RAPID, PCR-BASED METHOD FOR MEASURING *ENTEROCOCCI* AND *BACTEROIDES* IN WATER SAMPLES

A. SUMMARY

The following document describes a method for the detection of total *Enterococcus* and *Bacteroides* spp in water samples based on the collection of these organisms on membrane filters, extraction of their total DNA, and polymerase chain reaction (PCR) amplification of group-specific DNA sequences using the TaqMan^{TM} PCR product detection system and a real time PCR product detection instrument.

The TaqMan system signals the formation of PCR products by a process involving the nucleolytic degradation of a double-labeled fluorogenic probe that hybridizes to the target sequence at a site between the two PCR primer recognition sequences. The reactions are performed in a specially-designed thermal cycling instrument that automates the detection and quantitative measurement of the fluorescent signals produced by probe degradation during each cycle of amplification. These signals are stoichiometrically related to the quantities of PCR products produced. As is typical of PCR amplification, product formation initially follows a logarithmic curve, which subsequently falls off into a plateau. The ability to detect product formation during the initial logarithmic phase provides the basis for determinations of the starting quantities of target sequences in the reactions. During this phase, there is a log-linear relationship between these starting target sequence quantities and the number of amplification cycles required for the fluorescence signal to reach an instrument or user-defined threshold that is significantly above background. The instrument automatically calculates this cycle number for each reaction and reports it as a cycle threshold (C_T) value.

Procedures are further described for the use of C_T values generated by the instrument to enumerate target *Enterococcus* and *Bacteroides* cells in the water samples. The approach described is based on the comparative cycle threshold (C_T) method (PE Biosystems, 1997), which employs an arithmetic formula to determine target sequence quantities in DNA extracts from test samples relative to those in similarly-prepared DNA extracts from calibrator samples containing a known quantity of target organism cells. C_T values for an exogenous control sequence or reference organism, added in equal quantitities to both the test and calibrator samples before DNA extraction, are used to normalize results for differences in the amount of total DNA recovered from each sample (*e.g.*, caused by differences in DNA extraction efficiency between samples) or to signal potential PCR inhibition in test samples.

B. MATERIALS AND PROCEDURES

1.0 Preparation of glass bead extraction tubes **(performed by both Sampling and Analytical Labs)**:

(Note: it is recommended that preparation of these tubes be performed in advance of the water sampling and DNA extraction procedures described in sections 2, 3 and 4 below).

1.1 Materials:

1.1.1 Dedicated laminar hood or workstation for preparation of extraction tubes (Note: for the Analytical Lab, this can be the same hood or workstation as that is used for preparation of PCR reagents as described in section 6).

1.1.2 Weighing Balance (Note: can be outside hood but should be physically removed from any potential sources of DNA contamination).

1.1.3 Semi-conical, screwcap microcentrifuge tubes (PGC, #506-636), hereafter called extraction tubes.

1.1.4 Acid-washed glass beads (Sigma, # G-1277).

1.1.5 Autoclave

1.2 Procedures

Sampling Lab Note: For each sampling visit, prepare in advance a minimum of 1 extraction tube with glass beads for each water sample to be collected plus at least one additional extraction tube with glass beads to be used for filter blanks for each six water sample tubes. Tubes for multiple sampling visits can be prepared in advance.

Analytical Lab Note: For each batch of samples, prepare in advance a minimum of 6 extraction tubes with glass beads. Tubes for multiple analysis days can be prepared in advance.

1.2.1 Unscrew lids from extraction tubes and weigh 0.3 g $\pm$ 0.01 g of glass beads into each tube. Replace lids on the tubes and close tightly.

(Note: Make sure there are no glass beads on the O-rings of the caps that could stop the tubes from being sealed). Also visually check O-rings of the caps after closing to make sure they are seated properly.

1.2.2 Autoclave all extraction tubes with glass beads, prepared as described above, for 15 min at 121 PSI.

2.0 Water sample filtration **(Sampling Lab)**

2.1 Materials:

2.1.1 Dedicated workstation for water filtrations, preferably a laminar flow hood with UV light source.

2.1.2 Water filtration apparatus, including disposable or autoclaved filter funnels and bases for 47-mm filter; vacuum filter flask, or manifold and trap with appropriate tubing connected to vacuum source (line, aspirator or electric pump).

2.1.3 Autoclaved extraction tubes with glass beads, prepared as described in section 1.

2.1.4 Polycarbonate filters, 47-mm, 0.4-µm pore size (*e.g.,* Osmonics Inc., #K04CP04700).

2.1.5 PCR-grade water (e.g OmniPur water from VWR #EM-9610 or can be from in-house distillation or Milli-Q system if verified to be free of target DNA in negative control PCR reactions).

2.1.6 100 ml graduated cylinders.

