

BIOLOGY 124: LABORATORY MANUAL: ANTIBIOTIC DISCOVERY RESEARCH PROJECT



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Biology 124: Laboratory Manual: Antibiotic
Discovery Research Project

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Licensing

A detailed breakdown of this resource's licensing can be found in [Back Matter/Detailed Licensing](#).

1: Laboratory Safety and Best Practices

Laboratory Safety Overview

Learning Objectives

- explain safe microbiology procedures.
- practice safe microbiology procedures

The lab activities in this course involve the use of living organisms. Although the organisms that we use are not considered to be highly virulent, **all microorganisms should be treated as potential pathogens** (capable of causing disease). With proper laboratory procedures, the danger is minimal; however, carelessness can result in very serious accidents. For your safety and the safety of everyone in the lab and minimize the contamination of the lab environment, you must observe the following safety rules **at all times**.

This will be reinforced by practice and feedback in every lab.

Laboratory safety is our first priority and everyone (instructors and students) are expected to observe the following safety rules **at all times**.

Safety Rules

- Never place books, backpacks, purses, etc., on bench tops. Always place these in the assigned area. Always clean lab manuals, pens, pencils, etc after use to minimize transfer of microbes to other surfaces.
- Electronic devices should not be brought into the lab. This includes, but is not limited to computers, cell phones, and calculators.
- Clean your work area with 70% ethanol solution at the **beginning AND end** of each lab.
- Wash your hands with soap and dry with paper towels when entering and leaving lab.
- Use appropriate personal protective equipment at all times while working in the lab.
 - Closed-toe shoes (no sandals) are to be worn in the lab.
 - Long hair must be tied back to prevent exposure to flame and contamination of cultures.
 - Goggles must be worn if anyone in the laboratory is using a Bunsen Burner, caustic chemicals, a hot plate, or anything with a splash or fire risk.
- Do not place anything in your mouth or eyes while in the lab. This includes pencils, food, and fingers. Keep your hands away from your mouth and eyes.
 - Eating and drinking are prohibited in the lab at all times. This includes gum, cough drops, and candy. Do not apply cosmetics in the lab. This includes Chapstick™ and Blistex™.
 - Never pipet by mouth. Use a mechanical pipetting device.
 - You may bring a drink or a snack to leave outside the classroom.
- Use care around open flames.
 - Be aware of lit Bunsen burners and do not leave a lit burner unattended.
 - Tie back long hair and avoid wearing loose clothing around the flame.
 - Never spray ethanol in the vicinity of a lit burner. If a beaker of ethanol is used for sterilization in conjunction with a Bunsen burner, be sure that you have a fire-proof lid for the beaker within easy reach in case the ethanol catches fire.
- Do not remove media, equipment, or bacterial cultures from the laboratory except for authorized reasons (to autoclave).

- Do not place contaminated instruments such as inoculating loops, needles, and pipettes on bench tops. Loops and needles should be sterilized, and pipettes should be disposed of in designated receptacles of bleach solution or waste.
- Carry cultures in a test tube rack when moving around the lab or when keeping cultures on bench tops for use. This prevents accidents and contamination of your person or belongings.
- Immediately cover spilled cultures or broken culture tubes with paper towels and then saturate them with disinfectant solution. Notify your instructor that there has been a spill. After 15 minutes, dispose of the towels and broken items as indicated by your instructor.
- Report accidental cuts or burns to the instructor immediately.
- At the end of each lab session, dispose of biological waste properly. Biological waste consists of living organisms (such as microbes) and any objects that may have come in contact with them (such as gloves, pipets, etc.).
 - Solid disposable waste should be put in biohazard bags.
 - Liquid waste should be dumped in the collection beaker to be bleached by the lab staff before it is poured down the drain.
 - Test tubes and other glassware should be soaked in a 10% bleach solution for 20 minutes.
- Persons who are immune-compromised (including those who are pregnant or may become pregnant) and students living with or caring for an immune-compromised individual are advised to consult with your physician to determine the appropriate level of participation in the lab. Should your physician determine that you should not participate in this lab, please have him or her write a note stating the concerns. Alternative accommodations may be indicated.

OSHA Information

Safety Data Sheets (MSDS) are located: _____

The first aid kit is located: _____

The eyewash station is located: _____

The shower is located: _____

The fire extinguisher is located: _____

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2: Microscopy Experiment

We use a microscope to visualize objects that are too small to see with the naked eye. This is especially critical in Biology as all cells are quite small and require a microscope for visualization.

All living organisms are composed of cells. Most organisms are so small that they are one cell while some eukaryotic organism are much larger and are composed of somewhere between 2 and millions of cells. In this experiment you will learn to use a compound microscope and will measure and characterize single and multicellular eukaryotic samples as well as bacterial cells.

Learning Objectives

At the completion of this lab, students will be able to:

Content:

- Define magnification, resolving power, contrast, and field of view
- Recognize and give the function of the parts of a compound microscope
- Define cell, prokaryotic, eukaryotic, nucleus, and organelle
- Describe the similarities and difference between prokaryotic and eukaryotic cells
- Identify cell parts and describe their function
- Distinguish between plant and animal cells and know that they are both eukaryotic cells

Physical Skills:

- Properly care for a compound microscope
- Correctly use a compound microscope
- Make a wet mount
- Perform a simple stain

Scientific Communication Skills:

- Correctly label a microscopy image
- Format a figure legend

Introduction to Microscopy

The science of microbiology originated with the invention of the microscope in the late 16th century and its use by Robert Hooke and Antoni van Leeuwenhoek in the 17th century to view and describe microscopic living organisms. Microscopes remain one of the most important tools for modern microbiologists.

The microscopes we will use in class are compound bright field light microscopes (**Figure 1**). A light source in the base of the microscope is focused by the condenser and then passes through an experimental sample placed in the stage. The image is then magnified by the objective and ocular lenses (**Figure 2**). Our microscopes contain 10x ocular lenses, meaning this lens magnifies an image 10-fold. They are stereomicroscopes, so the two ocular lenses need not focus on the same focal plane. They also contain 4 objectives: a 4x (scanning), 10x (low power), 40x (high dry) and 100x (oil immersion).

Parts of the Compound Light Microscope

Use Figure 1 to identify the various parts of your microscope. Read each description below, find them on your microscope and on the lab handout and manipulate the parts only where indicated. Before you start, make sure that the shortest objective is in the light path.

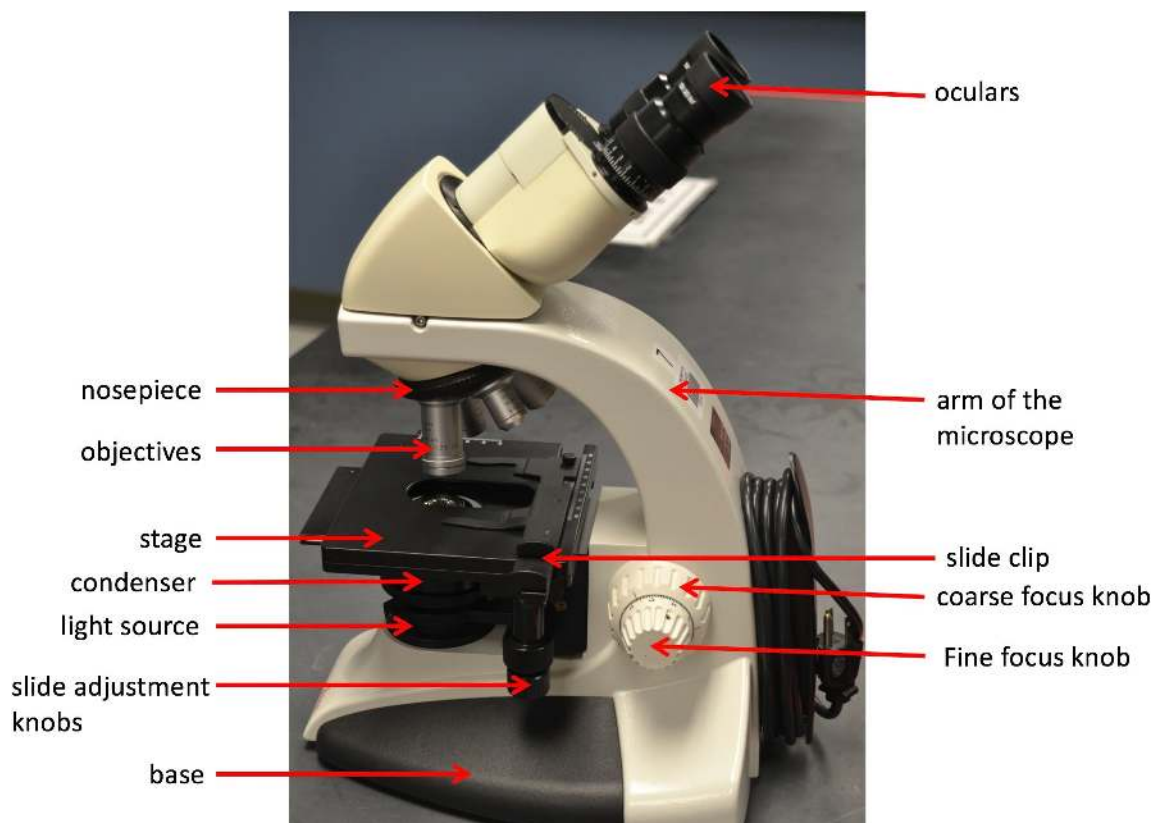


Figure 1. Parts of a compound light microscope

- *Light source.* The compound microscope uses transmitted light to illuminate a transparent (or super thin) sample mounted on a glass slide. Find the light source/illuminator, the off/on switch, and the rheostat which can vary the light intensity so that it is not too bright.
- *Condenser.* A condenser is composed of a *condenser lens* and *iris diaphragm* to focus the light on the specimen so that it is equally and fully illuminated. The lever for the iris diaphragm of the condenser is used to open and close the condenser to increase or decrease the area illuminated and thereby light intensity.
- *Stage.* Your slide containing a sample is placed on a mechanical stage over a central hole. Position a slide on the stage by releasing the tension in the spring loaded moveable arm and using this slide clip to hold your slide in place. There are two knobs to the right or left of the stage: one to move the specimen forward or backward and one to move it side to side.
- *Focusing knobs.* The coarse focus knob is for use with the low power objectives ONLY whereas the fine focus knob is critical for focusing especially with higher power objectives.
- *Objectives.* Our microscopes have four different objectives 4 objectives: a 4x (scanning), 10x (low power), 40x (high dry) and 100x (oil immersion). Note that they are labeled and color coded for ease of use. The other number written on the objective is the numerical aperture (NA). The higher the numerical aperture, the greater the resolving power.
- *Ocular.* The magnifying lens that you look into is called the ocular. Our microscopes have two 10x oculars (and are therefore stereomicroscopes). Since each objective has a different magnifying power, the total magnification is calculated by multiplying the magnifying power of the ocular by that of the objective.

Importantly, the objectives are **parfocal** meaning that once one objective has been focus, you can rotate another one into place without losing the focus. You will likely need to very finely adjust the fine focus, but it specimen will be very close to being in focus. Also, the center of the field of view will stay the same as you move from one objective to the next.

These features will allow you to generally focus at the low power (4x) where focusing is MUCH easier and then move to a higher power and only have minimal adjustments.

Magnification and Resolution

Magnification is the amount an image is increased in size; **resolution** is the ability to distinguish two distinct objects. Increasing the magnification does not help us see greater detail if the resolution is not sufficient to differentiate objects we are viewing at this high magnification. To be useful, an image must be clear, not just magnified.

The total magnification of your microscope is the product of the magnification of the objective and ocular lenses. For example, if you are looking at a slide through the 4x lowest power objective, the total magnification is $(4)(10)=40x$. If you switch to the 100x highest power objective, the total magnification is now $(100)(10)=1000x$. Remember you can select objectives of different magnification but on these microscopes your ocular lens is always 10x.

The resolution of a microscope is more difficult to control. Resolution depends on a built-in characteristic of the lens itself called the numerical aperture, as well as the wavelength of light. The theoretical maximum resolution of a compound light microscope is about $0.2\ \mu\text{m}$. Two objects (e.g. two bacteria or two subcellular structures) can only be distinguished as separate objects using a light microscope if they are at least $0.2\ \mu\text{m}$ apart, no matter how great the magnification.

One way to maximize the resolution is to use an oil-immersion lens. Since immersion oil has the same refractive index as glass, placing a small drop of oil between the coverslip and the objective reduces the refraction that would normally happen as the light travels through air. With less light scattered, the resolution of the lens is optimized.

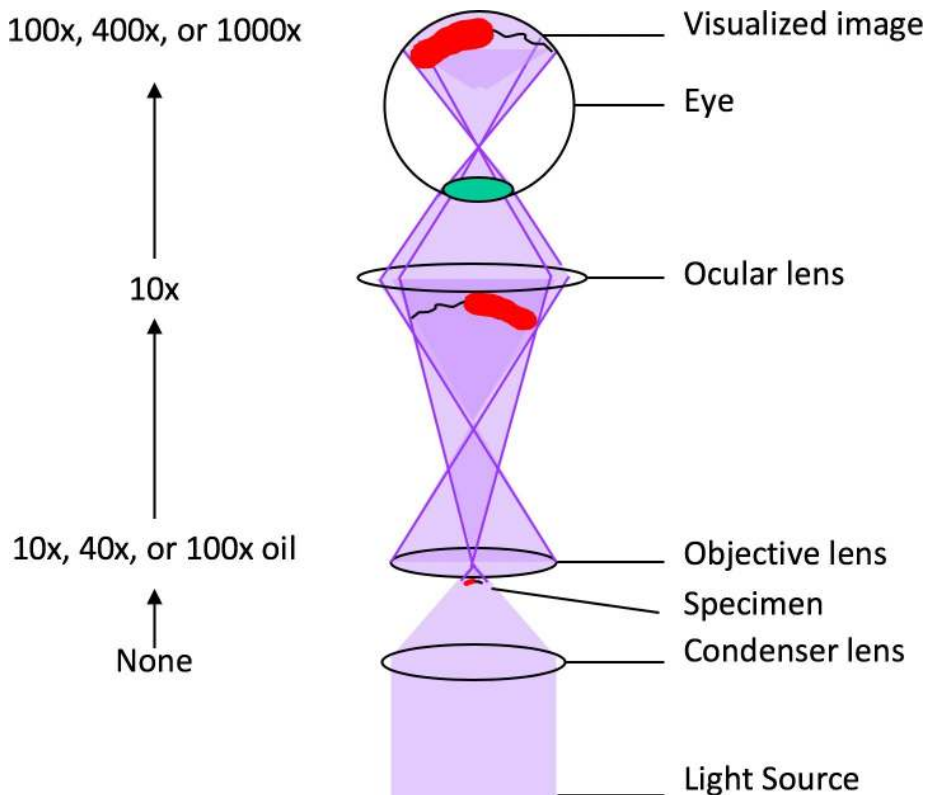


Figure 2. Light path through the microscope and magnification of a specimen.

The 100x objective and **ONLY the 100x** on your microscope is an oil-immersion lens. The three lower powered objectives are dry lenses – they cannot be used with immersion oil. *Getting oil on a dry lens will severely damage it.* If this happens, please notify your instructor ASAP so that the issue can be addressed and the objective thoroughly cleaned before it is damaged.



Types of Cells

All cells have four basic cellular components: cell membrane, DNA genome, cytoplasm, and ribosomes. Beyond that, however, cell sizes and structures can differ greatly between different organisms.

Based on ribosomal sequencing work conducted by Carl Woese et al. in the 1990s, all life on earth can be subdivided into three domains of life. Eukarya have nuclear membranes surrounding their DNA genome and can be multicellular, while Bacteria and Archaea are single-celled organisms and do not have a nucleus. Bacteria and Archaea can be differentiated based on their ribosomal DNA sequences and the components of their cell membranes.

Archaea (shown in Figure 3 in red) are microscopic single cellular organisms including halophiles (live in high salt environments) and hyperthermophiles (live at extremely high salt conditions). They have unusual membranes with ether linkages which can help them withstand these extreme conditions.

Bacteria (shown in Figure 3 in blue) are also microscopic single cellular organisms with a phospholipid membrane and are incredibly diverse organisms. We'll be focusing on these in our research project.

Eukarya (shown in Figure 3 in brown) includes all macroscopic organisms including plants, animals, fungi and protists. Eukaryotic organisms can be either single celled or multicellular. All eukaryotic cells have a nucleus and membrane-bound organelles for example mitochondria and chloroplasts.

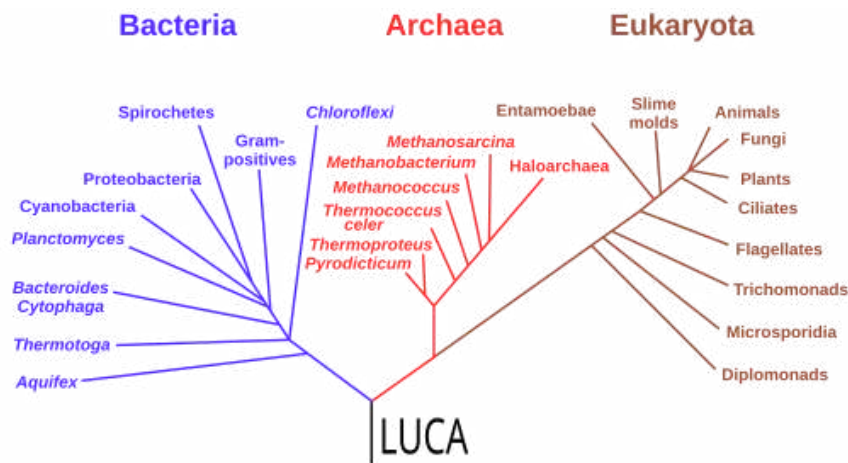


Figure 3: The universal tree of life. LUCA, at the base of the tree, refers to the **L**ast **U**niversal **C**ommon **A**ncessor (**LUCA**) - the original cell from which all cells and organisms have evolved. From this original cell, three domains of organisms derived - Bacteria (single celled organisms), Archaea (single celled organisms that are often found in harsh environments) and Eukarya (single and multicellular organisms that include plants and animals). by Chiswick Chap, [CC BY-SA 4.0](https://creativecommons.org/licenses/by-sa/4.0/), via Wikimedia Commons

Types of Eukaryotic Cells

Eukaryotic organisms have a great diversity in cellular composition where some are single celled and some are multicellular. We will look at protists, freshwater plants and algae, onion cells, and human cheek cells. All of the eukaryotic cells that you will observe are alive and you will need to make your slides (called a wet mount) for observation of each cell type.

Protists: You may see protists in the isolated protist samples or a mixed pond water sample. These are small single-celled organisms that can sometimes move quite rapidly and prefer to avoid the bright light of the microscope. To image these, put one drop of sample on the slide and add one drop of methylcellulose to slow their movement. Cover with a coverslip and observe. You will need to eventually observe these organisms with the 40x objective (but remember to start at the 4x). Your instructor will tell you which samples we have available.

Possibilities include: Paramecium (single celled ciliated organisms which move very rapidly and have a large central vacuole to maintain water balance), Euglena (freshwater, photosynthetic, single celled organisms that move with a long flagella), Amoebae (translucent blobby shaped organisms that move by extending finger-like pseudopods).

Plants: To observe the freshwater plant **Elodea**, make a wet mount from a small piece of a single thin leaf. These leaves are composed of only a few layers of cells, you can adjust your focus to see each of them as you scan through the depth of the leaf. For Elodea, notice how all of the cells are rectangular. This regular shape is a result of the rigid cellulose cell wall which provides structure for these cells. If you look carefully and are in a hypertonic environment, you can see the area of the plasma membrane immediately inside the cell wall where it sometimes pulls away a little. Additional structural features that you will notice include the chloroplasts (small and green organelles floating in the cytoplasm), the central vacuole (a section of the central portion of the cell without chloroplasts) and sometimes the nucleus (clear and egg-shaped near the periphery of the cell).

To observe the **onion** cell, make a wet mount of the onion epithelium. To do this, cut a small wedge-shaped piece of the onion. Notice how the onion curves, break the piece against this curve to pull off a thin layer of cells that lie along the inner surface of the curve. The layer is only a few cells thick and can look like plastic wrap. Use this thin epithelial layer for your wet mount. Try to lay this as flat as possible to prevent overlapping cells. Add one drop of stain to increase the contrast and cover with a coverslip. You will likely see a circular nucleus and possibly tiny mitochondria around the edges of the cell.

Animals: To make a wet mount of your **cheek** cells, gently scrape the lining of your cheek with a clean toothpick. Carefully wipe the end of this toothpick on a slide and add one drop of iodine or methylene blue dye. These dyes will increase the contrast and allow you to identify the nucleus, cytoplasm, and the cell membrane. You might be able to see additional organelles inside the cell too.

Viewing of Prokaryotic Cells (Bacteria and Archaea)

There are three domains of living organisms: **Bacteria**, **Archaea**, **Eukarya**. All three of these domains are composed of cells. Bacteria and Archaea are single cells and using the microscopy techniques available to us, Bacteria and Archaea are almost indistinguishable and together are categorized as **prokaryotic**.

Prokaryotes lack a nucleus (including nuclear membranes and histone proteins) and membrane bound structures that perform specialized functions called organelles. The genetic material in prokaryotes is found in one central area of the cell called a nucleoid. Although single celled and generally quite small, prokaryotic cells come in a variety of cellular morphologies (shapes).

The three most common shapes of bacteria are coccus, bacillus, and spirillum. Coccus, cocci for plural, refers to round, spherical bacteria that might be arranged singularly or in larger groups. Bacillus, bacilli for plural, indicates longer rod-shaped bacteria which sometimes appear as a long chain of linked rods. Spirillum are much less common in nature and look like corkscrews. Look at these slides carefully as often the bacteria are clumped together. Each fixed slide has all three types of bacteria. We'll make and fix some slides ourself as well.

NOTE: you will not be able to see any subcellular structures as they are too small for the resolution of our microscopes. You will need an electron microscope to see details including **ribosomes**, the **plasma membrane**, and the **nucleoid**.

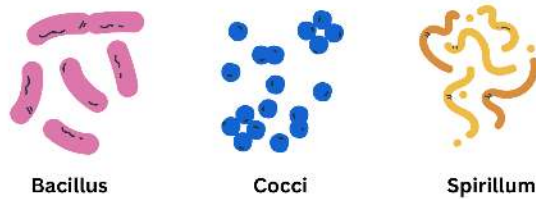


Figure 4: Examples of the most common bacterial cell morphologies. Bacillus are rod-shaped, cocci are shaped as spheres, and spirillum are corkscrew shaped. The average bacterial size is ~ 1 micron and are only visible under a microscope.

Protocols

Protocol 1: Using the Microscope

Initial set up

- Carry your microscope to and from your lab bench by grasping the arm with your dominant hand and supporting the base with your other hand. Make sure to always keep the microscope upright and *do not try to carry anything else at the same time*.
- Remove the dust cover and, if needed, clean the lenses with a piece of lens paper. Never use anything else to clean your microscope as it could scratch and damage your microscope.
- Unwind and plug in power cord
- Turn on the light source – switch on right side of microscope
- Dial or Slider on right side of microscope will adjust the light intensity, depending on the sample and magnification used.
- Adjust the ocular focus:
 - With your left eye closed and a slide on the stage (see below), focus your right eye on something on the slide using the normal focus knobs.
 - Then close your right eye and adjust the focus of the left eye using the focus ring on the ocular lens.
 - Adjust the spacing of the oculars so that you can look with both eyes simultaneously.

Viewing a Sample

- Select lowest power (4x) objective by turning the black ring just above where the objectives attach to the body of the microscope.
 - Important: Never rotate by grabbing or putting pressure on an objective lens itself.
- Set slide on stage and hold in place with clip.
- Use the coarse and fine focus knobs to focus on the sample at lower magnification before rotating the collar (not touching the objectives themselves!) to switch to a higher objective.
- Refocus at the desired magnification, using the fine focus knobs.
- If you want to use the 100x oil objective, *check with your instructor before using*. Turn the collar so that the slide is centered between the 100x oil lens and the adjacent objective lens (neither one is clicked into place).
 - Carefully put one small drop of immersion oil on the cover slip.

- Slowly rotate the collar so that the 100x objective clicks in place.
- The immersion oil should form a connection between the slide and the lens.
- Important: Use care never to get oil on any part of the microscope other than the 100x oil lens. Especially do not get oil on the other dry objectives – this can severely damage them.
- Adjust the level of illumination with the slider on the side of the microscope.

Tips For Focusing

- Always start by getting in focus at the lowest power objective (4x). Then increase objective strength one at a time, focusing before moving to a higher objective.
- If you have trouble finding the correct focal plane in which to view your sample, try focusing first on the edge of the cover slip.
 - Then, without moving the focus knob, scroll the stage over so that the specimen is in the field of view.
 - It now should be close to in focus, requiring only minor adjustment with the fine focus knob.

Putting the Microscope Away

- Clean the immersion oil off the 100x oil objective if used.
 - Tear off a piece of lens paper and gently swipe it across the lens.
 - Never use a Kimwipe or anything but lens paper on a lens: it could scratch the lens.
 - Rotate the paper to a clean portion or get a fresh sheet and gently swipe again.
 - Repeat until there is no visible oil coming off the lens onto the paper.
 - Never “scrub” on the lens by pressing with your finger or rubbing back and forth – any tiny pieces of dirt can scratch the lens.
- If desired, clean the other lenses in the same way, with clean lens paper.
 - They should not have any oil on them, but sometimes they will get a little dusty or dirty.
- Wipe up any oil that accidentally got on other parts of the microscope.
 - The stage is most likely to become contaminated by stray smudges of oil.
 - If necessary, moisten the corner of a Kimwipe with a little ethanol to help wipe up the oil. Remember, Kimwipes are never to be used on the lenses themselves.
- Turn the objectives so that either no objective points down toward the stage or the 4x objective points down (this helps to prevent damage to the objectives)
- Adjust the stage scroll knobs so that it doesn't stick out.
- Wrap the cord around the holders.
- Carefully store it in the appropriate place in the microscope cabinet.

Protocol 2: Using the Microscope

During the semester, we will perform multiple experiments with the microscope, so it is critical to feel comfortable using one. This first activity will familiarize you with how to use a microscope. Please refer to the microscope guide in your lab manual.

Using some of the fixed samples provided, practice viewing and focusing on your samples. Remember to start with low power and then step-by-step move to higher power. This will help you avoid colliding the objective into the slide and make focusing easier. As you practice, answer the following questions:

Materials

- microscope
- ruler (or letter e) slide

Protocol

- Using the ruler slide, measure the length of the field of view at each magnification and record in the lab handout. Which objective has the largest **field of view**? Which has the smallest?
- Using the ruler slide, compare the direction of the image. Is this image right side up or upside down with respect to the actual ruler? Is the image backwards or forwards? As you move the slide from right to left which way does the image move? As you move from front to back, which way does the image move?
- As you focus on the slides, which objectives can you adjust the focus the most? This objective has the greatest **depth of field**. The one with the narrowest band in focus has the least depth of field.

Protocol 3: Making a Wet Mount

Materials

- microscope
- slides
- coverslips
- protist samples
- ProtoSlo
- onion sample
- Elodea sample
- toothpick
- stains (iodine and methylene blue)
- blotting paper.

Protocol

- Gently place your sample on a clean slide, if needed, add a drop of water, dye to improve visibility, or Protoslo to slow rushing protists.
- Lower the coverslip slowly from one edge to avoid creating bubbles.
- If using dye, place the slide between a few pieces of blotting paper and press down gently (don't rub) to soak up extra dye.
- View the suspension under the microscope starting at low power.
- When finished, place the slide and coverslip in the bucket of soapy water to be washed.



Figure 3. Making a wet mount. Note how the hand is placing the coverslip by dropping it at an angle. This helps prevent some air bubbles. [Witia](#), [CC BY 3.0](#), via Wikimedia Commons

Protocol 4: Observing Bacterial Cells

Theory

Before bacterial cells can be stained, they must first be attached to a glass slide in a thin film called a smear. A smear can be prepared from a broth culture or from a single colony on a plate. Put a tiny drop of water ~2µl in the circle on your slide. You can

do this with either a pipette or using an inoculation loop. Then transfer a small amount of bacteria from a single colony to that drop of water, mix thoroughly, and spread it around into a thin film on the slide.

NOTE: Take care not to transfer too much bacteria or the cells will be clumped together and difficult to distinguish (this is a common challenge for beginning microbiologists). Allow the bacterial smear to air dry completely.

Now that you have prepared a smear of bacterial cells on the slide, you need to fix the cells. Fixing has two purposes. First, it kills the cells quickly and in a way that minimizes shrinkage and distortion so that you can accurately determine the cell morphology. Additionally, fixing sticks the cells to the slide so that they will not wash off during the staining process. We will be using heat fixation. To heat-fix the bacteria on your slides, pass the slide through the flame of a Bunsen burner. Be sure to hold the slide carefully with a slide clamp or clothespin so that you don't burn your fingers. Your heat-fixed smear is now ready for staining.

