POUR PLATE DETERMINATION OF BACTERIA NUMBERS

Principle and Purpose

Some microbiological investigations require the determination of the number of microbes present in a culture, solution, etc. There are several means of making this quantitative measurement. In this exercise, students will use two commons methods: the standard plate count and spectrophotometric analysis. The former is based upon the ability of viable organisms to grow on a medium. It will not measure the number of non-viable cells in a culture. In contrast, the spectrophotometric method measures turbidity, i.e., the density of a culture, which can be related to cell number. However, this latter method does not distinguish between live and dead cells. When a standard plate counts and turbidity measurements are performed in conjunction with one another, as will be done in this exercise, a standard curve can be developed to estimate the number of viable cells in a culture based upon spectrophotometry.

The standard plate count method involves the serial dilution of a sample in buffer, water, or broth media. Subsequently, a small volume of selected dilutions is placed on or in media, which is then incubated to permit the formation of single colonies. Presumably, each colony results from the rapid growth of a single viable cell. Using a standard size Petri dish (15 x 100 mm), a successful plate count of a given dilution will result in the formation of 25 to 250 colonies, or better termed ***c***olony-***f***orming ***u***nits, or CFU. This term is used because dead cells cannot be counted in this method, thereby presenting the number of viable cells in a given volume, in this case, milliliters (ml). When the dilution used to generate this value is taken into consideration, the original concentration of cells from a given tube can be calculated.

For example, if a 1 ml volume of a 1 x 10^{-4} dilution plated onto growth media resulted in the formation of 135 colonies, then the concentration of cells in the original tube would be 135 divided by the volume placed on the plate (1 ml), which would then be divided by the dilution factor (1 x 10^{-4}), giving a final answer of 1.35 x 10^{6} CFU per ml. [Note: Such calculations may appear complicated, but are really quite simple. College-level students should have been previously mastered this type of mathematical problem solving. However, for practice examples, see http://crcooper01.people.ysu.edu/microlab/dilution-problems.pdf].

Spectrophotometry measures the turbidity of a sample and is directly related to the density of cells in suspension. Light is transmitted through a sample, which is detected and converted to electrical energy. This energy is then read on a galvanometer or like detector, thereby providing a numerical data point. The greater the number of cells in suspension, the less light that travels through the sample. A decrease in the number of cells in suspension permits more light to travels through the sample. H ence, the reading indirectly reflects the number of cells in suspension. Spectrophotometry is faster than the standard plate count but is limited in sensitivity. Usually, concentrations greater than 1 x 10^{7} cells per ml restricts the amount of transmitted light.

In the following exercise, light will be measured as the Optical Density at 600 nm of light, or commonly referred to as “OD600”. For this purpose, the Model 200E spectrophotometer (“Spec 200E”) will be used for this purpose (for instructions on the use of the Spec 200E, see http://crcooper01.people.ysu.edu/microlab/Using-Spec200E.pdf).

In the present exercise, students will determine the cell number of a culture of *Escherichia coli*

by employing a plate count method. In particular, the pour plate method shall be employed. In addition, students will determine sample turbidities using the Spec 200E. Data from both types of measurements shall be used to develop a standard curve.

Learning Objectives

Upon completion of this exercise, a student will be able to demonstrate the ability to:

- Properly perform a serial dilution scheme;
- Prepare pour plates from aliquots of the serial dilution; and
- Accurately interpret the results of this experiment.

Materials Required

The following materials are necessary to successfully conduct this exercise:

Organisms

- TSB culture (24-48 hour) of *Escherichia coli* (ATCC 25922)

Media

- TSB in bottles
- Molten plate count agar, approx. 18 ml per 16 x 150 mm tube (held at 50-55°C)

Materials

- Sterile serological pipets (1 ml, 5 ml, 10 ml)
- Sterile 13 x 100 mm test tubes with caps
- Sterile plastic Petri dishes

Equipment

- Spectronic 200E spectrophotometer
- Electronic pipettor

Important Techniques/Skill Sets

Students are *strongly encouraged* to review the following videos which demonstrate various techniques. Also, the cited documentation provides important operational information.

Serological pipets. The following videos introduce students to the serological pipet and the various pipettor aides: https://youtu.be/WGLivRvsh5w and https://youtu.be/4VTTE_oWs58. These instruments shall be very important in performing serial dilutions.

