

VIROLOGY AND BACTERIAL GENETIC RECOMBINATION

A virus is a noncellular entity that can replicate within a permissible host cell. Viruses possess no autonomous metabolic activity; they do not carry on respiration or photosynthesis, and are incapable of ATP synthesis. Once a virus contacts a suitable host it begins to infect the cell, and uses the cellular machinery of the host for its own replication. After copies of the viral protein and genetic material are prepared, new virus particles spontaneously assemble. The cell is then ruptured and hundreds to thousands of new viruses are released. For many viruses, the stage of bacterial growth is important to their ability to infect and replicate in the host cell. Often, rapidly dividing cells, such as bacteria in the exponential growth phase, are required.

A virus that infects bacteria is called a **bacteriophage** (literally "bacteria eater"). There are many different kinds of bacteriophages, and each can only infect a single or limited number of bacterial types. Among the most thoroughly studied bacteriophages are those of the "T" group, such as T2, T3, T4, etc. In Part I of this lab exercise, you will study the specificity of a T-type bacteriophage for different bacterial hosts.

Even though we cannot see viruses with a light microscope, we can count viruses using some standard microbiology culture methods. Counting the number of viruses in a sample is called **titration**. The most common procedure used to count bacteriophage is the **plaque assay** procedure. You will use this technique in Part II.

Transformation is one of the three mechanisms by which bacteria can undergo genetic recombination. Through transformation, bacteria can take up naked DNA strands that occur in the medium surrounding the cells. Under natural conditions, bacteria use transformation to obtain new genes from DNA released from dead cells. You will observe bacterial transformation in the final part of this lab exercise.

Summary of exercise

1. You will examine the host specificity of a bacteriophage.
2. You will titrate a bacteriophage using the plaque assay procedure.
3. You will observe acquisition of antibiotic resistance by *E. coli* via bacterial transformation.

I. Host Specificity of Bacteriophages

Viruses are highly specific for the types of cells that they can infect. Most bacteriophage can only infect a single species of bacteria.

Bacteriophage typically will cause lysis of their host cell as the final stage of infection. When bacterial cells in a broth culture are lysed, the turbid medium becomes clearer. The host specificity of a bacteriophage can be determined, therefore, by inoculating the virus into cultures of different bacteria and looking for clearing of the media. The objective of this exercise will be to the host specificity of an unknown bacteriophage.

Supplies

E. coli strain B (light blue cap)

your bacterial unknown

Pseudomonas aeruginosa (green cap)

T2 bacteriophage suspension (violet cap)

6 tubes containing 3.0 ml of Tryptic Phosphate Broth (TPB, clear cap)

Procedure

1. Number the tubes of TPB and inoculate them with bacteria cultures as indicated below.
2. After the tubes have been inoculated with bacteria, use your transfer loop to aseptically inoculate the appropriate tubes, also as indicated below. Flame your loop between each transfer so not to contaminate the T2 suspension with bacteria.
3. Incubate all of the tubes at 37°C for 24 - 48 hours.
4. Record which tubes are clear and turbid in Table 1, and draw conclusions about the host specificity of the bacteriophage.

Inoculation procedure for bacteriophage host specificity exercise.

<u>Tube #</u>	<u>Inoculate with:</u>	<u>Inoculate with bacteriophage?</u>
1	<i>E. coli</i>	—
2	<i>E. coli</i>	Yes
3	Semester Unknown	—
4	Semester Unknown	Yes
5	<i>Pseudomonas</i>	—
6	<i>Pseudomonas</i>	Yes

