

## **THE MICROBIOLOGICAL EXAMINATION OF FOODS & WATER**

Foods and water contaminated with pathogenic microorganisms are major avenues for the spread of infectious diseases. In this country, strict state and federal guidelines regulate food and water processing industries, even so, many outbreaks of diseases linked to contaminated foods or water occur every year.

Municipal water and food products are routinely monitored for both the total number of microorganisms and presence of pathogens, but there are many challenges to doing so. Even nonpathogens, if present in large enough numbers, can cause deterioration of food products and distaste in water. And testing for all types of pathogens is not practical since we do not know which may be present.

The alternative to testing for many different types of pathogens is to test for bacteria that suggest the potential presence of pathogens. This is possible since most pathogen contaminants come from the digestive system of infected humans and animals, which also contain innumerable nonpathogenic bacteria. The presence of these other bacteria in food or water reveals that fecal contamination has occurred (undesirable enough), and a potential presence of pathogens. The most commonly used “indicator bacteria are fecal coliform and fecal enterococci.

### **Coliform Bacteria**

Certain groups of bacteria serve as INDICATORS of fecal contamination. Indicator bacteria are not themselves pathogenic, but are common denizens of the digestive systems of animals. As a result, indicator bacteria are abundant in feces. COLIFORM bacteria are a widely used group of indicator bacteria. Coliform are defined as Gram-negative aerobic or facultative anaerobes, nonspore-forming, rod shaped bacteria that ferment lactose with acid and gas production. *E. coli*, an abundant denizen of the human colon, is the most important indicator of human fecal contamination. Some coliform bacteria are of non-fecal origin, such as *Enterobacter aerogenes*, which may be naturally present in uncontaminated water of lakes and streams. The presence of indicator bacteria does not mean that human pathogens are definitely present, but their presence means that the fecal contamination has occurred, and that pathogens may be present.

### **Counting bacteria**

There are two standard methods of counting bacteria: the standard plate count (SPC) and the most probable number (MPN) methods. The SPC is routinely used for samples that have a relatively large number of bacteria, which can be diluted down and grown as a countable number of colonies in a petri plate. The MPN method is used when there is a small number of bacteria, such as in water, where only a few coliform per liter is unacceptable.

### **Summary of exercise**

1. You will count the total number of bacteria in a sample of spoiled milk using the STANDARD PLATE COUNT technique.
2. You will perform a coliform count of a water sample using the MOST PROBABLE NUMBER (MPN) technique.

## I. STANDARD PLATE COUNT OF BACTERIA IN FOOD PRODUCTS

The STANDARD PLATE COUNT is the most common method used to quantify bacteria in foods. To perform a standard plate count, the food to be tested is suspended in liquid and a sample is then spread over the surface of a solid medium in a petri plate. Bacterial cells present will form colonies that can be counted to determine the number of cells in the original sample. When the objective is to estimate the total number of bacteria, a complex medium called **Plate Count Agar** is commonly used since it will support growth of many different types of bacteria. We call the results the number of **Colony Forming Units (PFU)**, not total bacteria. This is because no single culture medium will support all different types of bacteria, we can only count those that do grow to form a visible colony.

### Serial Dilution of samples

When performing a bacteria count, **between 30 and 300 bacterial colonies** need to be on the plate. A minimum of 30 assures that the data is statistically reliable; however, if there are more than 300 colonies are present, competition for nutrients can suppress growth of colonies. For example, if a sample were to contain  $10^6$  cells/ml, a 1 ml sample would contain  $10^6$  bacteria – far more than the 300 cell limit of a standard plate count – and the sample must be serially diluted:

The standard way to dilute a sample in microbiology is through Serial Dilution. As shown in Figure 1, a sample is diluted step-wise in a series of tubes containing sterile diluent. Each step yields a particular dilution factor:

$$\text{Dilution factor for each step} = \frac{\text{Final volume in the tube}}{\text{Vol of the sample added to the tube}}$$

