S/N 7R1562211012; Maintained at 4 ± 2°C, Room B246A
PCR#10, Thermo TDE Freezer	S/N 1127920701221013; Maintained at -80 ± 10°C, Room B246
Or Equivalent	

3.3. Supplies/Consumables –

Supply/Consumable	Notes
Gloves, Latex or Nitrile	N/A
Dispersion Beads, 0.1 mm, Zirconium Silica	Weigh out 0.3 ± 0.01 g of dispersion beads into each extraction tube. Seal tube tightly, making sure the O-ring is seated properly. Autoclave at 121°C for 15 min (15 psi). Record in LIMS Autoclave Log.
Extraction Tubes, Conical, 2 mL Screw Cap with O-Ring Seal	
Centrifuge Tubes, 0.5, 1.5, and 2.0 mL	N/A
Sterile PCR Grade Filter Tips	N/A
96-Well Reaction Plate, Clear	N/A
Optical Adhesive Seal for Rection Plates	N/A
Or Equivalent	

4. REAGENTS AND STANDARDS

4.1. Reagents and Standards –

Reagent/Standard	Notes
Deionized (DI) Water	N/A
Molecular Biology Grade Water	Corning Molecular Biology Grade Water tested to USP Sterile Water (Catalog Number: MT46000CV)
Bleach Solution, 10% (v/v)	Prepare per SOP BG-05
Bovine Serum Albumin (BSA), 2 mg/mL	Prepare per SOP PCR-3.0
TaqMan™ Environmental Master Mix 2.0	Acceptable
Applied Biosystems™ TaqPath™ BactoPure™ Microbial Detection Master Mix	Acceptable and preferred due to better sensitivity
Salmon DNA Standards (10^3, 10^2, 10^1)	Prepare per SOP PCR-3.1
Salmon DNA Extraction Buffer, 0.2 µg/mL	
Salmon DNA Forward and Reverse Primers, 500 µM Stock	

Salmon DNA Probe, 100 µM Stock	
HoF597 gBlock Standards (10^6, 10^5, 10^4, 10^3, 10^2)	Prepare per SOP PCR-3.9
HoF597 gBlock DNA Spikes (10^6, 10^4)	
HoF597 Forward and Reverse Primers, 500 µM Stock	**Forward Primer, HoF597F:** 5'–CCAGCCGTAAAATAGTCGG–3' **Reverse Primer, HoF597R:** 5'–CAATCGGAGTTCTTCGTG–3' Prepare per SOP PCR-3.9
HoF597 Taqman Probe, 100 µM Stock	**Probe, HoF597Probe:** 5'–/6-FAM/CGGAACTCG/ZEN/TGGTGTA GCGGTGAAA/3IABkFQ/–3' Prepare per SOP PCR-3.9

5. CALIBRATION AND STANDARDIZATION

5.1. See DEP Laboratory Quality Manual, Section 5.5.

5.2. Check the calibration of thermometers annually. See SOP LB-035.

5.3. Check the calibration of micropipettes quarterly for accuracy and precision. See SOP PCR-2.4.

5.4. Refer to SOP PCR-2.3 for use and maintenance of the thermocycler.

5.5. Refer to SOP BB-035 for calibration, use, and maintenance of the balance.

5.6. Sterilization of the workstation:

5.6.1. UV workstation: After each use, wipe down all surfaces with 10% bleach, then with PCR grade water. Turn on UV lamps for at least 3 minutes. Wipe metallic surfaces with 80% ethanol weekly to prevent corrosion. Never mix bleach and ethanol.

5.6.2. Counters: Clean work area counters after use. Spray surface of work area with 10% bleach solution and wipe down after 15 minutes. Spray and wipe down with sterile DI water.

6. SAMPLE COLLECTION, PRESERVATION, AND HANDLING

6.1. See DEP Field SOP FS 2000 for sample collection and preservation.

6.2. See SOP PCR-5.0 for details on sample custody and printing sample labels.

7. SAMPLE PREPARATION

7.1. Prior to analysis, samples are filtered (per SOP PCR-4.0) and extracted (per SOP PCR-4.1). Inhibited or solid samples are purified (per SOP PCR-4.2).

