DETERMINATION OF MICROBIAL NUMBERS

Principle and Purpose

Many microbiological investigations require the determination of the number of microbes in a culture, aquatic environment, foodstuff, etc. In the research laboratory, this data is important in standardizing and comparing results from different experiments. In everyday life, knowing the number of microbial organisms in water and foodstuffs has a direct impact on the potential health and well-being of humans and other animals.

There are two general means of making quantitative measurements of microbes – direct or indirect. Direct counts involve the actual enumeration of individual cells. Such counting methods mainly employ a microscope to count cells within a known volume of fluid. It can be tedious, yet it is fairly exacting. By comparison, indirect counts use a method to derive cell numbers without actually enumerating individual cells. Such methods include growing a known volume of cells on agar media or the use of instruments to measure the density of cells in a given volume. Indirect methods are most efficient in determining microbial numbers when initially correlated to those data derived from direct counts.

In this exercise, three common methods will be used to count microbes, specifically yeasts, from a broth culture: direct counting using a hemocytometer and indirect counting using a standard plate count as well as spectrophotometric analysis. Each of these methods have nuances that need to be realized and which are presented in the pertinent sections describing these methods. In addition, it is significant to note that many original microbial samples contain far too many cells to be counted directly or indirectly by practical means. Hence, original sources are often diluted to reduce the concentration of cells to a level that can be reasonably quantified by direct or indirect counting methods. A standard method used to accomplish this is the 'serial dilution'.

Serial dilution. A 'serial dilution' consists of a series of stepwise dilutions performed in a sequence that results in a geometric decrease in cell concentration. This process reduces the density of cells in a sample to a more manageable concentration, thereby making it easy to count colonies or cells as well as to make turbidity measurements. Importantly, not only does the concentration decrease throughout the dilution series, but it is critical to know exactly what the total dilution is. The latter is the basis of the 'dilution factor' which is important in calculating the original concentration of cells in a sample.

Note: The following discussion describes concepts that involve simple mathematical calculations, including exponents. Yes, there is math in microbiology and, yes, exponents, are simple. It is essential that students grasp not only the basis of dilutions, but also how dilution factors facilitate the determination of cell numbers in a suspension or other sample. Students may wish to brush up on the use of scientific notation and how to add/multiply/divide exponents.

To understand a serial dilution series and what is involved in determining the dilution factor, consider the scheme shown in Fig. 1. In this example, transferring 10 ml of the original sample (first bottle on left) into the second bottle containing 90 ml of sterile water (or a buffer) results in a 10-fold decrease in the concentration of any organisms present. In effect, this is a 1 to 10 (1/10), or one-tenth, dilution. This can be written in scientific notation as 1×10^{-1}. By definition, the inverse of this number is the 'dilution factor'. In this case, the inverse of 1×10^{-1} is calculated as $1/(1 \times 10^{-1}) = 1 \times 10^{1}$, or 10.

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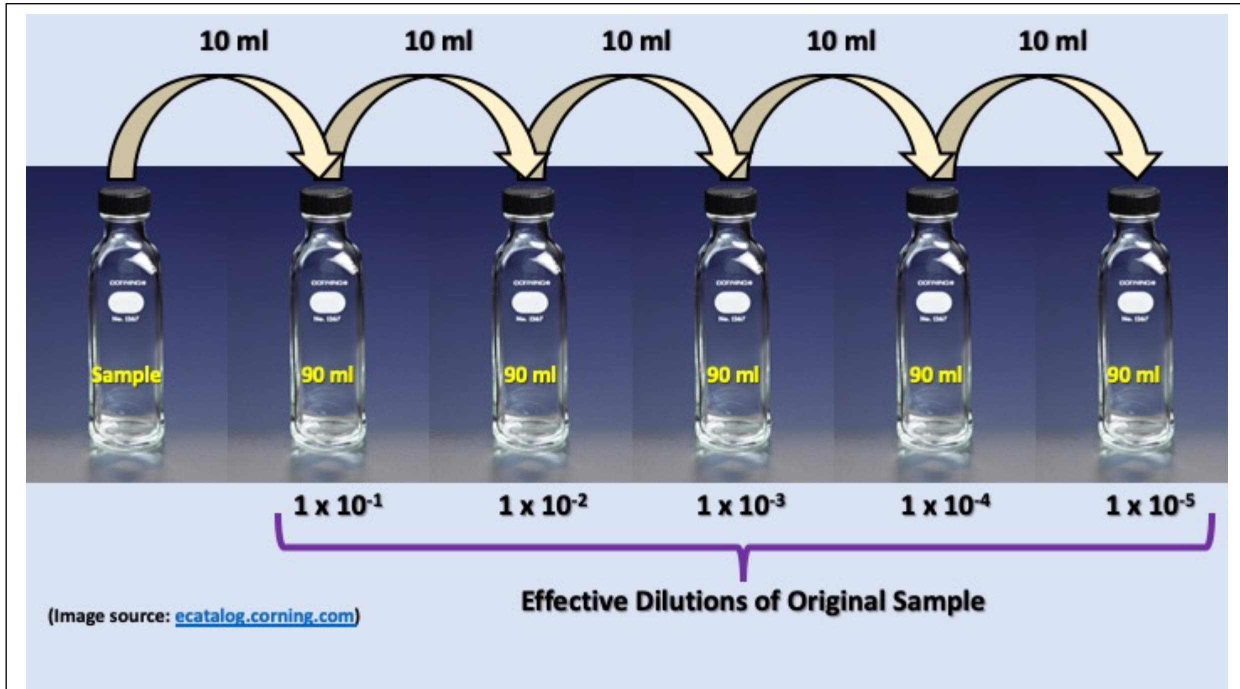

Figure 1. A serial dilution scheme. This scheme demonstrates the derivation of 1 x 10^{-1} through 1 x10^{-5} dilutions of a given sample in which each bottle contains 90 ml of diluent. (Figure by C. R. Cooper, Jr.)

Another way to calculate the dilution factor (DF) is to divide the final volume (V_f) produced by the combination of the aliquot (V_a; in this case, the 10 ml) to the diluent (V_i; i.e., original volume of fluid in the dilution bottle, 90 ml) by the volume of the aliquot (V_a). Thus, in the first dilution shown in Fig. 1, this is expressed as DF = (V_f /V_a) = ([V_a + V_i]/ V_a) = ([10 ml + 90 ml]/10 ml) = ([100 ml]/10 ml) = 10.

Consider the additional dilutions depicted in Fig. 1. If 10 ml of the 1 x 10^{-1} dilution is transferred to 90 ml of water in a second bottle, the sample has in effect been diluted by an additional 10 times, or 1/10, which is also written in scientific notation as 1 x 10^{-1}. However, at this point in the scheme, the actual dilution of the original sample is greater than 1 x 10^{-1}. This is simplecalculated by multiplying the first dilution by the second, i.e., (1 x 10^{-1}) x (1 x 10^{-1}) = 1 x 10^{-2} [simple math, just add the exponents]. Thus, any organisms in this second bottle have been diluted from the original sample by 1/100, or one-hundredth, or as calculated 1 x 10^{-2}. Hence, as shown above, the 'dilution factor' at this point in the scheme would be 1 x 10^{2}, or 100. Based upon this concept, continuation of the serial dilution scheme in Fig. 1 results in dilutions of 1 x 10^{-3}, 1 x 10^{-4}, 1 x 10^{-5} in each of the next three bottles, respectively. The inverse of these values would be the corresponding dilution factors: 1,000; 10,000; and 100,000.

