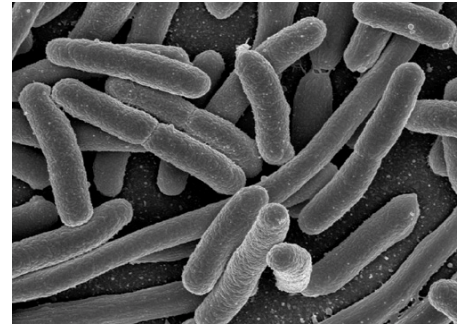


Lab #2 Growth of *E. coli* Bacteria

Escherichia coli (*E. coli*) is a rod-shaped (bacillus), enteric (gut) bacterium of the family Enterobacteriaceae. It is a normal resident of the human colon and therefore grows ideally at 37°C. In the laboratory, *E. coli* can be grown in solid or liquid culture media that contains nutrients such as carbohydrates, proteins, nucleic acids, salts and vitamins. We will be using LB broth and LB agar as our culture medium. LB media was designed by Salvador Luria and Guiseppe Bertrani in 1952.

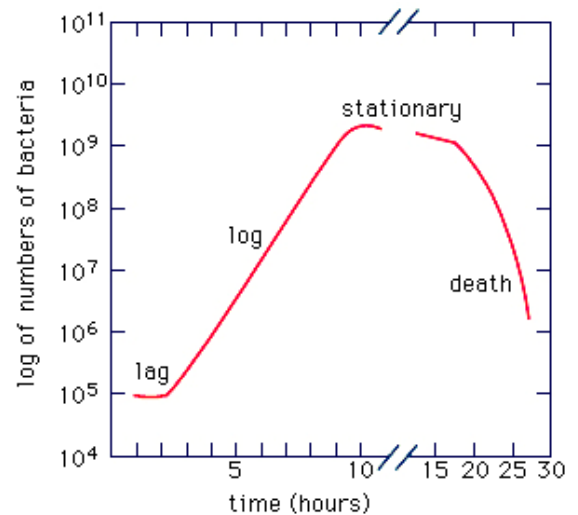


The growth of *E. coli* under ideal condition (LB broth at 37°C) follows a specific pattern as shown in Figure 1. When a small amount of bacteria is inoculated into a large amount of media (50:1) the bacteria enter into a lag phase. During this time, there is little to no growth because cells are adjusting to their new environment. During the logarithmic (log) phase, cells grow exponentially and the cell number doubles every 20-30 minutes. During the stationary phase, cell division matches cell death and the total cell number remains constant. After an extended period of time (>24 hours) cells enter the death phase and the culture slowly dies due to depletion of nutrients and build-up of wastes.

E. coli are used extensively in molecular biology labs as a means of manipulating DNA and cloning genes. It is often useful to know the concentration of cells in a bacterial culture and also to know the growth rate of that culture. There are several methods by which the number of bacterial cells in a culture can be determined. One method is to make serial dilutions of the culture, spread a known volume of the dilutions on LB agar plates and then incubate the plates at 37°C overnight. A single cell will divide many times; giving rise to a colony that is visible to the unaided eye. The number of colonies are counted and then multiplied times the dilution factor to determine the number of cells/ml in the original culture. This method is very accurate, but time consuming.

Spectrophotometry offers an alternative method to estimate the concentration of cells in a bacterial culture. Bacterial cells are small particles capable of absorbing and scattering light. When placed in a spectrophotometer the scattering of light by bacteria can be measured as apparent absorbance at a wavelength of 650 nm. Absorbance values gained in this way are also known as Optical Density or OD₆₅₀. The greater the density of the bacterial culture, the greater the OD₆₅₀ for the culture. Using OD₆₅₀ values and a standard curve (generated by our colony count experiment) we will estimate the number of cells/ml for a given culture of bacteria. We will also determine the growth rate of *E. coli* bacteria by measuring the OD₆₅₀ of actively dividing cells at regular time intervals.

