

## USEPA<sup>1</sup> A-1 Medium

## Method 8368

### Most Probable Number (MPN) Method

**Scope and Application:** For non-potable water and wastewater.

<sup>1</sup> Most Probable Number Method 8368, A-1 Medium, is USEPA-accepted for testing non-potable waters. Method 8368 meets or exceeds the specification criteria stated in *Standard Methods for the Examination of Water and Wastewater*, 18th edition, 9221 E. Fecal Coliform Procedure. *USEPA Manual for the Certification of Laboratories Analyzing Drinking Water* states “5.5.3. A-1 medium may be used as an alternative to EC Medium to enumerate fecal coliforms in source water, in accordance with the Surface Water Treatment Rule. A-1 Medium must not be used for drinking water samples.”

#### Before starting the test:

Make sure that all materials used for containing or transferring samples are sterile.

The bottles of dilution water contain 99 mL of sterile buffered dilution water. When 11 mL of sample is added to a 99-mL bottle of dilution water, the sample is diluted by a factor of 10 (10-fold or 10x dilution). Be sure to mix the bottles thoroughly before and after the sample is added.

Refer to Sample Dilution in the Introduction to Bacteria section to find the number of dilutions that must be made for the sample type. For example if Class A sludge, add a 100X sample dilution to five tubes, a 1000X sample dilution to five tubes and a 10,000X sample dilution to five tubes. If the coliform density is not known, add five different dilutions to 5 sets of MPN tubes.

If all the tubes are positive, dilute the sample several more times and repeat the test. If all tubes are negative, the sample was diluted too many times. Repeat the test with less serial dilutions.

If more than three dilutions were made, use three consecutive dilutions that contain both positive and negative tubes.

The dilution factor for an undiluted sample is 1.

No confirmation is needed when using A-1 Medium.

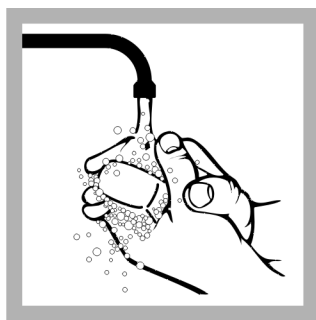
Disinfect the work bench with a germicidal cloth, dilute bleach solution, bactericidal spray or dilute iodine solution. Wash hands thoroughly with soap and water.

#### Collect the following items:

Description	Quantity
A-1 Medium Broth Tubes	15
Dilution Water, buffered, 99-mL, sterile	3 bottles
Incubator	1
Pipet, serological, 10-11 mL, sterile	3
Pipet filler	1
Coliform tube rack	1

See [Consumables and replacement items](#) for reorder information.

## Fecal Coliforms, A-1 Medium Broth, method 8368



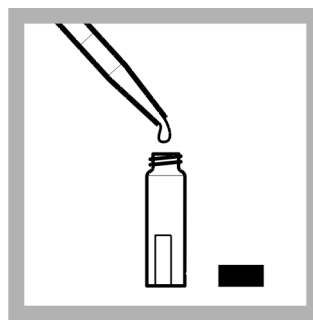
1. Wash thoroughly with soap and water.

Invert the sample for 30 seconds, approximately 25 times, to make sure it is well-mixed.



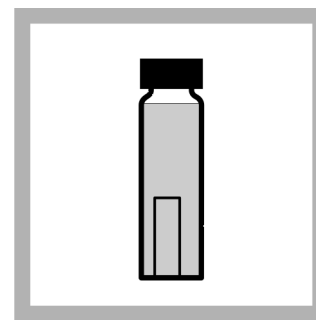
2. Using sterile buffered dilution water, prepare at least 3 serial dilutions of the sample.

Refer to [Sample dilution](#) for instructions.

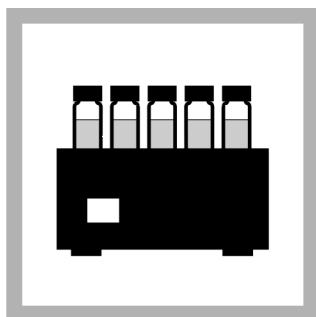


3. Remove the caps from 15 tubes of A-1 Medium Broth. Use a sterile pipet to transfer 10 mL of the first dilution into 5 tubes. Use a sterile pipet to transfer 10 mL of the second dilution into 5 tubes. Use a sterile pipet to transfer 10 mL of the third dilution into the remaining 5 tubes.

Do not touch the open end of the tubes or the inside of the caps.