The same results are derived using the formula DF = (V_f /V_a). As previously shown, DF = 10 for the first dilution bottle. The second dilution bottle would also have a DF = 10 because 10 ml was added to 90 ml in the second bottle. Hence, as before, DF = (V_f /V_a) = ([V_a + V_i]/ V_a) = ([10 ml + 90 ml]/10 ml) = ([100 ml]/10 ml) = 10. The total dilution factor at this point is then calculated as the DF of the first bottle multiplied by that of the second, which results in a total

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DF = 100. Likewise, similar calculations result in dilution factors of 1,000, 10,000; and 100,000 for the next three bottles.

These are not necessarily the only dilution factors to consider. In particular, because concentration is determined per unit volume, and since this volume is often a milliliter, then an aliquot of less than 1 ml is essentially a dilution as well. For example, if 0.1 ml of a dilution is used for the plate count method (see below), then this would be considered effectively a one-tenth dilution, or 1×10^{-1}, or a dilution factor of 10. If the 0.1 ml aliquot was taken from the third bottle in the scheme shown in Fig. 1, then the total dilution would be (1×10^{-1}) [effectively the dilution of the aliquot] x (1×10^{-2}) [the dilution of the third bottle] = 1×10^{-3}, or a dilution factor of 1,000. The concept that a volume of less than 1 ml effectively represents a dilution is integral to the use of a hemocytometer (see Direct Counting below).

As previously noted, serial dilutions and dilution factors are important in conducting proper direct and indirect cell counting methods. How these concepts are integral to quantifying microbes will be presented in the following sections describing specific direct and indirect methods for counting.

Direct Count Method. One method for the direct counting of microbial cells uses a special counting chamber known as a hemocytometer (Fig. 2). These slide-like instruments come in various styles including disposable and non-disposable models. Yet, each measures the number of all cells in a

Figure 2. Disposable hemocytometer. This particular hemocytometer has two sets of grids for counting cells. http://www.bulldog-bio.com/c_chip.html

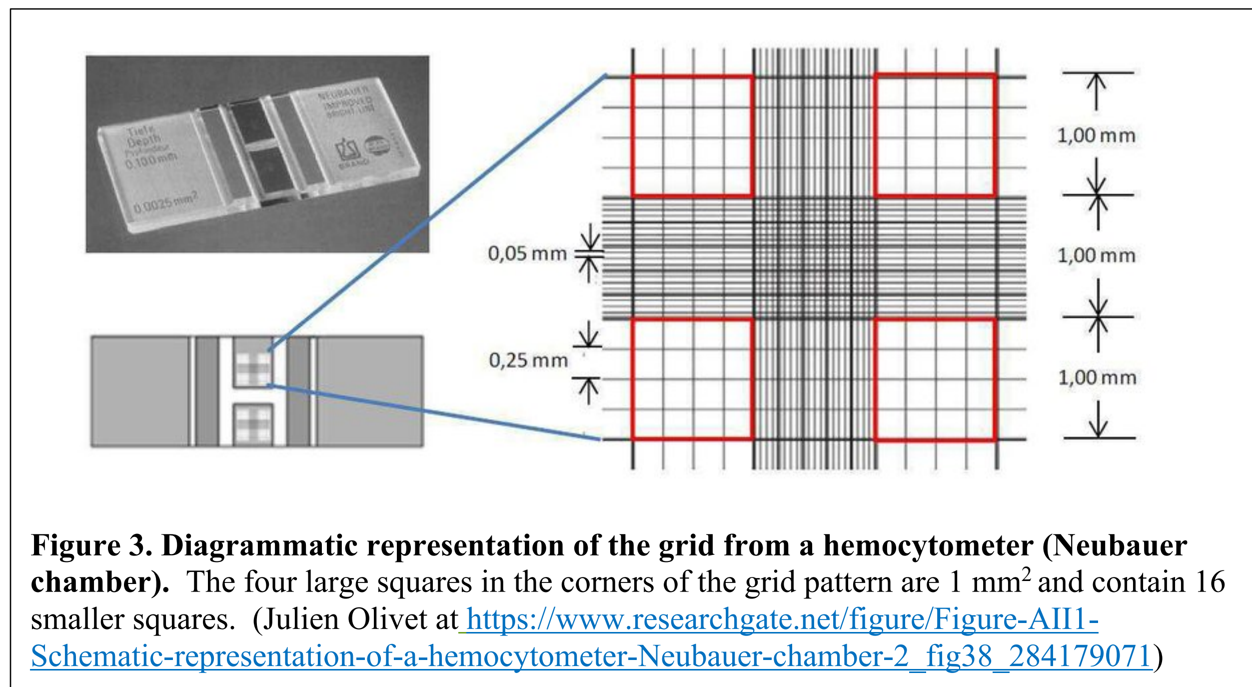

Figure 3. Diagrammatic representation of the grid from a hemocytometer (Neubauer chamber). The four large squares in the corners of the grid pattern are 1 mm^2 and contain 16 smaller squares. (Julien Olivet at https://www.researchgate.net/figure/Figure-AII1-Schematic-representation-of-a-hemocytometer-Neubauer-chamber-2_fig38_284179071)

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given volume. Discerning between live and dead cells requires that the cells be stained in a specific manner.

The chamber portion of the hemocytometer incorporates a special counting grid (Fig. 3). This grid has specific dimensions in length and width, typically 3 mm x 3 mm, with each grid divided into nine equal 1 mm^2 squares. The four large corner squares are further divided into 16 smaller squares. The chamber also holds a specific volume of fluid given that it is 0.1 mm in depth. Hence, the volume (which is calculated as depth x length x width) over one large square of the grid pattern is 1 mm x 1 mm x 0.1 mm, or a total of 0.1 mm^3. Taken further, if one understands that 0.1 mm^3 is one-ten thousandths of 1 cm^3, and that 1 cm^3 is equal to 1 ml, then the volume over one large grid square is 1×10^{-4} ml. In effect, the latter number is considered a dilution when counting cells with the hemocytometer. Thus, the dilution factor of one large corner square is 10,000.

Each clinical or research laboratory typically has a standard procedure for counting cells in a hemocytometer, e.g., how many squares are used in counting, which boundaries of a square are included, etc. In general, the procedures from laboratory to laboratory are similar, though they may differ depending upon the types of cells being counted. The following example demonstrates one such procedure.

