

Lab 2.

Serial Dilution and Plating of a Bacterial Culture

"Nature in her errors reveals herself unbidden."

-Francis Bacon, circa 1620

1. Background

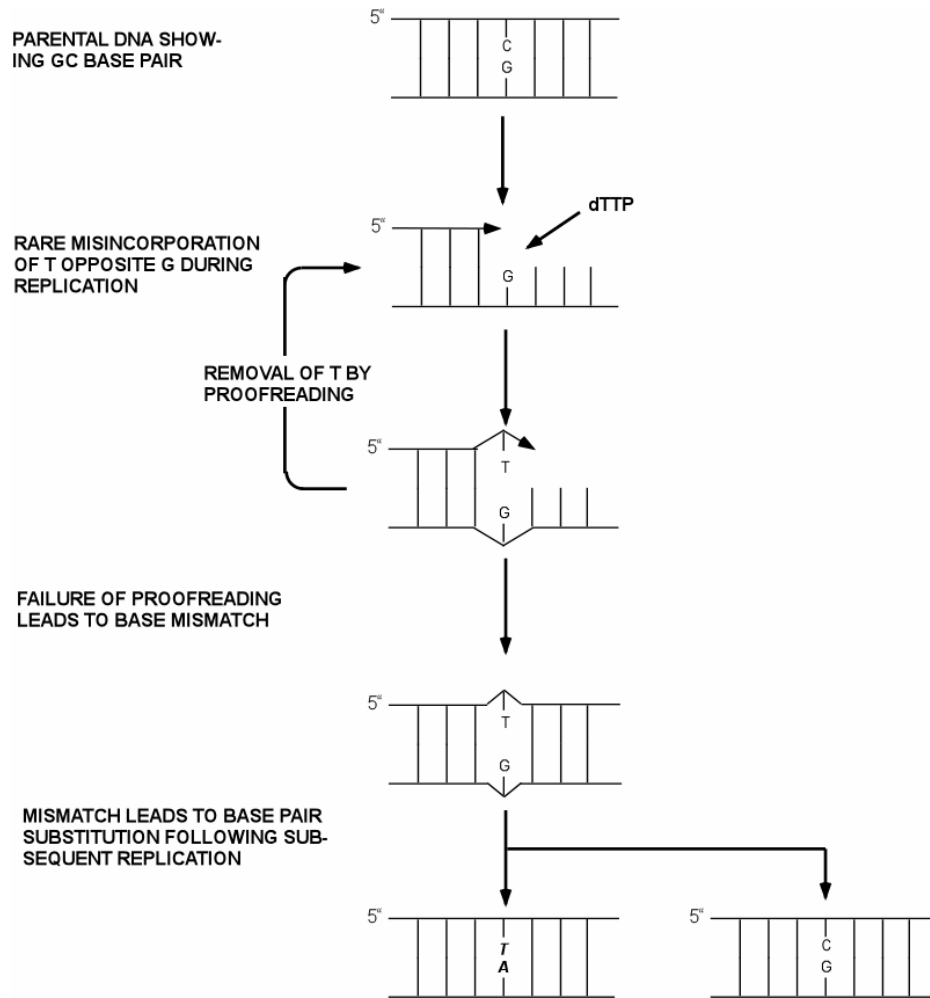
In this exercise you will apply the ability to perform dilutions gained in the previous exercise, along with some additional basic skills for manipulating bacteria, in order to conduct a simple, structured investigation. The conceptual goal of the investigation is to determine the effect of **DNA proofreading** on the frequency of antibiotic-resistant mutants in *E. coli*.

What is DNA proofreading?

During replication, an incorrect base is occasionally incorporated in a new DNA strand. Incorrect in this context means that the new base is not the one required by the standard base pairing rule of "A with T and G with C". Left uncorrected, the mismatched base would lead to a mutation in the DNA sequence. In the bacterium *E. coli*, the major DNA replicating enzyme (DNA Polymerase III) has the ability to recognize a mismatched base immediately after inserting it. The mismatched base is then removed. The ability of DNA polymerase III to immediately recognize and remove a misincorporated base is called **proofreading**.

