Multiplex HF183 Taqman with IAC and simplex Sketa22 with Environmental Master Mix 2.0 SOP

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 DNAEasy kit)

1. Calculate volume of the 10mg/uL salmon stock needed for making SPC-lysis buffer that contains 0.2ng/uL salmon testes DNA as sample processing control (include 2 extra samples)

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

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

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

Once SPC lysis buffer is made vortex and let settle (it might foam up during vortex) before use

2. Add 500uL spc lysis buffer to each beaded 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. The supernatant are crude DNA extracts that will be purified in the following steps.

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

7. From the microtube in step 6, pipet 300uL of crude DNA extracts from each sample adding this volume to their respective 1.5mL microtube in step 7 tube containing the binding buffer. 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. Carefully remove the spin column from the collection tube (make sure no wash buffer residual at the bottom of the spin column), and place the 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, this is purified DNA extracts). 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.

qPCR

Preparation of serial dilutions for 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 copy/2ul stock plasmid standard solution (see separate protocol for plasmid standard stock preparation) as Standard 1. Vortex and briefly microcentrifuge.
3) Pipet 10μL of 10^6 copy/2ul stock 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 making sure to maintain ratios; this is given as an example of making 100ul of each concentration and typically volumes this large are not necessary.

Calculating Number of QPCR Reactions to set up for Each Assay:

A duplicate 6-point standard curve will be included in each run of each assay. All samples will be run in duplicate. A no template control and the negative extraction control, each in triplicate, 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: HF183 duplex assay and Specimen Processing Control simplex Sketa22 QPCR Assays:

1) The accompanying excel sheet 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) All primers and probes are in a 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 Preparation:

1) The plate setup is at the end of this SOP. 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 assay master mix (see above), dipense 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, and only open one sample or standard tube at a time.
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 produced from CFX Machine, StepOne Plus, AB7500 fast:

Analyzing Data:

1) 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.

HF183 plate setup (an example assuming 1 unknown sample)

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std-1 hf183 IAC	Std-1 hf183 IAC	Std-2 hf183 IAC	Std-2 hf183 IAC	Std-3 hf183 IAC	Std-3 hf183 IAC	Std-7 sketa	Std-7 sketa	Std-8 sketa	Std-8 sketa	Std-9 sketa	Std-9 sketa
B	Std-4 hf183 IAC	Std-4 hf183 IAC	Std-5 hf183 IAC	Std-5 hf183 IAC	Std-6 hf183 IAC	Std-6 hf183 IAC	Std-10 sketa	Std-10 sketa				
C	Unk-1 FAM IAC	Unk-1 FAM IAC	Neg hf183 IAC NEC	Neg hf183 IAC NEC	NTC hf183 IAC NTC	NTC hf183 IAC NTC	Unk-2 sketa	Unk-2 sketa	Neg sketa NEC	Neg sketa NEC	NTC sketa NTC	NTC sketa NTC
D												
E												
F	Std-11 hf183 IAC	Std-11 hf183 IAC	Std-12 hf183 IAC	Std-12 hf183 IAC	Std-13 hf183 IAC	Std-13 hf183 IAC	Std-17 sketa	Std-17 sketa	Std-18 sketa	Std-18 sketa	Std-19 sketa	Std-19 sketa
G	Std-14 hf183 IAC	Std-14 hf183 IAC	Std-15 hf183 IAC	Std-15 hf183 IAC	Std-16 hf183 IAC	Std-16 hf183 IAC	Std-20 sketa	Std-20 sketa				
H	Unk-3 FAM IAC	Unk-3 FAM IAC	Neg hf183 IAC NEC	Neg hf183 IAC NEC	NTC hf183 IAC NTC	NTC hf183 IAC NTC	Unk-4 sketa	Unk-4 sketa	Neg sketa NEC	Neg sketa NEC	NTC sketa NTC	NTC sketa NTC