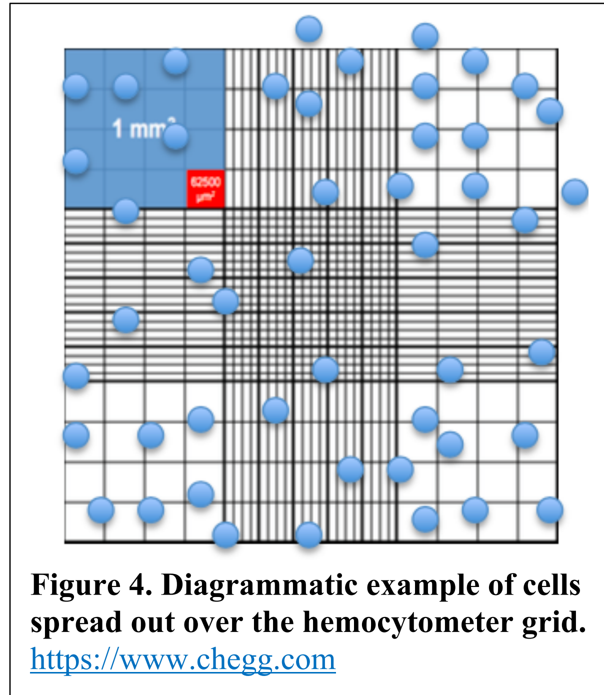

Figure 4. Diagrammatic example of cells spread out over the hemocytometer grid. https://www.chegg.com

In Fig. 4, the number of cells (blue circles) in each of the four large corner squares is as follows: 5 (upper left), 8 (upper right), 7 (lower left), and 7 (lower right). The standard practice used in this example excludes counting any cells that touch the right or lower boundaries of a given large square. Cells that are entirely within the square and are not touching these lines are counted. Conversely, cells that touch the upper and left boundary lines of a square are counted. Hence, in the four large squares a total of 27 cells were counted, or an average of 6.75 per large square.† Because the volume over one large square is equal to a dilution of 1×10^{-4}, i.e., a dilution factor of 10,000, then the number of cells in the sample used to fill the hemocytometer is 6.75×10^4 cells per ml. If the original sample had been diluted 1 ml into 99 ml (a dilution of 1×10^{-2}), then the original sample would have a cell concentration of 6.75×10^6 cells per ml.

Note: Carefully review the above example and be sure the concept of counting cells in a hemocytometer and the subsequent calculations of cell concentration are understood.

In addition, hemocytometer counts can be used to assess the proportion of viable cells among properly stained samples. For example, the viability of yeast cells in a given culture can be determined using the stain methylene blue. Initially, all cells in the sample exposed to this dye

† Normally, cell counts between 30 and 200 per large square are considered valid. Generally, counts outside this range are considered statistically suspect. The counts used in this example were for demonstration purposes only.

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are stained blue. However, metabolically active (viable) yeast cells convert methylene blue to a colorless substance. Therefore, live cells appear clear under the microscope. However, dead cells, which are not metabolically functional, remain blue in color. When used in combination with a hemocytometer, the concentration of both live and dead cells can be determined.

Indirect Counting: The Plate Count Method. The standard plate count relies upon the ability of viable organisms to grow on a medium. It will not measure the number of non-viable cells. In addition, the growth of the microbe may be dependent upon the type of medium or incubation conditions employed. That is, some microbes require specific nutritional requirements not included in all types of media (e.g., the pathogen *Legionella* requires the amino acid cysteine and the element iron), whereas others may not grow well at certain incubation temperatures (e.g., most strains of *Geobacillus stearothermophilus* do not grow at temperatures below 35°C).

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 within media, which is then incubated to permit the formation of single colonies. If the cells are mixed with molten agar, this is generally termed a “pour plate”. If the cells are applied across the surface of a solid agar plate, then this is generally termed a “spread plate”.

Presumably, each colony that forms by one of these methods results from the rapid growth of a single viable cell. In reality, this may not be the case. Two or more cells that clumped together or lay very close to one another shall form a single colony. Nonetheless, a plate count of a given dilution is considered valid if it results in the formation of 30 to 300 colonies, or better termed ***c***olony-***f***orming ***u***nits, or CFU (Fig. 5). This term is used because dead cells cannot be counted by this method nor can viable cells be detected that are unable to grow on the medium used due to the absence of some growth factor (e.g., vitamin) or physical parameter (e.g., osmolarity). Hence, the plate method method determines 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 cell concentration of a given sample can be calculated.

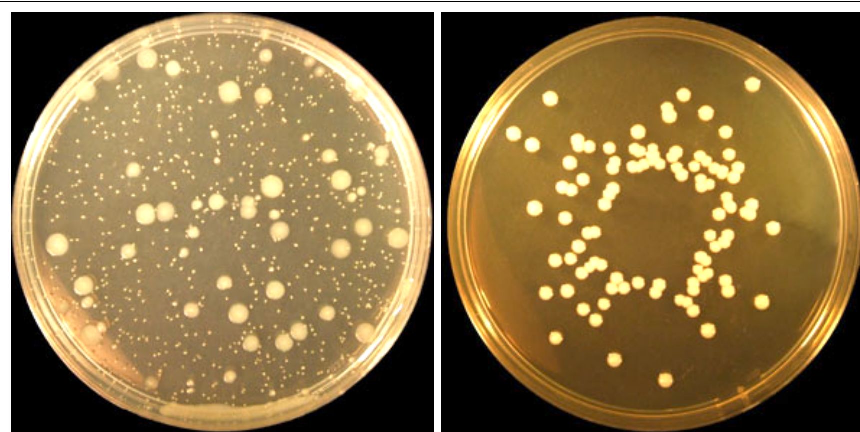

Figure 5. Two methods of performing the agar plate count. The left image depicts the formation of bacterial colonies that were mixed in molten agar (pour plate method). Note that pinpoint colonies are growing within the medium, whereas the larger colonies are growing on the agar surface. All the colonies on this plate, in the agar and on the surface would be counted. However, in practice, this plate probably has too many colonies (>300) to count. This would be designated as “TMTC”, i.e., ***T***oo ***M***any ***T***o ***C***ount. In contrast, the right image shows yeast colonies that were spread on the surface of the medium (spread plate method). The number of colonies fall within a reasonable range (30-300) and would be easy to count. http://www.labm.com

For example, if in the right image of Fig. 5, a 1 ml volume of a 1 x 10^{-4} dilution plated onto the

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growth media resulted in the formation of 95 colonies, then the concentration of cells in the original tube would be calculated as follows: 95 colonies divided by the volume placed on the plate (1 ml), which would then be multiplied by the dilution factor (10,000), giving a final answer of 9.5 x 10^5 CFU per ml. Easy, right? If a smaller volume of a dilution had been used in this example, say 0.1 ml, then this would have been an additional dilution factor of 10, making the final concentration 9.5 x 10^6 CFU per ml.

Note: To emphasize, most standard plate count methods require that data derived from a given dilution are only considered valid between 30 and 300 colonies form in/on a plate. Some laboratories/protocols vary (e.g., 25 colonies minimum, 250 colonies maximum). For the purposes of this exercise, the standard is 30 to 300 colonies per plate. Fewer than 30 colonies, shall be designated as "TFTC", i.e., ***T***oo ***F***ew ***T***o ***C***ount. Conversely, more than 300 colonies will be designated as "TMTC", i.e., ***T***oo ***M***any ***T***o ***C***ount.

Indirect Counting: The Turbidimetric Method. Spectrophotometry measures the turbidity of a microbial sample and is directly related to the density of cells in suspension. However, spectrophotometry does not distinguish between live and dead cells unless special methods and instruments are employed. The accuracy of spectrophotometric measurements, sometimes referred to as 'optical density', are dependent upon a variety of factors. Among those most relevant to the current exercise are the wavelength of light employed, the physiological state of the microbe being measured, the clumping of cells, and the species of the organism.

Functionally, the typical spectrophotometer operates by passing light of a specific wavelength through a sample. In a biological application, some of this light is blocked or reflected by the presence of cells. That light that does pass through the sample, termed transmitted light, is then detected and converted to electrical energy. This energy is then read on a galvanometer 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. Hence, the reading indirectly reflects the number of cells in suspension. Although spectrophotometry is notably quicker than the standard plate count, it is limited in sensitivity. Very high concentrations of cells restrict the amount transmitted light. Moreover, for measurements to be meaningful, they must be correlated to cell number or biomass. Once, this correlation has been accomplished subsequent measurements can be assumed to be generally accurate provided that the same culture conditions are employed.