The proofreading function resides in a specific protein subunit, "epsilon", of DNA polymerase III that is not necessary for the synthesis of DNA. That is, a molecule of DNA polymerase III lacking the epsilon subunit is capable of synthesizing DNA, but is no longer able to proofread. We expect that without proofreading, the frequency of mutations in *E. coli* would be higher.

The following diagram hopes to clarify this by showing an example in which T (rather than C) is incorporated opposite G during replication. If the G/T mismatch is not corrected by proofreading (or by any other repair mechanism) then the subsequent replication of the mismatch will lead to the substitution of an AT pair for a GC pair in the original DNA.



How can we possibly test the ability of proofreading to prevent mutation?

To test this we will work with two strains of *E. coli*. One strain has the normal form of DNA polymerase III (strain ϵ^+) while the other strain does not have a functioning epsilon subunit (strain ϵ^-), so it cannot proofread. Our experiment will attempt to measure the frequency of antibiotic-resistant mutants in cultures of the two strains.

This strategy is a specific example of a very popular and powerful approach to analyzing the physiological effect or function of a specific gene or gene product, i.e. compare the properties of two strains that are identical in genotype (“isogenic”) except that one strain lacks the gene or gene product (epsilon in this case) you are interested in. This is often referred to as the “genetic approach” or as “genetic analysis” of a gene function.

Why are we determining the frequency of antibiotic-resistant mutants rather than some other type of mutant?

One reason is that it should be very straightforward to detect antibiotic-resistant mutants by a simple plating technique. If we spread a large number of *E. coli* cells on an agar plate containing an antibiotic that the strain is sensitive to, then most of the cells will be killed. Only the resistant mutant cells within that population will grow to produce visible colonies. By counting those colonies we can know how many resistant cells were present in the culture sample we spread on the agar plate.

Another reason is that the evolution of antibiotic-resistant strains of bacteria, whether they arise from chromosomal mutations as described above, or by plasmid-encoded genes, is a very major public health concern. This exercise hopes to provide some food for thought on the clinical implications of spontaneous genetic mutations for evolution of antibiotic-resistance in the clinical arena.

That's cool. What antibiotics are we going to use?

We will use the antibiotics ampicillin and rifampicin. *E. coli* is usually sensitive to both. Here is some additional information about the action of these antibiotics.

Rifampicin

Rifampicin is a derivative of the natural antibiotic "rifamycin", which is produced by the soil bacterium *Streptomyces mediterranei*. Presumably, *S. mediterranei* uses rifamycin to kill competing soil bacteria (without somehow killing itself!).

The lethal effect of rifampicin is exquisitely potent and precise - a true "magic bullet". The antibiotic binds to the β' subunit of RNA polymerase, which is the single enzyme responsible for transcription of all 3 classes of RNA in *E. coli*. The RNA polymerase•rifamycin complex permanently locks onto the DNA template, blocking transcription. In other words, the *E. coli* cannot make RNA, and therefore it cannot make protein. Rifampicin is active against some bacteria at concentrations as low as 0.01 $\mu\text{g/ml}$!

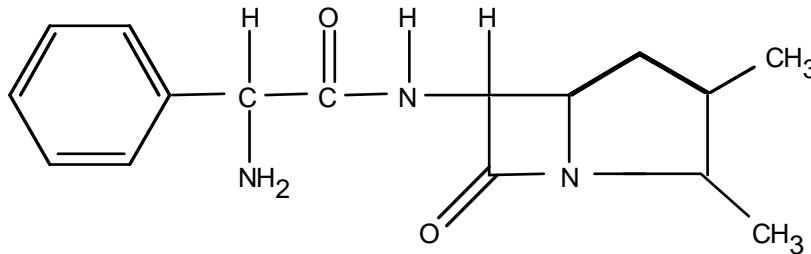
Nearly all the mutations leading to a rifampicin-resistant phenotype occur in the gene that codes for the β' subunit of RNA polymerase. These mutations apparently alter the structure of the β' subunit so that rifampicin is unable to bind to it, although the RNA polymerase retains its activity in transcription.

Because it may produce serious side effects, rifampicin is restricted to treating tuberculosis caused by strains of the disease organism, *Mycobacterium tuberculosis* which are resistant to other antibiotics. However, rifampicin is always administered simultaneously with some other antibiotic to reduce the

problem of spontaneous rifampicin-resistant mutants evolving within the patient during prolonged therapy.

