Coliforms, Total, Fecal and *E. Coli*

DOC316.53.01217

USEPA[1] Lauryl Tryptose Broth presumptive test with BGB, EC Medium and EC/MUG confirmation

Method 8001A

Most Probable Number (MPN) Method

Scope and application: For non-potable water and wastewater.

[1] Most Probable Number Method 8001A for wastewater is USEPA-accepted. Method 8001A meets or exceeds the specification criteria stated in *Standard Methods for the Examination of Water and Wastewater*, 19th edition, 9221 Multiple-Tube Fermentation Technique for Members of the Coliform Group.

Test preparation

Before starting

Wash hands thoroughly with soap and water.
Make sure that all of the materials that come in contact with samples are sterile.
Use a dilute bleach solution, bactericidal spray or dilute iodine solution to clean the work area.
Set the temperature of the incubator to 35 ± 0.5 °C (95 ± 0.9 °F). Let the incubator temperature become stable, then add the samples.
For the presumptive test, use Lauryl Tryptose broth. For the total coliform confirmation test, use Brilliant Green Bile (BGB) broth. For the fecal coliform confirmation test, use EC Medium broth. For the *E. coli* confirmation test, use EC Medium with MUG broth. The confirmation test is used to eliminate false-positive results that can occur with the presumptive test.
If all tubes are positive, dilute the sample several times then do the test again. Do this until the dilution series gives both positive and negative tubes. If all of the tubes are negative, the sample was diluted too many times. Do the test again with less serial dilutions.
If more than three dilutions are made, select the three dilutions that are the most equivalent to the sample.
The dilution factor for an undiluted sample is 1.
The bottles of dilution water contain 99 mL of sterile buffered dilution water. When 11 mL of the sample is added to a 99-mL bottle of dilution water, the sample is diluted by a factor of 10 (10x or 10-fold dilution). Before and after the sample is added, make sure to fully mix the bottles.
For USEPA reporting, it is necessary to inoculate the confirmation tubes with an inoculation loop. Cap transfer is not permitted.
To sterilize an inoculating needle, apply heat to the needle with an alcohol or a Bunsen burner until the needle is red hot. Let the needle cool before use.
Refer to Bacteria disposal on page 9 for instructions on correct bacteria disposal.

Items to collect

Description	Quantity
Lauryl Tryptose broth tubes	15
Brilliant Green Bile (BGB) broth tubes	varies
EC Medium broth tubes	varies
EC Medium with MUG broth tubes	varies
Dilution water, buffered, 99-mL, sterile	3 or more bottles
Incubator	1
Alcohol burner	1
Inoculating loop	1

Items to collect (continued)

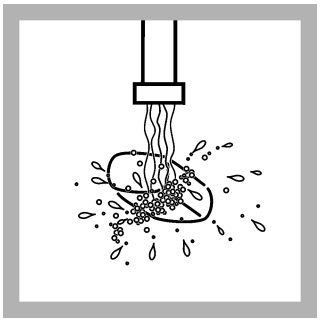
Description	Quantity
Pipet, serological, 10–11 mL, sterile	1
Pipet filler	1
Coliform tube rack	1

Refer to Consumables and replacement parts on page 10 for order information.

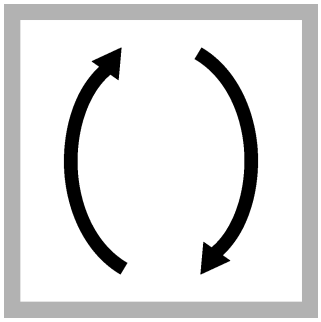
Sample collection

- Use a sterile glass or plastic container such as a Whirl-Pak® bag that contains sterilized sodium thiosulfate. The sodium thiosulfate is not necessary if the sample does not contain a residual disinfectant.
- Open the sample containers immediately before collection and close immediately after collection. Do not put the lid or cap down. Do not touch the lip or inner surfaces of the container. Do not rinse the containers before use.
- To collect a potable water sample from a faucet, spigot, hydrant or pump, let the water flow at a moderate rate for 2 to 3 minutes. Remove any screens or aerators. Do not use faucets or spigots that swivel or leak.
- To collect a non-potable sample from a river, lake or reservoir, remove the cap under water. As an alternative, remove the cap and push the container, mouth down, into the water to prevent the collection of surface scum. Fill the container entirely under water. Put the mouth of the container into the current. Put the cap back on the container.
- Collect a minimum of 100 mL of sample and keep a minimum of 2.5 cm (1 inch) of air space in the container.
- Write the sample information on the container and start the analysis as soon as possible.
- If the analysis cannot be started immediately, keep the sample at or below 10 °C (50 °F) for up to 6 hours. Do not let the sample freeze.
- Failure to collect and transport samples correctly will cause inaccurate results.

