Multiple Tube Fermentation Technique (MPN) for Fecal Coliforms

1. SCOPE AND APPLICATION

1.1. In the multiple tube fermentation technique, replicate tubes and dilutions are used to estimate coliform density. The result is reported as a most probable number (MPN) per 100 mL and is based on probability formulas used to estimate a mean density of coliforms in the sample. This method is based on Standard Methods SM9221E and is applicable to non-potable water.

1.2. This procedure is used for samples with LIMS test IDs of FCOLI-MPN.

2. SUMMARY OF METHOD

2.1. A water sample is thoroughly mixed and aliquots are dispensed into 15 tubes of LTB medium. The aliquots are typically 5 replicates of 10 mL, 1 mL, and 0.1 mL each. The tubes are incubated for up to 48 hours at 35°C. Any positive tubes (growth and gas production) are transferred to tubes containing EC medium and incubated for 24 hours at 44.5°C. EC tubes that have growth with gas production are considered positive for fecal coliforms. Fecal coliform density is determined using the MPN table, SM9221:IV and reported as MPN/100 mL.

2.2. Interferences

2.2.1. Inadequate mixing or bacterial clumping can lead to an underestimation of bacterial density. Samples must be thoroughly mixed before dispensing into tubes.

2.3. Definitions

2.3.1. Fecal Coliforms – facultative anaerobic, gram-negative, non-spore forming, rod-shaped bacteria that ferment lactose to produce gas within 24 ± 2 hours at 44.5°C.

2.3.2. MPN – Most probable number is an estimation of bacterial density derived from probability formulas that are based on the assumption of random dispersion of bacteria in the water sample.

3. EQUIPMENT AND SUPPLIES

3.1. Sterile gloves, latex or nitrile

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

3.3. Calibrated Repeater Pipette, plus tips, for sample volumes <1 mL

3.4. Test-tube racks

3.5. Test tubes with metal or plastic screw caps, autoclavable

3.6. Inoculating loops, sterile

3.7. Walk-in refrigerator, maintained at 1- 4 °C

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3.8. Heratherm Incubator (S/N 41784672), maintained at 35 ± 0.5 °C

3.9. Precision water bath (S/N 699081148), maintained at 44.5 ± 0.2 °C

4. REAGENTS AND STANDARDS

4.1. Buffered Water, prepared per SOP MB-02.24

4.2. Fermentation Tube Media

4.2.1. Prepare the following per SOP MB-02.33. Add 10 mL of media per tube.

4.2.1.1. EC medium (EC)

4.2.1.2. Lauryl Tryptose Broth (LTB)

4.2.1.3. Double strength Lauryl Tryptose Broth (LTB 2X)

4.2.1.3.1. Add double the amount (71.2 g) of dry ingredients per liter of water. Dispense media into large tubes. The inverted vial should be at least ½ to ⅔ covered by the media after sterilization.

4.2.1.3.2. Use double strength media for sample dilutions of 10 mL.

4.2.2. If fermentation media has been stored in the refrigerator, incubate at room temperature overnight prior to use. Discard any tubes that show growth and/or bubbles. Alternatively, media can be stored at room temperature if used within one week and evaporation has not caused a loss in media of >1 mL.

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

4.3.1. *Escherichia coli* (*E. coli*) ATCC #11775

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

5. CALIBRATION AND STANDARDIZATION

5.1. See FDEP Bureau of Labs Quality Manual, Section 5.5 and 5.6.

5.2. Check temperatures of incubators twice daily at least 4 hours apart. Record in LIMS Laboratory\Technical\Laboratory Logbook *Temperature_Log.*

5.3. Check thermometers annually against a NIST certified thermometer. Record in LIMS Laboratory\Technical\Laboratory Logbook *Thermo_Cal_Log.*

6. SAMPLE COLLECTION, PRESERVATION, AND HANDLING

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

7. SAMPLE PREPARATION

7.1. See SOP BB-30 for sample custody and labels.

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8. SAMPLE ANALYSIS

8.1. Presumptive Test

8.1.1. For each sample, set up 3 different dilution rows of 5 LTB tubes each. For 10 mL dilutions use double strength LTB. For <10 mL dilutions use single strength LTB. Typical dilutions are 0.1, 1, and 10 mL, but can be adjusted as necessary. If more dilutions are needed set up additional rows of tubes.

8.1.2. At the end of each dilution row, add one more tube to be used for the blank. The volume of buffered water added to each tube should correspond to the volume of sample added to the other tubes in that row. Inoculate the blank when one dilution set has been completely inoculated and before starting the next dilution.

8.1.3. Shake sample bottle vigorously, approximately 25 times. Inoculate each row of tubes with the appropriate dilution volume. Re-shake the sample after each dilution to ensure it remains well mixed. Use a new sterile pipette tip for each dilution and inoculate in increasing volume. For example, in a 0.1, 1, 10 mL dilution series, inoculate the first 5 tubes with 0.1 mL each. Then inoculate the second row of tubes with 1 mL each. Finish by inoculating the last row of tubes with 10 mL each. Gently agitate each tube to mix the sample into the medium. Repeat this process for each sample.