Ampicillin

Ampicillin is one of the most commonly used clinical antibiotics. It is derived from the natural antibiotic penicillin produced by the fungus *Penicillium* sp. We will use ampicillin again later in this course during our molecular biology exercise.



In bacteria such as *E. coli*, synthesis of the bacterial cell wall is a tremendously complex exercise in biosynthesis, requiring the careful orchestration of a number of different enzymes. Ampicillin binds to, and inhibits, several different enzymes required for cell wall biosynthesis. Inactivation of any one of several of these enzymes is sufficient to inhibit or kill *E. coli*.

When cell wall synthesis is disrupted, the cells die because they become sensitive to lysis by the osmotic uptake of water from the medium. (i.e. they swell and bust like a balloon, a water balloon).

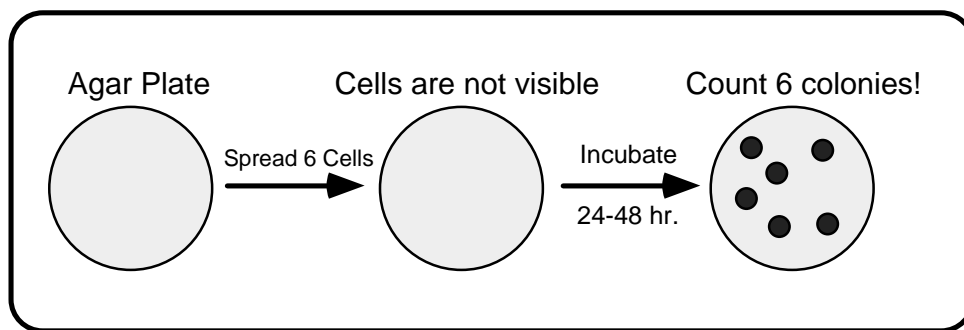
Reading (on reserve)

- Biology 6th ed. by Neil Campbell (the sections below):
 - Enzymes proofread DNA during its replication and repair damage in existing DNA (p. 299-301)
 - Point mutations can affect protein structure and function. (p. 322-325)
 - The short generation span of bacteria facilitates their evolutionary adaptation to changing environments (p. 340-341)
 - Nearly all prokaryotes have cell walls external to their plasma membranes (p. 528-529)
- Epidemiology of Drug Resistance: Implications for a Post-Antimicrobial Era
Cohen, Mitchell L. (1992) *Science* 257; 1051.
- Resistance to Antibiotics Mediated by Target Alterations
Spratt, Brian G. (1994) *Science* 264: 388.

2. General Description of Method

A. Spread Plating

The method presented here allows an investigator to determine the concentration of viable bacteria in a liquid sample. This is accomplished indirectly by spreading the cells on the surface of an agar plate and then counting the number of visible colonies after the plate is incubated. The inherent assumption of the technique is that each colony on the plate consists of millions of cells that arose, during incubation, from a single cell in the original sample.



Basis of the Spread Plating Technique

B. *E. coli* strains

The *E. coli* strains (ϵ^+ and ϵ^-) are provided as overnight cultures grown overnight in a standard culture medium called Luria-Bertani (LB) Broth. Each student pair will work with one of the 2 strains. Be sure to record which of the two strains you are given.

Consult the Safety Section at the end of this exercise for specific information on hazards/precautions for working with *E. coli* in the lab.

C. Serial Dilution

Before lab, do your best to devise a serial dilution procedure that will allow you to obtain dilutions of the overnight culture at dilution factors of factors of 10^{-1} , 10^{-5} , 10^{-6} , and 10^{-7} . The procedure must be planned so that you have approximately 1 ml, or more, of each of these dilutions for plating.

You should plan this dilution series ahead and come to lab with a proposed procedure written out and diagrammed in your notebook. The instructor will check this, make corrections, and offer advice as they deem necessary before allowing you to proceed. It is unlikely that your instructor will mark you down for proposing an unworkable or incorrect procedure.

The single most common cause of bogus results in this exercise (by far) is inadequate mixing of the dilution tubes, leading to non-representative sampling.

3. Procedure

When you and your instructor have agreed on a specific serial dilution procedure, go ahead and set up the dilution tubes and add the appropriate amounts of diluent (sterile 0.9% NaCl) to each tube. Remember to make aseptic transfers.