Simple Staining

Staining procedures that use only one type of stain are called simple stains. They increase the contrast between the bacterial cells and the slide background. Many different dyes can be used and we will use methylene blue. Methylene blue is a basic dye and binds to negatively charged cellular components, including the cell wall.

To stain a heat-fixed smear, hold the slide over a beaker. Using a dropper, put several drops of the dye directly on top of the fixed bacteria. Let the stain sit for about a minute, then tilt the slide over the beaker and rinse the stain off using the distilled water wash bottle. Rinse for 10 seconds or more, until the water running off the slide is clear.

Gently blot the slide dry. Don't wipe the slide or the fixed bacteria might wipe off. The simply stained bacteria are now ready to look at under the microscope. To view your sample, place a coverslip over the stained sample. The purpose of the coverslip is to help prevent contamination of the microscope with any of the bacterial sample.

I. Preparing a smear:



Inoculate a few bacteria in 2µl of water in a dime-sized area on a glass slide.

Allow to dry completely.

II. Heat fix and stain:



Pass slide through a flame to heat fix.

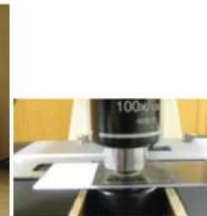


Flood slide with stain and let sit for 1 minute.



Rinse with water and allow to dry.

III. Microscopy



Place the slide on the microscope stage. Focus with the 4x objective and work up to examining the bacteria with 40x objective lens

Figure 6. Step-by-step image guide for simple staining a bacterial sample.

Materials

- microscope

- slides
- coverslips
- dH₂O
- bacterial strains
- sterile sticks, micropipette and/or inoculating loop
- clothespin
- methylene blue stain
- beaker
- Kimwipes or blotting paper

Protocol

- Label the slides with the identification information for your microbe.
- Make a dime sized smear with a plate culture of the your bacteria.
 - Put 2µl of water (or your bacterial sample if it is in broth) on a slide. You can use either an inoculating loop or a pipette
 - Spread the bacteria in this small volume
- Set the slide aside and allow the smear to dry completely before fixation.
- Heat-fix the smears by passing the slide through the flame of a Bunsen burner several times
- Stain the slide with 1-2 drops of methylene blue; let sit for 1 min
- Rinse off the stain with dH₂O
- Carefully blot slide dry between Kimwipes or using blotting paper
- Observe the bacterial morphology using the 40x dry objective (start at 4x and work your way up).
- Draw what you see in the field of view or take a photo. Be sure to label your drawing with the name of the sample, the total magnification, and the cellular morphology of that species
- Repeat the simple stain procedure for each bacteria.

Simple Stain Video



Discarding Materials

When finished:

- Clean off your microscope
- Lower the stage of the microscope to the bottom
- Turn the 4x (lowest power objective) to the down position
- Have your instructor sign off on your microscope - and then cover the microscope and return it to the correctly numbered space
- Return the ruler slides and any previously prepared slides the appropriate box

- Place the onion and Elodea slides in the tray of soapy water
- Place any cheek (and toothpick) and bacterial slides in the red biohazard sharps container.
- Using the 70% Ethanol or Sanizide sprayer on your bench, spray your bench space to disinfect it
- Wipe the bench down with a paper towel.
- Wash your hands prior to leaving.

References

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Perry et al., Lab Manual for Majors General Biology, 1st edition. Brooks Cole. 2008

Powell and Sullivan. MyMicrobe Project – A Guided Research Project Academex. 2015

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3: Reading and Citing Scientific Papers

Scientific research papers are the major way that scientists communicate information about their recent discoveries. As textbooks are very general and contain only a broad overview of a topic, scientists, students, and researchers regularly need to read more focused scientific papers to learn the most recent information about a topic. Through this course, we will be reading scientific papers which focus on topics that we are investigating in class.

Learning Objectives

At the completion of this lab, students will be able to:

Content:

- Know where to access library databases and how to perform a search for desired content.
- Understand what information can be found in sections of a typical research article in order to read and understand more efficiently.

Physical Skills:

- Cite material from a variety of sources

Scientific Communication Skills:

- Recognize the difference between research (primary), review (secondary) and textbook or encyclopedias (tertiary) sources and select appropriately for the purpose.

Types of Articles

Reading scientific papers and gathering information from them is a challenging skill which we'll practice regularly in class. There are three main types of scientific sources. Primary and secondary sources are peer reviewed, which means that other scientists have checked the work and support that the experiments and analysis were done well and that the work is reliable. The sources that you cite in this course should primarily be primary and secondary articles. Tertiary sources more general and are great places to start for some basic background.

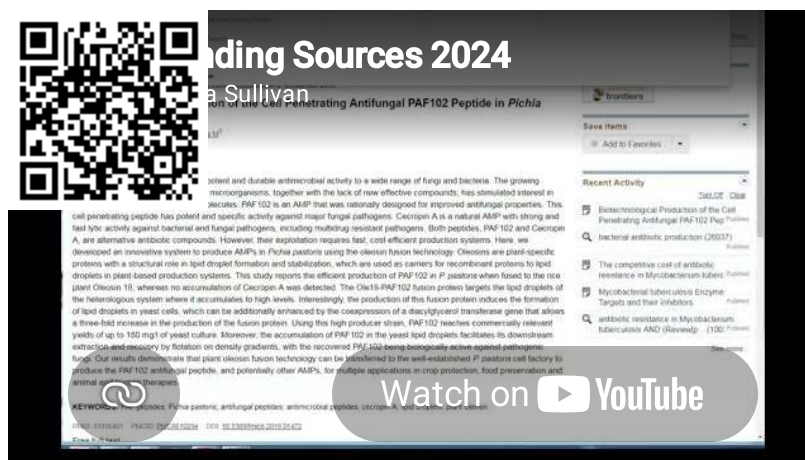
- A **primary research article** is written by the scientists who do the experiments and these articles contain data from the experiments that they complete. One way that you can tell that a paper is a primary article is that it contains a methods section describing how they did each of the experiments. Here is a link to a primary article that you can read. [Gagneux et al.Science.pdf](#)
- A **secondary (review) article** is written by scientists who have read MANY primary articles and combine all of that knowledge into a bigger picture discussion of the field. These articles don't have their own experiments, but discuss the experiments of others. Some ways to tell that a paper is a secondary article is that it uses the word "review" or contains only figures that are models rather than original experiments. These articles also have very long lists of references. Here is a link to a secondary article that you can read.
- A **tertiary (third level) source** could be a textbook or encyclopedia entry. These are great ways to get some basic background and find other resources, but are not good sources to cite in your research as you should always give credit to the authors who did the work themselves. Here is a link to a tertiary article related to the two above.
- Finally, popular press articles often bring in scientific research and write about experiments that scientists have performed. However, these are written for the general population and are therefore much easier to read. They often lack the experimental details, but help provide context for the research.



Finding Sources Using the Databases:

Here are the three databases that I recommend for finding primary and secondary sources related to Biology.

1. [PubMed Central\(opens in new window\)](#) - this is a government sponsored website that hosts full-text archive of literature from biomedical and life sciences journals. This database has easy ways to filter based on date, free open access, review articles, etc
2. [Google Scholar\(opens in new window\)](#) - a version of Google that specifically only searches for scholarly articles. These aren't always peer reviewed (therefore not always primary and secondary articles) and can sometimes include Masters and PhD thesis instead. GoogleScholar does have good ways of filtering the searches based on date and review articles etc.
3. [EBSCO Host\(opens in new window\)](#) - This is our libraries (Hayden Memorial Library) search database. You can select many different types of databases and filter based on publication date, type of article, journal, etc. The big benefit of this database is that it can directly provide you with access to articles through our library and if our library doesn't have it, you can complete an interlibrary loan request to get the document by e-mail in a few days.



Citing Sources Correctly:

It is critical to cite your sources correctly to give you credit to the scientists who completed the work that you are discussing. Unlike English or even History courses, citations in the sciences are not quotes!! Instead, we cite the scientists ideas and work in our own words. Therefore, we paraphrase the papers that we read. Because the ideas that we write about are NOT our own, we still cite that work. There are two types of citations and your document should always include **BOTH**.

1. **In-text Citation** - This is in the paragraph where you are talking about the article you read. After paraphrasing the ideas you'll give credit to the authors. There are two formats. If you don't name the authors in the sentence you'd write (Authors last name, Year), but if you list the author in the sentence, then you'd just include (Year). The last few minutes of the second video below will guide you through this process better in particular what to do with multiple authors etc.

2. **Bibliography/Works Cited Citation** - This is the part that goes at the end of your paper where you include all of the information about the article that you are citing. This format is much longer and includes all the info including Author Names, Year, Title, Journal, Volume, Issue, Page, Digital Object Identifier ... See the second video listed below for a step-by-step guide. Also, [this APA 7th edition guide is a good sample for citation style.](#) ([opens in new window](#))

Example Citation for a Primary or Secondary Source:

In-text/Parentetical

When I've found information that you want to include in your paper, I'll write that information in my own words and then give the authors credit. Note that **I did not quote**, I wrote out the information in my own words, but gave the scientists credit for their discovery. For example:

The structure of deoxyribonucleic acid can be modeled as a double helix where the phosphate and sugar backbone are on the outside and the base-pairs hydrogen bond in the middle, like rungs of a ladder (Watson and Crick, 1953).

Works Cited/Bibliography

Now, at the end of my assignment, I need to include the full bibliography for this work that I've cited above. I do this for all of the resources that I have used on the assignment.

Watson, J.D. & Crick, F.H.C., (1953). Molecular Structure of Nucleic Acids: A Structure for Deoxyribose Nucleic Acid. *Nature* 171, 737–738. <https://doi.org/10.1038/171737a0>

General Citation Formatting Guidelines:

In-text/Parentetical

- Summary sentence in your own words
- Do Not Quote
- General format for one author (**AuthorLastName, year**)
- General format for two authors (**Author1LastName & Author1LastName, Year**)
- General format for one author (**AuthorLastName et. al., year**) "et al.," means "and others" in Latin

Works Cited/Bibliography

- Write it yourself, or carefully check the work done by the "cite this" link and/or EasyBib. They regularly get it in the wrong formatting.
- For Journal Articles, the information is in the order: Author, (year) Article Title. *Journal Title*. Volume (issue), page#. DOI
- For books, websites, etc, refer to the citation handout from class.



The collage features several elements: a QR code in the top left; a newspaper clipping with the headline "Antibiotic resistance: A Sullivan" and sub-headline "New resistance enzyme inhibitors that breathe new life into old antibiotics must continue"; a document snippet discussing beta-lactamase inhibitors (Classes A, C, and D) and their hydrolytic mechanisms; and a YouTube video thumbnail at the bottom with the text "Watch on YouTube" and a description of the GAIN Act (Generating Antibiotic Incentives Now) and the Bacteria Accelerator Program.

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4: Pipetting and Probiotics Experiment

During the semester, we will perform many experiments that require us to transfer small volumes of liquid with precision and accuracy. Measuring small volumes (10 ml or less) requires that we use special equipment called pipettes. There are two major types of pipettes: serological pipet and micropipet. In this lab we will make some solutions, practice transferring small volumes of water (and aqueous solutions), and evaluate the quantity of bacteria in various probiotic samples.

Learning Objectives

At the completion of this lab, students will be able to:

Physical Skills

- Use serological and micropipettes accurately
- Perform a serial dilution series from a stock concentration
- Use sterile technique to transfer and plate bacterial samples

Scientific Communication Skills

- Calculate serial dilution concentrations
- Count colonies on a plate to determine CFU/gram of the initial sample.

Types of Pipettes

Glass serological pipet

- We will use 5 ml, 10 ml, and 25 ml sizes
- Controlled using a Pipet-Aid = a pump that draws up or dispenses liquid
- The pump may be electronic, scrolling, or bulb

Micropipette (aka pipetman or pipettor)

- We will use 2 μ l, 20 μ l, 100 μ l, and 1000 μ l sizes
- P2: measures volumes less than 2.00 μ L
- P20: measures volumes between 2.0 μ L (minimum) and 20.0 μ L (maximum)
- P100: measures volumes between 10.0 μ L (minimum) and 100.0 μ L (maximum)
- P1000: measures volumes between 100.0 μ L (minimum) and 1000.0 μ L (maximum)
- Draws up and dispenses liquid using a disposable plastic tip

Which pipet should you use?

- Generally, choose the smallest possible instrument to measure the volume you need all at once
 - example: if you need 3.5 ml of water, it is best to use a 5 ml serological pipet rather than multiple uses of a P1000
 - example: if you need 95 μ l of water, it is best to use a 100 μ l (P100) pipettor rather than a P1000
- The exception is if you need a precise amount that is slightly more than 1 ml, use a P1000 and pipet in 2 steps.
 - example: if you need precisely 1450 μ l of water, pipet 750 μ l then 700 μ l, or some other combination that totals 1450 μ l.
 - This situation is uncommon, however, and usually you should select the instrument that allows you to measure the entire volume at once.

Use of a Serological Pipet

There are three possible types of Pipete-Aids: electronic, scroll, and bulb (Figure 1). The basic operation is the same, but subtle differences will it easier to use them accurately.

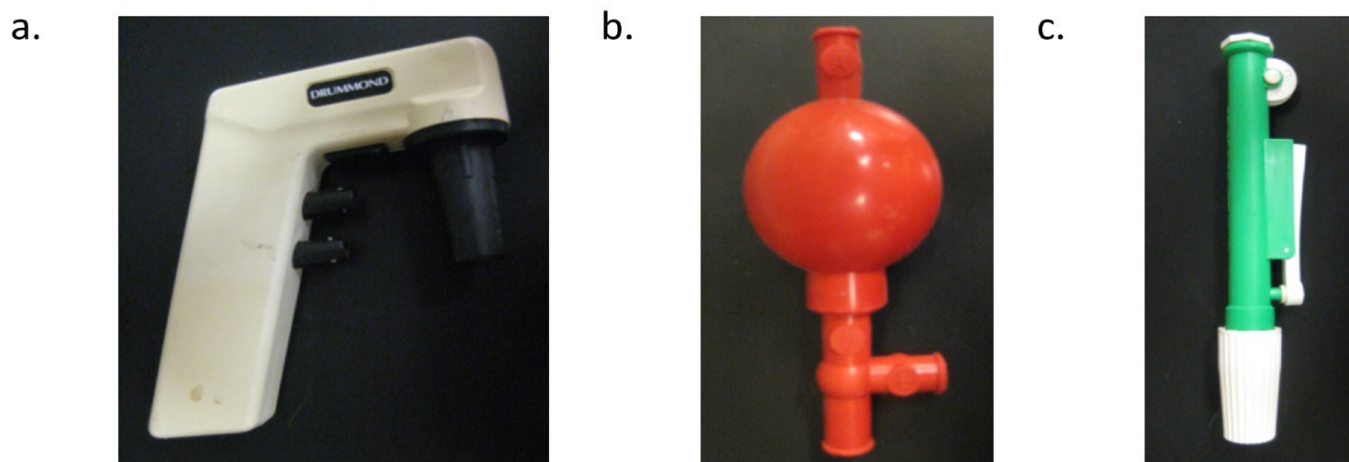


Figure 1. Three different styles of pipet-aids for transferring large volumes. Serological pipets are inserted into the hole on the bottom of each of these pumps. a. Electronic Pipet-Aid b. Bulb-style Pipet-Aid c. Scroll-style Pipet-Aid

For all Serological Pipette Usage:

- Carefully insert a clean serological pipet into the nose of the Pipet-Aid so that the numbers face you when you hold it.
 - Be sure to handle the pipet only near the top, so that your hands will not contaminate the portion that will enter the solution
- Insert the pipet into the solution and draw up the desired amount, measuring at the bottom of the meniscus
 - Note that usually the measurement numbers are in reverse order, so if you want to measure 2 ml using a 5 ml pipet, you may need to draw up your liquid to the mark that is labeled 3 ml. Some pipets are marked differently, so pay attention to each one you use.
 - **Caution:** Never allow any liquid to enter the nose of the Pipette-Aid
- Insert the filled pipet into the target container and dispense the liquid
- Carefully remove the used serological pipet from the Pipet-Aid and dispose as instructed by your professor.

Electronic Style Pipet-Aid

- These Pipet-Aids are cordless so you do not need to plug them in or turn them on
- Select the rate of drawing and dispensing by pressing the + and – buttons on the top back of the Pipet-Aid
- Depress the top finger button to aspirate (draw up) liquid and depress the bottom finger button to dispense liquid

Bulb-Style Pipet-Aid

- These Pipet-Aids look like balloons with three valves.
 - The largest appendage at the bottom attaches to the pipet while the valves on the other two can be used to control the rate of drawing and dispensing.
- To fill a pipet, start with a deflated bulb (squeeze the bulb and hold the "A" (air) valve at the top)
- Press the "S" (suck) valve on the side to allow air into the bulb, thereby filling the pipet with liquid.
- Press the "E" (expel) valve on the side to dispense the liquid.

Scroll-Style Pipete-Aid

- These Pipet-Aids are tube-shaped with a scroll wheel at the top
- Select the rate of drawing and dispensing by turning the scroll up and down.
- As the scroll moves, the plunger will move up and down. This will pull a vacuum, drawing liquid into the pipet. Scroll down to dispense.

Use of a Micropipetter

At the top of the micropipette is the push button that you will use to manipulate the liquid. Pressing this button will reveal that there are two stops, the first for sucking up the liquid and the second for expelling the liquid. Just below and off to the side is another button used to eject the disposable plastic tips during operation. The black movable ring, or thumbwheel, in the handle allows you to adjust the volume setting of the micropipette. As you turn the thumbwheel, look at the volumeter display. **Do NOT turn this wheel past the minimum and maximum volumes for the micropipette to prevent damaging the instrument.**

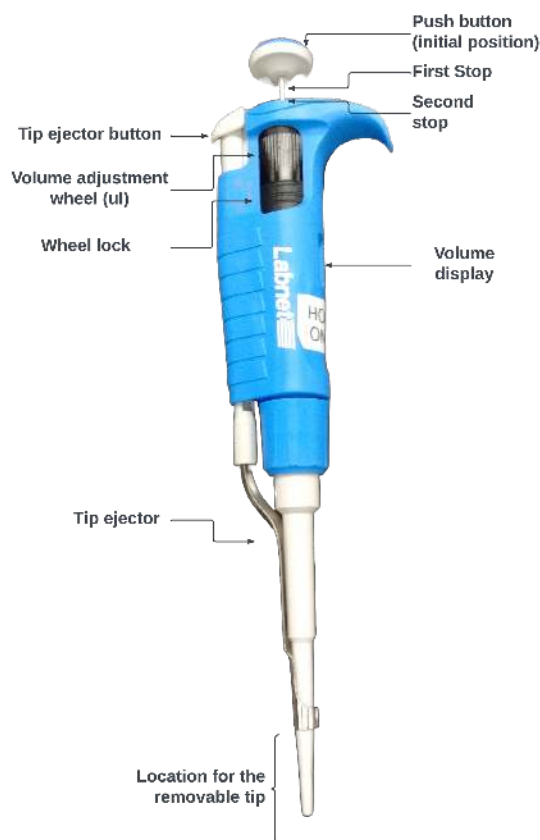


Figure 2. Diagram of a micropipette.

To determine the volume to which a micropipette is set, you can look at the volume display window on the side of the instrument. See Figure 3 for examples. These values are read vertically and interpreted based on the maximum capacity of the micropipette. In the example below, the left window is from the P20 micropipette and indicates that the instrument is set to measure 6.5 μL .

On the P20 micropipette, notice how the tenths place is shown in red. The center window is from the P100 micropipette and indicates that the instrument is set to measure 92 μL . The right window represents the P1000 micropipette and displays 726 μL . On the P1000 micropipette, notice how the thousands place is shown in red, and the ones place is not displayed.

P20	P100	P1000
0 Tens	0 Hundreds	7 Thousand
6 Ones	9 Tens	2 Hundreds
5 Tenth	2 Ones	6 Tens

Figure 3. Volume setting windows for the three most common pipettors used in lab. P20 is set at 6.5 μ l, P100 is set at 92 μ l, and P1000 is set at 726 μ l.

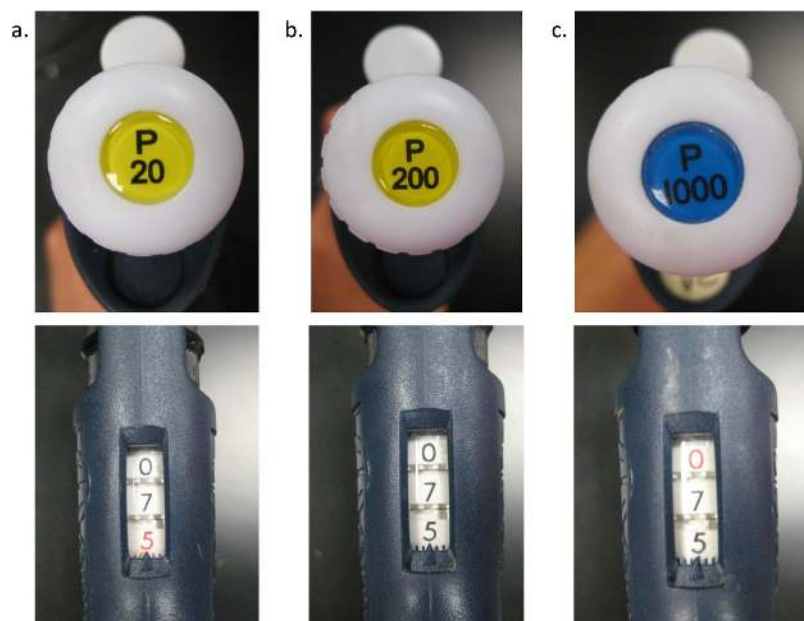


Figure 4. The three most common pipettors used in lab. (a) A 20 μ l pipettor (P20) can be used to accurately transfer between 2 μ l and 20 μ l of a liquid. Here the pipettor is set to 7.5 μ l. NOTE that the decimal place is red. (b) A 200 μ l pipettor (P200) can be used to accurately transfer between 20 μ l and 200 μ l of a liquid. Here the pipettor is set to 75 μ l. NOTE that all of the digits are black. (c) A 1000 μ l (1ml) pipettor (P1000) can be used to accurately transfer between 200 μ l and 1000 μ l (1ml) of a liquid. Here the pipettor is set to 750 μ l. NOTE that the thousands place is red.

NOTE: sometimes in lab we use a P100 and sometimes P200, they both have all black digits in the volume setting

To Set the Volume

Turn the knob on top of the plunger or use the volume adjustment wheel (Figure 2). You will probably need to release the scroll lock on the pipette before you can scroll. **Caution: Never try to set pipettor outside its proper range, which is printed on the top of the pipettor!**

To Put on the Tips

Gently but firmly press the end of the pipettor onto a pipet tip. P1000s use the large tips; P100s and P20s use the smaller tips; P2s use tiny tips. **You should always change tips between samples.**

To Suck Up (Aspirate) Liquid

Using your thumb, depress the plunger to the **first stop** or point of resistance. While holding the plunger down, insert the pipet tip into the solution. Slowly and smoothly release the plunger to draw up the desired amount. **Caution: Never allow any liquid to enter the barrel of the pipettor!**

To Squirt Out (Expel) Liquid

Insert the filled pipet tip into the target container, just below the surface of any existing liquid, and depress the plunger all the way to the second stop. To prevent bubbles, while holding the plunger down, remove the pipettor and tip from the target container, then release the plunger. Check the pipet tip to make sure you successfully dispensed all the liquid without leaving any drops stuck to the tip.

To Eject the Tip

Point the pipette tip over the waste container. Eject the tip into the waste container by pressing the tip ejector button.

How to Use a Pipette Video



Working with Solutions

Solutions are homogenous mixtures of two or more substances and can have various properties. In biology, many times solutions are liquid and composed partially of water. Your blood, for example, is a solution of water, red and white blood cells, platelets, nutrients (sugars, amino acids, ions including sodium and potassium, oxygen), hormones, waste products (carbon dioxide, urea) and many other particles.

There are many different examples of solutions including milk, blood, rainwater, urine, gasoline, air, and each have two things in common a **solvent** (the dissolving agent - water in our blood example) and at least one **solute** (the dissolved substance - sugars, for example).

Solubility refers to the ability of one substance (the solute) to dissolve or mix well with another (the solvent). In biology, the solvent is usually water, a small **polar** molecule and we can use the phrase, "like dissolves like" to help us predict which solutes might be soluble in the water. Molecules that are also polar would be expected to dissolve, making these molecules **hydrophilic** (water loving). This happens because the partial positive and negative charges on the polar water molecule can make hydrogen bonds with other partial positive and negative charges on the solute. These intermolecular (between molecules) interactions stabilize the mixture and help distribute or dissolve the solute evenly in the solvent. Non-polar molecules, on the other hand, would be insoluble in the water because they would not form the stabilizing intermolecular interactions and the two molecules would separate. These non-polar molecules would therefore be referred to as **hydrophobic** (water fearing). Since these hydrophobic molecules would dissolve well in oil, they would be referred to as **lipophilic** (lipid loving).

Concentration describes the amount of a solute dissolved in a solvent. The concentration is often represented either as a mass over a volume, such as milligrams per milliliter (mg/ml) or as a molarity, moles per liter (M). Some molecules have limited solubility in given solvent while others are highly soluble.

✓ Example

If 5g of methylene blue were dissolved in 1 liter of water to make a stain for your bacteria, what is the concentration of this dye solution? What is the solvent? the solute?

Answer

Solvent is water

Solute is methylene blue

Final concentration is 5g/L. (5mg of methylene blue in 1 L of water)

One important type of concentration measurement that impacts your health and daily life, is the detection and identification of bacteria. If you have ever drunk a glass of water, gotten a urinary tract infection, or cleaned your hands with hand sanitizer, you've benefited from our ability to measure the amount of microscopic bacteria in an environment. For your health and safety, drinking water must contain fewer than 1 viable bacteria/100ml of water. Your doctor will diagnose you with a UTI if the bacteria in your urine exceeds 100,000 bacteria / ml of urine. And the labels on hand sanitizer claim that they kill >99% of the bacteria. The units used to measure bacterial concentration are CFU (**Colony Forming Units**)/gram.

Probiotics

In an average 1 gram of soil sample or 1 ml of sea water there could be 10,000 to 100,000,000 bacteria. We humans also have an incredibly diverse and essential **microbiome** in/on our bodies. Your skin is estimated to have 1 million bacteria per cm² (Chen and Tsao, 2013) and your digestive tract is even more jam packed with bacteria and approximately half of your poop are bacteria (micropia)! We need these bacteria for our health and digestion and they are involved in protecting us from infection, contributing to our digestion, impacting our mental health, and are responsive to our treatment of them. Especially after taking antibiotics, which kill a large proportion of our natural microbiome alongside the dangerous pathogens, we might need to re-build our microbiome. One way of doing that is to consume **probiotics**.

In our lab today, we will measure the bacterial concentrations in two types of **probiotics**. Probiotics are live microorganisms (bacteria and fungi/yeasts) that are consumed intentionally to improve health. These organisms are often lactic acid producing bacteria including *Bifidobacterium* and *Lactobacillus* species which make the gut more acidic, increase the diversity of gut bacteria, and improve digestive health. Scientists are conducting clinical trials to learn more about the roles that probiotics can play in the treatment of human gut diseases and recent findings have suggested that probiotics and help shape the microbial community in the digestive tract leading to improved health and control of some digestive tract diseases (Kim et al., 2019). A variety of studies have also identified a link between consumption of probiotics and improved mental health including a reduction in depressive symptoms (Jarbrink-Sehgal and Andreasson, 2020).

Additionally, physicians often recommend that patients consume probiotics either as part of their diet or as supplements during or after antibiotic treatment. This recommendation comes because during a course of antibiotics, both the pathogenic (harmful) bacteria and the normal gut microbiome (helpful) bacteria are killed. Some patients have digestive-tract complications, which can be prevented by consumption of probiotics (McFarland, 2014). These probiotics can come in many forms either as over-the-counter supplements or as foods that naturally contain bacteria including yogurt, kefir, sauerkraut, kimchi, and kombucha. The advertisements on yogurt and probiotics list bacterial counts in the BILLIONS!!

However, not all probiotics are created equal. Because these are foods and supplements, they are not regulated by the FDA and vary widely in effectiveness. Different strains of probiotics may have different physiological effects on their human hosts and some might be better for some health concerns rather than others. Also, it is not always clear what concentration of bacteria are needed to be most effective. Finally, not all probiotics contain the number of bacteria stated on the label. Some manufactures may over-inflate their numbers, sometimes the units are hard to compare, and since these are living organisms, they can be impacted by changes in the environment like changes in temperature.