2.1.7 Two - Membrane filter forceps.

2.1.8 Ethanol, 95%.

2.1.9 Fisher or Bunsen burner.

2.1.10 Permanent marking pen for labeling tubes

2.2 Procedures:

(Note: this procedure can be performed simultaneously with multiple water samples depending on the type of filtration manifold in use. It is recommended that 1 negative control filter be prepared with each batch of 6 water samples (i.e. for each sampling visit) by the same procedure, omitting step 2.2.2. These negative control filters are referred to as filter blanks)

2.2.1 Remove funnel, place a polycarbonate filter on the filter base and replace funnel.

2.2.2 Shake the water sample bottle vigorously to suspend the bacteria uniformly, immediately measure 100 ml with a sterile graduated cylinder, and pour it into the filter funnel.

2.2.3 Apply vacuum to filter the sample, and rinse the sides of the funnel twice with 25 ml of PCR-grade water.

2.2.4 Turn off the vacuum and remove the funnel from the filter base.

2.2.5 Using sterile forceps, fold the filter on the filter base into a cylinder with the sample side facing inward, and insert filter into an extraction tube with glass beads, previously prepared as described in section 1.

(Note: Handle the filter with the forceps at the edges; do not touch the portion of the filter exposed to the water sample.)

2.2.6 Cap the extraction tube tightly and label.

2.2.7 Replace used filter funnels with new sterile funnels. Repeat steps 2.2.1 – 2.2.6 until all water samples have been processed.

3.0. Preparation and extraction of calibrator samples (**Analytical Lab**).:

3.1 Materials:

3.1.1 Dedicated laminar flow hood or workstation with UV light sources for work with DNA samples.

3.1.2 *Enterococcus faecalis*, American Type Culture Collection (ATCC) 29212, cell suspension: ~10^9 cells/ml in 10 µl frozen aliquots in 1.5 ml microcentrifuge tubes. Note: these cell suspensions may be supplied to the analytical lab in 100 µl aliquots. It will be the responsibility of the analytical lab to sub-aliquot these suspensions

3.1.3 *Lactococcus lactis*, American Type Culture Collection (ATCC) 19435, cell suspension, ~5 x 10^8 cells/ml in 20 µl frozen aliquots in 1.5 ml microcentrifuge tubes. Note: these cell suspensions may be supplied to the analytical lab in 100 µl aliquots. It will be the responsibility of the analytical lab to sub-aliquot these suspensions

3.1.4 *Bacteroides fragilis*, ATCC 25285 cell suspension: ~5 x 10^9 cells/ml in 10 ul frozen aliquots in 1.5 ml microcentrifuge tubes. Note: these cell suspensions may be supplied to the analytical lab in 100 µl aliquots. It will be the responsibility of the analytical lab to sub-aliquot these suspensions

3.1.5 Working stock of commercially available Salmon testes DNA (Sigma #D1626) dissolved in PCR grade water (e.g. OmniPur water from VWR #EM-9610) at a concentration of ~10 ug/ml as determined by A260 reading in spectrophotometer)

3.1.6 AE buffer (Qiagen, #19077)

3.1.7 Polycarbonate filters, 47-mm, 0.4-µm pore size (*e.g.,* Osmonics Inc., #K04CP04700).

3.1.8 Extraction tubes with glass beads only, prepared as described in section 1

3.1.9 Two membrane filter forceps.

3.1.10 Ethanol, 95%.

3.1.11 Fisher or Bunsen burner.

3.1.12 Single or 8-place mini bead beater (Biospec Corp., Bartlesville, OK).

3.1.13 Standard Microcentrifuge.

3.1.14 Vortex mixer.

3.1.15 Rainin Pipetmen: P-20, P-200 and P-1000 (or equivalent).

3.1.16 Rainin aerosol barrier pipet tips: 20, 200 and 1000 µl capacity (or equivalent).

3.1.17 1.7 ml low retention microcentrifuge tubes (e.g., #C-3228-1, GENE MATE)

3.1.18 Permanent marking pen for labeling tubes

3.1.19 4° C refrigerator

3.1.20 -20° or -70° C freezer.

3.1.21 10% (v/v) bleach solution and autoclaved, distilled water for work station cleaning

3.1.22 Disposible gloves

3.2 Procedures

(Note: To prevent contamination of water sample filtrates and filter blanks, these procedures should be be performed on the day prior to the filter blank and water sample filtrate extraction procedures described in section 4 but should employ the same batches of extraction buffer with salmon DNA and diluted *L. lactis* cell suspensions that will be used in section 4. These materials should be stored at 4° C overnight after preparing the calibrator samples.

3.2.1 With flame-sterized forceps, fold six polycarbonate filters into cylinders and insert each into an extraction tube prepared as described in section 1.

3.2.2 Dilute Salmon DNA working stock with AE buffer to make 0.2 ug/ml salmon DNA/extraction buffer. Note: prepare sufficient salmon DNA/extraction buffer for the number of samples to be extracted in both sections 3 and 4 x 600 µl plus some extra (e.g. for 18 samples plus 3 sampling filter blanks, plus 6 calibrator samples, dilute 350 µl of 10ug/ml working stock to 17.5 ml with AE buffer to make enough for 29 samples).

3.2.3 Remove one tube each of *E. faecalis*, *B. fragilis* and *L. lactis* stock cell suspensions from freezer and allow to thaw completely.