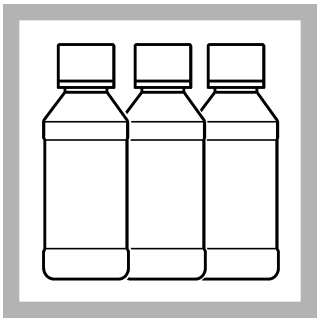
Presumptive test for coliform bacteria (Lauryl Tryptose Broth)



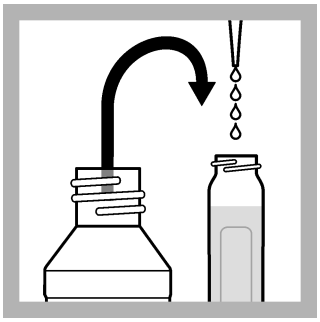
1. Wash hands thoroughly with soap and water.



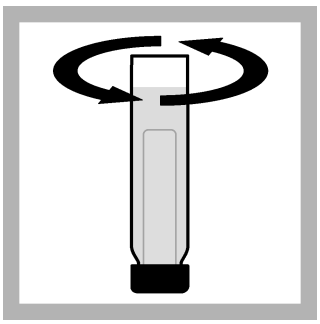
2. Invert the sample for 30 seconds (approximately 25 times) to make sure that the sample is mixed well.



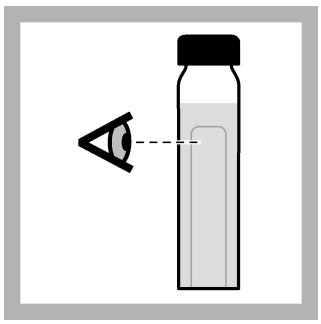
3. Prepare a minimum of three serial dilutions of the sample with sterile buffered dilution water. Refer to Sample dilution on page 8 for dilution instructions.



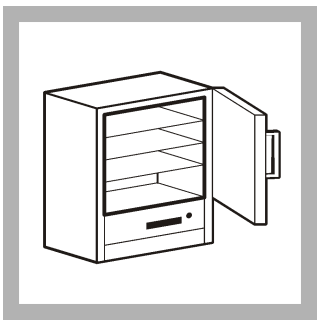
4. Remove the caps from 15 tubes of Lauryl Tryptose broth, one at a time. Use a sterile pipet to add 10-mL portions of each sample dilution into five Lauryl Tryptose broth tubes for the first dilution. Do this two more times for the second and third dilutions. Do not touch the open end of the tubes or the inner surface of the caps.
Immediately replace and tighten the screw cap on each tube.



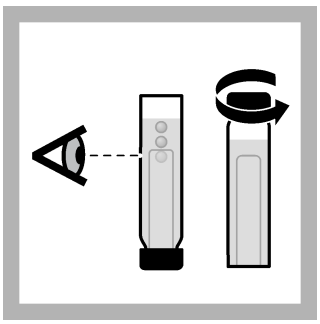
5. Invert the tube. While the tube is inverted, gently swirl until the sample is fully mixed with the nutrient medium.



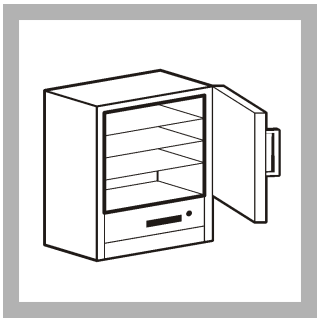
6. Examine the tubes to make sure that the inner vial is full of liquid with no air bubbles.



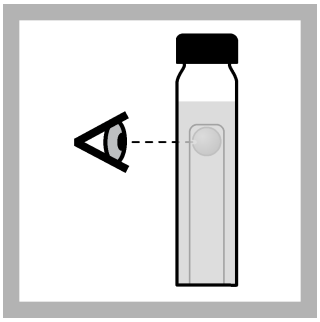
7. Incubate the sample at 35 ± 0.5 °C (95 ± 0.9 °F) for 1 hour.
Bubbles that form in the inner vials during the first hour are not from bacteria.



8. After 1 hour, invert the tubes to remove air from the inner vials. Make sure that there are no bubbles and keep the tubes in a vertical position. Loosen the caps only a little, then put the tubes in the incubator.