Our project today will be to look at two types of probiotics – one supplement and one yogurt with live and active cultures. In our experiment today, we will determine:

- The number of live bacteria: colony forming units/gram (CFU/g)

- Compare this to the advertised value
- Compare the supplement to the yogurt to determine which contains more living bacteria

To culture the bacteria in the probiotics, we will spread a diluted soil sample on a plate and look for the appearance of **colonies**. Just as you'll see spots of mold on an old piece of bread, you can see a bacterial colony on agar plates. A colony is a pile of genetically identical bacteria that arise from a single cell. Single cells that give rise to a colony are referred to as **CFUs (colony forming units)**. As this single cell reproduces, the mass of dividing cells eventually becomes large enough such that a macroscopic colony becomes visible to the naked eye. By the time you can see a colony on a plate it contains approximately 1 million cells. Colonies are our way of seeing into the microscopic world, and each bacterial type has specific colony features that can be recognized by experienced microbiologists.

The probiotics that we will be starting with will have billions of bacteria per gram in their initial source and therefore be way too concentrated to form individual colonies on a plate. If we tried, the bacteria would be so crowded that it would completely fill the plate that we were using and form a "lawn" of bacterial growth. We would never be able to count something this concentrated. Instead, we will dilute them and spread out the CFUs so that they grow into non-overlapping colonies. To do this, we will dilute the bacterial samples repeatedly in a solvent to decrease the number of cells in any sample. We will then spread a small volume (100µL) on an agar plate to separate and spread out the bacteria all over the surface of the plate where they will grow into single colonies (Figure 4). Our work today will use a particular type of media (food source for bacteria) that is specific for *Lactobacillus species* as these are the bacterial organisms most common in our probiotics.

Protocols

Protocol 1: Learning to Use the Pipettes

During the semester, we will perform experiments that will require us to transfer small volumes of liquid with precision and accuracy. Measuring small quantities (1 mL or less) of sterile broth, solutions, or bacterial cultures, can be accomplished using an instrument known as the **Micropipette**. This first activity will familiarize you with how to use a micropipette. Please refer to the pipetting guide in your lab manual.

Materials

- 3 pipettes (P20, P100, and P1000)
- pipette tips
- small squares of Parafilm
- dH₂O squirt bottle
- food dye solutions in dH₂O

Basic Technique Protocol

- With your partner, set the P20 to 20 µl, the P100 to 100 µl, and P1000 to 100 µl.
- Insert the end of the micropipette shaft into the correct size disposable plastic tip. Press down firmly before lifting up the micropipette with the tip. **Do not allow the plastic tip to touch any surface, including your hands and the desktop.** If you contaminate the tip, always replace it before putting it into your sample.
- To draw up the selected volume into the tip, press down on the push button until the first stop. Lower the plastic tip into your bottle of water ensuring that you do not submerge the micropipette beyond the protective plastic tip. Slowly release the push button and check to make sure that the liquid filled the tip without bubbles.
- Over the sink, slowly press down on the push button until the first stop. Watch as the liquid is dispensed into the sink. Continue to press the push button to the second stop to release the remaining liquid.
- After the liquid transfer, hold the micropipette over the waste beaker on your tabletop. Press the ejector button to release the plastic tip into the beaker.
- Switch micropipettes with your partner and repeat the procedure. Once you feel comfortable with the volume transfer procedure, continue to the next step.

Variable Volumes

- Retrieve two precut pieces of parafilm and one container of each colored solution (red, yellow, blue) from the side counter.
- Peel off the paper backing from the parafilm and lay it flat on your benchtop. Each partner should have his or her own piece of parafilm. You will be sharing the P100 micropipette for this challenge.
- Make a mixture of the three dye colors on a piece of parafilm. Due to the hydrogen bonding causing the surface tension of water, it will form a round droplet. **Switch tips between transfers to prevent contamination of our stock solutions.**
 - 45 μL of red solution
 - 65 μL yellow solution
 - 15 μL blue solution (you may want to use the P20, not P100)
- Compare your results to those of your lab partner and measure the total volume of the colored drop.
- Before disposing of your drop, confirm both the color and the size with your instructor.

Larger Volumes

- Repeat the color mixing with larger volumes:
 - 450 μL of red solution
 - 650 μL yellow solution
 - 150 μL blue solution
- Compare your results to those of your lab partner and measure the total volume of the colored drop.
- Before disposing of your drop, confirm both the color and the size with your instructor.

Protocol 2: Aseptic Technique

Aseptic or sterile technique is a central concept in microbiology and may be the most important part of working in a microbiology lab. The goal of aseptic technique is to promote practices that:

- prevent the contamination of cultures, lab supplies, and equipment with bacteria or fungi from the environment, and
- prevent the contamination of individuals working in the lab with potentially pathogenic bacteria.

Aseptic technique requires constant attention until it becomes second nature. Practicing these skills and paying full attention to the steps is crucial to making proper aseptic technique a habit. The health and safety of everyone in the lab and the accuracy of your experiments depend on these principles. Here are some general guidelines for practicing aseptic technique:

Do

- Remember that bacteria are everywhere (on you and in the environment)
- Sanitize your benchtop prior to starting lab.
- Have a Bunsen burner flame on. This will keep an up-draft in your immediate work area
- Keep your sterile items sterile until use (sticks, beads, plates, tips, media, tubes, and other sterile materials)
- Discard or set for re-sterilizing any sterile material (stick, tip, plate, etc) that accidentally was touched or otherwise contaminated.
- Discard any material (tip, stick, plate, tube) or set for re-sterilizing in the appropriate container after use.
- Get a new stick or tip for each new sample.
- Label all your plates and tubes before using them. (see protocol 3)
- Keep the lids on all your plates. This keeps unwanted bacteria out of your experiment and your bacteria out of the environment. If you need to remove a lid to inoculate or streak a plate, hold it over the plate to protect the plate as much as possible.
- When using tubes, keep the lids closed as much as possible. When you need to open them, keep that time as minimal as possible.
- When you open tubes, keep them at an angle, so less stuff can fall into the open tube. Also, work in the sterile zone of your Bunsen burner.

Don't

- DON'T work too far (or too close) to your Bunsen burner. Too close is dangerous for you, too far doesn't protect your work from contamination.

- DON'T have a cluttered or dangerous work area. Keep all papers and unnecessary materials in another area. You need space to work and don't want any fires.
- DON'T use non-sterile items to grow or transfer bacteria. This will mess up your experiment.
- DON'T use contaminated sticks, tips, or other materials as this can contaminate you and/or your experiment.
- DON'T put contaminated sticks, tips, or other material on your benchtop or in the sink. Always put them directly in the appropriate container. Putting them on your desk contaminates your desk and then you and anyone else who touches it.
- DON'T put any material in or on unlabeled plates or tubes. You might forget what is in there or get distracted.
- DON'T remove the lids from the plates and don't put your lids down on the bench. This could contaminate them and therefore your experiment.
- DON'T walk around the room with open plates, this will contaminate your plate for sure!
- DON'T leave tubes open on your bench, same!
- DON'T remove your contaminated material or anything from the experiment from the lab room.

Protocol 3: Labeling

Labeling materials and supplies are a critically important aspect of science. With multiple plates, tubes, and samples, it is easy to become confused and lose track of things between and within experiments without the proper labeling practices. Here are a few guidelines for labeling:

Materials

- Tubes or plates
- Sharpie Marker

Protocol

1. Always label the back (food side) of the Petri dish, never the lid, because the lid may become separated from the actual dish containing the growth medium (for example, if plates are dropped).
2. Always add a date. Dates help remind you about when the experiment was performed and provide information about the quality of materials.
3. Write as legibly as possible – others may need to find your materials or experiments.
4. Label experiments with your initials or name and the experiment number and number of trials
5. Label isolates with your initials or name, type of medium used, and an identifying number. For example: SS-PDA-5 stands for Sam Smith, potato dextrose agar medium, isolate number 5. If the identity of the organism is known, label with scientific nomenclature: genus, species, and type.
6. Here is an example of a labeled plate:



Protocol 4: Serial Dilution

Adapted from: [Serial Dilution Protocols](#): Jackie Reynolds – Microbe Library

The advertisements on our yogurt and probiotics list bacterial counts in the BILLIONS!! Because this density is so high, it would be almost impossible to get an exact count of the cells by examining them directly. Instead, microbiologists estimate the cell density through colony forming units (CFUs). To perform this calculation, the sample must first be diluted in water or a saline solution to keep the bacteria alive. An initial dilution of 1g of yogurt in 9mls of water (to make 10ml total) would create a 10-fold or 1:10 dilution. This will spread out the bacteria in 10x the volume, thereby making them further apart. This sample is still too concentrated with bacteria to be able to accurately count them. Therefore, we will continue separating the bacteria by making serial

dilutions of the sample in 10-fold increments until they are easier to work with and count. Once we reach a reasonable dilution, we can spread the sample out on a solid medium that will feed the bacteria. Each CFU (viable bacteria that can grow in this environment) in the sample will then grow into a colony and we can count them and, using the dilution and plating information, calculate the CFUs present in each initial sample. For example, if we serially dilute 1 g of yogurt by a factor of 10^3 , spread and incubate the dilution on a solid medium, and then observe 130 colonies, we would obtain 130×10^3 or 1.3×10^5 CFUs/g of soil. This number represents the number of **viable cells**, i.e., cells in an environmental sample that can survive lab conditions and grow in culture.

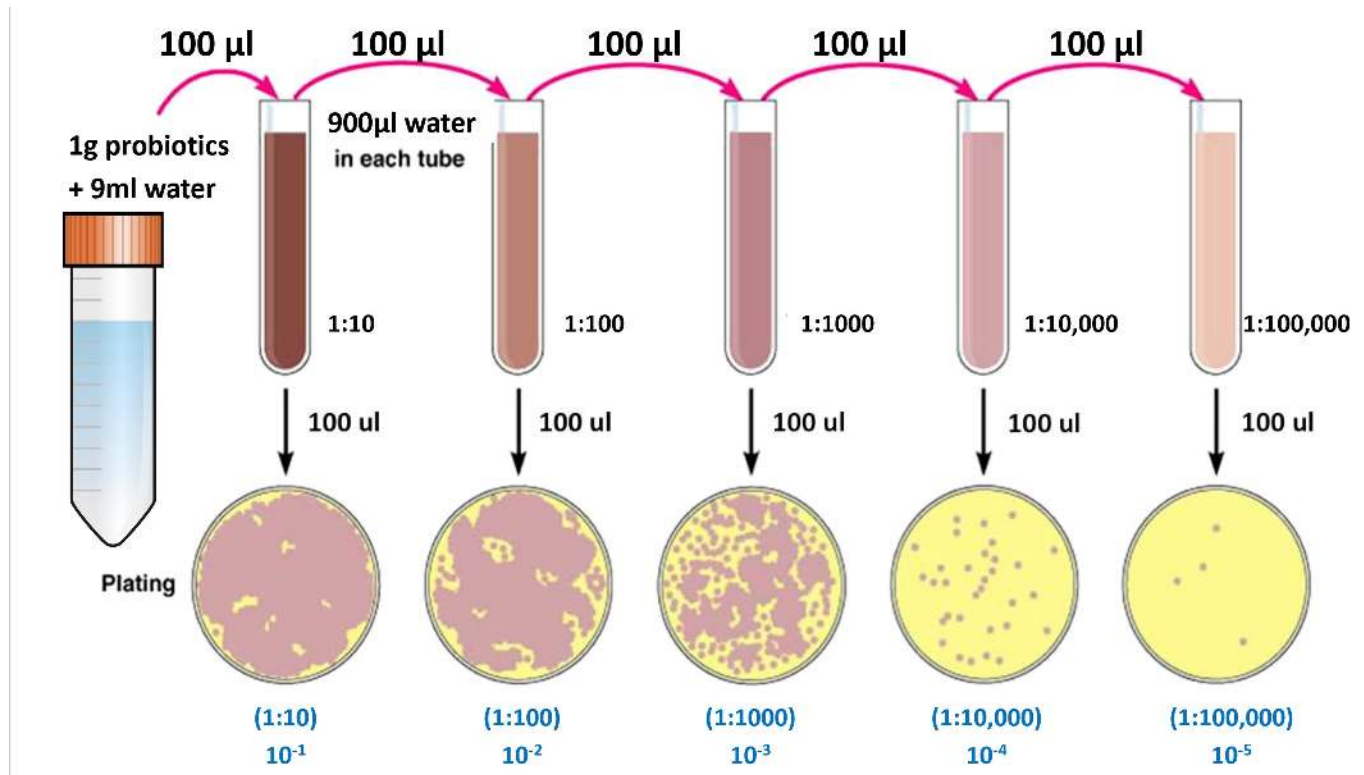


Figure 5. Serial dilution and plating schematic. A series of 10-fold dilution is made from the soil sample. Each plate therefore has 1/10 of the number of bacteria from the previous plate. This makes it much easier to count the colonies. Image modified from Alves and Cruvinel, 2016.

Materials

- 15ml conical tube
- probiotic sample (your group will be assigned either the supplement capsule or yogurt)
- Sterile water solution
- Vortex Machine
- 1.5 ml microfuge tubes
- Pipettors and tips
- Scale
- Sharpie Marker

Protocol

Use Aseptic Technique for this protocol

- Obtain and label appropriate number of plates and 1.5 mL microcentrifuge tubes, one for each subsequent dilution. Dilutions should be made in increments of 10 (1:10, 1:100, 1:1000, etc.)

- **NOTE:** For yogurt I recommend plating the 1:10 through 1:100,000 dilutions. For the supplement capsule, I recommend plating the 1:10,000-1:100,000,000.
- Add 9 mL of water to the conical tube.
- Measure the weight of your probiotic.
 - Place a 15ml conical tube on the scale and “tare” it. Add ~1ml of yogurt or empty the contents of the capsule into the tube.
 - **Record the mass of the probiotic.** You’ll need this later!
 - **HINT:** the sample will mix better if you put the water in the tube first, then add the yogurt/probiotic sample.
- Vortex soil/water mixture for 30–60 sec.

Determine the dilution series and calculate appropriate volumes for each. Dilutions should be made in increments of 10, thus add 900 μ L of solvent (water) into each dilution tube. 900 μ L solvent + 100 μ L sample transferred = 1000 μ L

- Put 900 μ L of sterile water into each labeled 1.5ml microcentrifuge tube
- Remove 100 μ L of probiotic+water with micropipette from 15mL conical tube and add to 900 μ L of water in microcentrifuge tube. This is 1:10 dilution.
- Mix with vortexer for 30 seconds.
- Remove 100 μ L of the 1:10 dilution and add to 900 μ L of water in the next microcentrifuge tube. This is the 1:100 dilution. Mix by vortexing.
- Continue to transfer 100 μ L of previous dilution to 900 μ L of water until reach desired dilution, vortexing between each sample

*Follow instructions on spreading a plate below.

Protocol 4: Spreading a Plate

One method of distributing bacteria evenly over the surface of an agar plate medium is commonly referred to as the spread plate method. Classically, a small volume of a bacterial suspension is spread evenly over the agar surface by using a sterile bent glass rod or glass beads as the spreading device. The goal is to distribute the bacteria evenly so that they are well separated and can be counted or sampled.

Some things to take note of in the plate spreading are:

- Be sure that your plates are dry before spreading. If they are too wet it will cause the bacteria to run into each other and smear.
- Choose the serial dilutions to plate thoughtfully. If you have too many bacteria on the plate you won't be able to count them next week. If you have too few, you won't have enough isolated colonies to get accurate counts.

NOTE: For yogurt I recommend plating the 1:10 through 1:100,000 dilutions. For the supplement capsule, I recommend plating the 1:10,000-1:100,000,000 dilutions.

- Plate the correct volume of sample. I recommend ~ 100 μ L (0.1 mL). This is enough to fill the plate so that the bacteria spread out, but not so much that the plate is drippy.
- Work swiftly, some bacteria attach to the plate surface quickly, so be sure to spread as soon as you put on the liquid or you will have all your bacteria clumped wherever your dropped them.
- Use aseptic technique to prevent contamination from bacteria in the air. Work near your flame, keep the tubes and plates closed, and change tips whenever they touch something.
- Start plating from the most dilute suspension to the most concentrated. This way, you don't need to change tips as each sample has more bacteria than the prior one.

Materials

- serially diluted probiotic probiotic samples
- Vortex Machine
- Pipettors and tips
- Lactobacillus MRS agar
- Sharpie Marker
- Beaker for used beads

Protocol

- Obtain 5 MRS plates and make sure that the plates are dry.
- Select the dilutions you wish to plate.

NOTE: For yogurt I recommend plating the 1:10 through 1:100,000 dilutions. For the supplement capsule, I recommend plating the 1:10,000-1:100,000,000 dilutions.

- Label the **BOTTOM** (food side) of each plate with: Group Name, Date, Type of Plate, Dilution, Incubation Temperature (30°C)
- Put 4-8 beads on the agar surface of each plate being sure to keep plates closed.
- Starting with the most dilute sample, transfer 100µl of the dilution to the center of the selected agar plate.
- Work backward spreading the most dilute samples first.
- Spread the sample around the plate by shaking as soon as the dilution has been added to the plate since some cells will rapidly attach to the agar, especially if the plate agar is dry.
- Pour off the beads into the used beads collection beaker, so that they can be re-sterilized for your future use.
- Let the plates sit on your bench for 10-20 minutes, so that all the sample soaks into the plate.
- Invert the plates (food side up) and place them at the selected temperature (30°C) to grow for the next week.

Video Demonstrating Serial Dilution



Hints for Next Week and the Weekly Written Assignment

Counting Colonies

Look at your plates in order of concentration and you should see a predictable drop in CFUs/plate as a result of your 10-fold dilution series. If your plates differ from this substantially, let your instructor know.

The goal is to have **between 30 and 300 colonies** on a plate. Too many and it is too hard to count, too few and the count can be very inaccurate.

We want to work backward from this to determine the INITIAL number of bacteria in the initial sample of probiotics. To do this, we have to account for each set of dilutions we did, but since we know all the volumes, this becomes a multiplication calculation.

How to Calculate CFU/g

How to calculate CFU/g:

$$\left(\frac{\text{ColonyNumber}}{\text{VolumePlated}(\mu\text{l})} \right) \times \left(\frac{1}{\text{DilutionFactor}} \right) \times \left(\frac{1000\mu\text{l}}{1\text{ml}} \right) \times \left(\frac{\text{InitialVolume}(\text{ml})}{\text{ProbioticWeight}(\text{g})} \right)$$

- **Colony number** - select a plate with 30-300 colonies, these are the most reliable.
- **Volume plated** - accounts for the small volume spread on the agar plate. For our work, this is 100 μl .
- **Dilution factor** - accounts for the amount of serial dilutions done. Use the scientific notation, e.g. 10^{-4} .
- 1000 μl /1ml - is a conversion factor because there are 1000 μl in 1ml.
- **Initial Volume** - this is that first big tube, before you start the dilutions. For our work, this is 10ml.
- **Probiotic Weight** - this is the weight of the sample you recorded in grams. It should be small (1g or less)

Assuming that you followed all the dilution and plating instructions correctly, only the highlighted numbers will change.

For example:

A yogurt sample weighing 1.0 grams on the 10^{-2} plate with 120 colonies.

Substitute in the values - yellow ones from your particular experiment, the rest from the standard values.

$$\left(\frac{120\text{colonies}}{100\mu\text{l}} \right) \times \left(\frac{1}{10^{-2}} \right) \times \left(\frac{1000\mu\text{l}}{1\text{ml}} \right) \times \left(\frac{10\text{ml}}{1.0\text{g}} \right)$$

Solve each fraction and cancel out the extra units.

$$1.2 \left(\frac{\text{colonies}}{\mu\text{l}} \right) \times 10^2 \times 1000 \left(\frac{\mu\text{l}}{\text{ml}} \right) \times 10 \left(\frac{\text{ml}}{\text{g}} \right)$$

Multiply across and convert to scientific notation. Either form of the answer is acceptable.

$$1200000 \left(\frac{\text{colonies}}{\text{g}} \right) = 1.2 \times 10^6 \left(\frac{\text{colonies}}{\text{g}} \right)$$

Discarding Material

When finished:

- dispose of the parafilm with the droplets in the regular trashcan.
- place your plates in the 30°C incubator with the food side (the labeled side that you wrote on) UP.
- clean off the shaft of each micropipette and return to the holders
- close the lids on the pipette tip boxes and return to the common supply
- dispose of all used tips and tubes in the biohazard trash (not strictly necessary this week, but a good habit to get into).
- consolidate all used spreading beads in the common beaker to be sterilized for re-use
- return all reagents and tools to the place that you got them
- Using the 70% Ethanol or Sanizide sprayer on your bench, spray your bench space to disinfect it
- Wipe the bench down with a paper towel.
- Wash your hands prior to leaving.

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5: Biochemistry and Macromolecules

In this experiment, you will test common food items for the presence of each of the major macromolecules.

Learning Objectives

At the completion of this lab, students will be able to:

Content:

- Define macromolecule, carbohydrate, monosaccharide, disaccharide, polysaccharide, lipid, protein, and amino acid
- Describe the basic structures of carbohydrates, lipids, and proteins
- Recognize and correctly analyze positive and negative tests for carbohydrates, lipids, and proteins
- Test substances to determine the presence of biologically important molecules

Physical Skills:

- Use serological and micropipettes accurately

Scientific Communication Skills:

- Design a controlled experiment

Introduction

Biochemistry is the study of all of the chemicals and chemical processes that occur in living organisms. Although there are virtually thousands of chemicals in a living cell, the vast majority of these chemicals are organic, or carbon-based, molecules termed macromolecules. Macromolecules can be divided into four main groups: carbohydrates (sugars), proteins (amino acids), lipids (fats, oils, sterols, phospholipids, and waxes), and nucleic acids (DNA and RNA). Most macromolecules consist of smaller units (**monomers**) that can be covalently bonded together to create larger molecules (**polymers**). Although all of these organic molecules are, for the most part, composed of the same elements (carbon, hydrogen, and oxygen), they all have distinctly different chemical and physical properties. The unique properties of any class of organic compounds can be attributed to its “functional groups”.

A functional group is part of a larger molecule; it is composed of an atom or a group of atoms that have a characteristic chemical behavior. A functional group can be as simple as a carbon-carbon double bond which occurs in many lipids such as those that make up the cell membrane, or as complicated as a carboxylic acid (a carbon double bonded to a single oxygen and single bonded to an OH group). The OH group itself is a functional group, it is an alcohol and is often referred to as a hydroxyl group. See *Figure 4* for the structures of these molecules.

Macromolecules

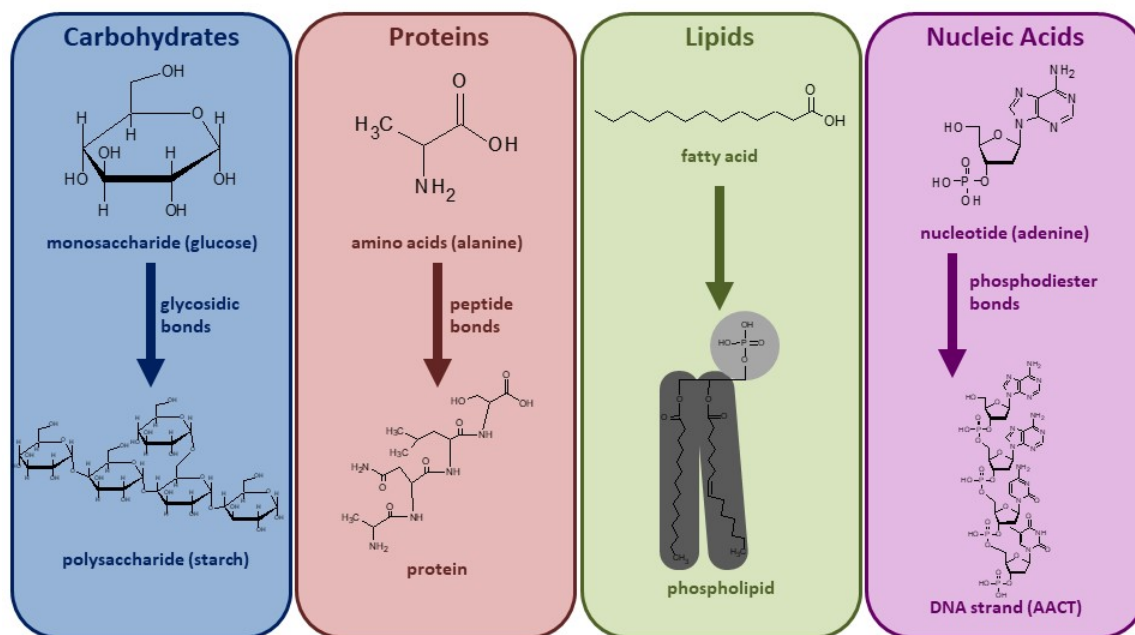


Figure 1: Composition of Organic Compounds. Carbohydrates are built from the monosaccharide glucose attached by glycosidic bonds to make a polysaccharide. Proteins are made by connecting amino acids together with peptide bonds. Lipids can have many structures, one of which is a phospholipid that has a polar head and hydrophobic fatty acid tails. Nucleic acids (DNA and RNA) are assembled from individual nucleotides attached by phosphodiester bonds.

In these experiments, you will learn how to identify lipids, carbohydrates, and proteins using various test reagents that recognize the unique functional groups (or lack of functional groups) on these three different types of organic compounds.

Nutrient and Growth Requirements for Bacteria

In our research project for the semester, we will grow bacteria in the lab. Bacteria grow on solid petri plates which serve as their food source. These petri plates contain mixes of essential nutrients suspended in water with a solidifying agent (agar or gelatin) added to the nutrients to make it solid.

Media ingredients range from dehydrated extracts of plant, animal, or fungal material to synthetically derived compounds. Each type of media contains different nutrients. Over the years, microbiologists have developed standard media mixtures.

Additionally, other physical parameters such as light exposure, temperature, and tonicity can drastically affect the composition of microbes that grow on plates. The composition and concentration of ions and molecules in a bacterium's environment not only influence their nutrition but also the overall stability of their cells. A medium with inadequate electrolytes may represent a hypotonic environment that will cause cells to swell up and burst. Conversely, a hypertonic medium with an excessively high concentration of solutes may cause cells to shrivel and die. Similarly, many bacteria cannot survive outside a relatively narrow pH range.

In our experiment today, we will be analyzing a few different types of media to determine the types of nutrients and macromolecules present in the bacterial growth media. As a comparison, we will use some human foods for controls.

Protocols

Carbohydrates function as the primary source of energy for living organisms and also serve to provide structural support. Remember, the monomer of a carbohydrate is a **monosaccharide** which consists of a single sugar molecule, while a **polysaccharide** consists of many sugar molecules covalently linked together. Some common monosaccharides include glucose, fructose, and galactose. Some common polysaccharides are starch and glycogen, which are used for carbohydrate storage in plant and animal cells, respectively. Additionally, cellulose and chitin are polysaccharides used for structural support in plant and animal cells.

The test reagents or chemicals used to identify carbohydrates are **Benedict's reagent** (recognizes monosaccharides) and **Lugol's reagent** (recognizes the polysaccharide starch). Benedict's reagent reacts with hydroxyl groups (-OH) of glucose and fructose. In the presence of heat, Benedict's reagent reacts with the OH group on monosaccharides and causes a color change.

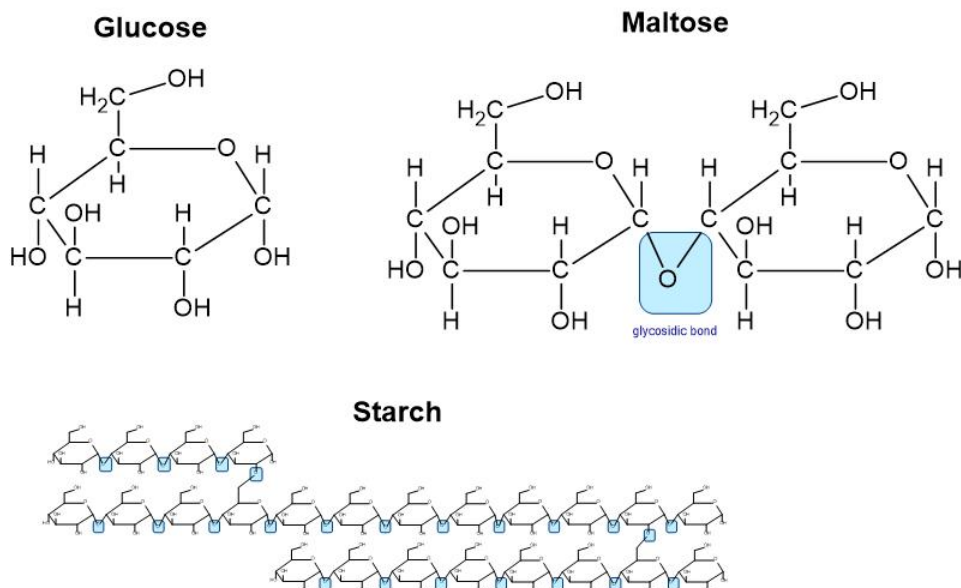


Figure 2: Molecular structure of the carbohydrates used in this experiment

The color results for the Benedict's Test are the following:

Benedict's Test Color Results

Color	Meaning
Blue	no monosaccharides
Green/Brown	some monosaccharides
Yellow/Red	large amounts of monosaccharides

In contrast to Benedict's solution, Lugol's solution does not recognize a specific functional group, but rather, it binds to the three-dimensional helical structure of starch that forms when the glucose molecules are assembled into a single polysaccharide.