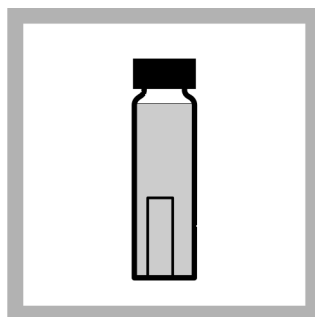


4. Replace the screw cap on each tube immediately after the sample is added. Invert and swirl the tube several times to thoroughly mix the sample with the nutrient medium. After the last inversion, make sure that the inner vial is full of liquid with no air bubbles.



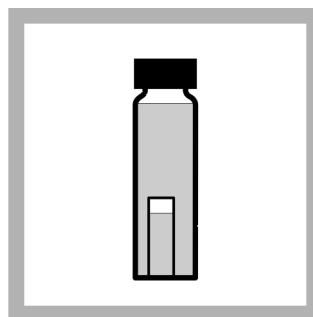
5. Place the tubes in the incubator at a temperature of  $35 \pm 0.5$  °C.

Any bubbles that form in the inner vials during this period are not from bacteria. Be sure to remove the bubbles by inverting the tubes. Make sure there are no bubbles and then carefully return the tubes to an upright position. Return the tubes to the incubator.



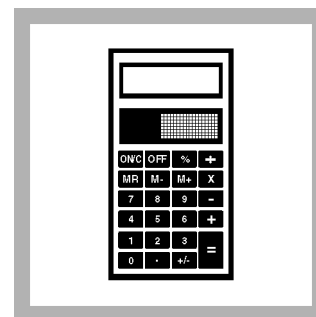
6. After 3 hours, invert the tubes to remove trapped air in the inner vials. Loosen the caps slightly before returning the tubes to the incubator.

Increase the temperature to  $44.5 \pm 0.2$  °C and incubate for an additional 21 hours. The tubes must be kept upright for the rest of the test.



7. After  $24 \pm 2$  hours, tap each tube gently and examine the inner vials for gas. If the inner vials contain gas bubbles, the test is positive for fecal coliform bacteria. Count the number of tubes that contain gas.

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



8. Find the MPN index per 100 mL from the [MPN index table](#).

Multiply the MPN index by the dilution factor in the first set of tubes (lowest dilution used in the test).  
(MPN index x lowest dilution factor = fecal coliform bacteria per 100 mL sample)

See the [Example calculation](#).

## Sample dilution

Complete the following procedure to make serial dilutions of the sample. Refer to the [Dilution guidelines by sample type](#) to find the number of times the sample must be diluted. Use the three dilutions from the [Dilution guidelines by sample type](#) for the test (step 3 of the fecal coliform procedure).

### Procedure

1. Wash hands.
2. Open a bottle of sterile Buffered Dilution Water.
3. Invert the sample container for 30 seconds, approximately 25 times.
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 the sample (for 30 seconds) 25 times. This is a 10-fold or 10x dilution (sample is diluted by a factor of 10).
6. Add 11 mL of the 10x dilution to another dilution bottle and mix well (100x dilution).
7. Add 11 mL of the 100x dilution to a third bottle and mix well (1000x dilution).
8. Continue to make dilutions until there are three bottles that contain the dilutions listed in the [Dilution guidelines by sample type](#).

**Note:** Shaking the sample too vigorously 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 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 effluent	10,000x	100,000x	1,000,000x
Raw sewage	100,000x	1,000,000x	10,000,000x

## Example calculation

A sample was diluted into 3 different buffered dilution bottles. The dilutions were 10-fold (10x), 100-fold (100x) and 1000-fold (1000x). 5 MPN tubes were filled from each dilution (15 tubes total). The first set of tubes (10x) had four tubes with gas, the second set (100x) had 2 tubes with gas and the third set (1000x) had 1 tube with gas.

1. Find the MPN index for the three sets of tubes from the [MPN index table](#).
2. Multiply the MPN index by the lowest dilution factor.

The MPN index from the [MPN index table](#) table for 4, 2 and 1 positive tubes is 26. The coliform result for the sample is  $26 \times 10 = 260$  coliforms per 100 mL of sample.

