Standard Operating Procedure for Measuring Human Contamination in Water by Quantitative Polymerase Chain Reaction

Version 1_1

Updated on 8/21/2013

qPCR assays included:
Multiplex HF183 Taqman with IAC and simplex Sketa22 with Environmental Master Mix 2.0

For questions, please contact

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Table of Contents

Supplies

Below is a list of supplies necessary for each processing step. Each item is essential to successfully completing the process.

Filtration

- equipment
    - manifold
    - carboy
    - tubing
    - vacuum pump
    - forceps
    - small beaker
    - burner
- consumables
    - funnels
    - polycabonate .4uM filters (Millipore, HTTP04700)
    - glass beaded 2.0mL screw cap tubes (GeneRite, S0205-50)
    - 2.0mL screwcap tubes (Sarstedt, 72.693.005)
    - freezer boxes
    - labels/sharpie
    - gloves
-
- Reagents
    - 70% ethanol
    - 10% Bleach
-

Extraction

- Equipment
    - Microtube racks
    - Bead beater (Biospec, 607)
    - Centrifuge with capacity of spinning 1.5-2mL microtubes up to 120000g
    - Minspin (VWR, 93000-196)
    - Vortex
    - 10, 100 and 1000uL pipets
- Consumables
    - Kimwipes
    - 1.7mL low bind snap cap microtubes (Fisher Scientific, 07-200-184)
    - Extraction kit (For purified extract, GeneRite K200-02C-50)
    - Falcon tube rack and falcon tubes (50mL recommended)
    - 10, 100, 1000uL barrier tips
    - gloves
- Reagents
    - 70% ethanol
    - 10% Bleach
    - DNaway (Fisher Scientific, 21-236-28)
    - 10ng/uL salmon sperm (Sigma, D1626)
-

qPCR Preparation

- Equipment
    - Thermal cycler
    - Microtube racks
    - Vortex
    - Minspin (VWR, 93000-196)

    - 10, 100, and 1000uL pipets
- Consumables
    - Kimwipes
    - 1.7mL low bind snap cap microtubes (Fisher Scientific, 07-200-184)
    - gloves
    - sharpie
    - 10, 100, and 1000uL barrier tips
- Consumables
    - 70% ethanol
    - 10% Bleach
    - DNaway (Fisher Scientific, 210236028)
    - Environmental Master Mix 2.0 (ABI 4396838)
    - BSA Fraction V (Invitrogen, 15260)
- Reagents
    - 70% ethanol
    - 10% Bleach
    - DNaway (Fisher Scientific, 21-236-28)
    - Molecular grade dI (Fisher Scientific, BP28194)
    - Environmental Master Mix 2.0 (ABI 4396838)
    - BSA Fraction V (Invitrogen, 15260)

Standard Preparation

- Equipment
    - Water bath
    - 10, 100 and 1000uL pipets
    - Centrifuge with capacity of spinning 1.5-2mL microtubes up to 120000g
    - Minspin (VWR, 93000-196)
    - Vortex

- Consumables
    - Qiagen Plasmid Mini kit (12123)
    - Kimwipes
    - 1.7mL low bind snap cap microtubes (Fisher Scientific, 07-200-184)
    - 10, 100, 1000uL pipets and barrier tips
    - gloves
- Reagents
    - 70% ethanol
    - 10% Bleach
    - DNaway (Fisher Scientific, 21-236-28)
    - Restriction enzyme (NotI, PvuI)

Standard Material

HF183Reference (i.e. sequence that can be inserted into a plasmid vector)
5’-cgtcaggtttgtttcggtattgagtatcgaaaatctcacggattaactcttgtgtacgctCTCGAGgaccagctaatg
Catataaataagttacgtgatgagaccggcgcacgggtgagtaacacgtatccaacctgccgtctactcttggccagccttctgaaagg
aagattaatccaggatgggatcatgagttcacatgtccgcatgattaaaggtattttccggtagacgatggggatgcgttccattagCTC
GAGatagtaggcggggtaacggcccacctagtcaacgatggataggggttctgagaggaagg-3’