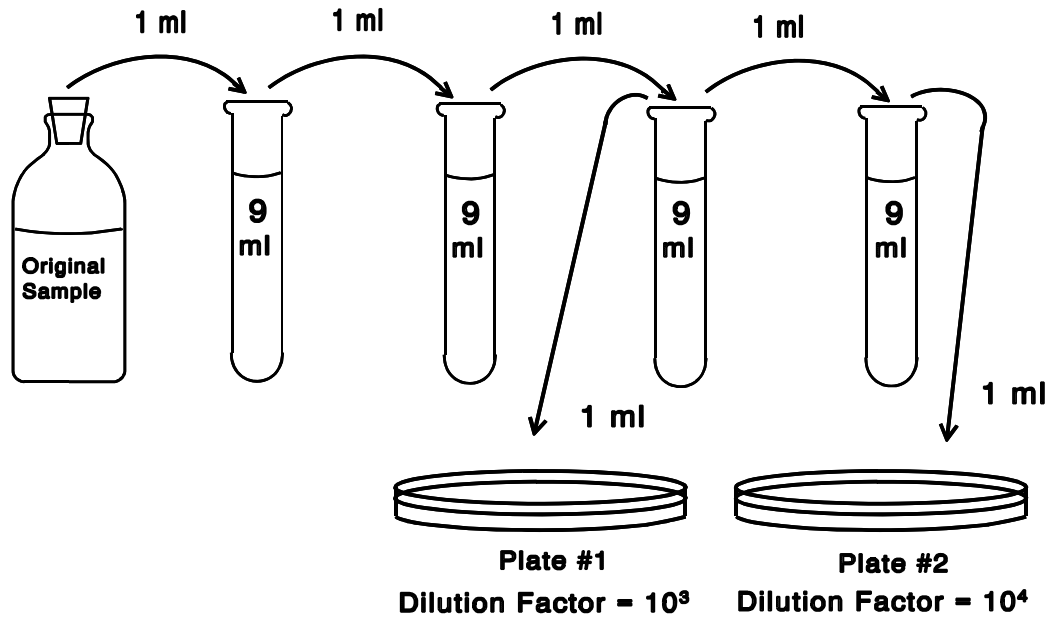
from which the total dilution of the sample can be calculated:

$$\text{Total dilution of a sample plated} = \frac{\text{Product of all dilution steps}}{\text{Vol of the sample plated}}$$

Since the original concentration of bacteria in the milk sample is unknown, we will plate several dilutions, and hope that at least one will yield between 30- 300 colonies on the plate.

**\*\*\*Do the practice problems at the end of this lab exercise; these must be turned in with the results of this lab exercise.\*\*\***

**Figure 1. Example dilution series for a sample.**



## Supplies

sterile 1 ml pipets

7 tubes containing 9 ml of sterile distilled water (yellow caps)

4 plates containing Plate Count bottom agar.

4 tubes containing 4 ml of overlay agar held @ 48°C, clear cap

## Procedures

### 1. Preparing the serial dilutions

As previously noted, some guesswork must be performed to estimate the number of bacteria in food samples. In this case we will estimate that the spoiled milk contains  $10^5$  -  $10^9$  bacteria/ml.

1. Plan your serial dilution clearly and copy them neatly in the space provided on the Results pages.
2. You will plate samples from samples diluted from  $10^4$  to  $10^7$ . (Note that 30 colonies on a plate at  $10^4$  dilution represents an original concentration of  $3 \times 10^5$ , and 300 colonies on a plate at  $10^7$  dilution represents an original concentration of  $3 \times 10^9$ .)

## HELPFUL HINTS

When performing your dilutions keep in mind the following points:

1. Be sure to observe aseptic technique, and use a new sterile pipet for each dilution step. Why?
2. THOROUGHLY MIX each tube before preceding with the next dilution step. However, do not mix so violently that the contents splash near the cap.

## 2. Plating the diluted samples

A 1 ml sample from the selected tubes will be plated using the AGAR OVERLAY METHOD. This technique helps to distribute the cells evenly over the plate surface.

1. Label along the edge of the bottom of 4 bottom agar plates with:
  - a. your name(s) and date
  - b. the dilution factor of the sample plated

Prepare one overlay at a time following this procedure.

