**Lab 3.1 Counting Bacteria and Measuring the Rate of Bacterial Cell Division[[1]](#footnote-1)**

“*Not everyone is mindful of it, but all cell biologists have two cells of interest: the one they are studying and* Escherichia coli*.*”[[2]](#footnote-2)

“*What is true for the bacterium, is true for the elephant.*”[[3]](#footnote-3)

### Introduction & Background

Modern cell biology has made tremendous strides toward understanding the inner workings of cells. Historically, bacteria have been organisms of choice for understanding the genetics and molecular & cellular biology of cell function, including metabolism and replication, although the focus has shifted toward eukaryotes in recent years. Nonetheless, studies of prokaryotes remain an important component of the overall picture, including biodiversity (millions of bacterial species, estimated), disease (microbial pathogens, *e.g.* *E. coli*/*Salmonella* outbreaks), and biotechnology (*E. coli* is a workhorse of genetic and protein engineering). *E. coli* is small, ~ 1 µm across and ~ 2 to 4 µm in length (Figure 1, right). It uses flagella for swimming, which facilitates movement in response to chemical gradients, chemotaxis (Figure 1, left).

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| ttp://upload.wikimedia.org/wikipedia/commons/4/4d/Ecoli_O157_trans_elect_micrograph.jpg | EscherichiaColi_NIAID*Above:* A scanning electron micrograph of *Escherichia coli*, grown in culture and adhered to a cover slip (Rocky Mountain Laboratories, NIAID, NIH, [http://en.wikipedia.org/wiki/File:EscherichiaColi\_NIAID.jpg](http://en.wikipedia.org/wiki/File%3AEscherichiaColi_NIAID.jpg), accessed 9/3/12).*Left*: Transmission electron micrograph of (a deflated) *E. coli* stained to generate contrast. The filaments are flagella. (CDC, [http://commons.wikimedia.org/wiki/File:Ecoli\_O157\_trans\_elect\_micrograph.jpg](http://commons.wikimedia.org/wiki/File%3AEcoli_O157_trans_elect_micrograph.jpg), accessed 5/22/15).  |

Figure 1. Electron microscope images of *Escherichia coli*.

### Goals for the lab

Fundamental questions are addressed in this unit, as well as technical objectives. These include:

* How fast do bacteria divide when given all the nutrients that are necessary for cell division?
* What is the mode of growth and how does this mode influence the rate of cell division?
* What are the advantages and disadvantages of the three different methods for counting cells?

These objectives are addressed over the next two lab periods:

#### First Week (Lab 3.1):

Week one has the technical objective to introduce three methods for counting cells: (1) with phase-contrast microscopy, (2) in the plate reader, (3) by spreading diluted cultures on nutrient agar and counting the colonies that appear after incubation.

#### Second Week (Lab 3.2):

You will use the conversion factors you calculated to measure the rate of cell growth under nutrient-rich conditions. How can these methods be used to assess the fundamental requirements for cell division?

### What you should do before your lab period:

* Read about the disposable hemocytometer (page 10).
* Ponder lowly *E. coli* and all that it must do to divide. (Do a web search?)
* Read the introduction to the plate reader.
* Read “making a dilution series.”

## Lab 3.1: Viewing and Counting Bacteria

***How dense is the starting culture?***

### The plate reader:

In Unit 1 we used three pieces of equipment (micropipettors, microsocopes, and computers) to quantify pollen tube growth. In this unit, you will quantify bacteria with a new piece of equipment: the plate reader. Before lab you should become familiar with how a plate reader works by reading the document entitled “Introduction to the plate reader.” You will also count bacteria directly using microscopy.

### Materials

***E. coli* cultures**. Tubes containing stationary phase *E. coli* are in the refrigerator. Your team will withdraw samples from these flasks to make your observations. Keep them cold to prevent cell division.

**Pipette Tips.**

**Luria-Bertani (LB) growth medium.** LB is a complete growth medium made from tryptone (trypsin-digested milk protein) for amino acids, yeast extract for carbohydrates and vitamins, and NaCl. It will also be used as a diluent and a spectrometer blank. Keep it cold to prevent cells from dividing until you want them to.

**Disposable hemocytometers**are cell-counting chambers that have calibrated volumes (the name derives from the fact that they are used to count blood cells). Hemocytometers allow you to determine the concentration of cells in solution under the microscope.

**96 well plate**. To measure the optical density (OD) of cell cultures.

**LB-agar petri dishes and glass bead spreaders**. Petri dishes, ~10 cm in diameter, are filled with 30 mL of LB-agar. LB-agar hardens as it cools after heat sterilization, into a stiff Jell-O (97% LB, 3% agar by weight). The spongy substrate is used to grow visible colonies of bacteria from single cells in an incubator overnight.