3.2.4 Using Pipetmen with P-1000 & P-20 aerosol barrier tips, respectively, transfer 980 µl AE buffer and 10 µl of *B. fragilis* stock cell suspension to a tube containing 10 µl of *E. faecalis* stock cell suspension and mix thoroughly by vortexing.

3.2.5 Using Pipetman with P-1000 aerosol barrier tips, transfer 980 µl AE buffer to one tube of 20 µl *L. lactis* cell suspension and mix thoroughly by vortexing.

3.2.6 Using a Pipetman with a P-20 aerosol barrier tip, spot 10 µl of the diluted *E. faecalis* & *B. fragilis* cell suspension from step 3.2.4 on the inside surface of each filter cylinder from step 3.2.1. (Note: Vortex the diluted cell suspension immediately before pipetting.).

3.2.7 Using a Pipetman with a P-20 aerosol barrier tip, spot 20 µl of the diluted *Lactococcus* cell suspension from step 3.2.5 on the inside surface of each filter cylinder from step 3.2.1. (Note: Vortex the diluted cell suspension immediately before pipetting).

3.2.8 Using a Pipetman with a P-1000 aerosol barrier tip dispense 600 µl of Salmon DNA/extraction buffer (prepared as described in 3.2.2) to each of the extraction tubes.

3.2.9 Cap the tube as tightly as possible. Note: Visually inspect O-rings of the caps after closing to make sure they are seated properly.

3.2.10 Shake the extraction tubes on bead-beater for 60 sec. at maximum rate.

3.2.11 Centrifuge in microcentrifuge at 12,000 *x g* for 1 min to pellet glass beads and debris.

3.2.12 Using Pipetman and P-200 aerosol barrier tip, carefully transfer **entire** supernatants above glass beads from each sample to correspondingly-labeled, 1.7 ml, low retention microcentrifuge tubes.

Note: The filter will normally remain intact during the bead beating and centrifugation process. Collect as much of the supernatant as possible from around the filter without disrupting the glass bead pellet at the bottom of the tube.

3.2.13 Using a Pipetman with a P-20 aerosol barrier tip, transfer 40 µl of each supernatant to another correspondingly labeled 1.7 ml, low retention microcentrifuge tube containing 160 µl of AE buffer (=5x dilution).

3.2.14 Refrigerate 5x-diluted samples for analysis the next day. For long term storage, freeze at -20° or -70° C. Store remainder of undiluted supernatants at -20° or -70° C for shipment to EPA lab.

3.2.15 When work is completed, treat all work surfaces in hood or work station with a 10% (v/v) bleach solution. Allow the bleach to contact the surfaces for at least 15 minutes prior to rinsing with autoclaved, distilled water. Turn UV lights on overnight..

4.0. Extraction of water sample filtrates and filter blanks (**Analytical Lab**).

4.1 Materials:

4.1.1 Dedicated laminar flow hood or workstation with UV light sources for work with DNA samples..

4.1.2 Extraction buffer containing 0.2 ug/ml salmon DNA in AE buffer, prepared in section 3.2.2.

4.1.3 Diluted *L. lactis* cell suspension, prepared in section 3.2.5.

4.1.4 AE buffer (Qiagen, #19077)

4.1.5 Single or 8-place mini bead beater (Biospec Corp., Bartlesville, OK).

4.1.6 Standard Microcentrifuge.

4.1.7 Vortex mixer.

4.1.8 Rainin Pipetmen: P-20 and P-1000 (or equivalent).

4.1.9 Rainin aerosol barrier pipet tips: 20 and 1000 µl capacity (or equivalent).

4.1.10 1.7 ml, low retention microcentrifuge tubes (e.g., #C-3228-1, GENE MATE)

4.1.11 Permanent marking pen for labeling tubes

4.1.12 4° C refrigerator

4.1.13 -20° or -70° C freezer.

4.1.14 Water sample filtrates and filter blanks (shipped from Sampling Lab)

4.1.15 10% (v/v) bleach solution and autoclaved, distilled water for workstation cleaning

4.1.16 Disposible gloves

4.1.17 Clean Lab Coat

4.2 Procedures

Note: Disposible gloves and clean lab coat should be worn for these procedures.

4.2.1 Using a Pipetman with a P-20 aerosol barrier tip, spot 20 µl of the diluted *L. lactis* cell suspension from section 3.2.5. on the inside surface of each rolled up water sample and blank filter. (Note: Vortex the diluted cell suspension immediately before pipetting.).

4.2.2 Using a Pipetman with a P-1000 aerosol barrier tip dispense 600 µl of extraction buffer containing 0.2 µg/ml Salmon DNA in AE buffer from section 3.2.2 to each filter blank & water sample filtrate extraction tube.

4.2.3 Close tubes as tightly as possible and label.(Note: Visually inspect O-rings of the caps after closing to make sure they are seated properly.