2. Working near one of the 45°C water baths, aseptically pipet 1 ml into a tube of soft agar.
3. Mix the overlay thoroughly by rotating the tube between the palms of your hands.
4. Pour the overlay onto the bottom agar, reposition the lid, and gently swirl the plate to spread the overlay.
5. Return the plate to your bench and allow the overlay to solidify.
6. Incubate all of the plates at 37°C for 24 to 48 hours.

## 3. Counting the colonies

The colonies can be easily counted with the **bacterial colony counters** present in the laboratory. Most of the colonies that form will occur within the agar overlay (i.e., below the surface); however, you will also see some colonies growing on the surface of the agar. may suppress formation of colonies and cause an underestimate of the actual bacterial numbers.

1. Place the plate upside down on the colony counter. Turn on the power switch; this will illuminate the plate from below.
2. Count the colonies while viewing through plate through the magnifying glass mounted above it.
3. Mark the petri plate over each colony with a marking pen to indicate which colonies have been counted. Use the Hand tally provided to record the number of colonies counted.
4. When you are done, record your results in Table 1, and properly discard the plates.

## 4. Calculating the original cell concentration

The concentration of bacteria in the original samples is calculated by multiplying the colony counts by the total dilution factor. Only plates that contain between 30 and 300 colonies should be used to calculate the original concentration. If multiple plates fall within this range, then average the concentrations calculated for each plate.

## II. USING THE MOST PROBABLE NUMBER TECHNIQUE TO COUNT COLIFORM IN WATER

In water, only a few coliform per liter can represent a potential health hazard. In this situation, the concentration of cells is too low to count with a standard plate count (no cells may be present within any particular 1 ml sample). The **most probable number (MPN)** technique is one solution to this problem.

### Theory of Most Probable Number counts

The MPN technique is a statistical method of estimating the concentration of bacteria. Imagine a situation where a sample of water contains 100 bacteria per 100 ml. If the bacteria were evenly dispersed throughout the sample, then each 1 ml sample withdrawn would contain 1 cell, and each 1 ml sample would yield visible bacterial growth when cultured in nutrient broth medium. For water containing 10 bacteria per 100 ml, bacterial growth would occur for each 10 ml sample cultured, but in only 1 tube in 10 if 1 ml samples were cultured. *Thus, the number of cultures that show bacterial growth will depend upon the concentration of bacteria and the volume of the sample being tested.* Theoretically, the concentration of bacteria in a sample could be determined by knowing the minimum volume that yields bacterial growth.

For example, suppose samples of different volumes were withdrawn from a sample of water, and these samples were each cultured in a broth medium. We might observe the following results:

<u>Volume cultured</u>	<u>Number of samples cultured</u>	<u>Number of cultures showing bacterial growth</u>
10	10	5
1.0	20	2
0.1	20	0

Since the smallest sample size that yielded growth was 1.0 ml, and growth occurred in 2 of 20 tubes, the number of bacteria in the original sample appears to be 2 cells per 20 ml (i.e., 0.1 cell / ml).

*In the real world bacteria are randomly but not necessarily evenly distributed in a water sample.* Thus, in our example above, if twenty 1 ml samples were cultured, growth might occur in fewer or more than 20 tubes. Some tubes in which growth occurs might actually start with 2 or more cells. If growth occurs, all we know is that at least one viable bacterial cell was present. What a bummer!

However, since the cells are *randomly* distributed, statistical techniques can be used to estimate the number of bacteria in the original sample, i.e., the "most probable number!" A statistical estimate of the number of bacteria can be obtained from an appropriate statistical table, such as the one shown below.

**Table I. MPN Index when five 10 ml samples, one 1 ml sample, and one 0.1 ml sample are**

used.

