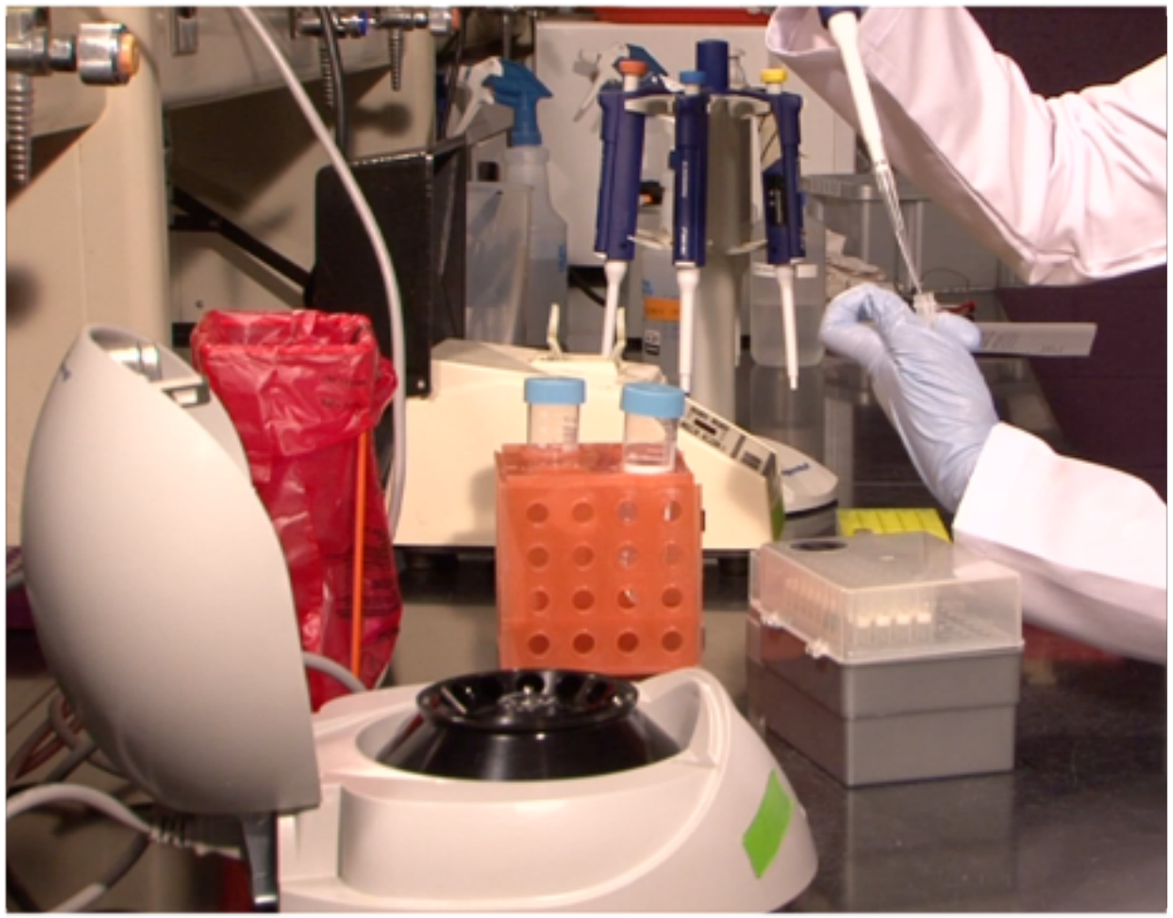
CHAPTER 6

Analytical Procedures for Method 1611



This chapter will provide detailed information on running the Enterococci 23S qPCR assay in recreational beaches routinely.

Running the Method 1611

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6.1. Membrane Filtration (Concentration of target DNA)

This step concentrates the target DNA from the sample onto a membrane filter prior extraction. Preparation of the method blank is also a part of this step. This step take place at the "Membrane Filtration Preparation Workstation".

Sterile conditions should be maintained during sample processing. Wear disposable gloves when handling the samples, equipment, and supplies.

