Standard Operating Procedure for:
Membrane Filter Method for Fecal Streptococcus

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1. SCOPE AND APPLICATION

1.1. This method is used to detect and count fecal streptococcus bacteria in non-potable water and is based on Standard Methods SM9230C. Since fecal streptococci are commonly found in the GI tract 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 Laboratory Information Management System (LIMS) test IDs: FSTREP-MF, FS-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 a mENT agar dish, which is a selective medium for fecal streptococci. The dish is then incubated for 48 hours at 35.0 °C. All positive colonies, light to dark red, 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 fecal streptococci difficult.

2.3. Definitions

2.3.1. **Fecal Streptococci** - Bacteria that produce light to dark red colonies after 48 hour incubation at 35.0 °C on mENT agar. This includes *Enterococcus faecalis*, *E. faecium*, *E. avium*, *E. gallinarium*, *E. bovis*, and *E. equinus*.

2.3.2. **Target Organism** – The target organism is the specific bacteria for which the method is testing. In this method the target organism is fecal streptococci.

2.3.3. **Positive Growth** – Bacterial growth that resembles a typical positive colony which is light to dark red in color.

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

3. EQUIPMENT AND SUPPLIES – Record any equipment repairs or maintenance in LIMS Laboratory Logbook, *Maintenance_Log*

3.1. Sterile gloves, latex or nitriles

3.2. Petri dishes, 47 mm, sterilized, with tight fitting lids

3.3. Flask, Erlenmeyer, 2 L

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

3.5. Graduated cylinder, 1 L

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3.6. Magnetic stir bar, medium size

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

3.8. Hot plate with stirrer

3.9. Autoclave, Market Forge Sterilmatic Model STM-E (S/N 226393) - Calibrate per SOP MB-03.2

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

3.11. Repeater pipette with 50 mL tips – Calibrate per SOP MB-03.3

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 – volume of each new lot is checked per SOP MB-03.3

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. Hydrogen Peroxide, 3%

4.3. Ethanol, 80% - Prepare per SOP IZ-09.

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

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4.5. Media - Prepare per SOP MB-02.33.

4.5.1. m Enterococcus Agar (mENT)

4.5.2. Brain Heart Infusion Broth (BHIB)

4.5.3. Brain Heart Infusion Agar (BHI Agar)

4.5.4. Bile Esculin agar (BEA)

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

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

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

5. CALIBRATION AND STANDARDIZATION

5.1. See 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-30 for sample custody and labels.

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

7.3. At the beginning of each day, 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 mENT agar dish lid. Write the volume to be filtered on the lower section of the lid with a black marker.

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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.

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 of sample with a sterile pipet into the funnel and filter. 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 mENT 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.

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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 35.0 ± 0.5 °C incubator for 48 ± 3 hours. Plates 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.

8.9. After incubation, count and record on dish above the sample label all colonies that have a light to dark red color as fecal streptococci. Verify any colonies that are questionable. 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 ± 3 hours at 35.0 ± 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 slant tube for 48 ± 3 hours at 35.0 ± 0.5 °C.

9.2.2. Incubate BHI broth tube for 48 ± 3 hours at 45.0 ± 0.5 °C.

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

9.3. Perform the catalase test by adding a few drops of 3% hydrogen peroxide onto a slide that has a smear of growth from the BHI agar slant. If bubbles form, the test is positive.

9.4. If the catalase test result was negative (no bubbles), Gram stain growth from BHI agar slant per SOP MB-01.10.

9.5. Colonies that are catalase negative, gram-positive cocci, hydrolyze esculin on the BEA slant, and grow in BHI broth at 45.0 °C are verified as fecal streptococci.

9.6. Record results in *Monthly Verification Tests Logbook.*

10. DATA ARCHIVAL

10.1. Record filter, incubation, and read dates/times, and media used for each sample in the 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-31.

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10.4. After results are uploaded to QCM, refer to SOP BB-32 for LIMS upload and data authorization.

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

10.6. Calculations (based on SM 9222B.6):

10.6.1. If at least one dilution is within the ideal colony count range of 20-60 colonies, use only the dilutions that fall within this range to calculate the final result in the equation below.