II. Plaque Assay of T2 Bacteriophage

In this exercise, you will use the **plaque assay** procedure to determine the concentration (or **titer**) of bacteriophage T2. To perform a plaque assay, a sample to be tested is first serially diluted. Agar overlays are then inoculated with the virus and a host bacterium and poured into petri plates. During the incubation period, the bacteria will grow throughout the agar overlay except near a bacteriophage. A small clear zone, called a **plaque**, will form around each bacteriophage. A plaque forms because the replicating virus lyses all the bacterial cells. A plaque is roughly analogous to a bacterial colony in that each plaque arises from an individual isolated virus, just as an isolated colony forms from a single bacterium in a standard plate count. Different types of viruses also produce plaques of distinctive sizes and margins. Initially, the bacteriophage infects and lyses a single bacterial cell. The new bacteriophages released from the lysed cell infect surrounding bacteria, and this cycle is repeated over and over, all of the cells surrounding the original virus particle become lysed, forming the plaque. The original titer of the bacteriophage is calculated by counting the number of plaques on the plate and multiplying this by the appropriate dilution factor. Usually, serial dilution of the sample is necessary to reduce the concentration of the virus so that they become widely dispersed in the agar medium. The agar overlay contains soft agar (0.7%) to allow the bacteriophage to diffuse away from the lysed cell and repeat the replication cycle in neighboring cells.

Not every bacteriophage in a suspension will create a plaque (some viruses may be structurally defective). In fact, the plaque forming efficiency of some bacteriophage is 50% or lower. Thus, the results of a plaque assay are expressed as **plaque forming units (PFU)** per ml, rather than the absolute number of bacteriophage.

Serial Dilution of Samples

The original sample of T2 contains approximately 10^7 PFU per ml – far too many than could be effectively counted on a single plate. Thus, the sample must first be diluted – for statistical and practical reasons, we need between 30 - 300 plaques on the plate to get a valid count. Thus, the T2 sample first must be 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.*****

Supplies

bacteriophage T2 suspension (violet cap) 10 sterile 1.0 ml pipets
2.0 ml of *E. coli* grown in TPB (light blue cap)
5 tubes containing 4.5 ml of sterile TPB (Black cap)
5 tubes of Tryptic Phosphate top agar (Pink cap, in 50°C water bath)
5 plates of Tryptic Phosphate Agar bottom agar

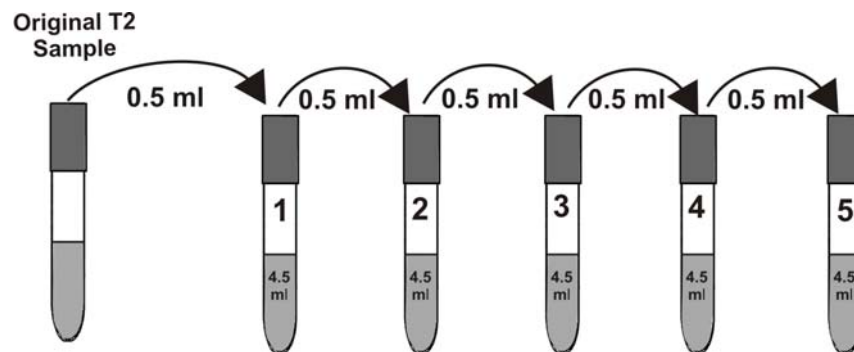
Procedure

The overall steps for serially diluting and pouring the plaque assay plates is shown in Figure 1. 1. WRITE IN the dilution factors for each plate. Prepare the agar overlays while the tubes are kept in the 50°C water bath. However, pour the overlays RELATIVELY QUICKLY after adding the bacteria and bacteriophage so that neither remains at the elevated temperature any longer than is absolutely necessary.

A. Serial dilution of bacteriophage. (Figure 1A)

1. Label the bases of 5 TPB plates with your names and date, and the dilution factor of the T2 suspension to be plated (Plate # 1 will receive no T2.).
2. Align 5 tubes of TPB in your test tube rack, and number them from 1 - 5.
3. Using a sterile 1.0 ml pipet, transfer 0.5 ml of the original T2 suspension to TPB tube #1, and mix THOROUGHLY.
4. Using a fresh pipet, transfer 0.5 ml of the diluted suspension to the next tube in the series, and again mix thoroughly.
5. Repeat step 4 four more times.
6. Calculate and write in the dilution factors for each tube in Figure 1B and in Table 2.