Prior to filtration, thorough cleaning of the surfaces is required to avoid cross contamination. Wipe down bench tops before and after sample processing with 10% bleach solution, allowing the bleach to contact the work surface for a minimum of 15 minutes prior to rinsing with sterile water. After decontamination, discard gloves and replace with new clean pair.

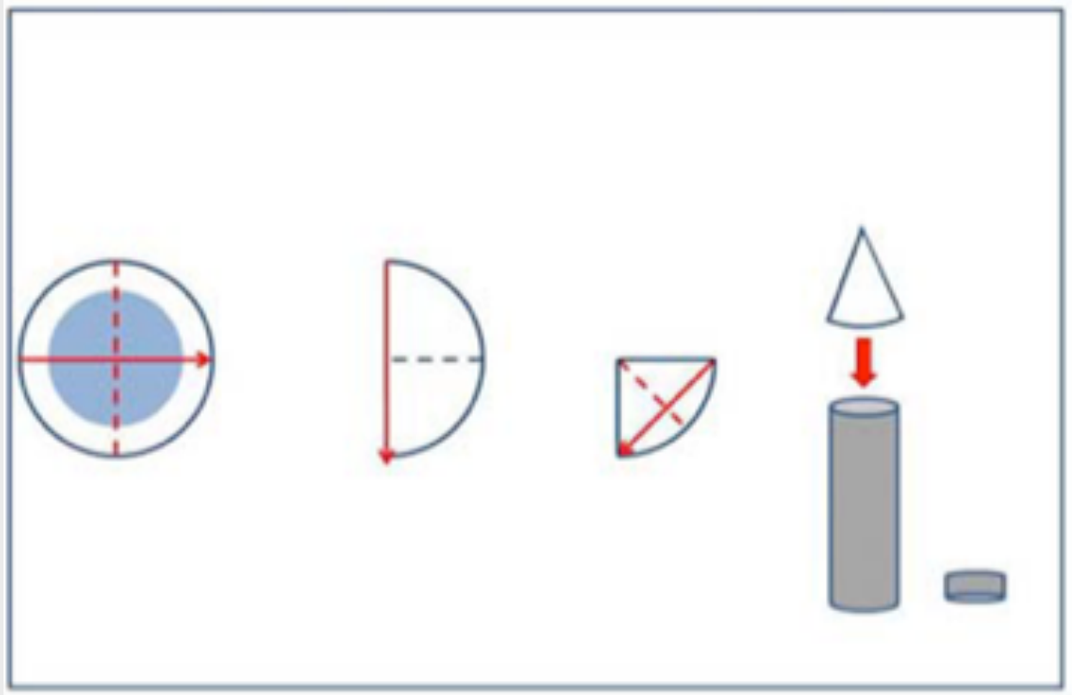
6.1.1. Method Blank Membrane Filtration Protocol

The method blank ensures there is no contamination during sample processing (e.g. membrane filtration and DNA extraction).

- Set up filtration apparatus.

- Either use an autoclaved or disposable funnel and base, or UV sterilize funnel and base for 3 minutes. If filtering multiple samples do not forget to dry the funnel and the base off with KimWipes™ prior to UV sterilization. Failure to do so will result in inadequate sterilization.
- Insert sterile funnels base and stopper into the filter manifold.
- Using flame sterilized forceps place a polycarbonate filter (47mm diameter, 0.4 µm pore size) onto a sterile funnel base.
- Assemble the funnel by attaching it to the base using the clamp, unless magnetized (i.e. PALL™).
- Add 100 ml of PCR grade molecular H_2O to the funnel.
- Turn the vacuum pump ON until the entire water sample has passed through the filter and funnel base, rinsing the sides of funnel with additional sterile PCR grade H_2O as it filters (~20 – 30 ml).
- Turn the vacuum pump OFF.
- Remove the funnel. Using flame sterilized forceps fold the filter (Figure 6.1.) and place it open side down into a labeled, sterilized DNA extraction tube, containing 0.3 g of acid–washed glass beads.

Figure 6.1. Diagram showing membrane filter folding.

