United States
Environmental Protection
Agency

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

May 2013

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Acknowledgments

This method was developed under the direction of Orin Shanks and Richard Haugland of the U.S. Environmental Protection Agency's (EPA) Office of Research and Development (Cincinnati, Ohio) and Kate Field of Oregon State University's Department of Microbiology (Corvallis, OR).

The following laboratories are gratefully acknowledged for their participation in the validation of this method in marine and fresh waters:

Participant Laboratories

Marine ambient water multi-laboratory validation

Fresh ambient water multi-laboratory validation

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1.0 Scope and Application

1.1 This method describes a quantitative polymerase chain reaction (qPCR) procedure for the measurement of two 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 DNA, and detection of human-associated target sequences in purified DNA extracts by real time polymerase chain reaction (qPCR) using TaqMan® Environmental master mix PCR reagent and the TaqMan® probe system. The TaqMan® system signals the formation of PCR products by a process involving the enzymatic hydrolysis of a labeled fluorogenic probe that hybridizes to the target sequence.

1.2 *Bacteroides* human-associated gene sequences are commonly found in the feces of humans and other sources of human fecal pollution (sewage and septage). Although DNA from these organisms is sometimes present in other animal sources in low concentrations, their presence in environmental water is typically an indication of human fecal pollution.

1.3 This method is recommended for the detection of human fecal pollution in ambient marine and fresh waters.

1.4 The potential for variable total DNA recovery from environmental water samples should be taken into consideration when analyzing results from this method. Guidance is provided in section 9 of this document on how laboratories can monitor the initial and ongoing performance of the method in their hands. It is expected and has been observed that the performance of the method will improve as laboratories gain additional experience with it through continued practice.

1.5 This method assumes the use of a Life Technologies Real-Time PCR System as the default platform. The user should refer to instructions for these instruments in the appendices. Users should thoroughly read the method in its entirety before preparation of reagents and commencement of the method.

2.0 Summary of Method

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

3.0 Definitions

3.1 Human-associated *Bacteroides*: refers to a subpopulation of microorganisms that harbor a gene sequence that is associated with human fecal material.

3.2 Target sequence: A segment of the human-associated *Bacteroides* gene containing nucleotide sequences that are homologous to both the primers and probe used in qPCR assays.

3.3 Sample processing control (SPC) sequence: A segment of the ribosomal RNA gene operon, internal transcribed spacer region 2 of chum salmon, *Oncorhynchus keta* (*O. keta*) and other salmon spp., containing nucleotide sequences that are homologous to the primers and probe used in the SPC qPCR assay. SPC sequences are added as part of a total salmon DNA solution in equal quantities to all water and control sample filters prior to extracting DNA from the samples. The purpose of this control is to monitor the efficiency of DNA extraction, and, if necessary, discard samples that fail acceptance criterion as described in Section 9.7. This control may also signal potential co-extraction of water sample components that interfere with the PCR analysis.

3.4 DNA reference standard: A purified, RNA-free and spectrophotometrically quantified plasmid DNA preparation. DNA standards are used to generate standard curves for determination of performance characteristics of the qPCR assays and instrument with different preparations of master mixes containing TaqMan® reagent, primers and probe as described in Section 9.6.

3.5 Internal amplification control (IAC): A purified, RNA-free and spectrophotometrically quantified plasmid DNA preparation spiked into samples to monitor for amplification interference. This process is described in Section 9.4.

3.6 No template control (NTC): A control where purified DNA extract is substituted with purified, RNA-free water prior to qPCR amplification. NTC are used to identify the presence of contaminating target sequences that may be introduced during preparation of the reagents or reactions.

3.7 Method blank control (MBC): A control where the environmental water sample is substituted with purified, RNA-free laboratory grade water. MBC are used to identify the introduction of contaminating target sequences that may be introduced during filtration, DNA extraction and/or preparation of the reagents or reactions.

3.8 Amplification efficiency (*E*): A measure of the average efficiency at which target sequences are detected by their respective primer and probe assays during each thermal cycle of the qPCR reaction. *E* values should range from 0.90 to 1.05 and are calculated from a standard curve as described in Section 9.6.

3.9 Coefficient of determination (R^2): A measure of the variability in the standard curve for each human-associated qPCR assay. R^2 values should range between 0.95 and 1.0 and are described in Section 9.6.

4.0 Interferences

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

5.0 Safety

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

5.2 Where possible, facial masks should be worn to prevent sample contamination.

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 sample filtering, DNA isolation, qPCR reagent assembly, and qPCR amplifications occur in four separate laboratories with dedicated equipment. In addition to physical barriers and dedicated equipment, qPCR analysis should progress in a single direction (**Figure 1**). Uni-directional progression prevents backtracking of purified DNA from environmental and reference samples, as well as qPCR amplicons generated from DNA amplification.

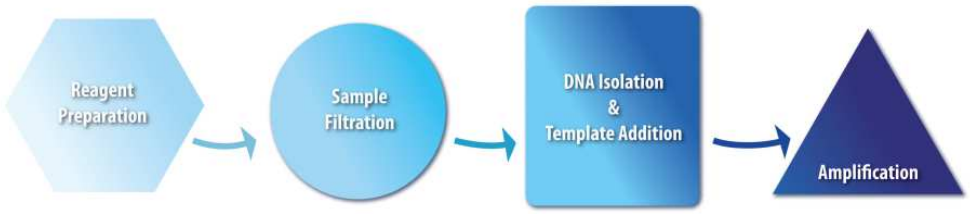

Figure 1: Recommended physical separation and uni-directional progression of analysis for a qPCR method.