4.2.4 Shake the extraction tubes on bead-beater for 60 sec. at maximum rate.

4.2.5 Centrifuge in microcentrifuge at 12,000 *x g* for 1 min to pellet glass beads and debris.

4.2.6 Using Pipetman and P-200 aerosol barrier tip, carefully transfer **entire** supernatants above glass beads to correspondingly-labeled, 1.7 ml, low retention microcentrifuge tube. Note: The filter will normally remain intact during the bead beating and centrifugation process. Collect as much of the supernatant as possible from around the filter but take care not to disrupt the glass bead and sediment pellet at the bottom of the tube. **It is recommended that this procedure be performed for filter blanks first and then water samples.**

4.2.7 Using a Pipetman with a P-20 aerosol barrier tip, transfer 40 µl of each supernantant to another correspondingly labeled 1.7 ml, low retention microcentrifuge tube containing 160 µl of AE buffer (=5x dilution).

4.2.8 Refrigerate 5x-diluted samples until ready for analysis as described in sections 5 & 6. For long term storage, freeze at -20° or -70° C. Store remainder of undiluted supernatants at -20° or -70° C for shipment to EPA lab.

4.2.9 When work is completed, treat all work surfaces in hood or work station with a 10% (v/v) bleach solution. Allow the bleach to contact the surfaces for at least 15 minutes prior to rinsing with autoclaved, distilled water.

5.0 TaqMan Analysis (**Analytical Lab**):

5.1 Materials:

5.1.1 At least two laminar flow hoods or workstations with UV light sources: #1] for preparation and aliquoting of PCR reagents to Smart Cycler reaction tubes (can be same hood or station as used for preparation of extraction tubes); #2] for additions of DNA extracts to reaction tubes (can be same hood or station used for DNA extractions, however see sections 3.2.15 and 4.2.9)

5.1.2 Different protective clothing (labcoats) for each workstation.

5.1.3 10% bleach and autoclaved, distilled water for each workstation.

5.1.4 Disposible gloves separate box for each workstation.

5.1.5 TaqMan Universal PCR Master Mix (Applied Biosystems, #4304437).

5.1.6 Bovine serum albumen (BSA), fraction V powder.

5.1.7 PCR-grade water (OmniPur water from VWR #EM-9610)

5.1.8 *Enterococcus* forward primer: ECST748F; 5'-AGAAATTCCAAACGAACTTG, (Ludwig and Schleifer, 2000).

5.1.9 *Enterococcus* reverse primer: ENC854R; 5'-CAGTGCTCTACCTCCATCATT, (Ludwig and Schleifer, 2000).

5.1.10 *Enterococcus* TaqMan probe:GPL813TQ; 5'-(FAM)-TGGTTCTCTCCGAAATAGCTTTAGGGCTA-3'(TAMRA or BHQ) (Ludwig and Schleifer, 2000).

5.1.11 *Bacteroides* forward primer: GenBactF3;
5'-GGGGTTCTGAGAGGAAGGT (Haugland, Unpublished)

5.1.12 *Bacteroides* reverse primer: GenBactR4;
5'-CCGTCATCCTTCACGCTACT (Dick and Field, 2004)

5.1.13 *Bacteroides* probe: GenBactP2;
5'-CAATATTCCTCACTGCTGCCTCCCGTA-3'(TAMRA or BHQ), (Dick and Field, 2004)

5.1.14 Salmon DNA forward primer: SketaF2;
5'-GGTTTCCGCAGCTGGG (Haugland *et al.* 2005).

5.1.15 Salmon DNA forward primer: SketaR3;
5'-CCGAGCCGTCCTGGTCTA (Haugland *et al.* 2005)

5.1.16 Salmon DNA TaqMan probe: SketaP2;
5'-FAM-AGTCGCAGGCGGCCACCGT-3'(TAMRA or BHQ) (Haugland *et al.* 2005)

5.1.17 1.7 ml, low retention microcentrifuge tubes (e.g., #C-3228-1, GENE MATE), separate container for each work station.

5.1.18 Test tube racks for 1.7-ml microcentrifuge tubes, separate racks for each work station .

5.1.19 Vortex mixer, one for each work station .

5.1.20 Standard microcentrifuge.

5.1.21 Smart Cycler 25 µl PCR reaction tubes (Cepheid, #900-0085).

5.1.22 Rack and microcentrifuge for Smart Cycler 25 µl PCR reaction tubes (provided with the instrument).

5.1.23 Rainin Pipetmen: P-10, P-20, P-200 and P-1000 (or equivalent), separate set for each work station.

5.1.24 Rainin aerosol barrier pipet tips: 10, 20, 200 and 1000 µl capacity (or equivalent), separate racks for each work station ..

5.1.25 Cepheid Smart Cycler® System

5.1.26 Printer (not mandatory)

5.1.27 Data archiving system (*e.g.*, ZIP drive and disks, LAN system connection to another computer, etc.).

5.2 Procedures:

5.2.1 To minimize environmental contamination with amplified products, routinely treat all work surfaces with a 10% (v/v) bleach solution. Allow the bleach to contact the surface for at least 15 minutes prior to rinsing with autoclaved, distilled water. Use disposible gloves, and wear a separate clean lab coat at each workstation.

