

VPC Viable Plate Count

Learning Objectives

The student will

- Use aseptic techniques in the safe inoculation of various forms of media.
- Follow oral and written instructions and manage time in the lab efficiently.
- Apply correct terminology regarding microbiological techniques when making observations.
- Use serial dilution to estimate the concentration of microorganisms in a broth culture.

Background/Theory

Estimating the number of bacterial cells in a sample, known as a bacterial count, is a common task performed by microbiologists. The number of bacteria in a clinical sample serves as an indication of the extent of an infection. Quality control of drinking water, food, medication, and even cosmetics relies on estimates of bacterial counts to detect contamination and prevent the spread of disease. Two major approaches are used to measure cell number. The direct methods involve counting cells, whereas the indirect methods depend on the measurement of cell presence or activity without actually counting individual cells. Both direct and indirect methods have advantages and disadvantages for specific applications. (OpenStax CNX, 2018)

The **viable plate count**, or simply **plate count**, is a count of viable or live cells. It is based on the principle that viable cells replicate and give rise to visible colonies when incubated under suitable conditions for the specimen. (OpenStax CNX, 2018) A measured amount of a liquid culture is inoculated onto a plate. The plate is incubated and the colonies that result are counted.

The results are usually expressed as **colony-forming units** per milliliter (CFU/mL) rather than cells per milliliter because more than one cell may have landed on the same spot to give rise to a single colony. Furthermore, samples of bacteria that grow in clusters or chains are difficult to disperse and a single colony may represent several original cells. Some cells are described as viable but nonculturable and will not form colonies on solid media. For all these reasons, the viable plate count is considered a low estimate of the actual number of live cells. These limitations do not detract from the usefulness of the method, which provides estimates of live bacterial numbers. (OpenStax CNX, 2018)

There are two common approaches to inoculating plates for viable counts: the pour plate and the spread plate methods. Although the final inoculation procedure differs between these two methods, they both start with a serial dilution of the culture. (OpenStax CNX, 2018) Serial dilution is necessary because the concentration of cells in even a slightly turbid culture is too large to produce discrete colonies that are countable on a plate.

The **serial dilution** of a culture is an important first step before proceeding to either the pour plate or spread plate method. The goal of the serial dilution process is to obtain plates with CFUs in the range of 30–300, and the process usually involves several dilutions in multiples of 10 to simplify calculation. The number of serial dilutions is chosen according to a preliminary estimate of the culture density. Figure 1 illustrates the serial dilution method. (OpenStax CNX, 2018)

A fixed volume of the original culture, 1.0 mL, is added to and thoroughly mixed with the first dilution tube solution, which contains 9.0 mL of sterile broth, the **dilution blank**. This step represents a dilution factor of 10, or 1:10, or 10^{-1} compared with the original culture. From this first dilution, the same volume, 1.0 mL, is withdrawn and mixed with a fresh tube of 9.0 mL of dilution solution. The dilution factor is now 1:100, or 10^{-2} compared with the original culture. This

