PREPARATIONS FOR BEACH SEASON 2020: PROTOCOL UPDATES FOR QPCR METHOD C

Prepared for the Michigan qPCR Network

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Updated 2 June 2020

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REAGENTS FOR BEACH SUMMER SEASON

Package content

1. primer/probe mix: EC23S & Sketa22
2. Bovine Serum Albumin
3. Salmon DNA (0.2 µg/mL)
4. Standards:
    - Sets of 5 concentrations of Method C standards
    - Labeled 1, 2, 3, 4, 5 going from highest to lowest concentration
5. *E. coli* Bioball Cells for calibrator samples at $5x10^6$ Cells/tube

Table 0. Reagents Shipped to All Laboratories

Item name	Volume (mL) / unit	Quantity of tubes or bottles	Require storage temperature
Primer probe mix **EC23S** **Sketa**	 1 mL 1 mL	 3 3	-20°C upon receiving the package & storage 4°C during experiment (achieved by storing reagents in a box full of ice)
BSA	1mL	3	-20°C upon receiving the package & storage 4°C during experiment (achieved by storing reagents in a box full of ice)
Standards	0.2 mL	6 per standard 30 total	-80°C upon receiving the package and before use 4°C during experiment (achieved by putting reagents in a box full of ice)
Salmon DNA stock (SAE buffer, 0.2 µg/mL)	245 mL	1	4°C upon receiving the package & for the entire season 4°C during experiment (achieved by storing reagents in a box full of ice)
E. Coli Bioball Cells for calibrator samples	at 5E06 Cells/tube	1 tube per lab	-80°C upon receiving the package For use, see part 2 of Appendix B

Note: If the shipment your laboratory received does not contain all the items listed above or if additional standards are required (e.g. >3 analysts per laboratory), please contact Michigan State University, the Water Quality, Environmental, and Molecular Microbiology Laboratory (Matt Flood email: floodmat@msu.edu)

PRE-SEASON PROTOCOL

A. EPA STANDARDS FOR E. COLI USED FOR STANDARD CURVES

- Sets of 5 concentrations of standards will be provided to each lab in frozen 200 uL aliquots. Tubes will be labeled 1, 2, 3, 4, 5 going from highest to lowest concentrations.
- Keep tubes frozen until ready to use. Once thawed, tubes can be stored in refrigerator (4°C) up to ~1 month. Laboratories are encouraged, however, to run all reactions with a given set of standard tubes within 1 week of thawing them to reduce the time of refrigeration and potential minor deterioration of the standards. Individual analysts can mix and match curves from different tube sets of the standards if necessary.
- Do not transfer standards to different tubes
- Use new frozen tubes of standards for running standard curves at the beginning of each beach season as described in section B of this protocol.

Required storage temperature for standards: -80°C

Do not refreeze the standards.

We highly encourage you to run curves within 48 hours after thawing standards.

B. PROPOSED LAB STANDARD CURVE AND PRELIMINARY POSITIVE AND NEGATIVE CONTROL REQUIREMENTS FOR 2020 BEACH SEASON

1. Run 6 data sets (standards and positive/negative controls) per analyst
2. Suggested plate layouts for 1 and 2 sets of analyses per plate shown in Figures 1 & 2 of this protocol.
   Note: if running two sets of analyses per plate, prepare different master mixes for each set
3. Run 6 no template controls (NTC) in each data set
4. Recommended that at least 3 different sets of 3 calibrators and 3 filter blank samples be extracted and run together with the standards (see section E for preparation of samples).
5. **For manual data transfer to Method C workbooks:** standards Ct data from each run should be copied and pasted (going from highest to lowest standards concentrations) into column E of "Lab Std Curves" tab of Method C template workbook(s) that will be provided to each of the labs in 2020. Also enter run date for each curve in column B and (if applicable) the concentrations of the standards in column C.
6. No template control Ct data should be copied and pasted into column C of "NTCs" tab of Method C template workbook. Also provide run date for each data set in column B.
7. *E. coli* and Sketa Ct data from filter blank samples from each run should be copied and pasted into columns E and F, respectively of "Filter Blanks" tab of Method C template workbook. Also provide run date for each data set in column B

8. *E. coli* and Sketa Ct data from calibrator samples from each run should be copied and pasted into columns E and F, respectively of "Initial Calibrators" tab of Method C template workbook. Also provide run date for each data set in column B.

For Automated Data transfer to Method C workbooks: A Method C Macro Tool that will be provided to each of the labs can be used to import the data if the plate layouts indicated in Figures 1 or 2 are followed exactly. Detailed instructions on how to use the Method C Macro can be found in Appendix D.

9. Each analyst that will be running beach samples in the coming beach season should complete steps 1-8 prior to running beach samples

After completing steps 1-8 above, labs are required to send Ct export files and completed Method C template workbook(s) to MSU (Erin Dreelin: dreelin@msu.edu) and EGLE (Shannon Briggs: BRIGGSS4@michigan.gov) with the following additional information provided in each export file:

1. Date
2. Environmental master mix lot#(s)
3. Analyst's name
4. Each plate well identified with sample name & *E. coli* or Sketa target
5. Plate layout using plate layout code provided in Figures 1 & 2
6. See Appendix C for recommended workbook naming conventions.

All standard curve runs should be completed and data sent ASAP.

Workbooks will be reviewed for acceptability of data from each lab/analyst. Labs/analysts will be notified of the acceptability of their data within 2 weeks and templates with any comments or corrections will be sent back to the Labs/analysts within same time period. Labs/analysts with unacceptable results will receive further guidance from MSU and may be requested to repeat the entire protocol.

Troubleshooting

After each run, please check QA results in following tabs: "NTCs (column D)"; "Filter Blanks (columns H-J)"; and "Initial Calibrators (columns G-J)" of the Method C template workbook to determine whether positive and negative controls pass QA (passing Ct values also shown in Table 1 below).

- If EC23S assay results for the calibrators are outside of range in Table 1, we advise you to adjust your volumes or dilutions of the cell suspensions used to prepare the calibrator samples.
- If Sketa22 assay results for the calibrators and filter blanks are outside of range in Table 1, we advise you to dilute your SAE buffer with more AE buffer/EB Buffer or add additional 10 ug/mL salmon DNA stock solution (remember a 2x increase or decrease in concentration should give a respective decrease and increase of about 1 Ct, (10x increase or decrease gives about a 3 Ct change).
- If Ct values are greatly different than these acceptance ranges, review protocols for making up *E. coli* cells and SAE buffer (salmon DNA in AE buffer/EB buffer) or contact Geoff Rhodes (rhodesg2@michigan.gov) and Matt Flood (floodmat@msu.edu).
- If any EC23S assay NTC or Filter Blank Ct values fail QA you may have a reagent or laboratory contamination problem. Contact Geoff Rhodes (rhodesg2@michigan.gov) and Matt Flood (floodmat@msu.edu) for consultation.

If the composite standard curve is not acceptable (i.e. no slope and intercept values shown with 6 data sets entered), workbooks can be sent to Geoff Rhodes (rhodesg2@michigan.gov) and Matt Flood (floodmat@msu.edu)for consultation prior to submission to MSU and EGLE.

Table 1. QA for positive and negative controls

Positive and Negative Controls	Passing Ct Values	Remedial action in case of QA failures
Calibrator positive control sample Ct values for EC23S (E. coli) assay	26.48 to 29.63	Increase or decrease *E. coli* cell concentrations in calibrator samples
Calibrator Positive Control and Filter Blank negative control sample Ct values for Sketa22 (SPC) assay	18.58 to 22.01	Increase or decrease salmon DNA concentrations in SAE extraction buffer
Filter Blank negative control sample Ct values for EC23S (E. coli) assay	> 35.17	Decontaminate sample and/or reagent preparation areas
NTC negative control Ct values for EC23S (E. coli) assay	> 35.17	Decontaminate reagent preparation area or test new tubes or lots of reagents. Tubes or lots of reagents can be tested by preparing 36 separate no template control reactions and confirming that the lower threshold of Ct values does not fall below 35.17.