5.2.2 Using a Pipetman with aerosol barrier tips, add PCR grade water to the dried probe and primers from the vendor to create primary stock solutions of 100 µM probe and 500 µM primer, and dissolve by extensive vortexing. Centrifuge briefly. Note: Avoid strong lighting when working with probe stock solutions, store stock solutions at -20° C.

5.2.3 Prepare working stock solutions of *Enterococcus*, *Bacteroides* and Salmon DNA probe/primer mixes by adding 4 µl of probe stock and 10 µl of each primer stock to 676 µl PCR grade water, and vortex. Centrifuge briefly. Use a Pipetman with aerosol barrier tips for all liquid transfers. Store working stock solutions at -20° C. Note: It is recommended that these working stock solutions be aliquoted for storage into sufficient volumes for a single day's use.

5.2.4 Using a Pipetman with aerosol barrier tips, prepare assay Master Mix working stocks of the *Enterococcus*, *Bacteroides* and Salmon DNA reactions in sterile labeled 1.6- ml microfuge tubes as follows:
- 1.5 µL of sterile H_2O/ reaction.
- 2.5 µL of BSA/reaction.
- 12.5 µL of TaqMan master mix/reaction.
- 3.5 µL of probe and primer working stock solution*/reaction.

[*This will give a final concentration of 1 µM of each primer and 80 nM of probe in the reactions]. Note: it is recommended that Master Mix working stocks be prepared at beginning of each day – see section 6.1. Preparing working stocks in advance and freezing is not recommended at this time because of uncertainty about the affects of freezing and thawing on the Taq polymerase enzyme.

5.2.5 Vortex the assay mix working stocks; then centrifuge briefly. Return unused primer/probe working stocks and the other reagents to the refrigerator.

5.2.6 Using a P-20 pipettor with aerosol barrier tips, add 20 µL of the assay mix working stocks to labeled 25 µl Smart Cycler tubes (Note: Try to avoid

generating air bubbles because they may interfere with subsequent movement of the liquid into the lower reaction chamber.). The same tip can be used for pipetting multiple aliquots of the same assay mix as long as it doesn't make contact with anything else.

5.2.7 Cap the Smart Cycler tubes loosely and transfer to the appropriate PCR set-up station (see section 5.1.1).

5.2.8 Using a P-10 pipettor with aerosol barrier tips, add 5 µL of DNA extracts, appropriately diluted in AE buffer (see section 6) to corresponding labeled Smart Cycler tubes containing the assay mix working stocks then close the tubes tightly.

5.2.9 When all Smart Cycler tubes have been loaded, place them in a Smart Cycler centrifuge, and run for 2-4 sec.

5.2.10 Transfer the tubes to the Smart Cycler, inspecting each tube to verify that sample has properly filled the lower reaction chamber. (Note: A small concave meniscus may be visible at the top of the lower chamber, but no air bubbles should be present. If the lower chamber has not been properly filled, carefully open and reclose the tube, and recentrifuge.)

5.3 Smart Cycler Operation (Note: This protocol is intended to provide only information about critical instrument settings required to perform the EPA method. Further details concerning the operation of the instrument can be obtained from the Smart Cycler Operation Manual, Cepheid Part # D0190 Rev. D):

5.3.1 Turn on the Smart Cycler; then the computer.

5.3.2 Double-click on the **Smart Cycler** icon on the computer desktop.

5.3.3 The following steps for defining a protocol are only required before the initial run of the instrument. The protocol that is defined in these steps is used in all subsequent runs of the instrument.

5.3.3.1 Click on the **Define Protocols** icon to go to **Define Protocols** screen.

5.3.3.2 Click on the **New Protocol** button to open the **Protocol Name?** dialog. Enter "TaqMan 15-120" for the new protocol name, and click **OK**. The protocol stages are defined in the series of boxes at the bottom of the **Define Protocol** screen. To define **Stage 1**, click on its drop-down box to display the menu of stage types; then select **Hold**. In the **Temp** column, enter 50, and in **Secs** column, enter 120, leaving the **Optics** setting as the default **Off** setting. (Note: This stage is performed to eliminate potential PCR

carryover products in the reactions using the Amp-Erase® UNG enzyme provided in the TaqMan Universal PCR Master Mix.).

5.3.3.3 To define **Stage 2**, click on its drop-down box to display the menu of stage types, and again select **Hold**. In the **Temp** column, enter 95, and in **Secs** column enter 600. Again, leave the **Optics** setting on **Off** (Note: This stage is performed to inactivate the Amp-Erase® UNG enzyme.).

5.3.3.4 To define **Stage 3**, select **2-Temperature Cycle** from its drop down menu. For the first step, enter 95 in the first row of the **Temp** column and 15 in the **Secs** column, **Optics** column **Off**. For the second step, enter 60 in the second row of the **Temp** column, 120 in the **Secs** column, and click on the **Optics** cell to select **On** from the drop-down menu. This sets the detection of the fluorescence signal to occur at the end of the second step in each cycle. Enter 45 in the **Repeat** field at the top of the **Stage 3** box to specify that it should be repeated for 45 cycles. Click the **Save Protocol** button.