Figure 1. Growth of *E. coli* in LB broth



I. Rate of growth of bacteria (Day1)

1. Calibrate the spectrophotometer

Turn on the spectrophotometer, set the wavelength to 650nm, the filter to 600-950 nm (lever on bottom, left) and allow it to warm up for 15 minutes. Be certain that the sample chamber lid is closed, and then adjust the left knob (0%T) until **transmittance** reads zero. Now place a test tube containing 3 ml LB broth into the sample chamber of the spectrophotometer (first remove the cap from the test tube) and adjust the right knob (100%T/0A) until the **absorbance** reads zero. You have now blanked the machine. The spectrophotometer will ignore the contribution of the LB broth to the absorbance of your samples.

2. There is a 37°C water bath in the room that has several flasks, each containing 30 ml of LB broth. Take one of these flasks back to your bench and label it with your initials.

3. Using sterile technique, add 0.6 ml of stock E. coli to the flask and mix.

4. Pipet 3 ml of the mixture into a glass culture tube (use your p1000), measure the OD₆₅₀ in the spectrophotometer and enter that data in Table 1 beside time zero.

5. Place the flask back in the shaking 37°C water bath. Note the time. Time Zero _____

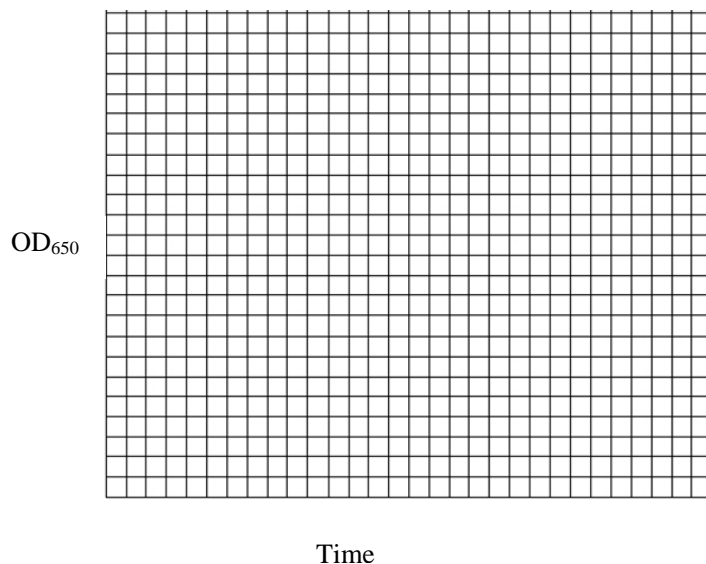
6. Determine the OD₆₅₀ for the culture every 30 minutes (by repeating step 4) and enter the values in Table 2.

7. Plot the data in Table 1 on the graph in Figure 2 and draw a best fit line.

Table 1. Time vs OD650

Time (min)	OD ₆₅₀
0	
30	
60	
90	
120	
150	
180	
210	
240	

Figure 2. Time vs OD650



II. Determining bacteria concentration by colony count (Day 1)

1. You will now make 10-fold serial dilutions of the stock bacterial culture.
2. Label 7 glass culture tubes 1A-7A at the top of the tube with a permanent marker
3. Using a 2 ml plastic pipet and pump, add 2.7 ml of LB broth to each tube.
4. Using sterile technique, add 0.3 ml of stock E. coli culture to tube #1A and mix.
5. Transfer 0.3 ml of diluted culture from tube #1A into tube #2A and mix
6. Transfer 0.3 ml of diluted culture from tube #2A into tube #3A and mix
7. Transfer 0.3 ml of diluted culture from tube #3A into tube #4A and mix
8. Transfer 0.3 ml of diluted culture from tube #4A into tube #5A and mix
9. Transfer 0.3 ml of diluted culture from tube #5A into tube #6A and mix
10. Transfer 0.3 ml of diluted culture from tube #6A into tube #7A and mix
11. Obtain 7 LB agar plates and label them as 1A-7A.
12. Using sterile technique, spot 0.1 ml of serial dilution #1A on plate #1A and spread cells out using a sterile glass spreader. Briefly, the glass spreader is dipped in alcohol and flamed. Allow the spreader to cool (5 seconds), then spread the cell culture uniformly across the plate. The instructor will demonstrate this technique. Repeat for the remainder of the dilutions.
13. Allow the plates to sit at room temperature undisturbed for 15 minutes, giving the liquid culture time to soak into the agar. Then bundle all the plate together with a piece of tape. Label with your initials. Incubate at 37⁰C upside-down overnight. The instructor will store these plates for you at 4⁰C until next week's lab. Next week you will count colonies and determine the cell concentration of the original stock culture.