10.6.2. If no dilutions fall within the ideal colony count range, use all the dilutions to calculate the final result in the equation below and add a "B" qualifier code. If a 100 mL dilution was filtered, and it has a count less than 20 colonies, use only this dilution and do not add a "B" qualifier.

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

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

10.6.5. 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 = Fecal Streptococci 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.

11.1.2. Maintain a control chart of duplicate results. Convert results to logarithms and calculate the range (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 and the comment "Result is J-qualified due to a lab replicate precision failure".

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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 a mENT 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 35.0 ± 0.5 °C for 48 ± 3 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 that are greater than MDL with a "V" qualifier and the comment "Result is V-qualified due to control blank failure" if their results are less than 10 times the blank. If the results are greater than or equal to 10 times the blank result, no V-qualifier is needed, but comment the result with "Control blank failure. Result is ≥ 10 times the blank result".

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 and shake vigorously. Filter a 1 mL volume of this suspension and place filter on a mENT agar dish. Place a media label on the dish and mark it with a "-" sign for negative control. Incubate at 35.0 ± 0.5 °C for 48 ± 3 hours.

11.3.2. To check viability of negative control, also add a small amount of the suspension to a BHI broth tube and incubate at 35.0 ± 0.5 °C for 24 ± 2 hours. If negative control does not grow in BHI broth, then the *P. aeruginosa* may not be viable and the negative control test on the media should be repeated with a new culture.

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 and shake vigorously. Filter a 1 mL volume of this suspension and place filter on a mENT agar dish. Place a

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media label on the dish and mark it with a "+" sign for positive control. Incubate at 35.0 ± 0.5 °C for 48 ± 3 hours.

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 mENT 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 a mENT 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 by incubating with a small amount of TSB 2X (See SOP MB-02.33) that has been swirled around to contact all surfaces at 35.0 ± 0.5 °C for 24 ± 2 hours. If there is growth, recheck lot or batch. If recheck also fails (shows growth), do not use the lot or rewash batch of glassware and contact a supervisor. Record results of sterility checks in the appropriate LIMS **Standard Preparation Tracker** module or **Laboratory Logbook** log. Sterility of sample containers should be checked before they are packed into kits and shipped to customers.

11.6. To enter media QC results, go to LIMS Laboratory/Technical/Standard Preparation Tracker. Choose the Group "BIO-MICRO". Click Media → .Media QC Pass/Fail. Highlight the media preparation, enter the Date QC Test Read, QC Test Read By, Sterility Results, and click Pass or Fail.

11.7. Sodium Thiosulfate Dechlorination Check

11.7.1. Check each lot of sample containers that have a sodium thiosulfate tablet for adequate dechlorination.

11.7.1.1. Dilute bleach to 0.1%. Check the concentration with the Hach Colorimeter II per SOP TA-06.22 and record in **Standard Preparation Tracker.**

11.7.1.2. Dilute the 0.1% solution to 16 mg/L Cl_2 (also check the concentration with the Colorimeter II) and record the new solution in Standard Preparation Tracker.

11.7.1.3. Add 100 mL of the 16 mg/L Cl_2 solution to the sample container with the tablet that is being tested. Shake until the tablet is

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dissolved. Use a chlorine test strip to determine if the solution has been dechlorinated. Record the date, analyst, SN of the 16 mg/L Cl_2 solution and its concentration reading from the Colorimeter II, the final concentration of the solution after dechlorinating, and the lot number of the chlorine test strips in the ENTRY_COMMENTS of the corresponding sterility check record for the sample container lot in LIMS **Laboratory/Technical/Laboratory Logbook** *Lab_Supplies_QC*. If the chlorine test strip shows that the solution still has chlorine, repeat the test. If the repeat test fails (does not dechlorinate), do not use the lot and contact a supervisor.

11.8. Colony Verification

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

11.8.2. Adjust final colony counts for verified samples.

11.9. Colony Counting Variability

11.9.1. Compare colony counts between two analysts from one positive sample monthly. Counts should be within 10% of each other. Comparisons should be performed by all analysts performing this test and recorded in the Microbiology QC Checklist.

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.