Number of tubes giving Positive Reaction (acid + gas)*			95% confidence limits		
<u>10 ml</u>	<u>1 ml</u>	<u>0.1 ml</u>	MPN cells per 100 ml	<u>Lower</u>	<u>Upper</u>
0	0	0	<2	0	5.9
0	1	0	2	0.050	13
1	0	0	2.2	0.050	13
1	1	0	4.4	0.52	14
2	0	0	5.0	0.54	19
2	1	0	7.6	1.5	19
3	0	0	8.8	1.6	29
3	1	0	12	3.1	30
4	0	0	15	3.3	46
4	0	1	20	5.9	48
4	1	0	21	6.0	53
5	0	0	38	6.4	330
5	0	1	96	12	370
5	1	0	240	12	3700
5	1	1		88	

\*This table only includes those combinations of positive tubes that occur with a significant frequency. If the other nine possible combinations occur with a greater frequency than 1%, than faulty technique should be suspected.

### The three stages of the MPN procedure

The MPN technique is used to estimate coliform bacteria (not total bacteria), and therefore selective and differential media must be used. There are 3 stages to this method:

**1. The Presumptive test.** The samples are first inoculated into **lactose broth** containing a Durham tube. In a positive culture, growth will occur with the production of BOTH acid and gas. Because few other types of bacteria can ferment lactose and yield acid and gas, a positive presumptive test is a very good indication of the presence of coliform.

**2. The Confirmed test.** Some non-coliform bacteria can yield a false positive in the presumptive test. Thus, all positive lactose broth cultures are subjected to the confirmed test: a loopful of each culture is streaked onto **Eosine Methylene Blue agar (EMB)** and inoculated into **Brilliant Green Lactose Bile Broth (BGLBB)**. EMB is a differential medium; eosine and methylene blue combine to yield a distinctive coloration pattern for coliform bacteria. The presence of bile and brilliant green in BGLBB make this medium selective for coliform bacteria. A confirmed test is positive when colonies with a green metallic sheen form on EMB and gas occurs in BGLBB.

**3. The Completed test.** Cells from an isolated colony on EMB are transferred to an agar slant and again transferred to lactose broth. If acid and gas are again observed, and Gram-negative rods are found in the slant culture, then the identification of coliform is considered positive.



## 2. CONFIRMED TEST

Normally confirmed and completed tests would be run for all positive presumptive tests. You will run a confirmed test for only one tube showing a positive presumptive test.

1. From a tube containing presumptive coliform, inoculate a tube of Brilliant Green Lactose Bile Broth (BGLBB) and streak a plate containing Eosin Methylene Blue (EMB) Agar. Both media are selective for Gram-negative bacteria.
2. Incubate at 37°C for 48 hours.
3. A positive confirmed test will show the presence of dark centered colonies with a metallic green sheen. Other non-coliform colonies have different appearances (see table on next page). Gas formation in BGLBB is also positive. Both tests must be positive for a positive confirmed test.
4. Record your results in Table 2.

### TYPICAL APPEARANCE OF SELECTED BACTERIA ON EMB AGAR

*E. coli* & *Klebsiella pneumoniae*: colonies with a dark center and green metallic sheen

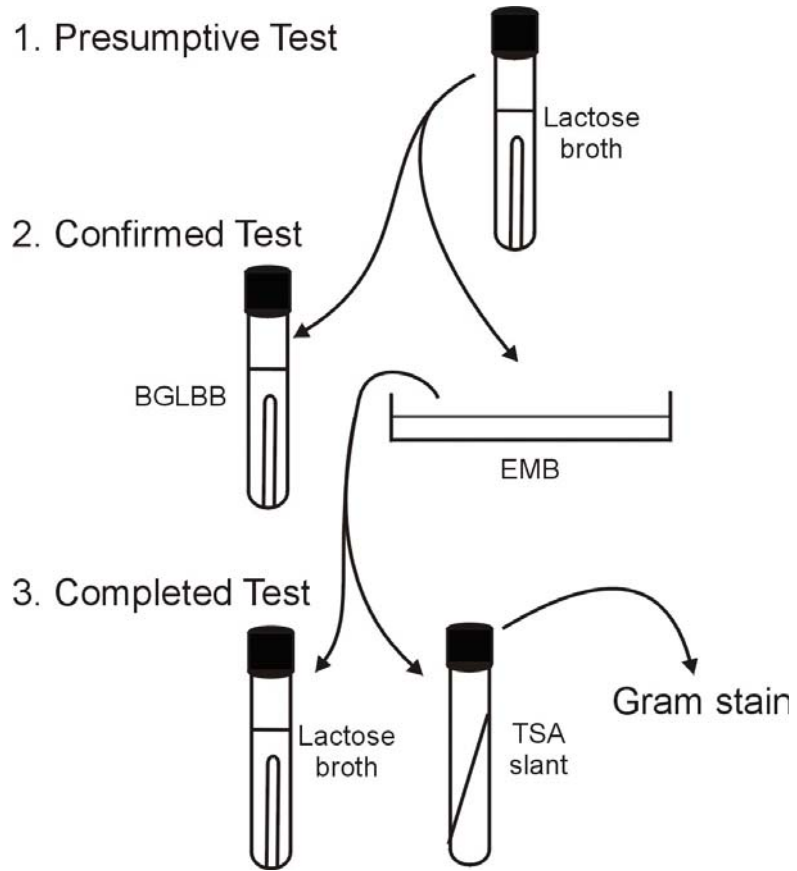
*Enterobacter aerogenes*: pink colonies with no sheen