6.2 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, etc.) is required.

6.3 Workstation for water filtrations with dedicated supplies that is physically separate from the reagent preparation and DNA isolation workstations.

6.4 Sterile bottles/containers for sample collection

6.5 Disposable membrane filtration units [filter base, Supor® Membrane (0.22 µM) or polycarbonate filter (0.45 µm) with 47 mm diameter, and 100 mL capacity funnel], individually bagged, and gamma-irradiated (MicroFunnels™ filter funnels Part Number: 4806 or equivalent).

6.6 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.

6.7 Flask, filter, vacuum, usually 1 L, with appropriate tubing

6.8 Filter manifold to hold a number of filter bases

6.9 Flask for safety trap placed between the filter flask and the vacuum source

6.10 Stainless steel forceps, straight or curved, with smooth tips to handle filters without damage, 2 pairs

6.11 Permanent ink marking pen for labeling tubes

6.12 Single or multi-place bead beater capable of 5000 reciprocations/minute.

6.13 Microcentrifuge capable of 12,000 × g

6.14 Micropipettors with 10, 20, 200 and 1000 µL capacity. Each workstation should have a dedicated set of micropipettors. Ideally, micropipettors should be calibrated monthly.

6.15 Micropipettor tips with aerosol barrier for 10, 20, 200 and 1000 µL capacity micropipettors. *Note*: All micropipetting should be done with aerosol barrier tips. The tips used for reagents not containing DNA should be separate from those used for reagents containing DNA and test samples. Each workstation should have a dedicated supply of tips.

6.16 Microcentrifuge tubes, low-retention, clear, 1.7 mL (GENE MATE C-3228-1 or equivalent) and 0.65 mL (GENE MATE C-3226-1 or equivalent).

6.17 Rack for microcentrifuge tubes, use a separate rack for each set of tubes

6.18 Vortex mixer (ideally one for each work station)

6.19 Dedicated lab coats for each work station

6.20 Molecular grade disposable powder-free gloves for each work station

6.21 Refrigerator, 4°C (ideally one for reagents and one for DNA samples)

6.22 Freezer, -20°C (ideally one for reagents and one for DNA samples)

6.23 Freezer, -80°C (for storage of filters)

6.24 Ice, crushed or cubes for temporary preservation of samples and reagents

6.25 Data archiving system (*e.g.*, flash drive or other data storage system)

6.26 UV spectrophotometer capable of measuring wavelengths of 260 and 280 nm using small volume capacity (*e.g.*, 0.1 mL) cuvettes or NanoDrop® (ND-2000 spectrophotometer or equivalent) capable of the same measurements at 2 µL sample volumes

6.27 Life Technologies StepOnePlus™ or ABI 7900

6.27.1 Optical 96 well PCR reaction tray (Catalog #N801-0560 or equivalent)

6.27.2 Optical adhesive PCR reaction tray tape (Catalog #4311971 or equivalent) or MicroAmp™ caps (Catalog#N8010534 or equivalent)

6.27.3 Life Technologies StepOnePlus™ or 7900 Real-Time PCR System

6.27.4 Aluminum-foil sealing films for storage, AlumaSeal96™ (Catalog #: 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 from VWR EM-9610 or equivalent). Water must be DNA/DNase free.

7.4 Isopropanol or ethanol, 95%, for flame-sterilization

7.5 AE Buffer (Qiagen #19077)

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 (Part #: 4596838)

7.9 Bovine serum albumin (BSA), fraction V (Invitrogen #15561-020 or equivalent)

Dissolve 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 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 HumM2 primer and probe set:

Forward primer (HumM2F): 5'- CGTCAGGTTTGTTTCGGTATTG -3'

Reverse primer (HumM2R): 5'- TCATCACGTAACTTATTTATATGCATTAGC – 3'

TaqMan® probe (HumM2P): [6-FAM]-5'- TATCGAAAATCTCACGGATTAACTCTTG TGTACGC -TAMRA

TaqMan® probe (UC1P1): [VIC]-5'- CCTGCCGTCTCGTGCTCCTCA -TAMRA

Note: **The HumM2 qPCR assay is patented by U.S. Environmental Protection Agency (EPA). Any use of this method is prohibited without a license granted by the EPA (United States Patent No. 7572584).**

7.10.3 Sketa22 primer and probe set:

Forward primer (SketaF2): 5'-GGTTTCCGCAGCTGGG

Reverse primer (SketaR2): 5'-CCGAGCCGTCCTGGTC

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

7.10.4 Preparation of primer/probes: Using a micropipettor with aerosol barrier tips, add PCR grade water to the lyophilized primers from the vendor to create stock solutions of 500 µM and dissolve by extensive 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 Sterile DNA extraction tubes containing glass beads from Generite LLC., (S02050-50 or equivalent).