### Cell counting techniques

The *technical objective* this week is to learn and compare different means for measuring the cell concentration (cells/mL). Some are more convenient than others; each method makes different assumptions. We will measure cell density by three techniques: (1) OD measurements in the plate reader, counting (2) cells in a hemocytometer and (3) colonies on an LB-agar plate.

### Procedures

#### Get cell cultures.

Take a tube of cells from the refrigerator and carry it ***on ice*** to your bench.

#### Make 2 dilution series.

(From practical experience, it has been found, sadly, that the culture must be diluted much more for plating on agar than for reading in a plate reader. That’s why we need two dilution series.)

Use cold LB and keep all tubes on ice at all times. Label 2 sets of tubes: 1A-10A and 1B-10B.

##### A. 2-fold dilution series:

* Put 0.5 mL of cold LB into each of 9 tubes (labeled 2A-10A), and put them on ice.
* Put 1 mL of cold cell culture into a tube (tube 1A) and place on ice.
* Transfer 0.5 mL from tube 1A to tube 2A. Mix gently.
* Repeat for the rest of the tubes in the series. Keep tubes on ice.

##### B. 10-fold dilution series

* Put 0.9 mL of cold LB into each of 9 (labeled 2B – 10B) tubes, and put them on ice.
* Put 1 mL of cold cell culture to a tube (tube 1B) and place on ice.
* Transfer 0.1 mL from tube 1B to tube 2B. Mix gently. Keep cold.
* Repeat for the rest of the tubes in the series. Keep tubes on ice.

#### 2. Measure cell optical density (OD) using the plate reader

OD is a general property of a solution that could refer to either the *absorption* of light, or light *scattering*, which also reduces the intensity of transmitted light and makes solutions cloudy or *turbid*. Bacterial cells scatter light, and the amount of scattering is proportional to the cell concentration. Typically, the OD of a bacterial culture is measured at 600 nm, but our plate reader measures OD at **595 nm**. When the response is linear, the cell concentration will be *proportional* to OD through a constant. The protocol **BUG\_OD** is set up to measure OD595.

* Dispense 250 µL of each culture in the 2-fold dilution series to wells A1 – A10 of your plate.
* Put 250 µL of LB into wells A11 and A12.
* Dispense 250 µL of each culture in the 10-fold dilution series to wells B1 – B10 of your plate.
* Put 250 µL of LB into wells B11 and B12.

**Using the plate reader:**

Take your plate to the plate reader, where the instructor will assist you in taking the measurements. The measurement method generated for this experiment is called “**Bug\_OD**.”

* If you have to wait in line for a plate reader, put your plate in the refrigerator until it is your turn.
* Read the OD at 595nm (OD595) with the protocol **Bug\_OD**.
* Open OPTIMA, click your group from the users menu, then click “RUN”.
* Open plate reader by pushing the button on the machine and place your plate on the tray so you can read the letters and numbers. **Remove your plate’s lid as it interferes with the reading.** Push the button on the machine again to have the tray retract back into the machine.
* When ready to take a reading, click the traffic light on the top left side of the program window.
* It will now ask you to select a test program: select the program called **Bug\_OD**; click ok.
* On the next menu screen, name the file (choose a file name specific to yourselves), select protocol and method as the secondary identifiers, and click start measurement.
* Where the traffic light was, there will be an absorbance graph. Click this once to see your data being collected in real time.
* Make sure the values that are showing up are absorbance values. This is on the bottom right hand side of the screen.
* When the reader has concluded collecting data, simply click close. (Do not push save – it doesn’t do what you hope it does. It turns your data into a jpeg file!).
* Your data should be in a .csv file in your Wahoo folder waiting for you to analyze it
* Open the file at your desktop computer. Save it as an Excel (xlsx) file to the desktop! Make a note in your notebook of the file name and what’s in it.
* Compile your two dilution series and plot the data.

### Compile and sort the data

* You will probably like columns better than rows for this activity.
* To turn your row data into column data, you will need the paste transposed command (Edit/paste special/transpose).
* Get all your OD595 readings in a single column, with their dilution magnitude to the left of them.
* Sort the data from lowest concentration to highest. (Use Data/sort.)
* Don’t forget to subtract the blank!

#### The relationship between concentration and OD595

Plot dilution vs. OD595 using a scatterplot (not a line graph, which isn’t what it sounds like). Read the section below on getting rid of noise. Create a new graph with just the data in the linear range.