Electronic pipettor. In this exercise the electronic pipettor, ThermoFisher S1 Pipet Filler, will be used as the pipet aide. The operating manual is available at the following URL: https://assets.thermofisher.com/TFS-Assets/LCD/manuals/S1-Pipet-Filler-1508880-User-Manual.pdf. The laboratory instructor shall review how to properly use this pipettor.

It is critical to properly control the electronic pipettor so that accurate volumes are transferred. If a student is unfamiliar with the use of a pipettor and serological pipets, it would be prudent to practice delivering a volume of water from one beaker to another. BE SURE NOT TO DRAW FLUID INTO THE ELECTRONIC PIPET! If this occurs, immediately notify the laboratory instructor.

Spectrophotometry. The following video describes the underlying basis of spectrophotometry: https://youtu.be/pxC6F7bK8CU. The Spectronic Model 200E shall be used in this exercise (http://crcooper01.people.ysu.edu/microlab/Using-Spec200E.pdf).

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Procedures

Students shall review and use the BIOL 3702L Standard Practices regarding the labeling, incubation, and disposal of materials.

1) Obtain ten (10) empty, sterile Petri dishes. On the bottom the dishes (NOT the lid), label two as '10^{-4}', two more as '10^{-5}', a third pair as '10^{-6}', the fourth pair as '10^{-7}', and the remaining two dishes as '10^{-8}'. Mark the plates with any additional information as appropriate. Set these plates aside. These will be used in steps 12-16 (see below)
2) Obtain eight (9) sterile 13 x 100 mm test tubes. Label eight of them sequentially from 10^{-1} to 10^{-8}. Label the ninth tube as "Blank".

Before proceeding with this step, as noted previously, students are *strongly encouraged* to view the videos at the following URLs as an introduction to the serological pipet and the various pipettors associated with their use: https://youtu.be/WGLivRvsh5w and https://youtu.be/4VTTE_oWs58.

3) Using the electronic pipettor and a sterile 5-ml serological pipet (or a 10-ml pipet), carefully and aseptically transfer 4.5 ml of TSB (in the bottles provided) to each of the nine test tubes.

Note: The same serological pipet can be used repeatedly in this step unless it becomes potentially contaminated, e.g., set on bench, touched by a hand, etc. If this happens, discard the pipet in the appropriate receptacle and use a new, sterile pipet.

4) Mix the TSB culture of *Escherichia coli* well by rolling it between the hands. To be sure that the bacterial cells are suspended, roll the tube in both palms ten times or more to suspend any sediment of cells that may have formed. Roll the tube quickly, but not so harshly that the broth splashes onto the tube cap or such that it rolls out of the hands causing leakage or breakage.
5) Using the electronic pipettor and a sterile 1-ml serological pipet, carefully and aseptically transfer 0.5 ml of *E. coli* culture to the tube labeled 10^{-1}.

   Set the *E. coli* culture to the side – it will no longer be needed for this exercise

Note: For steps 7-11 detailed below, the same 1-ml serological pipet can be used repeatedly unless it becomes potentially contaminated, e.g., set on bench, touched by a hand, etc. If this happens, discard the pipet in the appropriate receptacle and use a new, sterile 1-ml pipet.

6) Mix the contents of the tube prepared in step 5 above by rolling it between the hands. To be sure that the bacterial cells are suspended, roll the tube in both palms ten times or more to suspend any sediment of cells that may have formed. Roll the tube quickly, but not so harshly that the broth splashes onto the tube cap or such that it rolls out of the hands causing leakage or breakage.
7) Using the electronic pipettor and a sterile 1-ml serological pipet, carefully and aseptically transfer 0.5 ml of cell suspension prepared in the tube labeled 10^{-1} to the tube labeled 10^{-2}.
8) Mix the contents of the tube prepared in step 7 above by rolling it between the hands. To be sure that the bacterial cells are suspended, roll the tube in both palms ten times or more to suspend any sediment of cells that may have formed. Roll the tube quickly, but not so harshly that the broth splashes onto the tube cap or such that it rolls out of the hands causing leakage or breakage.
9) Similar to step 7, aseptically transfer 0.5 ml of the cell suspension prepared in the tube

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labeled 10^{-2} to the tube labeled 10^{-3}.