5.3.3.5 To display primary curve graphs, click **Define Graphs**. Check the box for **Automatically add to new runs**. Under **Graph Type** choose **Optics** from the pull down menu. Under **Channels** check the box for **Ch 1 (FAM)**. Under **Show** check the boxes for **Primary Curve** and **Threshold**. Under **Axes** check the box for **Fluorescence vs Cycle**.

5.3.4 Click on the **Create Run** icon to open the **Create Run** screen. For each new run, enter a unique name in the **Run Name** field. (Note: The software does not allow duplicate run names.).

5.3.5 Enter any additional information about the run in the **Notes** field. Click the arrow in the **Dye Set** box to display a drop-down menu of the possible selections. Select **FTTR25** (Note: This selects the dye set: FAM, TET, TAMRA, ROX, and a 25 µl reaction).

5.3.6 Click the **Add/Remove Sites** button. The **Select Protocols and Sites...** dialog will appear. Highlight (click on) the “TaqMan 15-120" protocol developed prior to the first run (See section 5.3.3) in the **Protocols** list. In the **Sites** list, highlight the sites on the instrument to be used with this protocol in the current run by clicking on them with the control key held down (Note: Sites refer to the I-core modules in the Smart Cycler processing block in which reaction tubes will be placed; a total of 16 are possible per block. When using multiple blocks, the site numbers will be preceded by the block letters, e.g. A, B C); then click the right pointing arrow to transfer the selected sites and protocol to the **Selections** table.

5.3.7 Click on the **OK** button to save the selections, and return to the **Create Run** screen.

5.3.8 Place the loaded Smart Cycler reaction tubes in the I-core module slots, selected above for current run. The tubes should snap into place. Either the front or back of the caps can face the front of the processing block.

5.3.9 In one of the **View** menus that is shown, select **Analysis Settings**. The displayed table includes one row for each of the four possible dye channels defined in the dye set. Click on the cell in the **FAM** row under the **Usage** column heading, and select **Assay** from the drop down menu. Set the **Usage** cells for all other dyes to **Unused** in the same manner. (Note: All assays in this protocol use FAM as the reporter dye.) All other cells in this table should be left at default settings (See Smart Cycler Operation Manual.). When finished editing the **Analysis Table**, click on the **Update Analyis** button.

5.3.10 In the other **View** menu that is shown, select the **Results Table**. Enter the sample identification information for each site in the **Sample ID** column (Additional information can be entered into the **Notes** column.). Leave the other columns as default settings (See Smart Cycler Operation Manual.).

5.3.11 Click on the **Start Run** button. The orange LEDs on the Smart Cycler processing block should turn on, and the software will automatically switch to the **View Results** screen.

5.3.12 To display the real time temperature profiles for all sites, click **Temperature** in either of the **View** menus. To display real time growth curves for all samples (*i.e.,* the fluorescence signal vs. cycle), click **Primary** in the other **View** menu.

5.3.13 At the end of the run, it is recommended to check the cycle threshold values calculated by the instrument for each sample by opening the **Results Table** window by clicking on this selection in the upper **View** menu. It is also recommended to inspect the growth curves in the **Primary** window which can be opened in the same manner from the lower **View** menu. The default threshold fluorescence value is shown in this window as a single horizontal red line and the cycle thresholds for each site are shown as vertical red lines. To view the data for individual sites in this window, click on that site number in the table to the right of the graph. If the default threshold fluorescence line is well above all of the growth curve lines prior to visible amplification, the threshold fluorescence value can be changed to a lower value. This is done by reopening the **Analysis Settings** window from the upper **View** menu and entering a new value in the **Manual Thresh Fluor Units** cell in the **FAM** row. Conversely if the

default threshold fluorescence line is below any of the growth curve lines prior to visible amplification, the threshold fluorescence value should be changed to a higher value in the same manner. Previous studies have indicated that a threshold value of 8 works well for most analyses. Click on the **Update Analyis** button to view the new threshold line in the **Primary** window. The cycle threshold values will be automatically updated in the **Results Table**.

5.3.14 Once the threshold fluorescence value is adjusted to an optimal value, click the **Save Run** button. (Note: The Smart Cycler Software does not give a prompt to save changes before printing or exporting. Therefore, it is possible to make changes to the Results Table or Analysis Settings, and immediately print or export the data, then close the run without saving the changes. In this case, the data saved in the Smart Cycler database will not match the printed or exported data. If no changes are made in the threshold fluorescence value, the run data is automatically saved as it is when the program is closed or a new run is created).

5.3.15 To save the **Results Table and Analysis setting**, containing the instrument-calculated cycle threshold values for each sample, click the **Export** button to display the **Export Data** dialog box. Check the box next to the heading **Export Results Table and Analysis Settings** by clicking on it, while leaving all other boxes unchecked. Enter a path and a unique file name (Note: This can be same file name as the run file) by editing the area below the heading, **Export File Name:** (*e.g.*, export\filename.cvs), and then click on the **Export** button at the bottom of the window. Data are exported as comma-delimited text (**.cvs**) files in MS Excel-compatible files to the Export folder in the Smart Cycler folder: **C:\Program Files\Cepheid Smart Cycler 1.2\Export**.