A common type of spectrophotometer often used in the laboratory is the Spec 200E (Fig. 6; see http://crcooper01.people.ysu.edu/microlab/Using-Spec200E.pdf). With this instrument light is measured either as "percent (%) transmission" or "absorbance". Percent transmission refers to the amount of light that passes through a sample. Conversely, absorbance refers to the amount of light that does not pass through a sample. The following mathematical formula defines the relationship between two values:

$$\text{Absorbance} = 2 - \log(\%T)$$

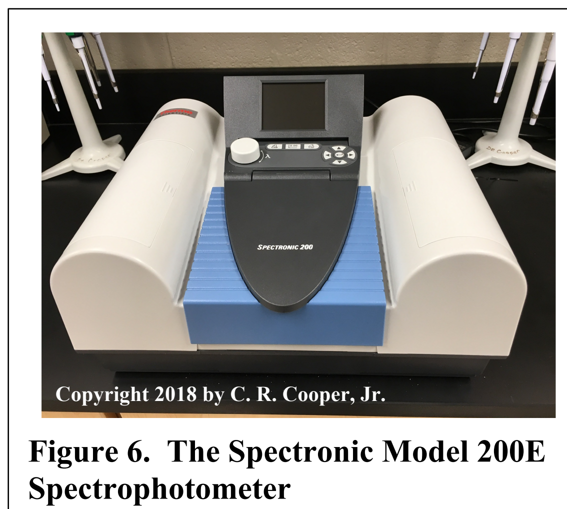

Figure 6. The Spectronic Model 200E Spectrophotometer

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When used in combination with plate or direct counts, a standard curve can be developed that relates the number of cells to an absorbance/percent transmission value.

In the present exercise, students shall be exposed to different techniques as well as critical thinking skills. Serial dilution, used in conjunction with a plate count method, shall determine the cell number of a culture of the budding yeast, *Saccharomyces kudriavzevii* (formerly termed *Saccharomyces cerevisiae*). In particular, the spread plate method shall be employed. In addition, students shall count the number of viable and non-viable cells using a hemocytometer to observe stained yeasts from this culture. Moreover, the turbidity of various dilutions of the yeast culture shall be measured using a spectrophotometer, specifically the Spec 200E. Data from these three types of measurements shall be compared and used to develop a standard curve relating cell number and sample turbidity.

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.

Micropipette. The proper use of a micropipette is important to the success of this experiment. Students having no or limited prior experience with a micropipette should watch the following video: https://www.labtube.tv/video/using-a-micropipet. Students may also wish to practice using the micropipette prior to initiating this experiment. The laboratory instructor can be a resource in helping students master this essential skill.

Serial dilutions and pour plates. The following video demonstrates how serial dilutions can be used to count the number of viable bacteria by employing the plate count method: https://youtu.be/pmRUBYIPMBM. This is one means of serial dilution plating, but it is not the protocol that will be used in this exercise. Nonetheless, it provides a general overview of the concept.

Spread plate technique. The following video demonstrates how serial dilutions can be used to count the number of viable bacteria by employing the spread count method (though this is not the exact method used in this exercise): https://youtu.be/pmRUBYIPMBM. A related video provides an excellent demonstration of the spread plate technique that is used in this exercise: https://www.micro.iastate.edu/video/microbiology-004-spread-plate-method.

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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Learning Objectives

The exercise described in this document will not only provide students the opportunity to develop new skill sets, but also students shall be expected to achieve multiple learning objectives. Upon completion of this exercise, a student should be able to:

- Understand the concept of serial dilutions;
- Distinguish between direct and indirect counting methods;
- Describe the advantages and disadvantages of different counting methods;
- Critically analyze the relationship between cell number and turbidity; and
- Accurately interpret the results of this exercise.

Materials Required

The following materials are necessary to successfully conduct this exercise:

Organism

- Sabouraud dextrose agar culture (3-5 days old) of *Saccharomyces kudriavzevii* (ATCC 2601; formerly designated as *Saccharomyces cerevisiae*) [abbreviated as *S. kudriavzevii*]

Media and Reagents

- Butterfield's Buffer (sterile; multiple bottles containing approximately 500 ml each)
- Dextrose Yeast Nitrogen (DYN) broth (multiple bottles of approximately 100 ml each)
- YPD agar plates
- Methylene blue solution (0.1%)

Materials

- Sterile (capped) 250-ml Erlenmeyer flask
- Sterile serological pipets (1 ml, 5 ml, 10 ml, 25 ml, and 50 ml)
- Sterile 1.5 ml microfuge tubes
- Micropipette tips (sterile; 10 µl volume minimum and 100 µl volume minimum)
- 13 x 100 mm test tubes
- 70% alcohol (in a wide-mouth jar/container)
- Alcohol wipes
- Disposable two-chamber hemocytometer slide (Model DHC-N01, C-Chip – Neubauer Improved; SKC, Inc., Covington, GA; https://www.skcfilms.com/wp-content/uploads/DHC-N01.pdf)

Equipment

- Spec 200E spectrophotometer (http://crcooper01.people.ysu.edu/microlab/Spec200-brochure.pdf)
- Electronic pipettor (ThermoFisher S1 Pipet Filler; https://assets.thermofisher.com/TFS-Assets/LCD/manuals/S1-Pipet-Filler-1508880-User-Manual.pdf)
- Rotary 25°C shaker incubator
- Vortex mixer

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- Micropipettes (capable of delivering 10 µl and 100 µl volumes)
- Cell spreader or bent glass rod ("hockey stick")
- Quebec Colony Counter (Reichert, Inc. Depew NY; https://www.reichertai.com/clientuploads/directory/download_pdfs/Quebec%20Colony%20Counter%20User%20Guide.pdf

Procedures

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

The following procedure contains various components. The section "Preparing the Yeast Culture" must be initiated 3-5 days prior to beginning the other parts of this exercise. Please plan accordingly. The other sections of this exercise are conducted nearly simultaneously but are dependent upon the yeast broth culture being ready to use.

Preparing the Yeast Culture

Three to five days prior to the actual start of this exercise, a broth culture of the yeast species *S. kudriavzevii* must be grown. This culture will be used in the sections "Preparing the Dilution Series" and "Hemocytometer Counting and Viability". Follow the steps below to prepare the yeast culture.

Note: The particular age of this broth culture is not critical. However, 4 to 5 days of growth of this yeast should provide a late exponential/early stationary phase culture that is significantly turbid, i.e., possesses 1-5 x 10^7 cells per ml.

1) Obtain a sterile 250-ml Erlenmeyer flask.

   The following instruction is an exception to typical practice: *use a tape label* on this flask with identifying information.

   DO NOT WRITE WITH PERMANENT MARKER ON THE FLASK.
2) Using an electronic pipettor and a sterile 25- or 50-ml serological pipet, aseptically transfer 50 ml of sterile DYN broth into the labeled flask.
3) Using aseptic technique, transfer a loopful of the *S. kudriavzevii* culture to the broth. Incubate the flask at 25°C for 4-5 days on a rotatory shaker operating at 125 rpm.