Figure 1. One data set per 96-well plate

Row/Mix	1	2	3	4	5	6	7	8	9	10	11	12
A/EC23S	Std 1	Std 1	Std 1	Std 2	Std 2	Std 2	Std 3	Std 3	Std 3	Std 4	Std 4	Std 4
B/EC23S	Std 5	Std 5	Std 5	NTC	NTC	NTC	NTC	NTC	NTC			
C/EC23S	Cal1	Cal1	Cal2	Cal2	Cal3	Cal3	FB1	FB1	FB2	FB2	FB3	FB3
D/Sketa22	Cal1	Cal1	Cal2	Cal2	Cal3	Cal3	FB1	FB1	FB2	FB2	FB3	FB3
E												
F												
G												
H												

Figure 2: Two data sets per 96-well plate

Row/Mix	1	2	3	4	5	6	7	8	9	10	11	12
A/EC23S	Std 1	Std 1	Std 1	Std 2	Std 2	Std 2	Std 3	Std 3	Std 3	Std 4	Std 4	Std 4
B/EC23S	Std 5	Std 5	Std 5	NTC	NTC	NTC	NTC	NTC	NTC			
C/EC23S	Cal1	Cal1	Cal2	Cal2	Cal3	Cal3	FB1	FB1	FB2	FB2	FB3	FB3
D/Sketa22	Cal1	Cal1	Cal2	Cal2	Cal3	Cal3	FB1	FB1	FB2	FB2	FB3	FB3
E/ EC23S	Std 1	Std 1	Std 1	Std 2	Std 2	Std 2	Std 3	Std 3	Std 3	Std 4	Std 4	Std 4
F/ EC23S	Std 5	Std 5	Std 5	NTC	NTC	NTC	NTC	NTC	NTC			
G/ EC23S	Cal1	Cal1	Cal2	Cal2	Cal3	Cal3	FB1	FB1	FB2	FB2	FB3	FB3
H/ Sketa22	Cal1	Cal1	Cal2	Cal2	Cal3	Cal3	FB1	FB1	FB2	FB2	FB3	FB3

C. REAGENTS PREPARATION

In preparation for each beach monitoring seasons, laboratories will need to estimate the number of samples that will be analyzed prior to and during the season to acquire and prepare sufficient materials and reagents for their analyses.

For the 2020 beach season, all reagents needed, except Environmental Master Mix, will be provided by Michigan State University's Water Quality, Environmental, and Molecular Microbiology Laboratory. If you have any questions, contact Matt Flood (floodmat@msu.edu) for consultation for consultation.

Determining total number of expected analyses and total volume needed: multiply expected number of analysis plates for study or monitoring season x 96 and add 30-50% to account for preliminary analyses, potential troubleshooting analyses and waste. Multiply above number times 0.0125 to estimate total volume in mL needed.

C1. REAGENTS AND MATERIALS TO BE OBTAINED OR PURCHASED

- DNA standards (5 concentrations provided in 2020, instructions are provided in the first section of the document)
- *E. coli* Bioball cells ($5x10^6$) cells/tube provided by MSU in 2020, instructions on further dilutions and storage are provided in Appendix B)
- Items listed in Table 2.

C2. PRIMER PROBE SEQUENCES

EC23S assay qPCR primers and TaqMan probe for E. coli

Forward primer: 5'-GGTAGAGCACTGTTTTGGCA
Reverse primer: 5'-TGTCTCCCGTGATAACTTTCTC
TaqMan probe: [6-FAM]-5'-TCATCCCGACTTACCAACCCG-TAMRA

Sketa22 assay qPCR primers and TaqMan probe for salmon DNA

Forward primer: 5'-GGTTTCCGCAGCTGGG
Reverse primer: 5'-CCGAGCCGTCCTGGTC
TaqMan probe: [6-FAM]-5'-AGTCGCAGGCGGCCACCGT-TAMRA

C3. ENVIRONMENTAL MASTER MIX

Environmental master mix will be purchased using a reserved batch number and lot number that has been pre-checked to minimize the presence of *E. coli* DNA in the master mix.

If you purchase additional master mix
Check with MSU on availability of new pre-checked lots in 2020 before purchasing and checking new lots. We recommend immediately testing any different lots that are purchased for *E. coli* DNA contamination. Analyses of several (>10) no template control (NTC) samples with just AE buffer or EB buffer added should either give "Undetermined" or Ct values that are higher than those obtained from standard 5. Generally, NTC Ct values of < 36 will indicate a problem.

Table 2. Other items to be purchased or acquired

Category	Supplies	Cat No.	Storage Temperature
DNA extraction	Micro-pipet aerosol barrier tips (200 and 1000 µL capacity)		Room temp
	Sample extraction tubes a. Semi-conical, screw cap micro- centrifuge tubes, 2.0 mL b. Acid washed, 212-300-µm glass beads	VWR 10025-752 or 10025-756 (free-standing) Sigma G-1277	Room temp
	1.7ml low retention micro-centrifuge tubes	MaxyClear (Axygen) VWR 22234-046	Room temp
	AE Buffer or EB Buffer	Qiagen #19077 or # 19086	4°C
	Salmon DNA (250 mg)	Sigma #D1626- 250MG	-20°C or - 80°C
qPCR	Optical 96 well PCR reaction plates	ThermoFisher	Room temp
	Optical adhesive PCR reaction tray tape	ThermoFisher	Room temp
	Micro-pipet aerosol barrier tips (10, 20, 200 and 1000 µL capacity)	Laboratory's choice*	Room temp
	PCR-grade water (10 mL) or PCR-grade water (500 mL)	VWR 10128-566 (item may be discontinued) VWR EM-9602	Room temp or 4°C
	Environmental Master Mix PCR reagent	ThermoFisher	-20°C and 4°C after opening
	EC23S and Sketa22 TaqMan probes	ThermoFisher	-20°C
	EC23S and Sketa22 assay primers	Sigma	-20°C
	Bovine Serum Albumen (BSA) fraction 5	VWR 97061-420	-20°C
Filtration	Polycarbonate membrane filter 0.4 um or Pall disposable filtration units	Millipore HTTP04700 VWR 97058-848	Room temp
	Phosphate Buffered Saline	ThermoFisher BP2438-4	4°C
Sample collection	1 liter bottles Write-On Blender Bag (sterile bags) (size 1L) Mfr. No. B01195WA Description 55-oz. 1,627-ml	ThermoFisher Cat# 312104-0032 Thomas Scientific Cat# 0677A02	Room temp

Note: *Check to see if different tips are required for single and 12-channel pipetting if applicable

C4. BSA (2MG/ML) WORKING STOCK

1. Multiply total number of expected EC23S and Sketa22 analyses for entire study or for monitoring season times 0.002 to estimate volume in mL needed.

2. Split total volume into 1 mL aliquots in 1.7 mL tubes and store at -20°C.

Determining total number of expected analyses and total volume needed: multiply expected number of 96 well plates for study or monitoring season x 96 and add 30-50% to account for preliminary analyses, potential troubleshooting analyses and waste. Multiply above number times 0.0125 to estimate total volume in mL needed.

Running out of BSA?
For 2020 beach season, 3 mL of BSA (2mg/mL) ready to be used will be shipped to each of the 10 network laboratories. If this volume is nearly used before the end of the beach season, laboratories are advised to contact Michigan State University (Matt Flood - floodmat@msu.edu) for consultation to receive an additional volume.

C5. PREPARATION OF SALMON DNA (SKETA) WORKING STOCK USING SIGMA SALMON DNA, 250MG (#D1626-250MG)

1. Transfer 250 mg of salmon DNA to a new 240 ml bottle of AE buffer or EB buffer (QIAGEN).

2. Cap tightly and dissolve by gentle stirring with a sterile magnetic stir bar, overnight until dissolved, followed by vigorous vortexing to obtain a homogeneous DNA solution. The concentration should be about 1 mg/ml.

3. Aliquot and freeze at -20°C.

4. Remove a 0.5 ml aliquot of 1 mg/mL stock and dilute to 50 ml with AE buffer or EB buffer (100-fold dilution). The concentration should be about 10 µg/mL. Check the actual concentration with Qubit or spectrophotometer. Adjust this working stock to 10 µg/mL if necessary, either by further dilution or by adding additional 1 mg/ml stock, vortexing and rechecking the concentration with Qubit or spectrophotometer. Aliquot 5 mL portions of the adjusted DNA working stock and freeze at -20°C.

Be sure to make enough working stock
Use 10 µg/mL working stock first to prepare 0.2 µg/mL SAE as detailed in the following section. When needed, follow step (4) above to prepare more 10 µg/mL working stock using aliquots of 1 mg/ml stock and aliquot for storage at -20°C. These salmon DNA stocks should be sufficient for many beach seasons.

C6. PREPARATION OF SAE EXTRACTION BUFFER

1. Multiply the total number of expected samples (including positive and negative controls) for entire study or for monitoring season times 0.6 to estimate volume in mL needed.

2. Prepare SAE buffer (containing 0.2 µg/mL salmon DNA) in a new 240 mL bottle of AE buffer or EB buffer (Qiagen) by adding /5 mL of 10 µg/mL salmon DNA working stock and mixing thoroughly. Store bottle in refrigerator (4oC). This stock should be enough to make ~ 400 sample extracts and can be used for up to one complete beach season. Aliquots can be poured into a sterile disposable tube for daily use. Be careful to avoid contaminating the original bottle!

To determine total number of expected samples and total volume of SAE extraction buffer needed: assuming a full plate will be run each analysis day, multiply expected number of analysis days times 24 and add 20-40% to account for preliminary analyses and potential troubleshooting analyses. Multiply above number times 0.6 to estimate total volume in mL needed. SAE extraction buffer can be stored in refrigerator (4O°C) for several months.

