Standard Operating Procedure for:
Single Step Membrane Filtration Procedure for the Detection and Enumeration of Enterococci using mEI agar

DEPARTMENT OF ENVIRONMENTAL PROTECTION
BUREAU OF LABORATORIES
BIOLOGY SECTION
TALLAHASSEE FLORIDA

The OFFICIAL COPY is the on-line version. All other copies are considered unofficial and uncontrolled.

TABLE OF CONTENTS

1. SCOPE AND APPLICATION
2. SUMMARY OF METHOD
    1.1. Interferences
    1.2. Definitions
3. EQUIPMENT AND SUPPLIES
4. REAGENTS AND STANDARDS
5. CALIBRATION AND STANDARDIZATION
6. SAMPLE COLLECTION, PRESERVATION, AND HANDLING
7. SAMPLE PREPARATION
8. SAMPLE ANALYSIS
9. VERIFICATION
10. DATA ARCHIVAL
11. QUALITY CONTROL
12. SAFETY/HAZARDOUS WASTE MANAGEMENT
13. REFERENCES
14. TABLES, DIAGRAMS
    Figure 14.1 Setup for Vacuum Filtration System
    Flowchart 14.2 Procedure for Verification of Enterococci

The OFFICIAL COPY is the on-line version. All other copies are considered unofficial and uncontrolled.

1. SCOPE AND APPLICATION

1.1. Based on EPA Method 1600, this method is used to detect and count enterococci bacteria in non-potable water. Since enterococci are commonly found in the feces of humans and other warm-blooded animals, they can be an indicator of fecal pollution and possible presence of enteric pathogens, but not all strains are related to fecal pollution.

1.2. This procedure is used for samples with LIMS test ID of ENT-24-MF and EN24-MF-CL.

2. SUMMARY OF METHOD

2.1. A water sample is filtered through a membrane that has a 0.45 µm pore size to capture bacteria. The membrane filter is placed on an mEI agar dish, which is a selective medium for enterococci. The dish is then incubated for 24 hours at 41°C. All positive colonies, ≥ 0.5 mm diameter with a blue halo, are counted and recorded.

2.2. Interferences

2.2.1. Suspended particulate materials in sample water can clog the membrane filter and cause spreading of bacterial colonies. This can make accurate identification and enumeration of enterococci difficult.

2.3. Definitions

2.3.1. ***Enterococci*** - Bacteria that produce ≥ 0.5 mm diameter colonies with a blue halo after 24 hour incubation at 41°C on mEI agar. This includes *Enterococcus faecalis, E. faecium, E. avium, E. gallinarium*, and others from the genus *Enterococcus*, formerly known as Group D fecal streptococci.

2.3.2. **Target Organism** – The target organism is the specific bacteria that the method is testing for. In this method the target organism is *Enterococci*.

2.3.3. **Positive Growth** – Bacterial growth that resembles a typical positive colony which is ≥ 0.5 mm diameter with a blue halo.

2.3.4. **Negative Growth** – Bacterial growth that does not have the characteristics of a typical positive colony.

3. EQUIPMENT AND SUPPLIES

3.1. Sterile gloves, latex or nitriles

3.2. Flask, Erlenmeyer, 2 L

3.3. Weighing Dish, Pour Boat Style, medium size and Weighing paper, medium size

3.4. Graduated cylinder, 1L

3.5. Magnetic stir bar, medium size

3.6. Balance, Mettler Toledo MS303S (S/N B247544008) - Calibrate per SOP PCR-2.1

The OFFICIAL COPY is the on-line version. All other copies are considered unofficial and uncontrolled.

3.7. Hot plate with stirrer

3.8. Autoclave, Market Forge Sterilmatic Model STM-E (S/N 226393)

3.9. pH meter, Accumet Excel XL 25 (S/N XL94005930) - Calibrate per SOP MB-03.4.

