Standard Operating Procedure for Measuring *Enterococcus* spp. in Water by Quantitative Polymerase Chain Reaction

Version 1_1

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qPCR assays included:
Simplex Entero1a 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)
    - AE Buffer (For crude extract, Qiagen 19077)
    - Extraction kit (For purified extract, GeneRite K200-02C-50)
    - Falcon tube rack and falcon tubes (50mL recommended)
    - 10, 100, 1000uL barrier tips
    - sharpie
    - gloves
- Reagents
    - 70% ethanol
    - 10% Bleach
    - DNaway (Fisher Scientific, 21-236-28)
    - 10ng/uL salmon sperm (Sigma, D1626)

qPCR Preparation

- Equipment
    - Thermal cycler
    - Microtube rack
    - 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
- 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)

qPCR primers

Entero F1A (GAGAAATTCCAAACGAACTTG)
Entero R1 (CAGTGCTCTACCTCCATCATT)
Entero Probe GPLQ813TQ ([6-FAM]-TGGTTCTCTCCGAAATAGCTTTAGGGCTA-[TAMRA])

Sketa 22 For (GGTTTCCGCAGCTGGG)
Sketa 22 Rev (CCGAGCCGTCCTGGTC)
Sketa 22 Probe ([6FAM]-AGTCGCAGGCGGCCACCGT-[TAMRA])

Summary of Method:

Water samples are filtered onto polycarbonate membranes in order to concentrate enterococci. DNA is released from the membrane by bead beating, and the released DNA is used for QPCR quantification using Taqman Environmental complexes targeting *Enterococcus* sp.

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 the 23S ribosomal DNA that contains the nucleotide sequence homologous to the primer/probe used in the QPCR assay. Specific to *Enterococcus* sp.

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.

Cell equivalents: the number of copies of the target gene sequence in the average *Enterococcus* cell

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.

Quantification Method:

The difference in Ct values is used to get the ratio of the *Enterococcus* cell equivalents in the sample to the known *Enterococcus* cell equivalents in the calibrator. By multiplying this ratio by the number of cell equivalents in the calibrator, we can calculate sample *Enterococcus* cell quantities. It is mathematically important for the QPCR amplification efficiency, which we calculate from the standard curve, to be over 87% in order for this method to work.

Using the Specimen Processing Control to Correct for Inhibition:

Many environmental compounds may interfere with the PCR reaction, reducing the amplification efficiency and invalidating the quantification method. We hope that by diluting all samples 1:5 with nuclease-free water we have effectively diluted out the inhibitory compounds. To test whether or not the diluted samples contain PCR inhibitors, we use a specimen processing control. Salmon testes DNA, which we do not expect to find in our water samples, is added in known quantities to each sample and calibrator. A QPCR assay targeting the salmon sperm is performed, and the Ct value of the samples is compared to that of the calibrator. Because we added the same amount of salmon testes DNA to each sample and the calibrator, we expect the Ct values to be close. If the sample Ct value is higher than the calibrator, we know that the QPCR reaction was not as efficient in the sample and that the sample likely contained some PCR inhibitors. When a sample's salmon testes Ct is greater than the calibrator Ct + 1.7, it is inappropriate to make management decisions on beach closure based upon *Enterococcus* QPCR results.

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: A filter spiked with a known quantity of *Enterococcus* cells (in this case 1e65cells) will be bead-beaten with the sample filters. QPCR results comparing the sample Ct values and the calibrator Ct values will be used to determine the relative amount of *Enterococcus* cells in the sample compared with the calibrator. .
- DNA Standard Curve: The bead beaten material from the calibrator will be serially diluted 1:10, 1:100, 1:1000 and possibly 1:10000. 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 Wet 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.