7.12 Purified, RNA-free quantified and characterized DNA reference material (section 11.1).

5'-cgtcaggtttgtttcggtattgagtatcgaaaatctcacggattaactcttgtgtacgctCTCGAGgaccagctaatg
catataaataagttacgtgatgagaccggcgcacgggtgagtaacacgtatccaacctgccgtctactcttggccagccttctgaaaggaag
attaatccaggatgggatcatgagttcacatgtccgcatgattaaaggtattttccggtagacgatggggatgcgttccattagCTCGAGa
tagtaggcggggtaacggcccacctagtcaacgatggataggggttctgagaggaagg-3'

7.13 Purified, RNA-free quantified and characterized IAC material (section 11.2).

5'-atcgcgtcaggtttgtttcggtattgagCCTGCCGTCTCGTGCTCCTCAtctcgaggaccagctaatg
catataaataagttacgtgatgaatgcgaccggcgcacgggtgagtaacacgtatccaacctgccgtctactcttggccagccttctgaaag
gaagattaatccaggatgggatcatgagttcacatgtccgcatgattaaaggtattttccggtagacgatgTGTAGCAACGGCGT
GTTatagtaggcggggtaacggcccacctagtcaacgatggataggggttctgagaggaagg-3'

7.14 Not1-HF™ RE-Mix® restriction endonuclease 10x master mix (New England BioLabs, Catalog # R5189S or equivalent). (*Note:* A different restriction endonuclease may be necessary if the plasmid vector does not contain a unique Not1 restriction site).

7.15 QIAGEN Plasmid Mini Kit (Catalog # 27104 or equivalent)

7.16 DNA extraction kit (Gene-Rite K102-02C-50 DNA-EZ® RW02)

7.17 Optional: 5 mL polystyrene round bottom test tubes with snap cap (BD Falcon™ #352003)

8.0 Sample Collection, Handling, and Storage

8.0 Sampling procedures are briefly described below. Adherence to sample preservation procedures and holding time limits is critical to the production of valid data. Samples not collected according to these procedures should not be analyzed.

8.1 **Sampling Techniques** - Samples are collected by hand or with a sampling device if the sampling site has difficult access such as a dock, bridge or bank adjacent to a surface water. Composite samples should not be collected. The sampling depth for surface water samples should be 6 − 12 inches below the water surface. Sample containers should be sterilized and 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.

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

8.3 **Holding Time Limitations -** Examine samples as soon as possible after collection. Do not hold samples longer than 8 hours between collection and initiation of filtration.

9.0 Quality Control

9.1 Quality control (QC) parameters are necessary to generate reliable estimates of genetic marker concentration in unknown samples. Errors can arise from numerous sources in the qPCR method protocol ranging from improper sample handling, degradation of DNA standards, laboratory technician difficulties, to interferences originating from the unknown sample itself. Each laboratory is required to operate a formal quality assurance (QA) program that addresses and documents instrument and equipment maintenance and performance, reagent quality and performance, analyst training and certification, and records storage and retrieval.

The minimum analytical QC requirements for the analysis of samples includes an internal amplification control (Section 9.4), no template controls (Section 9.2), method blanks (Section 9.3), DNA standard curves (9.5), and sample processing controls (9.7).

9.2 **No template controls** — The laboratory should analyze "No Template Controls" (NTCs) to ensure that reagents or laboratory environment introduced contaminants. On an ongoing basis, the laboratory should perform a minimum of three NTC reactions with every instrument run. If any of the NTC reactions for instrument run elicit true positive logarithmic amplification traces with C_q values below 40 (not from chemical degradation of probe with linear kinetics that exhibit rising baseline), the analyses should be repeated with new master mix working stock preparations.

9.3 **Method blank controls** — Filter 100 mL volume of PCR-grade water before beginning the sample filtrations. Remove the funnel from the filtration unit. Using two sterile or flame-sterilized forceps, fold the filter on the base of the filtration unit and place it in an extraction tube with glass beads as described in Section 11.4. DNA extraction as in Section 11.5. The absence of a fluorescence amplification growth curve for the HF183 and HumM2 assays during PCR analysis of these controls (reported as "undetermined" on ABI instruments) indicates the absence of contaminant target DNA. **Prepare at least three Method Blank filters for each batch of samples (note: Method blank controls contain salmon DNA, but should not yield C_q values < 40 with HF183 or HumM2 assays).**

9.4 **Screening test sample DNA extracts for amplification inhibition** — Substances inhibitory to qPCR amplification can persist after DNA purification. Therefore, an Internal Amplification Control (IAC) designed to evaluate the suitability of isolated DNA for qPCR-based amplification should be performed with each test sample DNA extract. The criterion for concluding no significant qPCR amplification interference can be established as a mean $C_q \pm 1$ based on IAC Cq values within the determined IAC range of quantification (ROQ) obtained from respective multiplex calibration curve reactions.

Test sample IAC C_q data can then be reviewed to determine if respective IAC Cq values are within the acceptable range of variability. IAC C_q values greater than the upper bound acceptance threshold can result from either amplification inhibition or competition between the IAC and native human-associated DNA targets in the multiplex qPCR reactions.

In order to establish a target competition threshold, users must generate a multiplex calibration curve, determine the IAC ROQ) and establish a threshold for native target/IAC competition (**Figure 2**). The IAC ROQ is determined by comparing raw IAC C_q measurements from the

multiplex calibration curve. The range of plasmid reference DNA standard concentrations where there is less than a ± 0.75 Cq shift from the IAC mean Cq at 10 copies is defines the IAC ROQ. The interference threshold is then calculated based on the mean and standard deviation of all IAC data points as follows: Mean IAC Cq + (4 * Standard Deviation). The competition threshold is the C_q value where the upper bound of the IAC ROQ intersects the multiplex calibration curve. For example, **Figure 2** depicts an IAC ROQ range from $\log_{10}$ 1 to 3 plasmid reference DNA standard concentrations. The competition threshold is then determined to be where the upper bound of the IAC ROQ ($\log_{10}$ 3 copy number) intersects the multiplex calibration curve (29.6 C_q).