#### The art of the standard curve

Add a linear trendline. Think about whether you should force this line through the origin by setting the intercept to 0 (you can do this in the options menu of the trend-line dialog box). (Hint: what should the OD595 of no bacteria be?) One of your trend-line options is to include the equation of the line on the graph. Another option, which may be useful in deciding which points to discard, is to display R2. This value is a measure of the goodness of fit of the points to the line. R2 is 1 for a perfect fit, and 0 for no discernable trend at all.

Figure 2 shows a different set of data. In this case it’s the absorbance of a dye in solution. The concentration is on the x-axis and the absorbance is on the y. The data points show a general positive correlation between concentration and absorbance, but don’t fall convincingly on a line (certainly not one that has been forced through zero).

Figure 2. Relationship between concentration and absorbance.

The two circled regions of points represent *noise*, that is, extraneous background signals that prevent detection of the actual relationship. There is no apparent relationship between concentration and absorbance at either the high (Figure **3**) or the low (Figure 2) end of the data range. Concentrations above 10 are apparently so dark that the detector cannot distinguish between them. Similarly, concentrations below 1 are also apparently indistinguishable from each other. Therefore, you ought not use absorbance values below 0.2 or above 0.9 to estimate the concentration of this particular substance.

Figure 3. The relationship between high concentration and absorbance.

Figure 2. The relationship between low concentration and absorbance.
(Note X axis range compared to previous graphs.)

Figure 3 contains the same mid-range data as Figure 2. The data points for concentrations above 10 or below 0.9 have been removed using “source data”, and a trendline has been fitted to the points and forced through the origin. This is now a standard curve, that is, a graph you can use to estimate the concentration of an unknown solution. The dotted lines show how you can interpolate a concentration from an absorbance value.

You can also use the equation of the line to calculate X from Y.

Estimated concentration of unknown

Measured absorbance of unknown

Figure 3. Standard curve. A straight line is fitted to points plotted of the absorbance readings from solutions of known concentration. The dashed line shows how to estimate the concentration of a solution giving a reading of about 7. Note that the line is used, not the individual data points.

#### 3. Count cells in the hemocytometer

The hemocytometer has precisely calibrated volumes that are very small, as illustrated in Figure 4. We can count how many cells there are in one of these areas and calculate cell density from that number.

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Figure 4. The iN Cyto disposable hemocytometer is a 1 x 3-inch microscope slide (left), but has an attached coverslip with gridlines that define the counting volume. Each slide has two counting areas (for two samples). When 10 µL of liquid cell suspension is introduced at the sample injection port, capillary action draws the culture liquid into the counting area (expanded at right). The counting area is an array of squares and rectangles. Volumes are calibrated in mm3 (length *x* width *x* depth). The depth of the counting area is 0.1 mm. (Image: [http://www.incyto.com](http://www.incyto.com/)).

**Calculate** the volume of the entire counting area. **Calculate** the volume of the tiniest area. Show these calculations in your notebook.

Make sure that you can focus on an empty hemocytometer and find the squares. Once you have successfully examined an empty hemocytometer with your microscope, proceed to the counting procedure:

***Keep all tubes with cells on ice!***

* Pick a culture with the highest concentration that is just barely out of the noise. Refer to the section on the art of the standard curve (p. 7) for a review of the meaning of *noise*.

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| dilution | OD | By way of illustration, here is a set of data and the lowest part of the graph created from them, including the trend line.Notice how the points below a cell culture fraction of about 0.03 (also bolded in the data table) don’t show a linear relationship between concentration and OD. This is known as *noise*, and it tells you something about the limits of the instrument. The plate reader cannot distinguish between concentrations this low. Start with the culture indicated by the arrow. |
| 0.000000001 | **0.2575** |
| 0.00000001 | **0.2508** |
| 0.0000001 | **0.247** |
| 0.000001 | **0.2458** |
| 0.00001 | **0.1955** |
| 0.0001 | **0.2553** |
| 0.001 | **0.2415** |
| 0.001953125 | **0.2904** |
| 0.00390625 | **0.2638** |
| 0.0078125 | **0.207** |
| 0.01 | **0.3156** |
| 0.015625 | **0.2341** |
| 0.03125 | 0.3012 |
| 0.0625 | 0.3813 |
| 0.1 | 0.4805 |
| 0.125 | 0.4495 |
| 0.25 | 0.6881 |
| 0.5 | 1.0594 |
| 1.0 | 1.7322 |
| 1.0 | 1.8613 |