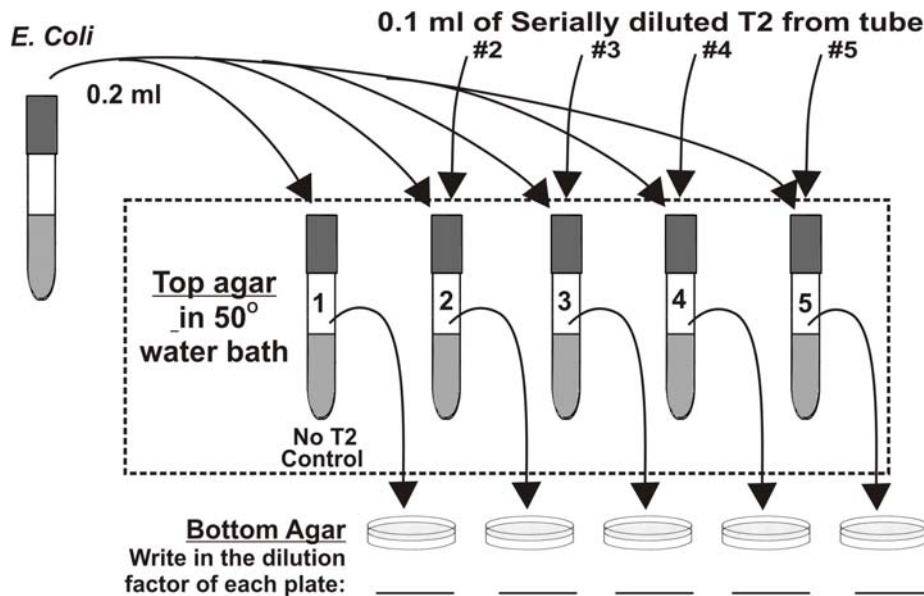
Figure 1A. Using tubes containing 4.5 ml of TPB, serially dilute T2 as shown below.



B. Addition of bacteria to top agar and pouring of control plate. (Figure 1B)

1. Align the 5 tubes of top agar in a rack in the 50°C water bath.
2. Using a single 1 ml pipet, aseptically transfer 0.2 ml of your *E. coli* culture to each tube.
3. Mix each tube thoroughly, and return to water bath.
4. Pour the contents of tube 1 onto plate #1, and replace the cover. Swirl the plate to allow the agar overlay to completely cover the bottom agar.
5. Set the plate aside to solidify. Plate #1 represents a CONTROL that has received no bacteriophage.

Figure 1B. Addition of *E. coli* and T2 to Top Agar, and plating of overlays. Do this at 50°C water bath on side bench.



Note: Review equations on page 3 for calculating individual dilution factors, and then write in the dilution factor for each tube.

C. Addition of bacteriophage to top agar and plating of remaining overlays. (Figure 1B)

1. From dilution tubes 2 - 5, add 0.1 ml of the diluted T2 to the appropriate tube of top agar.
2. After each addition, mix the tube thoroughly (rolling between your hands), and pour the top agar to the appropriately labeled petri plate, and swirl gently to distribute the overlay.
3. Proceed to the next tube in the dilution series, being sure to use a **sterile pipet for each dilution step**.
4. After all of the overlays have been poured and solidified, incubate them inverted at 37°C for 48 hours.

D. Counting plaques.

Observe the plates from the dilution series. Plaques should be clearly visible on one or more plates. Complete clearing may occur for the plate (#2) that received the highest concentration of T2. This is referred to as CONFLUENT LYSIS.

1. Count the number of plaques (use colony counter if necessary) on plates that contain between 30 - 300 plaques.
2. Record your data in Table 2, and calculate the titer (PFU) in the undiluted sample.

