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Multiple Tube Method Most Probable Number - Total and Fecal Coliforms and E. coli Standard Method 9221

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1. Scope and Application

1.1 This method is a lactose fermentation test used to detect the presence of total and/or fecal coliform or E. coli in a sample. It involves one to three media, two incubation temperatures and periods expanding 48-96 hours to complete. Since we now have the faster enzyme substrate test, it is no longer used in this lab as the primary method for drinking water. It is utilized always for the water quality samples and occasionally for drinking water samples when one of the two following conditions prevails:

•The sample is turbid and colored preventing it from being a candidate for the enzyme substrate method.

•The sample needs an MPN (Most Probable Number) and the enzyme substrate media is not available in the lab for immediate use.

Regardless of the sample type, the analysis begins with inoculating Lauryl Tryptose Broth for the presumptive phase. Drinking water is dispensed in 10 ml increments into 10 double strength LSTB tubes. Water quality is dispensed into 20 tubes of single strength LSTB (five times in each of the following four dilutions: 1.0 ml, 0.1 ml, 0.01 ml, and 0.001 ml). For drinking water samples, Brilliant Green is used as the confirmation media for total coliform and EC+MUG media is used to confirm E. Coli. Water quality requires the determination of fecal coliform, so EC media is inoculated. (Occasionally, it is requested that the water quality sample be tested for total coliform or E. Coli. as well.)

2. Definitions

2.1 Refer to Section 3 and Section 4 of the Georgia EPD Laboratory Quality Assurance Manual for Quality Control Definitions. (See reference 13.3)

3. Interferences

3.1 Method interferences may be caused by contaminants in reagents, media, test tubes, and glassware. To abstain from interferences, all reagents and media are tested for sterility prior to use. Also, all glassware is sterilized and tested prior to usage.

3.1.1 Each batch of media is aseptically prepared according to manufacturer's instructions, sterilized, and tested prior to use. Media used in this procedure are EC Broth, EC + MUG Broth, Lauryl Tryptose - Double Strength and Single Strength, and Brilliant Green.

3.1.2 Dilution water is aseptically prepared and sterilized according to prescribed methods. Each batch of water is tested for sterility.

3.1.3 All glassware must be washed, sterilized, and put in the hot air oven at 180°C for 2 hours. A pH check is performed on all batches of glassware using a 0.04% solution of bromothymol blue. After drying and cooling, seal, and store glassware in a clean environment to prevent any accumulation of dust or other contaminants.

4. Safety

4.1 Refer to Laboratory Chemical Hygiene Plan & Fire Safety Plan, online revision. (See SOP reference 13.4)

5. Apparatus and Equipment

5.1 Round - 250 ml (8 oz) or 125 ml (4 oz) plastic bottles
5.2 44.5º± 0.2ºC Water Bath
5.3 35º± 0.5ºC Incubator
5.4 Sterile Wooden Applicator Sticks (6''x 1/12")
5.5 Sterile 2 ml and 10 ml Transfer Pipettes
5.6 Pipette Holder
5.7 Hydro Marker (Grease Pencil)
5.8 Water Resistant Crayon Marker
5.9 Test Tube Racks
5.10 Reusable Borosilicate Glass Culture Tube without Rim –
25 X 200 mm
12 X 75 mm
20 X 150 mm
10 X 75 mm
16 X 125 mm
6 X 50 mm
5.11 UV lamp (Long wavelength – 365 nm)

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6. Reagents

6.1 Lauryl Tryptose Broth [Single Strength (SS) and Double Strength (DS)]
6.2 EC Broth
6.3 EC Medium with Mug
6.4 Brilliant Green Bile 2%
6.5 Dilution Water

7. Sample Collection

7.1 Refer to Chapter 5 of the Georgia EPD Laboratory Quality Assurance Manual for Sample Container, Sample Preservation and Sample Holding Times. (See SOP reference 13.3)

8. Calibration

8.1 There are no calibrations associated with this method. Maintain sterility with equipment, media, and technique. Maintain the water bath and incubator at their proper temperature. Media must be used prior to its expiration date and pass all quality control measures prior to use.