Lugol's Test Color Results

Color	Meaning
Brownish yellow	no starch
Dark blue / black	starch present

Materials

- glass test tubes
- Benedict's Reagent
- Iodine
- pipettes and tips
- water bath and hot plate
- agar plates
- soil sample
- knife or scalpel
- food samples

Protocol 1A: Benedict's Test for Monosaccharides

- Fill a 100ml beaker about half full with tap water, place on the hot plate and let it come to a boil. Once it comes to a boil reduce the heat and let simmer.
- Number the test tubes 1B -10B (B is for Benedict's)
- Label each agar plate clearly with the type of media present in the plate. All of the media can look similar, so be sure that you can tell one from the next.
- Using a knife or scalpel, slice each plate into 6 slices.
- Arrange test tubes 1B-10B in the front row of your test tube rack. These will be tested with Benedict's.
- For each test tube add 1 ml of the sample to be tested or one slice of the agar plate. You should select these samples based on the ones provided. Ensure that you have included a negative control, a positive control, each of the agar plates, the soil and 2-3 samples that interest you.
 - Record your sample selections on your lab handout.
- Place 2ml of Benedict's solution in the appropriate test tubes. Place in the simmering water for 3-5 minutes and then remove.
 - Record the colors before and after on your lab handout

Protocol 1B: Lugols Test for Starch (you'll do this at home)

- Place 10 samples on a plate or other surface (I don't recommend paper).
 - Take a photo of these samples and record their appearance
- Add one drop of the Lugol's Iodine reagent to each samples (heating is not necessary!)
 - Take another photo.
 - Record the colors and results on your lab handout.

Proteins consist of long chains of monomers called **amino acids** that are linked to one another by covalent bonds termed peptide bonds. There are twenty types of amino acids used to manufacture proteins. All of the amino acids are exactly the same with the exception of the "R" group, or side chain, that is attached to the central carbon. Therefore, it is the R-group that distinguishes one amino acid from another (**Figure 3**).

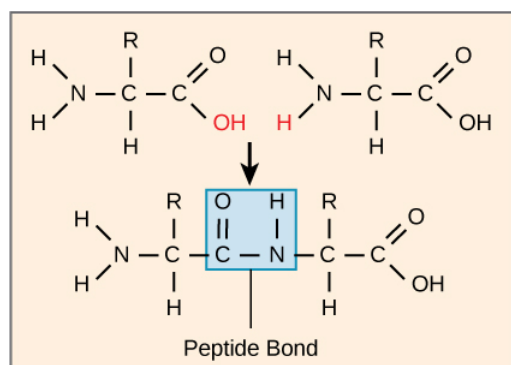


Figure 3: Proteins are formed by connecting two amino acids by a peptide bond using a dehydration synthesis reaction. From [OpenStax Biology 2e](#)

The R-group can be as simple as a single hydrogen atom (H, as is the case with glycine) or a very large organic subunit (as is the case with tryptophan). Polypeptide chains are folded into very specific 3D structures that allow the resulting protein to perform specific functions. One example of a protein is an enzymes which is important for the running of chemical reactions in living organisms.

In this experiment, you will use the **Biuret reagent** to detect the presence of proteins. This reagent works due to the binding of the copper in the reagent to the amino group ($-\text{NH}_2$) of the protein.

Biuret Test Color Results

Color	Meaning
Blue / no color change	no protein
Lilac	protein present

Materials

- glass test tubes
- NaOH
- Biuret's Reagent
- pipettes and tips
- agar plates
- soil sample
- knife or scalpel
- food samples

Protocol 2: Biuret's Test for Protein

- Number the test tubes 1P-10P with a Sharpie.
- For each test tube add 4.5 ml of the sample to be tested or two slices of the agar plate. As you select your samples, ensure that you have included a negative control, a positive control, each of the agar plates, the soil and 2-3 samples that interest you. Besides the agar and the soil, I recommend testing different samples from the earlier Benedict's and Lugols, but this is your choice.
 - Record your sample selections on your lab handout.
- Carefully add 3ml of sodium hydroxide (NaOH) to each of the tubes and mix.
- Add 150 μl of 1% copper sulfate solution (CuSO_4) to each of the tubes and carefully mix.
- The change in color from clear to a pale violet indicates the presence of proteins.
 - Record the colors and results on your lab handout.

Protocol 3: Biochemical Tests to Identify Lipids

Lipids are the only macromolecule that does not form true polymers. Although lipid molecules do not form covalent bonds with one another, they do tend to group together as they are all hydrophobic, or water-fearing. **Figure 4** shows the formation of a triglyceride, a simple fat containing three hydrophobic fatty acid tails and a glycerol backbone. Lipids serve a variety of functions for living organisms, including energy storage (fats and oils), controlling growth and development (sterols), and serving as a major component of cell membranes (phospholipids).

Color	Meaning
No Color Change	no lipids
Faint orange	some lipids present
Bright orange	lots of lipids present

Materials

- filter paper
- Sudan III
- dH₂O
- pipettes and tips
- food samples

Protocol 2: Sudan III's Test for Lipids

- Obtain a piece of filter paper and place in the large side of the disposable Petri dish.
- Using a pencil and a penny, make 6 equally spaced circles each about ½ inch from the edge as shown in **Figure 5**.
- Label each circle with a letter.
- Spread a thin layer of the correct substance in each of the spaces provided (wipe off any excess sample). If you are pipetting, 10uL is probably a good amount. Set aside and let dry for 10-15 minutes. I recommend testing only food samples here, the agar pieces do not adhere to the filter paper very well.
- Place some Sudan III solution in the Petri dish (fill the bottom). Add the filter paper, sample side down in the Sudan III and allow to soak for 3 minutes.
- Rinse the filter paper in the same Petri dish with distilled water and allow to dry for at least one minute before evaluation.
 - Record the colors and results on your lab handout.

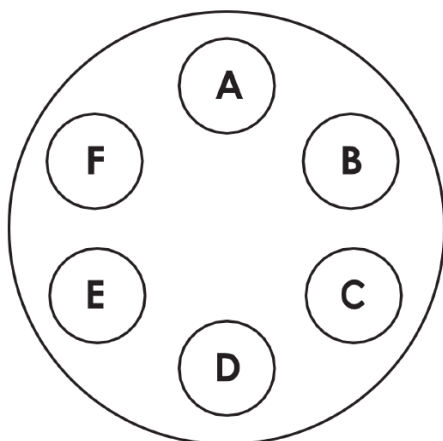


Figure 5: Sample layout for Sudan III test. The larger circle is the filter paper and the smaller labeled circles are for each of the 6 test samples.

Video of Biochemistry Lab Background



Biochemistry Lab Intro

a Sullivan

	Monomer	Polymer	What Reacts?	Biochemical Test
Carbohydrates	Monosaccharides (glucose, fructose, galactose)		-OH	Benedicts Test
		Starch	Looped/Helical structures	Lugols Test
Proteins	Amino acids	Polypeptides (proteins)	Peptide bond	Biuret's Test
Lipids	Glycerol + fatty acids	Phospholipids or fats/oils	hydrophobic	Sudan's Test
Nucleic Acids				

Watch on  YouTube

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6: Culturing and Isolating Microbes

Antibiotic Discovery Research Project

As a multi-week guided research project, you will isolate, identify, and perform a preliminary characterization of several soil microbes capable of producing antibiotics. You may choose the environmental site you want to sample and you will be responsible for isolation and characterizing an antibiotic producer for the duration of the project. Over the next several weeks, you will determine which of your isolates produce antibiotic activity, which organisms they inhibit, describe their cellular and colony morphologies. One isolate will be characterized further by using the latest bacterial identification technology to determine the species identity of your microbial strain. You will present your work in a written and oral form at the end of the project.

Learning Objectives

At the completion of this project, students will be able to:

Physical Skills:

- use pure culture and selective techniques to enrich for and isolate microorganisms
- properly prepare and view specimens for examination using microscopy
- estimate the number of microorganisms in a sample (using viable plate count)
- use appropriate microbiological and molecular lab equipment and methods
- practice safe microbiology, using appropriate protective and emergency procedures
- document and report on experimental protocols, results and conclusions

Scientific Communication Skills:

- formulate hypotheses and design experiments based on the scientific method
- analyze and interpret results from a variety of methods and apply these methods to analogous situations
- use mathematical reasoning and graphing skills to solve problems in biology
- effectively communicate fundamental concepts of biology as well as experimental protocols, results and conclusions in written and oral format

Introduction

The world is rapidly running out of usable antibiotics. This is a crisis worldwide as people are now dying of diseases that were previously treatable with antibiotics and that haven't been deadly in almost a century. This crisis is due to the rapid proliferation of bacterial strains that are resistant to antibiotics. According to the World Health Organization, 25,000 people die each year in Europe due to antibiotic resistant bacterial infections (WHO, 2011). In the United States, the Centers for Disease Control and Prevention (CDC) stated in 2013 that over 2 million people acquired serious antibiotic resistant infections and over 23,000 people died from these infections (CDC, 2013).

Unfortunately, we are not discovering antibiotics rapidly enough to counteract the rapid evolution. Many major drug companies have stopped or dramatically decreased their antibiotic discovery research as antibiotic discovery is challenging and not financially profitable. In the past 25 years, antibiotic drug approvals by the United States Food and Drug Administration has been cut in half (Spellberg, 2004). This leads to a larger problem - bacteria are developing resistance at precisely the same time that the number of new antibiotics are falling.

Antibiotic Discovery

Amazingly, most antibiotics on the market today were discovered as byproducts of other bacteria. This is because in complex environments (including soil and aquatic environments) bacteria must compete for limited resources. As a result one species of bacteria makes weapons that help it fight off neighboring bacteria. There is so much to be discovered about how and why and the

optimal conditions for these bacteria produce antibiotics, but we know that a small fraction of the bacteria in the soil do produce antibiotics under laboratory conditions. The key, and our goal this semester is to find some!

Can Students Make a Difference?

We certainly can!! Although the path of an antibiotic from soil bacteria to the pharmacy shelf takes much longer than a semester, we can start this process running and find some potential antibiotics -- and learn about the process of scientific research in the process. In my experience doing this project with students, approximately 50% of the research groups are successful in finding antibiotics. All of the other groups also have successful projects finding bacteria that are fascinating for their own reasons. Additionally, I save and store each antibiotic producer for further analysis and we contribute to the Tiny Earth database which helps identify trends in antibiotic production worldwide.

Through this research, we Citrus College students are participating in a global community of scientists helping to address this international antibiotic crisis.

We Live in a Bacterial Planet

Microbes live in incredibly variable environments and inhabit every environment on earth from as cold as Antarctic ice sheets to hot hydrothermal vents and as wet as the ocean to as dry as the Sahara desert. They also live in and on eukaryotic organisms (such as yourself) and on inanimate objects like door handles. Microbes are responsible for much of the change that our Earth has experienced over its 4.5 billion year history including making much of the oxygen we breathe, decomposing waste, fixing the nitrogen to biologically accessible forms, and helping to form raindrops and snowflakes!

Although microbes are microscopically small, they are diverse in evolution and in chemical abilities. Fascinatingly, microbes also communicate with each other—sharing information about the environment, providing food for their neighbors, and also battling each other with microbial warfare.

Definitions

Microbe refers to all organisms that cannot be seen without a microscope. These microbes include **bacteria** and **archaea** (**prokaryotes**), and microscopic **eukaryotes**, such as yeasts, molds, and protists. **Viruses** are also generally classified as microbes, although they are generally considered nonliving. As you know, prokaryotes and eukaryotes differ in their level of complexity and cellular organization. Eukaryotes package their genetic material inside a compartment called the **nucleus**, whereas prokaryotes do not. Prokaryotes do not contain any intracellular compartments and have their genetic material in the cytoplasm. The number of individual prokaryotic cells in soil across the planet has been estimated to be 26×10^{28} (260 billion billion billion) (Whitman, Coleman, & Wiebe, 1998).

Our research focuses on bacteria because:

- Bacteria are found in all environments on the planet
- Bacteria are simple but are able to make a diverse array of chemical compounds, many of which have human applications;
- Bacteria greatly impact our health—some may cause infectious disease and others protect our bodies from invasion by harmful bacteria.

Getting Started

In our *Tiny Earth* research project, we will design and execute experiments to answer questions related to microbial ecology and bacterial interactions focusing in particular on soil microbes abilities to create antibiotics and inhibit the growth of neighboring microbes. As microbiologists, we will use many techniques from biological fields including cell biology, biochemistry, and genetics. In this way, our research will tie in quite nicely with the topics that we are discussing in lecture.

The Earth Around You

Soil

Soil covers the earth's surface, supports plant life, and is an ecosystem for some of the most numerous, diverse, and dynamic organisms on earth—bacteria. The main characteristics defining soil are its physical structure, its chemical composition, and its association with plant roots or other resident organisms. The association of soil with plant roots creates a distinction between soil types. The **rhizosphere** is soil that is directly in contact with plant roots and **bulk soil** is everything else. The root systems in the rhizosphere create microenvironments that may favor the growth of specific bacteria, so although soil looks the same throughout, it is far more complicated and diverse.

Soil can be made of three major sediment types—sand, silt, and clay. These three components provide the structure for the soil and influence how permeable the soil is to water, how well it is aerated, and how well nutrients are maintained. Each of those features influence which microbes thrive in each environment. Coarser soils (like sand) hold air well, but not water resulting in them drying out rapidly and losing nutrients. Finer soils (like silt) and clay can pack densely limiting O₂ availability, but stay moist longer. To tell whether your soil is sand, silt, or clay, grab a small handful, make it moist and try to squeeze it together. If it holds, then your soil has a clay component. To look for sand vs silt, rub a small amount of the soil between your fingers. If it feels coarse and rough like sandpaper, you have sandy soil while a slick smooth feeling is silt instead.

In addition to the sediment types influencing the microbes present in the soil, the animal and plant life drastically impact the types and number of microbes in the environment. Environments with greater biodiversity—more animals and plants present—tend to have greater microbial diversity as well. The particular types of plant roots in the soil as well as soil pH and decaying organic matter create different mini ecosystems for the bacteria to inhabit.

Deeper in the soil, the physical, biological, and chemical properties of the soil changes. The soil is divided into distinct layers called **horizons** (**Figure 1**). Horizons are a good predictor of the nutritional characteristics of the microbes living within them. Generally, as we descend from one horizon to the next, the abundance of organic components, as well as the exposure to light and oxygen, plummets.

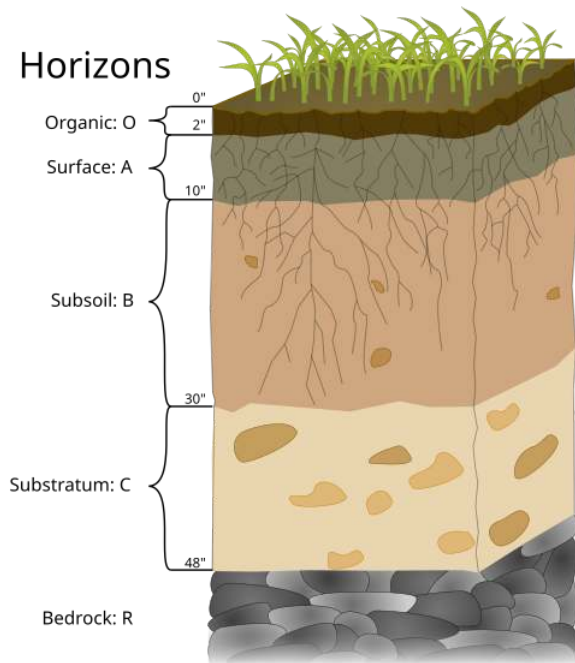


Figure 1. Soil Horizons: As you dig deeper into the soil, the amount of organic matter (microbes, animals, plants and decomposing material) decreases and the inorganic material (rock, minerals) increases. From [Wilsonbiggs](#) Vector: EssensStrassen, CC BY-SA 4.0 via Wikimedia Commons

The **O horizon** is a very thin layer at the surface and is the part of the soil that you usually see. Next is the **A horizon** (aka topsoil) which is quite rich in organic matter and microbial life. Here is where the microbes decompose organic material, interact with roots

and other organisms. Third is the **B horizon** (aka subsoil) where fewer microbes live and those that are present are often anaerobes and therefore don't use oxygen for cellular respiration. Next is the **C horizon** where microbes are present, but few. Finally, below all of these levels is bedrock. It is important to know which layer of soil microbes are isolated from since this greatly influences microbial growth conditions.

Soil Microbes and Antibiotics

Microbes are quite prevalent in soil in terms of both number and variety. Within one gram of soil, billions of bacteria from tens of thousands of distinct species are present (Curtis, Sloan, & Scannell, 2002; Schloss & Handelsman, 2006). Soil microbes are quite diverse in their physiology and chemistry.

Some microbes decompose plant or animal matter, others cycle nutrients, and they compete with each other for food and other resources. As part of this battle, bacteria produce **secondary metabolites** (organic compounds that enhance an organism's survival while not being essential for life). These secondary metabolites can provide the bacteria with pigments that make them

colored, signals that encourage plant growth in the area, and **antibiotics** to kill or limit the growth of competing microbes. In their natural habitat, microbes produce antibiotics to ward off competitors. Scientists and doctors have used this bacteria vs. bacteria weapon to save the lives of those who have an infectious disease.

Most antibiotics used to fight disease today are derived from soil bacteria. The most common bacterial species making these antibiotics are Actinomycetes, which are quite common in soil. Since such a small fraction of soil bacteria have been thoroughly studied, there are probably many more antibiotics produced by soil bacteria just waiting to be discovered. Because this environment is so poorly understood and easily accessed, our research project will begin by collecting soil samples so

that we can study it further and hopefully find antibiotic producing bacteria of our own. As you do this, keep in mind that your soil sample is a mini ecosystem and pay attention to the inorganic as well as biological features of this ecosystem so that we can better understand the microbes within it.

Our worldwide research collaborations through the *Tiny Earth* ranging from Malaysia to Kenya to Massachusetts, USA can result in a huge database of soil and microbial information. The data that you gather here from soil type, to microbial number, to number of antibiotic producing bacteria will help future students and scientists worldwide find new sources of antibiotics.

Bacterial Growth

Bacterial growth is generally used to describe bacterial reproduction or the increase in size of a microbial population. Bacteria are experts at reproduction and can double the size of the population as fast as every 20 minutes (although some species and environments result in slower growth). Bacterial growth/cell division is completed through a process called **binary fission** (**Figure 2**). This process begins with DNA synthesis in which the bacteria makes a copy of its chromosome, continues as the bacteria grows larger which separates the two identical chromosomes into the two halves of the cell, and ends by the cell membrane and cell wall constricting to cut the bacterial cell into two equally sized pieces. Through this process, the daughter cells are genetically identical to the parent cell.

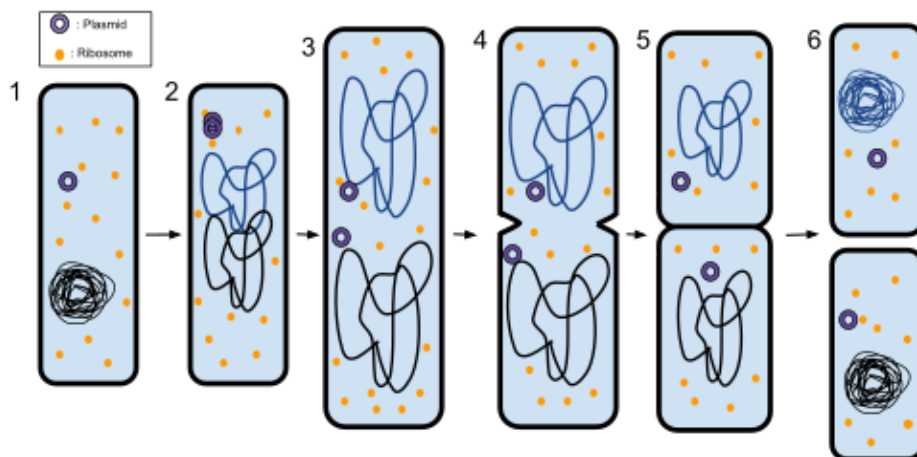


Figure 2. The process of binary fission results in the duplication of a bacterial cell. Each round of division doubles the size of the bacterial population. From Ecoddington14, CC BY-SA 3.0, via Wikimedia Commons

Bacteria grow at different rates depending on the identity of the bacterial species. Some, like *Escherichia coli*, are very fast and double in 20 minutes while others, for example, *Mycobacterium tuberculosis*, can take 16 hours. The optimal growth conditions can also be different for each organism as some prefer rich nutrient environments, others low nutrient, some warm temperatures and other bacteria cool environments. Additional diversity can be found in wet vs. dry, acidic vs. basic, etc. However, no matter what an organisms “optimal growth” condition is, it is unlikely to be found in a complex ecosystem where bacteria are kept in check by competing microbes and limited resources. Optimal growth conditions are typically accomplished in the lab, where microbiologists have developed nutrient-rich environments to support the growth of some bacteria.

Bacterial Growth in the Lab

To **culture** (grow) bacteria in the laboratory, scientists have developed standard **media** (bacterial food sources) which are a mixture of nutrients. Each particular bacterial species has its own preferences for the types of nutrients that it prefers, and, in our research, you will have the chance to select the media that you wish to use for your microbe. This media can come in either a liquid or solid form, though we will primarily be using solid media for our research. There is no one media choice that is best for all bacteria, and each week you will have choices to make related to the type of media that you use. The solid media that we are using is referred to as an agar plate. The agar is the solidifying agent used to make the nutrients solid in the Petri dish (**Figure 3**). As we work to culture our bacteria, you will carefully control and record the culture conditions—media type, temperature, humidity, oxygen levels, etc.

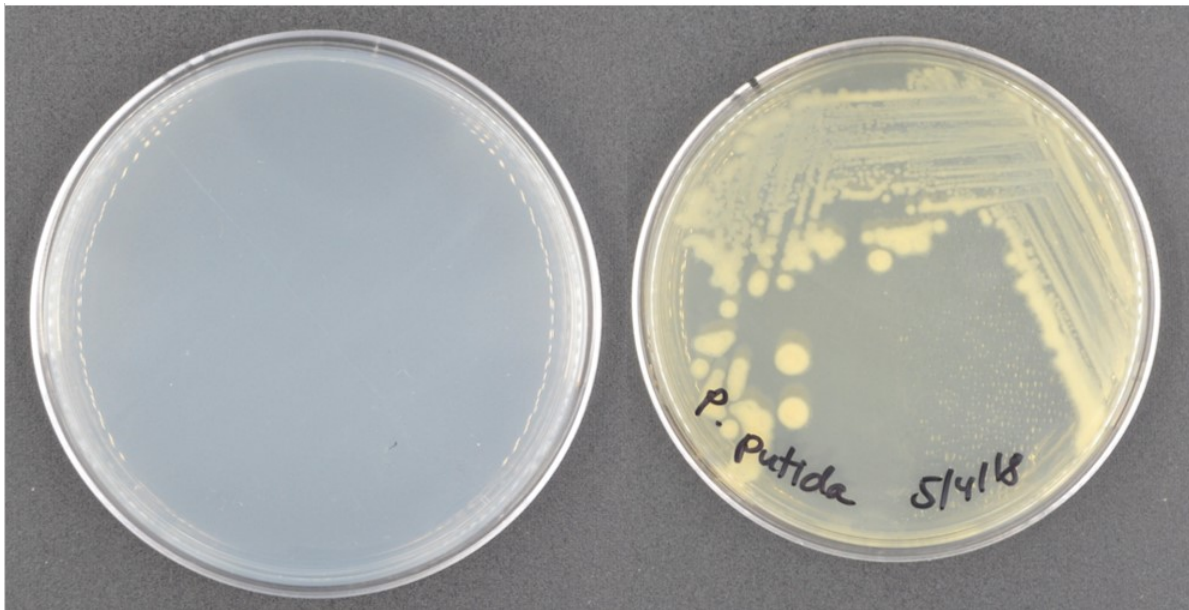


Figure 3. (A) A sterile, empty agar plate which contains nutrients, water, and the solidifying agent agar. (B) The same type of plate, with the bacteria *Pseudomonas putida*.

CFUs and Bacterial Colonies

It is important to note that most soil bacteria cannot be cultured in the lab. Most of these bacteria require growth conditions that are not easy to replicate in the lab. Therefore, although we will isolate and identify many different bacteria in the course of our project, these bacteria that we identify are a tiny fraction of the soil bacteria. It is estimated that only 0.3 percent of all bacteria in soil can be cultured in the lab (Amann, Ludwig, & Schleifer, 1995).

To culture the bacteria in the soil, we will spread a diluted soil sample on a plate and look for the appearance of **colonies**. Just as you'll see spots of mold on an old piece of bread, you can see a bacterial colony on an agar plates. A colony is a pile of genetically identical bacteria that arise from a single cell. Single cells that give rise to a colony are referred to as **CFUs (colony forming units)**. As a this single cell reproduces, the mass of dividing cells eventually becomes large enough such that a macroscopic colony

becomes visible to the naked eye. By the time you can see a colony on a plate it contains approximately 1 million cells. Colonies are our way of seeing into the microscopic world, and each bacterial type has particular colony features that can be recognized by experienced microbiologists.

Our goal this week is to isolate bacteria from the soil to obtain **single colonies**. These single colonies are distinct and not touching other colonies. They are also uniform in color and texture throughout indicating that they came from just one single bacterial cell. If we are successful in isolating single colonies, all of the bacteria within the colony are the same species. The trick to be able to do this successfully is to dilute them and spread out the CFUs so that they grow into non- overlapping colonies.

To do this, microbiologists dilute the soil samples repeatedly in a solvent to decrease the number of cells in any sample. We will then spread a small volume (100µL) on an agar plate to separate and spread out the bacteria all over the surface of the plate where they will grow into single colonies (**Figure 4**). It is absolutely essential, while examining these bacteria, that we ensure that these single colonies are not contaminated with other species. To help determine this, first do not use plates where bacteria have spread all over the surface creating a lawn, also be sure to select only colonies that are not touching any other colonies. Our goal is to obtain pure cultures that contain only one single species of bacteria. We will use these pure cultures in our subsequent experiments during the semester.

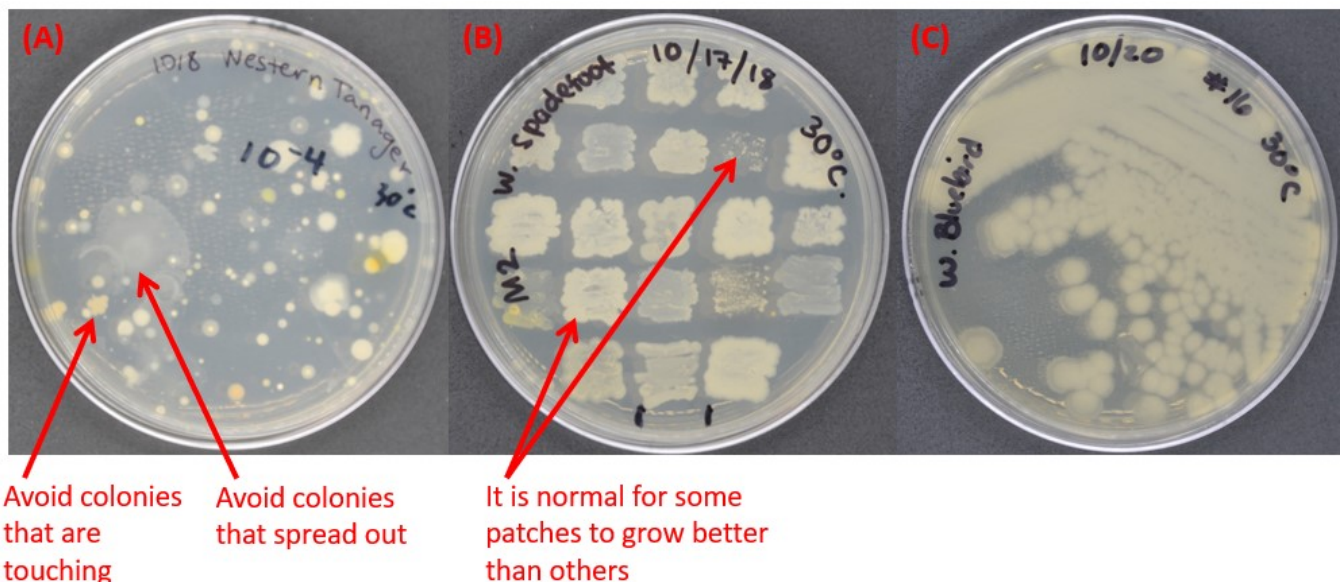


Figure 4. (A) A serial dilution plate of a rich soil sample. Bacteria in the soil sample were suspended in sterile water and diluted 10,000-fold (10⁻⁴) to spread the bacteria out well. After incubating this plate, single colonies can be picked from the dilution plate and smeared on a new plate to make the master plate. (B) The master plate is the collection of each of the distinct bacteria you isolated from the soil sample. Each of these patches was transferred from a dilution plate and then the plate was incubated to grow these patches. We can now test each of these patches. (C) The unique isolates on the master plate are “streaked” onto a new plate to isolate single colonies.