Running out of sketa?
For 2020 beach season, Sketa will be shipped to network laboratories in a volume of 245 mL SAE at 0.2 µg/mL concentration ready to be used. If the quantity provided becomes limited during the season, laboratories are advised to contact Michigan State University (Matt Flood - floodmat@msu.edu) for consultation to receive an additional volume.

C7. PREPARATION OF PRIMER/PROBE WORKING STOCKS FOR SKETA22 AND EC23S ASSAYS

1. Purchase Primers and probes using sequences provided above (section C2).

2. It is recommended to make sufficient quantities of the primer/probe working stocks for the entire beach season. Table 3 provides an example of preparing a Primer/Probe mix for 1100 reactions, enough for 10 plates.

To determine total number of expected analyses and total volumes needed: multiply expected number of analysis plates for study or monitoring season x 48 and add 30-50% to account for preliminary analyses, potential troubleshooting analyses and waste. Multiply above number times 0.003 to estimate total volume in mL needed.

Table 3. Preparation of primers and probe mix

Primer/probe	Initial concentration (µM)	Volume per reaction (µL)	Running 10 96-well plates 1100 reactions, Volume (µL)
Forward	500	0.05	55
Reverse	500	0.05	55
TaqMan probe	100	0.02	22
Water		2.88	3168
Total volume			3,300 or 3.3 mL

3. Prepare Sketa22 and EC23S assay primer/probe working stocks using mixing ratios as shown in the Table 3.

4. Split total volumes of each working stock into 1 mL aliquots in 1.7 mL low retention tubes and store aliquots at -20°C. After thawing each aliquot, refrigerate (4°C) until used up.

D. MASTER MIX PREPARATION

Table 4 and 5 demonstrate the reagents mix components needed to run one reaction of qPCR *E. coli* or Sketa. The same reagent mix ratio is used for the preparation of both salmon DNA (Sketa22) and *E. coli* assays. The difference is that you have to change primers and probes used in the reagent mix.

1. First mix reagents #1 to #4 in tables 4 and 5, and then distribute the reagent mix in wells prior to adding DNA template (#5).

Table 4. Reaction mix components (Assay master mix for E. coli (EC23S)

#	Component	**Vol (µL) Per Reaction
#1	2x Environmental Master Mix	12.5
#2	BSA (2mg/mL)	2.5
#3	Primer/Probe (EC23S)	3.0
#4	DNA-free water	2.0
#5	DNA - Template	5.0

** Multiply these volumes by the number of reactions to be prepared plus extra

Table 5. Reagents mix components for Sketa22

#	Component	**Vol (µL) per Reaction
#1	2x Environmental Master Mix	12.5
#2	BSA (2mg/mL)	2.5
#3	Primer/Probe (Sketa22)	3.0
#4	DNA-free water	2.0
#5	DNA - Template	5.0

** Multiply these volumes by the number of reactions to be prepared plus extra

E. POSITIVE AND NEGATIVE CONTROL SAMPLE PREPARATIONS

1. Prepare 3 calibrator samples and 3 filter blank samples with SAE extraction buffer as follows:
    a. Remove a tube containing 0.1 mL, $5x10^4$ CFU E. coli cell suspension (see Appendix B)
    b. Add 0.9 ml PBS to thawed E. coli cell tube.
    c. Transfer entire 1 mL volume to a 15 or 50 mL tube
    d. Add 4.1 mL PBS to 15 mL tube and vortex gently (final volume: 5.1 mL, ~10^4 E. coli CFU/mL)
    e. Set up 3 filtration units with polycarbonate filters at manifold station (see section F.5)
    f. Add 20 mL PBS to each filtration unit and filter under vacuum until no standing liquid is observed on the filters
    g. Transfer filters to bead tubes labeled FB1, FB2, & FB3 (filter blank samples)
    h. Set up 3 filtration unit bases with polycarbonate filters at manifold station
    i. Filter 1 mL aliquots of 5.1 mL *E. coli* cell suspension through each filter
    j. Transfer filters to bead tubes labeled Cal1, Cal2, & Cal 3 (calibrator samples)
    k. Add 0.6 ml of SAE extraction buffer to each bead tube with filter and cap tubes tightly

Need to adjust calibrator concentrations?

If E. coli cell concentrations in calibrator samples need to be increased, multiple tubes of $5x10^4$ CFU E. coli cell suspensions can be used and diluted with a correspondingly smaller volume of PBS. Try preparing three tubes of $5x10^4$ CFU E. coli cell suspension as described in Steps a. – c. Combine them together and in Step d. mix with 2 mL PBS instead of 4.1 mL PBS.

If E. coli cell concentrations in calibrator samples need to be increased or decreased, these final dilutions can be performed with smaller or greater volumes of PBS, respectively. However, a minimum final volume of 3.0 mL is needed for three calibrator samples.

2. Extract calibrator and filter blank samples as follows:
    a. Bead beat tubes for 1.0 min at the maximum rate (5,000 rpm)
    b. Remove the tubes from the bead beater and centrifuge at 12,000 × g for 1 minute to pellet the glass beads and debris.
        1. *Note: To prevent contamination, a new pair of gloves may be used.*
    c. Using the 200 µL micropipettor, transfer 400 µL of the supernatant (DNA extract) to corresponding labeled sterile 1.7-mL low detention tube labeled with sample name FB1, FB2, & FB3 or Cal1, Cal2, & Cal 3 , taking care to not mix FB and Cal samples (crude extracts).
        1. *Note: The filter will normally remain intact during the bead beating and centrifugation process. Generally, 400 µL of supernatant can easily be collected. Collect a minimum of 300 µL of supernatant.*
    d. Centrifuge crude extract for 5 minutes at 12,000 × g.
    e. Transfer ~350 µL of the clarified supernatant to another 1.7-mL low detention tube, taking care not to disturb pellet.
    f. These are the undiluted extracts, ready for analysis.

F. ENVIRONMENTAL DNA WATER SAMPLE COLLECTION

F1. EQUIPMENT STERILIZATION

If you have pall reusable units, those can be bleached or autoclaved to destroy DNA from previous samples and sterilized for the next use.

1. Soak, for a minimum of 10 minutes, the water sampling bottles and cylinders using 10% v/v bleach (e.g. 1:10 dilution or 1- part bleach for every nine parts of water)
2. Add 1 gram of sodium thiosulfate per liter of 10% bleach and mix well
3. Remove water sampling bottles and cylinders and pour off any remaining neutralized bleach solution.
4. Rinse three times with reagent-grade water or sterile nanopure water

Or

1. Rinse the water sampling bottles and cylinders three times with reagent-grade water or sterile nanopure water
2. Cover loosely with Aluminum foil
3. Autoclave at 120°C for 15 minutes (contact S. Briggs if you do not have autoclave)

Use of disposable filtration units is recommended.

F2. WHAT YOU NEED IN FIELD

- A cooler (size based on the number of samples intended to be collected)
- 1L sterile Nalgene bottles equal to the number of samples to be collected
- 1 Nalgene sterile bottle with autoclaved Milli-Q water as a field sample blank
- Ice-packs to keep samples under chilled conditions during transportation until filtration
- Thermometer for ambient and water temperature measurements
- Field sample collection sheet to record in-field necessary information (e.g. name of technician collecting the sample, time of sample collection, turbidity, temp. air & water, pH, DO, streamflow)

F3. WATER SAMPLE COLLECTION AND FILTRATION

1. Wear sterile gloves for sampling and do not touch the rim or inside of the cap or bottle
2. Place the bottle into the flow of water
3. After filling bottle (Volume 1000mL), replace the cap and tighten the cap to prevent leakage
4. Label sampling bottle with sample identification (e.g. Sample ID, Location, Date/Time, Collector) in permanent marker
    a. *Note: use a removable top for labeling to allow multiple uses of sample collection bottles*
5. Place the bottle in the cooler with ice pack, field sample collection blank, and transport back to laboratory within 6 hr.
6. Once arrived in the lab, shake and invert the sample bottle vigorously 25 times to distribute the bacteria uniformly and make 100 mL aliquots (using sterile 100mL bottles) of each collected sample as indicated below.
7. Place a fresh membrane filtration funnel assembly on the vacuum manifold base for each test sample aliquot.

8. Filter 100 mL aliquots of each sample for different qPCR tests:
    a. 100mL or 2x100mL for E. coli qPCR by the local lab
        i. Only 1 aliquot is necessary if only -80°C freezer storing the filters and still doing IDEXX testing of the water samples.
        ii. 2 aliquots are necessary if laboratory is doing same day qPCR testing. Aliquot 1 for the same day testing and Aliquot 2 stored in -80°C freezer.
    b. Optional additional filters for MST or EPA (see below after step 13)
9. After the water samples have passed through the filters, add 20 mL PBS to each filtration unit and filter until no standing liquid is observed on the filters.
10. After filtration is completed, turn off the vacuum and remove the funnel from the filter base.
11. Leave the filter on the filtration base and using sterile forceps, fold filter into cylinder with the sample side facing inward, being careful to handle the filter only on edge, where the filter has not been exposed to the test sample. Insert the rolled filter into a pre-labeled extraction tube with glass beads.
    a. Note: All extraction tubes should be labeled in a manner so that they can be identified by sample date, location, and Beach ID (see section H.2. for recommended data sheet labeling conventions). Freezer stored tubes should contain this information on the sides of the tubes.
12. Store the labeled extraction tubes with sample filters in -80°C until processed or shipped.
    a. Note: Frozen samples should be DNA extracted and qPCR analyzed on the same day they are thawed.
13. Required volumes of the remainder of the original sample should be used for E. coli culture testing with the IDEXX method by all Labs that are not doing same day qPCR testing. IDEXX test is optional for labs that are doing same day qPCR testing.