3.10. Petri dishes, 47 mm, sterilized, with tight fitting lids, 20 per 100 mL of mEI agar prepared

3.11. Repeater pipette with 50 mL tips

3.12. Low Temperature Incubator Model 815 (S/N WB64710726), maintained at 1-4 °C

3.13. White trays, for carrying samples

3.14. Chlorine test strips

3.15. Vacuum Filtration system (See Figure 14.1)

3.16. Membrane filtration unit, sterile wrapped in foil

3.17. UV Sterilizer unit

3.18. Forceps, sterile, straight or curved with smooth tips

3.19. Beaker, 30 mL

3.20. Glass serological disposable pipettes (1, 2, 5, 10 mL as needed), sterile and individually wrapped, with pipette pump

3.21. Membrane filters, sterile, white gridded, 47 mm diameter, 0.45±0.02 µm pore size

3.22. Inoculating loops, sterile

3.23. Test tubes, 16 X 125 mm, with screw caps

3.24. Stereo microscope, Fisher Scientific

3.25. Compound microscope, Leica

3.26. Thelco Precision Cat No 51221093 Incubator (S/N 699121205), maintained at 41.0 ± 0.5 °C

3.27. Thelco Model #4 Incubator (S/N 22-AH-9), maintained at 35.0 ± 0.5 °C

3.28. Thelco Model #625D Incubator (S/N 309N0143), maintained at 45.0 ± 0.5 °C

4. REAGENTS AND STANDARDS

4.1. Deionized Water

4.2. Ethanol - Prepare per SOP IZ-09.

4.3. Buffered Dilution Water - Prepare per SOP MB-02.24.

4.4. membrane-Enterococcus Indoxyl-β-D-Glucoside Agar (mEI Agar) – Prepare per SOP MB-02.33

4.5. Brain Heart Infusion Broth (BHI) - Prepare per SOP MB-02.33.

The OFFICIAL COPY is the on-line version. All other copies are considered unofficial and uncontrolled.

4.6. Brain Heart Infusion Broth with 6.5% NaCl (BHI 6.5%) - Prepare per SOP MB-02.33.

4.7. Brain Heart Infusion Agar (BHI Agar) - Prepare per SOP MB-02.33.

4.8. Bile Esculin agar (BEA) - Prepare per SOP MB-02.33.

4.9. Control cultures (See SOP MB-03.1.)

4.9.1. *Enterococcus faecalis (E. faecalis)* ATCC #19433

4.9.2. *Pseudomonas aeruginosa (P. aeruginosa)* ATCC #10145

5. CALIBRATION AND STANDARDIZATION

5.1. See FDEP Bureau of Labs Quality Manual, Section 5.5.

6. SAMPLE COLLECTION, PRESERVATION, AND HANDLING

6.1. See FDEP Field SOP FS 1000 for sample collection and preservation.

7. SAMPLE PREPARATION

7.1. See SOP BB-030 for sample custody and labels.

7.2. Turn on UV sterilizers and let warm up for at least 10 minutes.

7.3. Unwrap 6 funnels (3 with bases) and place 3 funnels in each UV sterilizer. Place bases in vacuum manifold. Use a new set of funnels daily.

7.4. Place forceps in a small beaker of 80% ethanol. Fill a new, clean, dry rinse bottle with buffered dilution water.

7.5. Place sample containers in a tray.

7.6. Select sample volumes that will produce 20-60 colonies, based on expected pollution level. A minimum of three dilutions should be analyzed for each sample, with the exception of field or equipment blanks, to ensure that a countable dish is obtained. For field or equipment blanks, one 100 mL aliquot should be filtered. If sample is turbid, filter 0.1, 1, and 10 mL. For most samples, 1, 10, and 50 mL dilutions should be sufficient. 50 mL of buffered dilution water should be filtered for method blanks. Also, consider the sample's previous results when choosing volumes.

7.7. Place sample label on the middle section of each mEI agar dish lid. Write the volume to be filtered on the lower section of the lid with black marker.

8. SAMPLE ANALYSIS

8.1. Use sterile forceps to place a sterile membrane filter, grid-side up, on the filter base and attach the funnel to the base. If analyzing sample volume <20 mL, add 20-30 mL of buffered dilution water to the funnel prior to adding sample.

The OFFICIAL COPY is the on-line version. All other copies are considered unofficial and uncontrolled.

Note: Keep forceps in a small beaker of 80% ethanol during filtration process. Dip in the ethanol and shake off excess before grasping each filter. If forceps are accidentally dropped or placed on counter, replace with a new pair of sterile forceps from the UV sterilizer.

8.2. Shake the sample container vigorously, approximately 25 times to uniformly distribute bacteria. Carefully open container. Avoid touching the top rim of the container to prevent contamination of the sample.