5.3.16 To archive a run, close all other software programs, and select **Archive Run(s)** from **Database Menu**. Click **Proceed**. Select the run to be archived by clicking on its name in the database list. Select a folder, and enter an archive **Filename**. Click **Save**. The **Archive** dialog box will appear when the run has been successfully archived.

6.0 Sample Processing, Analysis and Data Quality Acceptance Guidelines (**Analytical Lab**).

6.1 Sample Analysis

Note: These guidelines assume the availability of four thermal cycling blocks on the Smart Cycler instrument, that would permit analyses with three primer/probe sets (*Enterococcus, Bacteroides* and salmon DNA assay) of all samples from one day of sample collection (18 samples + 3 blanks) in a single run on the instrument. It is assumed that this is a reasonable estimate of the maximum

number of samples that can be extracted and analyzed in a single working day by the analytical lab, given the two hours required for each run of the instrument.. However, the analytical lab may, at its discretion, choose to extract all samples from two days of sample collection (36 samples + 6 blanks) which can be analyzed in two runs of the instrument in this configuration

Each day, determine the number of reactions to be performed for each assay and calculate volumes of master mixes needed as described in section 5. In dedicated work station #1 described in section 5, prepare master mix working stocks and transfer aliquots to Smart Cycler tubes which are then loosely capped until samples are added.

In workstation #1, set up 6 negative reagent control *Enterococcus* and *Bacteroides* assay reactions by adding 5 µl AE buffer to Smart Cycler tubes containing 20 µl of respective master mixes.

In workstation #2, set up *Enterococcus, Bacteroides* and salmon DNA assay reactions by adding 5 µl of each of the six, 5x-diluted calibrator extracts (prepared previous day and stored in refrigerator as described in section 3) to one Smart Cycler tube containing 20 µl of each of the respective master mixes. Next serially dilute 20 µl aliquots of three of the six, 5x-diluted calibrator extracts in AE buffer to make 50x and 500x dilutions. Set up *Enterococcus, Bacteroides* and salmon DNA assay reactions by adding 5 µl of each of the 50x and 500x dilutions to one Smart Cycler tube containing 20 µl of each of the respective master mixes.

Run negative reagent control and calibrator reactions in Smart Cycler blocks as described in section 5. At end of run, adjust fluorescence threshold as described in section 5.3.13 and inspect growth curves for each reaction to verify that C_T values are associated with true growth curves as opposed to spikes in the fluorescence background. If latter instances are observed, re-adjust manual threshold values and update analysis as described in section 5.3.13. Check results for QC acceptance as described in section 6.2 , save the run as described in section 5.3.14 and export the Results Table and Analysis settings as described in section 5.3.15.

While calibrator and reagent control samples are running, prepare filter blank and water sample filter extracts as described in section 4

Set up *Enterococcus, Bacteroides* and salmon DNA assay reactions by adding 5 µl of each 5-fold diluted filter blank and water sample extract to one Smart Cycler tube containing 20 µl of each of the respective master mixes.

Run water sample and filter blank reactions in Smart Cycler blocks as described in section 5.

At end of run adjust fluorescence threshold as described in section 5.3.13 and inspect growth curves for each reaction to verify that C_T values are associated with

true growth curves as opposed to spikes in the fluorescence background. If latter instances are observed, re-adjust manual threshold values and update analysis as described in section 5.3.13.

Save the run as described in section 5.3.14 and export the Results Table and Analysis settings as described in section 5.3.15.

Minimize the Smart Cycler window and open the newly-created export file from Windows Explorer (Note: the export file will be opened in the Excel spreadsheet program). Also open the Comparative C_T calculation template file in Excel. Copy the *Enterococcus, Bacteroides* and Salmon DNA assay C_T values for the calibrator samples, test samples and negative controls and paste in the appropriate cells of the Comparative C_T calculation template file (Note: this file should already contain appropriate formulas entered for all calculations). Copy the avg. calibrator ΔC_T value calculated by the spreadsheet into the corresponding cells of each test sample row. The spreadsheet will then automatically calculate and report the total number of *Enterococcus* and *Bacteroides* cells as Calibrator Cell Equivalents (CCE's) in the original water sample for each test sample. Concentrations can be calculated by dividing this number by the volume of water filtered. (Note: if another run of the Smart Cycler is to be performed immediately, copy the export file to a floppy disk or rewritable CD and transfer to another computer before doing these calculations. Cepheid, Inc. advises against opening additional programs while the Smart Cycler is in operation.)

6.2 Data Quality Acceptance

If greater than one-third of the reagent controls reactions with a particular master mix give positive signals (CT values below 45) or if any of the CT values are lower than 35, the analyses should be repeated with new Master Mix working stock preparations.

