Standard Operating Procedure for:
Membrane Filter Method for Total Coliforms

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1. SCOPE AND APPLICATION

1.1. This method is used to detect and count total coliform bacteria in non-potable water and is based on Standard Methods SM9222B. Total coliforms include species that inhabit the intestines of warm-blooded animals and can therefore be an indicator of fecal pollution. Total coliforms also include species that occur naturally in the soil, vegetation, and water.

1.2. This procedure is used for samples with Laboratory Information Management System (LIMS) test IDs: TCOLI-MF, TC-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 m-Endo dish, which is a selective medium for total coliforms. The dish is then incubated for 22-24 hours at 35.0 ± 0.5 °C. Positive colonies, which look pink to dark-red with a metallic surface sheen, are counted and recorded.

2.2. Interferences

2.2.1. Several elements can make accurate identification and enumeration of total coliforms difficult. Suspended particulate materials in sample water can clog the membrane filter and cause spreading of bacterial colonies. Waters with high numbers of non-coliform bacteria can crowd the m-Endo dish with non-target growth and interfere with the development of a metallic sheen on coliform colonies. Also, some strains of total coliforms can produce growth with no metallic sheen.

2.3. Definitions

2.3.1. **Total Coliforms** - Facultative anaerobic, gram-negative, non-spore forming, rod-shaped bacteria that produce a red colony with a metallic sheen within 22-24 hours of incubation at 35.0 ± 0.5 °C on m-Endo media. The amount of metallic sheen development can vary among different coliform strains. This includes *Escherichia*, *Citrobacter*, *Enterobacter*, and *Klebsiella* from the Enterobacteriaceae family.

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 total coliforms.

2.3.3. **Positive Growth** – Bacterial growth that resembles a typical positive colony which is a pink to dark-red colony with a metallic sheen. Positive colonies that do not produce a metallic sheen are considered atypical positive growth.

2.3.4. **Negative Growth** – Bacterial growth that does not have the characteristics of a typical positive colony and is considered a non-coliform.

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3. EQUIPMENT AND SUPPLIES

Record any instrument repairs or maintenance in LIMS Laboratory Logbook *Maintenance_Log*

3.1. Sterile gloves, latex or nitrile

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

3.5. Weighing paper, medium size

3.6. Graduated cylinder, 1 L

3.7. Magnetic stir bar, medium size

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

3.9. Hot plate with stirrer

3.10. Autoclave, Steris Amsco 250 LS Model 20VS (S/N031761851)

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

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

3.13. Isotemp Refrigerator, maintained at 1-5 °C

3.14. White trays, for carrying samples

3.15. Chlorine test strips

3.16. Vacuum Filtration system (See Figure 14.1)

3.17. Membrane filtration unit, sterile wrapped in foil

3.18. UV Sterilizer unit

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

3.20. Incinerator

3.21. Beaker, 30 mL

3.22. Serological disposable pipettes (1, 2, 5, 10, 50 mL as needed), sterile and individually wrapped, with pipette pump. Volume of each new lot is checked per SOP MB-03.3

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

3.24. Inoculating loops, sterile

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

3.26. Stereo microscope, Fisher Scientific

3.27. Incubator , maintained at 35.0 ± 0.5 °C

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4. REAGENTS AND STANDARDS

4.1. Deionized Water

4.2. Ethanol, non-denatured, 95% (190 proof)

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

4.4. Media - Prepare per SOP MB-02.33.

4.4.1. m-Endo medium (m-Endo or m-EndoA)

4.4.2. Lauryl Tryptose Broth (LTB)

4.4.3. Brilliant Green Lactose Broth (BGLB)

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

4.5.1. Escherichia coli (E. coli) ATCC #11775

4.5.2. Pseudomonas aeruginosa (P. aeruginosa) ATCC #10145

5. CALIBRATION AND STANDARDIZATION

5.1. See the Laboratory Quality Manual.

6. SAMPLE COLLECTION, PRESERVATION, AND HANDLING

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

7. SAMPLE PREPARATION

7.1. See DEP 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. Remove any bottles of Buffered Dilution Water from the refrigerator and allow them to reach room temperature before using.

> **Note:** Any opened bottles of Buffered Dilution Water must be refrigerated after sample filtration has been completed for the day.

7.5. Place forceps in a small beaker of 95% ethanol and turn on the incinerator. Fill a new, clean, dry rinse bottle with buffered dilution water.