*Proteus mirabilis* & *Salmonella typhimurium*: colorless colonies

## 3. COMPLETED TEST

1. From an ISOLATED colony with a typical coliform appearance from your EMB PLATE, reinoculate a tube of lactose broth and a TSA slant.
2. Incubate both tubes at 37°C for 24 - 48 hours.
3. A positive completed test is indicated by the formation of acid and gas in the lactose broth, and Gram-negative rods on the nutrient agar.
4. Record your results in Table 2.

**Figure 2. Steps of the MPN procedure**





NAME: \_\_\_\_\_

In a fashion similar to Figure 1, diagram (neatly) the dilution series you used for your Standard Plate Count, and identify the dilution factor for each tube and the total dilution factor for each plate.

**Do the practice problems on pages 14 & 15 first.**

**\*\*Indicate where your results do not agree with your expectations, and write a brief explanation of the discrepancy\*\***

NOTE: The subsurface colonies are very small; they look like little dots in the medium; count these and any surface colonies.

Table 1. Standard plate count of milk sample

	Plate 1	Plate 2	Plate 3	Plate 4
Dilution Factor				
Colony Count				
Cell concentration* (viable cells / ml)				

**Note: express values using appropriate scientific notation (e.g.,  $2.3 \times 10^5$ )**

Which plates had a countable (between 30 - 300) number of colonies?

What is the concentration of bacteria in the original milk sample? (average plates if appropriate)

If you did not get 30 - 300 colonies on any of your plates, what dilutions do you think might have yielded an appropriate number of colonies?

**Results of MPN tests**

**Table 2. Presumptive Test**

	Tube 1		Tube 2		Tube 3		Tube 4		Tube 5	
Tubes receiving:	ACID	GAS	ACID	GAS	ACID	GAS	ACID	GAS	ACID	GAS
10 ml samples										
1.0 ml sample										
0.1 ml sample										

Number of positive tubes (acid + gas)

10 ml samples \_\_\_\_\_

1.0 ml sample \_\_\_\_\_

0.1 ml sample \_\_\_\_\_

MPN (from Table I) = \_\_\_\_\_ x 100 (dilution factor) = \_\_\_\_\_ cells/100 ml

RESULTS OF CONFIRMED TEST

Did any colonies on EMB have a green metallic sheen?  
yes / no

Was gas produced in BGLBB?  
yes / no

Was your confirmed test positive?  
yes / no

RESULTS OF COMPLETED TEST

Were acid and gas produced in the lactose broth? yes / no

Appearance of cell on the agar slant:  
cell shape: \_\_\_\_\_  
Gram-reaction: \_\_\_\_\_

Was your completed test positive?  
yes / no

**Also turn in an answer to this question: (typed)**

The SPC and MPN techniques have advantages and disadvantages for different types of samples. For which situations is each technique best suited (and explain why), and why would the other technique be less useful?