8.1.4. Incubate tubes at 35 ± 0.5 ˚C for 24 ± 2 hr.

8.1.5. Swirl each tube and examine for growth and gas.

8.1.5.1. If no gas or growth is apparent, re-incubate and re-examine after another 24 hours. (48 ± 3 hr total incubation time).

8.1.5.2. For tubes where growth and gas are present at 24 hours, the confirmed test can be started. See Step 8.2.

8.1.6. In the *Microbiology MPN Data Sheet* logbook, record tubes with growth and gas formation as positive (+) and tubes with no gas formation as negative (-).

8.2. Confirmed Test

8.2.1. For each positive LTB or LTB 2X tube, transfer a loopful of growth to a corresponding EC medium tube.

8.2.1.1. To help keep track of which LTB tube corresponds with which EC tube, set up the EC tubes in the same order as the LTB tubes—3 rows of 5 tubes each, 0.1, 1, and 10 mL dilutions. Label the tubes with numbers or some other identification.

8.2.2. Place EC tubes into a water bath within 30 minutes after inoculation. Incubate inoculated EC tubes at 44.5 ± 0.2˚C for 24 ± 2 hr. Make sure the water level reaches the upper level of the medium.

8.2.3. In the *Microbiology MPN Data Sheet* logbook, record tubes with growth and gas formation as positive (+) and tubes with no gas formation as negative (-).

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9. DATA ARCHIVAL

9.1. In the *Microbiology MPN Data Sheet* logbook, add up the number of positive EC tubes for each dilution (row) and record this as the positive tube combination in the logbook. The positive tube count for the largest sample volume should be the first number, the smallest volume should be the last, with all other volumes falling in between in descending volume order.

9.1.1. For example, if there are 4 positive tubes for the 10 mL dilution, 3 positive tubes for the 1 mL dilution, and 1 positive tube for the 0.1 mL dilution, the positive tube combination is 4-3-1.

9.2. Using the positive tube combination, look up the corresponding MPN value in Standard Methods Table 9221:IV. Record the value in the logbook.

9.3. Record the dilution factor in the logbook as: 10/largest volume tested

9.4. Multiply the dilution factor by the MPN value to calculate the final result as MPN/100 mL

9.5. If more than three decimal dilutions are used, the positive tube combination is not in the table, or if the dilutions used are not decimal, follow the guidelines in SM9221C.2 to determine the MPN.

9.6. Upload results into LIMS using the Results Editor module.

10. QUALITY CONTROL

10.1. Method blanks run on each dilution to ensure proper handling of the samples.

10.2. Sample Replicates

10.2.1. Analyze a laboratory duplicate for 10% of samples with at least one duplicate per batch.

10.2.2. Report the geometric mean of the sample and its laboratory duplicate. Report with an “A” qualifier.

10.2.3. 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.

10.3. Negative Controls

10.3.1. For each batch of prepared media (EC or LTB tubes), transfer a small amount of *P. aeruginosa* to a media tube using an inoculating needle and gently mix. Place a media label on the tube and mark it with a “-” sign for negative control. Incubate at the appropriate temperature/duration for the media being tested.

10.3.2. To check viability of negative control, also add a small amount of the bacteria to a BHI broth tube and incubate at 35 ± 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.

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10.3.3. Absence of growth in negative control indicates that the medium does not support growth of non-target organisms.

10.3.4. 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.

10.4. Positive Controls

10.4.1. For each batch of prepared media (EC or LTB tubes), transfer a small amount of *E. coli* to a media tube using an inoculating needle and gently mix. Place a media label on the tube and mark it with a "+" sign for positive control. Incubate at the appropriate temperature/duration for the media being tested.

10.4.2. Growth and gas production indicates that the medium does support growth of target organisms.

10.4.3. If no growth occurs, re-run the positive control with a new culture of *E. coli*. 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 tested media, then the media batch does not support growth of target organisms. Discard the batch and prepare a new media batch.

10.5. Sterility Checks

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

10.5.2. Check sterility on each lot of sample containers and each sterilized batch of glassware.

10.6. Refer to QA manual for corrective action and handling out-of-control data.

11. SAFETY/HAZARDOUS WASTE MANAGEMENT

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

11.2. Refer to the Laboratory Health and Safety Manual.

11.3. Read MSDS information for reagent handling and disposal.

12. REFERENCES

12.1. Standard Methods SM 9020—2015

12.2. Standard Methods SM 9221 E—2006

12.3. NELAC Quality Systems Manual

12.4. Bureau Quality Manual http://www.dep.state.fl.us/labs/library/progplan.htm

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12.5. Laboratory Health and Safety Manual