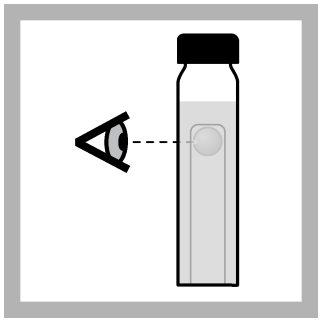


9. Incubate the inoculated confirmation media at 35 ± 0.5 °C (95 ± 0.9 °F) for 24 (±2) hours.
Note: *It is necessary to keep the tubes in a vertical position for the remainder of the test.*



10. After 24 (±2) hours, tap each tube gently and examine the inner vials for gas.
If the broth is cloudy and the inner vials contain gas bubbles, coliform bacteria are likely in the sample. Gas in the inner vial is an indication of coliform bacteria.
If no gas can be seen, put the tubes in the incubator for 24 (±2) hours (for a total of 48 (±3) hours) and examine the tubes again.

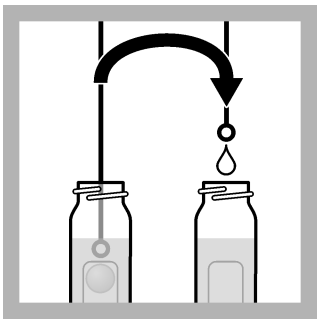
If any gas can be seen, coliform bacteria are in the sample.



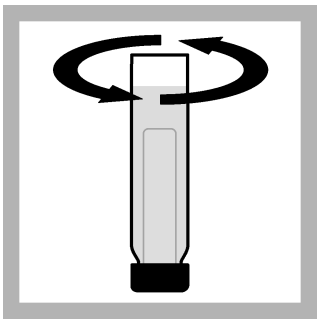
11. Count the number of tubes that contain gas in the inner vial.
Complete a confirmation test for the tubes that contain gas. The confirmation test determines if total coliforms, fecal coliforms or *E. coli* are in the sample. The confirmation test is used to remove false-positive results that can occur with the presumptive test.

If none of the tubes contain gas, the test is negative for coliform bacteria.

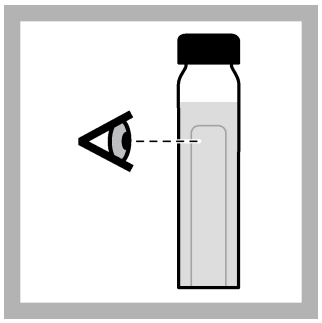
Confirmation test for total coliforms (Brilliant Green Bile Broth)



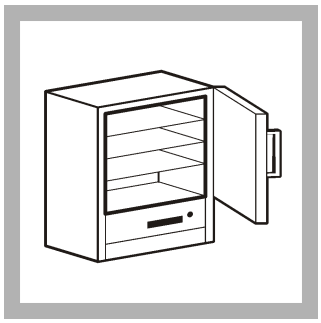
1. From each positive Lauryl Tryptose broth tube, inoculate a Brilliant Green Bile (BGB) broth tube. Use a sterile, disposable inoculation loop or a flame-sterilized, nichrome wire. Put the loop into the positive Lauryl Tryptose broth tube and immediately into a BGB broth tube. Do not touch the rim of the tubes with the loop/wire.
Immediately replace and tighten the screw cap on each tube.



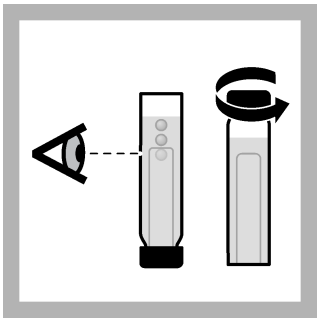
2. Invert the tubes to remove air from the inner vials. Gently swirl, if necessary.



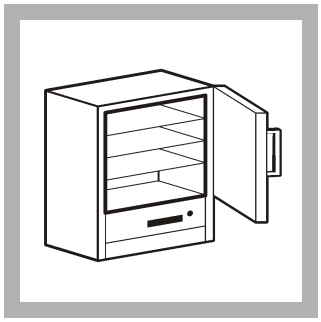
3. Examine the tubes to make sure that the inner vial is full of liquid with no air bubbles.



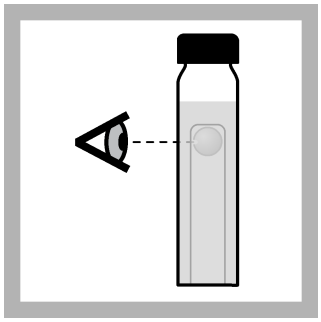
4. Incubate the inoculated confirmation media at 35 ± 0.5 °C (95 ± 0.9 °F) for 1 hour.
Bubbles that form in the inner vials during the first hour are not from bacteria.