Cleanliness and Preparation of Work Area:

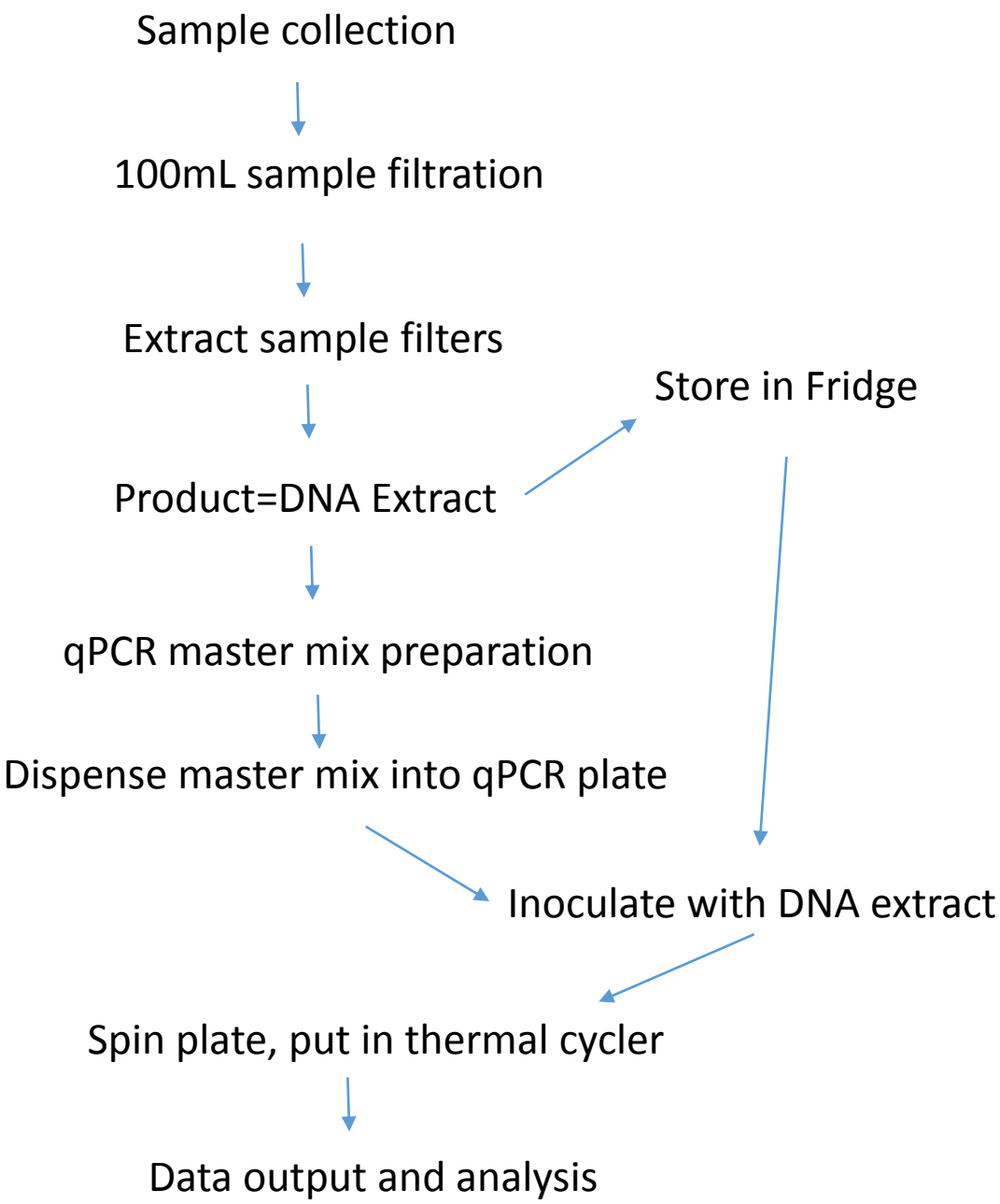
QPCR requires technical precision and careful lab practice. Contamination can be a serious problem. To decrease the risk of contamination, gloves should be changed regularly and pipette tips and tubes must be sterile. Change your pipette tip after every single use! It is also a good idea to use filtered 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
Store in Fridge
Product=DNA Extract
qPCR master mix preparation
Dispense master mix into qPCR plate
Inoculate with DNA extract
Spin plate, put in thermal cycler
Data output and analysis

Filtration:

1) On the 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 and remove the funnel from the base.
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) Samples must be processed immediately or stored at -80°C.