At this point we suggest that you set up 1 or 2 additional practice dilution tubes. Use a colored dye solution to practice pipetting and mixing. The colored dye gives a good visual indication of whether or not your mixing technique is adequate.

If you are confident of your technique you can proceed to complete the dilution series. Remember to change pipettes or pipette tips at each step of the dilution series.

Label all your agar plates if you have not already done so.

When the dilution series is complete, make spread plates using 50 μl samples from the 10^0 , 10^{-5} , 10^{-6} , and 10^{-7} dilutions on LB Agar. (LB Agar is the same standard growth medium as LB Broth except that the addition of agar transforms it to a gel.)

Also, plate 50 μl samples of the undiluted cell culture and of the 10^{-1} dilution, on LB agar plates containing rifampicin, and plate 50 μl samples of the undiluted cell culture on LB agar plates containing ampicillin.

The plating scheme is summarized in this table:

Dilution	LB	LB+Amp	LB+Rif
10^0	X	X	X
10^{-1}			X
10^{-5}	X		
10^{-6}	X		
10^{-7}	X		

Put your 7 plates in the indicated tray for incubation. The plates will be incubated for 1-2 days, long enough for visible colonies to develop. Then the plates will be refrigerated to stop growth until the next lab meeting.

4. DATA COLLECTION AND CALCULATIONS

Examine all your plates and record your general observations (directly in your notebook). Are the number of colonies on the LB Agar plates roughly consistent with the dilutions?

For the LB Agar plates (without antibiotic), choose the ONE plate that has between 30 - 300 colonies. Count the number of colonies on this ONE plate ONLY and use that ONE value to calculate the cell concentration in the original culture. Count the colonies on the plates using the semi-automatic counter.

Counting colonies on plates with < 30 makes the method imprecise because it introduces significant statistical error. Counting colonies on plates with > 300 colonies is inaccurate because there would be significant coincidence counting (i.e. 2 cells close enough together that they grew into what appears to be a single colony). If there is more than one LB Agar plate with between 30 - 300 colonies than something is wrong, isn't it?

Use the result (i.e. # colonies on a plate) to calculate the total cell concentration (cells/ml) in the overnight culture we gave you. For this calculation you need to consider the dilution factor of the overnight culture that was spread on the plate that you counted, and the volume (50 μ l) of the dilution that was plated.

Next, count the colonies, if any, on the LB Agar plates containing antibiotics and calculate the concentration (cells/ml) of antibiotic resistant mutants in the original culture. You should have two values here, one for each antibiotic.

IMPORTANT!!!

Report cell concentration data with 2 significant figures only. The precision of the method allows no more. A calculated value of 2.06×10^8 cells/ml should be rounded to 2.1×10^8 .

If you have antibiotic plates with no colonies on them, then you report your result as a "<" value. It is not valid to enter "0". That is, if you examine 100 cells and none are resistant you cannot claim that the frequency is zero, only that it is less than 1/100. To calculate the appropriate concentration value in this case you must take into account the volume of cell suspension spread on the plate. The value would be $< 1 \text{ cell/volume}$. (EXAMPLE: If you spread 0.1 ml and observe 0 colonies, your calculated value for cell concentration would be $< 10 \text{ cells/ml}$.)

Your instructor may ask you to enter your data in an EXCEL spreadsheet . You will need to provide the following information:

- strain (ϵ^+ or ϵ^-)
- total cell concentration in the original culture (cells/ml)
- concentration of RIF^R mutants in the original culture (cells/ml)
- concentration of AMP^R mutants in the original culture (cells/ml)

In EXCEL, scientific notation is formatted in an unusual way. 2.1×10^8 is entered as 2.1E+08, and so on.

5. Discussion and Analysis

You should consider the data from the entire class. The first issue would be to evaluate the reproducibility of the results for the different groups working with the same strain. Inasmuch as several groups essentially repeated the same experiment, their results should be approximately identical. Is this the case? If not, how do you deal with it? (Mindlessly “taking the average” may not be a robust approach.)