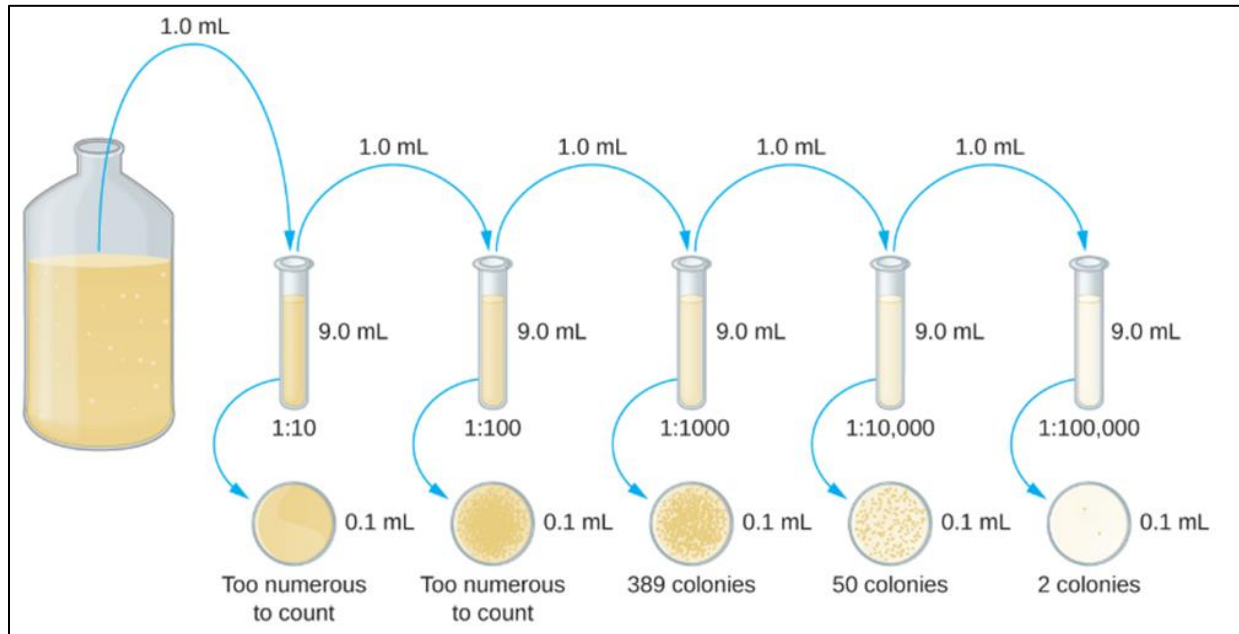


Figure 1 Serial dilution involves diluting a fixed volume of cells mixed with dilution solution using the previous dilution as an inoculum. The result is dilution of the original culture by an exponentially growing factor. (credit: modification of work by "Leberecht"/Wikimedia Commons) (OpenStax CNX, 2018)

process continues until a series of dilutions is produced that will bracket the desired cell concentration for accurate counting. (OpenStax CNX, 2018)

From each tube, a sample (usually either 0.1 mL or 1.0 mL) is plated on solid medium using either the **pour plate method** or the **spread plate method** (figure 2 below). The plates are incubated until colonies appear. Thorough mixing of samples with the dilution medium (to ensure the cell distribution in the tube is random) is paramount to obtaining reliable results. (OpenStax CNX, 2018)

The dilution factor is used to calculate the number of cells in the original cell culture. In our example, an average of 50 colonies was counted on the plates obtained from the 1:10,000 dilution. Because only 0.1 mL of suspension was pipetted on the plate, the multiplier (final dilution factor or FDF) required to reconstitute the original concentration is $10 \times 10,000$ (final dilution factor 1:100,000 or 10^{-5}). The number of CFU per mL is equal to $50 \times 100 \times 10,000 = 5,000,000$. The number of bacteria in the culture is estimated as 5 million cells/mL. (OpenStax CNX, 2018)

Another way to look at it is using this equation:

$$OCD = \frac{CFU}{\text{volume plated}} \text{ or } OCD = \frac{CFU}{FDF}$$

This gives you a result with the unit CFU/mL. The volume plated can also be expressed as the final dilution factor on the plate.

In figure 1, the colony count obtained from the 1:1000 dilution was 389, well below the expected 500 for a 10-fold difference in dilutions. This highlights the issue of inaccuracy when colony counts are greater than 300 and more than one bacterial cell grows into a single colony. (OpenStax CNX, 2018) In other words, with too much growth, colonies begin to become confluent and individual CFUs are not represented accurately as discrete colonies.

Microbiologists typically count plates with 30–300 colonies. Samples with too few colonies (<30) do not give statistically reliable numbers because of sampling error, and overcrowded plates (>300 colonies) make it difficult to accurately count individual colonies. Furthermore, counts in this range minimize occurrences of more than one bacterial cell forming a single colony. Thus, the calculated CFU is

closer to the true number of live bacteria in the population. (OpenStax CNX, 2018) We refer to plates with colony counts in this range as **countable** plates.