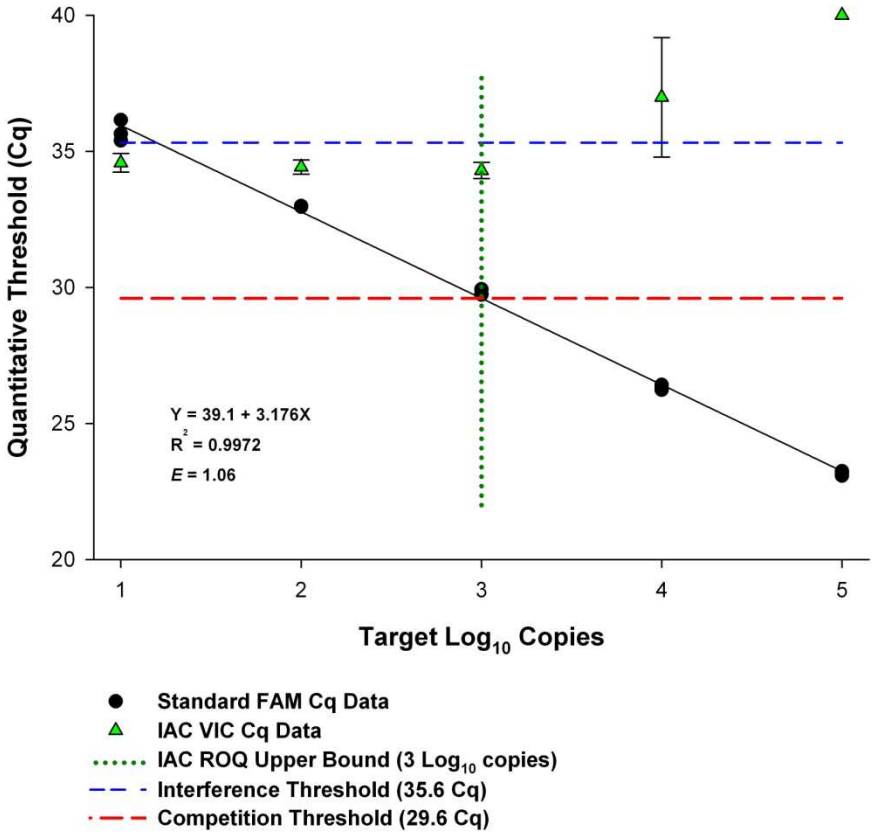

Figure 2: Plot of HumM2 multiplex calibration curve single instrument run, determination of IAC range of quantification (IAC ROQ), and establishing a competition threshold.

The combination of establishing an interference threshold based on IAC C_q measurements and a competition threshold both derived from the IAC ROQ and multiplex calibration curve provide the tools to differentiate between IAC inhibition (IAC C_q > upper bound interference threshold and > than competition threshold) and competition (IAC C_q > upper bound interference threshold, but < than competition threshold). It is recommended that data associated with a particular DNA extract where IAC C_q values are observed outside the accepted range are either discarded from the study or the DNA extract is diluted and re-tested.

9.5 **Reference DNA standards and calibration curves –** A composite calibration curve should be generated from triplicate analyses of each dilution of reference DNA using respective HF183 and HumM2 primer and probe assays and subjected to linear regression analysis. A calibration curve generated from reference DNA dilutions (triplicate reactions) must be included with each instrument run.

Two criteria used to determine the suitability of the calibration curve for transforming raw quantification cycle (C_q) data into sample concentration estimates include amplification efficiency (*E*) and the coefficient of determination (R^2). *E* is derived from the slope parameter in the fitted curve and is defined as follows:

$$E = (10^{-1/\text{slope}})-1 \qquad \textit{Equation 1}$$

It is recommended that an *E* value should be within the range of 0.90 to 1.05. R^2 is the proportion of variability in the reference DNA standard C_q measurements that is accounted for by the regression model and it can range from 0 to 1. A value of $\geq$ 0.95 is recommended for applying a calibration curve for estimating unknown sample concentrations. In the event that values from a subsequent calibration curve regression are outside of the acceptance ranges, the diluted standards should be re-analyzed. If this difference persists, new working stocks of reference DNA material should be obtained and tested.

9.7 **Salmon DNA SPC analyses** — Loss of DNA, interference from the environmental matrix, and laboratory technician error can all impact the efficiency of DNA recovery during extraction from an environmental sample filter. To monitor for variability in sample processing efficiency, each environmental sample filter is spiked with a fixed concentration of salmon sperm DNA and the resulting DNA elute is tested with the Sketa22 qPCR assay. The demonstration of consistent recovery efficiency from one sample DNA extract to the next is achieved by establishing an acceptance threshold based on repeated control experiments. The sample processing efficiency threshold should be based on a minimum of three Method Blank controls (see Section 11.4) containing salmon DNA spike (triplicate C_q measurements for each extraction blank DNA extract) per extraction batch. A mean is then calculated from resulting Method Blank Control Sketa22 qPCR C_q data. A commonly used acceptance threshold range is $\pm$ 3 C_q of the established Method Blank Control mean C_q **(Figure 3).** For test sample DNA extracts that elicit Sketa22 qPCR C_q values outside the acceptance threshold, respective human-associated qPCR C_q values (HF183 and HumM2) should be discarded from the data set.