9. Quality Control

9.1 Refer to Table 14.1 for Quality Control Acceptance Criteria associated with this method.

10. Procedure

10.1 Multiple Tube Methods for Drinking Water

10.1.1 Set up a rack of 10 DS Lauryl Tryptose tubes with metal caps.
10.1.2 Write the assigned Bac-T number on the first tube only.
10.1.3 Shake the water sample approximately 25 times.
10.1.4 Carefully remove cap and pipette 10 ml of sample into each tube.
10.1.5 Incubate rack at 35º±0.5ºC for 24 hours ± 2 hours.
10.1.6 Interpretation of results:

- Swirl tubes gently and examine each for growth, gas, and acid reaction.
- Slightly turbid tubes may be gently shaken to facilitate or increase any gas displacement that may be present. Note: Care must be taken not to over shake the tube.
- Lactose fermentation and gas production represents a positive reaction. In the reading process, gas displacement in the inverted vials of the positive tubes are assigned a number corresponding to the approximate amount of displacement, ex: 10, 20, 30, etc. or "Tr" for trace amount.
- If the tube is clear, record results as negative. Any negative tubes are placed back in the incubator. They are incubated for an additional 24 hours and re-examined at the end of 48 ± 3 hrs.
- All positive presumptive tubes must then be transferred to confirmation media. Brilliant Green is confirmatory for total coliform presence and EC+MUG media confirms E. coli.
  Make sure to label the first tube of Brilliant Green and EC+MUG with the appropriate Bac-T Lab number before transferring positives.
  NOTE: When performing a double inoculation using both confirmation medias, inoculate EC+MUG first, then Brilliant Green.
- Gently swirl positive tubes to re-suspend the organisms. Insert a sterile wooden applicator stick into culture, promptly remove, and plunge applicator stick to bottom of confirmation tube. Remove and discard applicator stick. Repeat for all other positive presumptive tubes.
- Once inoculated, Brilliant Green is incubated at 35º±0.5ºC for 24 ± 2 hours. If tubes are negative, they are incubated an additional 24 hours and read at 48 hours ± 3 hours. Lactose fermentation and gas production denotes a positive.
- Once inoculated, EC+MUG is placed in the water bath at 44.5º±0.2ºC for 24 ±2 hours. Check all tubes with lactose fermentation and gas production for fluorescence using UV lamp. Presence of bright blue fluorescence is considered positive. A clear or non-fluorescent tube indicates a negative.
- Any positive tube in Brilliant Green confirms the sample as positive for total coliform. Any positive tube in EC+MUG confirms the sample as positive for E. coli.
- Invalidation of total coliform negative drinking water samples: All samples that produce a turbid culture (i.e. heavy growth) in the absence of gas/acid production in LSTB must be invalidated. Request that the system collect another sample within 24 hrs. from the same location as the original sample.
- **For batches that contain Multiple Tube samples only: use a blank tube as a negative control and inoculate one tube with 0.1 ml E. coli as a positive control.**

10.2 Multiple Tube Method for Water Quality

10.2.1 Assign a Bac-T number to the stream sample, stamp time and date received.
10.2.2 Set up a rack of 20 SS Lauryl Tryptose tubes (4 rows of 5 tubes).
10.2.3 Label the first tube of each row with the last 3 numbers of our assigned Bac-T number and an "X" or "D" for duplicates. (For Quality Assurance purposes, every tenth stream sample must have a duplicate test run on it.) Also label these first tubes, 1.0, 0.1, 1_1, 1_2, respectively.
10.2.4 Shake stream sample bottle approximately 25 times.
10.2.5 Pipette 1 ml of original sample into first row of 5 tubes labeled "1.0".
10.2.6 Pipette 0.1 ml of sample into second row labeled "0.1".
10.2.7 Next pipet 1 ml of original sample into a leveled dilution bottle. Label it with the assigned Bac-T

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number and an "X" and shake the dilution bottle, to mix thoroughly. (Note: Before using dilution bottle check to make sure there is no soap or black sediment in water. Additionally, you may have to level water in bottle if necessary.)