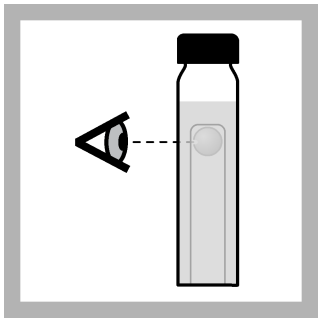
5. After 1 hour, invert the tubes to remove air from the inner vials. Make sure that there are no bubbles and keep the tubes in a vertical position. Loosen the caps only a little, then put the tubes in the incubator.



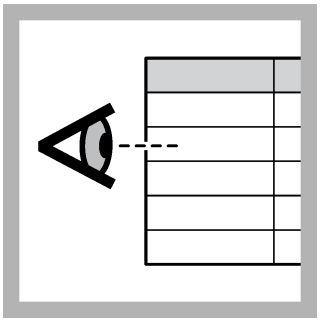
6. Incubate the inoculated confirmation media at 35 ± 0.5 °C (95 ± 0.9 °F) for 24 (±2) hours.
Note: *It is necessary to keep the tubes in a vertical position for the remainder of the test.*



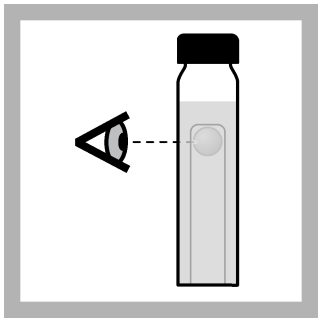
7. After 24 (±2) hours, remove the samples from the incubator. Tap each tube gently and examine the inner vials for gas.
If the broth is cloudy and the inner vials contain gas bubbles, coliform bacteria are likely in the sample. Any gas that shows is an indication of coliform bacteria.
If no gas can be seen, put the tubes in the incubator for 24 (±2) hours (48 (±3) hours total) and examine the tubes again.



8. After 48 (±3) hours, gently tap each tube and examine the inner vials for gas. If the inner vial contains gas bubbles, the test is positive for total coliform bacteria.
If none of the tubes contain gas, then the test is negative for total coliform bacteria.

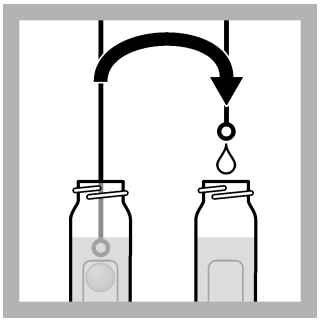


9. Count the number of tubes that contain gas. Refer to Table 2 on page 8 to find the MPN index for each 100 mL sample.

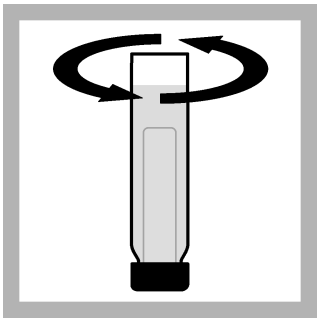


10. If the test is positive for total coliform bacteria, complete a confirmation test for fecal coliform or *E. coli* bacteria (required for USEPA reporting).

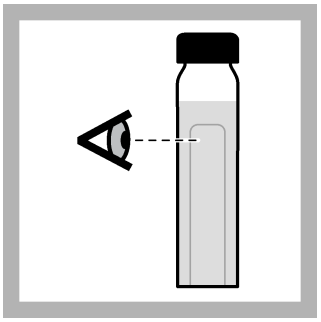
Confirmation test for fecal coliforms (EC Medium)



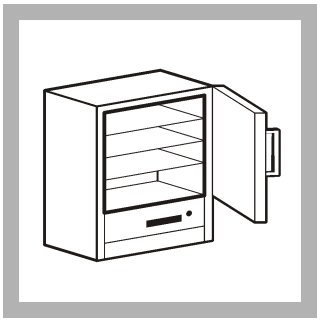
1. From each positive Lauryl Tryptose broth tube, inoculate an EC Medium broth tube. Use a sterile, disposable inoculation loop or a flame-sterilized, nichrome wire. Put the loop into the positive Lauryl Tryptose broth tube and immediately into an EC Medium broth tube. Do not touch the rim of the tubes with the loop/wire. Immediately replace and tighten the screw cap on each tube.



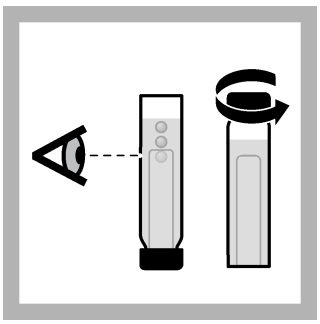
2. Invert the tubes to remove air from the inner vials. Gently swirl, if necessary.