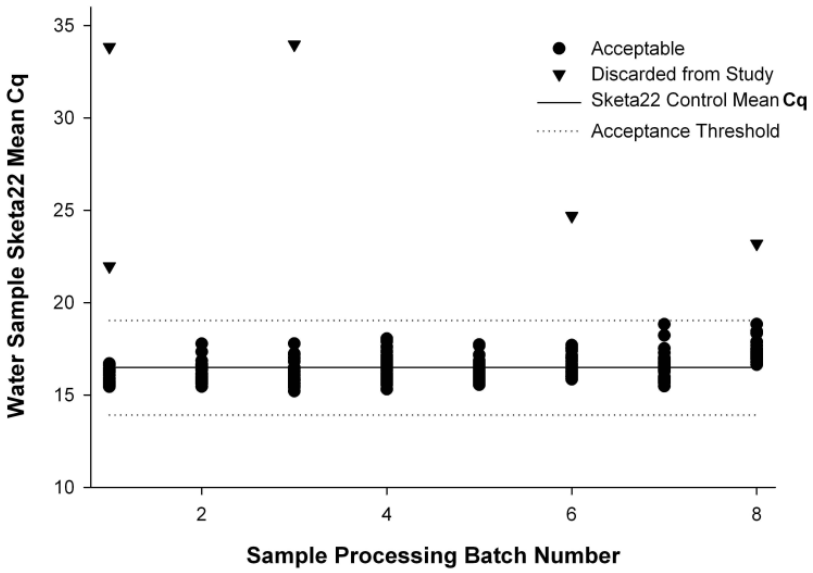

Figure 3: Interpretation of sample processing control data. The solid line denotes the Sketa22 control mean C_q. The dotted line indicates the acceptance threshold range based on the Sketa22 Method Blank Control mean ± 3 C_q. Circles represent environmental samples with acceptable sample processing efficiency. Triangles represent samples that fail SPC metric and should be discarded from future data interpretation. Note: Sketa control mean Cq can vary from one salmon DNA working stock solution (0.2 µg ml^{-1}) to another (see Section 11.3). To avoid discontinuity within a study, prepare single salmon DNA working stock solution at the appropriate volume need to complete a particular study.

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. Check columns for breaks.

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

11.1 Preparation of Plasmid-Derived Reference DNA standards

11.1.1 Highly recommended that plasmid reference DNA standards should be prepared in a separate laboratory from work areas used for reagent mixing, sample filtration, and DNA

isolation (Section 6.0) using dedicated supplies and instruments to prevent cross-contamination with test samples.

11.1.2 Plasmid-derived DNA standards contain a sequence corresponding to a human-associated primers and hydrolysis probe and can be constructed in-house or ordered from companies specializing in custom gene synthesis. (*Note:* Plasmid reference DNA standard construct should be sequenced to confirm that primer and probe sequences are correct).

11.1.3 Digest 2 µg of plasmid with the Not1-HF™ RE-Mix® (Section 7.14) 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 Not1 restriction site).

11.1.4 Clean digested product using the QIAGEN Plasmid Mini Kit (Section 7.15) according to manufacturer directions. Final elution volume should be 50 µl.

11.1.5 Measure spectrophotometric absorbance of cleaned product at 260 nm (A_{260}) in triplicate and average the readings.

11.1.6 Use plasmid size to determine plasmid copies per gram as follows:

$$\frac{6.023 \times 10^{23} \text{ molecules/gram}}{(\text{X bp})(650 \text{ Daltons/bp})} = \text{molecules/gram} \qquad \textit{Equation 2}$$

,where X indicates the total number of base pairs in the plasmid including the standard DNA construct insert.

11.1.7 Use absorbance reading to calculate the concentration of the plasmid in copies per 2 µl. Use this value to make the following plasmid reference DNA dilutions: 10^6 copies/2 µl, 10^5 copies/2 µl, 10^4 copies/2µl, 10^3 copies/2 µl, 10^2 copies/2 µl, and 10^1 copies/2 µl.

11.1.8 Prepare aliquots of each dilution and store in low-retention plastic microcentrifuge tubes at -20ºC. Aliquots should be discarded after three freeze/thaw cycles to minimize the effect of template degradation.

11.2 Preparation of Internal Amplification Control (IAC)

11.2.1 Highly recommended that the IAC should be prepared in a separate laboratory from work areas used for reagent mixing, sample filtration, and DNA isolation (Section 6.0) using dedicated supplies and instruments to prevent cross-contamination with test samples.

11.2.2 Internal amplification control plasmids contain a sequence corresponding to the human-associated primers and the UC1P1 (HumM2) or Bac234IAC (HF183) probe sequences and can be constructed in-house or ordered from companies specializing in custom gene synthesis. (*Note:* Plasmid DNA standard construct should be sequenced to confirm that primer and probe sequences are correct).

11.2.3 Digest 2 µg of plasmid with the Not1-HF™ RE-Mix® (Section 7.14) 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 Not1 restriction site).