Based on the class data, derive the best values for

- total cell concentration in the original culture (cells/ml)
- concentration of RIF^R mutants in the original culture (cells/ml)
- concentration of AMP^R mutants in the original culture (cells/ml)

It would not be valid to compare to concentration of mutant cells in the two cultures directly. Those values might be different for the trivial reason that the cultures had different total cell concentrations. Therefore you should use the cell concentration values derived from the class data to calculate the following frequency values:

Frequency RIF^R mutants in the ϵ^+ strain

Frequency RIF^R mutants in the ϵ^- strain

Frequency AMP^R mutants in the ϵ^+ strain

Frequency AMP^R mutants in the ϵ^- strain

Remember that frequency values are always unitless and are always between 0 and 1.0.

We expected that the ϵ^- strain would have a higher frequency of mutants. Is this the case? If so, how much higher?

Were the frequencies of resistant mutants the same for the two antibiotics? If not, why might they be different?

A few further off the wall questions you may wish to think about and explore:

What result do you think we would have gotten if we plated the culture on LB Agar plates that contained both antibiotics?

Another aspect of interest would be to consider the implications of this exercise for the clinical use of these antibiotics. For example, human bacterial infections sometimes cannot be treated with ampicillin because the bacteria are resistant to the antibiotic. How do you reconcile this with the results of your exercise?

How do the concentrations of the antibiotics used in our agar media compare to the concentrations of the antibiotics that are achieved in the tissues of human patients following administration of typical doses?

5. Lab Safety Section

Guidelines for Laboratory Work with *E. coli*

E. coli is by far the most commonly encountered microorganism in biology research and teaching laboratories and therefore a special discussion of the potential health and environmental hazards of working with *E. coli* is warranted, particularly in the wake of recent well-publicized outbreaks of *E. coli*-caused hemorrhagic colitis (Jack in the Box, Odwalla, Hudson Foods, etc.).

E. coli is a normal and ubiquitous inhabitant of the large intestine of healthy vertebrates. On the other hand, *E. coli* is known to cause a variety of clinical diseases including mild, self-limiting diarrhea; severe invasive gastro-intestinal infections such as dysentery and hemorrhagic colitis; urinary tract infections; septicemia; and meningitis. This apparently schizophrenic behavior on the part of *E. coli* with respect to pathogenicity is due primarily to extensive genetic variation among different strains of *E. coli*. Most *E. coli* strains, including the typical laboratory strains, have very rarely, if ever, been directly implicated in causing disease; whereas other strains are out and out pathogens because they carry genes for specific virulence factors such as toxins or siderophores. Thus, the potential hazard associated with laboratory strains is considerably less than with strains freshly isolated in a clinical setting, even though they are all technically *E. coli*.

Ultimately, though, the factors that influence whether a particular strain, in particular circumstances, should be regarded as a pathogen or not are so diverse that it is generally considered good practice to treat all microorganisms as presenting some degree of hazard in one way or another. However, economic considerations limit the extent of precautionary measures adopted when dealing with organisms of little or no demonstrated hazard, such as laboratory strains of *E. coli*. Moreover, an environment where research productivity is impaired by intolerably complicated and rigorous procedures is counterproductive because workers would then be tempted to take shortcuts. The following safety guidelines are therefore a pragmatic compromise.

This class will only work with standard laboratory strains of *E. coli*. Nevertheless we will consider all cultures to be "potential pathogens" and will rigorously adhere to the following precautions:

- No eating, drinking or smoking are allowed in the laboratory.
- Do not pipette cell suspensions by mouth.
- Notify the instructor if you have an open cut or burn.
- Notify the TA or instructor of any spilled cultures so they can be properly decontaminated.

- After each lab period wipe your benchtop down with disinfectant and wash your hands.
- Cultures are not ever poured down the drain or thrown in the ordinary trash.

Agar plates (and only agar plates) are placed in the designated biohazard waste can at the front of the room.

Flasks, tubes and bottles are placed in the autoclaving pan provided. Pipettes, swabs, kimwipes and micropipette tips are collected for autoclaving.

The prep staff will autoclave these materials before they are discarded.

- Further information on *E. coli* and biosafety can be found on the course web site.
- Review the directions for handling of hazardous waste given in Appendix VI.

Bunsen burners

The Bunsen burner presents a flammability hazard in this exercise, particularly used in conjunction with ethyl alcohol.

- Turn your Bunsen burner off whenever you are not using it.
- If your hair is long wear it up or tied back during this lab.
- Keep your ethanol jar away from the flame and cover the jar if you are not using it.