Determining bacteria concentration by colony count (Day 2)

1. Pick up your plates (1A-7A) from the teacher's bench.
2. Choose a plate that has more than 25, but less than 250 colonies. Count the number of colonies on this plate and enter the data on this line _____.
3. Using dilution factors calculate the number of cells/ml in the original stock culture and enter that data in column 3 of Table 2. Show your calculations in the space below.

III. Relating Cell Concentration with OD₆₅₀ (Day 1)

We have been using OD₆₅₀ as a relative measure of bacterial concentration. We know that as the bacterial culture becomes more concentrated the OD₆₅₀ rises. Now we will determine the absolute relationship between cell concentration and OD₆₅₀ by assigning specific cells/ml concentrations to specific OD₆₅₀ values.

Procedure

1. You will now make 2-fold serial dilutions of the original stock E. coli culture.
2. Label 6 culture tubes as 1B-6B at the top of the tube with a permanent marker.
3. Pipet 3 ml of LB into each tube
4. Pipet 3 ml of stock E. coli culture into tube 1B and mix (cover with parafilm and invert 3X)
5. Transfer 3 ml of culture from tube #1B to tube 2B and mix
6. Transfer 3 ml of culture from tube #2B to tube 3B and mix
7. Transfer 3 ml of culture from tube #3B to tube 4B and mix
8. Transfer 3 ml of culture from tube #4B to tube 5B and mix
9. Transfer 3 ml of culture from tube #5B to tube 6B and mix
10. Measure the OD₆₅₀ of tubes 1B-6B and measure the OD₆₅₀ of the original culture. Enter those values in Table 3.
11. You will be able to complete Table 2 and Figure 3 next week after the colony count data is available.

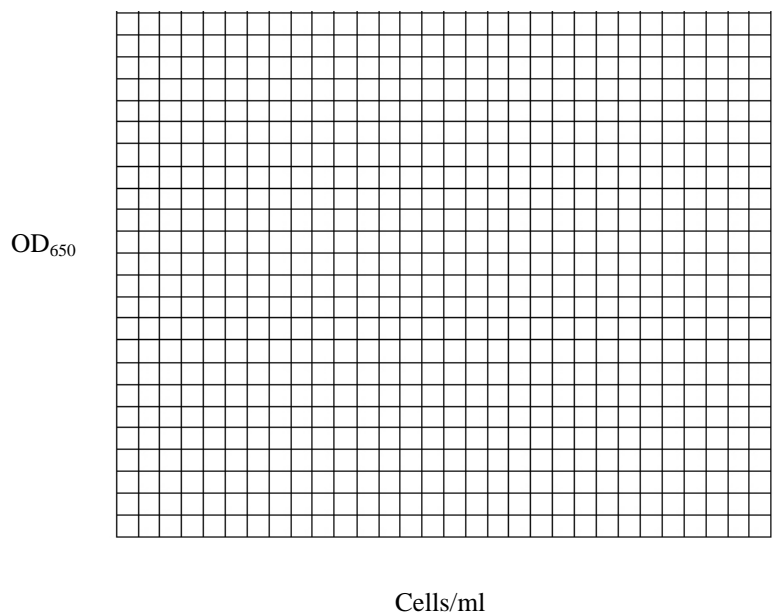
Relating Cell Concentration with OD₆₅₀ (Day 2)

1. By now you should have determined the concentration of the stock E. coli culture using the colony count method. And that data was entered in Table 2 below.
2. Knowing that tubes 1B-6B were 2-fold serial dilutions, you can now use simple mathematics to complete column 3 of Table 2.
3. Plot the data from Table 2 on the graph in Figure 3 and draw a best-fit line.