12.6. FDEP SOP-001/01 FS 1000, *General Sampling Procedures*

12.7. FDEP SOP BB-30: *Sample Custody, Preparation Labels and Worksheet Instructions for Bench Biology Samples*

12.8. FDEP SOP MB-02.33, *Preparation of Dehydrated Culture Media*

12.9. FDEP SOP MB-03.1, *Maintenance of Bacterial Cultures*

12.10. FDEP SOP MB-03.4: *Accumet Excel XL 25 pH Meter Calibration and Use*

12.11. Table 9221:IV MPN Reference Table

Combination of Positives	MPN Index/100 mL	Confidence Limits		Combination of Positives	MPN Index/100 mL	Confidence Limits	
		Low	High			Low	High
0-0-0	<1.8	—	6.8	4-0-3	25	9.8	70
0-0-1	1.8	0.090	6.8	4-1-0	17	6.0	40
0-1-0	1.8	0.090	6.9	4-1-1	21	6.8	42
0-1-1	3.6	0.70	10	4-1-2	26	9.8	70
0-2-0	3.7	0.70	10	4-1-3	31	10	70
0-2-1	5.5	1.8	15	4-2-0	22	6.8	50
0-3-0	5.6	1.8	15	4-2-1	26	9.8	70
1-0-0	2.0	0.10	10	4-2-2	32	10	70
1-0-1	4.0	0.70	10	4-2-3	38	14	100
1-0-2	6.0	1.8	15	4-3-0	27	9.9	70
1-1-0	4.0	0.71	12	4-3-1	33	10	70
1-1-1	6.1	1.8	15	4-3-2	39	14	100
1-1-2	8.1	3.4	22	4-4-0	34	14	100
1-2-0	6.1	1.8	15	4-4-1	40	14	100
1-2-1	8.2	3.4	22	4-4-2	47	15	120
1-3-0	8.3	3.4	22	4-5-0	41	14	100
1-3-1	10	3.5	22	4-5-1	48	15	120
1-4-0	11	3.5	22	5-0-0	23	6.8	70
2-0-0	4.5	0.79	15	5-0-1	31	10	70
2-0-1	6.8	1.8	15	5-0-2	43	14	100
2-0-2	9.1	3.4	22	5-0-3	58	22	150
2-1-0	6.8	1.8	17	5-1-0	33	10	100
2-1-1	9.2	3.4	22	5-1-1	46	14	120
2-1-2	12	4.1	26	5-1-2	63	22	150
2-2-0	9.3	3.4	22	5-1-3	84	34	220
2-2-1	12	4.1	26	5-2-0	49	15	150
2-2-2	14	5.9	36	5-2-1	70	22	170
2-3-0	12	4.1	26	5-2-2	94	34	230
2-3-1	14	5.9	36	5-2-3	120	36	250
2-4-0	15	5.9	36	5-2-4	150	58	400
3-0-0	7.8	2.1	22	5-3-0	79	22	220
3-0-1	11	3.5	23	5-3-1	110	34	250
3-0-2	13	5.6	35	5-3-2	140	52	400
3-1-0	11	3.5	26	5-3-3	170	70	400
3-1-1	14	5.6	36	5-3-4	210	70	400
3-1-2	17	6.0	36	5-4-0	130	36	400
3-2-0	14	5.7	36	5-4-1	170	58	400
3-2-1	17	6.8	40	5-4-2	220	70	440
3-2-2	20	6.8	40	5-4-3	280	100	710
3-3-0	17	6.8	40	5-4-4	350	100	710
3-3-1	21	6.8	40	5-4-5	430	150	1100
3-3-2	24	9.8	70	5-5-0	240	70	710
3-4-0	21	6.8	40	5-5-1	350	100	1100
3-4-1	24	9.8	70	5-5-2	540	150	1700
3-5-0	25	9.8	70	5-5-3	920	220	2600
4-0-0	13	4.1	35	5-5-4	1600	400	4600
4-0-1	17	5.9	36	5-5-5	>1600	700	—
4-0-2	21	6.8	40				

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Appendix of Changes

07/18/2008	Updated to SM 9221B 20th Edition and added a references section.
07/16/2009	Removed the use of LIMS logbook and replaced with MPN Data sheet log.
07/14/2010	Removed reference to older version of safety plan and added new version
06/15/2011	Converted SOP to new format and added Summary of Method section.
05/22/2013	Updated calibration and standardization section.
06/15/2015	In Equipment and Supplies, added repeater pipette with tips, corrected "Refrigerator" to "Low Temp Incubator" and added Serial Number, added Model Number and Serial Number to water bath; switched the order of Sections 4.2.2. and 4.2.3.; in Data Archival, better explained how to determine the positive tube combination for determining MPN; removed all "whirl-paks" and replaced with "sample containers."
12/30/2016	Updated incubators in Section 3; removed "Deionized water" and replaced with "Buffered water," corrected amount of media to add per tube and re-ordered the steps in Section 4; added method blanks and clarified Confirmed Test in Section 8; added method blank to Section 10; removed Lab Health and Safety "burlabs" link from Section 12.
04/12/2019	Added incubation time after inoculation under the confirmed test section. Added SM 9221E-17 and Table 9221:IV to reference section for easy access.

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