Spread Plate Procedure

In this lab, you will plate the samples using the spread plate technique.

1. Aseptically remove the cap from the dilution tube. Because this requires extra dexterity, your partner may hold the cap. Flame the mouth of the tube as usual.
2. With a sterile pipet, draw up 0.1 mL into the pipette, re flame the test tube mouth and replace the cap. Set the tube in a rack.
3. With the plate right-side-up, lift the lid enough to allow the pipet to dispense the liquid on to the center of the plate. Discard the pipet in the disposal container.
4. Remove the bent glass rod or triangular spreader from the jar of EtOH and replace the jar lid. Pass the rod briefly through the burner flame to ignite the alcohol. Hold the horizontal length of the rod below your hand so that the burning alcohol does not run down and burn your fingers. Do not hold the rod over paper or other ignitable materials. Do not shake the rod. The alcohol will sterilize the rod. The flame merely burns off the excess.
5. Spread the sample over the surface of the agar with a back and forth motion while turning the plate. Your non-dominant hand will hold the plate lid above the plate and rotate the base while your dominant hand will use the bent rod.
6. Immediately after spreading the inoculum, place the glass rod back into the alcohol jar and replace the plate's lid. Do not set it on the bench.
7. The plate should remain right-side-up for a few minutes to allow for the inoculum to soak into the agar. It should be incubated up-side down.

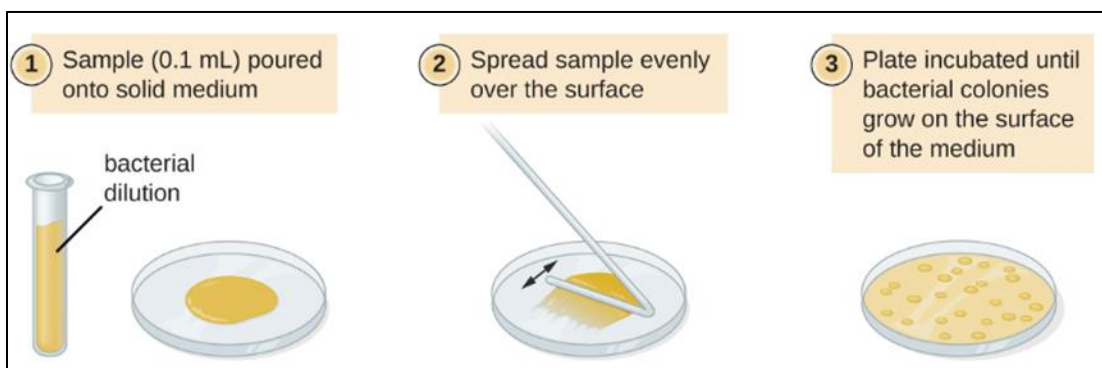


Figure 2. In the spread plate method of cell counting, the sample is poured onto solid agar and then spread using a sterile spreader. This process is repeated for each serial dilution prepared. The resulting colonies are counted and provide an estimate of the number of cells in the original volume samples. (OpenStax CNX, 2018)

Experiment/Exercise

Materials per student pair

- Test tube rack
- 5 sterile tubes
- 6 1.0 mL pipettes
- 1 10.0 mL pipette
- Small pipette pump (blue)
- Large pipette pump (green)

4 TSA plates
 1 Jar EtOH with lid
 1 bent glass rod or metal spreader
 Container of sterile saline

Cultures

Fresh overnight broth

E. coli

Procedure Lab 1

- The protocol. The procedure will follow the diagram in figure 3. Fill in the tube dilution factors and the final dilution factors on the plates in the diagram. There will be a prelab question about this diagram!