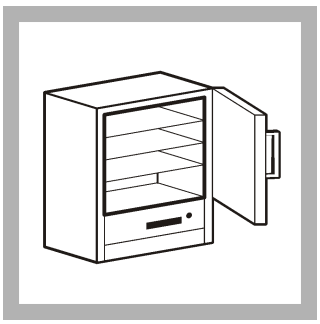
3. Examine the tubes to make sure that the inner vial (if Durham tubes are used) is full of liquid with no air bubbles.



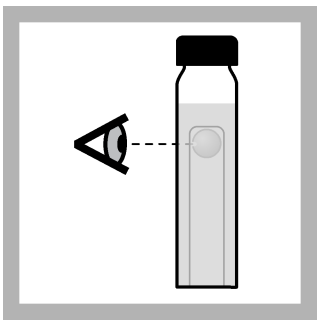
4. Incubate the inoculated confirmation media at 44.5 ± 0.2 °C (112.1 ± 0.5 °F) for 1 hour.
Bubbles that form in the inner vials during the first hour are not from bacteria.



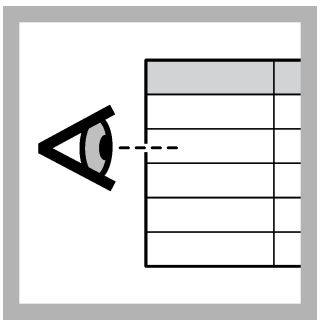
5. After 1 hour, invert the tubes to remove air from the inner vials. Make sure that there are no bubbles and keep the tubes in a vertical position. Loosen the caps only a little, then put the tubes in the incubator.



6. Incubate the inoculated confirmation media at 44.5 ± 0.2 °C (112.1 ± 32.36 °F) for an additional 24 (±2) hours.
Note: *It is necessary to keep the tubes in a vertical position for the remainder of the test.*



7. After 24 (±2) hours, remove the tubes from the incubator. Tap each tube gently and examine the inner vials for gas. If the inner vial contains gas bubbles, the test is positive for fecal coliform bacteria.
If none of the tubes contain gas, the test is negative for fecal coliform bacteria.



8. Count the number of tubes that contain gas in the inner vial. Refer to MPN results to find the MPN for each 100-mL sample.

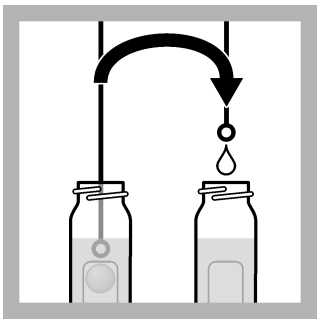
Confirmation test for *E. coli* (EC Medium with MUG broth)

⚠CAUTION

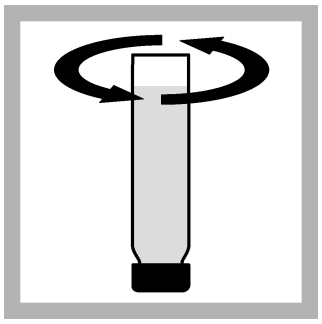
Ultraviolet (UV) light exposure hazard. Exposure to UV light can cause eye and skin damage. Protect eyes and skin from direct exposure to UV light.

When the nutritional media contains MUG, use a long-wave (e.g., 365 nm) UV lamp to confirm the presence of *E. coli*. The sample will fluoresce if *E. coli* is in the sample. No additional confirmation procedure is necessary.

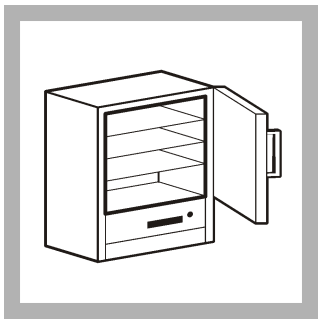
Note: *The sample container can fluoresce slightly. To help with fluorescence detection, use an E. coli Fluorescence Standard. Compare the fluorescence from the sample and the standard.*



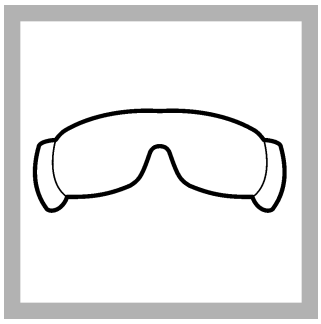
1. From each positive Lauryl Tryptose broth tube, inoculate an EC Medium with MUG broth tube. Use a sterile, disposable inoculation loop or a flame-sterilized, nichrome wire. Put the loop into the positive Lauryl Tryptose broth tube and immediately into an EC Medium with MUG broth tube. Do not touch the rim of the tubes with the loop/wire. Immediately replace and tighten the screw cap on each tube.