Optional Additional Filters for Microbial Source Tracking (MST) and EPA Study

Each lab will need to decide which, if any, of the below additional filters to run. Table 6 summarizes the potential filters to prepare for the 2020 beach season.

1. MST Samples
    a. Only 1 aliquot of 100 mL sample is necessary if a laboratory in the network plans to run MST assay targeting either exclusively animal or exclusively human fecal sources. Two aliquots of 100 mL each of sample are necessary if a laboratory in the network plans to run MST assay targeting both human and animal fecal sources. Samples have to be stored in -80°C before analysis.
    b. 100mL for MST for local lab – One aliquot for a human fecal source target
        i. This sample should be collected:
            (1) If E. coli results have consistently tested high and potential source was not identified by the sanitary survey performed on sampled beach
            (2) If human fecal pollution has been suspected as a potential source by the sanitary survey
    c. 100mL for MST at MSU Lab – One aliquot for an animal fecal source target
        i. You can opt to have MSU run MST for animal targets if:
            (1) If your laboratory is new and has not yet been trained in MST methods
            (2) If the sampling site has been identified to have animals (e.g. dogs or birds) as potential sources by the sanitary survey
            (3) If the above two points do not define the scenario observed on the beach being sampled, please contact Shannon Briggs and the MSU Lab for further instructions on what to be done.
2. Specific labs: 4x100mL for Rich at USEPA
    a. Labs will be contacted individually to see if they wish to participate in an independent study to evaluate atypical E. coli culture vs qPCR quantitative estimates and sample matrix

interference mitigation techniques at selected beaches. More details on procedures and sampling schedules for this study will be provided to labs that are willing to participate after further discussions with these labs about their sample collection capabilities.

3. Prior to shipping to MSU or EPA, check to ensure that all field information has been recorded in the field sample collection form. A copy of the field sample collection form should be shipped with samples packaged for processing at a different lab.

Table 6. Summary of potential filters for the 2020 Beach season

Filter Type	Use	Lab Doing Analysis	Required or Optional*
100mL EC - qPCR	qPCR E. coli analysis	Trained Local lab	Required: All labs filter at least 100 mL for qPCR analysis
100mL EC - qPCR	qPCR E.coli analysis	Trained Local lab	Required for labs running qPCR real time
100mL MST – local lab	MST for human markers	Trained Local lab	Optional
100mL MST – MSU	MST for animal markers	MSU	Optional
4 x 100mL EC - qPCR USEPA	EPA study	EPA	Optional- specific labs will be contacted by EPA

*Optional refers to samples collected in addition to what is required for implementing Method C for beach monitoring. This includes all samples for MST and the EPA study.

G. WATER SAMPLE FILTER ANALYSES

Except as otherwise indicated, this protocol is for one full 96-well reaction plate containing duplicate analyses of 3 calibrator and 3 filter blank samples and either duplicate analyses of 18 test samples or triplicate analyses of 12 test samples with both EC23s and Sketa assays (see Notes under Section H #2 regarding analysis of less than maximum number of test samples on a plate). It also assumes the analysis will be performed on an Applied Biosystems StepOnePlus instrument.

1. Groups of three calibrator samples can be prepared and extracted (as described in section E) and analyzed to determine whether they pass QA (as described in section B) in advance of test sample analyses. Refrigerated extracts of acceptable calibrator samples can be reanalyzed as the positive controls with multiple test sample batches for at least one week (DNA stability in the extracts with longer storage times is possible, but has not been confirmed).
    a. *Note: Three filter blank samples should be prepared, extracted and analyzed with each batch of test samples as indicated below.*

2. In DNA-free laboratory workstation, prepare master mixes containing primer/probe working stocks for EC23S assay and Sketa22 assay with 2x Environmental Master Mix sufficient for 48 reactions (+ 6 extra) each.
    a. ***Tip**: take into account that actual volumes of reagents that are pipetted may vary, so excess volumes of master mixes normally should be prepared, e.g. for 48 reactions, it may be necessary to make up sufficient master mix for 50 or more reactions. For multichannel pipetting, even larger excess volumes will be required. It is suggested that each lab/analyst experiment with using*

different volumes of 5% glycerol in water (to mimic reaction mix) to load a spare 96 well reaction plate to determine the minimum excess volume needed to reproducibly prepare 48 reactions.

b. ***Tip**: If multiple plates will be run on same day, multiply 48 reactions by the number of plates to be run during that day and prepare reaction mixes for all of the plates at same time. Amount of excess master mixes needed per plate when multichannel pipetting should decrease with increasing number of plates prepared at one time. All prepared plates should be immediately stored in refrigerator and used on the day they are prepared.*

3. Transfer 20 µl aliquots of EC23S and Sketa22 master mix to 48 wells each of a 96 well reaction plate (suggested options for plate layouts shown Figures 3 or 4) and cover the plate with aluminum foil or adhesive foil tape for transport to main laboratory and immediately store in refrigerator until ready to add DNA samples
    a. ***Tip**: It is currently thought that the same tips can be used for all transfers of the same reagent. P20 single channel or L50 multichannel pipets can be used.*

    b. *Note: Different tips may need to be purchased for single and multi-channel pipettors.*

4. In main laboratory, filter 100 mL aliquots of 18 water samples or 12 water samples (for duplicate or triplicate analyses, respectively) and rinse filters with 20 mL PBS as indicated in section F.3.

5. Transfer filters to bead tubes labeled with sample identification and store in refrigerator.

6. Prepare and filter 3 filter blank samples (and optional* 3 calibrator samples) as described in Section E.2.
    a. *See step G.1 above regarding recommended advance preparation, extraction and analysis of calibrator samples*

7. Remove water sample filters from refrigerator and add 0.6 mL of SAE extraction buffer to each bead tube with filter, cap tubes tightly.

8. Extract calibrator (if applicable), filter blank and water sample filters as indicated in section E.2.

9. Transfer 5 µL aliquots of calibrator, filter blank and water sample DNA extracts to wells of the reaction plate containing EC23S and Sketa22 master mixes as indicated in Figure 3* when performing single-channel pipetting.
    a. *Note: Use a separate P10 pipet tip for each sample transfer.*

    b. **Other sample layouts and less than 18 or 12 samples per plate are acceptable if correct sample naming conventions are followed – see section H.*

10. For 12-channel multi-channel pipetting, transfer ~30 µL of calibrator and filter blank extracts and ~50 µL of test sample extracts to an empty 96 well plate (extract plate) and then transfer replicate 5 µL aliquots of the extracts from the extract plate to the reaction plate as suggested in Figure 4*.
    a. *Note: Use a separate pipet tip for each sample transfer. Different tips may need to be purchased for multi-channel pipettors.*

    b. **Other plate layouts and less than 12 samples per plate are acceptable but may not be applicable for multi-channel pipetting.*

11. After all DNA extracts are loaded in the reaction plate, seal the reaction plate with optical adhesive tape and centrifuge the plate using a salad spinner or plate centrifuge. Inspect each of the wells in the plate to confirm all liquid is in the bottom of the wells and all wells contain equal volumes. Load plate in StepOnePlus and run reactions after setting up the instrument as described in Appendix A.
    a. *Note: Sample names can be entered for each well as part of the instrument run set up or afterward as indicated in section H.2 or Appendix C.*

Figure 3. Recommended plate layout options for single channel sample pipetting of A: duplicate test sample analysis plates and B: triplicate test sample analysis plates.