8. ASSAY PREPARATION

NOTE: Primer/Probe Mix, Master Mix, and Standard preparation for each plate is documented in the PCR-PLATEPREP form. These are saved at \\Floridadep\data\Labs\Lab3\Biology\qPCR\RxnPlatePrepWS. The Primer/Probe Mix and Master Mix are prepared in PCR Workstation #1.

8.1. Primer/Probe Mix Preparation –

8.1.1. HoF597 Assay –

Forward Primer, 500 µM	2.4 µL
Reverse Primer, 500 µM	2.4 µL
Probe, 100 µM	8 µL
PCR Grade Water	587.2 µL

In a 1.5 mL centrifuge tube, add 2.4 µL of each primer stock and 8 µL of probe to 587.2 µL of PCR grade water to make the primer/probe mix. Mix by tapping then centrifuge briefly.

Label with the date, assay name, and "PP" and store at 4°C. Use within one week.

8.1.2. SKETA Assay –

Forward Primer, 500 µM	10 µL
Reverse Primer, 500 µM	10 µL
Probe, 100 µM	9 µL
PCR Grade Water	571 µL

In a pink 1.5 mL centrifuge tube, add 10 µL of each primer stock and 9 µL of probe to 571 µL of PCR grade water to make the primer/probe mix. Mix by tapping then centrifuge briefly.

Label with the date, assay name, and "PP" and store at 4°C. Use within one week.

8.1.3. Primer/Probe Mix Summary –

	HoF597 Assay	SKETA Assay
Forward Primer (500 µM Stock)	2.4 µL	10 µL
Reverse Primer (500 µM Stock)	2.4 µL	10 µL
Probe (100 µM Stock)	8 µL	9 µL
Sterile H_2O	587.2 µL	571 µL

8.2. Master Mix Preparation –

8.2.1. Count the number of wells to determine the total volume of reagents needed for the assay. Add an additional 3-4 wells to ensure enough Master Mix is available, as some can be lost in mixing and pipetting. Enter the number of wells in the '#Rxns,' column in the Master Mix Preparation section of the reaction plate worksheet.

8.2.2. For each assay, add 2 µL X total wells of BSA, 10 µL X total wells of the master mix (TaqPath™ BactoPure™ Microbial Detection Master Mix/ TaqMan™ Environmental Master Mix 2.0), and 3 µL X total wells of primer/probe mix (PP). Use a pink 1.5 mL centrifuge tube for the SKETA Master Mix and another color 1.5 mL centrifuge tube for the HoF597 Master Mix. Label the tubes "MM".

