Entero1a and Simplex Sketa22 with Environmental Master Mix 2.0 SOP
Quick step by step guide

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) Samples must be processed immediately or stored at -80°C.

Extraction (non purified 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 bead beating buffer 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 bead beating buffer 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 stock (10ug/mL) aliquot, and add appropriate volume to AE buffer.
4) Store at 4°C.

Bead Beating:

1) Remove one frozen filter calibrator tube (containing *Enterococcus* 100,000 cell/filter) 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) Recover 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) Recover as much supernatant as possible without disturbing the pellet. This supernatant is the crude DNA extract. Aliquot the crude extract if necessary.
8) Proceed with qPCR analysis.

qPCR

Preparation of serial dilutions for 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 crude DNA extract from the 100,000 cell/filter calibrator 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) 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 of making 100ul of each concentration and typically volumes this large are not necessary

NOTE: In the future standards will be ordered from ATCC and are used in a 4 point dilution curve

Calculating Number of QPCR Reactions to Prepare for Each Assay:

A duplicate 5-point (4-point when using the ATCC genomic standard) 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, in duplicate, 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 Assay Master Mixes for the *Enterococcus* qPCR assay (Entero1a) and the Specimen Processing Control (Salmon Testes DNA, Sketa22) qPCR assay:

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) All primers and probes are ready to use in a prepared P/P mix.
3) To create the master mix add the correct amount of nuclease free water to a 1.7mL low bind microtube.
4) Next, the Environmental master mix should be taken from the fridge, vortexed and added to the tube.
5) 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).
6) Vortex, spin and add BSA. The assay master mixes are ready for use in the next step.

QPCR Plate setup:

1) The plate setup is at the end of this SOP, for setting up the plate manually. Electronic plate setup files ready to be imported to various qPCR platforms are also available for downloading from the automatic data analysis portal (http://data.sccwrp.org/b13micro/).
2) Briefly vortex and spin the sketa22 and entero1a 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), crude extracts from each sample, the calibrator, 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 Processing and Data Analysis:

Analyzing QPCR Results produced from CFX Machine, StepOne Plus, AB7500 fast:

Analyzing Data:

Once the run is complete, export the results in .csv file as instructed (instruction available http://data.sccwrp.org/b13micro/), and upload the file for data analysis to http://data.sccwrp.org/b13micro/. A pdf report will be returned depicting QC results and concentration of HF183 targets.

Entero Plate Setup (an example assuming 1 unknown sample)

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std-1 entero	Std-1 entero	Std-2 entero	Std-2 entero	Std-3 entero	Std-3 entero	Std-7 sketa	Std-7 sketa	Std-8 sketa	Std-8 sketa	Std-9 sketa	Std-9 sketa
B	Std-4 entero	Std-4 entero	Std-5 entero	Std-5 entero	Pos-1 entero	Pos-1 entero	Std-10 sketa	Std-10 sketa	Pos-2 sketa	Pos-2 sketa		
C	Unk-1 entero	Unk-1 entero	Neg entero NEC	Neg entero NEC	NTC entero NTC	NTC entero NTC	Unk-2 FAM	Unk-2 FAM	Neg sketa NEC	Neg sketa NEC	NTC sketa NTC	NTC sketa NTC
D												
E												
F	Std-11 entero	Std-11 entero	Std-12 entero	Std-12 entero	Std-13 entero	Std-13 entero	Std-17 sketa	Std-17 sketa	Std-18 sketa	Std-18 sketa	Std-19 sketa	Std-19 sketa
G	Std-14 entero	Std-14 entero	Std-15 entero	Std-15 entero	Pos entero	Pos entero	Std-20 sketa	Std-20 sketa	Pos sketa	Pos sketa		
H	Unk-3 FAM	Unk-3 FAM	Neg entero NEC	Neg entero NEC	NTC entero NTC	NTC entero NTC	Unk-4 sketa	Unk-4 sketa	Neg sketa NEC	Neg sketa NEC	NTC sketa NTC	NTC sketa NTC