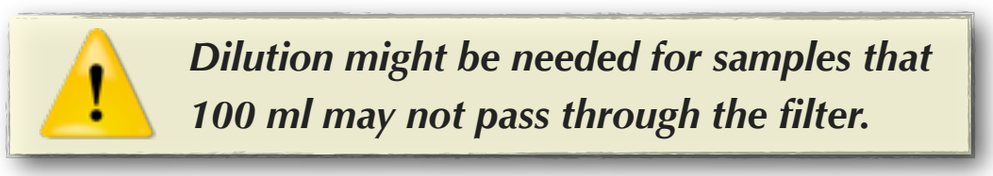


- The filter now can be extracted for DNA or stored at –80 °C.

Please see the training video at http://cws.msu.edu/videos/videos.php

6.1.2. Water Sample Membrane Filtration Protocol

- Set up filtration apparatus.
- UV sterilize funnel and the base for 3 minutes. If filtering multiple samples do not forget to dry the funnel and the base off with KimWipes™ prior to UV sterilization. Failure to do so will results in inadequate sterilization.
- Insert sterile funnels base and stopper into the filter manifold.
- Using flame sterilized forceps place a polycarbonate filter (47mm diameter, 0.4 µm pore size) onto a sterile funnel base.
- Assemble the funnel by attaching it to the base using the clamp, unless magnetized (i.e. PALL™).
- Shake the sample vigorously 25 times in a 1–foot arc within 60 seconds to evenly distribute the bacteria.
- Add the appropriate amount of the sample to the funnel (typically 100 ml).



- For a diluted sample, 25 ml of PCR grade H_2O should be added to the funnel first (enough to fill the smaller end of the funnel where it attaches to the funnel base). The desired amount of sample may then be pipetted and the total volume brought up to 100ml using PCR grade H_2O.
    - For example, for a 1:10 dilution; fill funnel near base with 25 ml PCR grade H2O, pipette in 10 ml of water sample, fill funnel to 100 ml with PCR grade H_2O.
- Turn the vacuum pump ON and apply a vacuum until the entire water sample has passed through the filter and funnel base. Rinse the sides of funnel with sterile PCR grade H_2O as it filters, passing an additional 20–30 ml through the filter.
- Turn the vacuum pump OFF.
- Remove the funnel. Using flame sterilized forceps fold the filter and place it open side down into a sterilized DNA extraction tube, containing 0.3 g of acid–washed glass beads.
- Important note: When folding the filter try to only touch the edges of the filter and fold sample side in.
- The filters now can be extracted for DNA or stored at –80 °C.

6.2. Nucleic Acid Extraction

This section takes place at the dedicated workstation or at the membrane filtration workstation. To minimize environmental DNA contamination, routinely treat the work surfaces with a 10% bleach solution, allowing the bleach to contact the work

surface for a minimum of 15 minutes prior to rinsing with sterile water. After decontamination, discard gloves and replace with new clean pair.

This section will primarily focus on Crude DNA Extraction as described in Method 1611.

6.2.1. Preparation of Enterococcus Calibrator Sample Extract

1. Remove a tube containing a 10 µl aliquot of frozen Enterococcus stock cell suspension (as prepared in Section 4.3.3) and thaw (preferably on ice, or placed at 4 °C until thawed).

2. 2. While cell stock is thawing, using sterile (or flame-sterilized) forceps, place one sterile polycarbonate filter in each of three extraction tubes with glass beads.

    2.1. Alternatively, BioBalls can be purchased and if using BioBalls for calibrators add a single BioBall® to each tube.

3. Dispense 590 µL of Salmon DNA/extraction buffer into each of the three extraction tubes with glass beads and filters. Label tubes as Replicate 1, Replicate 2 and Replicate 3.

    3.1. Calibrators contain the same amount of extraction buffer and starting amount of Salmon DNA as the samples.

4. When *E. faecalis* stock cell suspension has thawed (Step 1), transfer 990 µL AE buffer to the 10 µL E. *faecalis* stock cell tube and mix thoroughly by vortexing. Pulse microcentrifuge tube briefly (1 – 2 sec.) to coalesce droplets in tube.

5. Immediately after vortexing the E. *faecalis* stock cell suspension, spot 10 µL onto the polycarbonate filter in the extraction tube (Step 2).

6. Tightly close the tube, making sure that the O-ring is seated properly and label tubes appropriately.

7. Repeat this procedure for the other two replicate filters to prepare a total of three *E. faecalis* calibrator samples.