7.6. Select sample volumes that will produce 20-80 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 and 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.

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Also, consider the sample's previous results when choosing volumes or refer to Standard Methods Online Edition Table 9222: I.

7.7. Place sample label on the middle section of each m-Endo dish lid. Write the volume to be filtered on the lower section of the lid with a black marker. If using m-Endo broth, add 2 mL of the broth to the absorbent pad in each dish.

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 95% ethanol during filtration process. Dip in the ethanol and flame-sterilize in the incinerator before grasping each filter. The incinerator is extremely hot. Use caution. 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 the 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. Rinse the walls of the funnel with 20-30 mL of buffered dilution water (rinse all the way around 3 times).

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 m-Endo agar or broth-soaked pad. 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 the surface of the medium. 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.

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8.8. Place inverted dishes in a 35.0 ± 0.5 °C incubator for 22 - 24 hours. Dishes should be incubated no later than 30 minutes after they are filtered. Do not wait for all the samples to be filtered before incubation.

8.9. After incubation, count and record on dish above the sample label all colonies that are pink to dark-red with a metallic surface sheen. The metallic sheen may vary from pinpoint in size to covering the entire colony. Some positive colonies will show atypical growth and may not have a metallic sheen but are nucleated in the center of the colony and should be counted. Verify any colonies that are questionable. Use a fluorescent lamp and dissecting microscope for better visibility of colonies and sheen.

9. VERIFICATION

9.1. Before verification, remove LTB broth and BGLB broth tubes from the refrigerator and incubate for 24 hours at 35.0 ± 0.5 °C. Wrap the plate that will be verified with aluminum foil and place in the refrigerator. Let the plate reach room temperature before inoculating. After the broth tubes have been incubated, use a sterile inoculating loop to transfer a loopful of growth from a well-isolated colony to an LTB broth tube and into a BGLB broth tube (discard each inoculating loop after it has been transferred from both LTB to BGLB broth tube before moving onto the next set of tubes). Document the type of isolated colonies that will be transferred into the broth tubes in the *Monthly Verification Tests Logbook* (for example: colonies #1-3 are smooth, shiny green colonies #4-5 are metallic colonies).Incubate all tubes for 24 ± 2 hours at 35.0 ± 0.5 °C. Observe for growth (turbidity) and gas formation in the Durham tube. If there is no gas formation, incubate another 24 hours and recheck for gas formation.

9.2. Colonies that produce gas in both LTB and BGLB within 48 ± 3 hours of incubation are verified as positive for total coliforms.

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

10. DATA ARCHIVAL

10.1. Record filter, incubation, read dates/times, analyst, buffered water and media used, QC record numbers for consumables used, and blanks and dups with their respective Test IDs 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. Review the prep time, media and reagents, incubation times, read times, and prep and read analysts for accuracy and ensure the correct number of blanks and dups are on the worksheet and are labelled correctly. Comment as necessary. 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.

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

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

10.6. Document all support equipment and consumables used for sterility QC in Micro-QC Preparation Worksheet.

10.7. Document all QC results in LIMS lab logbook in the appropriate sections.

10.8. Calculations (based on SM 9222B.5):

10.8.1. If at least one dilution is within the ideal colony count range of 20-80 colonies, use only the dilutions that fall within this range to calculate the final result in the equation below.

10.8.2. 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.

10.8.3. 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.

10.8.4. If there are >200 target colonies in all dilutions, use the upper limit of the ideal range (80) and the smallest filtration volume in mL to calculate an estimated final result. Report with a "Z" qualifier.

10.8.5. 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 = Total coliform 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.

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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".

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 m-Endo 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 22 - 24 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 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."

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 an m-Endo 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 22 - 24 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.

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.of positive controls. Positive Controls

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11.3.4. For each batch of prepared media, prepare a known concentration of ATCC or NCTC derived E.coli per manufacturer instructions. Filter volumes of this suspension that will yield results in the countable range and place filter on an m-Endo dish. Place a media label on the dish and mark it with a "+" sign for positive control. Incubate at 35.0 ± 0.5 °C for 22 - 24 hours.

11.3.5. Positive growth results in the range of acceptance criteria according to the culture COA indicates that the medium does support growth of target organisms and produces the expected reaction (growth resembles typical positive colonies).