8.2.1. If the test ID includes –CL, check the sample for chlorine with a chlorine test strip. Pour a small volume of sample into a small container (such as a Durham tube), just enough to submerge the pads at one end of the strip. Swirl the test strip in the sample for 30 seconds. If the pads remain colorless, then the sample has already been dechlorinated and should be analyzed. If the pads turn purple, chlorine is present. Do not run the sample. Report as "No Result" with a "Y" qualifier for improper preservation. Record the lot number and result of the chlorine test strip in the **Chlorine Residual** column in Prep Worksheet Manager. See SOP BB-30 for instructions on using Prep Worksheet Manager.

8.3. Measure appropriate volume with a sterile pipette into the funnel and filter sample. If the funnel has been previously calibrated to the 50 and 100 mL marks, 50 or 100 mL volumes can be poured directly into funnel. Rinse the walls of the funnel with 20-30 mL of buffered dilution water (rinse all the way around 3 times).

8.3.1. To check the calibration of funnels prior to first use, place a sterile membrane filter on the filter base and attach the funnel to the base. Measure 50 mL of DI water with a Class A volumetric flask or cylinder and pour it into the funnel. If the meniscus lines up with the 50 mL mark, the funnel passes the 50 mL volume check. If it is above or below the 50 mL mark, use an engraver to make a mark in the proper location. Repeat this process with a 100 mL volume and record the results in LIMS **Laboratory/Technical/Laboratory Logbook** *Pipet-Fun_Cal_Log.*

8.4. Turn vacuum off and aseptically remove filter with sterile forceps.

8.5. Place filter, grid side up, on the petri dish by rolling it onto the mEI agar. Avoid creating bubbles between the agar and membrane. If bubbles do occur, lift and reset the membrane. Also, run forceps along outside edge of filter to help it bind to agar surface. Close dish.

8.6. Place the three used funnels into the UV sterilizer and place the other three that have been sterilized onto the funnel bases.

8.7. Repeat Steps 8.1.-8.6. until all dishes for all samples are filtered. Make sure funnels have been UV sterilized for at least 2 minutes before starting each new sample.

8.8. Place inverted dishes in a 41 ± 0.5 °C incubator for 24 ± 2 hours. Dishes should be incubated no later than 30 minutes after they are filtered. Do not wait for all of the samples to be filtered before incubation.

The OFFICIAL COPY is the on-line version. All other copies are considered unofficial and uncontrolled.

8.9. After incubation, count all colonies that are ≥ 0.5 mm in diameter with a blue halo as enterococci and record on dish above the sample label. Use a fluorescent lamp and dissecting microscope for better visibility of colonies.

9. VERIFICATION (SEE FLOWCHART 14.2)

9.1. Use a sterile inoculating loop to transfer a loopful of growth from a well-isolated colony to a BHI broth tube and onto a BHI agar slant. Incubate broth for 24 ± 2 hours and the slant for 48 ± 2 hours at 35 ± 0.5 °C. Observe for growth (turbidity).

9.2. After 24 hour incubation transfer a loopful of growth from the BHI broth tube to each of the following tubes and incubate as shown

9.2.1. Incubate BEA and BHI w/6.5% broth tubes for 48 ± 3 hours at 35 ± 0.5 °C.

9.2.2. Incubate BHI broth tube for 48 ± 3 hours at 45 ± 0.5 °C.

9.2.3. Observe all tubes for growth (turbidity) and production of black/brown precipitate on BEA slants.

9.3. After 48 hour incubation, Gram stain growth from BHI agar slant per SOP MB-01.10.

9.4. Colonies that are gram-positive cocci, hydrolyze esculin on the BEA slant, grow in BHI 6.5%, and grow in BHI broth at 45 °C, are verified as enterococci.

9.5. Record results in *Monthly Verification Tests Logbook*

10. DATA ARCHIVAL

10.1. Record filter, incubation, read dates/times, and media used for each sample in Prep Worksheet Manager per SOP BB-30.

10.2. When dishes are read, open the worksheet and record the date, time and analyst. Click the **Complete** button and close the worksheet.

10.3. Enter counts into the MICRO_DATA_LOG in the LIMS Laboratory Logbook. Upload results to the QC Manager per SOP BB-031.

10.4. After results are uploaded to QCM, refer to SOP BB-032 for LIMS upload and data authorization.

10.5. Document all verification of colonies in the *Monthly Verification Tests Logbook*.

10.6. Calculations:

10.6.1. If only one dilution is within the ideal colony count range of 20-60 colonies, calculate the final result as in the equation below using only the count and volume of that one dilution.