III. Genetic Recombination in Bacteria

In this exercise you will induce genetic recombination of *E. coli* via bacterial transformation. Through the process of transformation, the cells will acquire resistance to the antibiotic **ampicillin**. The gene for ampicillin resistance is carried on a **plasmid** (a small circular DNA molecule) called ‘pBLU’, which also carries a gene for an enzyme called ‘**β-galactosidase**’. You will select for transformed cells by plating the cells on medium containing ampicillin and ‘Xgal’ a chemically modified sugar. Only cells that have taken up the plasmid (said to be ‘transformed’) and have acquired ampicillin resistance will be able to grow on this medium. The transformed cells will also produce the β-galactosidase enzyme, which will convert “X-gal” into a blue product (see Figure 2). Thus, as a further indication of genetic transformation, the colonies will turn blue. The basic steps of the procedure are as follows:

1. Cells from an *E. coli* colony are combined with the pBLU plasmid in an ice-cold solution of calcium chloride (CaCl₂) for 15 minutes.
2. The cell suspension is then **heat-shocked** by incubating it a 42°C water bath for 60 seconds.
3. The cells are then incubated on ice for a few minutes.
4. A sample of the cell suspension is then spread over the surface of an Luria Agar medium containing ampicillin and X-gal.

Incubation with CaCl₂ and the heat-shock alter the properties of the bacterial cell wall and render the cells **competent**, i.e., receptive to the uptake of the plasmid DNA.

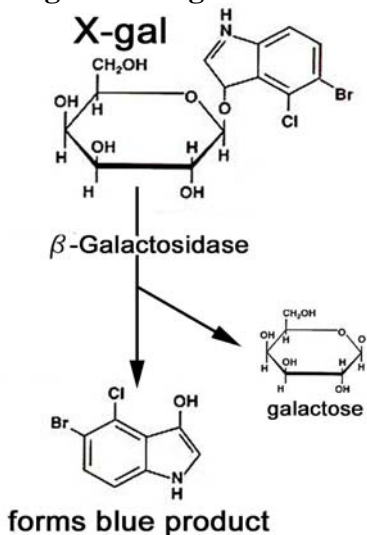
In this lab exercise, each group will treat two cell suspensions. Only one cell suspension will be exposed to the pBLU plasmid during the recombination procedure, the other one will serve as a "control." A sample from both suspensions will be subsequently plated on agar medium either lacking or containing ampicillin.

*** Before beginning, complete Table 3 to predict the expected outcome of the lab exercise. ***

Supplies

2 empty sterile 13 mm test tubes (white cap)	4 sterile 1 ml pipets	vortex
1 tube of sterile 50 mM CaCl ₂ (red cap)	3 plastic 10 μl inoculating loops	
1 tube of sterile Luria Broth (blue cap)	Scissors	
4 Luria agar overlays (brown cap; in 50°C bath)	Plate of <i>E. coli</i> (DH5-α)	
2 plates of Luria bottom agar w/out AMP or X-gal (‘- AMP’)		
2 plates of Luria bottom agar containing AMP+X-gal (‘+ AMP’)		

Figure 2. X-gal Reaction



Procedure (See overview in Figure 3)

A. Labeling of tubes and media

1. Label one of the sterile 13 mm tubes "+pBLU" and the other one "- pBLU."
2. Label the 4 bottom agar plates along the edge with your group name and date.
3. Label one - AMP plate and one +AMP agar plate as "+ pBLU."
4. Label the other -AMP and +AMP agar plate as "- pBLU."

B. CaCl₂ treatment of cells.

1. Using a sterile pipet, aseptically transfer 0.5 ml of CaCl₂ into each of the empty 13 mm test tubes.
2. Place both test tubes in an ice bucket.
3. Using a plastic inoculating loop, obtain a loopful of *E. coli* growth from a starter plate. **Do not scrape media so hard as to transfer any agar; even imperceptible amounts of agar can inhibit transformation.** Transfer the sample by cutting with scissors so that the loop and bacteria fall into one of the test tubes. This is necessary to transfer the sample completely.
4. Completely suspend the cells in the liquid on a vortex machine (present in the lab) for 5 - 10 seconds. Place the tube back in the ice bucket.
5. Repeat steps 3 and 4 for the other test tube.
6. Using a sterile plastic 10 µl inoculating loop, add 1 loopful of the pBLU solution directly into the +pBLU cell suspension. Gently swirl the suspension to mix the plasmid. You have just added 0.05 µg (0.00000005 g) of the pBLU plasmid. Dispose of the pipet in your disposal jar.
7. Incubate both test tubes on ice for 15 minutes.