10.2.8 Then pipette (using a new pipette) 1 ml of dilution "X" sample into third row of tubes labeled 1_1.

10.2.9 Finally, pipette 0.1 ml of diluted sample into fourth row of tubes labeled 1_2.

10.2.10 Incubate for 24 hours ± 2 hours at 35º±0.5ºC.

10.2.11 Interpretation of results:

- Swirl tubes gently and examine each for growth, gas, and acid reaction.
- A slightly turbid tubes may be gently shaken to facilitate or increase any gas displacement that may be present. Note: Care must be taken not to over shake the tube.
- In the reading process, gas displacement in the inverted vials of the positive tubes are assigned a number corresponding to the approximate amount of displacement, ex: 10, 20, 30, etc. or "Tr" for trace amount.
- If a tube is negative (clear in appearance and no gas displacement), then it is re-incubated and re-examined at the end of 48 ± 3 h. The tubes then undergo a final reading. If the tubes are still negative, they are discarded.
- All positive tubes are transferred to EC (to confirm fecal coliform) and/or to EC + MUG (to confirm E.coli) using a sterile wooden applicator stick. Positive presumptive tubes are cloudy and show gas production and displacement.
- EC and/or EC + MUG tubes must be marked with the appropriate assigned Bac-T number on the first tube and marked with an appropriate letter of the alphabet, "a, b, c, etc." corresponding to the number of tubes. After inoculation, EC and/or EC + MUG is placed in a 44.5º±0.2ºC water bath for 24 ± 2 hours.
- Interpretation of results. EC and/or EC + MUG is read as either positive or negative. EC which is positive shows cloudiness and gas displacement. A positive EC result confirms fecal coliform. EC + MUG positive tubes exhibit growth and fluorescence when exposed to a long-wavelength UV lamp. The presence of bright blue fluorescence is considered a positive response for E.coli.
- Invalidation of **total coliform** negative source water samples: All samples that produce a turbid culture (i.e., heavy growth) in the absence of gas/acid production in LSTB must be invalidated. Request a replacement sample from the same location as the original invalidated sample.
- Information Only Samples – samples with holding time greater than 24 and less than 48 hours. Analyze sample.
- Too Old Samples – samples with holding time greater than 48 hours. Do not analyze sample.

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11 Calculations

11.1 For drinking water samples reported as positive or negative, in keeping with The Revised Total Coliform Rule, there are no calculations. For the drinking water samples that require an MPN count the total number of positive tubes in the confirmative medias refer to Standard Methods Table 9221. III for the MPN Index for 10 tubes. For water quality count the total number of positive tubes in the confirmative medias and refer to Standard Methods Table 9221.IV for the MPN Index for 5 tubes. Note: Table for Drinking Water (10 Tube) and Table for Water quality (5 Tube) Below:

Table 11.1 EPA9221.III
MPN Index and 95% Confidence Limits for
Various Combinations of Positive and Negative
Results When Ten (10) ml Portions are Used

No. of Positive Tubes Out of 10 Of 10 mls Each	MPN Index/ 100 ml	95% Confidence Limits (Approximate) Lower	Upper

0	<1.1	0	3.0
1	1.1	0.03	5.9
2	2.2	0.26	8.1
3	3.6	0.69	10.6
4	5.1	1.3	13.4
5	6.9	2.1	16.8
6	9.2	3.1	21.1
7	12.0	4.3	27.1
8	16.1	5.9	36.8
9	23.0	8.1	59.5
10	>23.0	13.5	Infinite

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Table 11.2 EPA 9221.IV
MPN Index and 95% Confidence Limits for Various Combination of Positive
When Results Five Tubes Are Used per Dilution (10ml. 1.0ml and 0.1ml)