10.6.2. If more than one dilution is within the ideal colony count range of 20-60 colonies, use only the dilutions that fall within this range to calculate individual results in the equation below and then average the results to obtain the final reporting result. Do not report the result with an “A” qualifier.

The OFFICIAL COPY is the on-line version. All other copies are considered unofficial and uncontrolled.

10.6.3. If dilutions have counts that do not fall within the ideal colony count range of 20-60 colonies:

10.6.3.1. If all dilutions have counts below the ideal colony count range (< 20 colonies), use the dilution with the count that is closest to the ideal colony count range (closest to 20 colonies) in the equation below to calculate the final result and report the result with a "B" qualifier. If the dilution is 100 mL, do not report with a "B" qualifier.

10.6.3.2. If dilutions have counts above and below the ideal colony count range of 20-60 colonies, use the dilution with the count that is closest to the ideal colony count range in the equation below to calculate the final result and report the result with a "B" qualifier. If the dilution is 100 mL, do not report with a "B" qualifier.

10.6.3.3. If all dilutions have counts above the ideal colony count range (> 60 colonies), use the smallest filtration volume in the equation below to calculate an estimated final result and report the result with a "B" qualifier.

10.6.4. If all dilutions have a colony count of <1, use 1 as the total number of colonies counted and use the largest filtration volume to calculate the final result in the equation below and add a "U" qualifier.

10.6.5. If there are >200 target colonies in all dilutions, use the upper limit of the ideal range (60 colonies) and the smallest filtration volume in mL to calculate an estimated final result. Report with a "Z" qualifier.

10.6.6. If there are >200 non-target colonies, or if the colonies are not distinct enough to count (confluent growth) in all dilutions, report as "No Result" with a "Z" qualifier. A comment can be added, such as "Smear" or "Confluent", to provide a reason for the "No Result".

$$CFU\ /100mL = \frac{C \times 100}{V}$$

Where,
CFU/100 mL = Enterococci colony forming units per 100 mL of sample.
C = Total number of positive colonies counted on all acceptable dilutions.
V = Total volume (sum of all acceptable dilutions)

11. QUALITY CONTROL

11.1. Sample Replicates

11.1.1. Analyze a laboratory replicate for 10% of samples with at least one replicate per filtration batch. The acceptance limit is <25% RPD.

11.1.2. Maintain a control chart of duplicate results in \\fldep1\DEAR\Labs\Lab5\Biology\Bench Common\Micro\Control Charts All Tests 032514.Convert results to logarithms and calculate the range

The OFFICIAL COPY is the on-line version. All other copies are considered unofficial and uncontrolled.

(difference of logarithms of a replicate set). The range should be < 3.27 times the mean range of the last 15 replicate sets.

11.1.3. Corrective Actions: Report laboratory replicate results that do not meet the acceptance criteria with a "J" qualifier.

11.2. Method Blanks

11.2.1. Filter a 50 mL volume of buffered dilution water for each funnel used at the beginning and end of a sample filtration batch. Place the filter on an mEI agar dish and label the lid with "CB" (Control Before) or "CA" (Control After), funnel number, and date/time. If more than 30 minutes elapses between filtration batches, run a new set of controls. Incubate blanks with samples at 41.0 ± 0.5 °C for 24 ± 2 hours.

11.2.2. Absence of growth in method blanks demonstrates that media, filtration equipment, filters, and dilution water are free of contamination of the target organism from improper handling, inadequate sterilization or environmental exposure.

11.2.3. Corrective Actions: If method blanks have growth, determine the cause of contamination. The rinse water batch, media and equipment can be checked for sterility. If the cause of contamination has also affected samples, report all samples in the filtration batch with a "V" qualifier.

11.3. Negative Controls

11.3.1. For each batch of prepared media, transfer a small amount of *P. aeruginosa* to a 99 mL phosphate buffered water bottle. Shake vigorously and filter a 1 mL volume of this suspension and place filter on an mEI agar dish. Place a media label on the dish and mark it with a "-" sign for negative control. Incubate at 41.0 ± 0.5 °C for 24 ± 2 hours.

11.3.2. To check viability of negative control, filter a 1 mL volume of the suspension, place filter on a media that targets *P aeruginosa*, such as TSA, and incubate at appropriate temperature and duration.