Note: If a screw-capped flask is used, be sure not to tighten the lid. The screw-cap lid should be loose, but not to the degree at which it can fall off.

Preparing the Dilution Series

Using the three-to-five-day-old culture of *S. kudriavzevii* (see "Preparing the Yeast Culture" above), the following dilution series will be developed. The dilutions prepared below shall be used in the "Standard Plate Count" and the "Hemocytometer Counting and Viability" portions of this exercise.

1) Obtain six (6) sterile dilution bottles.

   DO NOT WRITE WITH PERMANENT MARKER ON THE DILUTION BOTTLES. The following instruction is an exception to typical practice: *using tape for labeling*, mark one bottle as '10^{-1}', another as '10^{-2}', the third as '10^{-3}', a fourth as '10^{-4}', a fifth as '10^{-5}', and the remaining bottle as '10^{-6}'.

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Note: In Step 2, the same serological pipet can be used to fill each bottle provided that it is not otherwise contaminated. If ever in doubt regarding contamination, use a new, sterile pipet.

2) Using an electronic pipettor and a sterile 25- or 50-ml serological pipet, aseptically transfer 90 ml of sterile Butterfield's Buffer into each of the labeled dilution bottles.
3) Gently swirl the DYN yeast broth culture to thoroughly suspend the cells.
4) Using an electronic pipettor and a sterile 10-ml serological pipet, transfer 10 ml of this culture to the dilution bottle marked 10^{-1}. With the cap securely tightened, vigorously shake the bottle 10-15 times.

Note: In Steps 5-9 below, the same serological pipet can be used to sequentially transfer aliquots of diluted cells provided that it has not been otherwise contaminated. If ever in doubt regarding contamination, use a new, sterile pipet.

5) Use an electronic pipettor and a sterile 10-ml serological pipet to transfer 10 ml of suspension prepared in the dilution bottle marked 10^{-1} (Step 4) to the bottle marked 10^{-2}. With the cap securely tightened on the latter bottle, vigorously shake it 10-15 times.
6) Use an electronic pipettor and a sterile 10-ml serological pipet to transfer 10 ml of suspension prepared in the dilution bottle marked 10^{-2} (Step 5) to the bottle marked 10^{-3}. With the cap securely tightened on the latter bottle, vigorously shake it 10-15 times.
7) Use an electronic pipettor and a sterile 10-ml serological pipet to transfer 10 ml of suspension prepared in the dilution bottle marked 10^{-3} (Step 6) to the bottle marked 10^{-4}. With the cap securely tightened on the latter bottle, vigorously shake it 10-15 times.
8) Use an electronic pipettor and a sterile 10-ml serological pipet to transfer 10 ml of suspension prepared in the dilution bottle marked 10^{-4} (Step 7) to the bottle marked 10^{-5}. With the cap securely tightened on the latter bottle, vigorously shake it 10-15 times.
9) Use an electronic pipettor and a sterile 10-ml serological pipet to transfer 10 ml of suspension prepared in the dilution bottle marked 10^{-5} (Step 8) to the bottle marked 10^{-6}. With the cap securely tightened on the latter bottle, vigorously shake it 10-15 times.

Standard Plate Count

The serial dilutions prepared in the "Preparing the Dilution Series" procedure above shall be used in the following a standard plate count procedure. The particular protocol performed in this exercise is the 'spread plate technique' in which a suspension of cells is smeared across the surface of a nutrient medium.

1) Obtain eight (8) YPD agar plates. In pairs, label the bottom (the agar containing half) of the plates '10^{-4}', '10^{-5}', '10^{-6}', and '10^{-7}'. Mark the plates with any additional information as appropriate.

Note: The plates are labeled with the final dilution of cells to be spread upon them. Remember, a portion of a ml is essentially a dilution. Hence, when 0.1 ml from a known dilution is placed on/in agar, this is equivalent to an additional dilution of 1×10^{-1}.

2) Shake the dilution bottle labeled 10^{-3} vigorously 10-15 times. Use an electronic pipettor and a sterile 1-ml serological pipet to *carefully and accurately* transfer 0.1 ml of the diluted cell suspension to the near center of both of the YPD agar plates labeled 10^{-4}.

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Note: It is critical to properly control the electronic pipettor so that an accurate volume of suspended cells is placed on the agar surface. If necessary, use the pipettor, a 1-ml pipet, water, and an empty Petri dish to practice delivering the required volume of fluid.

3) Sterilize a cell spreader (or "hockey stick") by dipping it in 70-80% alcohol. Remove the alcohol, allow excess alcohol to drain off the spreader, then eliminate any remaining alcohol by touching the spreader to an open flame being careful not to drip any burning fluid on the bench, or worse, into the jar of alcohol.

Note: *Use extreme caution with alcohol and open flames*. Burning alcohol is often not visible. Also, be sure that the spreader is cool before using it to smear cells on the agar surface.

4) For one of the pairs of agar plates labeled 10^{-4}, set it on the bench top and remove the lid holding it between the forefinger and thumb. Continue to hold the lid in one hand and the cell spreader in the other hand. While holding the lid over the Petri dish of medium, place the cooled spreader gently onto the agar surface. Quickly, yet carefully, gently press the spreader to the surface as it is moved across the agar surface while rotating the plate. With the forefinger and thumb holding the Petri dish lid, use the other part of the and to continuously turn the plate while smearing the liquid across agar until the entire surface has been evenly covered. Replace the lid and sterilize the spreader by placing it in alcohol.

Note: Setting the lid on the bench may lead to unwanted contamination. Good practice is to learn to hold the lid and the agar portion of the dish with one hand while spreading the cells and rotating the plate. Sounds easy, right?

5) Repeat Step 4 for the second of the pair of YPD agar plates labeled 10^{-4}.
6) Repeat Steps 2-5 for the dilution bottle labeled 10^{-4} by transferring 0.1 ml of the diluted cell suspension to the YPD agar plates labeled 10^{-5}.
7) Repeat Steps 2-5 for the dilution bottle labeled 10^{-5} by transferring 0.1 ml of the diluted cell suspension to the YPD agar plates labeled 10^{-6}.
8) Repeat Steps 2-5 for the dilution bottle labeled 10^{-6} by transferring 0.1 ml of the diluted cell suspension to the YPD agar plates labeled 10^{-7}.
9) After all plates have been spread, leave them sit on the lab bench for 5-10 minutes to allow the liquid to absorb into the medium.
10) Incubate all spread plates inverted (agar side of the Petri dish upwards) at 37°C for 3-5 days.

Remove the plates from the incubator and count the number of colonies on each plate. It may be helpful to use a colony counter (Fig. 7; see Appendix A for instructions).

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

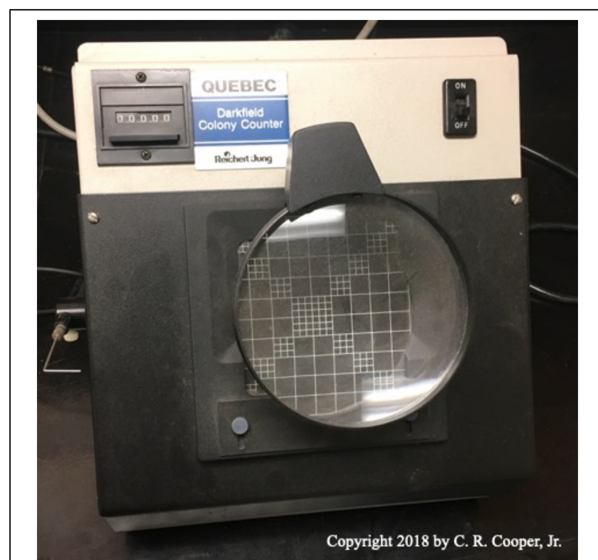

Figure 7. Colony Counter. The colony counter can be used with spread or pour plates to count the number of colonies.