References

Protocols

Protocol 1: Obtaining a Soil Sample

Soil harbors an incredible number and diversity of bacteria. The goal of this experiment is for you to select a local ecosystem, study it, and bring back a soil sample to study in the laboratory. Try to select an ecosystem that will have great bacterial diversity.

Materials

- Container: conical tube or sandwich bag
- Lab Marker
- "Scientific Soil Sample Collection Device" (plastic knife or spoon)
- Alcohol pads (to disinfect collection device, thermometer, and ruler)
- Thermometer
- Plastic ruler
- Soil collection worksheet (paper or electronic)

Protocol

- Write down a set of criteria for picking a soil sample and choose what location(s) on campus you wish to study.
- Obtain 1 container to collect a soil sample and a soil collection worksheet.
- Fill in your soil collection worksheet with important information about your soil sample: location, date and time, weather conditions, habitat, surrounding plant and animal life, and other descriptive information.
 - For location and weather, feel free to use maps or weather app to get an estimate.
 - Take note of the environment and be sure to take a picture of your sample site.
 - Checkout ["Guide of Texture by Feel"](#) to help you determine the soil type.
 - Take a picture of your sample location site. I recommend a close up of your hole as well as a larger format showing the general area.
- Go out into the environment and collect a small soil sample—pick 5–10 g of soil or roughly a fistfull of soil.
 - For conical tube, take the lid off and use the open tube to scoop soil directly.
 - For sandwich bag, turn the bag inside out, grab a fistful of soil, and wrap bag around soil.
- Close your container and bring it back to the laboratory.

SOIL SAMPLE DATA COLLECTION SHEET

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SOIL SAMPLE DATA COLLECTION SHEET	
Location:	
Collected By:	
Date of Collection:	
Depth of Sample:	
Type of Soil:	
Temperature of Air (°C):	
Temperature of Soil (°C):	
Weather Conditions on the Date of Collection	
General Location:	
GPS Coordinates via Google Earth:	
Sample Site Description:	
pH:	

Additional Documentation:

- Photo of sample site
- Identify any plant species present near site
- Photo of soil in collection tube

Protocol 2: Making Serial Dilutions of Your Soil Sample

In an average 1 gram of soil sample or 1 mL of sea water there could be 10,000 to 100,000,000 bacteria. Because this density is so high, it would be almost impossible to get an exact count of the cells. Instead, microbiologists estimate the cell density through colony forming units (CFUs).

To perform this calculation, the sample must first be diluted in water or a saline solution to keep the bacteria alive. An initial dilution of 1g of soil in 9mLs of water (to make 10mL total) would create a 10-fold or 1:10 dilution. This will spread out the bacteria in 10x the volume, thereby making them further apart. This sample is still too concentrated with bacteria to be able to accurately count them.

Therefore, we will continue separating the bacteria by making serial dilutions of the sample in 10-fold increments until they are easier to work with and count. Once we reach a reasonable dilution, we can spread the sample out on a solid medium that will feed the bacteria. Each CFU (viable bacteria that can grow in this environment) in the sample will then grow into a colony and we can count them and, using the dilution and plating information, calculate the CFUs present in each gram of soil. For example, if we serially dilute 1 g of soil sample by a factor of 10³, spread and incubate the dilution

on a solid medium, and then observe 130 colonies, we would obtain 130×10^3 or 1.3×10^5 CFUs/g of soil. This number represents the number of viable cells, i.e., cells in an environmental sample that can survive lab conditions and grow in culture.

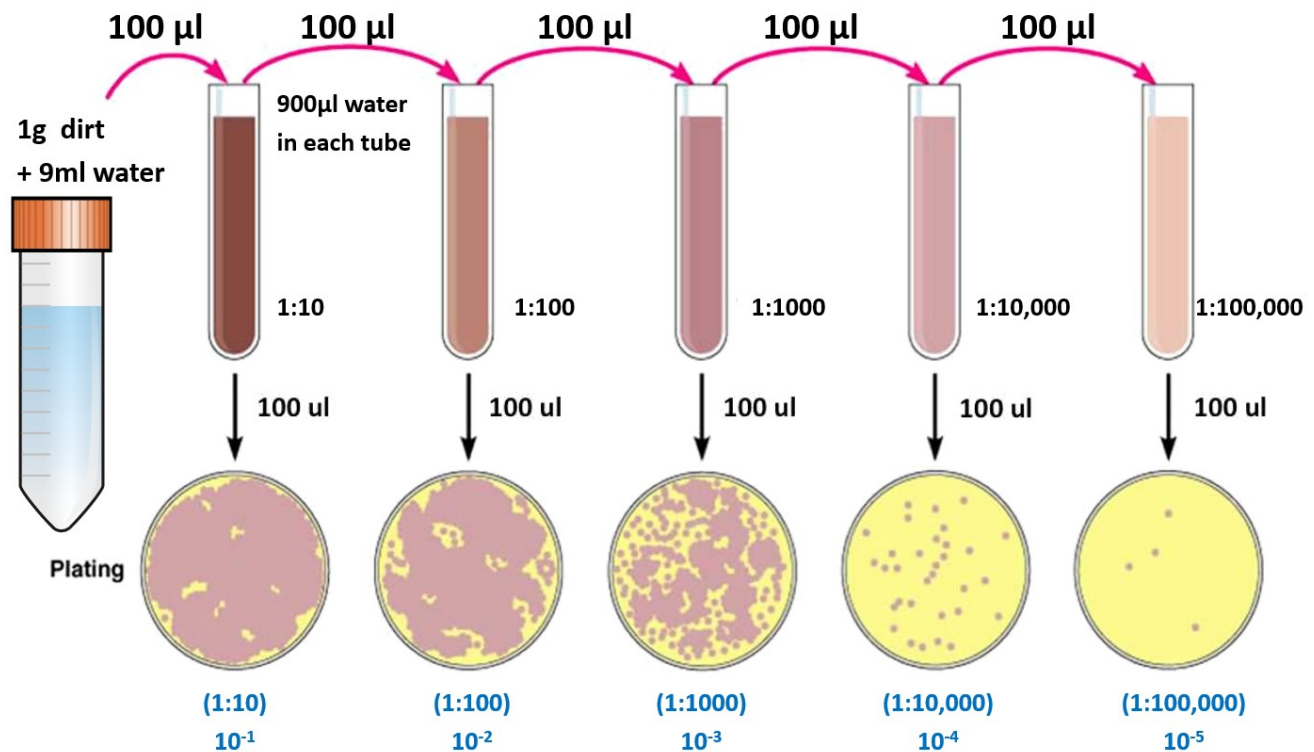


Figure 5. Serial dilution and plating schematic. A series of 10-fold dilution is made from the soil sample. Each plate therefore has 1/10 of the number of bacteria from the previous plate. This makes it much easier to count the colonies.

Materials

- 15 mL Conical tube
- Soil sample
- Sterile water solution
- Vortexer
- 1.5 mL microfuge tubes
- Pipettors and tips
- Scale

Protocol

- Obtain and label appropriate number of plates and 1.5 mL microcentrifuge tubes, one for each subsequent dilution. Dilutions should be made in increments of 10 (10^{-1} , 10^{-2} , 10^{-3} , etc.). **NOTE: I recommend going up to 10^{-6} dilution.**
- Take soil sample and weigh out 1 g.
- Transfer soil to 15-mL conical tube and add 9 mL of water.
- Vortex soil/water mixture for 30–60 seconds.
- Determine the dilution series and calculate appropriate volumes for each. Dilutions should be made in increments of 10, thus add 900 μL of diluent (water) into each dilution tube. 900 μL diluent + 100 μL specimen transferred = 1000 μL
- Remove 100 μL of soil+water with micropipette from 15mL conical tube and add to the 900 μL of water in a 1.5 mL tube. This is 10^{-1} dilution.
- Mix with vortexer for 30 seconds.
- Remove 100 μL of 10^{-1} dilution and add to 900 μL of water in another microcentrifuge tube. Mix by vortexing.
- Continue to transfer 100 μL of previous dilution to 900 μL of water until reach desired dilution, vortexing between each sample.

*Follow instructions on spreading a plate on the next page.

Protocol 3: Spreading a Plate

One method of distributing bacteria evenly over the surface of an agar plate medium is commonly referred to as the spread plate method. Classically, a small volume of a bacterial suspension is spread evenly over the agar surface by using a sterile bent glass rod or glass beads as the spreading device.

The goal is to distribute the bacteria evenly so that they are well separated and can be counted or sampled.

Some things to take note of in the plate spreading are:

- Be sure that your plates are dry before spreading. If they are too wet it will cause the bacteria to run into each other.
- Choose the serial dilutions to plate thoughtfully. If you have too many bacteria on the plate you won't be able to count them or isolate them next week. If you have too few, you won't have enough isolated colonies to continue the project. You would therefore need to repeat week 1. **In my experience, the 10^{-3} , 10^{-4} , and 10^{-5} dilutions are the best.**
- Plate the correct volume of sample. I recommend $\sim 100 \mu\text{L}$ (0.1 mL). This is enough to fill the plate so that the bacteria spread out, but not so much that the plate is drippy.
- Work swiftly, some bacteria attach to the plate surface quickly, so be sure to spread as soon as you put on the liquid or you will have all of your bacteria clumped in the middle.
- Use aseptic technique to prevent contamination from bacteria in the air. Work near your flame, keep the tubes and plates closed, and change tips whenever they touch something.
- Start plating from the most dilute suspension to the most concentrated. This way, you don't need to change tips as each sample has more bacteria than the prior one.

Materials

- Serially diluted soil samples
- Pipettors and tips
- NA (nutrient agar) or TSA (Trypticase Soy Agar): any mixture of types for a total of 6-8 plates/groups
- Spread beads or "hockey stick" spreaders
- Beaker for used beads

Protocol

- Chose the media type that you wish to use (NA or TSA or a combination) and collect 6–8 plates total. Make sure that the plates are dry.
- Select the dilutions you wish to plate. In my experience, the 10^{-3} , 10^{-4} , and 10^{-5} dilutions are the best. Consider doing duplicates of some plates.
- Label the BOTTOM (food side) of each plate with: Group Name, Date, Media Type, Temperature, Dilution Factor
- Put 4–8 beads on the agar surface of each plate being sure to keep plates closed.
- Starting with the most dilute sample (e.g. 10^{-6}), transfer 100 μ l of the dilution to the center of the selected agar plate. Work backward spreading the most dilute samples first.
- Spread the sample around the plate by shaking as soon as the dilution has been added to the plate since some cells will rapidly attach to the agar, especially if the plate agar is dry.
- Let the plates sit on your bench for 10–20 minutes, so that all of the sample soaks into the plate.
- Invert the plates (food side up) and place them at the selected temperature to grow for the next week.



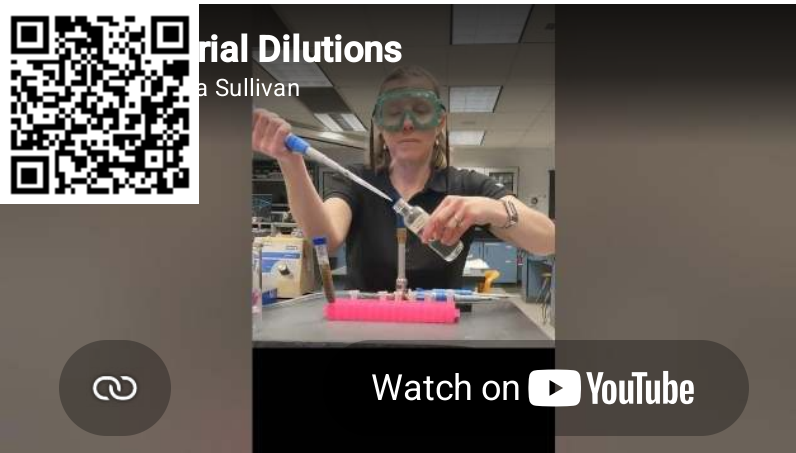
Discarding Materials

When you are finished plating your samples, please put your plates food side up at the appropriate temperature. The spreading beads should go in the appropriate container to be sterilized for re-use. All tubes and tips that you used can be thrown in the regular trash this week. Please return all reagents and tools to the place that you got them

Using the 70% ethanol sprayers on your bench, spray down your bench space to disinfect it and wipe it clean with a paper towel.

Tubes and agar plates should be discarded properly in a biohazard container for proper sterilization. Use aseptic (sterile) technique in the transfer of microorganisms from tube to tube and from tube to plates to prevent contamination.

Video of Serial Dilutions



Hints For NEXT WEEK AND THE WEEKLY WRITTEN ASSIGNMENT

Counting Colonies

Look at your plates in order of concentration and you should see a predictable drop in CFUs/ plate as a result of your 10-fold dilution series. If your plates differ from this substantially, let your instructor know.

The goal is to have between 30 and 300 colonies on a plate. Too many and it is too hard to count, too few and the count can be very inaccurate.

How to Calculate CFU/g

How to calculate CFU/g:

$$\left(\frac{\text{Colony Number}}{\text{Volume Plated}(\mu\text{l})} \right) \times \left(\frac{1}{\text{Dilution Factor}} \right) \times \left(\frac{1000\mu\text{l}}{1\text{ml}} \right) \times \left(\frac{\text{Initial Volume}(\text{ml})}{\text{Probiotic Weight}(\text{g})} \right)$$

- **Colony number** - select a plate with 30-300 colonies, these are the most reliable.
- **Volume plated** - accounts for the small volume spread on the agar plate. For our work, this is 100 μl .
- **Dilution factor** - accounts for the amount of serial dilutions done. Use the scientific notation, e.g. 10^{-4} .
- 1000 μl /1ml - is a conversion factor because there are 1000 μl in 1ml.
- **Initial Volume** - this is that first big tube, before you start the dilutions. For our work, this is 10ml.
- **Probiotic Weight** - this is the weight of the sample you recorded in grams. It should be small (1g or less)

Assuming that you followed all the dilution and plating instructions correctly, only the highlighted numbers will change.

For example:

A yogurt sample weighing 1.0 grams on the 10^{-2} plate with 120 colonies.

Substitute in the values - yellow ones from you particular experiment, the rest from the standard values.

$$\left(\frac{120 \text{ colonies}}{100\mu\text{l}} \right) \times \left(\frac{1}{10^{-2}} \right) \times \left(\frac{1000\mu\text{l}}{1\text{ml}} \right) \times \left(\frac{10\text{ml}}{1.0\text{g}} \right)$$

Solve each fraction and cancel out the extra units.

$$1.2 \left(\frac{\text{colonies}}{\cancel{\mu\text{l}}} \right) \times 10^2 \times 1000 \left(\frac{\cancel{\mu\text{l}}}{\text{ml}} \right) \times 10 \left(\frac{\cancel{\text{ml}}}{\text{g}} \right)$$

Multiply across and convert to scientific notation. Either form of the answer is acceptable.

$$1200000 \left(\frac{\text{colonies}}{\text{g}} \right) = 1.2 \times 10^6 \left(\frac{\text{colonies}}{\text{g}} \right)$$

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7: Selecting Microbes for Testing

Bacterial Nutrition

Like all organisms, microorganisms must use energy to build new cells, divide, and respond to their environments. The types of organic molecules produced by bacteria are the same as eukaryotic organisms: proteins, nucleic acids, lipids and carbohydrates (**Table 1**). All cells ultimately capture and store released energy in the form of **adenosine triphosphate (ATP)**, the “energy currency” common to all life, however, the processes by which they obtain this energy can vary.

Bacterial metabolism is very much more diverse than eukaryotic cells. We can classify bacteria based on how they obtain carbon and energy from their environments to make biological molecules. Some organisms, autotrophs or the producers in the food chain, produce organic molecules by obtaining or “fixing” carbon from inorganic sources such as CO₂. Some examples are photosynthetic organisms such as plants, algae, and cyanobacteria. On the other hand, **heterotrophs** obtain carbon from organic molecules formed by other organisms. Animals, protozoa, fungi, as well as many bacteria and archaea are heterotrophs. Because we are providing our bacteria with agar plate/media based food sources, the isolates we are studying are primarily heterotrophic. Another major distinction in bacterial classification relates to their tolerance for oxygen. Most bacteria that are in contact with the air are **aerobic**, meaning that they need oxygen (O₂) to survive. Bacteria that do not require oxygen or find O₂ toxic are referred to as **anaerobic**.

Macromolecules and their constituents

Organic Molecules	Elemental Components	Macromolecules
Amino acids	C, H, O, N, S	Proteins
Nucleic acids	C, H, O, N, P	DNA and RNA
Fatty acids	C, H, O	Lipids
Sugars	C, H, O	Carbohydrates

Media and Culture Conditions

In the lab, microbes are grown on many different types of media that contain mixes of essential nutrients suspended in water (e.g., broths, infusions, etc.). Some media like the ones that we are using, are solidified through the addition of agents such as agar or gelatin. Media ingredients range from dehydrated extracts of plant, animal, or fungal material to synthetically derived compounds. Media can be very nutrient rich, containing large concentrations of proteins, amino acids, simple or complex sugars, and/or salts. Media can also be kept minimal, composed of lower concentrations of simple sugars, salts, and water. In our laboratory, we will have multiple choices of media and you will have the opportunity to select the types you wish to use in your experiments.

Other physical parameters such as light exposure, temperature, pH, and salt concentration can drastically affect the composition of microbes that grow on plate. Bacteria can be quite sensitive to the ions, pH, and temperature that they are exposed to and photosynthetic bacteria will only grow in the light.

Growth on a plate indicates that the bacteria are living within an acceptable range of conditions that allows them to survive and proliferate. It does not mean that all of their needs are met or that they are making all of their possible compounds. In previous semesters we have seen examples where one bacterial species grows at multiple temperatures, but only make a pigment at one of them. We've also seen one bacteria make antibiotics on one type of media, but not another. This fascinating finding is related to induced **gene expression**, or the production of proteins based on signals received by the cell. Finding the right combination of growth conditions and environmental signals to stimulate both growth and antibiotic production is one of the most challenging parts of this research. There are many variables we can consider when choosing the right growth conditions and the more we experiment with conditions, the more we will be able to observe and learn from our samples—so use your imagination.

Growth Conditions: Solid vs Liquid Cultures

Colony Morphology

As you look at your dilution plates you will notice that the colonies display striking differences in physical appearance such as color, texture, shape, and margin or edges. Entire books, like the

thorough *Bergey's Manual of Systematic Bacteriology* contain descriptions of these features and help microbiologists identify and characterize bacteria. One of the easiest characteristics to observe is the shape of their colony, or **colony morphology** (**Figure 1**).

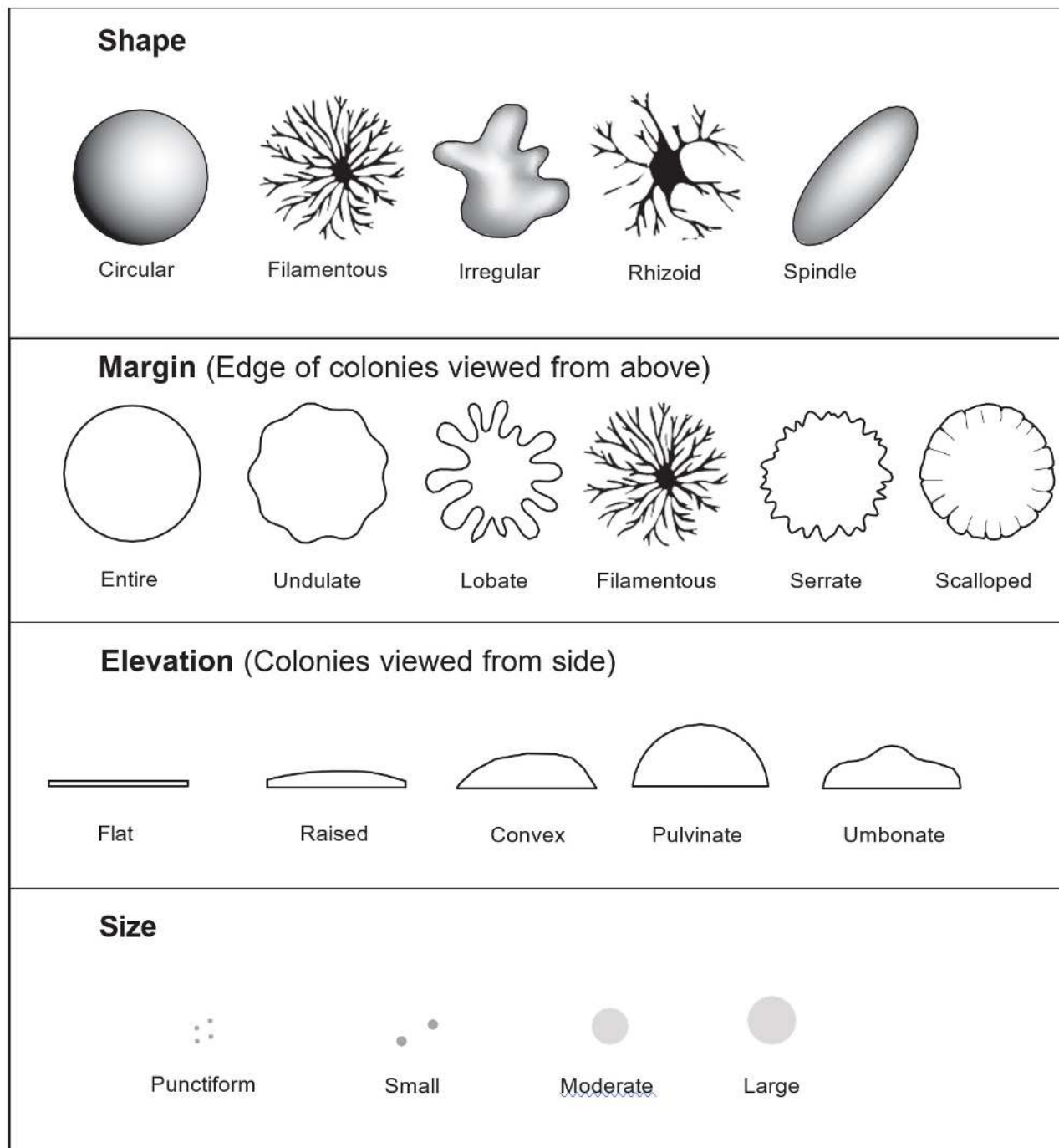


Figure 1. Diagram illustrating the various shapes, edges, and elevations of bacterial colonies. This is a useful guide for describing our soil microbes and being able to tell them apart.

Different types of bacteria tend to demonstrate characteristic morphologies – for example, *Lysobacter* and *Pseudomonas* colonies are typically slimy, irregular in shape, and may fluoresce under UV light (**Figure 2A**). *Streptomyces* colonies have bold colors on

the base of the colony and develop a white fuzzy top, which contains spores (**Figure 2B**). *Bacillus* colonies may sprawl thinly over the agar and typically show relatively dull, bland colors.

Unfortunately, we can't rely on visual appearance alone to identify our bacteria as we can get easily misled. However, this provides us a good clue about a possible bacterial identity which we will build on with future tests and experiments. The most precise and accurate way to identify our bacteria is using DNA sequencing, which we will do later in the semester.



Figure 2. Three microbes with distinctive colony morphologies, typical of their genus. (A) *Serratia marcescens* is red colored and can form colonies with white edges. The colonies are irregular in shape with entire edges. (B) *Acinetobacter baylyi* forms translucent white or clear colonies. The bacteria is gooey and stringy when lifted up off the plate. (C) *Micrococcus luteus* forms convex circular yellow colonies with an entire edges. These colonies are often small sized.

Growth Phases

Solid cultures are advantageous because culturable cells will grow where they are placed. This will allow us to isolate pure cultures. Liquid media is the opposite - the bacteria move around freely and randomly in the medium. If multiple types are present in liquid media, they are impossible to separate. Therefore, we will focus on using solid media to grow our many types of bacteria.

Bacterial growth typically follows a particular pattern. When put on/in a new media, bacteria typically grow very slowly at first (lag phase), as they become familiar with the new environmental conditions. After a few hours, the growth rate takes off and the cells begin to divide rapidly (log phase) or exponential phase. As nutrients are depleted and waste products accumulate, growth

slows such that the number of cells dividing is approximately equal to those dying (stationary phase). Secondary metabolites such as pigments and antibiotics are normally produced toward the end of the log phase, as cell concentration peaks and nutrients become depleted. Bacteria in the genus *Streptomyces* have been found to start making their secondary metabolites after they finish making their essential cellular components such as DNA (Bibb, 1996).

Protocols

Protocol 1: "Pick" and "Patch"

Our project this semester is to try to isolate and characterize antibiotic producers from soil found on campus. To do this we want a large variety of microbes to test to increase our chances of finding interesting antibiotic producers. Today, we will look at the growth of bacteria we plated on agar plates last week and try to isolate a large number of distinct single bacterial types to test next week.

You completed the first step last week where you used serial dilution and plating to spread out your bacteria on an agar plate. After allowing them to grow for a week, you will see many individual colonies. Unfortunately, you will also see many instances where colonies grew too close together, cells moved and spread across the plate, and/or cultures got contaminated. As you isolate colonies today from your plates, you will want to select colonies that are distinct and avoid those that are mixed or potentially contaminated.

Sterile conditions in your work environment and proper techniques are very important to isolating a bacterium and starting a pure culture. The isolation approach we will use is aptly called "pick and patch" and involves precisely those things: "picking" bacteria

from a mixed culture (e.g., usually a

dilution plate) and “patching” them onto a fresh plate, which may be a pure culture or a “master plate” containing all your unique bacteria of interest for your study. The slight touch of a colony with a sterile toothpick or a metal rod picks up thousands of bacteria that can be transferred and smeared or patched onto a fresh plate. At the end of this procedure you will have a “master plate” that will serve as a bacterial catalog for your experiments.

In choosing which colonies of bacteria to follow up with, there are several things that we will need to consider.

Choosing Colonies of Bacteria

1. Only select one species at a time	Select ones that don't touch & don't pick spreaders.
2. Choose a wide variety of bacteria	Select ones that look different in size, color, texture
3. Keep the single species isolated on the master plate	Put each species in a different square & leave space
4. Select enough bacteria to find an interesting one	Try to get 2 full plates of bacteria

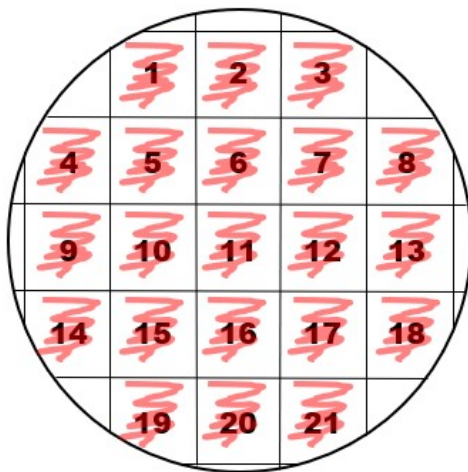


Figure 3. Master plate layout. This example shows how your plate should look after incubation and allowing the isolates to grow. Patches (in red) were oriented on the plate using a grid containing 21 colonies. **Notice how patches do not touch—this is critical to prevent cross-contamination.**

Materials

- Serial dilution plates from last week
- 2+ media plates of choice
- Square grid template
- Sterile Toothpicks
- Lab marker

Protocol

- Allow serial dilution plates of bacterial isolates or master plate to grow for 3-7 days
- Photograph your plates and describe them fully in your notebook
- Obtain 2 sterile agar plates containing your media of choice. Label the bottom (agar side) with:
 - Group Name
 - Date
 - Media Type
 - Temperature

- Plate number
- and mark the orientation of the grids

and draw a small vertical line at the edge of your plate as point of orientation

- Observe and describe the color, size, surface texture, elevation, margin, and shape each colony you select in your lab notebook. Note: You are not limited to using the terms in Figure 1. Once the colony is recorded, use a sterile toothpick to pick the unique colony from the dilution plate (10^{-3} or 10^{-4})

Note: Keep both plates covered whenever possible.

- Patch (gently zigzag) the colony smear on the toothpick onto fresh media plate within the boundaries of the square on your grid. Be careful not to puncture the agar as you smear. Also be careful to stay well within your gridlines so that you don't contaminate another square.
- Continue to pick and patch colonies onto media plate, each time into a new square. Make sure patches do not overlap or touch. Try to find as many morphologically unique colonies (i.e., different textures, colony margins, pigments), but do not go over 21 colonies per plate to avoid overcrowding.
- If possible, try to pick and patch 42 colonies (2 master plates). This collection of colony patches will be your "master plate."