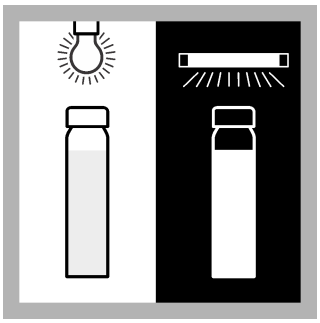
2. Invert the tubes to mix. Gently swirl, if necessary.



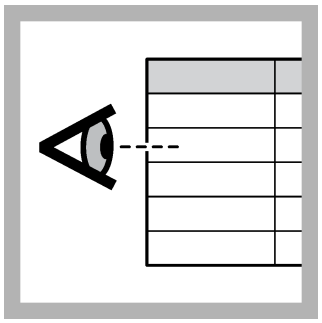
3. Incubate the inoculated confirmation media at 44.5 ± 0.2 °C (112.1 ± 32.36 °F) for 24 (± 2) hours.



4. Put on UV safety goggles



5. Apply UV light to the incubated sample that contains MUG broth with a long-wave UV lamp. Examine the tubes in a dark area. Compare the fluorescence of the sample tubes to a tube that contains a known *E. coli* positive confirmation.
If the sample fluoresces, *E. coli* bacteria are in the sample.
If the sample does not fluoresce, the test is negative for *E. coli*.



6. Count the number of tubes that show fluorescence. Refer to Table 2 on page 8 to find the MPN of the sample.

Sample dilution

Do the steps that follow to make serial dilutions of the sample.
Example: For Class A sludge, add 10 mL of the 100x sample dilution into five tubes, 10 mL of the 1000x sample dilution into another five tubes and 10 mL of the 10,000x sample dilution into the last five tubes. If the coliform density is not known, add five separate dilutions to five sets of five MPN tubes.

1. Wash hands thoroughly with soap and water. Gloves are optional.
2. Vigorously mix the sample for 30 seconds.
3. Open a bottle of sterile buffered dilution water.
4. Use a sterile pipet to add 11 mL of sample into the dilution water bottle.
5. Put the cap on the dilution water bottle and invert for 30 seconds (25 times). This is a 10-fold dilution (sample is diluted by a factor of 10).
6. Add 11 mL of the 10-fold dilution to another dilution bottle (100x dilution). Mix well.
7. Add 11 mL of the 100-fold dilution to the third bottle (1000x dilution). Mix well.
8. Continue to make dilutions until there are three bottles that contain the dilutions listed in Table 1.
   Note: *Do not vigorously shake the sample because this will injure or stress the organisms.*

Table 1 Dilution guidelines by sample type

Sample type	Dilution 1	Dilution 2	Dilution 3
Swimming pool water, chlorinated	undiluted (1x)	10x	100x
Bathing beach water	10x	100x	1000x
Lake water	10x	100x	1000x
Unpolluted river water	10x	100x	1000x
Final wastewater effluent, chlorinated	100x	1000x	10,000x
River water, polluted	1000x	10,000x	100,000x
Storm water	10,000x	100,000x	1,000,000x
Unchlorinated final wastewater effluent	10,000x	100,000x	1,000,000x
Raw sewage	10,000x	1,000,000x	10,000,000x

Example calculation

Do the steps that follow to find the MPN index:

1. Find the MPN index from the positive tubes of the three sets of dilutions. Refer to Table 2.
2. Multiply the MPN index by the Lowest Dilution Factor (LDF).

Example: A sample was diluted into three different buffered dilution bottles with these dilutions: 10x, 100x and 1000x. Five tubes were filled from each dilutions with 15 tubes total. The first group of tubes with the 10x dilution had four tubes with gas. The second group of tubes with the 100x dilution had two tubes with gas. The third group of tubes with the 1000x dilution had one tube with gas. The MPN index from Table 2 for four, two and one positive tubes = 26. The coliform result for the sample is: 26 × 10 = 260 coliforms for each 100 mL of sample.