A. Duplicate analyses

Option 1												
Plate Rows	1	2	3	4	5	6	7	8	9	10	11	12
A/EC23S	Cal 1	Cal 1	Cal 2	Cal 2	Cal 3	Cal 3	FB 1	FB 1	FB 1	FB 1	FB 3	FB 3
B/EC23S	sample 1	sample 1	sample 2	sample 2	sample 3	sample 3	sample 4	sample 4	sample 4	sample 4	sample 6	sample 6
C/EC23S	sample 7	sample 7	sample 8	sample 8	sample 9	sample 9	sample 10	sample 10	sample 10	sample 10	sample 12	sample 12
D/EC23S	sample 13	sample 13	sample 14	sample 14	sample 15	sample 15	sample 16	sample 16	sample 16	sample 16	sample 18	sample 18
E/Sketa22	Cal 1	Cal 1	Cal 2	Cal 2	Cal 3	Cal 3	FB 1	FB 1	FB 1	FB 1	FB 3	FB 3
F/Sketa22	sample 1	sample 1	sample 2	sample 2	sample 3	sample 3	sample 4	sample 4	sample 4	sample 4	sample 6	sample 6
G/Sketa22	sample 7	sample 7	sample 8	sample 8	sample 9	sample 9	sample 10	sample 10	sample 10	sample 10	sample 12	sample 12
H/Sketa22	sample 13	sample 13	sample 14	sample 14	sample 15	sample 15	sample 16	sample 16	sample 16	sample 16	sample 18	sample 18

Option 2												
Plate Rows	1	2	3	4	5	6	7	8	9	10	11	12
A/EC23S	Cal 1	Cal 1	Cal 2	Cal 2	Cal 3	Cal 3	FB 1	FB 1	FB 1	FB 1	FB 3	FB 3
B/Sketa22	Cal 1	Cal 1	Cal 2	Cal 2	Cal 3	Cal 3	FB 1	FB 1	FB 1	FB 1	FB 3	FB 3
C/EC23S	sample 1	sample 1	sample 2	sample 2	sample 3	sample 3	sample 4	sample 4	sample 4	sample 4	sample 6	sample 6
D/Sketa22	sample 1	sample 1	sample 2	sample 2	sample 3	sample 3	sample 4	sample 4	sample 4	sample 4	sample 6	sample 6
E/EC23S	sample 7	sample 7	sample 8	sample 8	sample 9	sample 9	sample 10	sample 10	sample 10	sample 10	sample 12	sample 12
F/Sketa22	sample 7	sample 7	sample 8	sample 8	sample 9	sample 9	sample 10	sample 10	sample 10	sample 10	sample 12	sample 12
G/EC23S	sample 13	sample 13	sample 14	sample 14	sample 15	sample 15	sample 16	sample 16	sample 16	sample 16	sample 18	sample 18
H/Sketa22	sample 13	sample 13	sample 14	sample 14	sample 15	sample 15	sample 16	sample 16	sample 16	sample 16	sample 18	sample 18

B. **Triplicate analyses**

Option 1												
Row/Assay	1	2	3	4	5	6	7	8	9	10	11	12
A/EC23S	Cal1	Cal1	Cal2	Cal2	Cal3	Cal3	FB1	FB1	FB2	FB2	FB3	FB3
B/Sketa22	Cal1	Cal1	Cal2	Cal2	Cal3	Cal3	FB1	FB1	FB2	FB2	FB3	FB3
C/EC23S	Sample1	Sample1	Sample1	Sample2	Sample2	Sample2	Sample3	Sample3	Sample3	Sample4	Sample4	Sample4
D/Sketa22	Sample1	Sample1	Sample1	Sample2	Sample2	Sample2	Sample3	Sample3	Sample3	Sample4	Sample4	Sample4
E/EC23S	Sample5	Sample5	Sample5	Sample6	Sample6	Sample6	Sample7	Sample7	Sample7	Sample8	Sample8	Sample8
F/Sketa22	Sample5	Sample5	Sample5	Sample6	Sample6	Sample6	Sample7	Sample7	Sample7	Sample8	Sample8	Sample8
G/EC23S	Sample9	Sample9	Sample9	Sample10	Sample10	Sample10	Sample11	Sample11	Sample11	Sample12	Sample12	Sample12
H/Sketa22	Sample9	Sample9	Sample9	Sample10	Sample10	Sample10	Sample11	Sample11	Sample11	Sample12	Sample12	Sample12

Option 2												
Row/Assay	1	2	3	4	5	6	7	8	9	10	11	12
A/EC23S	Cal1	Cal1	Cal2	Cal2	Cal3	Cal3	FB1	FB1	FB2	FB2	FB3	FB3
B/EC23S	Sample1	Sample1	Sample1	Sample2	Sample2	Sample2	Sample3	Sample3	Sample3	Sample4	Sample4	Sample4
C/EC23S	Sample5	Sample5	Sample5	Sample6	Sample6	Sample6	Sample7	Sample7	Sample7	Sample8	Sample8	Sample8
D/EC23S	Sample9	Sample9	Sample9	Sample10	Sample10	Sample10	Sample11	Sample11	Sample11	Sample12	Sample12	Sample12
E/Sketa22	Cal1	Cal1	Cal2	Cal2	Cal3	Cal3	FB1	FB1	FB2	FB2	FB3	FB3
F/Sketa22	Sample1	Sample1	Sample1	Sample2	Sample2	Sample2	Sample3	Sample3	Sample3	Sample4	Sample4	Sample4
G/Sketa22	Sample5	Sample5	Sample5	Sample6	Sample6	Sample6	Sample7	Sample7	Sample7	Sample8	Sample8	Sample8
H/Sketa22	Sample9	Sample9	Sample9	Sample10	Sample10	Sample10	Sample11	Sample11	Sample11	Sample12	Sample12	Sample12

Figure 4. Recommended plate layouts for 12 channel sample pipetting of A: duplicate test sample analysis plates and B: triplicate test sample analysis plates.

A. Duplicate analyses

12-channel extract plate

	1	2	3	4	5	6	7	8	9	10	11	12
row A	Cal1	Cal2	Cal3	FB1	FB2	FB3	Sample1	Sample2	Sample3	Sample4	Sample5	Sample6
row B												
row C	Sample7	Sample8	Sample9	Sample10	Sample11	Sample12	Sample13	Sample14	Sample15	Sample16	Sample17	Sample18
row D												
row E												
row F												
row G												
row H												

12 channel reaction plate (option1)

Row/Assay	1	2	3	4	5	6	7	8	9	10	11	12
A/EC23S287	Cal1	Cal2	Cal3	FB1	FB2	FB3	Sample1	Sample2	Sample3	Sample4	Sample5	Sample6
B/EC23S287	Cal1	Cal2	Cal3	FB1	FB2	FB3	Sample1	Sample2	Sample3	Sample4	Sample5	Sample6
C/EC23S287	Sample7	Sample8	Sample9	Sample10	Sample11	Sample12	Sample13	Sample14	Sample15	Sample16	Sample17	Sample18
D/EC23S287	Sample7	Sample8	Sample9	Sample10	Sample11	Sample12	Sample13	Sample14	Sample15	Sample16	Sample17	Sample18
E/Sketa22	Cal1	Cal2	Cal3	FB1	FB2	FB3	Sample1	Sample2	Sample3	Sample4	Sample5	Sample6
F/Sketa22	Cal1	Cal2	Cal3	FB1	FB2	FB3	Sample1	Sample2	Sample3	Sample4	Sample5	Sample6
G/Sketa22	Sample7	Sample8	Sample9	Sample10	Sample11	Sample12	Sample13	Sample14	Sample15	Sample16	Sample17	Sample18
H/Sketa22	Sample7	Sample8	Sample9	Sample10	Sample11	Sample12	Sample13	Sample14	Sample15	Sample16	Sample17	Sample18

12 channel reaction plate (option2)

Row/Assay	1	2	3	4	5	6	7	8	9	10	11	12
A/EC23S287	Cal1	Cal2	Cal3	FB1	FB2	FB3	Sample1	Sample2	Sample3	Sample4	Sample5	Sample6
B/EC23S287	Cal1	Cal2	Cal3	FB1	FB2	FB3	Sample1	Sample2	Sample3	Sample4	Sample5	Sample6
C/Sketa22	Cal1	Cal2	Cal3	FB1	FB2	FB3	Sample1	Sample2	Sample3	Sample4	Sample5	Sample6
D/Sketa22	Cal1	Cal2	Cal3	FB1	FB2	FB3	Sample1	Sample2	Sample3	Sample4	Sample5	Sample6
E/EC23S287	Sample7	Sample8	Sample9	Sample10	Sample11	Sample12	Sample13	Sample14	Sample15	Sample16	Sample17	Sample18
F/EC23S287	Sample7	Sample8	Sample9	Sample10	Sample11	Sample12	Sample13	Sample14	Sample15	Sample16	Sample17	Sample18
G/Sketa22	Sample7	Sample8	Sample9	Sample10	Sample11	Sample12	Sample13	Sample14	Sample15	Sample16	Sample17	Sample18
H/Sketa22	Sample7	Sample8	Sample9	Sample10	Sample11	Sample12	Sample13	Sample14	Sample15	Sample16	Sample17	Sample18

B. Triplicate analyses

12-channel extract plate

	1	2	3	4	5	6	7	8	9	10	11	12
row A	Cal1	Cal1	Cal2	Cal2	Cal3	Cal3	FB1	FB1	FB2	FB2	FB3	FB3
row B												
row C	Sample1	Sample2	Sample3	Sample4	Sample5	Sample6	Sample7	Sample8	Sample9	Sample10	Sample11	Sample12
row D												
row E												
row F												
row G												
row H												

12 channel reaction plate (option1)