8. Place the tubes in the mini bead beater and shake for 60 seconds at 5000 rpm or the maximum rate.

9. Remove the tubes from the mini bead beater and centrifuge at 12,000 × g for one minute to pellet the glass beads and debris.

10. Using a 200 µL micropipettor, transfer the crude supernatant to the corresponding labeled sterile 1.7 mL microcentrifuge tube. Transfer 400 µL of supernatant without disrupting the debris pellet or glass beads at the tube bottom.

    10.1. Generally, 400 µL of supernatant can be easily collected. Collect an absolute minimum of 100 µL of supernatant. Record volume of supernatant collected.

11. Centrifuge at 12,000 × g for 5 minutes and transfer clarified supernatant to a clean, labeled 1.7 mL tube, taking care not to disturb the pellet.

12. Label the tubes as undiluted or 1x *E. faecalis* calibrator extracts.

13. Label two additional sets of three 1.7- mL tubes for 5 and 25 fold dilutions. Label them as 5X, 25 X, 125X (Figure 4).

14. Using a micropipettor, add a 50 µL aliquot of each 1x *E. faecalis* calibrator extract to each of the three 5 X-labelled tube s and dilute each with 200 µL AE buffer to make 5 fold dilutions. Vortex the tubes (Figure 4.1).

15. Using a micropipettor, remove a 50 µL aliquot out of each 5-X labelled tube and dispense into each of the three 25X labelled tubes. Add 200 µL AE buffer in each of the three tubes to make 25 fold dilutions. Vortex the tubes (Figure 4).

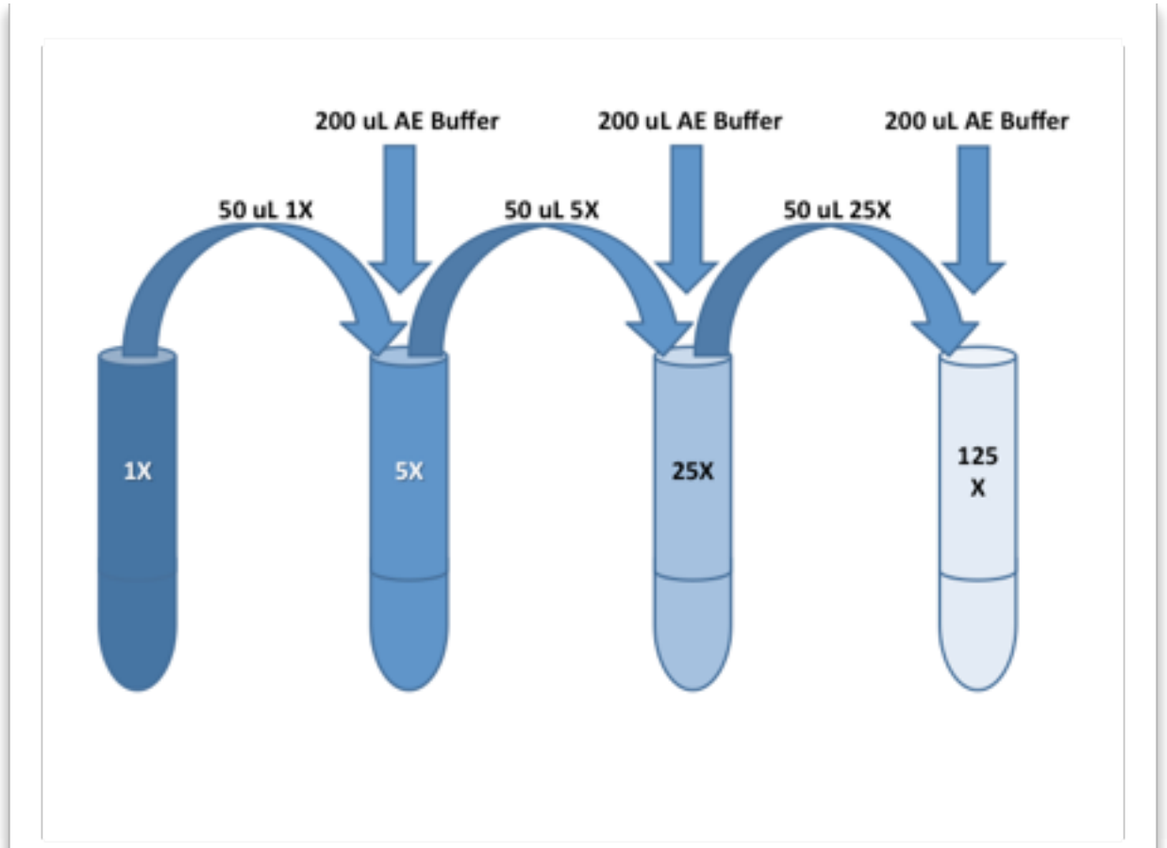
16. Using a micropipettor, remove a 50 µL aliquot out of each 25-X labelled tube and dispense into each of the three 125X labelled tubes. Add 200 µL AE buffer in each of the three tubes to make 125 fold dilutions. Vortex the tubes (Figure 4).