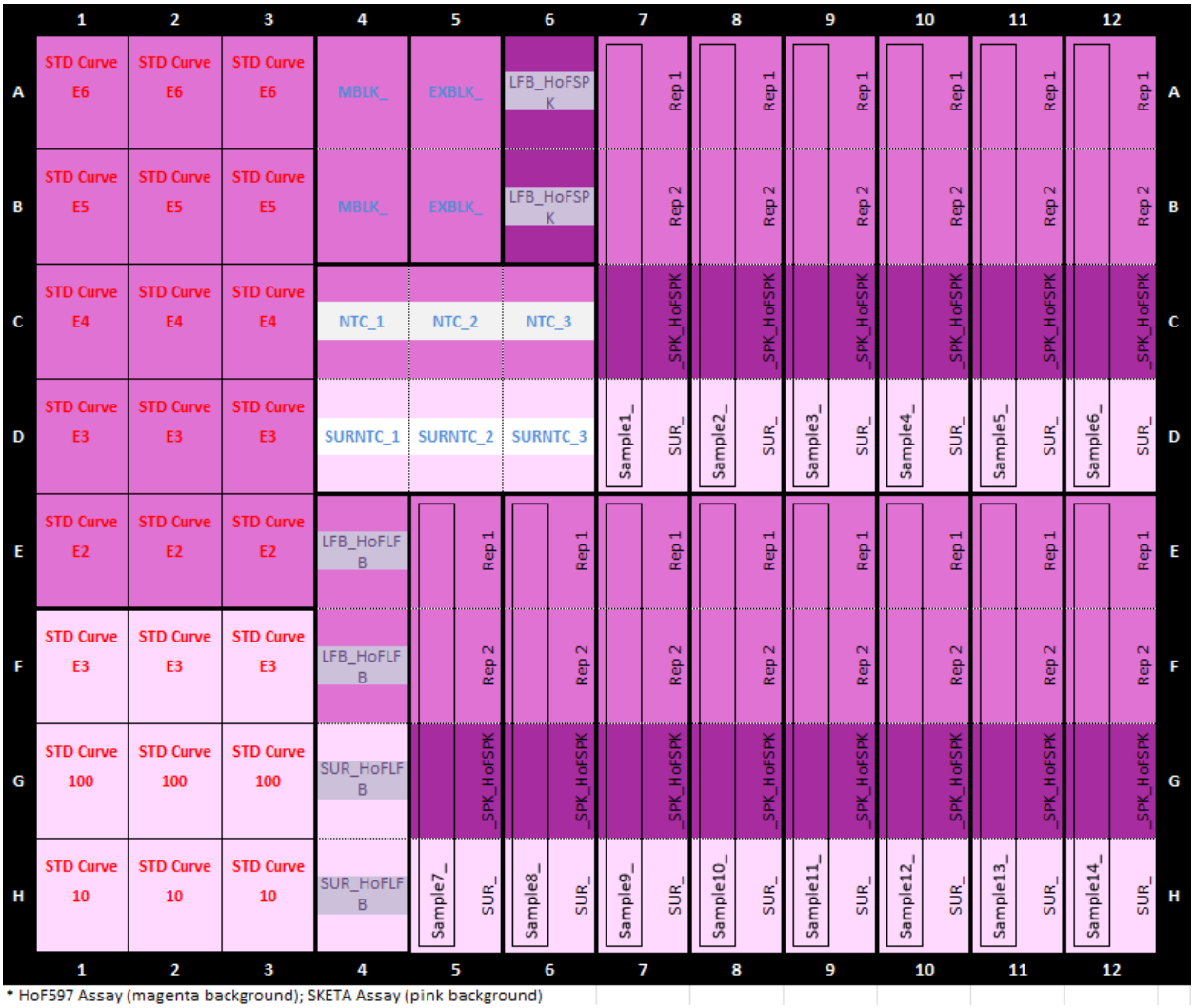
8.2.3. Master Mix Summary –

	HoF597 Assay	SKETA Assay

Reagent	Volume per reaction (µL)	Volume (µL) per full plate (68 + 4 extra wells)	Volume per reaction (µL)	Volume (µL) per full plate (28 + 3 extra wells)
BSA 2 mg/mL	2	144	2	62
MM	10	720	10	310
Primer/Probe Mix	3	216	3	93
Total of 15 µL of Master Master Mix + 5 µL of DNA template= total volume of 20 µL per reaction				

9. REACTION PLATE PREPARATION

9.1. Plate Layout Map –



* HoF597 Assay (magenta background); SKETA Assay (pink background)

9.2. Collect a 96 well plate and holder. In PCR workstation #1, add 15 µL of appropriate qPCR Master Mix assay to each well of the reaction plate. Use the figure in Section 9.1. as a guide. Add the HoF597 Master Mix to the magenta wells and the SKETA Master Mix to the pink wells. Place the Master Mix, BSA, and any extra labeled PP mix back in PCR#9 refrigerator. Carefully move the plate to PCR workstation #2.

9.2.1. Make sure to re-mix the Master Mix occasionally by tapping the tube, followed by a pulse spin in the minicentrifuge. This ensures that the Master Mix is evenly distributed throughout the plate.