Table 2. Cell Number vs OD₆₅₀

Tube #	OD ₆₅₀	Estimated Cells/ml
Stock culture		
1B		
2B		
3B		
4B		
5B		
6B		

Figure 3. Cell Number vs OD₆₅₀



IV. The isolation of single colonies (Day1)

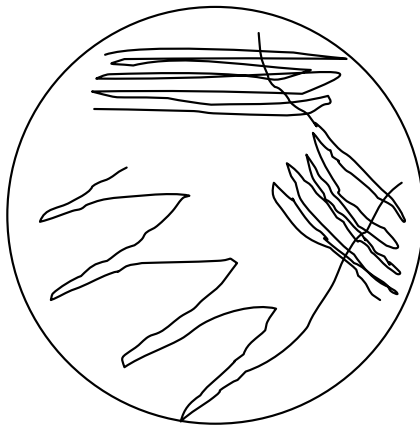
When using bacteria in molecular biology experiments it is best to work with a clonal population of cells that were derived from a single isolated cell. A pure, clonal population ensures consistent results in one's experiments. In the next exercise you will be asked to isolate single colonies of bacteria that were derived from a single cell and thus represent a clonal population of cells.

Procedure:

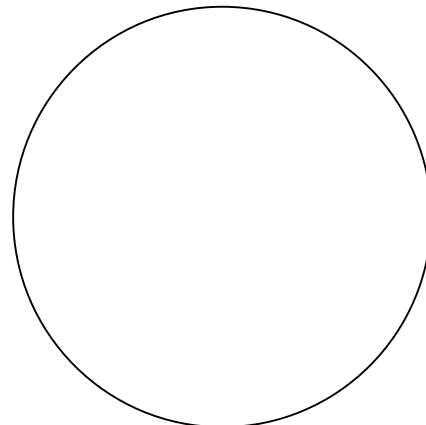
1. Label an LB agar plate with your name and the date. (label bottom portion of plate towards the edge)
2. Sterilize a wire loop by flame, and then allow it to cool (as demonstrated by the instructor)
3. Obtain a loop full of E. coli bacteria from your original stock culture
4. Spread the bacteria back and forth on one portion of the plate
5. Flame the loop again, allow to cool, and then draw out bacteria on to another portion of the plate.
6. Repeat step 5 again, then incubate inverted at 37°C overnight.
7. Your streak pattern should be something like the "example" shown in the circle below.

The isolation of single colonies (Day 2)

1. Obtain your streaked plate from the instructor's bench.
2. Make a rough sketch of the pattern of colonies obtained in the circle below.



Example



Observed result

V. Microscopy of Bacterial Cultures - Bacterial Gram Stain

The gram stain is a differential stain that allows you to classify bacteria as either gram-positive or gram-negative. These 2 groups differ in the structure of their cell walls. The cell wall of a gram positive bacterium is a thick peptidoglycan layer, while the cell wall of a gram negative bacterium is a thin peptidoglycan layer bound to a lipopolysaccharide layer.

Crystal violet is the primary stain used in the gram stain procedure. It passes through the cell walls and plasma membranes of all types of bacteria and thus will stain any bacteria purple. Next, one applies Mordant (Gram's iodine), which diffuses into the cell and combines with crystal violet to form an iodine/crystal violet complex. This complex is large and can't be washed out of gram positive bacteria because it can't pass through the thick peptidoglycan cell wall. However, the iodine/crystal violet complex can be washed out of gram negative bacteria because the cell wall is thin and more permeable. Applying decoloring agent (ethyl alcohol-acetone) will cause gram negative bacteria to lose their purple color, while gram positive bacteria will retain their purple color. Finally a counterstain (safranin) stains the decolorized bacteria red.

Procedure:

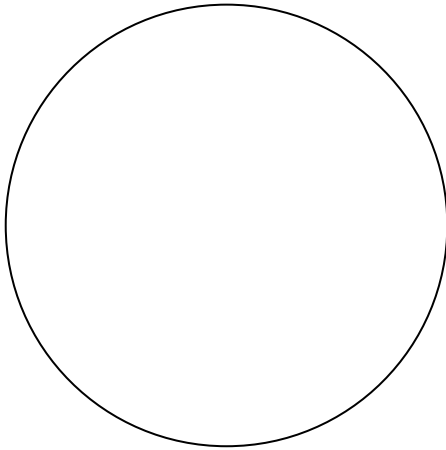
- 1) Prepare a bacterial smear of 2 cultures of bacteria, *Staphylococcus aureus* and *Escherichia coli*.
 - a) Using a permanent marker, draw 2 circles the size of dimes on the bottom of a well-cleaned slide
 - b) label the circles as one or the other bacterial culture (label outside the circles)
 - c) Sterilize an inoculating loop with a Bunsen burner.
 - d) Allow the loop to cool then spread 1 loopful of bacteria within one of the circles (on the unmarked side of the slide). Repeat for the other culture. Allow the slide to dry completely.
 - e) Hold the slide with a clothes-pin. Heat-fix the smear by quickly passing the slide, bacteria side up, through the blue portion of the flame 2-3 times. The back of the slide should feel warm, but not hot enough to burn. This glues the bacteria to the slide so that they won't be washed off during the staining process.
 - f) When the slide has cooled it can be stained.
- 2) Prepare a gram stain of your heat-fixed smear.
 - a) Place the smear in the crystal violet container and leave for 30 seconds.
 - b) Wash off the crystal violet with distilled water from a water bottle. DO NOT squirt the smear directly or you may wash off the bacteria.
 - c) Cover the smear with Gram's Iodine for 10 seconds.
 - d) Wash off the Gram's Iodine with distilled water as before.
 - e) Decolorize with 95% acetone/alcohol. Allow the alcohol to wash over the smears until there are no longer large amounts of purple dye washing off (10-20 seconds). Do not over-decolorize.
 - f) Immediately wash with distilled water.
 - g) Place the slide in the safranin container for 30 seconds.
 - h) Wash with distilled water and blot dry between absorbent bibulous paper.
 - i) Observe the slide under the microscope using low, high-dry and oil-immersion objective lenses. Place oil directly on the smear. You will not need a cover-slip.
 - j) Draw the bacteria as seen under oil-immersion on the next page. Use colored pencils. Identify the bacteria as either gram positive or gram negative in the "Gram Stain result" line below your drawings

Basic rules of microscopy

- Always begin examination of the specimen under the lowest power objective first and then increase magnification sequentially from 4X to 10X to 40X to 100X objective, bringing the specimen into clear focus with each lens.

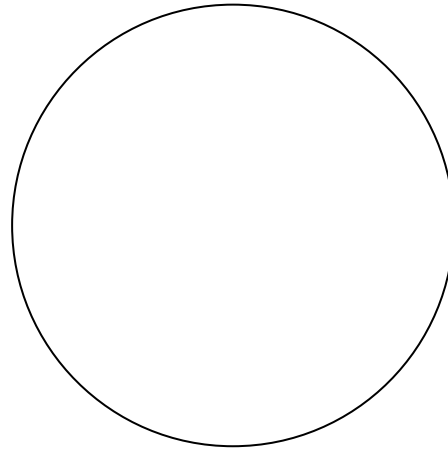
- The coarse focus knob is only used for the low power objectives (4X and 10X).
- The objective lenses are parfocal (focus is maintained when switching between objective lenses), thus only the fine focus knob is required for focusing when high power objectives lenses (40X and 100X) are in place.
- Keep all lenses clean. Use only lens paper to clean the lenses. Always clean your lenses before putting the scope away.

Drawings:



E. Coli

Gram Stain result _____



S. aureus

Gram Stain result _____

Questions

1. When a dilute liquid culture of *E. coli* is grown under ideal conditions it progresses through 4 phases of growth. List these phases and briefly describe the rate of growth during each phase.
2. How can we use a spectrophotometer and OD_{650} to estimate the concentration of a liquid *E. coli* culture?
3. You wish to determine the cells/ml concentration of a bacterial culture. You do three 100-fold serial dilutions and plate out 0.1ml of each dilution on LB plates. The next day you count 100 colonies on the 3rd (most dilute) plate. What is the concentration of the original culture? Show your calculations in the space below.
4. What does it mean to say that the objective lenses on microscopes are parfocal?
5. What is the only type of paper used to clean microscope lenses?
6. How does the structure of a bacterium's cell wall determine its color in a gram stain?

Notes