Extraction (crude extracts)

Preparation of Bead Beating Buffer:

The bead beating buffer should be prepared once weekly and stored at 4°C. Calculate the number of samples to be processed each week and in case of spills or pipetting errors, make enough buffer for 10 extra filters. 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.

1) Estimate the number of samples, standard, and negative extraction controls to be run over the course of the week. Add 10 extra to cushion for any spills or pipetting errors. This is the total number of filters we are preparing master mix for. (Use Excel template to assist with calculations).
2) Calculate the amount of AE buffer to use by multiplying 490μL by the total number of filters. Add AE buffer to 50mL tube.
3) Calculate the amount of salmon testes DNA to use by multiplying 10μL by the total number of filters. Defrost a salmon testes DNA aliquot, and add appropriate volume to AE buffer.
4) Store at 4°C.

Bead Beating:

1) Remove one tube of *Enterococcus* 100,000 cell standard from the freezer.
2) Apply 500μL of bead beating buffer to each sample, standard, and negative control. Be sure to change tip between each tube in order to avoid cross-contamination.
3) Close the tubes tightly, and place in bead beater. Beat for 2 minutes at the maximum rate.
4) Centrifuge tubes for 1 minute at 12000 x g (12,000 rcf).
5) Remove as much of the supernatant as possible and place in a clean microcentrifuge tube.
6) Centrifuge for 5 minutes at 12000 x g (12,000 rcf).
7) Remove as much supernatant as possible without disturbing the pellet. Aliquot the supernatant if necessary.
8) Store bead beaten material at 4°C for up to 1 week.

OR

Extraction (purified extracts using GeneRite)

1. Calculate salmon stock needed for lysis buffer (include 2 extra samples)

#samples for extraction + 2extra = S
500uL(volume of lysis buffer added to each sample) x S = Total Volume

(Total volume x0.2ng/uL)/10ng/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. Pour spc-lysis buffer and beads into sample tubes.

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 (can vary or remain constant for samples in a batch) 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 4 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 bead beaten material from 100,000 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 1:10. Vortex and briefly microcentrifuge.
4) Pipet 10μL of Standard 1:10 into second tube containing 90μL of nuclease-free water. Label tube as Standard 1:100. Vortex and briefly microcentrifuge.
5) Pipet 10μL of Standard 1:100 into third tube containing 90μL of nuclease-free water. Label tube as Standard 1:1000.
6) **If** doing a 5 point standard curve pipet 10μL of Standard 1:1000 into third tube containing 90μL of nuclease-free water. Label tube as Standard 1:10000

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.

NOTE: In future ATCC genomic standard will be ordered for a 4 point standard curve

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 *Enterococcus* assay on the Entero tab.
2) Each assay uses two primers (Forward and Reverse) and one probe. 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.
3) The rehydrated 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 Entero 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-A8 outline the components of the master mix. Cells G5-G8 outline what volume of each reagent is needed/reaction.
5) The number of samples can be entered into cell B11. This will automatically calculate the volume needed for each reagent in cells B12-B15 for the master mix. Remember two

master mixes are being made, one for entero 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) The plate setup tab on the excel file will tell you the layout of the plate. It is important to note the plate setup will remain the same for the duration of this study. The only items changing will be the samples. Therefore, it is imperative to keep track of which samples are done on which plate.
2) Briefly vortex and spin the sketa and entero assay master mixes, then add 23µL to each well that will be used for each assay. 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), bead-beaten or extract material from each sample diluted 1:5, the calibrator diluted 1:5, and each standard curve dilution to the appropriate wells. Be sure to change the pipette tip between each sample.
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 10-20 seconds.
6) Transfer plate to the QPCR machine making sure that it is properly aligned. Close lid.
7) Open the software and load the cycling protocol. Be sure to note any comments before clicking ‘Start Run.’

qPCR Data processing and analysis

1) Once the run is complete, export the results in .csv file as instructed, upload the file for data processing to http://data.sccwrp.org/b13micro/. A pdf report with all QC results, and Enterococcus concentration estimates will be returned.