IAC Reference (i.e. sequence that can be inserted into a plasmid vector)
5’-atcgcgtcaggtttgtttcggtattgagCCTGCCGTCTCGTGCTCCTCAtctcgaggaccagctaatg
catataaataagttacgtgatgaatgcgaccggcgcacgggtgagtaacacgtatccaacctgccgtctactcttggccagccttctgaa
aggaagattaatccaggatgggatcatgagttcacatgtccgcatgattaaaggtattttccggtagacgatgTGTAGCAACGGC
GTGTTatagtaggcggggtaacggcccacctagtcaacgatggataggggttctgagaggaagg-3’

qPCR primers

HF183
Forward primer (HF-183): 5'- ATCATGAGTTCACATGTCCG -3’
Reverse primer (BacR287): 5'- CTTCCTCTCAGAACCCCTATCC -3’
TaqMan® probe (BacP234MGB): [6-FAM]-5'- CTAATGGAACGCATCCC –MGB
TaqMan® probe (Bac234IAC): [VIC]-5'- AACACGCCGTTGCTACA –MGB

Sketa
Forward primer (SketaF2): 5'-GGTTTCCGCAGCTGGG
Reverse primer (SketaR2): 5'-CCGAGCCGTCCTGGTC
TaqMan® probe (SketaP2): [6-FAM]-5'-AGTCGCAGGCGGCCACCGT-TAMRA

NOTE: Those primers with MGB should be ordered from ABI. Others can be ordered from Operon or IDT. Reference material should be ordered as plasmid (with the corresponding insert) from IDT.

Summary of Method:

Water samples are filtered onto polycarbonate membranes. DNA is released from the membrane by bead beating, and the released DNA is used for QPCR quantification using Taqman Environmental complexes.

Definitions:

Threshold: the level of fluorescence defined to be appreciably greater than the background level.

Ct: the cycle at which a statistically significant increase in fluorescence is first detected in a sample. Point at which sample's fluorescence curve crosses the threshold.

Target sequence: the segment of DNA that contains the nucleotide sequence homologous to the primer/probe used in the QPCR assay.

Amplification efficiency: a measure of the efficiency at which the segments of target DNA are copied during the PCR. If the assay is performing at 100%, then there should be a doubling of the target DNA with each cycle.

Internal Amplification Control (IAC): Evaluates if inhibition is occurring within samples by determining CT values of a predetermined control are outside the suitable range.

Background:

Polymerase chain reaction (PCR) reproduces target sections of DNA. Quantitative PCR (QPCR) combines this reproduction of target DNA with the release of fluorescent signals by a probe. A QPCR machine monitors the emission of fluorescence during the reaction. By comparing the fluorescent signal growth in samples with that of known quantities of the target DNA (given in cell equivalents), we can calculate the amount of target DNA in the sample at the beginning of the reaction.

Quality Control:

A series of controls need to be included in every QPCR run in order to ensure that the QPCR reaction is working properly and that there has been no cross-contamination between samples. Cross-contamination will invalidate all QPCR results! A description of each control is detailed below.

- Negative Extraction Control: Sterile AE Buffer is filtered onto a polycarbonate membrane. The membrane is bead beaten with the sample filters. If *Enterococcus* is measured during the QPCR, we know that there was cross-contamination at the filtration and bead-beating steps.
- No Template Control: Sterile water is used in place of the bead-beaten material during the QPCR reaction. If *Enterococcus* is measured during the QPCR, we know that there was contamination during the QPCR step.
- Calibrator: Standard of known target DNA. QPCR results comparing the sample Ct values and the calibrator Ct values will be used to determine the relative amount of *target* in the sample compared with the calibrator.
- DNA Standard Curve: The calibrator will be diluted in a 1:10 dilution series. QPCR will be run on each dilution. A linear regression analysis of the Ct values at each dilution will be used by the QPCR machine to calculate the QPCR amplification efficiency. If the amplification efficiency is ≥87%, the run is acceptable. If the amplification efficiency is <87%, the QPCR assay must be re-run.