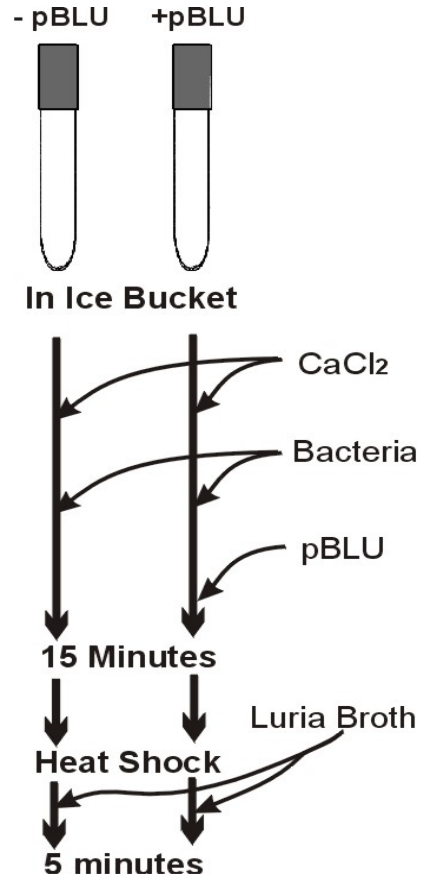


Figure 3. Overview of Transformation procedure

C. Heat shock treatment

1. After the 15 minute cold treatment, "heat shock" the cells by placing both tubes in the 42°C water bath for 60 seconds. Carry your tubes in the ice bucket, and then transfer them directly to the water bath, and after 60 seconds, transfer them directly back into the ice bucket.
2. Incubate the tubes on ice for another 5 minutes.
3. Using a sterile 1 ml pipet, add **1 ml of Luria Broth** to each of the cell suspensions, swirl to mix, and then place both tubes in your test tube rack at room temperature for 5 minutes.

D. Plating of bacteria

The cells can now be plated on the Luria broth agar medium.

1. Using sterile 1 ml pipets, add 0.5 ml of the '+pBLU' cell suspension to two Luria Top Agar overlays (in 50°C water bath), mix thoroughly by rolling between your palms, and then spread over Luria Bottom Agar plates + or - Amp and X-gal, as shown in Figure 4.
2. Repeat the above procedure for the '- pBLU' cell suspension.
3. After the overlays have solidified invert and incubate the plates for 24 to 48 hours at 37°C.

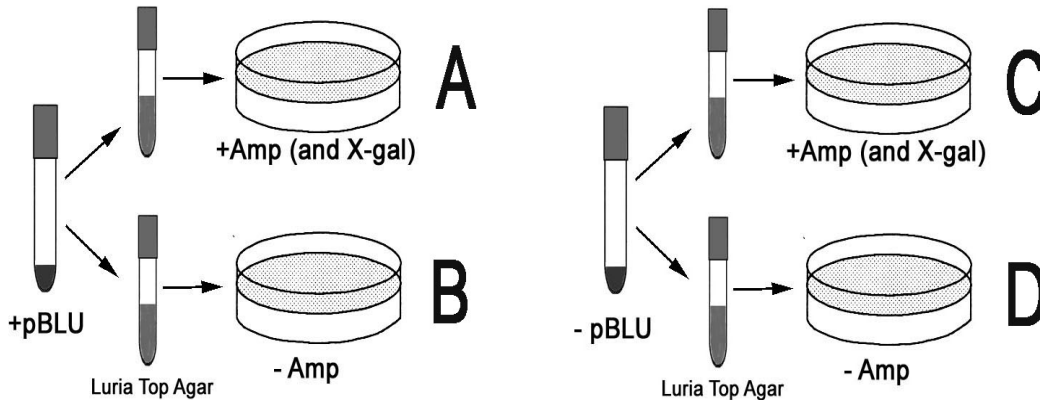


Figure 4. Plating of cell suspensions on bottom agar.