10) Mix the contents of the tube prepared in step 9 above by rolling it between the hands. To be sure that the bacterial cells are suspended, roll the tube in both palms ten times or more to suspend any sediment of cells that may have formed. Roll the tube quickly, but not so harshly that the broth splashes onto the tube cap or such that it rolls out of the hands causing leakage or breakage.

11) Sequentially, similar to steps 9 and 10, continue transferring 0.5 ml of the cell suspension from the prior dilution to the next labeled dilution tube (i.e., 10^{-3} to the tube labeled 10^{-4}, then 10^{-4} to the tube labeled 10^{-5}, etc.) until the final dilution tube (10^{-8}) receives 0.5 ml of the cell suspension from the 10^{-5} dilution. Be sure to appropriately mix each tube to the subsequent transfer.

Note: DO NOT TRANFER ANY CELL SUSPENSION TO THE "BLANK" TUBE.

At this point, the "Blank" and all test tubes labeled 10^{-1} through 10^{-7} will possess 4.5 ml of cell suspension, except for the tube labeled 10^{-8} which will contain 5 ml of the cell suspension.

Note: In steps 12-16 below, the same serological pipet can be used repeatedly unless it becomes potentially contaminated, e.g., set on bench, touched by a hand, etc. If this happens, discard the pipet in the appropriate receptacle and use a new, sterile pipet. In addition, start the pipetting with the highest dilution (lowest cell concentration, i.e., 1×10^{-8}) working up through the lower dilutions (highest cell concentration, i.e., 1×10^{-4}).

12) Obtain a new, sterile 1-ml serological pipet. From the tube labeled 10^{-8}, use the electronic pipettor and a sterile 1-ml serological pipet to transfer 1 ml of the cell suspension from this dilution to the center of one Petri dish labeled as '10^{-8}'. Repeat this for the second Petri dish labeled as '10^{-8}'. (**Note:** The effective dilution factor is 1×10^{-8}).

13) From the tube labeled 10^{-7}, use the electronic pipettor and a sterile 1-ml serological pipet to transfer 1 ml of the cell suspension from this dilution to the center of one Petri dish labeled as '10^{-7}'. Repeat this for the second Petri dish labeled as '10^{-7}'. (**Note:** The effective dilution factor is 1×10^{-7}).

14) From the tube labeled 10^{-6}, use the electronic pipettor and a sterile 1-ml serological pipet to transfer 1 ml of the cell suspension from this dilution to the center one Petri dish labeled as '10^{-6}'. Repeat this for the second Petri dish labeled as '10^{-6}'. (**Note:** The effective dilution factor is 1×10^{-6}).

15) From the tube labeled 10^{-5}, use the electronic pipettor and a sterile 1-ml serological pipet to transfer 1 ml of the cell suspension from this dilution to the center one Petri dish labeled as '10^{-5}'. Repeat this for the second Petri dish labeled as '10^{-5}'. (**Note:** The effective dilution factor is 1×10^{-5}).

16) From the tube labeled 10^{-4}, use the electronic pipettor and a sterile 1-ml serological pipet to transfer 1 ml of the cell suspension from this dilution to the center one Petri dish labeled as '10^{-4}'. Repeat this for the second Petri dish labeled as '10^{-4}'. (**Note:** The effective dilution factor is 1×10^{-4}).

17) Discard the serological pipet in the appropriate receptacle.

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Note: Prior to adding agar to each Petri dish (step 19 below), students may find the following short video useful in understanding the procedure: https://youtu.be/0e2F6sP12wI.

Also, work as quickly as possible once the molten agar tubes are removed from the incubator so that it does not solidify before you pour it into the plates (step 19).

18) Remove ten (10) tubes containing molten plate count agar from the 50-55°C incubator.

Note: If the incubator is a water bath, use paper towels to remove the excess fluid from the outside of the tube. If properly incubated at 50-55°C, the tube should be slightly uncomfortable to hold. At this point, the molten agar can be used in step 19.

19) For each of the Petri dish prepared in steps 12-16, remove the cap from one tube of molten agar aseptically and carefully pour it into bottom portion of the plate. When the agar is poured, it will be somewhat viscous in nature. Immediately, but gently, swirl the plate several times on the benchtop to mix and disperse the cell suspension previously place din the Petri dish. DO NOT SPLASH THE MOLTEN AGAR! Gently swirl the plate!

    Allow the dish to then sit on the bench until the agar has solidified completely (about 10 minutes).