9.2.2. Master Mix is more viscous than water and should be pipetted slowly to ensure accuracy. Bubbles can easily form in the pipette tip if pipetted too quickly. If many bubbles form in the tube when pipetting, tap the tube to remix followed by a pulse spin.

9.3. In PCR workstation #2, add 5 µL of DNA sample extract or control to each well using a 10 µL pipette. Use the figure in Section 9.1. as a guide. To ensure proper mixing, tap mix then centrifuge briefly. Switch tips for each sample or control. If any liquids falls outside of the well or sprays, the plate should be discarded and hood thoroughly cleaned. Use the following order for addition of the template to wells:

9.3.1. Add the HoF597 Standard Curve controls (see section 12.1.) to wells A1-E3.

9.3.2. Add the SKETA Standard Curve controls (see section 12.2.) to wells F1-H3.

9.3.3. Add the method blank (MBLK) control (see section 12.3.2.) to wells A4-B4.

9.3.4. Add the extraction blank (EXBLK) control (see section 12.3.3.) to wells A5-B5.

9.3.5. Add PCR grade water for the No Template Controls (NTC, SURNTC; see section 12.3.1.) to wells C4-D6.

9.3.6. Add 1 µL of the HoF597 spike standard (LFB_HoFSPK, _SPK_HoFSPK; see section 12.4.3.) for the spike control and 3rd well sample spikes to wells A6-B6, C7-C12, and G5-G12. Then, add 5 µL of PCR Grade water to control wells A6 and B6.

9.3.7. Add the extraction control (LFB_HoFLFB, SUR_HoFLFB; see section 12.4.1.) to wells E4-H4.

9.3.8. Add the sample extracts to appropriate wells (4 wells per sample). Add the sample to the unspiked wells then add the sample to the spiked 3rd well.

9.4. Seal plate with optical adhesive. Vortex slowly, on Speed 1, for 30 seconds to mix.

9.5. Centrifuge plate at 1300 g for 2 minutes (Program 1 on the MegafugeST4F Plus Centrifuge). Check to make sure the reaction mix is at the bottom of each well and the wells are bubble free. If not, centrifuge another minute.

10. SAMPLE ANALYSIS

10.1. Place plate in the thermocycler and analyze. Refer to SOP PCR-2.3 for setup and use of the thermocycler. Use the default thermocycler cycling conditions for a standard ramp speed (See Table Below).

		PCR	
Step	**Enzyme Activation**	**Cycle (40 cycles)**	
	Hold	**Denature**	**Anneal/Extend**
Temperature (°C)	95	95	60
Time	10 min	15 sec	1 min

11. DATA ARCHIVAL

11.1. Refer to SOP PCR-2.3 for instrument file setup.

11.2. Refer to SOP PCR-5.0 for worksheet instructions.

11.3. Refer to SOP PCR-5.1 for data upload instructions.

12. QUALITY CONTROL

12.1. HoF597 Assay Standard Curves

12.1.1. Analyze a HoF597 Assay standard curve per plate. Run five serially diluted standards in triplicate at the following concentrations: 10^6, 10^5, 10^4, 10^3, 10^2 target sequence copies (TSC)/5 µL. Dilute standards fresh from the 10^6 standard each time in TE buffer. Refer to SOP PCR-3.9 for preparing standard dilutions.

12.1.2. Standard curve target efficiency is 90-115% and R^2 should be ≥0.99. Reanalyze samples and notify supervisor if efficiency is <70% or R^2 is <0.97.

12.2. SKETA Assay Standard Curves

12.2.1. Analyze a SKETA Assay standard curve per plate. Run three serially diluted standards in triplicate at the following concentrations: 10^3, 10^2, and 10^1. Refer to SOP PCR-3.1 for preparing standard dilutions.

12.2.2. Standard curve efficiency should be between 80-120% and R^2 should be ≥0.99. Samples are not affected if the efficiency or R^2 are outside limits.

12.3. Negative Controls

12.3.1. No Template Control (NTC)

12.3.1.1. For each Master Mix used on a reaction plate, add 15 µL of Master Mix and 5 µL of sterile water to three wells.