Combination of Positives	MPN Index/ 100 ml	95% Confidence Limits		Combination of Positives	MPN Index/ 100 ml	95% Confidence Limits	
		Lower	Upper			Lower	Upper
0-0-0	<2	----	----	4-2-0	22	9.0	56
0-0-1	2	1.0	1.0	4-2-1	26	12	65
0-1-0	2	1.0	1.0	4-3-0	27	12	67
0-2-0	4	1.0	13	4-3-1	33	15	77
1-0-0	2	1.0	11	5-0-0	23	9.0	86
1-0-1	4	1.0	15	5-0-1	30	10	110
1-1-0	4	1.0	15	5-0-2	40	20	140
1-1-1	6	2.0	18	5-1-0	30	10	120
1-2-0	6	2.0	18	5-1-1	50	20	150
				5-1-2	60	30	180
2-0-0	4	1.0	17	5-2-0	50	20	170
2-0-1	7	2.0	20	5-2-1	70	30	210
2-1-0	7	2.0	21	5-2-2	90	40	250
2-1-1	9	3.0	24	5-3-0	80	30	300
2-2-0	9	3.0	25	5-3-1	110	40	300
2-3-0	12	5.0	29	5-3-2	140	60	360
3-0-0	8	3.0	24	5-3-3	170	80	410
3-0-1	11	4.0	29	5-4-0	130	50	390
3-1-0	11	4.0	29	5-4-1	170	70	480
3-1-1	14	6.0	35	5-4-2	220	100	580
3-2-0	14	6.0	35	5-4-3	280	120	690
3-2-1	17	7.0	40	5-4-4	350	160	820
4-0-0	13	5.0	38	5-5-0	240	100	940
4-0-1	17	7.0	45	5-5-1	300	100	1300
4-1-0	17	7.0	46	5-5-2	500	200	2000
4-1-1	21	9.0	55	5-5-3	900	300	2900
4-1-2	26	12	63	5-5-4	1600	600	5300
				5-5-5	>/=1600	-----	-----

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12. Waste Management

12.1 See GA EPD Laboratory SOP-EPD Laboratory Waste Management Standard Operating procedures, SOP 6-015, online revision.

13. References

13.1 Standard Methods for the Examination of Water and Water quality, 19th Edition, American Public Health Association: Washington, D.C., 1995.

13.2 Manual for the Certification of Laboratories Analyzing Drinking Water, EPA/815-R-05-004, January 2005.

13.3 GA EPD Laboratory Quality Assurance Plan, online revision.

13.4 GA EPD Laboratory Safety/Chemical Hygiene Plan & Fire Safety Plan, online revision.

13.5 GA EPD Laboratory SOPs – Initial Demonstration of Capability SOP 6-001, online revision and/or Continuing Demonstration of Capability SOP 6-002, online revision.

14. Practice Quantitation Limits (PQLs), Precision and Accuracy Criteria and Quality Control Approach

Table 14.1 Summary of Data Quality Objectives

Method	Parameter	QC Check	Min. Frequency	Accepted Criteria	Corrective Action	Flagging Criteria
SM 9221-Multiple Tube Method-Most Probable Number	BG, EC, LSTB-positive control	E. coli (EC)	Weekly	Positive w/gas	Discard Media	
	BG, EC, LSTB-positive control	Klebsiella aerogenes (KA)	Weekly	Positive w/gas	Discard Media	
	BG, EC, LSTB-negative control	Staphylococcus aureus (Staph)	Weekly	Negative	Discard Media	
	EC + MUG	Autofluo-rescence of media	Weekly	No fluorescence when exposed to UV lamp	Discard Media	
	EC + MUG	E. coli (EC)	Weekly	Growth. Gas displacement and fluorescence with UV light	Discard Media	
	EC + MUG	Pseudomonas aeruginosa (PA)	Weekly	Negative	Discard Media	
	EC + MUG	Klebsiella pneumoniae (Kleb)	Weekly	Growth. Gas displacement and no fluorescence with UV light	Discard Media	
	LSTB-any sample		Each Tube	No growth	None	Negative
	LSTB-Positive Drinking Water Sample		Each Tube	Growth with gas displacement	None	Positive transferred to Brilliant Green and EC+MUG
	LSTB-Positive Water quality Sample		Each Tube	Growth with gas displacement	None	Positive. Transferred to EC
	BG		Each Tube	No growth	None	Negative for total coliform and refer to Table 9221.III for MPN.
	BG		Each Tube	Growth with Gas Displacement	None	Positive for total coliform and refer to Table 9221.III for MPN.
	EC+MUG		Each Tube	No Growth	None	Negative for E. coli. and refer to Table 9221.III for MPN
	EC+MUG		Each Tube	Growth with Gas Displacement and Fluorescence	None	Positive for E. coli and refer to Table 9221.III for MPN.
	EC+MUG		Each Tube	Growth with Gas Displacement and no Fluorescence	None	Negative for E. coli and refer to Table 9221.III for MPN.
	EC		Each Tube	No Growth	None	Negative for fecal coliform and refer to Table 9221.IV for MPN.
	EC		Each Tube	Growth with Gas Displacement	None	Positive for fecal coliform and refer to Table 9221.IV for MPN.

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15.0 Updates to previous version:

Updated online revision