Storage of Primers and Probe:

Multiple frozen aliquots of the primers and probe should be made because they are very sensitive to multiple rounds of freeze/thaw. After you have removed an aliquot from the freezer, store it in a 4°C for up to 1 week. Discard after 1 week. This assay uses a Primer/Probe mix that is then added to the Master Mix (See excel spreadsheet).

Cleanliness and Preparation of Work Area:

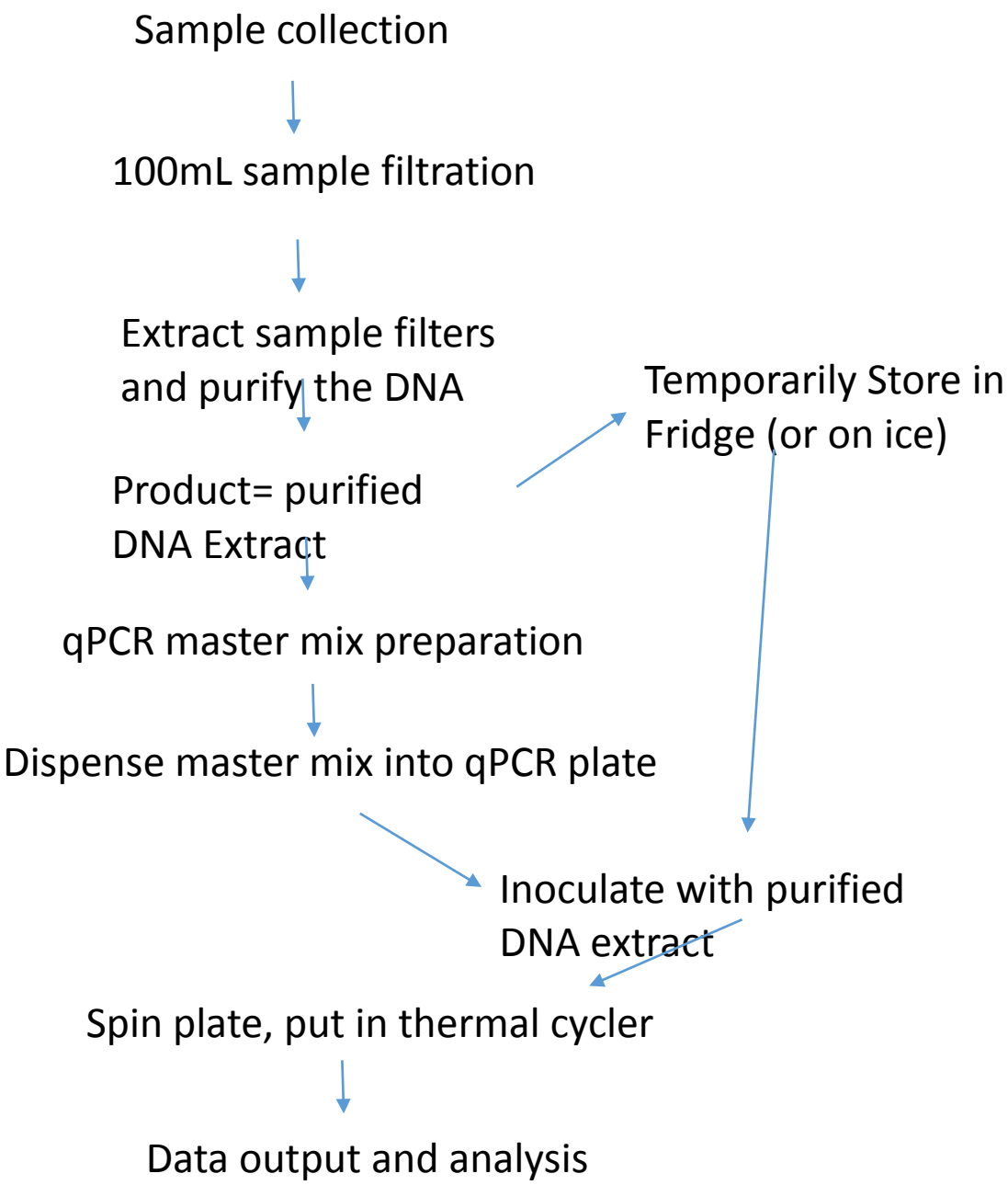
QPCR requires technical precision and careful lab practice. Contamination can be a serious problem. To decrease the risk of contamination, gloves should be changed regularly and pipette tips and tubes must be sterile. Change your pipette tip after every single use! It is also a good idea to use filtered pipette tips whenever you will be working with samples.