11.2.4 Clean digested product using the QIAGEN Plasmid Mini Kit (Section 7.15) according to manufacturer directions. Final elution volume should be 50 µl.

11.2.5 Use plasmid size (base pairs) to determine plasmid copies per gram as described above in Section 11.1.5.

11.2.6 Use absorbance reading to calculate the concentration of the plasmid in copies per 2 µl. Use this value to make the following plasmid dilution: 50 copies/2 µl.

11.2.7 Prepare aliquots of dilution and store in low-retention plastic microtube at -20ºC. Aliquots should be discarded after three freeze/thaw cycles to minimize the effect of template degradation on results.

11.3 Preparation of Salmon DNA Sample Processing Control (SPC)

11.3.1 Highly recommended that salmon DNA SPC preparations should be prepared in a separate laboratory from work areas used for reagent mixing, sample filtration, and DNA isolation (Section 6.0) using dedicated supplies and instruments to prevent cross-contamination with test samples.

11.3.2 Dilute salmon DNA (Section 7.2) to 1 mg ml^{-1} by adding 0.5 ml of the 10 mg ml^{-1} stock to 4.5 ml AE buffer.

11.3.3 Make a 10 µg ml^{-1} salmon DNA solution by using a 1:1000 dilution of the 10 mg ml^{-1} stock (Section 7.2). To prepare the 10 µg ml^{-1} salmon DNA solution, mix 25 µl of 10 mg ml^{-1} stock into 24.975 ml of AE buffer.

11.3.4 Make 1 ml aliquots of the 10 µg ml^{-1} solution and store in low-retention plastic microcentrifuge tubes at 4°C. (*Note:* Recommend preparing fresh salmon DNA solution at least every 6 months).

11.3.5 Select single 1 mL aliquot of 10 µg ml^{-1} solution and measure spectrophotometric absorbance at 260 nm (A_{260}). (*Note:* If a blank measurement is required to calibrate instrument, use AE.)

11.3.6 Use absorbance reading (1 OD = 50 µg ml^{-1}) to calculate volume needed to make 0.2 µg ml^{-1} salmon DNA working stock.

$$\frac{(A_{260}\ \text{value}) \times (50\ \mu g\ ml^{-1})}{0.2\ \mu g\ ml^{-1}} = \text{required volume} \qquad \textit{Equation 3}$$

11.3.7 Dilute 10 µg ml^{-1} solution to 0.2 µg ml^{-1} according to calculation using AE buffer. For example, for 40 samples, including 3 Method Blanks, dilute 500 µl of 10 µg ml^{-1} solution to 25 mL AE buffer.

11.3.8 Store salmon DNA working stock solution (0.2 µg ml^{-1}) in 50 mL conical tube or equivalent at 4ºC until used. (*Note*: Prepare the working stock salmon DNA fresh daily as needed).

11.4 Test sample filtration and method blank preparation

Note: It is required that minimally three sample method blanks be analyzed for every batch of test samples (Section 9.7).

11.4.1 Place a fresh microfunnel assembly (Section 6.5) on the filter base. (*Note:* Depending on the disposable filtration unit used, the membrane filter included may need to be removed and replaced with appropriate filter).

11.4.2 Shake the sample bottle vigorously 25 times to distribute the bacteria uniformly, and measure 100 mL of sample into the funnel.

11.4.3 After filtering the sample, turn off the vacuum and remove the funnel from the filter base. [*Note:* Method blanks are prepared by substituting 100 mL of water sample with 100 mL of PCR-grade water (Section 7.3)].

11.4.4 Label an extraction tube containing glass beads (Section 7.11) to identify test sample. Leaving the filter on the filtration unit base and 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 test sample. Insert the rolled filter into the labeled extraction tube with glass beads. Prepare three filters for each sample filtered in this manner.

11.4.5 Cap the extraction tube and store bead tubes containing folded filters at -80ºC until time of DNA isolation. (*Note:* Filters can be stored at -80°C for a maximum of 12 months before DNA isolation).

11.5 DNA extraction from test filters and method blank controls

11.5.1 Using a 1000 µL micropipettor, dispense 600 µL of the Salmon DNA/extraction buffer (Section 11.3.6) into each labeled extraction tube with glass beads containing Test sample or method blank filters from Section 11.4. Extract the method blank controls last.

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

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

11.5.4 Remove the tubes from the bead beater and centrifuge at 12,000 × g for 3 minutes to pellet the glass beads and debris. *Note*: To help prevent contamination, a new pair of gloves should be donned for this step.

11.5.5 Using the 200 µL micropipettor, carefully transfer a minimum of 400 µL of the supernatant to a corresponding 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, replace entire volume back into bead mill tube, centrifuge again, and repeat). Recover method blank supernatants last.

11.5.6 Centrifuge microcentrifuge tubes containing supernatant for 1 minute at 12,000 × g. Transfer 380 µL of the clarified supernatant to another low-retention 1.7 mL microcentrifuge tube, taking care not to disturb the pellet (*Note*: Pellet may not be visible in water samples). (*Note*: Important to pipet exact amount (380 µL) to yield reliable SPC data. Recover the method blank supernatant last.

11.5.7 Purify DNA extracts by adding 760 µL of 2X binding buffer to the clarified supernatant and mix gently by pipetting.

