MEASUREMENT OF THE CONCENTRATION OF VIABLE CELLS IN A SUSPENSION OF BACTERIA

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Introduction

There are a number of ways to estimate the density of a suspension of cells. Because the cells reflect light, the suspension appears turbid. Instruments are available to measure turbidity, and that measure is directly proportional to the concentration of cells in the suspension. However, it can say nothing about whether the cells are alive or dead or whether other factors contribute to the turbidity, such as precipitation of salts from the nutrient fluid or contamination by cells of a different type. Another way to measure is to sample a droplet of the suspension to a special microscope slide that has a chamber of known volume on its surface. When a coverslip is placed over the chamber, you can see the cells in the volume through a microscope and count them. You can focus on cells, ignoring salt crystals or cells that look different from the ones that interest you, but you still cannot tell whether the cells are alive or dead. The best way to do that is to carry out a viable cell count, a procedure that systematically dilutes the suspension to a point that individual living cells can divide and form a colony.

This procedure is used in many applications in bacteriology and cell biology. For example, if you want to monitor the effectiveness of an antibiotic on a culture of cells, you could suspend the cells in a fluid that contains the antibiotic and withdraw samples periodically for a viable cell count. If you wanted to verify that an illness is caused by a particular bacterium, you would culture a sample of diseased tissue (say, tonsils) and identify bacteria that grow from the sample, in part by the appearance of single colonies.

Our purpose today is simply to generate some data to illustrate statistical variability. We will all start with the same population of living *E. coli* bacteria. From its turbidity we can tell that it is very concentrated, but we cannot tell by looking just how concentrated it is. If we were to place a small amount on the surface of nutrient agar, individual cells would land in particular positions on the surface and start to grow. Over 18 to 24 hours, the colony resulting from that single cell will become visible to the naked eye. By counting the number of colonies and recognizing that they came from a defined volume of the original suspension, you can calculate how many of those cells were found in the suspension. For example, suppose 0.1 mL of suspension gave rise to 76 colonies. You would then know that the original suspension had about 760 colonies/mL (a concentration). If you also knew that the original suspension had a volume of 15 mL, you could easily calculate that there were a total of 11,400 cells in the population (a frequency).

Two issues complicate this interpretation. The first is biological. Ordinarily if a population of bacteria is dense enough to appear turbid, it will have so many cells/mL that even the smallest droplet on an agar plate will form a continuous lawn of bacteria, with all the individual colonies merging. No information can be obtained from that. Thus it is necessary to prepare dilutions of the original suspension by carefully taking a sample of known volume and mixing it with a known volume of nutrient broth to reduce the concentration by a known factor. Plating the diluted sample might reduce the concentration to the point where single colonies form and can be counted. The second issue is statistical. In the interpretation of your result, you have assumed that 76 colonies is an accurate estimate. Your neighbor, doing exactly the same series of manipulations, might get 83 or 65 colonies. Some of the variation comes from subtle differences in procedure: imprecise pipetting, poorly mixed initial sample, failure to deposit all of the droplet of suspension onto the plate, irregular spreading of the droplet over the plate, or differences of opinion as to what counts as a colony. Even if the procedure is automated,

however, seldom will two plates give identical counts of colonies. This is the element of random variation, the component that statistics can help us understand.

Today we will demonstrate these principles by taking a single starting sample of bacteria, diluting it tenfold, mixing, and diluting again a total of ten times. At some point in this series we expect to find two or three successive dilutions that give countable colonies, and that the numbers of colonies should be roughly ten times more abundant in the less dilute sample than in the more dilute one. By collecting the data from all the lab groups, we will be able to make a much better estimate of the actual density of cells in the original sample. One additional feature that makes this more of an experiment: some of the groups will use one kind of nutrient agar, and the others will use a different kind. This will illustrate genetic variability even in a population of bacteria.

Procedure

Your stations have been equipped with the following materials:

- A test tube rack with 10 sterile plastic snap-cap tubes
- A larger snap-cap tube containing 10 mL of nutrient broth (LB broth)
- A marking pen
- A sample of an overnight bacterial culture
- A box of sterile blue pipettor tips
- A box of sterile yellow pipettor tips
- P200 and P1000 automatic pipettors
- A sterile blue plastic spreader

At the front of the room are stacks of nutrient plates. If your marking pen is blue, take 10 of the unmarked plates; if it is black, take 10 plates that have a mark on the side of the lid.