11.3.3. Absence of growth in negative control indicates that the medium does not support growth of non-target organisms. Any negative growth indicates that non-target organisms will not resemble typical positive colonies.

11.3.4. Corrective Actions: If there is positive growth on a negative control, determine cause of contamination. If the cause of contamination has affected the media, discard media batch and prepare a new batch.

11.4. Positive Controls

11.4.1. For each batch of prepared media, transfer a small amount of *E. faecalis* to a 99 mL phosphate buffered water bottle. Shake vigorously and filter a 1 mL volume of this suspension and place filter on an mEI agar dish. Place a media label on the dish and mark it with a "+" sign for positive control. Incubate at 41.0 ± 0.5 °C for 24 ± 2 hours.

The OFFICIAL COPY is the on-line version. All other copies are considered unofficial and uncontrolled.

11.4.2. Positive growth indicates that the medium does support growth of target organisms and produces the expected reaction (growth resembles typical positive colonies).

11.4.3. Corrective Actions: If no growth occurs, re-run the positive control with a new culture of *E. faecalis*. Also, run a pour plate with HPC (See SOP MB-01.5.) on the new culture to check for viability. If there is growth on the HPC medium but no growth on the mEI medium, then the media batch does not support growth of target organisms. Discard the batch and prepare a new media batch.

11.5. Sterility Checks

11.5.1. Check sterility on each batch of media by incubating an mEI dish along with positive and negative control dishes. Place a media label on the dish and mark it with and "S" for sterility control. If growth occurs, discard media batch and prepare a new batch.

11.5.2. Check sterility on each lot of membrane filters, each lot of sample containers, and each sterilized batch of glassware with TSB 2X (see SOP MB-02.33). If there is growth, recheck lot or batch. If recheck also fails (shows growth), discard lot or rewash batch of glassware. Sterility of sample containers should be checked before they are packed into kits and shipped to customers.

11.6. Colony Verification

11.6.1. Verify at least 10 typical colonies per month. Verify atypical colonies to check for false negatives. See Section 9. for verification procedure.

11.6.2. Adjust final colony counts for verified samples.

11.7. Colony Counting Variability

11.7.1. Compare colony counts between two analysts from one positive sample monthly. Counts should be within 10% of each other.

11.8. Ongoing Precision and Recovery (OPR)

11.8.1. Precision and recovery is performed to demonstrate acceptable method performance.

11.8.2. One spiked reference sample should be run for every 20 samples with at least one per week. See SOP MB-01.13 for spiking procedure. The acceptance criterion for recovery is between 27% - 131%.

11.8.3. Maintain a control chart of OPR samples and calculate average percent recovery (R) and standard deviation (S). Method accuracy is calculated as a recovery interval of $R \pm 2S$.

11.9. Matrix Spikes (MS)

11.9.1. Matrix spikes are performed to test the effects of a specific matrix on enterococci recoveries.

The OFFICIAL COPY is the on-line version. All other copies are considered unofficial and uncontrolled.

11.9.2. Run a MS once every 20 samples for each specific wastewater source along with the unspiked sample. See SOP MB-01.13 for spiking procedure. The acceptance criterion for recovery is between 29% - 122%.