Clean all tube racks between uses. To clean tube racks:

1) Prepare a 10% bleach solution of household bleach and deionized water and store in suitable container. This solution should be discarded every week.
2) Prepare 1% solution of sodium thiosulfate in deionized water—1g of sodium thiosulfate per 100mL water. Store in suitable containers. This solution should also be discarded every week.
3) Soak tube racks in bleach solution overnight.
4) Dip racks in sodium thiosulfate solution.
5) Rinse racks with deionized water.

It is also important that the lab bench and pipettes are thoroughly cleaned between each step of this method. To clean bench and pipettes:

1) Prepare bleach solution fresh weekly by diluting 50mL of household bleach in 450mL of deionized water in a squirt bottle. Label as 10% Bleach.
2) Cover benchtop and pipettes with 10% Bleach solution and wipe with paper towels. Discard gloves.
3) Wearing a new pair of gloves, use a spray bottle to apply deionized water to benchtop and pipettes. Wipe with paper towels. Discard gloves.
4) Wearing a new pair of gloves, use a spray bottle to apply 70% ethanol to benchtop and pipettes. Wipe dry with paper Kimwipes.


Sample collection
100mL sample filtration
Extract sample filters and purify the DNA
Product= purified DNA Extract
Temporarily Store in Fridge (or on ice)
qPCR master mix preparation
Dispense master mix into qPCR plate
Inoculate with purified DNA extract
Spin plate, put in thermal cycler
Data output and analysis

HF183 and IAC Reference DNA Preparation:

HF183 reference DNA

1) Prepare standards in a separate area from sample processing and qPCR areas.
2) In the tube from IDT add 100uL DNase RNase free dI water. Let sit for ~40min. Use 50ul for restriction enzyme digestion, and save the other 50ul for digestion later (you can digest the full 100ul in one batch if needed).
3) Digest with Not1 following manufacturer's instructions (or equivalent restriction enzyme, i.e. PVU1)
4) Clean the digested product with the QIAGEN Plasmid Mini Kit following manufacturer's instructions.
5) Measure the final product's A260 absorbance reading in triplicate. The absorbance reading from above can be used to calculate the plasmid concentration in ng/ul.
6) Calculate copy/ul using the plasmid size (provided by IDT) and the ng/ul concentration from above:

   $\frac{6.023 \times 10^{23} \text{ molecules/gram}}{(\text{X bp})(650 \text{ Daltons/bp})} = \text{Y plasmid molecule molecules/gram}$

   X=total # of bps in plasmid

   Use the absorbance to calculate the concentration of the plasmid in copies/2uL

7) Prepare stock solution of 10^7copy/2ul based on the concentration above, and aliquot in small volumes for future use.

IAC reference DNA

Use the same procedure to prepare the IAC reference material as the HF183 reference material.

Salmon DNA Preparation:

1) Prepare salmon stocks in a separate area from sample processing and qPCR areas.
2) Create a 10mg/mL stock of the salmon solution by adding salmon DNA to AE buffer and vortexing until solution is homogenous (~4hrs). Note that this DNA material is fibrous and it can take a while to fully dissolve.
3) Quantify the stock solution just created and prepare a 10ug/mL salmon solution by mixing 25uL of 10mg/mL stock into 24.975 mL of AE buffer (just an example concentration). Make 1mL aliquots of this solution for storage at -20°C (need to make new stock every year) or 4°C (need to make new stock every 6 months).