Table 2 MPN index for dilution groups (for each 100 mL)

Number of positive tubes			MPN index	Number of positive tubes			MPN index
Dilution group 1	Dilution group 2	Dilution group 3		Dilution group 1	Dilution group 2	Dilution group 3	
0	0	0	< 2	4	2	1	26
0	0	1	2	4	3	0	27

Table 2 MPN index for dilution groups (for each 100 mL) (continued)

Number of positive tubes			MPN index	Number of positive tubes			MPN index
Dilution group 1	Dilution group 2	Dilution group 3		Dilution group 1	Dilution group 2	Dilution group 3	
0	1	0	2	4	3	1	33
0	2	0	4	4	4	0	34
1	0	0	2	5	0	0	23
1	0	1	4	5	0	1	30
1	1	0	4	5	0	2	40
1	1	1	6	5	1	0	30
1	2	0	6	5	1	1	50
2	0	0	4	5	1	2	60
2	0	1	7	5	2	0	50
2	1	0	7	5	2	1	70
2	1	1	9	5	2	2	90
2	2	0	9	5	3	0	80
2	3	0	12	5	3	1	110
3	0	0	8	5	3	2	140
3	0	1	11	5	3	3	170
3	1	0	11	5	4	0	130
3	1	1	14	5	4	1	170
3	2	0	14	5	4	2	220
3	2	1	17	5	4	3	280
4	0	0	13	5	4	4	350
4	0	1	17	5	5	0	240
4	1	0	17	5	5	1	300
4	1	1	21	5	5	2	500
4	1	1	26	5	5	3	900
4	2	0	22	5	5	4	1600
—	—	—	—	5	5	5	≥1600

Controls for coliform bacteria tests

Positive and negative controls validate that the test gives a positive result when coliform bacteria are in the sample and a negative result when coliform bacteria are not in the sample. *Pseudomonas aeruginosa* is recommended as a negative control and *Escherichia coli* is recommended as a positive control.

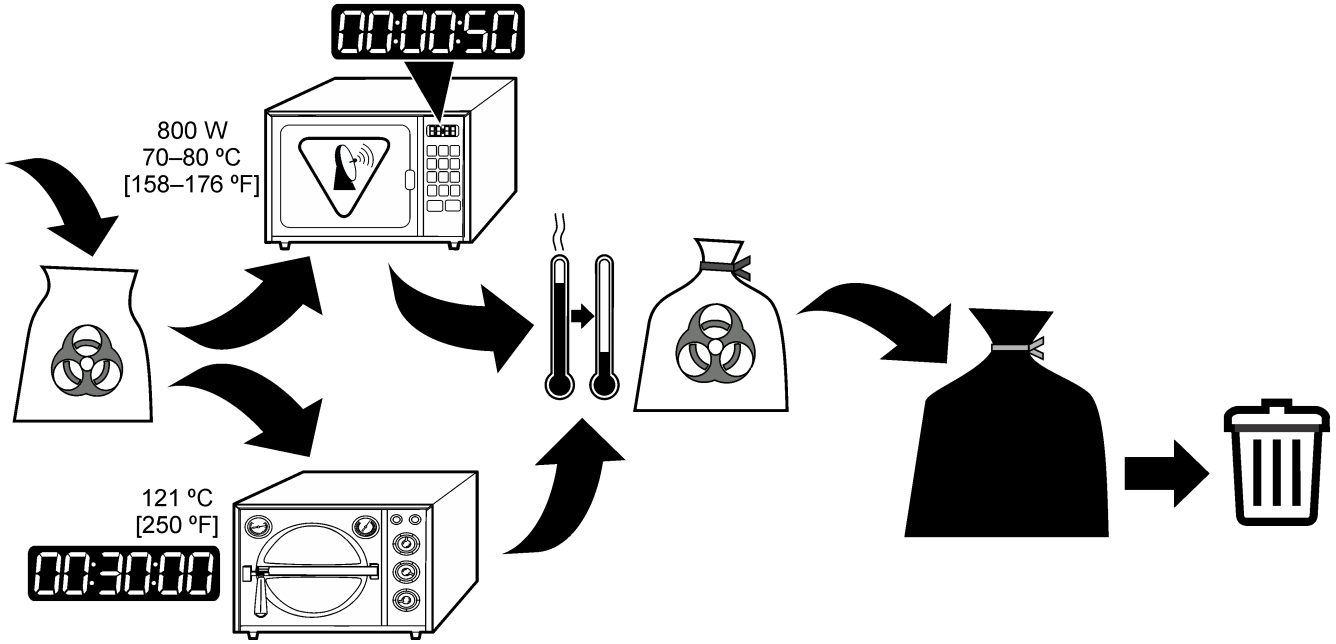
Bacteria disposal

Make sure to kill the cultured bacteria before disposal. Refer to Figure 1 to sterilize with a microwave or an autoclave.
Use one of the methods that follow to kill the cultured bacteria before disposal:

- Hypochlorite (bleach) solution can also be used. Add 1–2 mL of hypochlorite (bleach) solution to each test container. If a container has a lid, do not close it too tightly. Put the container in the microwave at 70–80 °C (158–176 °F) for 50 seconds. Wait 10 to 15 minutes. Pour the liquid down the drain.