Observing results

Record in Table 4 whether blue, white, or no colonies appeared on each plate. Count the number of colonies in the overlays using a colony counter. Record ">300" if the number of colonies exceeds the countable range.

NOTE: The AMP-resistance gene codes for a B-lactamase enzyme that is secreted from the cell into the surrounding medium, where it then inactivates the ampicillin. After an extended incubation period, so much ampicillin in the medium may become inactivated that non-transformed cells begin to reproduce and form colonies. These unrepressed cells generally form a "halo" of smaller colonies around the original amp-resistant colony.

Tryptose Phosphate Broth		Luria Broth and Agar	
	<u>g/L</u>		<u>g/L</u>
Tryptose	20	Trytone	10
Glucose.	2	Yeast extract. . . .	5
NaCl	5	NaCl	5
Na ₂ PO ₄	2.5	Glucose.	1
		[+ 15 g agar for Luria agar]	

Names: _____

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

NOTE: Swirl the medium to resuspend cells if they have settled out.

Table 1. Results of bacteriophage specificity exercise

Tube Number	Test bacteria	Bacteriophage added	Is the medium turbid? (yes/no)
1	<i>E. coli</i>	no	
2	<i>E. coli</i>	yes	
3	Unknown	no	
4	Unknown	yes	
5	<i>Pseudomonas</i>	no	
6	<i>Pseudomonas</i>	yes	

Which bacteria can serve as a host for this bacteriophage?

Explain the results obtained for your unknown:

Do the practice problems on pages 11 first.

Table 2. Results of plaque assay of T2 bacteriophage

Plate #	# of plaques	Total dilution factor*	Calculated PFU in original sample
1	control (no T2)	-----	
2			
3			
4			
5			
Calculate the PFU in the original sample. -----> Average values for plates with 30 - 300 plaques. Calculate an average if appropriate and circle the data that were used to calculate average. Use scientific notation.			

*See note in manual about plating of 0.1 ml volume of serial diluted samples.

Table 3. Predicted outcome of transformation experiment

Treatment	Indicate the type of bacterial growth expected on medium:	
	containing X-gal & ampicillin	lacking X-gal & ampicillin
Cells exposed to the pBLU plasmid	A	B
Cells not exposed to the pBLU plasmid	C	D

"B" = Blue (transformed) colonies expected

"W" = Growth/colonies will be white

"C" = Confluent growth expected

"-" = no growth expected

Table 4. Results of bacteria transformation exercise

Treatment	Results for plates (colony counts):	
	containing AMP and X-gal Color and colony count	lacking AMP and X-gal Color and colony count
Cells exposed to the pBLU plasmid	A	B
Cells not exposed to the pBLU plasmid	C	D

"B" = Blue (transformed) colonies occurred

"W" = Growth/colonies will be white

"C" = Confluent growth occurred

"-" = no growth occurred

For each plate, describe whether the results meet your predictions; if they do, explain why this result was as expected, if not offer a possible explanation.

Plate

A.

B.

C.

D.

Also turn in answers to these questions. (Typed)

1. Identify as many ways as you can think of that a viral plaque and bacterial colony are A) similar and B) different?

2. How and why would growth on the plates be different in the pBlu transformation experiment if:

A) ampicillin were left out of the culture medium, and

B) if you included the ampicillin but forgot to add X-gal?