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A Word About Yeasts and Counting Them Under a Microscope . . .

Yeast cells reproduce by the process known as budding. Briefly, the mother (original) cell forms a bleb-like extrusion that continues to enlarge in a round-to-oval shape. This is termed the daughter cell. As the daughter cell grows, a constriction forms between it and the mother cell. Eventually, the constriction breaks separating the mother and daughter cells. This generally happens when the daughter cell is at least half the size of the mother cell. Hence, reproduction has occurred upon separation – one cell became two. Now the daughter, as well as the mother cell, can continue to grow and divide making more cells. The figure on the left below depicts several yeast cells at different stages of budding. Regularly, depending upon the growth conditions, daughter cells may not fall away from the mother cell. Also, the mother cell sometimes initiates the formation of a new daughter cell while the prior one is still attached. This results in a chain or cluster of yeast cells.

The right figure below shows both live (clear) and dead (blue) yeast cells on one large square of a hemocytometer grid. When counted using the guidelines in Step 11 of this section, there are 75 total cells, 65 of which are viable and 10 that appear dead. Hence, approximately 13% of the cells are non-viable in this sample. Given the dilution factor of this one square, then there are 6.5 x 10^5 viable cells per ml. This does not take into consideration any of the dilution factors from which this sample was taken.

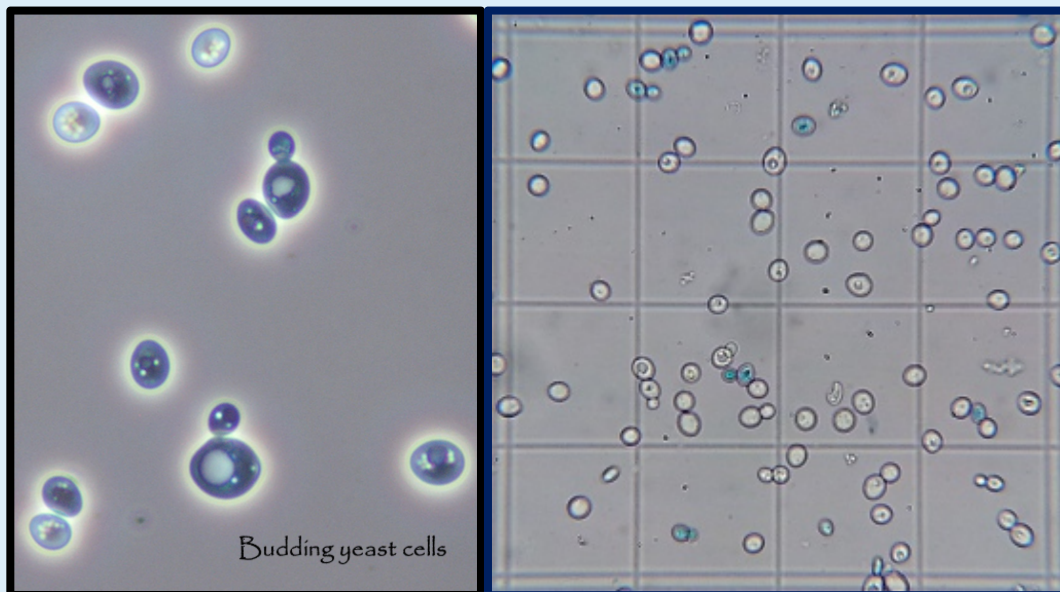

Figure Sources: http://www.rci.rutgers.edu/~microlab/applied%20micro/schedulelinks/budding%20yeast%20cells.htm and http://braukaiser.com/blog/blog/category/science/yeast/

Hemocytometer Counting and Viability

The serial dilutions prepared in the “Preparing the Dilution Series” procedure shall be used in calculating number of budding yeast cells in the original culture by direct counting. In addition, methylene blue staining will be used to determine the proportion of dead cells in the culture. A brief discussion on the growth and morphology of yeast cells, as well as an example of counting live and dead yeasts using a hemocytometer is presented in the box below.

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1) Obtain a packet containing a disposable hemocytometer slide. Open the packet and place the hemocytometer on the lab bench top. Note that there are two counting chambers on slide – one labeled 'A' and the other 'B' (see Fig. 2).
2) Obtain two sterile 1.5-ml microfuge tubes. Label one 'A' and the other 'B'.
3) Shake the dilution bottles labeled 10^{-1} and 10^{-2} vigorously 10-15 times each.
4) Using a micropipette and sterile pipette tip, transfer 100 µl of the cell suspension from the dilution bottle labeled 10^{-1} to a sterile 1.5 ml microfuge tube labeled 'A'.
5) Using a micropipette and sterile pipette tip, transfer 100 µl of the cell suspension from the dilution bottle labeled 10^{-2} to a sterile 1.5 ml microfuge tube labeled 'B'.
6) To each microfuge tube, use a micropipette and separate sterile pipette tips to add 100 µl of the 0.1 % methylene blue solution. Mix the contents of each tube for 5-10 seconds on a vortex operating at the highest setting. Allow both microfuge tubes to remain on the bench top at room temperature for 5-10 minutes.

Note: The mixing of the diluted cell suspension with an equal volume of stain is another dilution factor to be considered after counting the cells.

7) Again, mix the contents the microfuge tube labeled 'A' for 5-10 seconds on a vortex operating at the highest setting. From this tube, use a micropipette and a sterile pipette tip to slowly and carefully transfer 10 µl of the stained yeast cell suspension to the sample injection port of chamber A. If done properly, the fluid will fill the chamber by capillary action. Avoid introducing air bubbles into the chamber.
8) Again, mix the contents the microfuge tube labeled 'B' for 5-10 seconds on a vortex operating at the highest setting. From this tube, use a micropipette and a sterile pipette tip to slowly and carefully transfer 10 µl of the stained yeast cell suspension to the sample injection port of chamber B. If done properly, the fluid will fill the chamber by capillary action. Avoid introducing air bubbles into the chamber.
9) Allow the hemocytometer slide to set on the bench for 5 minutes.

Note: For the next step in this exercise, use the guidelines presented below to count the number of viable and non-viable yeast cells. There may be some instances where cells may not exactly fall within the guidelines pertaining to cell shape/morphology presented below. Regardless, count these cells but do so in a consistent manner.

- Count single cells as 1 cell.
- Count budding yeasts as 1 cell if daughter cell is less than half the size than mother cell.
- If a daughter cell is half the size or approximately equal in size as the mother cell, count this structure as 2 yeast cells.
- If a group consists of three or more cells, always count the larger cells as 1 cell and cells smaller than half the size of the larger ones should not be counted.
- Count all yeast cells located completely within a square.
- Count all yeast cells touching left and upper borders of the square.
- Do not count yeast cells touching right and lower borders.
- Count colorless (clear) yeast cells as viable.
- Count blue colored yeast cells as non-viable.