Filtration:

1) On the disposable filter funnel place a 0.4μm polycarbonate filter. Attach the funnel to the base.
2) Shake sample bottle vigorously. Apply 100mL of sample into filter funnel. For the negative extraction control, filter 100mL of sterile PBS. Apply vacuum.
3) When filter is visibly dry, rinse funnel with sterile PBS. When filter dries again, turn off vacuum.
4) Use 2 sets of forceps and being careful to handle only the edges of the filter, fold filter in half with sample side facing inward. Fold in half again. Insert folded filter into labeled bead-beating tubes pre-loaded with glass beads. Cap the bead-beating tube.
5) Sample filters must be processed immediately or stored at -80°C.

Extraction (purified extracts using GeneRite)

1. Calculate volume of the 10mg/uL salmon stock needed for lysis buffer (include 2 extra samples)

#samples for extraction + 4extra = S
500uL(volume of lysis buffer added to each sample) x S = Total Volume of lysis buffer needed

(Total volume x0.2ng/uL)/10mg/uL = ul Salmon stock needed

Total Volume- Volume Salmon Stock = uL DNEasy lysis Buffer needed

Once SPC lysis buffer is made vortex before use

2. Add 500uL spc lysis buffer to each bead tube containing a filter.

3. Tighten caps and bead beat samples for 2min.

4. Centrifuge microtubes at 12000rcf for 1min.

5. Pipet out all the liquid into a 1.5mL microtube. Centrifuge for 1 minute at 10,000rcf.

6. Add 1000uL binding buffer to the next 1.5mL microtube.

7. From the microtube in step 6, pipet 300- 400uL liquid from each sample adding this volume to their respective 1.5mL microtube in step 7 tube. Pipet up and down to mix solution and then gently vortex.

8. Add 690uL of DNA/binding buffer mixture to column and centrifuge for 1 minute a 10,000 rcf (discard flow through). Place column in new collection tube and add in the last of DNA/binding buffer mixture, centrifuge at 10,000rcf for 1 minute (discard flow through).

9. Place column in new collection tube. Add 500uL EZ wash buffer and centrifuge for 1 minute at 10,000rcf (discard flow through). Repeat.

10. Put column into final 1.5mL microtube. Add 50uL elution buffer, let sit for 1 minute, centrifuge for 1 minute at 10,000 rcf (keep flow through). Repeat.

11. Vortex final 100uL elution buffer/DNA solution (distribute to multiple if necessary).

12. Place tubes in –20C freezer. Note what box number they are placed in, and if DNA is to be used for PCR that afternoon, place in refrigerator and after use place in freezer.
The master mix should contain 490μL of sterile AE buffer and 10μL of salmon testes DNA (10μg/mL) for each sample, standard, and negative extraction control.

qPCR

Preparation of Standard Curve:

1) Add 90μL of nuclease-free water to 5 clean microcentrifuge tubes. Note: the same tip can be used for pipetting multiple aliquots of nuclease-free water as long as it does not make contact with anything else.
2) Label material from 10^6 cell standard as Standard 1. Vortex and briefly microcentrifuge.
3) Pipet 10μL of Standard 1 into first tube containing 90μL of nuclease-free water. Label tube as Standard 10^5. Vortex and briefly microcentrifuge.
4) Pipet 10μL of Standard 10^5 into second tube containing 90μL of nuclease-free water. Label tube as Standard 10^4. Vortex and briefly microcentrifuge.
5) Pipet 10μL of Standard 10^4 into third tube containing 90μL of nuclease-free water. Label tube as Standard 10^3.
6) Pipet 10μL of Standard 10^3 into third tube containing 90μL of nuclease-free water. Label tube as Standard 10^2.
7) Pipet 10μL of Standard 10^2 into third tube containing 90μL of nuclease-free water. Label tube as Standard 10^1.