Patching Video



Discarding Materials

When you are finished patching your samples, please put your new plates side up at the appropriate temperature. Stake your serial dilution plates and put them in the plate bag provided by your instructor. Label the correctly colored tape provided by your instructor with your group name and the date. These plates will be stored in the fridge in case you need them next week. The used sticks should go in the appropriate container to be sterilized for reuse. Any tubes or tips that you used can be thrown in the red biohazard bin. Please return all reagents tools to the place that you got them.

Using the 70% ethanol sprayers on your bench, spray down your bench to disinfect it and wipe it clean with a paper towel.

Meet the ESKAPE Pathogens

Understanding Infectious Disease

For centuries, people thought that disease was due to "bad air" or miasmas and as a result had little understanding of infectious disease. John Snow, a London physician, made a huge leap forward and traced the epidemic and transmission of cholera to a contaminated communal water pump.

The water source had been contaminated by the sewage thrown into the street and backyards of sick individuals. As a result of this discovery, the pump was closed, saving hundreds of lives, but more importantly began to connect disease to a physical source. Around the same time, Louis Pasteur, a French chemist and microbiologist demonstrated that sterile broth (like the media we use in lab) needs contact from the air or another external source to become contaminated. This began to draw attention to the microbes and resulted in the pasteurization process now used to make milk, juice, and beer safe to drink and increase their shelf-life.

Another 19th century scientist, the German physician Robert Koch developed four criteria (postulates) that we use to identify pathogens, or disease-causing microorganisms.

1. The microbe is always present in diseased individuals but not healthy individuals.
2. The microbe is isolated from the infected individual and grown in a pure culture.
3. The microbe in pure culture is introduced into a healthy individual and the same disease occurs.
4. The microbe is re-isolated and shown to be the same as the original.

These ideas might seem obvious, but were revolutionary to 1800s doctors and helped Koch identify the bacteria causing anthrax (*Bacillus anthracis*) and tuberculosis (*Mycobacterium tuberculosis*) and earn the Nobel Prize in 1905.

Unfortunately, many pathogens that were easily treated with antibiotics are now emerging in deadly and drug resistant infections. As a result, we are rapidly running out of treatments especially for hospital acquired "superbugs" and need to both slow the rate of the development of antibiotic resistance as well as discover new antibiotic treatments. Our research focuses on the discovery portion. As you isolate individual microbes, hopefully some will produce antibiotics. Initially, for practical reasons, researchers assay these microbes for the ability to inhibit the growth of only a few test organisms. Next week, we will describe experiments to screen our isolates for their ability to produce antibiotics. But first, we must decide which microbe to test our isolates against.

Choosing Test Organisms - The ESKAPE Pathogens

Since our goal is to fight human pathogens, it makes sense to select a test organism that is clinically relevant. There are six organisms that are today considered to be major threats, because they comprise the majority of antibiotic-resistant infections seen in health care settings. They are *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and several species of *Enterobacter* (ESKAPE).

Because prokaryotes differ in their anatomical and physiological traits, different organisms may be more or less susceptible to the effects of any particular antibiotic. One anatomical feature that plays a significant role in the susceptibility of a microbe to a particular antibiotic is its cell wall composition. Gram Positive bacteria have one layer of membrane and are surrounded by a thick layer of the cell wall made of peptidoglycan. Gram Negative bacteria, on the other hand, have two layers of cell membrane with a thinner layer of cell wall in between. As a result of this additional cell membrane, it is harder for antibiotics to get into Gram Negative cells and they tend to be more antibiotic resistant. We can't use the ESKAPE pathogens in lab because of safety concerns and worries that we might sicken ourselves or others. Instead, we will use their safe relatives in our tests: closely related species that do not cause disease. The names of the ESKAPE species, their safe relatives, and whether they are Gram Positive or Negative are listed in **Table 2**.

Table 2. The ESKAPE pathogens and their safe relatives, used as tester strains by Tiny Earth.

ESKAPE Pathogen	Safe Relative	Gram
<i>Enterococcus faecium</i>	<i>Bacillus cereus</i>	Positive
<i>Staphylococcus aureus</i>	<i>Staphylococcus cohnii</i>	Positive
<i>Klebsiella pneumonia</i>	<i>Escherichia coli</i>	Negative
<i>Acinetobacter baumannii</i>	<i>Acinetobacter baylyi</i>	Negative
<i>Pseudomonas aeruginosa</i>	<i>Pseudomonas putida</i> or <i>fluorescence</i>	Negative
<i>Enterobacter</i> species	<i>Enterobacter aerogenes</i>	Negative

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8: Antibiotic Discovery

Discovery of Antibiotics

Prior to the discovery of antibiotics, huge numbers of people died from bacterial infections. For example, during the American Civil War one third of the deaths were due to battle while two thirds died of infection. Treatment had improved by World War One, however, approximately one third of the fatalities were still due to infection. Scientists were actively looking for better **antiseptics**, cleaning solutions to prevent bacterial growth. However, most of these antiseptics were very corrosive and damaged the patient tissue while preventing the bacterial growth.

In 1928, Alexander Flemming returned from vacation to his messy lab and discovered a mold contaminant that had killed the neighboring staphylococci bacteria on one of his plates. There was a **zone of inhibition** or area of non-growth surrounding the mold contamination area. Fleming was intrigued by this observation and eventually showed that it was caused by a compound that he named penicillin after the fungus that produces it: *Penicillium notatum*.

To test the effectiveness of penicillin, Fleming tested it against several common pathogens. Penicillin inhibited the growth of Gram-positive bacteria associated with scarlet fever, pneumonia, gonorrhea, meningitis, and strep throat, while leaving eukaryotic cells unharmed (Brown, 2005). It exhibited the qualities of the miracle drug Fleming had been pursuing for years!

Work on penicillin lay dormant for ten years until scientists with extensive chemistry experience read Fleming's paper and used their background to purify the active compound and test it on infectious bacteria in patients (Amyes, 2001). This allowed penicillin to be mass-produced and administered orally and systematically to patients. Penicillin was commercialized in time to reach wounded soldiers in the battlefields of World War II, and significantly contributed to the Allied victory. The collaborative development of the first natural product antibiotic won Fleming, Florey, and Chain the Nobel Prize in Physiology or Medicine in 1945.

Characteristics of Antibiotics

- Selectively toxic toward microbes
- Generally antibacterial
- Small molecules (secondary metabolites)
- May be a synthetic or a natural product

Antibiotics

Antibiotics describe any small molecule produced by a microbe that kills or inhibits the growth of other microbes (Clardy, Fischbach, & Currie, 2009). “Small molecules” refers to the fact that antibiotics are smaller than the macromolecules that constitute a cell. Antibiotics greatly vary in their chemical structure and those with similar structures are grouped into **classes of antibiotics**. New classes of antibiotics are even more challenging to discover than new antibiotics themselves. One common class of antibiotic is the β -lactams, all of which have a four-member ring structure. This core structure allows β -lactams like penicillin and penicillin-derived antibiotics to inhibit bacterial cell wall synthesis (**Figure 1**).

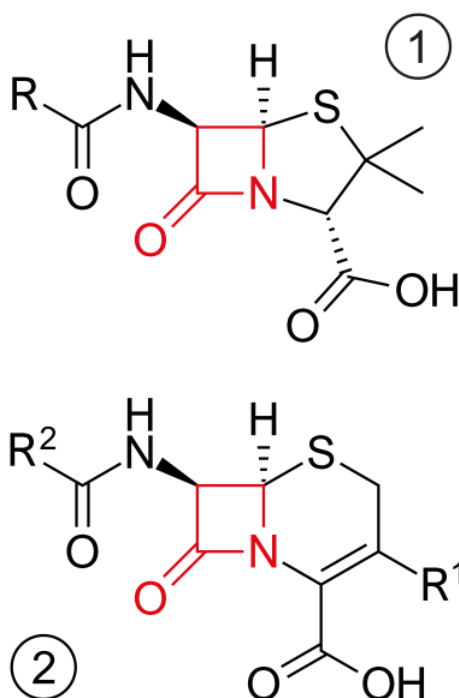


Figure 1. The β -lactam class of antibiotics, such as penicillin (1) and cephalosporin (2), have the important core of a four-member β -lactam ring (in red). This structure allows β -lactam antibiotics to inhibit bacterial cell wall synthesis. From: [Fvasconcellos](#) 19:02, 23 October 2007 (UTC), [Public domain](#), via Wikimedia Commons

Strictly speaking, true antibiotics are naturally-occurring antimicrobial compounds including penicillin, streptomycin, and chloramphenicol, which were originally isolated from living microorganisms. Natural product antibiotics make up 60–80% of all antimicrobials and most of the rest have been developed synthetically based on the natural product versions. Interestingly, the genus of bacteria, *Streptomyces*, which have complex lifecycles and look like fungi on solid media are the source of ~60% of the clinical antibiotics used today.

Antibiotic Targets

Antibiotics that interrupt the same target in the bacterial cell are also typically classified by their **mechanism of action**. Antibiotics need to target features that are specific to bacterial cells and are either not present in eukaryotic cells or are different enough that the drug doesn't disrupt on the eukaryotic version. One common target absent in eukaryotic cells is the bacterial cell wall. This cell wall is made of **peptidoglycan** (a macromolecule containing amino acids and a complex sugar). This material surrounds the cell, provides structure to the cell, defines the cell shape and is tough enough to prevent the cell from changes in osmotic potential or mechanical force. **Gram-positive** bacteria have a thick peptidoglycan layer as the outer surface of the cell, while **Gram-negative** bacteria have only a thin layer of peptidoglycan positioned in between two phospholipid membranes.

Some common antibiotics, including penicillin and vancomycin, inhibit **cell wall synthesis** resulting in eventual cell lysis. Another common antibiotic target is the bacterial ribosome which completes the process of **translation**. Although both bacteria and eukaryotic cells both do translation using a very similar process, the physical machinery of the ribosome is different enough that it can be targeted specifically. The antibiotics erythromycin and streptomycin specifically bind to the bacterial ribosome and prevent it from accurately building proteins. In a similar way, the antibiotic ciprofloxacin binds to the bacterial DNA gyrase enzyme involved in **DNA replication** preventing it's function while leaving the eukaryotic DNA gyrase unharmed. Similarly, rifampin inhibits RNA polymerase, which is necessary for the process of **transcription**.

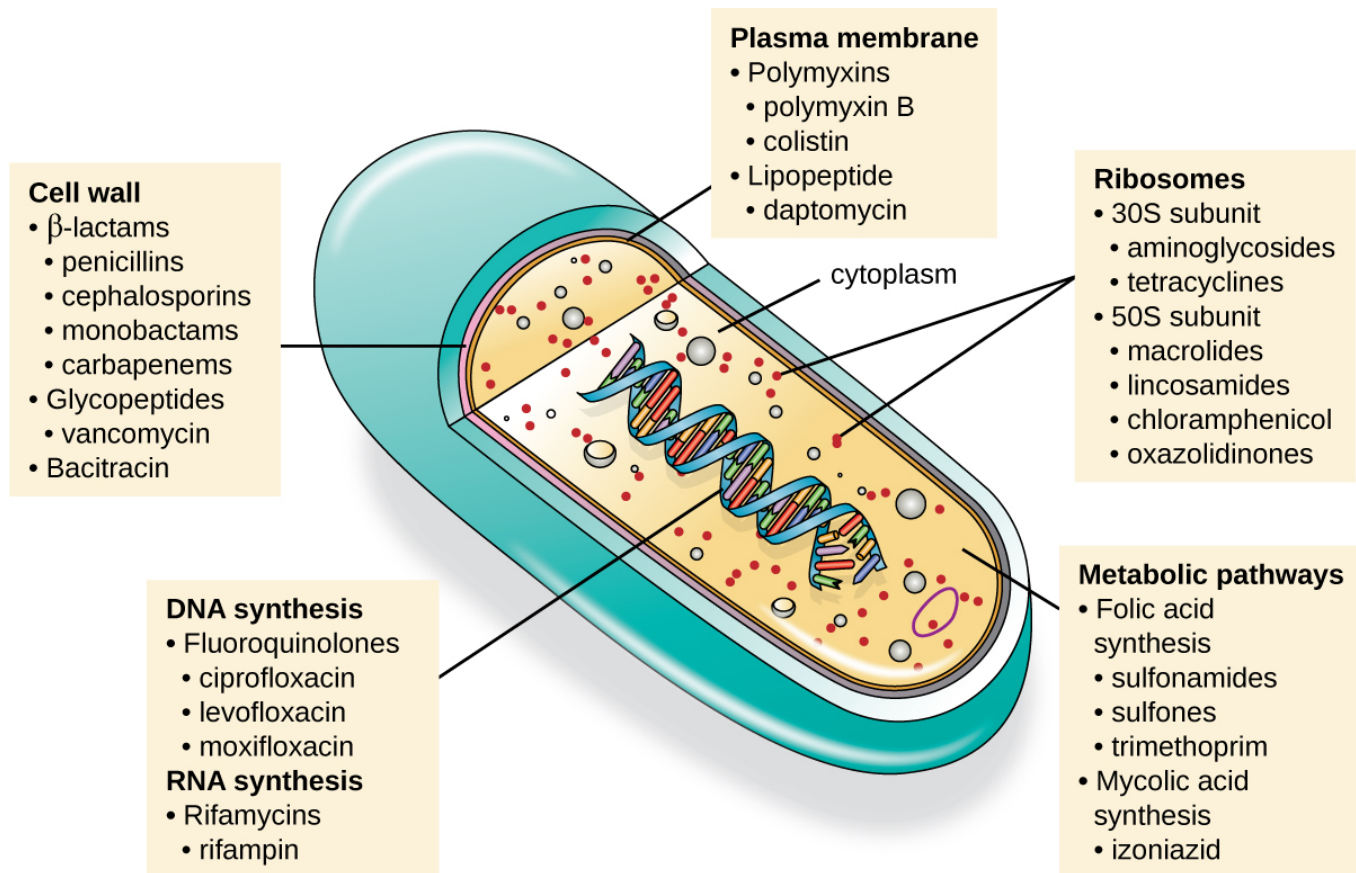


Figure 2. Schematic of antibiotic cellular targets (modes of action) and major classes of antibiotics (and examples of drugs). From [OpenStax Microbiology](#)

However, many bacteria have evolved mechanisms to render these antibiotics useless. There are two common mechanisms by which bacteria can acquire resistance to an antibiotic:

1. Bacteria may acquire a new resistance gene as the result of a random mutation, or
2. Bacteria pick up a preexisting resistance gene passed to them from other bacteria in the environment.

This rapid increase in antibiotic resistance is a major threat to human health. Unfortunately, due to evolution, the development of resistance is inevitable, but we can change our behavior to slow the rate while simultaneously developing new antibiotics.

Mechanisms of Antibiotic Resistance

- Cellular target (e.g., the bacterial cell wall) is altered in the resistant population so that antibiotics that would normally target them are no longer able to bind properly (e.g., vancomycin).
- Antibiotics may be actively secreted out of the resistant bacterium to prevent them from reaching their target (e.g., tetracyclines).
- The resistant bacterium may block the antibiotic entry into the cytoplasm (e.g., antibiotics).
- The antibiotic may be modified or destroyed by the resistant bacterium (e.g., β -lactams).

Protocols

Finding New Antibiotics

You have isolated a collection of soil bacteria with the hopes that some of them will produce antibiotics and now it is time to test them to see if they do indeed produce antibiotics. You will be conducting an assay (aka test) to identify which of your bacteria are antibiotic producers.

Before doing this, it is critical that you make a copy of your master plate so that you have an uncontaminated sample of each of your bacteria.

Protocol 1: Replicate Your Master Plate

To keep your bacterial isolates happy and healthy, you need to feed them regularly. To do that, the first thing that you will do today is to replicate your master plate by picking up a **very small amount** of bacteria from each patch and transferring them to the same spot on a new sterile

master plate. Make sure to note the phenotype for each colony in your lab notebook (use the same colony descriptors as last week). Also be sure to note any contamination or if you have two different appearances within one patch. Use sterile technique to be sure to keep everything sterile.

Materials

- Media plates of choice
- Master plates from last week
- Square grid
- Sterile toothpicks

Protocol

- Obtain your master plate(s) and as many new sterile plates as you already have for your master(s).
- Label your new plates with the same information as last week:
 - Group Name
 - Media Type
 - Temperature
 - Plate Number
 - and mark the orientation on your plate
- Line up the square grid with your new plate.
- Using one sterile toothpick, pick up a **VERY** small amount of the patch. Just touch the patch, you don't need to see the bacteria on the toothpick and you'll still have thousands!
- Touch it to the new plate (in the same spot as the first patch) and color in the square being sure not to contaminate between two neighbors.
- Choose the temperature you wish to grow at, record your decision in your notebook, and place your plates upside down at that temperature (food side up).



Protocol 2: Spread/Patch Screen for Isolate Antibiotic Production

It is possible to make modifications to this suggested protocol (your imagination is the limit), be sure to accurately report your techniques in your lab notebook.

Theory

The spread-patch protocol assays for bioactivity in bacteria that are in close physical contact with the tester strain. In this protocol, the tester strain is spread on a plate, dried, and then the isolates are patched on top the spread. If the isolate is active against the tester strain, it should theoretically have no trouble growing on the spread. This protocol assumes that microbe-microbe interactions may induce antibiotic production and will increase our chances of identifying a producer. One challenge you may run into is that an antibiotic producer may need time to establish itself on the medium or may not be successful at invading a growing culture of susceptible bacteria.

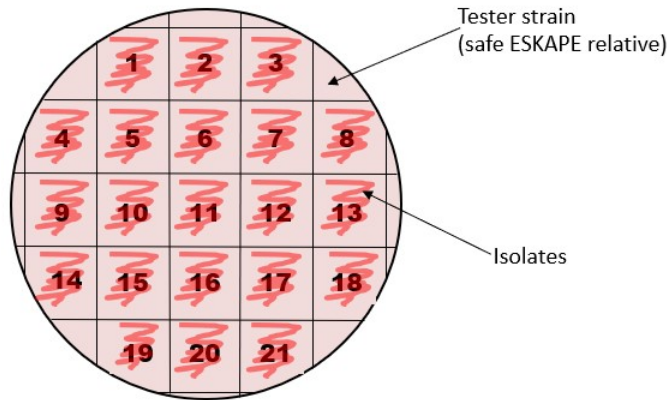


Figure 3. Spread-patch schematic. Isolates are patched (in the same grid arrangement as master plate) onto an agar plate that has been spread with the ESKAPE pathogen.

This assay is an elegant way to find antibiotic producers as they seem to appear overnight. This approach takes advantage of the fact that microbes are constantly interacting with each other in the environment. For a long time, scientists have been working to find the signals that bacteria use to communicate and identify their neighbors. Because these bacteria are so close together, they can directly compete which will hopefully result in one of your isolates producing antibiotics.

Materials

- Media plates (same as master plate)
- Liquid culture of safe ESKAPE relative
- Master plates with isolates
- Cotton swabs
- Square grid
- Sterile toothpicks

Protocol

- Obtain your master plate and the cultures containing your safe ESKAPE relative of choice. I recommend choosing at least one Gram positive and one Gram negative ESKAPE relative to test against. This will increase your chances of finding an antibiotic producer.
- Based on the number of patches you have successfully growing on your Master plate, determine how many fresh medium plates you need to test all of your strains.
- Label each plate with:
 - Group Name
 - Date
 - Media Type
 - ESKAPE relative used
 - Incubation Temperature
 - Plate Number
- Obtain a liquid culture of your safe ESKAPE relative.
- Dip your cotton swab into the liquid culture and swipe the swab along the edge of the tube to prevent dripping and remove extra liquid.
- Completely but gently (don't rip the plate) color over the surface of the plate to spread the ESKAPE pathogen over the whole surface. Turn the plate 90 degrees and recolor with the same swab completely. Finally, turn the plate an additional 45 degrees



and color for a third and final time. This will ensure that you've gotten the ESKAPE pathogen onto every surface on the plate evenly. The liquid should be absorbed into medium within minutes.

- Wait for the plate to dry 10–15 minutes.
- Place the dry plate spread with your ESKAPE relative face-up on top of the grid and align line of orientation with grid.
- Pick isolates from the master plate and patch onto safe ESKAPE relative spread arranged in the same orientation as on the master plate.
- Place in incubator at the temperature of your choice and allow them to grow for one week.



Caution

When you are finished patching your samples, please put your new plates food side up at the appropriate temperature. You should have 1-2 newly made duplicate master plates, and at least 2 antibiotic production test plates.

Stack your initial master plates from last week and put them in a plate bag with your dilution plates that have been stored from last week. Label the correctly colored tape with your group name and the date and use this to seal your bags. These plates will be stored in the fridge in case you need them next week. The used sticks should go in the appropriate container to be sterilized for re-use. Any tubes, tips, and cotton swabs that you used can be thrown in the red biohazard bin. Please put the ESKAPE pathogen tubes in the labeled area and return all reagents and tools to the place that you got them.

Using the 70% ethanol sprayers on your bench, spray down your bench space to disinfect it and wipe it clean with a paper towel

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9: Microbial Microscopy

Research Project Week 4: Microbial Microscopy

Microbes Under the Microscope

In the 17th century, Robert Hooke invented the compound microscope. In fact, Hooke coined the term "cell" because the cell walls of plant cells (specifically those in cork) reminded him of the rooms/ cells in monasteries. This magnificent new tool revolutionized the field of Biology as it allowed scientists to see microscopic organisms for the first time. Antoine Van Leeuwenhoek, inspired by Hooke's work examined, carefully illustrated, and reported on the microorganisms in water as well as those present on his teeth!

Although 21st century light microscopes are much more powerful than those from the 17th century, there are physical limitations to the amount of detail that we can observe. We can only tell apart objects that are more than $0.2\text{ }\mu\text{m}$ (micrometers, 1×10^{-6} or microns) apart. More advanced techniques such as electron microscopy open the window for observing even smaller structures such as viruses and subcellular components, but these microscopes are incredibly expensive and very difficult to use.

Bacterial Cell Morphology

Bacterial cells come in a variety of shapes and sizes (though all are incredibly small). There are three major categories of shape 1) cocci - round ball shaped cells; 2) bacilli - longer rod shaped cells; and 3) spirilli - even longer spiral shaped cells (**Figure 1**). Within these broad groupings, bacterial cells may be connected to each other in different ways depending on how they divide.

Bacterial cells come in a variety of shapes; the shape of a bacterial cell is characteristic of the species. Two of the most common shapes are cocci (spheres) and bacillus (rods). Additional shapes include spiral, stalked, and filamentous cells (Figure 1).

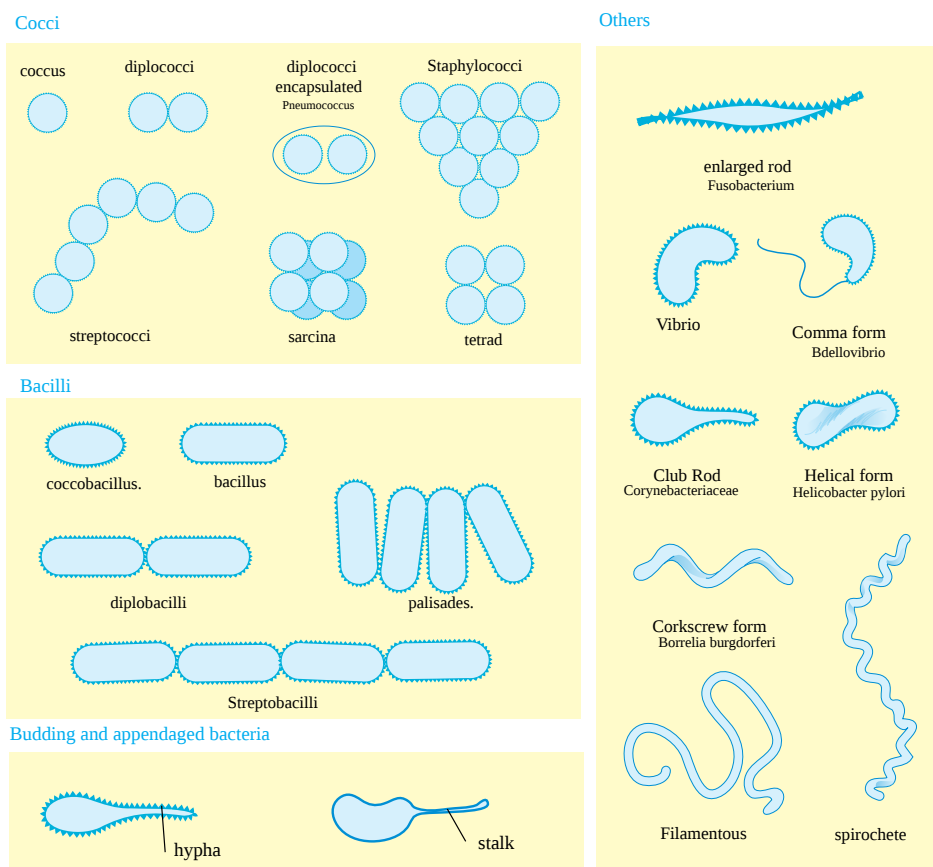


Figure 1. Examples of bacterial cell morphologies. Bacteria can range in size from $0.5\mu\text{m}$ to $5\mu\text{m}$. From [LadyofHats](#), Public domain, via Wikimedia Commons

The morphology (shape) of bacterial cells can be determined by looking at them under the microscope. However, since bacteria are translucent they are impossible to see on their own. In order to better see these bacteria, they need to be stained before microscopic analysis. Stains bind to the cellular components especially cell membranes and cell walls and thereby enhance our ability to see them under the microscope. The staining protocol can be found in **Figure 3**.

Protocols

Protocol 1: Streak Your Favorite Bacteria for Single Colonies

After observing and recording the results of last week's experiment, choose your favorite bacteria. This favorite strain will be the one that you plan to fully characterize and identify using DNA sequencing next week. If you are undecided, you can select up to three strains and then decide for sure next week.

Materials

- Media plates of choice
- Master plates from last week
- Sterile sticks

Protocol

- Obtain your master plate(s) and as many new sterile plates as the strains you want to streak.
- Label your new plates with the same information as last week:
 - Group Name
 - Date
 - Media Type
 - Temperature
 - Plate number
 - and mark orientation on your plate
- Using one sterile toothpick, pick up a VERY small amount of the patch. Just touch the patch, you don't need to see the bacteria on the toothpick and you'll still have thousands!
- Streak this stick in one line across one quarter of the plate. This is your primary streak.
- With a NEW sterile stick, start by touching the primary streak and then color the next quarter of the plate in a back and forth motion (**Figure 2**). This is your secondary streak.
- With another NEW sterile stick, start by touching the secondary streak and then color the next quarter of the plate in a back and forth motion (**Figure 2**). This is your tertiary streak.
- Finally, with another NEW sterile stick, start by touching the tertiary streak and then color the last quarter of the plate in a back and forth motion (**Figure 2**). Be careful not to overlap the primary streak as you do this. This is your final streak.
- Repeat this for each of the strains (up to 3) that you may wish to test next week.
- Choose the temperature you wish to grow at, record your decision in your notebook, and place your plates upside down at that temperature (food side up).