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10) Place the slide on the microscope stage and align the grid from chamber A underneath the low power objective. Focus on the slide until the grid and the yeast cells become clearly visible. Change objectives until one large square in the corner of the grid (the square consists of 16 smaller squares) takes up the entire field of vision (usually this is the 10X objective). Roughly estimate the total number of cells (viable and non-viable) in this one corner square. It may be necessary to use the 40X objective to get a better estimate, but only one small square of the 16 would be visible at this magnification. Scan several of these squares to make the estimate of the total number of cells in one large square. That is, if four small squares are counted and contain a total of 10 cells, then the estimated number of cells for the one large square (which is composed of 16 small squares) would be 40.
    - If the estimate falls between 25 and 250, then proceed with a detailed counting as indicated in Step 11.
    - If the estimate falls below 25, then a new sample must be prepared directly from the broth culture. Repeat the staining procedure as described above and use a new hemocytometer to count the cells as described below (Step 11).
    - If the estimate falls above 250, then observe the grid in chamber B. Roughly estimate the total number of cells (viable and non-viable) in one large corner square. If the estimate falls between 25 and 250, then proceed with a detailed counting as indicated in Step 11.
    - If the estimate in chamber B is above 250, prepare a newly stained sample as described above for the dilution bottle labeled 10^{-3}. If the estimated number of cells in this diluted sample falls within the 25-250 range, then count these cells using a new hemocytometer (see Step 11). *If this diluted sample fails to bring the number of cells into the proper counting range, then one should suspect that there is something seriously wrong with the serial dilution series*. It is highly unlikely that the concentration of yeasts in the original culture is greater than 1×10^9 cells per ml. If this occurs, review the dilution procedure to determine if a mistake was made. If so, repeat the dilution series. Otherwise, consult the laboratory instructor.

11) Using the guidelines presented above, count the number of viable and non-viable yeast cells in the four large corner squares of the hemocytometer grid that each contain between 25 and 250 cells.

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

Measuring Turbidity

The following procedure will use the Spec 200E spectrophotometer to assess the turbidity of the original yeast broth culture. This data will be correlated with those derived from the plate count and hemocytometer count methods. Specific instructions regarding the use of the Spec 200E can be found at the following URL: http://crcooper01.people.ysu.edu/microlab/Using-Spec200E.pdf. Students should review this document prior to using the Spec 200E.

Note: Before proceeding, turn on the Spec 200E and allow it to warm up.

1) Obtain seven (7) 13 x 100 mm test tubes. At the top of one tube, use a Sharpie marker (NOT tape) to label it ‘OC’ for Original Culture. At the top of each of six other tubes, label them sequentially as 1 through 6 using a Sharpie marker. Similarly, label the eighth tube ‘B’ for “Blank”. Set the tube labeled ‘OC’ to the side for now.

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2) Using the electronic pipettor and a sterile 5-ml serological pipet (or a 10-ml pipet), carefully and aseptically transfer 3.0 ml of DYN to the test tubes labeled 1 through 6 as well as 'B'. Do not place media in the tube labeled 'OC'.

Note: In Step 2, the same serological pipet can be used to fill each bottle provided that it has not been otherwise contaminated. If ever in doubt about a contaminated pipet, discard it, then use a new, sterile pipet.

3) Gently swirl the DYN yeast broth culture to thoroughly suspend the cells.
4) Using an electronic pipettor and a NEW sterile 5-ml (or 10-ml) serological pipet, transfer 3.0 ml of the DYN yeast culture to the test tube labeled 'OC'. Set this tube aside for now.
5) Using an electronic pipettor and a NEW sterile 5-ml (or 10-ml) serological pipet, transfer 3.0 ml of the DYN yeast broth culture to the test tube labeled '1'. Mix this solution for 5-10 seconds using a vortex operating at a *middle-speed setting*.

   DO NOT VORTEX THIS TUBE OR THOSE PRODUCED IN STEPS 6-9 AT A HIGH SPEED OR THE LIQUID WILL COME OUT OF THE TUBE!

Note: In Steps 5-10 below, the same serological pipet can be used to sequentially transfer aliquots of diluted cells provided that it has not been otherwise contaminated. If ever in doubt regarding contamination, use a new, sterile pipet.

6) Using an electronic pipettor and a sterile 5-ml (or 10-ml) serological pipet, transfer 3.0 ml of cell suspension from tube '1' to the test tube labeled '2'. Mix this solution for 5-10 seconds using a vortex operating at a *middle-speed setting*.
7) Using an electronic pipettor and a sterile 5-ml (or 10-ml) serological pipet, transfer 3.0 ml of cell suspension from tube '3' to the test tube labeled '4'. Mix this solution for 5-10 seconds using a vortex operating at a *middle-speed setting*.
8) Using an electronic pipettor and a sterile 5-ml (or 10-ml) serological pipet, transfer 3.0 ml of cell suspension from tube '4' to the test tube labeled '5'. Mix this solution for 5-10 seconds using a vortex operating at a *middle-speed setting*.
9) Using an electronic pipettor and a sterile 5-ml (or 10-ml) serological pipet, transfer 3.0 ml of cell suspension from tube '5' to the test tube labeled '6'. Mix this solution for 5-10 seconds using a vortex operating at a *middle-speed setting*.
10) Using an electronic pipettor and a sterile 5-ml (or 10-ml) serological pipet, remove 3.0 ml of cell suspension from tube '6' and appropriately discard the pipet with its contents.

Note: In effect, Steps 5-9 have created dilutions of the original yeast broth culture. It is these dilutions that will be measured using the Spec 20D spectrophotometer as detailed below. It is important that students calculate the dilution factors for each of these tubes.

11) If the Spec 200E has not been turned on, do so now. Follow the instructions provided (http://crcooper01.people.ysu.edu/microlab/Using-Spec200E.pdf) to prepare the instrument for use.
12) Use the tube labeled 'B', i.e., the "Blank" containing DYN broth, to 'zero' the instrument.
13) Begin reading the absorbance (or percent transmission) of each of the tubes labeled 'OC' and 1 through 6. Be sure to mix the contents of each tube either by rolling it between the hands or using a vortex operating at a *middle-speed setting* prior to placing the tube in the Spec 200E.

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

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Appendix A – Use of the Quebec Darkfield Colony Counter

More specific information regarding the Quebec Colony Counter is available in the following manual: https://www.reichertai.com/clientuploads/directory/download_pdfs/Quebec%20Colony%20Counter%20User%20Guide.pdf. Though this manual is for models the differ from the one in the laboratory, all operate in the same manner.

The following instructions refer to the instrument shown Fig. 8 below

1) Disinfect both probe tips with an alcohol wipe. Appropriately discard the wipe.
2) Place the agar plate to be counted on the gridded glass counting plate.
3) On the plate holder at the bottom counting plate, loosen the two thumbscrews to center the culture plate.
4) Plug the ground contact and the counting probe into the electric receptacles on the back of the instrument.
5) Remove the Petri dish lid and set it aside.
6) Position the ground contact with magnetic base near the Petri dish culture to be counted. Place the L-shaped probe tip on the agar surface that is devoid of any microbial growth.
7) Turn on the instrument to back illuminate the agar plate, then zero the automated counter by pushing the bar beneath the counter.
8) Using the probe tip, touch each colony to be counted. Counts shall be automatically registered on the counter.
9) When counts have been completed, replace the lid on the culture plate.
10) Disinfect both probe tips with an alcohol wipe. Appropriately discard the wipe.
11) Repeat Steps 5-10 for each additional culture plate to be counted.
12) When all plates have been counted, be sure to appropriately disinfect both probes with alcohol wipes, discard all unwanted culture plates, and turn off the colony counter.