Row/Assay	1	2	3	4	5	6	7	8	9	10	11	12
A/EC23S	Cal1	Cal1	Cal2	Cal2	Cal3	Cal3	FB1	FB1	FB2	FB2	FB3	FB3
B/EC23S	Sample1	Sample2	Sample3	Sample4	Sample5	Sample6	Sample7	Sample8	Sample9	Sample10	Sample11	Sample12
C/EC23S	Sample1	Sample2	Sample3	Sample4	Sample5	Sample6	Sample7	Sample8	Sample9	Sample10	Sample11	Sample12
D/EC23S	Sample1	Sample2	Sample3	Sample4	Sample5	Sample6	Sample7	Sample8	Sample9	Sample10	Sample11	Sample12
E/Sketa22	Cal1	Cal1	Cal2	Cal2	Cal3	Cal3	FB1	FB1	FB2	FB2	FB3	FB3
F/Sketa22	Sample1	Sample2	Sample3	Sample4	Sample5	Sample6	Sample7	Sample8	Sample9	Sample10	Sample11	Sample12
G/Sketa22	Sample1	Sample2	Sample3	Sample4	Sample5	Sample6	Sample7	Sample8	Sample9	Sample10	Sample11	Sample12
H/Sketa22	Sample1	Sample2	Sample3	Sample4	Sample5	Sample6	Sample7	Sample8	Sample9	Sample10	Sample11	Sample12

12 channel reaction plate (option2)

Row/Assay	1	2	3	4	5	6	7	8	9	10	11	12
A/EC23S	Cal1	Cal1	Cal2	Cal2	Cal3	Cal3	FB1	FB1	FB2	FB2	FB3	FB3
B/Sketa22	Cal1	Cal1	Cal2	Cal2	Cal3	Cal3	FB1	FB1	FB2	FB2	FB3	FB3
C/EC23S	Sample1	Sample2	Sample3	Sample4	Sample5	Sample6	Sample7	Sample8	Sample9	Sample10	Sample11	Sample12
D/EC23S	Sample1	Sample2	Sample3	Sample4	Sample5	Sample6	Sample7	Sample8	Sample9	Sample10	Sample11	Sample12
E/EC23S	Sample1	Sample2	Sample3	Sample4	Sample5	Sample6	Sample7	Sample8	Sample9	Sample10	Sample11	Sample12
F/Sketa22	Sample1	Sample2	Sample3	Sample4	Sample5	Sample6	Sample7	Sample8	Sample9	Sample10	Sample11	Sample12
G/Sketa22	Sample1	Sample2	Sample3	Sample4	Sample5	Sample6	Sample7	Sample8	Sample9	Sample10	Sample11	Sample12
H/Sketa22	Sample1	Sample2	Sample3	Sample4	Sample5	Sample6	Sample7	Sample8	Sample9	Sample10	Sample11	Sample12

H. DATA ANALYSIS

1. At the conclusion of the run, manually set threshold values for all wells to 0.03, ensure correct assay target names and reporter and quencher dyes have been selected, and export Ct results as indicated in Appendix A.

2. If sample names were not entered during the run set up, enter sample names for each well in column B of the exported Excel .xls file "results" tab.

   Excel spreadsheets with sample names listed in the order that they will be run can be prepared ahead of time so that sample names can be copied and pasted into the export file as blocks of data.

   It is highly recommended that all test sample naming be performed using the following convention:

   S_Date(yyyymmdd)_Location (0=composite,1=left,2=center,3=right facing the water)_Beachname (can be a short name or an identifiable number)

   Example: S_20190622_1_Dunes

3. **For manual data transfer to Method C workbooks**: a copy of the Sheet "TestSamples" should be made in the appropriate 2020 Method C Template Workbook, and renamed to reflect pertinent sample information
   a. Plates run in triplicate should use the "3rep" 2020 Method C Template Workbook, and samples run in duplicate should use the "2rep" 2020 Method C Template Workbook.
   b. More information on naming conventions can be found in Appendix C.
   c. Additional information can be manually entered into Cells A3 and B3

4. E. coli Ct data from each calibrator sample should be copied from column J of the export file and pasted into cells B7-B12 of the copied "TestSamples" sheet. The paired Sketa Ct data for each calibrator sample should be copied from Column J of the export file, and pasted into cells C7-C12 of the copied "TestSamples" sheet.

5. The same should be done for the paired E. coli Ct data and Sketa Ct data for each filter blank sample. E. coli Ct data should be copied from column J of the export file and pasted into cells B21-B26, and the paired Sketa Ct data for each filter blank sample should be copied from Column J of the export file, and pasted into cells C21-C26 of the copied "TestSamples" sheet

6. Sample names and data from test sample replicates should be pasted into block A33 to C68. Replicate samples should be arranged next to each other, with their names being pasted or manually entered into cells A33-A68. Paired E. coli and Sketa Ct values should be copied from column J of the export file, and pasted into cells B33-B68 and C33-C68 respectively, corresponding to the appropriate sample names.

7. **For automated data transfer to Method C workbooks:** a Method C Macro Tool that will be provided to each of the labs can be used.

   a. Detailed instructions on how to use the Method C Macro Tool can be found in Appendix D.

<u>Important notes on Method C Macro Tool use:</u>
For correct data transfer using the Macro Tool, confirm sample names are in column B, target names (e.g. *E coli* and Sketa) are in column C and Ct values are in column J of the "results" tab of the export file.

Macro Tool will accommodate sorting and analysis of up to 18 duplicate or 12 triplicate test samples with both EC23s and Sketa assays on a plate, duplicate analyses of 3 calibrators and 3 filter blank samples with both EC23s and Sketa assays are always required.

For correct sorting of the samples using the macro tool, the following sample naming conventions should always be used:

- Start calibrators sample names with “C”
- Start filter blank sample names with “F”
- Start test sample names with “S”

8. Click on the new tab to see results.

9. Results and QA analysis results of the individual replicate calibrator and filter blank sample analyses in the run are shown in rows 7-12 and 21-26, respectively, in the spreadsheet.

10. Results and QA analysis results of the individual test sample analyses in the run are shown in rows 33-68 in the spreadsheet.

11. Final compiled results and QA analyses from the replicate analyses of the filter blanks and each test sample are shown in rows 74-86 of the spreadsheet.

12. Column J of rows 74-86 shows mean estimated log10 copies per reaction for each sample or the primary QA test failure that caused such estimates not to be determined.
    a. Note: The different QA criteria are summarized in Table 1, Method C template workbook instructions and in Sivaganesan et al., 2019. Lower limits of quantification are based on the analyst’s individual composite standard curve rather than on a global value.

13. Column K of rows 74-86 shows median Method C MPN equivalents for each sample giving a numerical quantitative estimate in column J based on the global fitted linear relationship between Method C log10 copies per reaction and E. coli MPN estimates from the ColilertTM method established for recreational beach water samples in Michigan.

14. Column L of rows 74-86 indicates whether the median Method C MPN equivalent value for each sample is above the Michigan beach notification value of 300.

APPENDIX A. APPLIED BIOSYSTEMS STEPONEPLUS™ OPERATION

Applied Biosystems StepOnePlus™

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.

2. On the StepOnePlus™ home screen select New Experiment from File drop down menu and then select Advanced Set Up.

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

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).

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.

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 (e.g., Ecoli, Sketa). The selected targets will then be added on to the define target and sample screen.

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 FAM (default). From the **Quencher** dropdown menu, **select TAMRA** (NFQ-MGB is the default). Leave the default in the color field. Click on Save Target.

8. Click on the tab **Assign Targets and Samples** to see the screen view of the plate layout with 96 wells.
    a. Select the wells, based on the plate set up, by highlighting, one target at a time.
    b. 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.
    c. 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.
        i. 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. Click on Sample 1 or assign sample information to each selected well.

9. Click on **Run Method**

a. 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**.
b. Make sure the reaction volume per well field displays 25 µL, this is not the default setting.
c. Set the thermal profile to the following holding and cycling stages: Holding Stage 1: 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. Use instrument default of 40 cycles. **Important note**: The second step of the Cycling Stage should be changed from the default temperature of 60.0°C to 56.0° for Method C analyses.
d. **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.

10. Click on **Run** and load the plate into the instrument.

11. Click on **Start Run** (the green button in the upper right hand corner of the screen).
    a. 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.
    b. 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.**

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

13. Analyze the run
    a. Click on the tab in the top right corner of screen to **Analyze**.
    b. Click on **Analysis Settings** to get to CT settings (default CT settings).
    c. Click on **Edit Default Settings**. Click on **Automatic Threshold Box** to uncheck it. Change threshold from 0.2 to 0.03. Click on **Save Changes** box and then click on **Apply analysis settings** box.

14. Click on the **Export** tab in the menu bar at top of screen.
    a. Check the Results box in the Export Property screen
    b. Click on the browse button to find the correct place to **export the data** and click on open and then click on **Start Export**. After the export is done **close export tool**.

15. Click on **Save** to save changes

Did you remember to change these default settings?