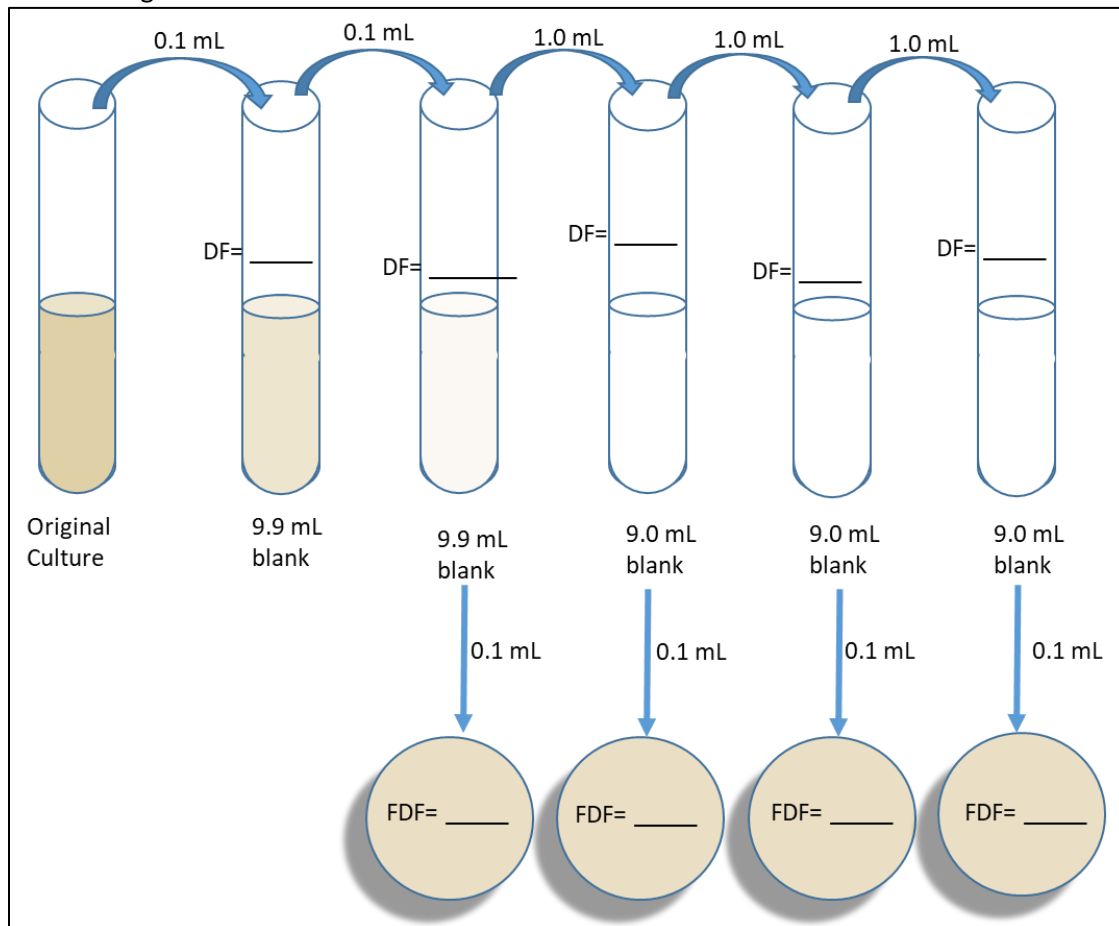


Figure 3 Dilution scheme for this exercise.

- Preparation of materials.
 - Label four plates with the DF indicated in the scheme in figure 3. To each plate, add the information required on every label in this course.
 - Label five sterile tubes with the DF indicated in the scheme in figure 3 and place them in order in the rack. This is the only labeling required because these tubes will not be incubated.
 - Into the 10⁻² and the 10⁻⁴ sterile tubes, aseptically pipet exactly 9.9 mL sterile saline using a 10.0 mL pipet and the green pipet pump.