Table 2 MPN index table

Number of positive tubes			MPN index per 100 mL	Number of positive tubes			MPN index per 100 mL
First dilution set	Second dilution set	Third dilution set		First dilution set	Second dilution set	Third dilution set	
0	0	0	< 2	4	2	1	26
0	0	1	2	4	3	0	27
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	í 1600

### Sample collection, preservation and storage

- Collect at least 100 mL of sample in sterilized Whirl-Pak® bags, sterilized disposable bottles or autoclaved glass or plastic bottles.
- Do not fill sample containers completely. Leave at least 2.5 cm (approximately 1 inch) of air space to allow adequate space for mixing the sample prior to analysis.
- Make sure that the samples are representative of the sample source. Fill sample containers from a tank or reservoir entirely under water.
- Start the analysis as soon as possible after collection. Allow no more than 6 hours to elapse after collection. If the test cannot be started immediately, cool the sample to below 10 °C. Do not freeze. Failure to properly collect and transport samples will cause inaccurate results.

## Controls

Positive and negative controls are important. *Pseudomonas aeruginosa* is recommended as a negative control and *Escherichia coli* as a positive control. Use the AQUA QC-STIK™ Device for quality control procedures. Instructions for use come with each AQUA QC-STIK Device. Potable water samples from municipal treatment facilities should be negative for total and fecal coliforms.

## Bacteria disposal

To safely dispose of bacterial cultures left in the broth tubes, use one of the following methods:

### Bleach

Sterilize used test containers with household bleach. Add 1–2 mL of the bleach to each test container. Allow 10 to 15 minutes contact time with the bleach. Pour the liquid down a drain.

### Autoclave

Place used test containers in a contaminated-items bag or a biohazard bag to prevent leakage into the autoclave. Autoclave the used test containers in the unsealed bag at 121 °C for 30 minutes at 15 pounds pressure. When cool, seal the bag, place it in another garbage bag and tie tightly.

## Summary of method

The Most Probable Number (MPN) method (also referred to as the Multiple Tube Fermentation Technique) uses screw-capped tubes containing sterile broth medium. The tubes contain an inverted inner vial (Durham tube) for gas collection. Sample is diluted, added to the tubes and incubated. If coliforms are present, gas is produced and is trapped in the inner vial.

The number of tubes that form gas is used to estimate the number of coliform organisms in the sample. The MPN method allows for the analysis of highly turbid samples by dilution prior to analysis. No filtering is necessary.

## Consumables and replacement items

### Required media and reagents

Description	Unit	Catalog number
A-1 Medium Broth Tubes	15/pkg	2560915
Dilution Water, buffered, 99-mL, sterile <sup>1</sup>	25/pkg	1430598

<sup>1</sup> Buffered Dilution Water is prepared with magnesium chloride and potassium dihydrogen phosphate.

### Required apparatus

Description	Unit	Catalog number
Bags, Whirl-Pak®, without dechlorinating agent, 207-mL	100/pkg	2233199
Incubator, 12-well Dri-Bath, 120 VAC, 50/60 Hz	each	2281400
Incubator, Culture, 120 VAC, 50/60 Hz	each	2619200
Pipet, serological, 10-11 mL, sterile, disposable	25/pkg	209798
Pipet safety bulb	each	1465100

## Optional media and reagents

Description	Unit	Catalog number
Powder Pillows for buffered dilution water (25 of each) <sup>1</sup>	50/pkg	2143166

<sup>1</sup> Add the contents of one potassium dihydrogen phosphate and one magnesium chloride powder pillow to one liter of distilled water and autoclave (sterilize) to prepare American Public Health Association buffered dilution water.

## Optional apparatus

Description	Unit	Catalog number
Adapter for rechargeable battery pack, 230 VAC (for 2580300)	each	2595902
Bags for contaminated items	200/pkg	2463300
Bags, Whirl-Pak®, with dechlorinating agent, 180-mL	100/pkg	2075333
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, polysulfone, autoclavable (use for buffered dilution water)	12/pkg	2245300
Bottle, sample, sterilized, 100-mL fill-to line, disposable	12/pkg	2495012
Bottle, sample, sterilized, 100-mL fill-to line, disposable	50/pkg	2495050
Bottle, sample, sterilized, 100-mL fill-to line, disposable with dechlorinating agent	50/pkg	2599150
Dechlorinating Reagent Powder Pillows	100/pkg	1436369
Germicidal Cloths	50/pkg	2463200
Incubator, Portable, 12 VDC	each	2569900
Marker, Laboratory	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, sterile, individually wrapped	200/pkg	2558996
Pipet Aid, 110 VAC Recharger, 4 replacement filters (UL, CSA approved)	each	2551701
Rack, Coliform Tube	each	221500



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