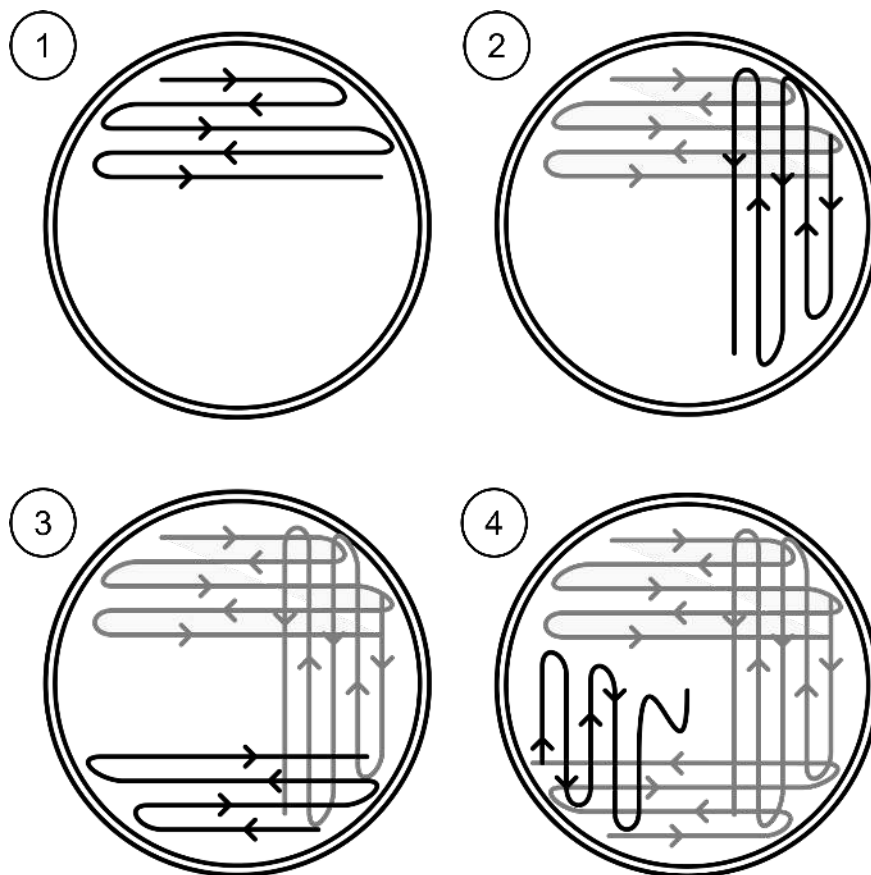


Figure 2. Streaking plate method. To isolate individual bacteria to grow single colonies, we use a quadrant streaking technique. (1) a stick is used to touch the initial colony on the patch plate and is gently brushed back and forth on one quarter of the plate. (2) a clean sterile stick is used to overlap the initial streak in the first quadrant and fill in a second quadrant rather than being used to collect more bacteria from the initial culture. (3) The process from step 2 is repeated in the third quadrant with a new sterile stick. (4) Finally step 4 pulls from zone three. From [Microbiology Manual \(Heartline\)](#) shared under a [CC BY-NC-SA 4.0](#) license and was authored, remixed, and/or curated by [Rosanna Hartline](#)

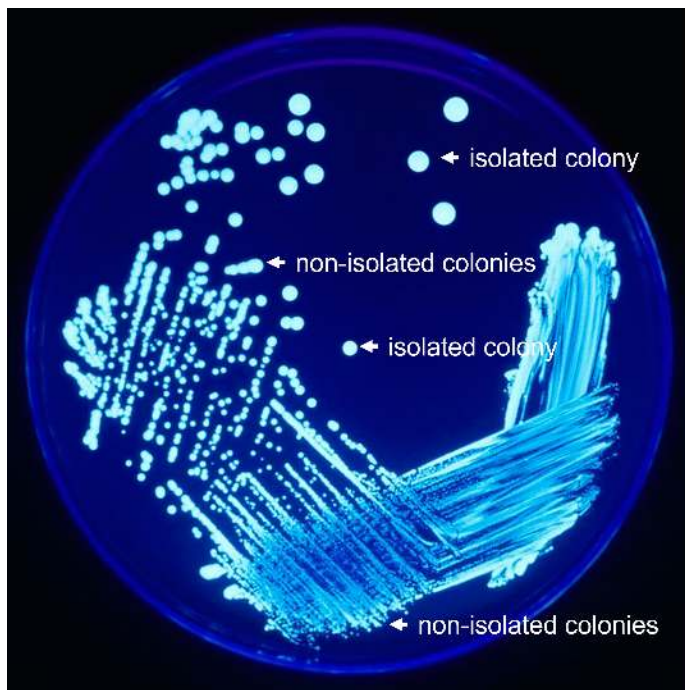


Figure 3. Streaking plate method results. This image shows a successful streak plate, where there are many isolated colonies in the third and fourth quadrants of the plate. From [Microbiology Manual \(Heartline\)](#) shared under a [CC BY-NC-SA 4.0\(opens in new window\)](#) license and was authored, remixed, and/or curated by [Rosanna Hartline](#)

Protocol 2: Repeat Your Screen of Any Interesting Strains

- After observing and recording the results of last week's experiment, identify any of your microbes that are interesting.
- If you have an antibiotic producer:
 - Retest each of these interesting microbes against the same ESKAPE Pathogen relative(s) that you tested last week. This will help you confirm the antibiotic production ability of your strain.
 - Retest them against all of the ESKAPE relatives. This will help you determine whether your antibiotic has a broad or narrow spectrum of activity (whether it can kill all bacteria or only a small subset).
 - Maybe test them under any other conditions (different medium, different temperatures, different testing protocols. The decision is yours, you have plenty of time to test things, but don't go overboard!
- For each of these tests, use the protocol from Week 3. If you do NOT have an antibiotic producer yet:
 - Retest all of your isolates against at least 4 more ESKAPE Pathogen relatives. This will hopefully help you find an antibiotic producer.
 - Maybe test them under any other conditions (different medium, different temperatures, different testing protocols. The decision is yours, you have plenty of time to test things, but don't go overboard!
 - For each of these tests, use the protocol from Week 3.

Protocol 3: Simple Stain

Before bacterial cells can be stained, they must first be attached to a glass slide in a thin layer called a smear. Some people find it helpful to draw a circle on the slide where they will put the bacteria to make them easier to find later. To spread out your bacteria, it is useful to start with a small drop of water on the slide (~2 μ L) to help move the bacteria around. As you do this, be especially careful not to transfer too much bacteria or the cells will be clumped together and difficult to distinguish (this is a common challenge for beginning microbiologists). Allow the bacterial smear to air dry completely.

Now that you have prepared a smear of bacterial cells on the slide, you need to fix the cells. Fixing has two purposes. First, it kills the cells quickly and in a way that minimizes the cells shrinking and changing shape so that you can identify the shape accurately. In this process, cellular enzymes are denatured so they cannot damage or destroy the cells after they have been fixed. In addition, fixing glues the cells to the slide so that they will not wash off during the staining process. There are several ways cells can be

fixed. One possibility is chemical fixation, which is accomplished by putting a chemical fixative such as 95% methanol on the cells. Instead, we will be using heat fixation. To heat-fix the bacteria on your slides, pass the slide through the flame of a Bunsen burner. Be sure to hold the slide carefully with a slide clamp or clothespin so that you don't burn your fingers. Your heat-fixed smear is now ready for staining.

Simple Staining

Staining procedures that use only one type of stain are called simple stains. They increase the contrast between the bacterial cells and the slide background, allowing you to see the cells and their morphology easily. Many different dyes can be used as simple stains; today we will use methylene blue. Methylene blue is a basic dye and binds to negatively charged cellular components, including the cell wall.

To stain a heat-fixed smear, hold the slide over a beaker. Using a dropper, put several drops of the dilute stain solution directly on top of the fixed bacterial smear. The circle you drew around the area of the smear will help guide you to the location of the bacteria. Let the stain sit for about a minute, then tilt the slide over the beaker and rinse the stain off using the dH₂O wash bottle. Rinse for 10 seconds or more, until the water running off the slide is clear.

Gently blot the slide dry between Kimwipes. Don't wipe the slide or even the fixed bacteria might wipe off. The simply stained bacteria are now ready for microscopic observation. To view your sample, place a coverslip over the stained sample; any residual water on the slide will help hold the coverslip in place. The purpose of the coverslip is to help prevent contamination of the microscope with any of the bacterial sample.

I. Preparing a smear:



Inoculate a few bacteria in 2µl of water in a dime-sized area on a glass slide.

Allow to dry completely.

II. Heat fix and stain:



Pass slide through a flame to heat fix.

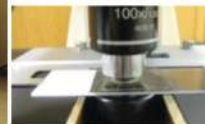


Flood slide with stain and let sit for 1 minute.



Rinse with water and allow to dry.

III. Microscopy



Place the slide on the microscope stage. Focus with the 4x objective and work up to examining the bacteria with 40x objective lens

Figure 3. Staining is required to increase the contrast between the microbes and the slide.

Materials

- Glass microscope slides
- Inoculating loop
- Clothespin
- Methylene blue
- dH₂O
- Kimwipes

- Beaker
- Lens paper

Protocol

- Draw a dime-sized circle on a clean glass slide and label the slide with the identification information of your “interesting” microbe.
- Sterilize the inoculating loop by passing it through the flame until red hot and then allow to cool.
- Use the sterile loop to pick up a drop of water. It should look like a bubble wand with bubble solution in it. Put this drop of water inside the circle on your labeled slide.
- Sterilize the inoculating loop again and use either this or a sterile stick to transfer a very small amount of bacteria from the patch on the agar plate to water drop on the glass slide.
 - Spread the bacteria out across the circle.
 - Allow the bacteria and water to dry completely.
- Heat-fix the smears by passing the slide through the flame of a Bunsen burner several times
- Stain the slide with 1–2 drops of methylene blue; let sit for about 1 minute.
- Rinse off the stain with dH₂O.
- Carefully blot slide dry between Kimwipes.
- Observe the bacterial morphology using the 40x dry objective. (Ask your instructor).
- Once you know that your slide is good, bring it up to the instructor microscope to take a picture of your bacteria using the 40x dry or 100x oil objective.
- In your lab notebook, be sure to label your drawing with the name of the sample, the total magnification, and the cellular morphology of that species.
- Repeat the simple stain procedure for each of your interesting microbial strains.

Using the Microscope Video





Discarding Materials

When you are finished streaking, retesting, and examining your bacteria under the microscope, please put your new plates food side up at the appropriate temperature. You should have >1 newly made streak plate and >4 antibiotic test plates.

Stack your master plates from last week and put them in a plate bag with the old plates. Label the correctly colored tape with your group name and the date and use this to seal your bags. There is no reason to store your antibiotic test plates, so after taking good photos of these plates, you can discard them in the biohazard plate waste tub.

The used sticks should go in the appropriate container to be sterilized for re-use. Any tubes, tips, and cotton swabs that you used can be thrown in the red biohazard bin. Please put the used ESKAPE pathogen tubes in the labeled area and return all reagents and tools to the place that you got them.

Using the 70% ethanol sprayers on your bench, spray down your bench space to disinfect it and wipe it clean with a paper towel.

References

Biology 105 Lab Manual

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10: Research Project Week 5-7- Molecular Sequence Identification

Microbial Characterization

At this point in your research project, you have isolated an antibiotic producing microbe from the soil, grown it in isolation (away from the community of microbes with which it shares the soil. It is also possible that you haven't yet found an antibiotic producer and instead have selected another cool bacteria that you wish to learn more about. Either way, we are about to change directions from looking at the growth and capacity of many different bacteria to focusing specifically on one species. So . . . where do we go from here?

We are still a long way off from marketing your potential antibiotic. Before we can use your antibiotic on patients we, the scientific community, must first produce large quantities of the molecule, ensure that it is effective in infected people, the antibiotic must not be harmful to eukaryotic/human cells, and it must continue to be active if ingested in pill form. Obviously, we won't be marketing your drug at the end of the semester! The first thing is that we need to know that this antibiotic is a **NEW** one— one that hasn't previously been discovered and is currently used.

To identify this antibiotic, we are going to begin by determining which bacterial species is producing it. The reason for this is that we know which bacteria are known to produce particular antibiotics. For example, *Acremonium chrysogenum* is known to produce cephalosporin c (a β -lactam). If an active soil isolate can be classified as *Acremonium* or a close relative, we might predict that the antibiotic is cephalosporin and we would begin our testing there.

Using Systematics to Identify Our Bacteria

Systematics is a sub-field of biology that works to classify organisms in an organized and logical fashion. The living organisms are classified in a hierarchal system where each **species** is given a name and grouped with closely related species into a larger group called the **genus** and multiple genera (plural of genus) are grouped in one **family**. This classification system reflect evolutionary relatedness meaning that two species in the same genus are be more closely related to each other than two species in different genera.

Table 1. Systematics Hierarchy. Important terminology/formatting note: The Genus and species names are always in italics, and Genus is capitalized while species is lowercase.

Systematics Hierarchy

Domain	Bacteria
Kingdom	Eubacteria
Phylum	Firmicutes
Class	Bacilli
Order	Bacillales
Family	Staphylococcaceae
Genus	<i>Staphylococcus</i>
Species	<i>aureus</i>

Historically, scientists used to classify bacteria on visible properties (cell shape, colony morphology, number or flagella, nutrient requirements, etc. However, sometimes these features are misleading and not particularly helpful in sorting such a diverse array of bacteria. Additional data, especially metabolic and genetic information is incredibly valuable in characterizing and classifying bacterial species. Using the genetic information, scientists have determined the **universal tree of life** (**Figure 1**). In our research on your antibiotic producer, we will attempt to identify the location of your species on this tree of life.

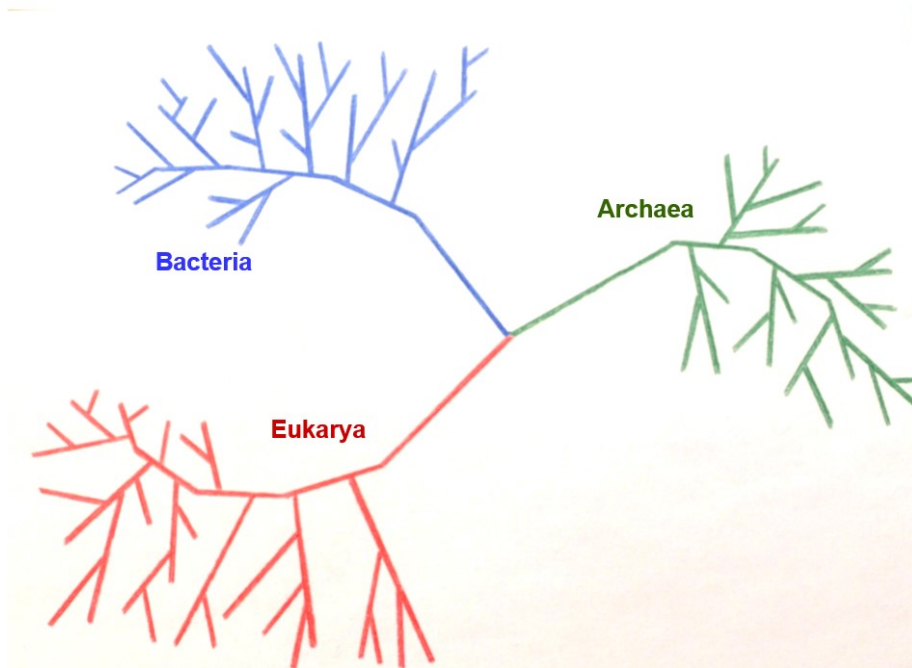


Figure 1. The universal tree of life.

With genetic (DNA sequence) information, scientists can compare the sequences of multiple organisms. Because two species evolved from a common ancestor, scientists can compare the similarities and differences of their DNA sequences to see how divergent they are. As the two new lineages evolved away from each other, their DNA sequences mutated and became less similar. The greater the difference in DNA sequence between two species, the greater the evolutionary distance between them. The more similar the two DNA sequences are, the more closely related these two species are.

Although sequencing technology has improved dramatically, it is still not efficient or cost-effective to sequence the entire genome of a new strain of bacterium to identify the species. Instead, scientists focus on a few genes possessed by all species of bacteria. The most important of these is the **16S rDNA gene**. The 16S rRNA is an absolutely essential component of the bacterial ribosome, so all bacterial species have this gene. Although many other genes could be used for this analysis, the ribosomal 16S subunit is the gold standard because it is 1) present in all cells, 2) is not too large (~1500 base pairs in the gene), 3) has regions of incredible variability as well as regions that don't change much. An additional helpful feature is that so many bacterial species have sequenced 16S rRNA genes, there are many species to compare it to.

Instead of sequencing the entire genome, a scientist typically uses a technique called PCR (polymerase chain reaction) to generate many identical copies of the 16S rDNA for the unknown species, sends this copied DNA to a sequencing company to determine the sequence of the gene, and compares that sequence with the publicly available established databases of all known bacterial 16S rDNA sequences. A "hit", or match, in the database indicates that the species is likely known. Even if there are no hits, comparing the sequence of the 16S rDNA of the potentially new species gives valuable information about the identities of its closest relatives.

Copying the DNA

Polymerase Chain Reaction

The process of DNA sequencing requires a large amount of a specific region of DNA to accurately determine the sequence. Just isolating the DNA from the bacteria does not provide a high enough concentration of DNA, so alternative methods must be used. The most common method for amplifying (aka copying) the DNA is a procedure called **polymerase chain reaction (PCR)**.

This technique uses a purified DNA Polymerase enzyme to copy small specific pieces of DNA exponentially and make millions of copies. PCR has made the field of molecular biology possible.

A PCR reaction requires a few ingredients: 1) the template (DNA being copied), 2) a thermostable DNA polymerase, 3) a pair of specific single stranded DNA primers (to identify the particular region to copy), 4) free nucleotides, and 5) a buffer. The reaction

mimics the DNA replication present in cells (**Figure 2**).

First, as in DNA replication, the **template** is dsDNA, although it can be from virtually any source and does not necessarily have to be the genome of a cell. This template must be denatured, or separated into two single strands, so that it can be copied by synthesizing the complementary strand to each single strand. While cellular DNA replication uses a helicase enzyme to break hydrogen bonds between the bases and keep the two strands separate, PCR does the same thing by heating the dsDNA template.

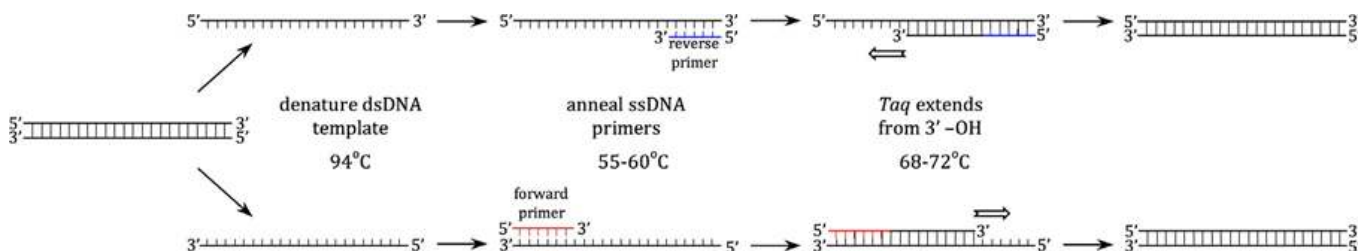


Figure 2. A single cycle of PCR. Each cycle of PCR replicates a region of the dNA template *in vitro*. First, the DNA is heated to denature the two strands. Primers anneal to the complementary regions of the template and provide the free 3' -OH from which a thermostable DNA polymerase such as *Taq* polymerase can extend the complementary strand. The result is two double stranded DNA copies of the region between the forward and reverse primers.

Second, in both types of replication a **DNA polymerase** enzyme synthesizes the new DNA strands in the 5' --> 3' direction. However, since the DNA polymerase used in PCR is exposed to the high heat necessary to denature the dsDNA template, it must be very heat stable and molecular biologists typically use the DNA Polymerase enzyme from a bacterium that lives in the very hot geyser waters of Yellowstone National Park. Since this bacterium is named *Thermus aquaticus* the enzyme is called *Taq* Polymerase.

Third, in the cell, DNA Polymerase uses an RNA **primer** made by the enzyme primase as its starting point for replication. In contrast, when we do PCR we scientists provide a pair of DNA primers that are very specific for the section of the DNA that we want to copy. The sequence of the primers is designed to only bind to the DNA right around the gene being copied. As a result, *Taq* polymerase only copies the region between the pair of primers. It is important to note that to copy a region of DNA via PCR, a scientist must know the sequence of at least a small section of the DNA surrounding that target region.

The final two ingredients of PCR are the nucleotides that get added by the enzyme and a buffer to maintain the appropriate pH and concentration of salts and cofactors. Note that the other pieces required for DNA replication in the cell are not required. No primase (we provide the primer instead), no DNA Pol I (there is no RNA to remove), no topoisomerase (the DNA being copied is short enough to not get tangled), no helicase (the heating separates the two strands instead).

One additional important difference between *in vivo* DNA replication and PCR is that in PCR we only want to copy a small region of the DNA many many many times rather than just once in DNA replication. This process, called **amplification**, is done by cycling the temperature pattern of the reaction repeatedly. There are three different temperatures used for PCR and each plays a critical role in the copying process. First, the temperature is raised to near boiling (typically about 94°C) to

separate the two strands of the DNA template. Second, the temperature is lowered to approximately 55–60°C so that the primers can stick to the complementary sequences in the template. Third,

the temperature is raised slightly to 68–72°C and the polymerase synthesizes the complementary strands. This cycle of three temperatures is repeated multiple (about 25–40) times. With each cycle, the quantity of target DNA is theoretically doubled, so after n cycles with a starting point of x copies of template, there will be $x2^n$ copies of the target sequence (**Figure 3**).

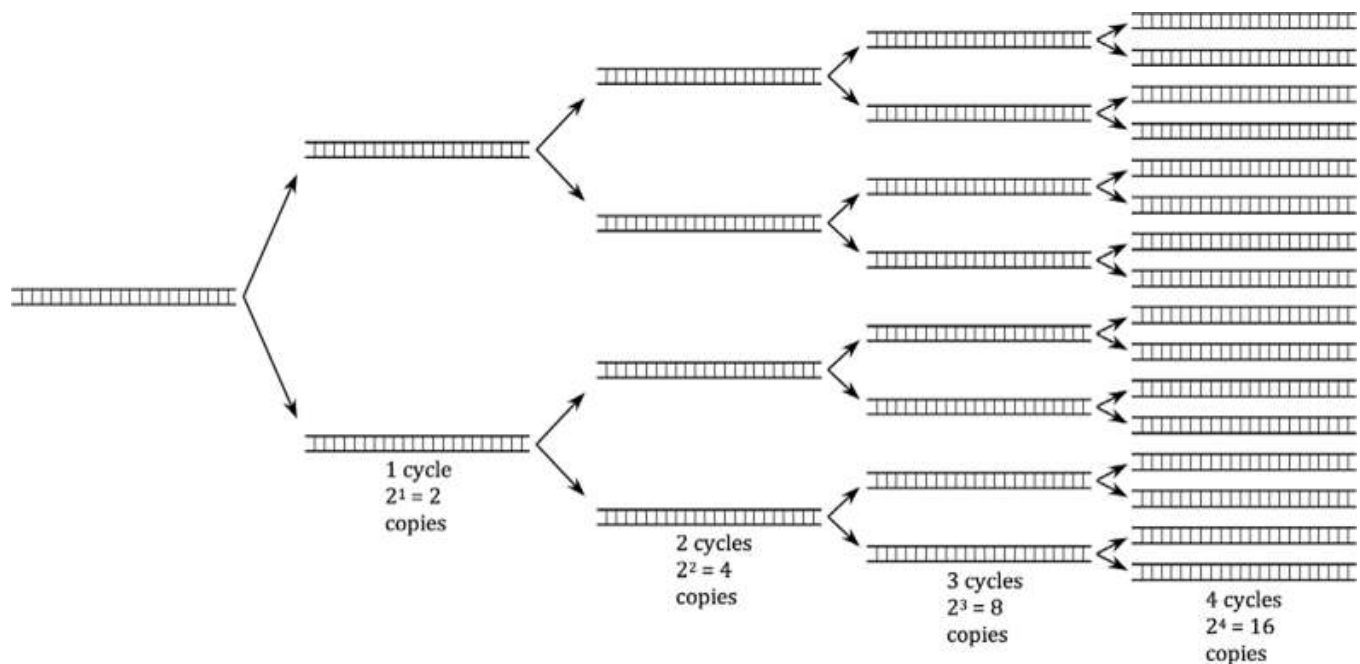


Figure 3. Amplification by PCR. Each cycle of PCR doubles the number of copies of the DNA between the forward and reverse primers. After n cycles, there are 2^n molecules, where x is the initial number of molecules present.

16S rDNA PCR

Although the 16S rDNA gene is present in all bacteria, the sequences are slightly different in each organism as the bacteria have evolved away from each other. Some parts of the 16S rDNA gene have evolved slower than others and most species are nearly identical sequences at these regions. Other regions are divergent, meaning that mutations have accumulated at a higher rate and therefore these regions are less similar among species. Because we want to use the sequence of the 16S rDNA as a unique identifier of a species, these divergent regions are the ones whose sequence will be most helpful to us. Therefore, we will design primers to match the sequences that are unchanged and use those primers to copy the section that is variable. That will make the primers "universal," so that they work on almost all bacteria. The pair of primers we will use for this experiment is:

oNS018: 5'-ACGCGTCGACAGAGTTTGATCCTGGCT-3'

oNS019: 5'-CGCGGATCCGCTACCTTGTACGACTT-3'

These primers amplify the majority of the 16S rDNA gene in most species of bacteria. The exact size of the PCR may vary slightly depending on the species, but should be approximately 1500 bp.

Separating the DNA by Size

Gel Electrophoresis

After amplifying a region of DNA by PCR, we need verify the size of the amplified fragment and estimate the quantity produced by the PCR. The most common way to do this is by gel electrophoresis, or "running a gel."

Gel electrophoresis is used to separate molecules based on their size and their charge as they move through a gel matrix. DNA has a net negative charge due to the phosphate groups in its backbone, so when an electric current is applied to a gel, DNA travels through the gel toward the positive electrode (anode). **Agarose** is a linear polymer extracted from seaweed that is composed of galactose sugars (**Figure 4**) and is typically used to separate DNA molecules. When melted agarose cools (or gels) it forms a three-dimensional mesh, like Jell-O. DNA moves through agarose at a rate inversely proportional the number of base pairs. Longer molecules of DNA migrate more slowly since they cannot worm their way through the pores of the gel as well as shorter DNA molecules. The rate at which the DNA travels depends also on the conditions of the gel itself (% agarose in the gel, amount of current applied). If a gel is run for a longer period of time, the DNA in that gel will migrate a greater distance.

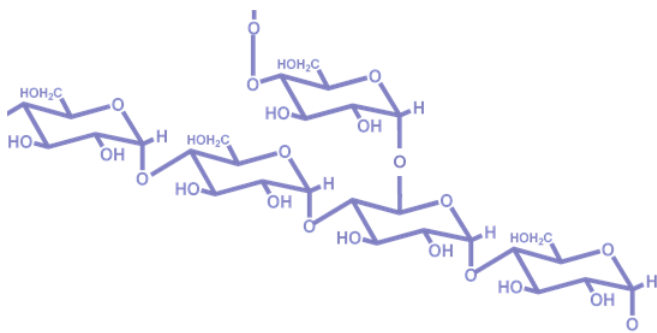


Figure 4. Structure of agarose. Agarose is a polymer of sugar molecules that connects to form a 3-dimensional mesh gel. Created by [user:ideru](#), [CC BY-SA 3.0](#), via Wikimedia Commons

To run a gel, a sample of DNA is first mixed with a special **loading dye** and then loaded in a well or small hole at one end of the gel. This dye has two purposes. First, the dye contains glycerol which makes it heavier than the water buffer for the gel, so the sample sinks to the bottom rather than floating away. Second, it makes it colored so that it is easier to see and manipulate. The color also remains visible while the gel is running so that we can tell when the gel has run for long enough. If a gel is run too long, the DNA molecules will continue to migrate off the end of the gel and into the buffer.

DNA can be visualized in a gel by staining it with a fluorescent DNA-binding molecule. These DNA intercalate, or insert themselves, between the bases of double stranded DNA (Figure 5). When exposed to UV light, the dye fluoresces, which allow us to see where the DNA is located. **Always use caution when working with these dyes because anything that binds to DNA has the potential of binding to YOUR DNA and may cause mutations and cancer.** We will use the dye **Gel Red**, which stains the DNA well, but is relatively safe (much safer than earlier dyes). Despite its relative safety, scientists must also treat GelRed with caution because it is a potential mutagen and carcinogen. **ALWAYS WEAR GLOVES** when working with DNA stains and keep the work area clean. We will dispose of anything that came in contact with GelRed as hazardous (non-biological waste).

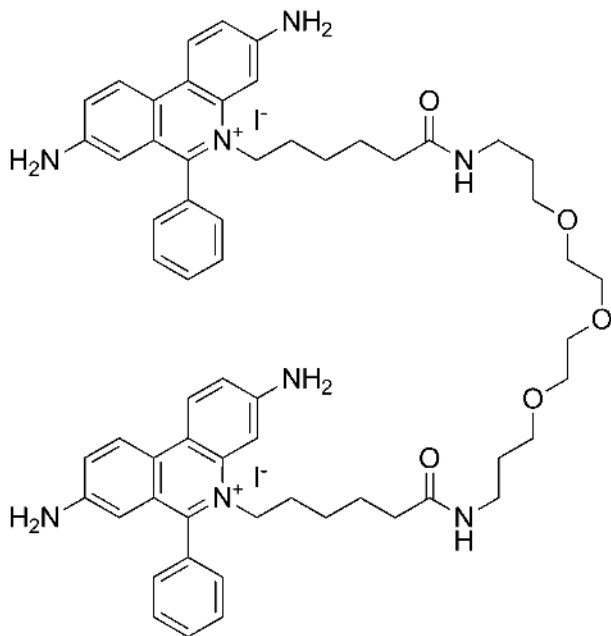


Figure 5. Chemical structures of the DNA stain GelRed. The flat planar structure allows it to intercalate between the bases of the DNA double helix. From: [Cacycle](#), Public domain, via Wikimedia Commons

Often a DNA sample loaded on a gel contains a mixture of different sized DNA molecules. All the DNA molecules of a given size, say 1 kb, migrate through the gel at the same rate. Therefore, when the current is turned off, they will be at the same location and form a **band** (stripe) of DNA in the gel. DNA molecules of a different size, say 2 kb, migrate through the gel at the same rate as

each other but at a different rate from the 1 kb molecules. Remember that larger pieces of DNA migrate more slowly through the gel matrix than smaller pieces of DNA. Thus, the 1 kb band will be farther from the well than the 2 kb band.