Mark the plates by writing on their lids with your initials. Then write on one plate and one tube the designation 1:10, on another pair 1:100, on a third $1{:}10^3$, a fourth $1{:}10^4$, etc. until 9 of the tubes and plates have been labeled. Mark the tenth plate "undiluted" and put it at the front of the series.

Reserve the tenth tube for initial practice opening and closing the tubes efficiently (note the two positions of closure; only the snapped down position will not leak if you turn it upside-down.) You can also practice putting tips on the pipettors and pipetting water. Fill the tube half way or so with tap water. To withdraw a sample, set the volume of the sample by twisting the knurled knob until the correct volume is displayed in the window. Pick up a tip by pushing the end of the pipettor into one of the tips in the box. (The tips are sterile, so keep the box closed unless you are obtaining a tip). Now depress the plunger to the first stop. Open the tube of water and immerse the tip below the surface. Smoothly allow the plunger to come out again, bringing the sample with it. Now pretend that the tube of water is the destination for the sample. Open it and press the tip against the inside of the tube below the mouth. Depress the plunger to the first stop; when most of the liquid has drained, depress it quickly to the second stop to "blow out" the remaining liquid. Recap the tube and discard the used pipettor tip by pointing it at a wastebasket and pushing on the button to the side of the plunger. (This part often leads to student competitions, so we sometimes remove the ejector mechanisms.) Now that you are familiar with the pipettors, we'll begin.

Set the P1000 pipettor to 900 µL and insert a sterile blue tip. Do not touch the tip with your fingers or touch it to any nonsterile surface. Open the tube of LB broth long enough to remove 900 µL. Replace the cap and remove the cap from one of the tubes. Dispense the 900 µL into the tube. Repeat for each of the labeled tubes.

Set the P200 pipettor to 100 µL and insert a sterile yellow tip. Do not touch the tip with your fingers or touch it to any nonsterile surface. Open the bacterial suspension and withdraw 100 µL. Transfer it to the 1:10 tube, replace the cap, and briefly shake briskly. Get a fresh tip for the P200 and transfer 100 µL from the 1:10 tube to the 1:100 tube. Recap, shake, and repeat this “serial transfer” down the line of the nine tubes.

Each time you add 0.1 mL (100 µL) to 0.9 mL (900 µL), you are diluting the concentration by a factor of 10 (1 part into 10 parts total).

When all the dilutions have been prepared, you are ready to plate your samples. Start with the most dilute sample. Use the P200 to remove 100 µL; dispense it into the center of the plate with the same marking on it. Put the cover down on the counter while you gently spread the droplet over the surface of the agar. Do it as quickly but as thoroughly as possible. If one person holds the spreader, you can use the same one as you proceed up the line of concentrations, putting 100 µL of each dilution onto its designated plate and spreading. It is best to use a separate tip for each sample, so broth carryover does not dilute the next sample. As long as you go from most dilute to most concentrated, a single spreader will work, because all that will be transferred is a little extra broth with only 1/10 the number of bacteria as on the previous plate. The tenth plate should get 100 µL taken straight from the undiluted culture.

Allow a few minutes for the broth to soak into the agar. Then invert the plates, stack them, and place them in the 37°C incubator. Tomorrow we will check for growth and count colonies.

Data analysis

Prepare a table with the headings: “dilution,” “number of colonies,” “viable cells / mL of dilution,” and “viable cells / mL in original culture.” Fill in the “dilution” column according to the dilutions you prepared, starting with undiluted. Count the colonies on plates that have reasonable numbers of distinguishable colonies. If you turn the plate upside down over a light box, you can see the colonies easily and mark the plate for each one you count (to prevent counting twice). Don’t forget to check the sides (some of the agar adheres to the sides). Write the count on the lid of the plate and in the column of your table labeled “number of colonies.” If you aren’t sure, ask someone else to read the same plate, perhaps circling the colonies where you have used a dot. Also enter your data on the table at the front of the room for the aggregated class data. For blank plates, the correct interpretation is “<1,” not “0.” For those that have too many colonies, if you are a masochist, you can divide the plate into quadrants and count one quadrant, multiplying by 4 to estimate the plate as a whole. Alternatively designate it as “Too many to count,” or “lawn.” Ideally you should have three successive plates that are countable: one with <10, one with <100, and one with <1000.

To estimate the concentration of viable cells in the dilution tube, multiply the number of cells in 0.1 mL by 10. To estimate the concentration of viable cells in the original culture, multiply this number by the dilution factor. Ideally all three countable samples should lead to an original concentration of the same magnitude. If not, it may reflect dilution error, sampling, or simply randomness.