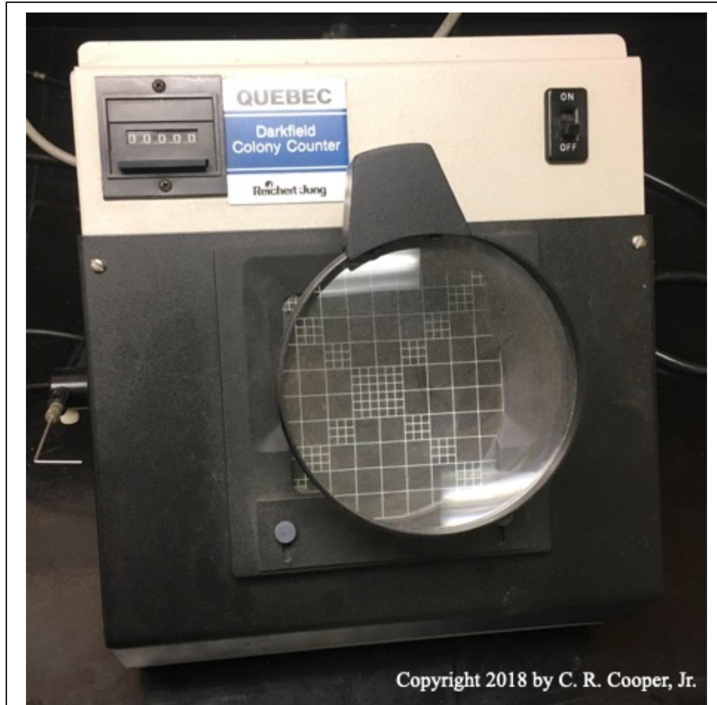

Figure 8. Quebec Colony Counter. The colony counter can be used with spread or pour plates to count the number of colonies.

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Student Name: ________________________________

COMPLETE THE FOLLOWING TABLES BASED UPON YOUR OBSERVATIONS

Standard Plate (Viability) Counts

Effective Dilution Factor	Plate #1 Colony Count	Plate #2 Colony Count	Average Plate Count	Calculated CFU/ml in Original Culture

Hemocytometer Counts

Effective Dilution Factor	Viable Cells/ml	Non-Viable Cells/ml	Percent Viability	Calculated Total Cells/ml in Original Culture

Turbidity Measurements vs. Calculated Cell Number

Tube Number	Effective Dilution	Estimated Cells/ml		Percent (%) Transmission	Absorbance*
		Hemocyto-meter Counts	Viable Counts		
‘OC’	---				
1					
2					
3					
4					
5					
6					

*Absorbance = 2 – log(%T)

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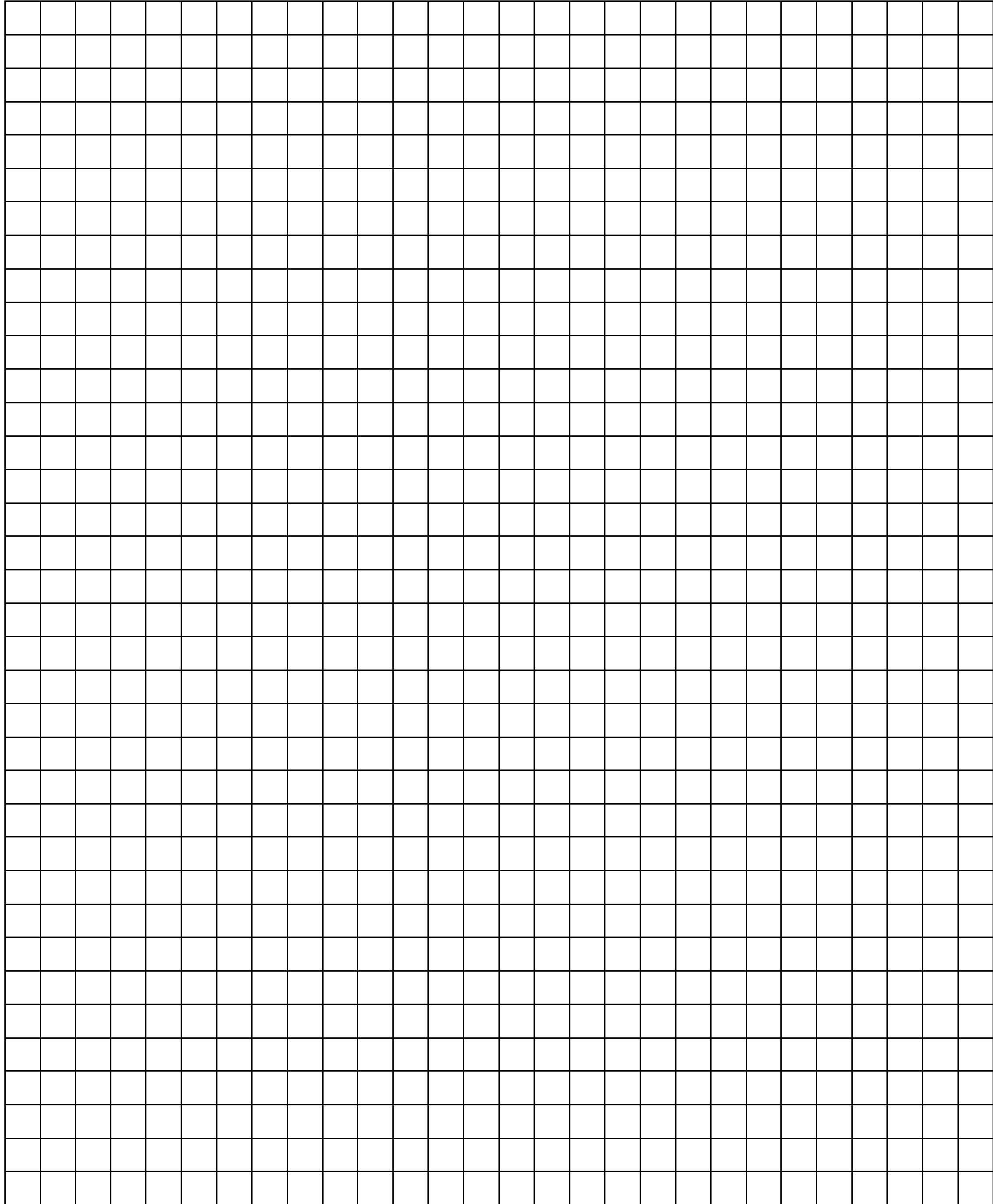
From your data, construct a standard curve by plotting the $\log_{10}$ of the CFU/ml of each dilution on the x-axis and the absorbance of the respective dilution on the y-axis. Be sure to present a figure legend for this graph.



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Discussion Questions

1) Based upon the graph generated above, what would the approximate absorbance 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?

2) Based upon the graph generated above, what would the approximate CFU/ml be for a sample having an absorbance of 0.3? If this cell count came from a sample of the original culture which had been diluted 1×10^{-2} times, what is the number of CFU/ml of the original culture?

3) If the 'percent transmission' value would be used instead of the 'absorbance' for the y-axis, how would the above graph appear?

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4) How would cell clumping affect turbidity measurements?

5) How would cell clumping affect the determination of CFU?

6) How does the presence of non-viable cells affect turbidity? CFU?

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