- d. Into the 10^{-5} , 10^{-6} and 10^{-7} sterile tubes, aseptically pipet exactly 9.0 mL sterile saline using a 10.0 mL pipet and the green pipet pump.
3. Make the first two tubes and the first plate transfers
 - a. Using a 1.0 mL pipet and the blue pipet pump, aseptically transfer exactly 0.1 mL of the original culture into the 10^{-2} dilution blank. Discard the pipet in the appropriate container.
 - b. Roll the 10^{-2} tube between your hands to mix it. Then, with a **fresh pipet**, mix the culture further by drawing up some of the liquid at the bottom of the tube and releasing it at the top of the tube. Repeat the mixing several times.
 - c. With the same pipet, aseptically transfer exactly 0.1 mL of the 10^{-2} dilution to the 10^{-4} blank. Discard the pipet in the appropriate container.
 - d. With a **fresh pipet**, mix the newly created 10^{-4} dilution tube by rolling it between your hands and by drawing the liquid up and down as described above.
 - e. With the same pipet used for mixing, aseptically transfer exactly 1.0 mL of the 10^{-4} dilution into the 10^{-5} dilution blank.
 - f. With the same pipet, transfer exactly 0.1 mL of the 10^{-4} dilution onto the 10^{-5} plate. Discard the pipet in the appropriate container.
 - g. Spread the inoculum on the 10^{-5} plate according to the spread plate method.
4. Make the next set of transfers from the 10^{-5} tube.
 - a. With a **fresh pipet**, mix the newly created 10^{-5} dilution tube by rolling it between your hands and by drawing the liquid up and down as described above.
 - b. With the same pipet used for mixing, aseptically transfer exactly 1.0 mL of the 10^{-5} dilution to the 10^{-6} blank.
 - c. With the same pipet, transfer exactly 0.1 mL of the 10^{-5} dilution onto the 10^{-6} plate. Discard the pipet in the appropriate container.
 - d. Spread the inoculum on the 10^{-6} plate according to the spread plate method.
5. Next set from the 10^{-6} tube.
 - a. With a **fresh pipet**, mix the newly created 10^{-6} dilution tube by rolling it between your hands and by drawing the liquid up and down as described above.
 - b. With the same pipet used for mixing, aseptically transfer exactly 1.0 mL of the 10^{-6} dilution to the 10^{-7} blank.
 - c. With the same pipet, transfer exactly 0.1 mL of the 10^{-6} dilution onto the 10^{-7} plate. Discard the pipet in the appropriate container.
 - d. Spread the inoculum on the 10^{-7} plate according to the spread plate method.
6. Next set from the 10^{-7} tube.
 - a. With a **fresh pipet**, mix the newly created 10^{-7} dilution tube by rolling it between your hands and by drawing the liquid up and down as described above.
 - b. With the same pipet used for mixing, aseptically, transfer exactly 0.1 mL of the 10^{-7} dilution onto the 10^{-8} plate. Discard the pipet in the appropriate container.
 - c. Spread the inoculum on the 10^{-8} plate according to the spread plate method.
7. Allow the plates to stay right-side up for 5 minutes. Stack the plates in order and tape them together with masking tape. Place them upside-down in the location designated for plates to be incubated.

Procedure Lab 2

1. Count the colonies on each plate and record the number in the data table. Colonies are best counted by placing a dot on the bottom of the plate for each colony with a marker. If you get to 100 colonies and have counted less than $\frac{1}{4}$ of the plate, and the growth is fairly even, you can assume that there are more than 300 on the plate. Record "**TNTC**," too numerous to count in the colony column. If the area covered by 100 colonies is greater than $\frac{1}{4}$ of the plate, keep counting. If you reach 300 and still have more uncounted colonies, you can stop and record TNTC.
2. Because each colony represents one CFU originally plated last week, you can determine the number of CFUs. For plates with less than 30 colonies, you should record "**TFTC**," too few to count, in the CFU column. If the number of colonies was TNTC, then the number of CFUs is also TNTC.
3. Calculate the Original Culture Density, OCD. If you have two countable plates, calculate an OCD for each and then average the numbers. (Do not average the CFU numbers first.) Be sure to include the units in the answer.