* Withdraw 10 µL of that culture and dispense it into the sample injection port of the hemocytometer (see Figure 4).
* Locate the counting area in the microscope and bring it into focus under low magnification (10× objective). Make sure the appropriate phase ring is in place.
* Increase the magnification to 40× (remember also to switch to the matching phase ring). ***Important tip: Do not touch the focus knob before switching lenses, as these microscopes are parfocal. If you are afraid of hitting the slide with the lens, push the stage down before you switch lenses.***
* Count the cells in the square(s) you have chosen, keeping careful record of width (w) and length (l) of the grid section. (Make a table in your lab notebook with headings of w, l and number of cells for each grid section.)
* Repeat this procedure until you have counted at least 100 cells. (The object is to gain a precise estimate of the number of cells, and the more you count, the greater your confidence in the concentration you calculate.)
* Compute the volume (l × w × d) for each grid section in which cells were counted, using recorded values of l and w and d = 0.1 mm.
* Calculate the cell concentration in units of cells/mL. (Useful conversion factors: 1 µL = 1 mm3, 1 mL = 1000 µL.)
* If you have time, repeat this procedure with a second 10 µL aliquot. Choose a higher or lower cell density according to how crowded the first sample was. (If it was hard to find cells, choose a more concentrated sample; if they were too numerous to count, go down.)

#### 4. Spread cells on an agar plate to count colonies.

Spreading cells onto an LB-agar petri dish is a counting method in which each colony that becomes visible after an overnight incubation corresponds to one *viable* cell. For cell plating, the desired number of colonies per plate is ~ 50 to 200. Since we don’t yet know just how concentrated our cells are, we will have to plate from more than one dilution.

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| * Label 8 agar plates around the margins of the ***bottom*** dish, leaving plenty of room for you to view the colonies, and no doubt as to whose plate it is, which dilution.
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* Dispense 100 µL of each of **the 8 most dilute cell cultures from the 10-fold dilution** series onto its own agar plate, and add a few sterile glass beads. Stack the plates right side up, and shake back and forth until the liquid has dried.
* Remove the beads: Turn each dish upside down in turn, tapping it to knock the beads into the lid, remove the lid while keeping the bottom upside down, and tap the beads into the waste receptacle. Replace the lid.
* Put the plates in the 37°C incubator, ***bottom side up*** to incubate overnight.
* Come back the next morning to count colonies (or put the LB-plates in the refrigerator to stop growth). Don’t bother trying to count the colonies on overcrowded plates. An overcrowded plate is any plate with >100 cells on it. It might be wise to take a picture of the plates you count.

### Notebook

In addition to all of the usual, your notebook should contain:

1. A table with the dilution factor in each well of your multi-well plate, and the OD595 values of each well.
2. Your graphs comparing dilutions to OD595 – all of the data and with the noise removed.
3. Cell counts, volume and cells/mL calculations for each hemocytometer sample, as well as an indication of which tube was used (and its dilution factor)
4. Colony counts for the countable agar plates (entered the next morning, obviously), and the dilution factor for each.

### Clean up

1. Throw microtubes in the trash.
2. Give your liquid waste to your instructor for disposal.
3. Put all plastic items contaminated with bacteria into the red biohazard waste bin.
4. Add bleach to bacterial cultures in glass, rinse once, and put in the glass trash.
5. Throw out your bench pad and all your gloves.
6. Clean your bench top with 70% ethanol.
7. Wash your hands.
8. Call home, they worry.
1. This lab was designed by Dr. Bob Weis. The protocol was written by Dr. Katherine Dorfman and Bob Weis with additions by Dr. Caleb Rounds [↑](#footnote-ref-1)
2. Schaechter M, Niedhardt FC in *Escherichia coli* and *Salmonella typhimurium*: *Cellular and Molecular Biology*. (Niedhardt FC, editor) Washington, DC: American Society of Microbiology, 1987, p 2. [↑](#footnote-ref-2)
3. This quote, or something like it, is attributed to Jacque Monod, who by it implied that the cellular machinery of bacteria is used by eukaryotes. However, the reverse may not true as frequently; see [Wolin, SL (1994) From the Elephant to *E. coli*: SRP-Dependent Protein Targeting 77 (6), 787-790.](http://www.sciencedirect.com/science?_ob=ArticleURL&_udi=B6WSN-4C5PPSP-2&_user=1516330&_rdoc=1&_fmt=&_orig=search&_sort=d&view=c&_version=1&_urlVersion=0&_userid=1516330&md5=cd4106c1a80e1e054bc197df454ef818) [↑](#footnote-ref-3)