1. What is the equation used to calculate

... an individual dilution factor:

... a total dilution factor:

2. When \_\_\_\_ of a sample is combined with \_\_\_\_ of media the dilution factor is \_\_\_\_

↓	↓	↓
1 ml	9 ml	_____
4 ml	4 ml	_____
5 ml	15 ml	_____
2 ml	8 ml	_____

If the above were performed as a series, the total dilution factor would be \_\_\_\_\_.

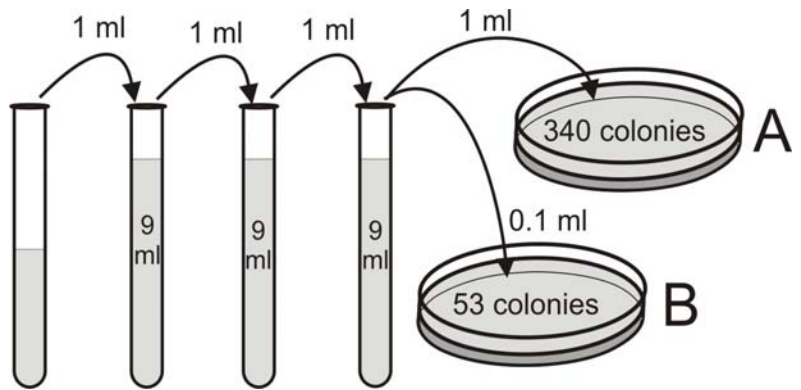
**3. Based upon the dilution series shown here:**

A. The total dilution factors are: (show calculation)

Plate A: \_\_\_\_\_

Plate B: \_\_\_\_\_

B. Which plate has a countable number of colonies? \_\_\_\_



C. What would have been the original concentration of bacteria?

\_\_\_\_\_ (Show calculation)

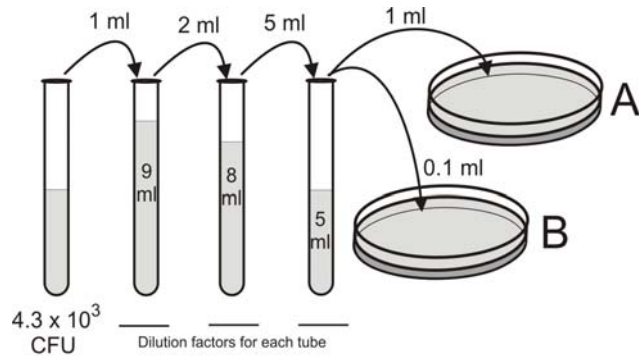
**4. Based upon the dilution series shown here:**

A. Write in the dilution factor for each tube.

B. What is the total dilution factor for each plate?  
(show calculations)

Plate A: \_\_\_\_\_

Plate B: \_\_\_\_\_



C. What is the expected number of colonies on each plate?  
(show calculations).

Plate A: \_\_\_\_\_

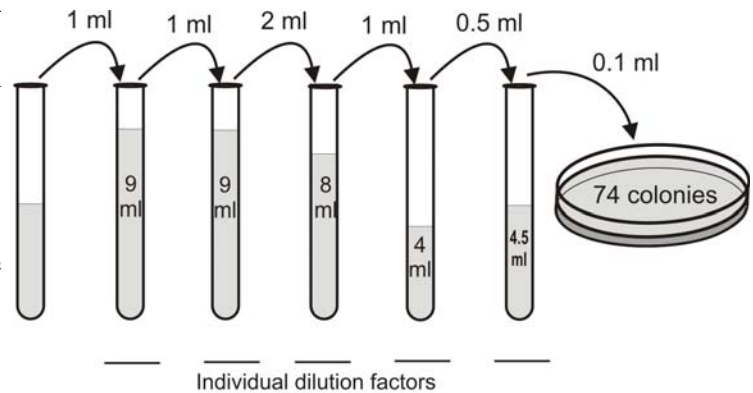
Plate B: \_\_\_\_\_

Which plate has a countable number of colonies? \_\_\_\_\_

**5. Based upon the dilution series shown here:**

A. Write in the dilution factor for each tube.

B. What is the total dilution factor for the plate? (show calculation)



C. What would be the bacteria concentration in the original tube? \_\_\_\_\_  
(show calculation)