12.3.1.2. The HoF597 NTCs are labeled NTC_1, NTC_2, and NTC_3. The SKETA NTCs are labeled SURNTC_1, SURNTC_2, and SURNTC_3.

12.3.1.3. If any of the NTCs have a result of less than 35 C_q, or more than one NTC well amplifies, the reaction plate may have

contamination. Re-run if samples are affected and notify supervisor.

12.3.2. Method Blank (MB) – labeled MBLK_#

12.3.2.1. Analyze a MB for each batch of samples filtered (maximum of 13 samples). Filter 50 mL of sterile PBS at the beginning of each sample batch. Extract and analyze with the same batch of samples. Add 5 µL of the MB extract to duplicate wells containing 15 µL HoF597 Master Mix.

12.3.2.2. If the MB has a result above the MDL, there may be possible sample contamination. Discuss with supervisor or senior analyst. All sample results in the batch that are less than ten times the amount of contamination should be "V" qualified, and the following comment added: "Result is V-qualified due to a control blank failure." For samples that are greater than ten times the blank, add the following comment: "Control blank failure. Result is >= 10 times blank result." No "V" qualifier is needed.

12.3.3. Extraction Blank (EB) – labeled EXBLK_#

12.3.3.1. Analyze an EB for each batch of samples extracted (maximum of 13 samples). Place a sterile filter in an extraction tube labeled EB. Add 600 µL of salmon DNA extraction buffer and extract along with the samples. Add 5 µL of the EB extract to duplicate wells containing 15 µL HoF597 master mix.

12.3.3.2. If the EB has a result less than 35 C_q, there may be possible contamination at the extraction step. Samples may be affected and should be re-analyzed. Discuss with supervisor or senior analyst.

12.4. Positive Controls

12.4.1. Extraction Control (EC)

12.4.1.1. Analyze an EC for each batch of samples extracted (maximum of 13 samples). Place a sterile filter in an extraction bead tube labeled EC. Pipette 5 µL of 10^6 HoF597 gBlock standard onto the filter and add 595 µL of salmon extraction buffer.

12.4.1.2. The HoF597 assay EC is labeled LFB_HoFLFB# and the SKETA assay EC is labeled SUR_HoFLFB#.

12.4.1.3. The EC helps determine extraction recovery. Percent recovery of extraction control for both the HoF597 and SKETA assay should be 50-150%. If recovery is low, extraction efficiency may be low.

12.4.2. Sample Processing and Inhibition Control (SPC) (SKETA Assay)

12.4.2.1. The SKETA assay serves as a SPC. Salmon DNA is added to the extraction buffer and each sample is analyzed for both the HoF597 and SKETA assays. The SKETA assay measures the

amount of recovery of Salmon DNA and therefore the efficiency of the extraction process. Low recovery indicates an inefficient extraction process or the presence of inhibition. This control is reported as a surrogate.

12.4.2.2. Analyze each sample, replicate, and EC with the SKETA assay alongside the HoF597 Assay.

12.4.2.3. Compare EC Salmon DNA recovery to the Salmon DNA recovery in each of the samples. If sample recovery is <50% of the expected recovery, then the sample may be inhibited.

12.4.3. Inhibition control (3rd well spike)

12.4.3.1. A known amount of HoF597 DNA is added to each sample as a matrix spike to check for inhibition and DNA recovery.

12.4.3.2. For each sample and replicate, spike the 3rd well of the HoF597 assay with 1 µL of E4 HoF597 gBlock spike standard. Also add 1 µL of the standard to the spike control wells.

12.4.3.3. Compare spike control recovery to sample matrix recovery. If sample recovery is <50% of the control recovery, the sample matrix may have inhibitory effects.

12.4.4. If both the SPC and 3rd well spike show inhibition, re-run the sample from a diluted or purified extract. The purification kit may remove inhibitors. See SOP PCR-4.2.

12.4.4.1. If only one of the two controls show possible inhibition, consult a supervisor prior to dilution or reanalysis.