12. SAFETY/HAZARDOUS WASTE MANAGEMENT

12.1. All media/bacteria must be autoclaved for 45 minutes before disposal.

12.2. Refer to the Health and Safety Plan. Read MSDS information for reagent handling and disposal.

13. REFERENCES

13.1. EPA Method 1600 (December 2009)

13.2. Standard Methods Online Edition (1997), Sections 9020, 9222.

13.3. NELAC Quality Systems Manual

13.4. Difco/BBL Manual

13.5. FDEP Bureau of Labs Quality Manual
http://www.dep.state.fl.us/labs/library/progplan.htm

13.6. Health and Safety Plan http://depnet/burlabs/safety.htm

13.7. DEP SOP IZ-09, *Preparation of 80% Ethanol*

13.8. DEP SOP PCR-2.1, *Calibration, Use and Maintenance of Mettler Toledo MS303S Balance*

13.9. DEP SOP MB-02.24, *Preparation of Buffered Dilution Water and Stock Components*

13.10. DEP SOP MB-02.33, *Preparation of Dehydrated Culture Media*

13.11. DEP SOP MB-03.1, *Maintenance of Bacterial Cultures*

13.12. DEP SOP FS 1000, *General Sampling Procedures*

13.13. DEP SOP MB-03.4, *Accumet Excel XL 25 pH Meter Calibration and Use*

13.14. DEP SOP BB-030, *Sample Custody, Preparation Labels and Worksheet Instructions for Bench Biology Samples*

13.15. DEP SOP MB-01.10, *Gram Staining of Bacterial Cells*

13.16. DEP SOP BB-031, *LIMS QC Manager Data Uploading Instructions for Bench Biology and Microbiology Samples*

13.17. DEP SOP BB-032, *Test Level Authorization of Uploaded Results for Bench Biology Samples*

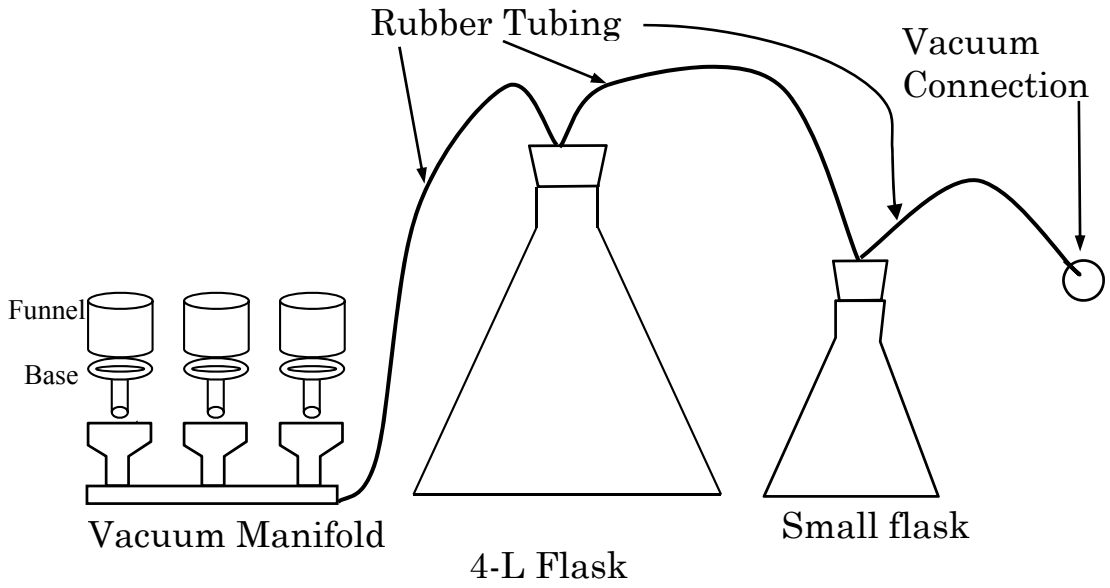
13.18. DEP SOP MB-01.5, *Pour Plate Method for Heterotrophic Plate Count*

13.19. DEP SOP MB-01.13, *Procedure for the Preparation and Analysis of Spiked Reference and Matrix Samples*

The OFFICIAL COPY is the on-line version. All other copies are considered unofficial and uncontrolled.