Lab Report: Viable Plate Count Part 1

Name _____

Lab Section _____

Data and Observations

Plate Dilution Factor	Number of colonies	Number of CFUs represented

	Countable plate DF	Countable plate CFU	Original Cell Density (OCD)
Second countable plate if applicable			

OCD _____

Post Lab Questions

1. Theoretically, how many countable plates should you have? Explain.
2. Give one case in which you could have two countable plates, theoretically.

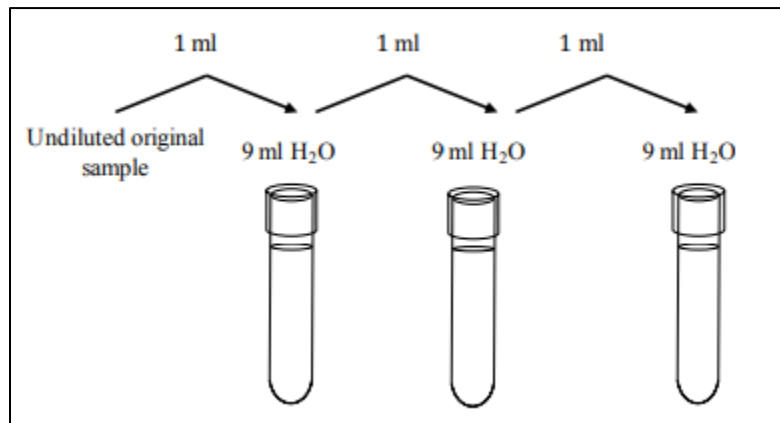
3. Note the results of a standard plate count below.

Plate Dilution Factor	Number of colonies	Number of CFUs represented
10^{-4}	1852	
10^{-5}	231	
10^{-6}	12	

- Calculate the OCD of the culture. Show your work.
- Explain the reasons that the 10^{-6} plate cannot be used in an accurate calculation of OCD.
- Explain why the 10^{-4} plate cannot be used in an accurate calculation of OCD.

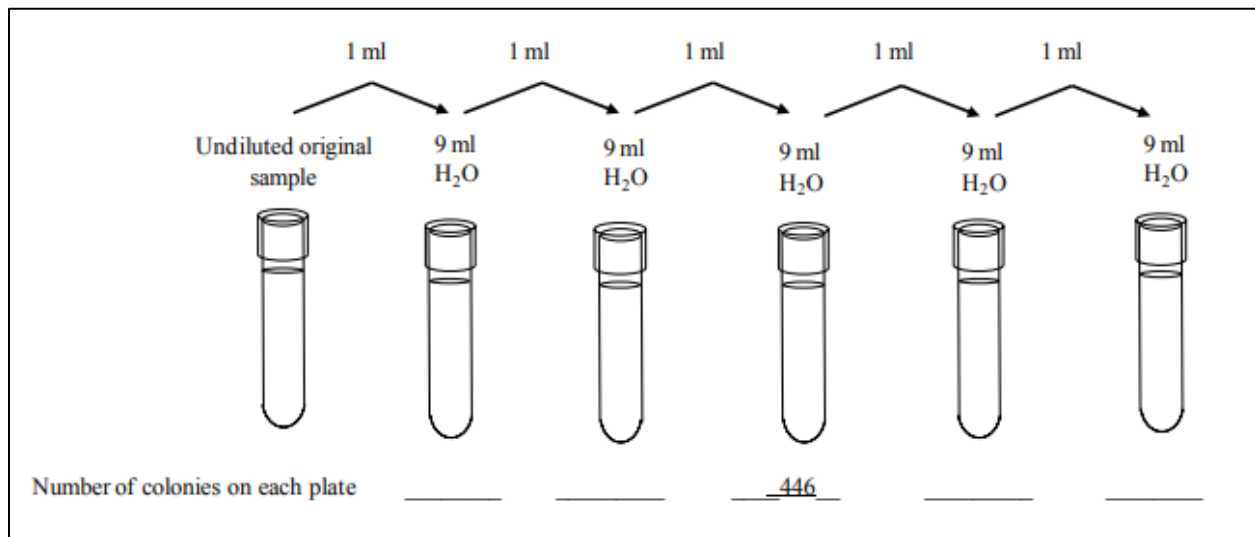
Post Lab Questions (Petersen, 2016)