11.5.8 Place columns from DNA-EZ kit into elution tubes.

11.5.9 Transfer approximately 600 µl of the mixture to the column and centrifuge at 12000 x g for 1 min.

11.5.10 Discard flow through and repeat step 11.5.9 using the remaining mixture and the same column.

11.5.11 Add 500 µl of wash buffer into the column and centrifuge at 12000 x g for 1 min. Discard flow through and repeat (total of two wash buffer rinses per column).

11.5.12 Using sterile forceps, remove the column and place into a fresh sterile low-retention 1.7 ml microcentrifuge tube.

11.5.13 Add 50 µl of warm (60°C) elution buffer into the column and centrifuge tube at 12,000 x g for 1 min.

11.5.14 Repeat step 11.5.13 for a total elution volume of 100 µl.

11.5.15 Transfer to a sterile, fresh 200 µl low-retention plastic microtube.

11.5.16 Label tube with respective sample identification notation. These are the water sample filter extracts. Also label tubes for method blanks.

11.5.17 Store purified DNA at 4°C. qPCR analysis should be performed within 24 hours of extraction.

11.6 Preparation of qPCR assay mixes

11.6.1 Initial qPCR reagent mixing should be performed in a separate workspace using dedicated supplies and equipment (Section 6.0). 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.

11.6.2 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.

11.6.3 Prepare working stocks of HF183, HumM2, and Sketa22 primer/probe mixes as outlined in Table 1.

Table 1: Preparation of primer and probe mix for qPCR assays

Reagent	**Stock Solution**	**Volume in Primer/Probe Mix**		
		HF183	*HumM2*	*Sketa22*
Forward Primer	500 µM	10 µl	10 µl	10 µl
Reverse Primer	500 µM	10 µl	10 µl	10 µl
6FAM Probe	100 µM	4 µl	4 µl	4 µl
VIC Probe	100 µM	4 µl	4 µl	---
UltraPure H_2O	---	572 µl	572 µl	576 µl

11.6.4 Remove TaqMan® Environmental Master Mix, and BSA reagents from storage. Gently mix each reagent microtube and pulse microcentrifuge to coalesce any droplets.

11.6.5 Using dedicated micropipettors for reagent mixing, prepare a master mix for each assay in separate, sterile, labeled 1.7 mL low-retention microcentrifuge tube as described in **Table 2**. **Note:** For mastermix preparations containing more than 70 reactions total, a 5 mL polystyrene round bottom test tube with snap cap is recommend (BD Falcon™ #352003). Prepare sufficient quantity of master mix for the number of test samples, reference DNA standards, NTCs, extraction blanks, and any other controls to be analyzed per instrument run plus an additional 10% of total reactions (ie. if 50 reactions expected, then prepare master mix with additional 10 reactions (50 x 0.10 = 5) for a total of 55 reactions). Note: Additional volume added due to master mix loss during mixing. Prepare assay mixes each day before handling DNA test samples.

Table 2: Preparation of master mix for each qPCR assay

Reagent	Final Concentration	Volume for one 25 µl reaction		
		HF183	*HumM2*	*Sketa22*
TaqMan® Environmental Master Mix	1 X	12.5 µl	12.5 µl	12.5 µl
BSA	0.2 mg ml^{-1}	2.5 µl	2.5 µl	2.5 µl
Primer/probe working stock solution	1 µM/80 nM	3.0 µl	3.0 µl	3.0 µl
UltraPure H_2O	---	4.0 µl*	4.0 µl*	5.0 µl*
IAC plasmid†	---	1 µl	1 µl	---

* Volumes may change depending on specifics of individual experiment.

† IAC plasmid should be added to mastermix in same work area as DNA template addition (Section 6.0)

11.6.6 Place cap firmly on tube (either 1.7 mL microcentrifuge tube or 5 mL polystyrene tube) containing Environmental master mix, BSA, primer/probe working stock solution, and UltraPure H2O and transfer to DNA template addition work area (Section 6.0). Store on ice until ready to add IAC plasmid and DNA templates.

11.6.7 In DNA template addition work area, pipet appropriate volume of IAC plasmid into mastermix tube and mix gently. Note: Important to mix thoroughly.

11.6.8 Pipet 23 µl of master mix into each well of a 96-well FAST qPCR reaction plate.

11.6.9 Lightly cover plate with aluminum adhesive qPCR tape (do not seal tape onto plate).

11.6.10 Label and store on ice in dark for transport to dedicated Laminar flow hood in DNA purification workstation for the addition of DNA template.

11.6.11 Add 2 µl of DNA template into appropriate wells using a dedicated pipette. Include a minimum of three no template controls (2 µl of PCR grade water substituted for DNA template), three extraction blanks, and two positive controls on each plate. Analyze environmental water samples in triplicate.

11.6.12 In addition, add 2 µl of each plasmid DNA standard in triplicate for each concentration ranging from 10 to $1x10^6$ copies on each plate. (*Note:* Current protocol recommends generating a standard curve with each instrument run. For studies requiring more than six instrument runs, it may be beneficial to use a master standard curve approach produced by a minimum of six standard curves in separate instrument runs (See Section 12.0).

11.6.13 Seal plate with optical adhesive PCR tape.

11.6.14 Place plate into real-time qPCR instrument according to manufacturer's instructions. Set amplification conditions for to 50°C for 2 min followed by 95°C for 10 min and then forty cycles of 95°C for 15 sec and 60°C for 1 min. Analyze data with the Manual C_q Threshold set at 0.03 for HF183 and Sketa22 or at 0.08 for HumM2.