12.5. Replicate Sample

12.5.1. Analyze at least one replicate per filtration batch (maximum of 13 samples). Filter and analyze the replicate in the same manner as original sample. Replicate samples are named SAMPLE ID_DUP_A and SAMPLE ID_DUP_B and 100 mL is filtered for each.

12.5.2. The target replicate Relative Percent Difference (RPD) should be ≤25% If RPD>25%, the duplicate should be “J” qualified at the job authorization step. A comment regarding precision is automatically added to the report in LIMS.

12.5.3. If a replicate sample is not run, all samples in the batch should have the comment “Precision data unavailable for HoF597-qPCR component” or “Precision data unavailable for HoF597-purified-qPCR component” added to the results.

12.6. Refer to SOP PCR-5.1 for further details on uploading quality control data into LIMS, including calculations and control limits.

13. SAFETY/HAZARDOUS WASTE MANAGEMENT

13.1. All hazardous waste including discarded samples, biological reagents, media, and bacterial cultures must be autoclaved for 45 minutes before disposal.

13.2. Refer to the Health and Safety Plan. Read MSDS information for reagent handling and disposal.

14. REFERENCES

14.1. Ahmed, W., S. Payyappat, M. Cassidy, N. Harrison, and C. Besley. 2023. Microbial source tracking of untreated human wastewater and animal scats in urbanized estuarine waters. Science of The Total Environment, 877: 162764.

14.2. Ballesté, E., K. Demeter, B. Masterson, N. Timoneda, L. Sala-Comorera L, and W.G. Meijer. 2020. Implementation and integration of microbial source tracking in a river watershed monitoring plan, Science of The Total Environment, Volume 736: 139573.

14.3. Dick, L.K., A.E. Bernhard, T.J. Brodeur, J.W. Santo Domingo, J.M. Simpson, S.P. Walters, and K.G. Field 2005. Host Distributions of Uncultivated Fecal Bacteroidales Bacteria Reveal Genetic Markers for Fecal Source Identification. Appl Environ Microbiol. 71: 3184-3191.

14.4. EPA 815-B-04-001, Quality Assurance/Quality Control Guidance for Laboratories Performing PCR Analyses on Environmental Samples (October 2004)

14.5. DEP Laboratory Quality Assurance Manual

14.6. Health and Safety Plan

14.7. DEP SOP FS 2000, *General Water Sampling*

14.8. DEP SOP LB-035, *Calibration of Lab Thermometers using a Constant Temperature Calibration Bath*

14.9. DEP SOP BB-035, *Calibration, Use and Maintenance of Mettler Toledo MS303S Balance*

14.10. DEP SOP PCR-2.3, *Use and Calibration of StepOnePlus™ Real-Time PCR System and QuantStudio 3™ Real-Time PCR System*

14.11. DEP SOP PCR-2.4, *Calibration and Use of Mechanical Pipettes using the Artel® PCR®3 Pipette Calibration System*

14.12. DEP SOP PCR-3.0, *Preparation of Reagents for quantitative Polymerase Chain Reaction (qPCR)*

14.13. DEP SOP PCR-3.1, *Preparation of SKETA Assay Reagents and Standards for qPCR Analysis*

14.14. DEP SOP PCR-3.9, *Preparation of HoF597 Assay Reagents and Standards for qPCR Analysis*

14.15. DEP SOP PCR-4.0, *Preparation of Samples for qPCR Analysis*

14.16. DEP SOP PCR-4.1, *Crude Lysate Extraction Method for qPCR Analysis*

14.17. DEP SOP PCR-4.2, *Purification of Filtered Samples using the Qiagen DNeasy PowerSoil® Pro Kit*

14.18. DEP SOP PCR-5.0, *Sample Custody, Labels, and Worksheet Instructions for Molecular Biology Samples*

14.19. DEP SOP PCR-5.1, *LIMS QC Manager Data Upload, Verification, and Sample Authorization Instructions for qPCR Samples*