NOTE: Feel free to alter volumes; this is given as an example and typically volumes this large are not necessary but ensure that the ratio is maintained.

Calculating Number of QPCR Reactions to Prepare for Each Assay:

A duplicate standard curve will be included in each run of each assay. All samples and the calibrator will be run in duplicate. A no template control and the negative extraction control will also be included in each run. The excel spreadsheet labeled as Entero will allow you to determine how many reactions are needed for each plate. Additionally, be sure to add in extra reactions to account for pipetting error.

Preparation of Specimen Processing Control (Salmon Testes DNA, SPC, Sketa) QPCR Assay Master Mix and the *Enterococcus* QPCR Assay Master Mix:

1) The accompanying excel spreadsheet outlines the primers and probes that are used for the salmon assay (spc, sketa) and the HF183 assay with IAC on the OSTD2 tab.
2) Each assay uses two primers (Forward and Reverse) and one probe (sketa) or 2 probes (HF183 with IAC). Once ordered and delivered, the primers and probes will need to be rehydrated with nuclease free water (primers) and TE buffer (probe) to the correct concentrations of 500uM for primers and 100uM for probes if ordered through Operon. If probes are ordered though ABI then each will already be hydrated in a 100uM solution.
3) The primers and probes will then be added into a Primer Probe mix (P/P mix) in the volumes outlined in column I of the excel spreadsheet. Once the P/P mix is made for the Human assay, another P/P mix needs to be made for the sketa assay.
4) The P/P mixes are then ready to be used in creating the two master mixes for the qPCR reaction. Cells A5-A9 outline the components of the human master mix and cells A42-A45 outline the reagents for the sketa master mix. Cells G5-G9 (and G35-38 for sketa) outline what volume of each reagent is needed/reaction.
5) The number of samples can be entered into cell B12 (and B41 for sketa). This will automatically calculate the volume needed for each reagent in cells B13-B17 (and B42-45 for sketa) for the master mix. Remember two master mixes are being made, one for human and one for sketa, so be sure to keep track of which P/P mixes are being used. This excel sheet can be used to calculate the master mixes for both assays.
6) To create the master mix add the correct amount of nuclease free water to a 1.7mL low bind microtube.
7) Next, the Environmental master mix should be taken from the fridge, vortexed and added to the tube.

8) The P/P mix should then be vortexed, spun and added (the P/P mix can be stored in the fridge for a week to avoid freeze/thaw).
9) Vortex, spin and add BSA.

QPCR Plate Preparation:

1) In the Excel file provided with this SOP there is a tab named 'qPCR plate setup.' The first plate layout corresponds to Entero and its corresponding Sketa assays. The second plate layout corresponds to HF183 and its corresponding sketa and IAC assays. The plate setup will be consistent throughout this study. The only changes will be the different samples that are run on each plate. Therefore, it is imperative to keep a record of which samples are run on which plates.
2) Once all reagent have been thawed, mixed well and combined, briefly vortex and spin the assay final master mix, then add 23µL to each well that will be used on the plate. Note: the same tip can be used for pipetting multiple aliquots of the same master mix as long as it does not make contact with anything else. Alternatively, a repeat pipette can be used for this step.
3) Add 2 µL of nuclease-free water (for the no template control), extract from each sample, and each standard curve dilution to the appropriate wells. Be sure to change the pipette tip between each well.
4) Apply adhesive seal to the top of the plate. Apply pressure across the entire plate to ensure that each well is completely sealed.
5) Put the plate in the plate-spinner, making sure that the plate-spinner is balanced with a second plate. Spin for 20 seconds.
6) Transfer plate to the QPCR machine making sure that it is properly aligned. Close lid. Open the software and program in the cycling protocol as outlined on the Excel spreadsheet. Be sure to note any comments that are pertinent to the run.

qPCR Data processing and analysis

Analyzing QPCR Results on CFX Machine:

Analyzing Data:

1) Once the run is complete save the file to your local drive and upload the file for data processing to http://data.sccwrp.org/b13micro/