    16.1. For example, if the initial concentration is 100000 CFU/100 ml, than the 5X would have 20000 CFU/100 ml, 25X would have 4000 CFU/100 ml and 125X would have 800 CFU/100 ml.

17. Divide the undiluted cell suspensions into (10 µL) and freeze at -20°C.

18. Store all diluted and undiluted extracts in refrigerator.

19. If the extracts are not analyzed immediately, refrigerate. For long term storage, freeze at -20°C or -80°C.



6.2.2. Preparation of the Samples and Method Blank Extracts

- Using a 1000 µL micropipettor, dispense 590 µL of the 0.2 µg/ml Salmon DNA extraction buffer into each labeled extraction tube with glass beads containing the water sample or method blank. Extract the method blank last.
- Tightly close the tubes, making sure that the O-ring is seated properly.
- Place the tubes in the mini bead beater and shake for 60 seconds at 5000 rpm or the maximum rate.
- Remove the tubes from the mini bead beater and centrifuge at 12,000 × g for 1 minute to pellet the glass beads and debris.
- Using a new pair of gloves and the 200 µL micropipettor, transfer 400 µL of the supernatant to a corresponding labeled sterile 1.7-mL microcentrifuge tube, taking care not to pick up glass beads or sample debris (pellet). Recover the method blank supernatant last.
    - The filter will normally remain intact during the bead beating and centrifugation process. Generally, 400 µL of supernatant can easily be collected. Collect an absolute minimum of 100 µL of supernatant.
- Centrifuge crude supernatant for 5 minutes at 12,000 × g. Transfer 350 µL of the clarified supernatant to another 1.7-mL tube, taking care not to disturb pellet. Note that pellet may not be visible in water samples. Recover the method blank supernatant last.
- Label the tubes as undiluted (or 1x) water sample extracts and with the sample ID. Label additional tubes for method blanks and for 5 and 25 fold dilutions of sample extracts.
- Make the 5X and 25X dilutions as follows:
    - In 5X labelled tubes, using a micropipettor, add a 50 µL aliquot of each 1x water sample extract and dilute each with 200 µL AE buffer to make 5 fold dilutions.
    - In 25X labeled tubes, using a micropipettor, add a 50 µL aliquot of each 5 fold dilution and dilute each with 200 µL of AE buffer to make 25 fold dilutions.
- Dilute the method blank supernatant last.
- Store all diluted and undiluted extracts in refrigerator.
- Use of 5 fold diluted samples for analysis is currently recommended if only one dilution can be analyzed. Analyses of undiluted water sample extracts have been observed to cause a significantly higher incidence of PCR inhibition while analyses of 25 fold dilutions may unnecessarily sacrifice sensitivity.
- If the extracts are not analyzed immediately, refrigerate. For long term storage, freeze at -20°C or -80°C.

6.3. Mastermix preparation

This section takes place at the dedicated workstation. To minimize environmental DNA contamination, routinely treat all work surfaces with a 10% bleach solution, allowing the bleach to contact the work surface for a minimum of 15 minutes prior to rinsing with sterile water. If available, turn on UV light for 15 minutes. After decontamination, discard gloves and replace with new clean pair.