Dilution Practice problems – turn in with lab results

Practice problems:

1. Convert to scientific notation
 A. 348600 _____ B. 7925000000 _____

2. Reduce to simplest scientific notation
 A. 135×10^5 _____ B. 0.023×10^6 _____

3. What is the equation used to calculate
 ... an individual dilution factor:

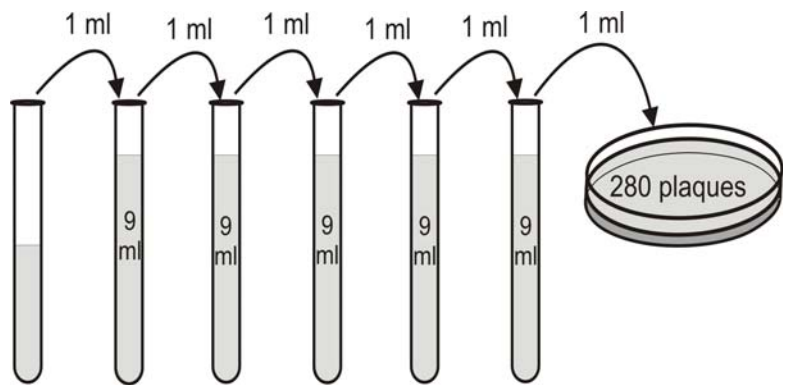
... a total dilution factor:

4. When _____ of a sample is combined
 with _____ of media the
 dilution factor is _____
- | | | |
|------|------|-------|
| ↓ | ↓ | ↓ |
| 1 ml | 9 ml | _____ |
| 2 ml | 8 ml | _____ |
| 3 ml | 6 ml | _____ |
| 5 ml | 5 ml | _____ |

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

5. Based upon the dilution series shown here:

- A. Each step in this series has a dilution factor of _____
- B. The total dilution factor is _____
- C. The original concentration of virus is _____ (Show calculation)



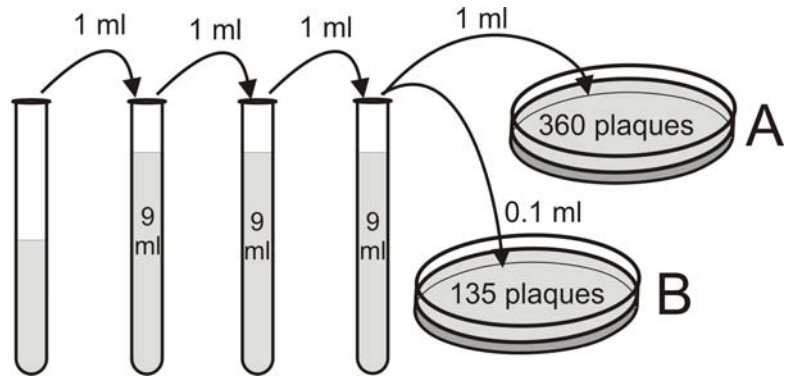
- D. If instead of 1 ml, 0.1 ml of the final dilution were plated, the total dilution would be _____

6. Based upon the dilution series shown here:

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

Plate A: _____

Plate B: _____



B. Which plate has a countable number of plaques? _____

C. What would have been the original concentration of viruses?
_____ (Show calculation)

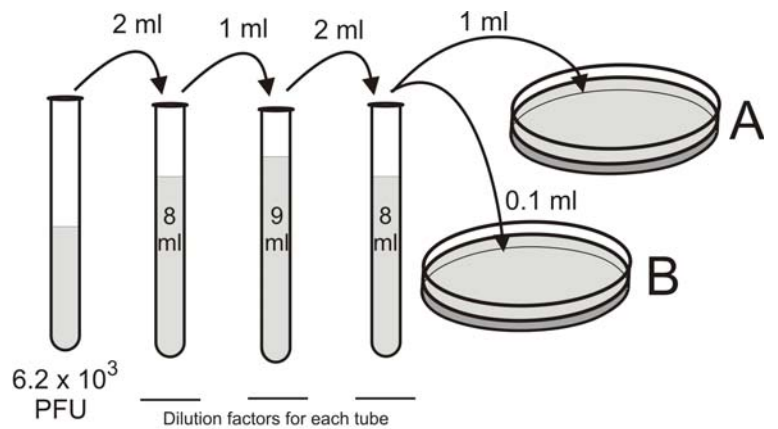
7. Based upon the dilution series shown here:

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 plaques on each plate? (show calculations)?

Plate A: _____

Plate B: _____

D. Which plate yields a countable number of plaques? _____