12.3. Read MSDS information for reagent handling and disposal.

12.4 The UV sterilizer unit has an automatic shutoff of the lamp when opened. In the event this shutoff malfunctions or needs to be overridden to perform maintenance or repairs on the unit, wear UV blocking safety glasses. Do not look at the UV bulbs without eye protection as serious eye damage can result.

13. REFERENCES

13.1. Standard Methods Online Edition (1993), SM9230C

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

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

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

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

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

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

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

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

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

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

13.15. DEP SOP MB-03.2, *Market Forge Sterilmatic Autoclave Calibration and Use*

13.16. DEP SOP MB-03.3, *Calibration and Use of Mechanical and Disposable Pipettes*

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

13.18. DEP SOP BB-30, *Sample Custody, Preparation Labels and Worksheet Instructions for Bench Biology Samples*

13.19. DEP SOP BB-31, *LIMS QC Manager Data Uploading Instructions for Bench Biology and Microbiology Samples*

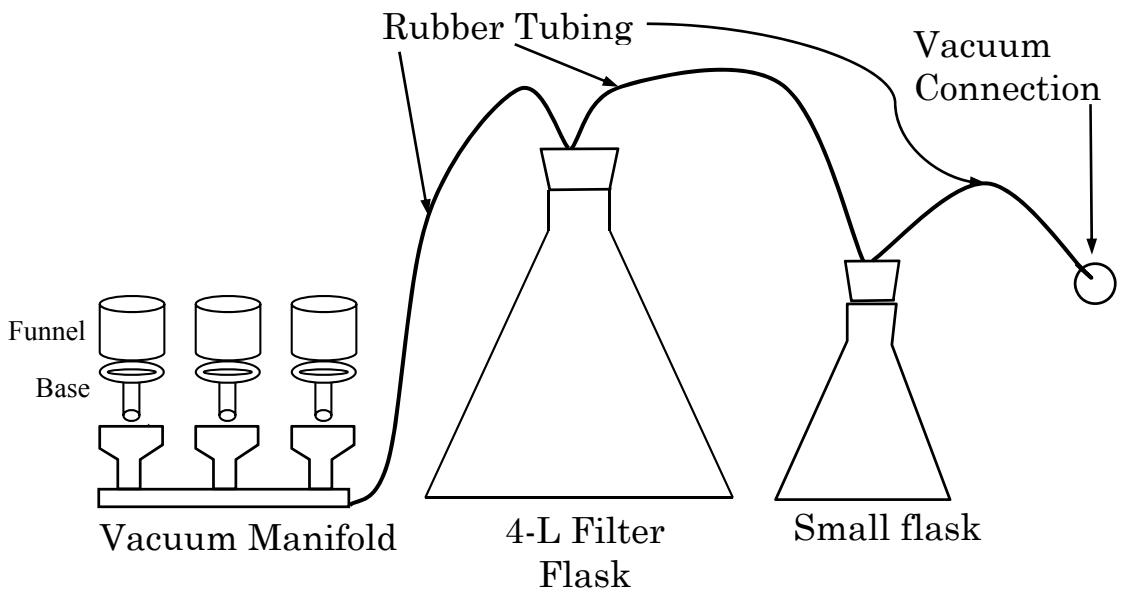
13.20. DEP SOP BB-32, *Test Level Authorization of Uploaded Results for Bench Biology Samples*

13.21. DEP SOP TA-06.22, *Measuring Total Residual Chlorine using the Hach Pocket Colorimeter and Colorimeter II Test Kits*