Corrective Actions: If the average result is outside the acceptance criteria according to the COA , re-run the positive control. . Also, run a pour or spread plate with PCA (See SOP MB-01.5.) on the new culture to check for viability. If there is growth on the PCA but no growth on the m-Endo medium, then the media batch does not support growth of target organisms. Discard the batch and prepare a new media batch.

11.4. Sterility Checks

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

11.4.2. 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. Check sterility of 1 funnel per sterilized batch. 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 the results of sterility checks in the **Laboratory Logbook** log. **Sterility (and de-chlorination) of sample containers should be checked before they are packed into kits and shipped to customers.**

11.5. 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.

11.6. Sodium Thiosulfate De-chlorination Check

Check each lot of sample containers that have a sodium thiosulfate tablet for adequate dechlorination Make sure the concentrated bleach solution has not expired. Purchased bleach should be logged into **Standard Prep. Tracker**. Dilute bleach to 1.0%. Add 1 ml concentrated bleach to 99 ml of DI water. (solution 1) Prepare another solution of 1 ml of the stock 1.0 % and add to 24 ml of DI water. (solution 2) Make sure to swirl the solutions to fully homogenize. Take 1 ml of

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the second solution and place into a 10 ml vial. Using a different pipette, add 9 ml of DI water to the same vial. Check the concentration with the Hach Colorimeter II per SOP TA-06.22. Using M1V1 = M2V2, determine the amount of Cl2 in the original concentration. Record results of each prepared bleach solution in mg/L in Standard Preparation Tracker. Add the Hach kit ID in Additional information. Record volume QC ID of pipettes used and add SN of mechanical pipette if used.

11.6.1.1. Determine how much of the original solution is needed to make X amount of a 16 mg/L solution by multiplying the number of containers by 100 ml (or 220 for the larger containers)

11.6.1.2. Measure the prepared 16 mg/L solution. If this value is not at least 15 mg/L or if it is markedly over your projected value, retest. If it is still not within this range, remake the solution and re-test on the Hach. Add this solution to standard prep tracker as previously stated.

11.6.1.3. Add 100 mL(or 220 mL) of the 16 mg/L Cl2 solution to the sample container with the tablet that is being tested. Shake until the tablet is dissolved. Use a chlorine test strip to determine if the solution has been dechlorinated. In **Lab_Supplies_QC** enter the lot # of the container in the **lot # box**. Select "dilute bleach" in **Entry Type** box. In **Entry Description** select "NaTS de-chlorination check". Record the date and analyst. Enter the SN of the 16 mg/L Cl2 solution in the **batch ID** box and Enter the final concentration of the solution after dechlorinating **in the entry value**. Enter the lot number of the chlorine test strips in **data source box**. 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.

11.7. Colony Verification

11.7.1. Verify at least 5 typical and 5 atypical colonies per month. See Section 9 for verification procedure.

11.7.2. Adjust final colony counts for verified samples using the following formula: (number of verified positive colonies/total number of colonies subjected to verification) * total number representative colonies. Example: (4 verified positive colonies/5 colonies tested) *30 metallic colonies + (0 verified positive colonies/5 colonies tested) *10 shiny green colonies = 24 CFU/100 ml

11.8. Colony Counting Variability

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Each trained analyst must compare colony counts against another trained analyst from one positive sample monthly. Counts should be within 10% of each other. . When comparing counts, each trained analyst will write their count on the back of the plate along with their initials. The counts will then be recorded into the MB-PARCOUNTLOG.