- Quantitation-Comparative Ct (ΔΔCt): Step 3b
- Standard-2hours: Step 3d
- Select appropriate quencher if adding new target: Step 7
- Set temperature for second step of Cycling Stage to 56: Step 9c
- Change threshold from 0.2 to 0.03: Step 13c

APPENDIX B. PREPARATION OF E. COLI CELL SUSPENSIONS FROM BIOBALLS

Note: This protocol is divided in two parts. Laboratories should only need to perform Part 2 in 2020.

Part 1

Steps under part 1 (from a to d) will be performed by Michigan State University. Cell suspensions created in Step (d) will be shipped to the labs on dry ice.

a. Transfer 2.2 mL (from two tubes) of rehydration fluid to a 5 mL tube.

b. Transfer one 10E8 BioBall to the tube with rehydration fluid.

c. Tube is held for 3 min to dissolve BioBall and then vortexed for 5 sec to thoroughly mix and suspend cells

d. Transfer 100 µL aliquots of cell suspension to twenty-one, 1.7 mL micro-centrifuge tubes (label as $5x10^6$ cells). Any excess suspension is discarded.

Note: Bioballs are supplied by Biomerieux USA (Part #: 56146, Description: BioBall MultiShot-10E8, Escherichia coli NCTC 12923). Rehydration Fluid is supplied by Biomerieux USA (Part #: 56021, Description: Rehydration Fluid).

Part 2

Once 5x10E6 cell tubes are received by the labs, they should be immediately stored in freezer (-80°C recommended). The labs should perform the rest of the steps (e to g)

e. Add 0.9 mL of PBS to one $5x10^6$ cell tube and vortex gently to thoroughly mix and thaw the cells.

f. Transfer entire 1 mL volume of diluted 5x10E6 cell tube to a 50 mL tube and dilute with 9 mL PBS (final concentration: $5x10^5$ cells/per ml)

g. Transfer 100 µL aliquots of $5x10^5$ cells/per ml suspension to 100 micro-centrifuge tubes ($5x10^4$ cell tubes). Freeze all $5x10^4$ cell tubes at -80°C.

Note: Make sure you have made sufficient aliquots of these cell suspensions for all preliminary runs with standards and for entire beach monitoring season and store them frozen (required temperature: -80°C; please notify Shannon Briggs if -80°C freezer is not available).

APPENDIX C. NAMING CONVENTIONS

SAMPLE NAME

S_SamplingDate(YearMonthDay)_BeachLocation_BeachName.
Example: S _20170622_1_Dunes

Ideally, new beach locations would be designated such that when facing the water from the beach, sampling locations are named from left to right starting at 1. A composite sample should be named as 0. However, since most monitoring locations have been used for the past 20 years, we can continue using the current labeling for monitoring locations at a beach---as long as it is consistently used.

FILENAMES FOR QPCR MACHINE EXPERIMENTAL FILES (.EDS FILES) AND EXPORT FILES (.XLS FILES)

There are four options for the filenames:

1. If data are to be analyzed on a "per beach" basis, where there is a range of dates included, the filename should be as follows:
   BeachName_SamplingDate-SamplingDate
   Example: RossLake_20160612-20160820

2. If data are to be analyzed on a "per beach" basis, where the samples are run on the same day as collection, the filename should be as follows:
   BeachName_SamplingDate
   Example: RossLake_20170713

3. If data are to be analyzed on a "sampling date" basis, where there is a range of dates included for multiple beaches, the filename should be as follows:
   SamplingDate-SamplingDate
   Example: 20160612-20160820

4. If data are to be analyzed on a "sampling date" basis, where the samples are run on the same day as collection for multiple beaches, the filename should be as follows:
   SamplingDate
   Example: 20170713

   *If necessary, amend with the initials of the lab analyst
   Example: RossLake_20160612-20160820_gr
   Example: RossLake_20170713_gr
   Example: 20160612-20160820_gr
   Example: 20170713_gr

The default export filenames should be identical to the experimental filenames, with "_data" added to the end.

DRAFT METHOD C WORKBOOK NAME

MethodC_template(6-10-20)_2rep_protected _ XXXX
or
MethodC_template(6-10-20)_3rep_protected _ XXXX

or
MethodC_template(6-9-20)_Combined_2020_XXXX

Where XXXX represents the lab name abbreviation.

*Initials of separate lab analysts should be included as in the following example:
Method C_template(4-25-19)_3rep_XXXX_xx

Where xx represents analyst's initials. This would be consistent with previous years.

TEST SAMPLES SHEET NAMES (WITHIN THE DRAFT METHOD C WORKBOOK)

Use the same conventions for naming Test Samples sheets as indicated above for "Experimental Files".

Note: The maximum number of characters that can be in a Test Samples sheet name is 31. The maximum number of characters for the Beach name is therefore 13 when a range of dates, as shown in the example above, is used and when not including analyst's initials. Including analyst initials should not be necessary in Test Samples sheets since it is assumed that each analyst will maintain their own workbooks.

APPENDIX D. METHOD C MACRO INSTRUCTIONS

A. General information

1. The following Macro tool file will be provided to each of the labs:
    a. "MethodC_Macro_Combined_2020.xlsm"
    b. The guidance in this 2020 SOP MUST be followed for the Macro to work correctly.
2. The Macro will automatically copy and paste information from an export file into a 2020 Method C template workbook. The Macro can also save the changes made (the addition of preseason or test sample data) to the workbook.
3. The 2020 Method C template workbook will automatically calculate Method C values.
    a. MethodC_Macro_Combined_2020.xlsm workbook can be used for samples run in duplicate or triplicate.
4. When setting plates for analysis, use the plate setups recommended in Sections B and G of this SOP. If the recommended plate setups are not used, alterations may need to be made to the export file, or the data must be entered manually.
    a. Recommended plate setups for preseason data (Standard Curves, NTCs, Calibrators and Filter Blanks) are shown in Section B, Figures 1 & 2.
    b. Recommended plate setups for test sample data are shown in Section G, Figures 3A, 3B, 4A, & 4B.
5. Additionally, it is important to follow the recommended sample naming conventions indicated in Section H and Appendix C of this SOP.

C. Using the Method C Macro and the 2020 Method C template workbooks

Don't forget to Locate the 2020 Method C workbook, Instrument export file(s) and the Macro in the same folder in your computer

1. Open the Macro
    a. When opening, a security message may appear: click on "enable content".
2. Press "Ctrl" "A" to start the macro
3. Select "OK" when asked to select a MethodC template file
    a. Select the 2020 MethodC template "MethodC_template(6-9-20)_Combined_2020_XXXX"

C. Preseason Data Import Instructions

1. If you are importing preseason data, select "Yes" when asked "Importing a std curve file?"
2. Press "ok" when asked to "Select an imported file for std curve"
3. Navigate to the folder where you placed the export file containing your preseason data and the macro and the MethodC template file.
4. Select the appropriate export file
5. Press "open".

6. Enter the run number
    a. The run number can be any integer 1 to 10 if you are running one data set per plate (using the plate setup in Section B Figure 1).
    b. The run number can be 1, 3, 5, 7 or 9 if you are running two data sets per plate (using the plate setup in Section B Figure 2).
    c. Note: If a run number is used more than once, the data entered previously will be over-written
7. Press “OK”.
8. When asked to “Enter 1 or 2: the number of standard curves for this run” enter the number of data sets included on the plate.
    a. If you are running one data set per plate (using the plate setup in Section B, Figure 1) enter 1.
    b. If you are running two data sets per plate (using the plate setup in Section B, Figure 2) enter 2.
9. Press “OK”.
10. When asked “Are you done importing std curve files?” Select “No” if more preseason data sets need to be imported.
    a. If “No” is selected, the process will repeat itself, starting at Step 2 above.
11. If you are done importing preseason data sets, select “Yes” when asked “Are You done importing std curve files?”
12. If you are not importing test samples, select “No” when asked if you will be importing a test sample file.
    a. Press “OK” when asked to “Select a destination and file name to save this file”.
    b. Select a destination folder to save the updated workbook to (default will be the folder where the starting workbook is located).
    c. Edit the filename for this workbook or accept the current filename indicated.
    d. Use the file naming conventions indicated in Appendix C.
    e. Press “Save”.
    f. Note: if accepting the current file name, the previous file by this name will be over-written if the destination folder is the same as that of the starting file. Select “Yes” if you want to replace the starting file.
    g. Press “OK” when you are told the file is saved.
    h. Your preseason data will be copied to the Lab Std Curves, NTCs, Filter Blanks, and Initial Calibrators sheets. The data will be automatically analyzed by the 2020 Method C template workbook for data quality acceptability and determination of standard curve parameters.
13. If you are importing test samples, follow the instructions below.

D. Test Sample Data Import Instructions

1. If you are importing test sample data, select “No” when asked “Are You importing a std curve file?”
2. If you are importing test samples, select “Yes” when asked “importing a test sample file?”
3. When prompted to “Select an imported file for Test Samples” Press “OK”.