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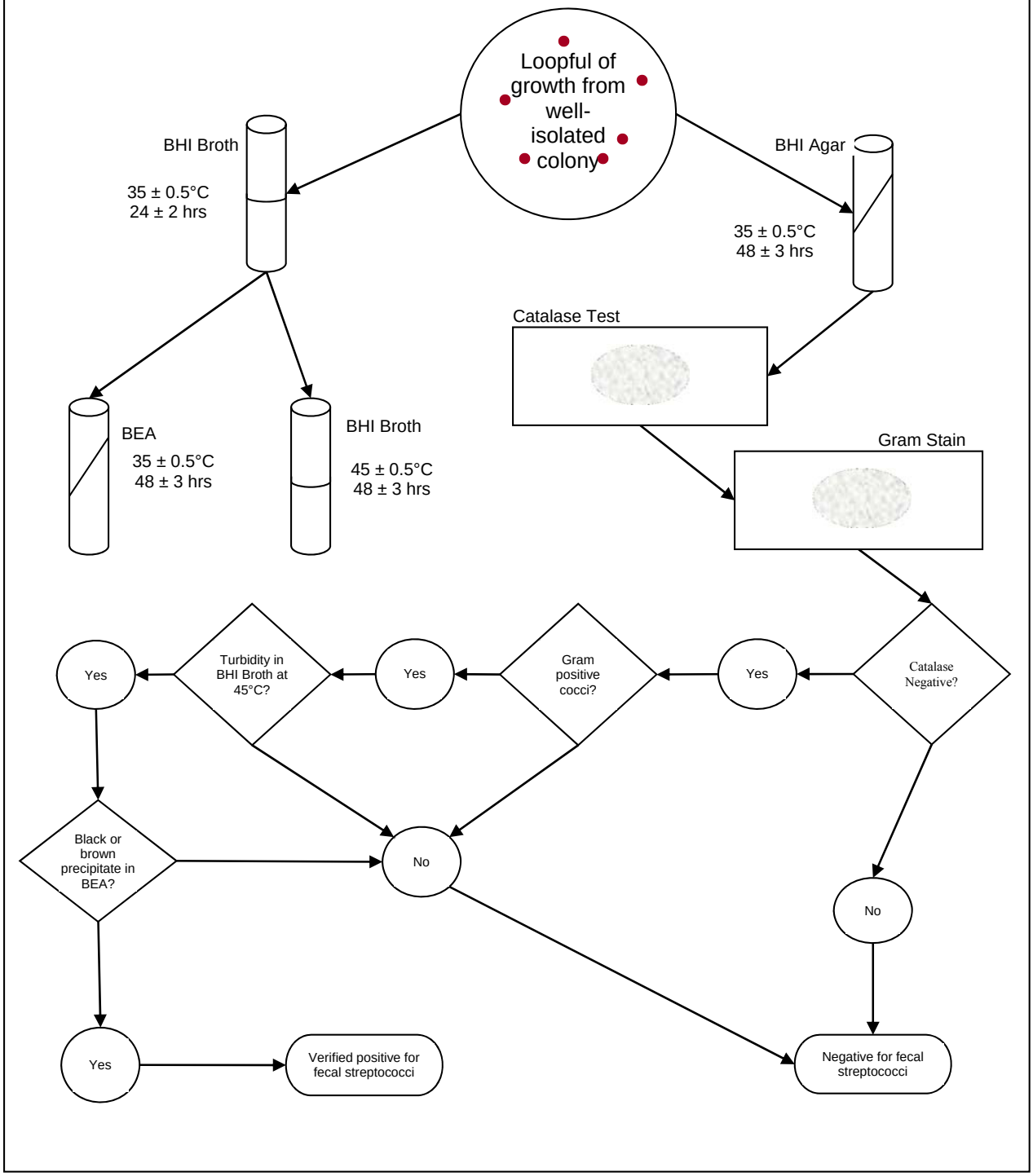
14. TABLES, DIAGRAMS

Figure 14.1 Set up for Vacuum filtration system



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Flowchart 14.2 – Procedure for Verification of Fecal Strepcocci



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APPENDIX OF CHANGES

08/22/2008	Converted to new format and updated to Standard Methods Online Edition.
08/27/2009	Added new LIMS test ID and prep worksheet documentation.
08/27/2010	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.
01/09/2013	Corrected serial number for incubators. Added decimal place for temperatures. Removed duplicate listing of mENT media. Added reference to SOP for Gram staining. Added reference to SOPs for balance and autoclave calibration and use.
01/02/2014	Added note to incubate plates no more than 30 minutes after filtration and removed references to whirl-pak bags. Updated to the use of new balance.
12/15/2014	Updated to reflect new pH meter.
01/26/2015	Formatted for 508 compliance
03/10/2015	Added procedure for checking volume on funnels and doing QC on sodium thiosulfate tablets.

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