1. You have a urine sample from a patient that you suspect has a urinary tract infection. You make ten-fold dilutions of this sample as shown below, and then plate 0.1 ml (100 μ L) of the last dilution on a TSA plate. There are 45 colonies on the plate. How many CFUs/ml were in the original urine sample?



2. You have received a sample from a sewage treatment plant, and have been asked to determine how many CFUs/ml are in this sample. You want to make a 1/100,000 fold dilution, but the smallest volume you can measure is 1.0 ml, and the tubes available to you only hold 10 ml. Draw a scheme showing how you would do this.

3. You do a series of dilutions as shown below, and you plate 1.0 ml of each dilution. Given the information below, fill in the number of colonies you theoretically would expect on each of the plates.



4. You do serial dilutions on a water sample, and plate the dilutions on TSA plates. You count the colonies on each of the plates as follows: (Note: TNTC = too numerous to count)

Plate Dilution Factor	Number of colonies	Number of CFUs represented
10^{-2}	Confluent	
10^{-3}	2044	
10^{-4}	352	
10^{-5}	50	
10^{-6}	6	
10^{-7}	0	

Based on these results, what is your estimate for the total number of CFUs/ml in the original sample? Show your calculation.

Lab Report: Viable Plate Count Part 2

Name _____

Lab Section _____

Post Lab Questions

1. If your culture has 2.77×10^4 CFU/mL, what is the FDF that will yield a countable plate? (Hint: Work backwards.) If you plate 0.1 mL, what tube dilution would it come from?

2. If your culture has 6.5×10^7 CFU/mL, what FDF will yield a countable plate? If you plated 0.1 mL, what tube dilution would it come from?

3. A sample has between 9.41×10^5 and 9.41×10^8 CFU/mL. Devise a (one) dilution scheme (with no extra plates) that will ensure you will get a countable plate. This should be a scheme doable with the supplies and measuring devices that we use in LSMCRB 121L. Make sure your scheme is legible! Be sure to show the following:
 - ☐ Amounts in each dilution blank
 - ☐ Amounts transferred to each blank
 - ☐ Amounts transferred to each plate
 - ☐ Dilution factor of each dilution tube
 - ☐ Final dilution factor on each plate.
 - ☐ Count the minimum number of transfer pipettes by designating each pipette with a circled number and showing which transfers are made with each pipette as demonstrated in class.

4. A sample has between 2.64×10^5 and 2.64×10^8 CFU/mL. Devise a (one) dilution scheme (with no extra plates) that will ensure you will get a countable plate. This should be a scheme doable with the supplies and measuring devices that we use in LSMCRB 121L. Include all the information listed above.

5. A sample has between 1.27×10^6 and 6.86×10^9 CFU/mL. Devise a (one) dilution scheme (with no extra plates) that will ensure you will get a countable plate. This should be a scheme doable with the supplies and measuring devices that we use in LSMCRB 121L. Include all the information listed above.
6. Given the parameters in question 5 above, devise a scheme that uses a minimum number of dilution tubes and pipettes.

References

- OpenStax CNX. (2018, Mar 19). OpenStax Microbiology. Retrieved from <http://cnx.org/contents/e42bd376-624b-4c0f-972f-e0c57998e765@4.24>
- Petersen, J. a. (2016). Laboratory Excercises in Microbiology: Discovering the Unseen World Through Hands-On Investigation. CUNY Academic Works. Retrieved from http://academicworks.cuny.edu/qb_oers/16