20) During the time that the agar is solidifying (step 19), use the Spec 200E spectrophotometer to measure the OD600 of the tubes labeled 10^{-1} through 10^{-8} with the "Blank" tube serving to zero the apparatus.

    NOTE: See http://crcooper01.people.ysu.edu/microlab/Using-Spec200E.pdf for instructions on the use of the Spec 200E.

    Record your observations on the report sheet attached to this exercise.

21) When the pour plates have solidified completely, incubate all at 35-37°C for 36-48 hours.

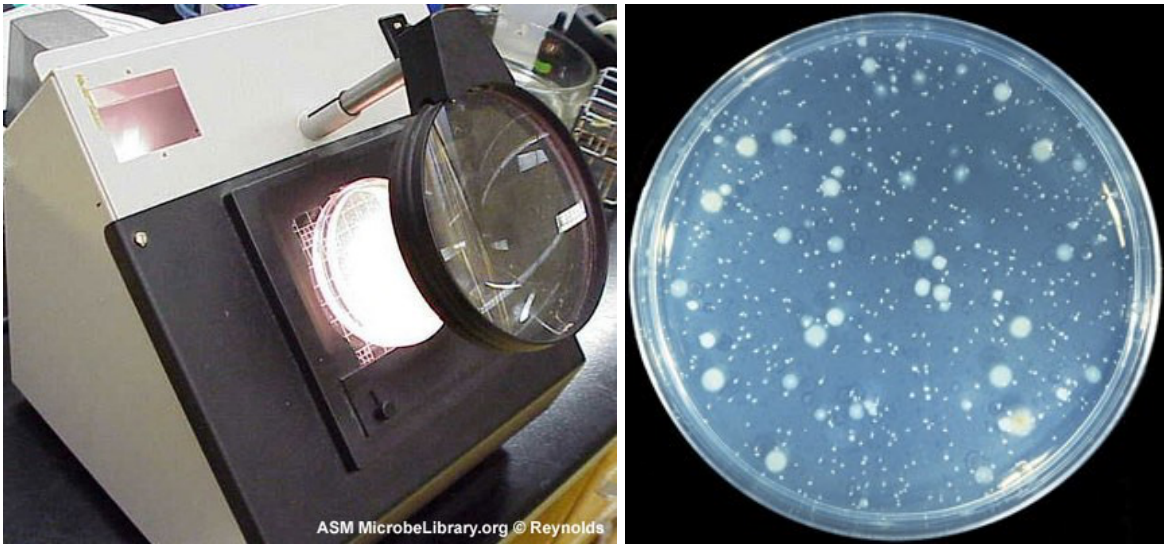

Figure 1. Bacterial Colony Counter and Pour Plate. The bacterial colony counter (left image) is used with pour plates (right image) to help determine cell numbers in a sample. Note that two types of colonies arise in and on the pour plate. The larger round colonies form on the surface of the medium, whereas the small ovoid colonies are growing within the agar medium.

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22) After incubating for 36-48 hours, remove the plates from the incubator. Using a bacterial colony counter (Fig. 1), count the number of bacterial colonies that form on the surface of the agar as well as within the agar. Note that the two types of colonies will appear somewhat different (Fig. 1; right image). Both types should be included in the total colony count per plate.

Record your observations on the report sheet attached to this exercise.

Special Note: Valid plates counts are those that contain between 25 and 250 colonies. Plates that have fewer than 25 colonies are termed "Too Few To Count" or TFTC. Such numbers are statistically unacceptable. Plates containing greater than 250 colonies are likely too close to be accurately counted and are termed "Too Many To Count" or TMTC. Hence, there is no need to count plates have fewer than 25 colonies or those greater than 250.