12. SAFETY/HAZARDOUS WASTE MANAGEMENT

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

12.2. Refer to the Laboratory 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 9222 B-2006

13.2. Standard Methods 9020-2017

13.3. NELAC Quality Systems Manual

13.4. Difco/BBL Manual

13.5. FDEP Laboratory Quality Manual http://www.dep.state.fl.us/labs/library/lab_sops.htm

13.6. FDEP Laboratory Health and Safety Plan http://www.fldepnet.org/content/dear/bureau-laboratories-safety-documents

13.7. DEP SOP-001/01 FS 1000, *General Sampling Procedures*

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

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

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

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

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

13.13. DEP SOP TA-06.18, *AMSCO Renaissance Series 3021 Autoclave Calibration and Use*

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

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

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

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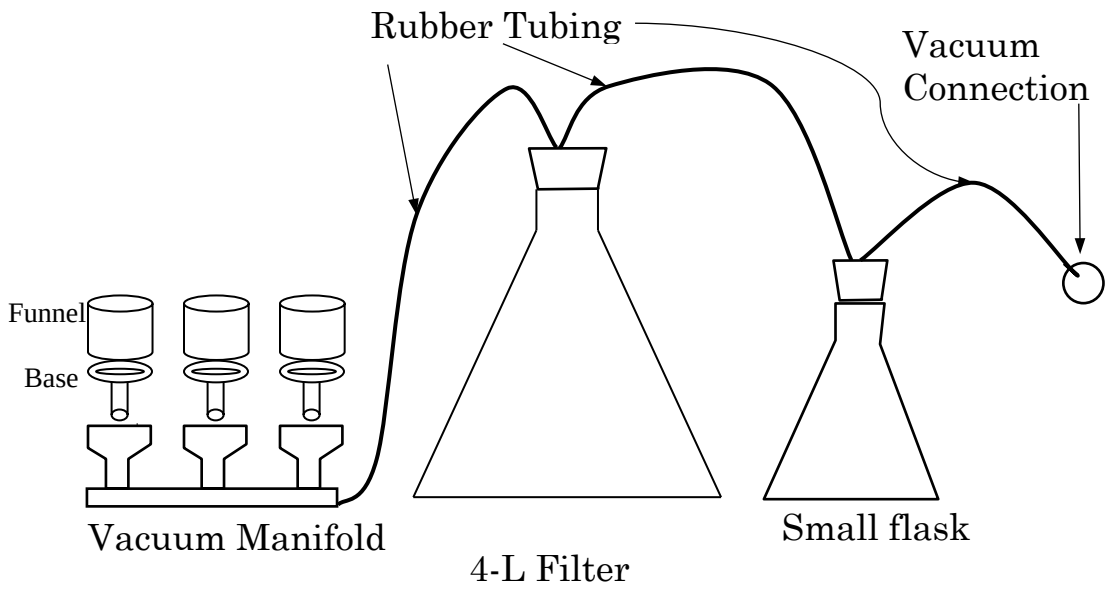
13.17. DEP SOP BB-31, *LIMS QC Manager Data Uploading Instructions for Bench Biology and Microbiology Samples*

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

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

14. TABLES, DIAGRAMS

Figure 14.1 Set up for Vacuum filtration system



APPENDIX OF CHANGES

08/20/2008	Converted to new format and updated to Standard Methods online edition.
08/19/2009	Added new LIMS test ID and prep worksheet documentation.
08/18/2010	Removed reference to older version of safety plan and added new version. Added version/year of SM online methods.
02/02/2011	Updated references to current version of quality manual
02/02/2012	Updated incubator and microscope information. Added corrective actions and removed reference to QAM for corrective actions.
01/28/2013	Added decimal place to some temperatures. Added items to supplies list.

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01/15/2014	Removed old balance and added new balance to Equipment and Supplies; replaced “whirl-pak” with “sample container;” added a separate step for changing out dirty and sterilized funnels in Sample Preparation; added comment about checking sample container sterility in Sterility Checks.
12/16/2014	Corrected the Sterilmatic autoclave Model ID and the temperature of Incubator Thelco Model #4. Updated DATA ARCHIVAL to reflect the changes made in LIMS Prep Worksheet Manager. Clarified “V” qualifier in Corrective Actions for Method Blanks failures.
03/10/2015	Added procedure for checking volume on funnels and doing QC on sodium thiosulfate tablets. Formatted for 508 compliance.
03/14/2017	Updated equipment information, removed funnel calibration, added prep worksheet review, removed “> 10 x blank” instructions for blank failures, updated parallel count instructions, and corrected hyperlinks.
03/13/2018	Updated equipment information in sections 3 and 13.
09/11/2018	Updated Standard Methods version numbers in section 13.
08/27/2019	Updated 10.6. Calculations SM reference.
02/14/2020	Added procedure for open bottles of Buffer water in section 7.3. Added information for broth tubes used for verification before inoculation; added procedure of discarding inoculating loops during each transfer; added documenting type of isolated colonies used for verification in section 9. In section 11.9, removed “and signed off on in Microbiology QC checklist;” corrected on where analyst will write parallel count.
04/23/2020	Updated Equipment, Verification, Data Archival, and Quality Control, and Count Variability sections

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