- Kill the cultured bacteria with autoclave pressure. Put the used test containers in a contaminated items bag or biohazard bag to prevent leaks. Do not seal the bag. Put the bag in the autoclave at 121 °C (250 °F) for 30 minutes at 15 lb of pressure. When the bag is cool, seal it and put it into a garbage bag. Make sure to tie the garbage bag tightly.

Figure 1 Bacteria disposal



Summary of method

The Most Probable Number (MPN) method, which is also referred to as the Multiple Tube Fermentation (MTF) technique, uses screw-capped tubes that contain sterile broth medium. The tubes contain an inverted inner vial (a Durham tube) for gas collection. Sample is added to the tubes and incubated. If coliforms are in the sample, then gas is formed in the inner vial.

The number of tubes that form gas is used as an estimate of the number of coliform organisms in the sample. When the EC Medium with MUG broth is used, fluorescence under a long-wave UV lamp shows if *E. coli* is in the sample.

Consumables and replacement parts

Required media and reagents

Description	Quantity/Test	Unit	Item no.
Lauryl Tryptose broth tubes	1	15/pkg	2101415
Brilliant Green Bile (BGB) broth tubes	1	15/pkg	32215
EC Medium broth tubes	1	15/pkg	1410415
EC Medium with MUG broth tubes (without Durham tubes)	1	15/pkg	2471515
Dilution water, buffered, 99 mL, sterile[1]	1	25/pkg	1430598

[1] Buffered dilution water is prepared with magnesium chloride and potassium dihydrogen phosphate.

Required apparatus

Description	Quantity/Test	Unit	Item no.
Alcohol burner	1	each	2087742
Sampling bags, Whirl-Pak® without dechlorinating agent, 207 mL	1	100/pkg	2233199
Inoculating loop, nichrome wire	varies	each	2112100
UV lamp, long-wave, 115 VAC	1	each	2184300
UV lamp, long-wave, 230 VAC	1	each	2184302
Laboratory incubator, culture, 110 VAC	1	each	2619200
Laboratory incubator, culture, 230 VAC	1	each	2619202
Pipet, serological, 10–11 mL, sterile, disposable	1	25/pkg	209798
Pipet, safety bulb	1	each	1465100
Rack, coliform tube	1	each	221500

Optional reagents and apparatus

Description	Unit	Item no.
Adapter for rechargeable battery pack, 230 VAC (for 2580300)	each	2595902
Autoclave, 120 VAC	each	2898600
Biohazard bag	200/pkg	2463300
Sampling bags, Whirl-Pak® with dechlorinating agent, 180 mL	100/pkg	2075333
Sampling bags, Whirl-Pak® without dechlorinating agent, 207 mL	500/pkg	2233100
Battery eliminator	each	2580400
Battery pack, rechargeable, for portable incubator 12 VDC	each	2580300
Bottle, sample, sterilized, 100-mL fill-to line, disposable with dechlorinating agent	12/pkg	2599112
Bottle, sample, sterilized, 100-mL fill-to line, disposable with dechlorinating agent	50/pkg	2599150
Bottle, sample, sterilized, 100-mL fill-to line, disposable	12/pkg	2495012
Bottle, sample, sterilized, 100-mL fill-to line, disposable	50/pkg	2495050
E. coli fluorescence standard	each	2361100
Inoculating loops, sterile, disposable	25/pkg	2749125
Isopropyl alcohol	500 mL	1445949
UV Lamp, long-wave, portable, 4 watt	each	2415200
Laboratory marker	each	2092000
Pipet, serological, 1 mL, sterile, disposable, individually wrapped	50/pkg	2092835
Pipet, serological, 10 mL, sterile, disposable, individually wrapped	50/pkg	2092628
Pipet, TenSette®, 1.0–10.0 mL	each	1970010
Pipet tips, TenSette, 1.0–10.0 mL, sterile, individually wrapped	50/pkg	2558996
Pipet Aid, 110 VAC recharger, four replacement filters (UL, CSA approved)	each	2551701
Powder Pillows for buffered dilution water (25 of each)	50/pkg	2143166
Sterilization Indicator, Sterikon®	15/pkg	2811115
Sterilization Indicator, Sterikon®	100/pkg	2811199
Wicks, replacement, for alcohol burner (2087742)	10/pkg	2097810

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