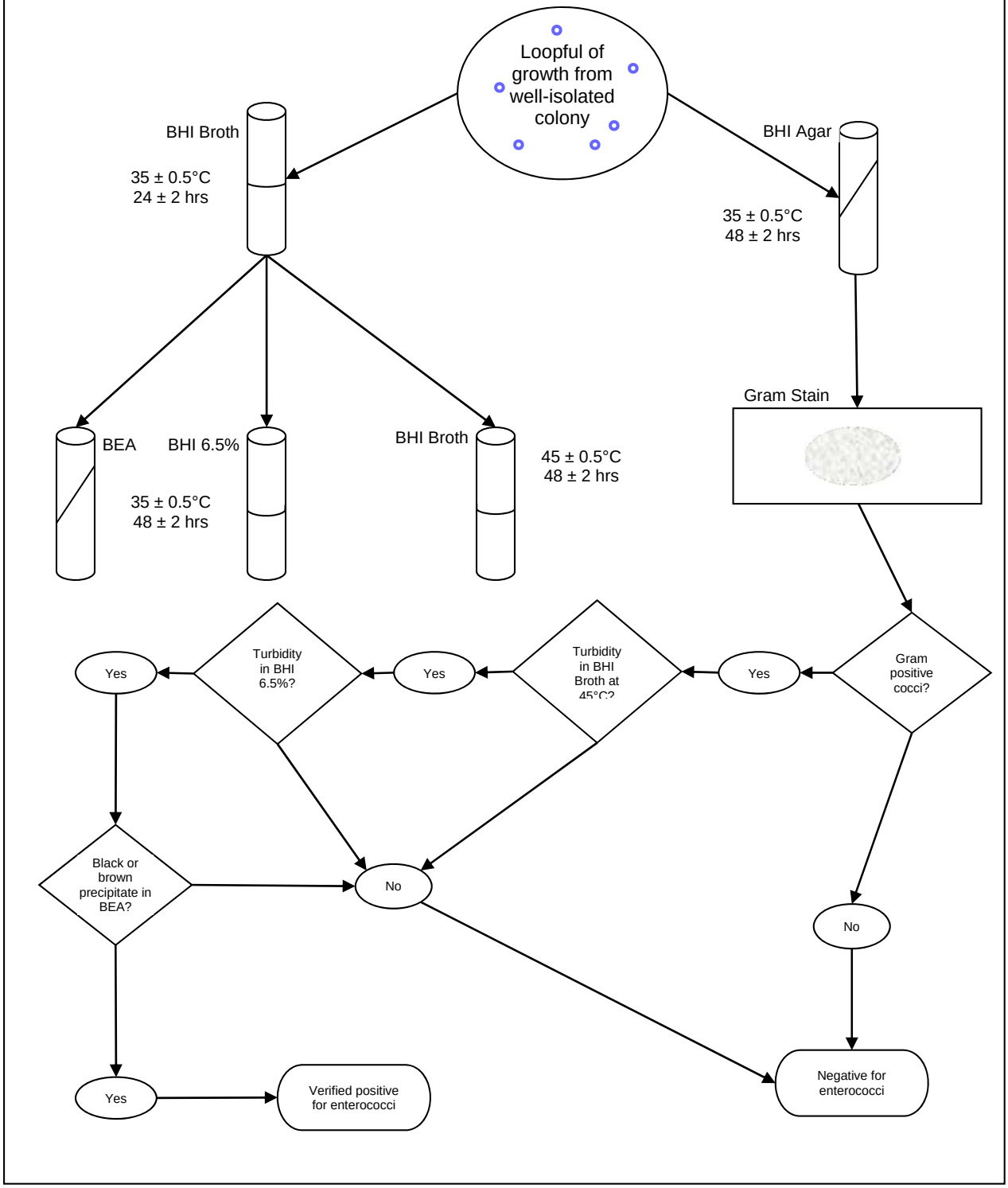
14. TABLES AND DIAGRAMS

Figure 14.1 Set up for Vacuum filtration system



The OFFICIAL COPY is the on-line version. All other copies are considered unofficial and uncontrolled.

Flowchart 14.2 – Procedure for Verification of Enterococci



The OFFICIAL COPY is the on-line version. All other copies are considered unofficial and uncontrolled.

APPENDIX OF CHANGES

07/16/2008 Added Calibration and Standardization section, added more specific references in Safety/Hazardous Waste Management section.

07/16/2009 Added new test ID. Added references to new SOP for media preparation and calibration of pH meter.

07/23/2010 SOP reviewed and edited to update according to current procedures. Removed reference to older version of safety plan and added new version. Changed the negative control test organism.

02/02/2011 Updated references to current version of quality manual.

01/25/2012 Updated incubator and microscope information. Added corrective actions and removed reference to QAM for corrective actions.

01/07/2013 Corrected serial numbers for incubators. Deleted duplicate listing of mEI in Section 4. Added reference to DEP SOP MB-01.10 for gram staining.

01/15/2014 Added separate step for changing out dirty and sterilized funnels in Sample Preparation; added documentation of colony verification in *Monthly Verification Tests Logbook* to Data Archival; added examples of possible blank contamination sources in Method Blanks.

01/13/2015 Updated to reflect new pH meter.

02/26/2015 Added Serial Number to Market Forge Autoclave; added current directions to Control Chart File in Quality Control Section.

04/10/2015 Changed calculations to reflect EPA 1600 method instead of SM 9222B.6. Formatted for 508 Compliance

The OFFICIAL COPY is the on-line version. All other copies are considered unofficial and uncontrolled.