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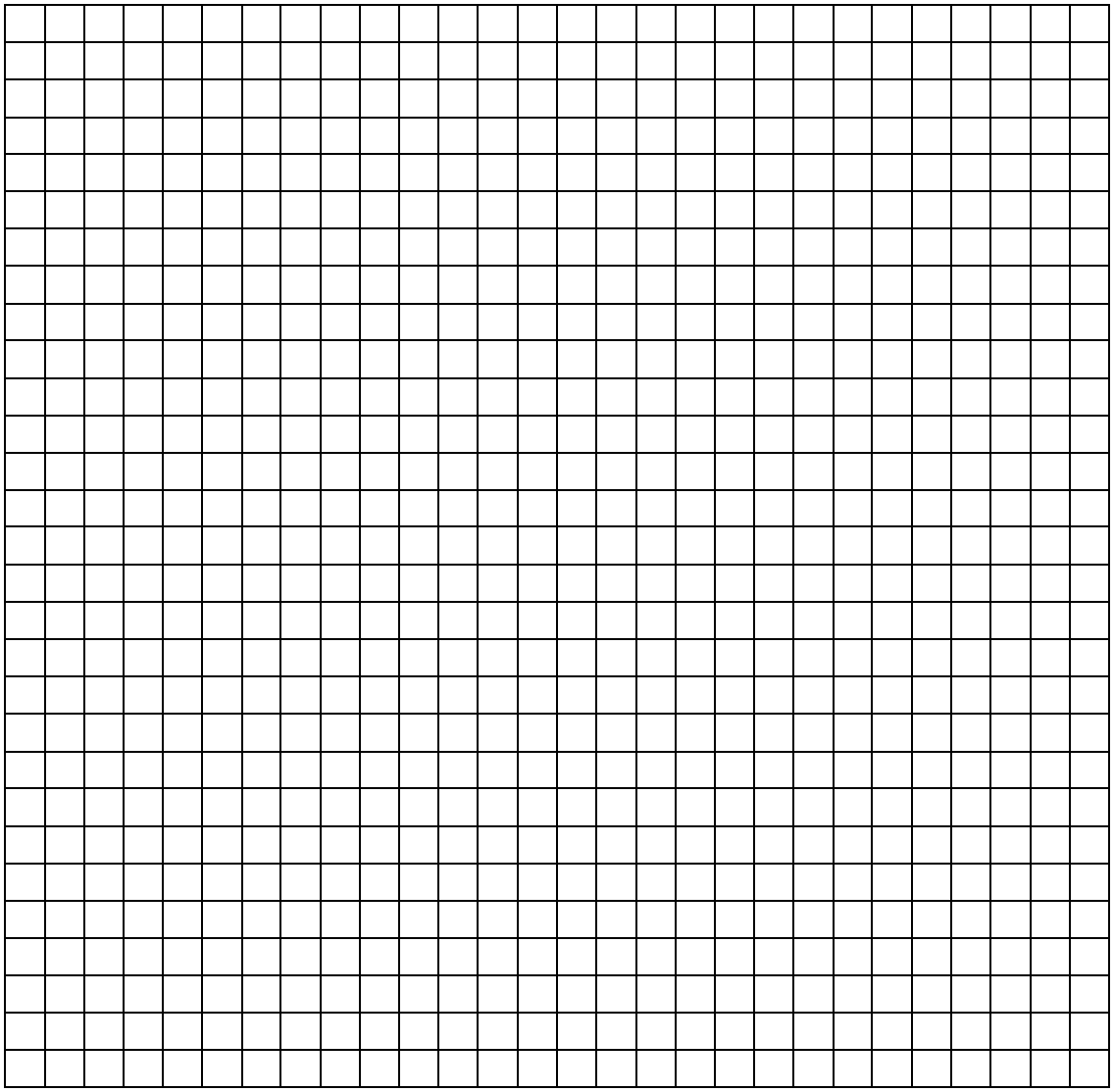
Student Name: _____

COMPLETE THE FOLLOWING TABLES BASED UPON YOUR OBSERVATIONS

Effective Dilution	Plate #1 Colony Count	Plate #2 Colony Count	Average Plate Count	Calculated CFU/ml in Original Culture
1×10^{-4}				
1×10^{-5}				
1×10^{-6}				
1×10^{-7}				
1×10^{-8}				

Effective Dilution	OD600
1×10^{-1}	
1×10^{-2}	
1×10^{-3}	
1×10^{-4}	
1×10^{-5}	
1×10^{-6}	
1×10^{-7}	
1×10^{-8}	

From your data, construct a standard curve by plotting the $\log_{10}$ of the CFU/ml of each dilution on the x-axis and the OD500 of the respective dilution on the y-axis. Be sure to present a figure legend for this graph.



Discussion Question

Based upon your graph above, what would the approximate OD600 be of a sample having 3.5×10^5 CFU/ml? If this cell count came from a sample of the original culture which had been diluted 1×10^{-4} times, what is the number of CFU/ml of the original culture?

Y and proud.

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