11.6.15 Run reactions in ABI StepOnePlus™ or ABI 7900 (all in non-Fast mode) Real-Time PCR Systems. For platform-specific operation see Appendix A.

12.0 Data Analysis and Calculations

Will be included in final method.

13.0 Acronyms

BSA	bovine serum albumin
C_q	quantification threshold
DNA	deoxyribonucleic acid
NTC	no template control
QA	quality assurance
QC	quality control
qPCR	quantitative polymerase chain reaction
SPC	sample processing control
UV	ultraviolet radiation
ROQ	range of quantification
IAC	internal amplification control
E	amplification efficiency
NIST	National Institute of Standards and Technology
C	Celsius
ANOVA	analysis of variance

Appendix A:
Life Technologies StepOnePlus™ and Life Technologies 7900 Real-Time PCR System Operation

StepOnePlus™ and 7900 Real-Time PCR System Operation

1.0 StepOnePlus™

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

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

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

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

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

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

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

1.0.7 Optional: If a new target is to be added click the **Enter Target Name** cell and type the name. From the **Reporter** dropdown menu, select appropriate reporter for a particular assay (see Table 1). From the **Quencher** dropdown menu, select appropriate quencher for a particular assay (see Table 1). Leave the default in the color field.

Table 1: Reporter and quencher settings for qPCR assays for the StepOnePlus™

Assay	Detector	Reporter	Quencher
HF183*	HF183 FAM	FAM	Non-fluorescent
	HF183 VIC	VIC	Non-fluorescent
HumM2*	HumM2 FAM	FAM	TAMRA
	HumM2 VIC	VIC	TAMRA
Sketa22	Sketa22 FAM	FAM	TAMRA

*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.

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

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

1.0.9 Run the Method

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

1.0.10 Load the plate into the instrument.

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

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

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

1.0.13 Analyze the run

- Click on the tab in the top right corner of screen to **Analyze the Run**.
- Highlight all of the sample wells and click on **Analysis Settings** to get to C_q settings (default C_q settings).

- Highlight all the targets running in the plate; uncheck the default setting for automatic threshold and **set the threshold to 0.03**. Keep the Automatic Baseline. Click on **Apply Analysis Settings** to save changes. The new settings can be confirmed by viewing the threshold line on the amplification plot.

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

1.1 7900 Real-Time PCR System Operation

1.1.1 Turn on the ABI 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.

1.1.2 Under File menu, select **New**.

1.1.3 In resulting **New Document** window that appears, change container selection from **384 well clear plate** to **96 well clear plate** using drop down menu. Accept default selections of **Absolute Quantification** and **Blank Template**. Click **OK** to display a new plate document.

1.1.4 Click, hold and drag mouse over all PCR reaction tray wells containing samples in upper left window. Selected wells will be outlined with a bold line and their position numbers should appear in the results table in the lower left window. To unselect wells, repeat above process while holding down control key.

1.1.5 Above right hand window, click on **Setup** tab.

1.1.6 Click on **Add Detector** button at the bottom of the setup screen. *Note*: Before any analyses are performed, a specific detector for the method must be created. To do this, go to step 1.1.7. Once the detector has been created, go to step 1.1.8.

1.1.7 Click on **New** in the pop-up window that appears. Another pop-up window will appears. 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 (see Table 2). Under **Quencher** select TAMRA (see Table 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 1.1.8).

Table 2: Reporter and quencher settings for qPCR assays for the 7900 system

Assay	Detector	Reporter	Quencher
HF183*	HF183 FAM	FAM	Non-fluorescent
	HF183 VIC	VIC	Non-fluorescent
HumM2*	HumM2 FAM	FAM	TAMRA
	HumM2 VIC	VIC	TAMRA
Sketa22	Sketa22 FAM	FAM	TAMRA

*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.

1.1.8 In pop-up window that was opened in step 1.1.6, select the desired detector under Names menu and click on **Copy to Plate Document** button. Click on **Done** button to return to setup screen.

1.1.9 Click on **Use** box next to FAM detector in right hand window. This box should become marked with an X. Name and color code for FAM detector should appear in each of the selected well positions in the upper left window and a data column for this detector should be created for each of the selected well positions in the results table in the lower left window. Repeat for VIC detector depending on assay (see Table 2).

1.1.10 Click on **Instrument** tab right hand window.

1.1.11 In instrument screen, change **sample volume** to 25 µL and choose **9600 emulation**.

1.1.12 Still in instrument screen, click on **Connect,** then click on **Open/Close** button in lower right hand "Real Time" window to open PCR reaction tray holder door on instrument.

1.1.13 Insert PCR reaction tray with prepared reactions in holder.

1.1.14 Click on **Open/Close** button to close PCR reaction tray holder door on instrument.

1.1.15 Click on **Start** button in lower right hand "Real Time" window to start thermal cycling in instrument.

1.1.16 Name run file at prompt.

1.1.17 At termination of the run, instrument-calculated cycle threshold values should automatically appear for each well position and detector entry in the lower left hand results table window.

1.1.18 At termination of the run success complete, choose **Analysis Settings** from the toolbar. In that box enter a value for the **Manual Cq Threshold**. Click on **OK**. Click on **Analyze** from the toolbar. You should see C_q values in the Results Table.

1.1.19 Calculated C_q 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.

1.1.20 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**.