4. Navigate to the folder where you placed the test sample file and containing the macro and the MethodC template file.
    a. MethodC_template(6-9-20)_Combined_2020.xlsx works for both triplicate and duplicate sample entry when using the 2020 MethodC Macro.
5. Select the appropriate test sample file and press “Open”.
6. When prompted, enter the number of samples in this analysis run.
    a. note: 96 well plates run in triplicate will have a max of 12 samples, and 96 well plates run in duplicate will have a max of 18 samples.
7. Press “OK”.
8. Enter a name for the Test Samples Sheet.
    a. Note: it is highly recommended to use the sheet naming conventions indicated in Section H and Appendix C of this SOP.
9. Press “OK”.
10. Press “OK” when notified that a copy of test samples sheet is saved.
11. If you are not done importing qPCR results, select “No” when asked “Are You done importing test sample files?”
    a. If you select “No”, the process repeats itself starting at Step 3 above, so that other export files can be auto imported and analyzed.
12. If you are done analyzing qPCR results, select “Yes” when asked if you are done importing test sample files.
13. Press “OK” when asked to select a destination to save the 2020 Method C template workbook to
14. Select a destination folder to save the updated workbook to (default will be the folder where the starting workbook is located).
15. Edit the filename for this 2020 Method C template workbook or accept the current file name indicated.
    a. note: it is highly recommended to use the sheet naming conventions indicated in Appendix C of this SOP.
16. Press “Save”.
    a. Note: if accepting the current file name, the previous file by this name will be over-written if the destination folder is the same as that of the starting file. Select “Yes” if you want to replace the starting file.
17. Press “OK” when you are told the file is saved.
18. Your results can be viewed in the Test Samples Sheet that you named and saved above.

APPENDIX E. GUIDE TO PROPER PIPETTING TECHNIQUE & PIPETTE QUICK CHECK

The pipette is a reliable, precise instrument that has been used and trusted for many years. However, as with many forms of instrumentation, a pipette will perform only as well as the operator's technique allows. Differences in technique can alter delivery volume and impact data integrity.

1. *Pre-wet the pipette tip*

Aspirate and expel sample liquid at least three times before aspirating a sample for delivery.

Evaporation within the tip can cause a significant loss of sample before delivery. Pre-wetting increases the humidity within the tip, thus reducing both the amount of and variation in sample evaporation.

Using the same tip (without pre-wetting) to deliver multiple samples results in lower volume for the first few samples. The need to pre-wet increases when working with volatile samples such as organic solvents.

2. *Work at temperature equilibrium*

Allow liquids and equipment to equilibrate to ambient temperature. The volume of sample delivered by air displacement pipettes varies with air pressure, relative humidity and vapor pressure of the liquid, all of which are temperature dependent. Working at a single, constant temperature minimizes this variation.

3. *Use standard mode pipetting*

Choose standard mode pipetting rather than "reverse mode" for all but viscous samples, if accurate and precise results are desired.

In reverse mode pipetting, the plunger is depressed completely (past the first stop) to aspirate the sample and then depressed only to the first stop to deliver the sample. If reverse mode is used with normal aqueous fluids, the pipette tends to deliver more than the calibrated volume. On the other hand, using normal mode with viscous samples, especially when liquid is retained in the tip, results in under delivery.

4. *Pause consistently after aspiration*

Pause with the tip in the liquid for about one second after aspirating the sample.

It takes a moment for the liquid in the tip to finish moving after the plunger stops, so failure to pause will cause the volume to be too low. Slow and even plunger release and a consistent, brief pause after aspiration minimize this error.

5. *Pull the pipette straight out*

Pull the pipette straight out of the container after aspirating a sample. Do not touch the tip to the sides of the container.

This technique is especially important when pipetting small volumes (<50 ul). Surface tension effects cause the sample volumes to vary if the exit angles vary. Touching the tip against the container walls results in loss of sample.

6. *Minimize handling of the pipette and tip*

Set the pipette down between sample deliveries and avoid handling the tip.

Body heat transferred to equipment during handling disrupts temperature equilibrium. As explained in Tip #2, the volume of sample delivered varies with temperature.

7. *Immerse the tip to the proper depth*

During sample aspiration, immerse the tip 2-5 mm below the meniscus and well clear of the container walls and bottom.

Inserting the tip too deep into the liquid causes excess droplets to cling to the outside of the tip. Pressing or resting the tip against the container walls or bottom restricts entry of the sample.

8. *Use the correct pipette tip*

For accurate volume delivery, choose a tip that is designed for use with the type of pipette being employed and securely attach it. Mismatching a tip and pipette or using poor quality tips can result in an inadequate seal between the pipette and tip. Quality tips are flexible and have thin walls, providing airtight seals and dependable sample delivery.

9. *Use consistent plunger pressure and speed*

Depress and release the plunger smoothly and with consistent pressure and speed when dispensing each sample. Pipettes, like all precision instruments, produce more reproducible results when operated with attention to detail and proper technique.

Common Pipetting Errors

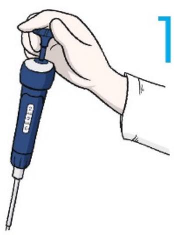
- Working too quickly.
- Removing the pipette tip before sample aspiration is complete.
- Dragging the pipette tip along the side of the container when exiting the sample.
- Releasing the plunger too rapidly.
- Not pre-wetting a new tip, particularly when working with volatile samples.

METTLER TOLEDO

Quick Check

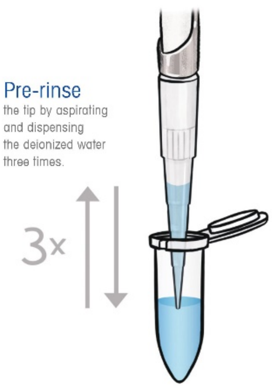
Evaluate a pipette's performance using this easy gravimetric Quick Check. You'll need a balance and vessel, some deionized water and an area to work in that's free from drafts, direct sunlight and vibration.

For 100 µL pipettes or larger, use a 5-place balance. Less than 100 µL use a 6-place balance.

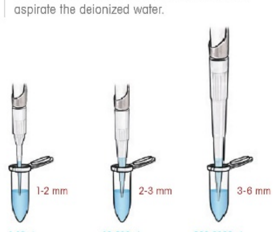


1 First, after preparing the vessel and taring the balance, adjust the pipette to 100 percent of its nominal volume and load the manufacturer's recommended tip.

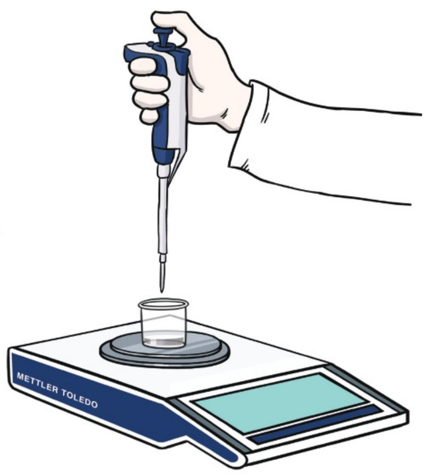
2 Pre-rinse the tip by aspirating and dispensing the deionized water three times.



3 Holding the pipette vertically immerse the tip to the appropriate depth and aspirate the deionized water.



4 Carefully move the pipette over the vessel on the balance and dispense the water. Slide the tip up the side wall to remove any excess liquid.

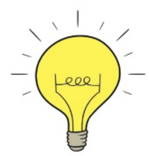


5 To avoid the effects of evaporation, as soon as the balance stabilizes, record the mass of the water in milligrams.

6 Repeat the process three more times using the same tip. Recording the mass each time.

7 Eject the tip after the final dispense.

8 Now, adjust the pipette down to 50 percent of its nominal volume, pre-rinse a new tip three times, and repeat steps 3-7.



9 With the data you have collected, you can now calculate the pipette's mean volume, accuracy (mean error) and precision (standard deviation).

10 Now, compare your results to the manufacturer's specifications.

Volume
Expressed in mL.

$$v_i = (w_i)Z$$

v_i = individual volumes
w_i = individual weighings
Z = Z factor

Mean Volume
The mean weight result with correction for Z-factor. Expressed in µL.

$$\bar{v} = \frac{\sum_{i=1}^{n} v_i}{n}$$

$\bar{v}$ = mean volume
n = number of weighings
v_i = individual volumes

Accuracy
Mean error is the difference between the mean volume of actual measurements and the true value as specified by the volume setting of the pipette. Expressed in µL.

$$E = \bar{v} - v_s$$

E = mean error
$\bar{v}$ = mean volume
v_s = volume setting

Precision
Standard deviation quantifies the magnitude of scatter due to random error.

$$s = \sqrt{\frac{\sum_{i=1}^{n} (v_i - \bar{v})^2}{n-1}}$$

s = standard deviation
n = number of weighings
v_i = individual volumes
$\bar{v}$ = mean volume

If your results don't match the pipette's specification, your pipette needs service or calibration.

For more Quick Check tools and white papers, visit mt.com/gpp-qc

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