Always briefly mix, and then centrifuge all qPCR reaction components just before combining the reagents. Gently swirl enzyme-containing master mixes and briefly (1–2 seconds) vortex primers and probes. Because qPCR master mixes are typically denser than the other qPCR reaction components, it is important to adequately blend reaction mixtures, otherwise precision could be compromised. However, avoid over-vortexing because it can cause bubbles which could interfere with fluorescence detection, and can reduce enzyme activity which could reduce amplification efficiency. Prior to pipetting, always centrifuge briefly to collect the contents at the bottom of the container and eliminate any air bubbles from the solutions.

- Remove primers and probe stock solutions from the freezer.
- Prepare working stocks of Enterococcus, and Salmon DNA primer/probe mixes by adding 10 µL of each Enterococcus or Salmon DNA primer stock and 4 µL of respective probe stock to 676 µL of PCR grade water, and vortex. Pulse centrifuge for a few seconds to create a pellet. Use a micropipettor with aerosol barrier tips for all liquid transfers. Transfer aliquots of working stocks for single day use to separate tubes and store at 4°C.
- Using a micropipettor, prepare assay mix of the Enterococcus, and Salmon DNA reactions in separate, sterile, labeled microcentrifuge tubes. Add sterile PCR grade H_2O 1.5 µL, BSA 2.5 µL, TaqMan® master mix 12.5 µL and Primer/probe working stock solution 3.5 µL. Prepare sufficient quantity of assay mix for the number of samples to be analyzed per day including calibrators and negative controls plus at least two extra samples. Prepare assay mixes each day before handling DNA samples. To calculate the number of reactions for each run, use Table 6.1.

Table 6.1. Calculation of the number of reactions for each run.

	Number of Samples/Controls	Number of Replicates	Number of Reactions
Samples	A	2	2*A
Calibrator	3	2	6
No template control (NTC)	1	2	2
Method Blank (MB)	1	1	1
		Total Number of Reactions:	9 + 2*A

- Vortex the assay mix working stocks; then pulse microcentrifuge to coalesce droplets. Return the primer/probe working stocks and other reagents to the refrigerator.
- Transfer 20 µL of mastermix to tubes or the individual wells of aPCR reaction tray equal to the number of samples to be analyzed including calibrator and negative control samples.
    - The same tip can be used for pipetting multiple aliquots of the same assay mix as long as it doesn't make contact with anything else).
- When the samples are loaded in the wells cover tray with adhesive sealers, or close tubes, and transfer to refrigerator at 4°C (if being stored for later analysis) or transfer directly to the reaction preparation workstation.

6.4. Template, Positive and Negative Control Preparation

This part of the analysis takes place at the reaction preparation workstation. To minimize environmental DNA contamination, routinely treat all work surfaces with a 10% bleach solution, allowing the bleach to contact the work surface for a minimum of 15 minutes prior to rinsing with sterile water. If available, turn on UV light for 15 minutes. After decontamination, discard gloves and replace with new clean pair.

Recording the positions of each sample, transfer 5 µL each of the DNA extracts of method blanks, samples and corresponding dilutions of calibrators to separate tubes or wells of the PCR reaction tray or tubes containing the Enterococcus mix.

Recording the positions of each sample, transfer 5 µL each of the DNA extracts of method blanks, samples and corresponding dilutions of calibrators to separate tubes or wells of the PCR reaction tray or tubes containing the Salmon DNA mix.

Transfer 5 µL aliquots of AE buffer to tubes or wells of PCR reaction tray containing Enterococcus mix. (Repeat these for separate tubes or wells of the PCR reaction tray or tubes containing the Salmon DNA mix too). These will be the no-template controls. Record positions of these samples.

Cap tubes or seal wells of PCR reaction tray containing samples tightly or cover tray and seal tightly with optical adhesive.

Centrifuge your PCR reaction tray before running reactions in a qPCR machine.

6.5. Quantification

Proceed to the amplification and product detection workstation. Set up the instrument and follow the instructions of the manufacturer for analysis.

- Run reactions in the thermocycler following instrument specific manufacturer's instructions.
    - See Method 1611, Appendix B for ABI 7500 and 7900 or Cepheid Smart Cycler.
    - Follow manufacturer's instructions when using other brands of instruments.