Results from 5x, 50x and 500x serially diluted calibrator sample reactions for each assay should be subjected to regression analysis of the $\log_{10}$–transformed calibrator cell equivalents (estimated calibrator cells in the extracts divided by dilution factor, not including initial five-fold dilution) on CT values. Note: salmon DNA assay regressions can be based on the $\log_{10}$–transformed dilution factors. Analyses can either be performed directly on the Smart Cycler or the C_T values can exported to Excel for analysis. The slopes of these regressions should be consistent with laboratory's historical averages. In the event of differences greater than 0.3 units from these averages, new serial dilutions should be prepared and the samples should be reanalyzed. If the differences persist new master mixes should be prepared and tested. If the differences still persist the amplification factor values used for calculations of target cell numbers, as described in section 7, should be modified based on the new slope values.

In general, target cell concentrations can be calculated from the results of the 5x diluted samples as described above. However, salmon assay results for each test

sample should be within 3 CT units of the average of the correspondingly-diluted calibrator sample results. Higher values may indicate PCR inhibition. Repeat *Enterococcus, Bacteroides* and Salmon DNA assay PCR reactions of any samples giving a Salmon DNA assay CT value greater than 3 CT units higher than the mean of the calibrator sample results using 5 µl of an additional 10x dilution of the already 5x-diluted extracts in AE buffer. The result from the original 5x-diluted sample can be accepted if its salmon assay CT value is lower than that of the corresponding 50x-diluted sample (assumption here is that there is negligible inhibition but poor recovery of total DNA in the extract - this is corrected for by the calculation method). If the CT value of the 50x diluted sample is lower, then the result from this sample is probably more accurate but should be reported as questionable.

7.0 Calculation of target cell numbers in test samples from TaqMan analysis Cycle Thresholds **(Analytical Lab)**.

TaqMan analysis permits simplified determinations of the ratios of the target sequences in a test sample compared to a calibrator sample using an arithmetic formula, referred to as the Comparative Cycle Threshold Method. These ratios can be converted to measurements of calibrator cell equivalents in test samples through the use of calibrator samples containing a known quantity of the target organism cells.

7.1 Procedure:

7.1.1 Subtract the reference assay C_T value ($C_{T,ref}$) from the target assay C_T value ($C_{T,target}$) for the calibrator sample extract to obtain $\Delta C_{T,cal}$.

7.1.2 Subtract the reference assay C_T value from the target assay C_T value for the water sample extract to obtain $\Delta C_{T,test}$.

7.1.3 Subtract $\Delta C_{T,cal}$ from $\Delta C_{T,test}$ to obtain $\Delta\Delta C_T$.

7.1.4 Calculate the ratio of the target sequences in the water and calibrator samples using the formula: F^(-ΔΔCT), where F = amplification factor of the target organism PCR assay. [Note: current amplification factor values observed at the USEPA laboratory in Cincinnati are listed in Table 1, however, it is recommended that each analytical laboratory confirm these values by generating standard curves of assay CT values from serially diluted DNA extracts of the target organisms and regression analysis of log cell equivalents on CT. Amplification factors can be calculated from the formula: af = 10^(1 / -X coefficient of the regression)]

Table 1

Target Organisms	PCR Master Mix	amplification factor
Enterococcus	TaqMan	1.9
Bacteroides	TaqMan	1.9
Salmon DNA	TaqMan	1.85

8.1.5 Multiply the ratio of the target sequences in the test and calibrator samples by the number of target organism cells in the calibrator sample to obtain the number of target organism cells in the test sample.

8.1.6 (Note: This calculation can be applied without modification to the analyses of diluted extracts if both the water sample and calibrator extracts are equally diluted.)

Table 2. Example Calculations (Amplification factor = 2):

Target Cells in Sample	Sample Type	$C_{T,target}$	$C_{T,ref}$	ΔC_T	$\Delta\Delta C_T$	Measured Cells in Test Sample ($2^{-\Delta\Delta CT}$ x cells in Calib[1])
10000	Calib[1]	21.4	18.3	3.1	---- ----	
Unknown	Test	23.9	17.4	6.5	3.4	0.089 x 10000 = 890
Unknown	Test	27.5	17.7	9.8	6.7	0.0096 x 10000 = 96

[1] Calib, Calibrator.

8.1.7 The geometric mean of the measured target cells and associated coefficients of variation in multiple water samples can be determined from replicate calibrator sample and water sample C_T values using the following procedures:

8.1.7.1 Calculate the mean ΔC_T values for the replicate calibrator sample extracts.

8.1.7.2 Use ΔC_T value for each individual water sample extracts and the mean calibrator ΔC_T value to calculate the measured cell numbers in each water sample, as described above.

8.1.7.3 Calculate the $\log_{10}$ of the measured cell numbers in each sample log(N).

8.1.7.4 Calculate the mean (M) and standard deviation (S) from the values of log(N) obtained in the previous step for the all of the filter extracts from the sampling period.

8.1.7.5 Calculate the geometric mean as 10^M.

8.1.7.6 The implied coefficient of variation (CV) is calculated, based on the lognormal distribution, as the square root of $10^{V/0.434} - 1$, where $V = S^2$.

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