To estimate the size of a band in a gel, we use a molecular weight standard called a **DNA ladder** (*Figure 6*). A ladder contains multiple bands of DNA fragments with known sizes. A scientist can compare the position of the DNA band to the ladder “eyeball” the size of the bands in a gel by looking where they are with respect to the different bands of the ladder. This ladder helps scientists measure the size of their DNA molecule.

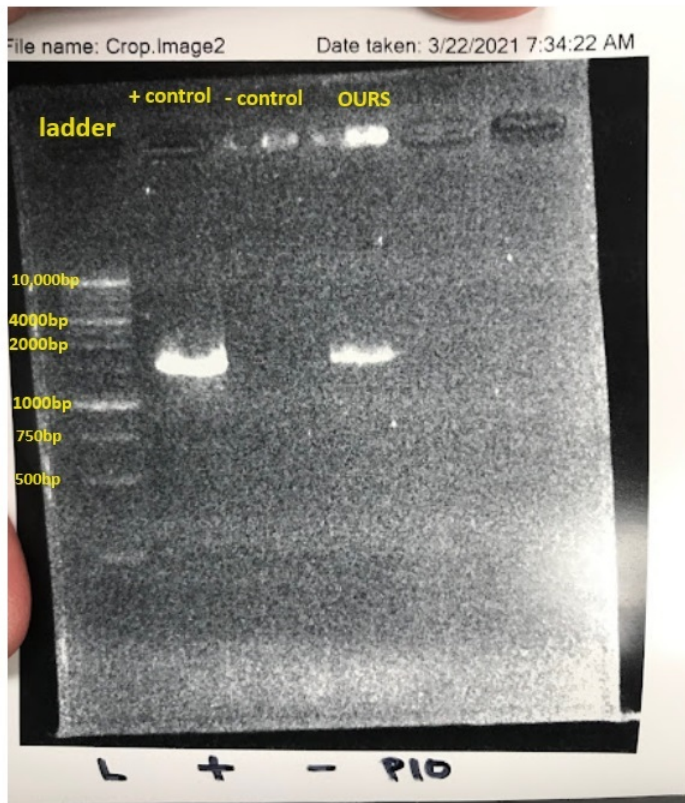


Figure 6. Agarose gel. The wells where the DNA samples are loaded are visible near the top of the gel. The lane on the far left is the DNA ladder, which is used as a standard to compare the sizes of the bands in the two sample lanes. Larger fragments move more slowly from the wells and are closer to the top of the gel. Smaller fragments move more quickly and so are closer to the bottom of the gel. Samples are loaded into the first lane (ladder) second lane (positive control), third lane (negative control) and fourth lane (experimental sample).

DNA Sequencing

A variety of methods can be used to determine the sequence of a fragment of DNA; the method chosen depends on a variety of factors including type and number of samples, desired length of the sequencing read, etc. You will prepare your samples and we will mail them to a company for Sanger DNA sequencing. Sanger sequencing works by the selective incorporation of dideoxynucleotides (ddNTPs) by DNA polymerase. These ddNTPs are chain-terminating and marked with fluorescent tags to distinguish one base from the next. This allows all four bases to be terminators in the same reaction. The reaction then can be run through a capillary tube and the band type and order can be identified to determine the sequence of the DNA sequence. This information is recorded by the company in a trace chromatogram and the sequence data can be “read” off the chromatogram by reading the peaks in order (*Figure 7*).



Figure 7. A portion of a trace chromatogram from an unknown sequence. Each peak corresponds to a base in that position in the sequence of the 16S rDNA.

Protocols

Protocol 1: Extract Genomic DNA

Each group will extract the genomic DNA of one of your antibiotic producer. In consultation with your instructor you may also be extracting the DNA of one of the positive or negative control samples and/or an additional bacterial strain. Confirm your plan with your instructor before you begin.

Materials

- Plate cultures of your antibiotic producer
- Sterile dH₂O
- Sterile 1.5 mL microfuge tubes
- Pipettes and tips

Protocol

- Label your 1.5 mL microfuge tubes containing 200 µL of sterile water.
- Using a micropipette tip, carefully touch the colony on the streak plate. A small, visible dab of cell that fills the end of the pipette tip will provide enough DNA template for the reaction.
- Dip pipette tip into a microfuge tube the dH₂O and gently swirl for 5–10 seconds to dislodge cells.
- Vortex the tube for 30 seconds to suspend the colony completely in dH₂O.
- Lyse these cells by heating them at 95°C for 10 minutes.
- Remove the lysed cells and store on ice until needed for the PCR.

Protocol 2: Polymerase Chain Reaction

Material

- Genomic DNA from Protocol 1
- PCR bead tubes (2-3 depending on number of samples)
- Primer tubes (2-3 depending on number of samples)
- Pipettes and tips

Protocol

- Obtain your PCR bead tubes, which contain Taq polymerase (heat-resistant enzyme) and other necessary reagents.
- Label your tiny PCR bead tubes. This is critical—you need to tell yours apart from everyone else's and know what is in the next week.
- Carefully and accurately add 15 µL of PCR primer mix into the PCR bead tube. The bead will start to dissolve and slightly effervesce.
- Carefully and accurately add 10 µL of your lysed resuspended cells to this PCR bead tube. This will bring the volume of the reaction up to 25 µL.
- Cap the PCR tube, vortex VERY gently.
- Spin the tubes in a microfuge for a few seconds to collect all liquid at the bottom of the tube.
- Transfer the tubes to the thermal cycler (PCR machine).
- Select the appropriate program to start cycling (~2 hours).
- Once cycling is complete, remove tubes and freeze until next week when you will run your agarose gel and prepare your sample for DNA sequencing. **Be sure that your tube(s) are clearly labeled so that anyone can identify their contents.**

Table 1. The conditions for PCR amplification.

Cycles	Temperature	Time	Purpose
1x	94°C	10 minutes	Breaking open cells/ denaturing DNA
36x	94°C 58°C 72°C	30 seconds 30 seconds 1.5 minutes	Denaturation Annealing Elongation
1x	72°C	10 minutes	Final elongation (finish up any incomplete DNA pieces)
1x	4°C	forever	Keep the samples cold until needed

PCR Set Up Video



Discarding Materials

When you are finished running the PCR of your samples, please put these PCRs into the freezer rack provided by your instructor.

While your PCR is running, discard the old plates from your experiment. You should put any old plates that you no longer need in the biohazard plates tub these plates would include your serial dilution plates, the initial master plate, and antibiotic test plates. Be sure that you have good pictures in your lab notebook of any plate you discard. In case your PCR does not work, be sure to keep your streak plate in the plate bag stored in the fridge.

Any used sticks should go in the appropriate container to be sterilized for re-use. Any tubes, tips, and cotton swabs that you used can be thrown in the red biohazard bin. Please put any used ESKAPE pathogen tubes or plates in the labeled area and return all reagents and tools to the place that you got them.

Using the 70% ethanol sprayers on your bench, spray down your bench space to disinfect it and wipe it clean with a paper towel.

Protocol 3: Agarose Gel Electrophoresis

You will need to run a **small** sample of each of the PCR reactions, including both controls, on a gel. **Be sure to save most of the PCR from your favorite bacteria for sequencing. If you put it all on the gel, you won't have enough to sequence.** Every gel

must have at least 1 lane containing the DNA ladder. Plan which samples will go in each well of your gel. I suggest drawing a picture, so that you know what is where for certain! Your PCR product(s) from last week will have been stored in the freezer and will be returned to you.

Materials

- PCR Product(s)
- 25 mL of 1% Agarose
- GelRed DNA stain (10,000x GelRed)
- 1x running buffer [Tris-borate-EDTA (TBE) or Tris-acetate_EDTA (TAE)]
- 6x Loading dye
- DNA ladder
- Ice bucket with ice

Protocol

- Put a gel tray into the pouring tray with the shaded end where the comb will go.
- Using gloves, measure 25 μ L of GelRed stain and have it prepared.
- Obtain a tube of 1% agarose from the hot water bath and pour it quickly into the gel tray. Each of these tubes contain 25 mL, so you don't need to measure them. **NOTE: the agarose hardens very quickly, so work fast and be prepared before you remove the agarose from the hot water bath.**
- Add the 25 μ L of Gel Red to your agarose in the tray, mixing it quickly with your pipette being sure to finish before it hardens, but don't make bubbles.
 - If there are any bubbles, use a clean pipet tip to pop or move them up onto the sides of the tray.
- Allow the gel to cool at room temperature until it solidifies (~5 min).
 - Be careful not to bump the gel tray or table until it is completely solid or the gel will have waves that can affect how well the samples run.
 - The gel will appear opaque when it is solid.
- Transfer 5 μ L of each of your PCR products to a separate piece of parafilm. Label them so you know which is which.
 - Keep the original PCR product on ice until later. **DO NOT USE IT ALL.** If you make a mistake, ask for help!
- Transfer 5 μ L of the DNA Ladder to a different piece of parafilm. Label this one too.
- Add 1 μ L of 6x loading dye to each of your samples **on the parafilm** and mix well by carefully pipetting up and down.
- After the gel is completely cool, remove the gel tray using gloves and insert the gel tray and gel in the gel box.
 - **Be sure the end of the gel with the wells is connected to the negative electrode.** This is VERY important as the DNA is negatively charged and will migrate to the positive electrode. If you run it backwards the DNA will run into the buffer and your DNA will be gone.
- Pour in enough 1x running buffer so that it covers the surface of the gel and fills the chambers on either end of the gel box up to the faint line.
- Carefully remove the comb by slowly lifting straight up.
- Load 6 μ L of the DNA ladder in the gel, as planned.
- Load 6 μ L of each of the controls.
- Load 6 μ L of your PCR sample with loading dye.
- Close the lid of the gel box and plug in the gel box.
- Turn on the power supply and set the voltage to 120 V.
- Allow the gel to run until the loading dye is near the end of the gel, 20–40 min.
- When the gel has run long enough, turn off the power supply and disassemble the gel box
 - The 1x running buffer should be collected to use again.
- Carefully place gel into a carrying box.
- With the assistance of your professor, photograph your gel under UV light.

- Record the photograph of the gel in your lab notebook and be sure to label the contents of each lane as well as the size of all bands on the gel.

Protocol 4: Purify Your PCR product

While your gel is running, you will prepare your PCR sample for DNA sequencing. Although most of the DNA in your PCR reaction will be the product that you are interested in, there will also be some of the original template as well as leftover primers and dNTPs. All of these will get in the way of the DNA sequencing reaction, and need to be removed before sequencing. To do this, we will use an enzymatic method to specifically chew up and destroy single stranded DNA (primers) and individual nucleotides (dNTPs). These enzymes are active at 37°C. When it has completed its activity, we will denature it at 80°C so that it will no longer be active.

Materials

- PCR Project
- 1.5 mL microfuge tube
- Ice bucket with ice
- ExoSAP-IT reagent
- PCR machine or heat blocks

Protocol

- Label your 1.5 mL tube.
- Working with your tubes on ice, transfer 10 µL of your PCR product to a new 1.5 mL tube.
- Add 4 µL of ExoSAP-IT reagent to your sample **KEEP ExoSAP-IT ON ICE!**
- Cap the PCR tube, vortex VERY gently, and then spin in a microfuge for a few seconds to collect all liquid at the bottom of the tube.
- Return the ExoSAP-IT reagent to your instructor.
- Transfer the tube to the heat block set at 37°C and leave them there for 15 minutes.
- Transfer the tube to the other heat block set at 80°C for another 15 minutes.
- Once cycling is complete, take the tube to your instructor to send for sequencing.

If the heat blocks are not available, we will use the PCR machine instead. Follow these directions.

- Label a 0.2 mL PCR tube.
- Transfer your PCR + EXOSAP-IT to 0.2 mL tubes and gently mix.
- Place these tubes in the PCR machine.
- Select the appropriate program to start cycling (30 min). The cycle program is listed below in Table 2.
- Once cycling is complete, take the tube to your instructor to send for sequencing.

Table 2. The conditions for PCR clean-up.

Temperature	Time	Purpose
37°C	15 minutes	Enzymes hydrolyze primers and degrade dNTPs
80°C	15 minutes	Enzymes are denatured

Protocol 5: Submit Sample for Sequencing

We will use a commercial company to sequence our 16S rDNA genes for us. To do this, we will need to package up each of the samples and the sequencing primer to prepare them all for sequencing.

Materials

Materials

- ExoSAP-IT treated PCR Product
- Bar codes
- Sequencing spreadsheet

Protocol

- Once our PCR product has been purified, take it to your instructor.
- Obtain one of the bar codes from your instructor and carefully place it around your tube.
- Fill in the sequencing spreadsheet with the appropriate information associated with your bar code.
- Give your sequencing tube to your instructor for submission.

Protocol 6: Housekeeping and Organization

It is critical as scientists that we carefully and accurately record and organize all of the products of our research. We will store your PCR samples until we get a good DNA sequence result back on your strain. We will also store your bacterial strain so that we can do additional research on it. Before leaving lab today, ensure that all of your materials are taken care of.

Materials

- ExoSAP-IT treated PCR Product
- Original PCR Product
- Master plates
- Individual isolates
- Sterile agar plates

Protocol

Clean up and organize all your material

- Fully and carefully label your original (dirty) PCR product. Make sure that it is clear that this is the “dirty” one.
- Give this PCR product tube to your instructor for safe keeping.
- Look through all of the lab plates and identify all of the plates that belong to your group.
- Using the most recent version of your master plate or individual isolate, restreak your antibiotic producer (or interesting strain) for individual colonies. **Ensure that this plate is VERY well labeled.**
 - Group Name
 - Date
 - Media Type
 - Incubation Temperature
 - Species name (genus and species)
 - List of ESKAPE relatives inhibited by this strain
- Give your newly streaked plate to your instructor.
- Dispose of all other plates in the biohazard bin.

Protocol 7: DNA Sequence Analysis

The portion of the genome that we submitted for DNA sequencing is the 16S rDNA gene which encodes one of the ribosomal RNAs. Since this particular gene is related in all living organisms, it makes this gene a particularly powerful way to identify,

characterize, and classify an organism. Organisms that are more closely related share more DNA base pairs in this gene than organisms that are distantly related. We can therefore use this gene as a DNA fingerprint to identify your unknown bacterial isolate.

However, each rDNA gene in bacteria is ~1500 base pairs long and it would be virtually impossible to read through the bases in your sequence and then compare them by hand to all other sequenced bacterial strains. Instead, we will use an online database (GenBank) and search algorithm (BLAST) to help to identify your microbe and its closely related species. BLAST or (Basic Local Alignment Search Tool) is a bioinformatics tool that allows us to navigate through huge databases and compare sequences with the collected library of published or submitted sequences (Altschul et al., 1990).

Materials

- 16S rDNA gene sequence file
- Personal Computer (or lab computer)
- BLAST online program at NCBI

Protocol

- Obtain your sequence files.
 - The sequence files for the whole class will be uploaded on Canvas
 - Locate the files that correspond to your unknown sample and the relevant controls. A description of the file names will be posted on the class website; if you have trouble determining which files are yours, contact your professor immediately.
 - Each sequence file is sent in different formats:
 - The .seq file is the sequence itself—the As, Ts, Gs, and Cs that were read by the sequencing machine. This is the file you will primarily work with.
 - The .ab1 file is the trace file. When the sequencing machine is gathering sequencing data, it appears as multiple peaks of different colors, which the machine reads and interprets as the As, Ts, Gs, and Cs that are in the .seq file. This is a great place to look quickly and see how your read "looks." Tall distinct peaks are a good thing, low or overlapping ones are not.
 - Some sequencing companies also send a .pdf file. The .pdf file has both pieces of information overlapped.
 - **Locate your unknown sequence files and download them.** Be sure to download all of the file types.
- Copy and paste the sequence information from the .seq file into your lab notebook along with any additional relevant information supplied by the sequencing company.
- **Analyze your unknown sequence** for the length and quality of the sequences.
 - Open the .seq file that you wish to examine.
 - **First** determine the length of your sequence
 - A good sequencing run should give you more than 500 base pairs of information.
 - An ok sequencing run would give you greater than 300 base pairs.
 - A bad/failed sequencing run will typically be less than 300 base pairs. This suggests that you did not have enough DNA in the sample or it was a mixture of two sequences. You will likely need to redo the experiment.
 - Second determine the quality of your sequences.
 - If the sequencer cannot determine the identity of a base, it will list an N for that position of the sequence.
 - The very beginning and end of a fragment of DNA cannot be decoded by sequencing, and so your sequence should start and end with a series of Ns. (Do not worry, this is normal and expected.)
 - Typically the first 60 or so bases will contain a large proportion of Ns.
 - After the first ~60 bases the sequence should consist of about 400–1000 bases of As, Gs, Ts, and Cs with ideally no Ns.
 - If the sequence you are examining contains more than 10 Ns between bases 60 and 500 the sequence is not considered to be of high quality.
 - A sequence with a few Ns may still be interpretable, but if there are too many (more than 30), you will likely have difficulty identifying the sequence.

- Record the sequence quality in your notebook.
- BLAST your unknown and control sequences.
 - **Open the NCBI homepage** (National Center for Biotechnology Information) homepage at www.ncbi.nlm.nih.gov.
 - NCBI is the primary site for molecular biology information. It is a part of the National Library of Medicine, as part of the National Institutes of Health. NCBI houses many important resources, including PubMed, the premier citation index for biomedical literature.
 - **Click on BLAST** under the list of popular resources on the right side of the NCBI landing page.
 - The Basic Local Alignment Search Tool (BLAST) is the most important way to search for DNA and protein sequences within a large collection of databases.
 - **Select nucleotide BLAST** under the Basic BLAST subheading.
 - **Paste your sequence into the search box.** At the top of the Standard Nucleotide BLAST page, you will see a section entitled Enter Query Sequence. In this section is a box labeled Enter accession number(s), gi(s), or FASTA sequence(s).
 - Copy and paste the sequence you wish to BLAST from the .seq file into this box
 - Select your entire sequence for BLAST analysis because BLAST will automatically remove the series of Ns at the beginning at and of your sequence (copy paste is your friend).
 - You can enter the sequence even if there are some Ns in this central sequence and the sequence is not of high quality.
 - **Set search parameters.** In the next section, entitled Choose Search Set, you will see a set of radio buttons and a pull down menu labeled Database.
- On the pull down menu and/or selection circles, select 16S ribosomal DNA sequences (Bacteria and Archaea).
 - Leave all other options blank or set to their default value.
- **Run BLAST.** Click on the blue button at the bottom of the page labeled BLAST.
 - You will be directed to a job processing page that will automatically update itself every few seconds until the search is complete.
 - Be patient; depending on network traffic this may take only a few seconds or several minutes.
- Analyze your unknown BLAST results.
 - At the top of the BLAST results page, you will see a summary of the basic Query information followed by a Graphic Summary that contains a row of black, blue, green, purple, and red boxes with some red and/or other colored lines underneath.
 - We will not use the Graphic Summary for this analysis, so you can collapse this section by clicking on the (–) button to the left of the Graphic Summary section heading.
 - The Descriptions section gives a list of all the “hits” from your BLAST search. These are the species and strains for which there was significant sequence identity with your query sequence. The list is often quite lengthy!
- **Identify your Microbe's relatives!** Scan down the Description column of this section, noticing the genus and species names for the top hits. Frequently the top several hits will be different species in the same genus.
 - This makes sense because two species that are closely related enough to be placed in the same genus likely also have very similar rDNA sequences to each other and therefore the same degree of homology with your query sequence.
 - **Analyze the quality of your hits.** *The lower the E value, the stronger the match.*
 - Notice also the values in the Max score column. *The higher the Max score, the better the match.*
 - After the Descriptions section is the Alignments section. This section shows an alignment of your query sequence with each of the hits listed in the descriptions section.
- **Analyze the first alignment.** You will see two sequences aligned: your query sequence on top and the sequence of the best hit below it, labeled Sbjct. The alignment will likely be multiple rows long, so each row will have the Query on top and Sbjct below.
 - A vertical line between two bases means there is an identical match between the Query and Sbjct at that position. No line means there is a mismatch. A horizontal dash in the sequence means that there is an insertion/deletion of a nucleotide.
 - Notice how many mismatches there are in the alignment. A perfect hit will have none, but a very close hit may have a few.

- Notice also what the mismatches are. If there is an N in your query sequence, then the mismatch may not “count” because an inability to determine that particular base in your sequence does not necessarily mean it doesn’t match with the Sbjct sequence.
- BLAST usually automatically removes the many Ns at the beginning and end of the sequence, but there may be a few dispersed Ns at the end that weren’t removed automatically.
 - Optional: open the .ab1 file to examine the sequence trace file for your sequence. Find the N that shows a mismatch in the sequence alignment you are analyzing. Occasionally the automated sequencer is not able to determine the identity of a base but you can if you examine the trace by eye. You may need to use the y slider on the right side of the window to increase the amplitude of the peaks. Based on the color of the peak, can you determine with high confidence the identity of the peak? If there are two peaks overlapping, there may be contaminating DNA and no identification is possible. If you need assistance interpreting the sequence trace, consult your instructor.
 - If you can determine with high confidence the identity of the peak that the automated sequencer called an N, then you may edit your sequence to exchange that N for the correct nucleotide. **Be sure you save the original and edited sequences as separate files.**
- Analyze the alignments the best several of the hits. Pay special attention to the 4–5 hits at the top of the list.
- Be sure you record these results and observations in your notebook.
- Make a figure and table for your notebook (and final project)
 - One figure of your notebook should be one or more alignments between your unknown bacterial sequence and the best BLAST hit(s).
 - Copy the alignment from the BLAST results page into Word.
 - Format the alignment appropriately to keep the spacing and information correct.
 - Use a monospace font (Courier) for this figure rather than a variable-width font such as Cambria or Times. If you do not, the spacing will be off and the vertical lines won't be in the right places.
 - Make the font type smaller (e.g. 8 or 10, so that a whole line will fit on one page)
 - At minimum you should have a figure with the alignment of the top BLAST hit. For your final project, you will evaluate the strength of the match based on how well the two sequences align.
 - If you wish to discuss multiple hits in your final project—for example if the top several BLAST hits are of similar strength be sure to include alignments for these too. They can be parts (a), (b), (c), etc. of the same figure.
 - When referring to specific mismatches, use the position number within the query sequence. For ease in determining this, the position numbers of the first and last base of each row are located next to the Query and Sbjct labels at the beginning and again at the end of the rows.

Sequencing Analysis Instruction Video



The thumbnail for the video shows a QR code on the left and a screenshot of a BLAST alignment on the right. The alignment is titled "Sequencing Analysis" and shows a query sequence (a Sullivan) aligned with a subject sequence. The alignment is displayed in a monospace font. Below the alignment, there is a YouTube logo and the text "Watch on YouTube". At the bottom of the thumbnail, it says "It is 1189 characters long, which is great."

Discarding Material

You are finished and have the sequence identity of your bacteria-congratulations!

It is time to completely clean up all of your laboratory material. If your sequencing is successful, you can discard all of your DNA and PCR samples in the biohazard trash. You can also discard your plates in the biohazard plate bin. The only plate that you need to save is the final streak plate. Please be sure that this final streak plate is labeled well and give it to your instructor.

Any used sticks should go in the appropriate container to be sterilized for re-use. Any tubes, tips, and cotton swabs that you used can be thrown in the red biohazard bin. Please put any used ESKAPE pathogen tubes or plates in the labeled area and return all reagents and tools to the place that you go them.

Using the 70% ethanol sprayers on your bench, spray down your bench, spray down your bench space to disinfect it and wipe it clean with a paper towel.

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11: Using the Microscope

Learning Objectives

Content Skills

- Recognize and give the function of the parts of a compound microscope

Physical Skills:

- Properly care for a compound microscope
- Correctly use a compound microscope

We use a microscope to visualize objects that are too small to see with the naked eye. This is especially critical in Biology as all cells are quite small and require a microscope for visualization.

Parts of the Compound Light Microscope

Use Figure 1 to identify the various parts of your microscope. Read each description below, find them on your microscope and on the lab handout and manipulate the parts only where indicated. Before you start, make sure that the shortest objective is in the light path.

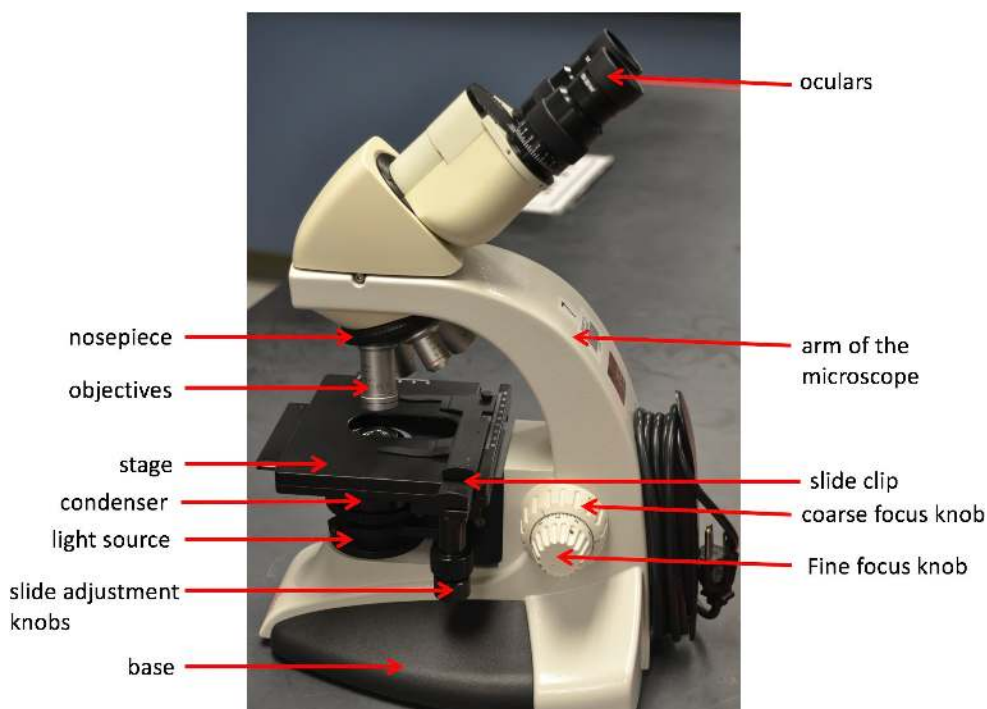


Figure 1. Parts of the Compound Microscope.

- **Light source.** The compound microscope uses transmitted light to illuminate a transparent (or super thin) sample mounted on a glass slide. Find the light source/illuminator, the off/on switch, and the rheostat which can vary the light intensity so that it is not too bright.
- **Condenser.** A condenser is composed of a *condenser lens* and *iris diaphragm* to focus the light on the specimen so that it is equally and fully illuminated. The lever for the iris diaphragm of the condenser is used to open and close the condenser to increase or decrease the area illuminated and thereby light intensity.

- **Stage.** Your slide containing a sample is placed on a mechanical stage over a central hole. Position a slide on the stage by releasing the tension in the spring loaded moveable arm and using this slide clip to hold your slide in place. There are two knobs to the right or left of the stage: one to move the specimen forward or backward and one to move it side to side.
- **Focusing knobs.** The coarse focus knob is for use with the low power objectives ONLY whereas the fine focus knob is critical for focusing especially with higher power objectives.
- **Objectives.** Our microscopes have four different objectives 4 objectives: a 4x (scanning), 10x (low power), 40x (high dry) and 100x (oil immersion). Note that they are labeled and color coded for ease of use. The other number written on the objective is the numerical aperture (NA). The higher the numerical aperture, the greater the resolving power.
- **Ocular.** The magnifying lens that you look into is called the ocular. Our microscopes have two 10x oculars (and are therefore stereomicroscopes). Since each objective has a different magnifying power, the total magnification is calculated by multiplying the magnifying power of the ocular by that of the objective.

Importantly, the objectives are **parfocal** meaning that once one objective has been focus, you can rotate another one into place without losing the focus. You will likely need to very finely adjust the fine focus, but it specimen will be very close to being in focus. Also, the center of the field of view will stay the same as you move from one objective to the next.

These features will allow you to generally focus at the low power (4x) where focusing is MUCH easier and then move to a higher power and only have minimal adjustments.

Using the Microscope

Initial set up

- Carry your microscope to and from your lab bench by grasping the arm with your dominant hand and supporting the base with your other hand. Make sure to always keep the microscope upright and *do not try to carry anything else at the same time.*
- Remove the dust cover and, if needed, clean the lenses with a piece of lens paper. Never use anything else to clean your microscope as it could scratch and damage your microscope.
- Unwind and plug in power cord
- Turn on the light source – switch on right side of microscope
- Dial or Slider on right side of microscope will adjust the light intensity, depending on the sample and magnification used.
- Adjust the ocular focus:
 - With your left eye closed and a slide on the stage (see below), focus your right eye on something on the slide using the normal focus knobs.
 - Then close your right eye and adjust the focus of the left eye using the focus ring on the ocular lens.
 - Adjust the spacing of the oculars so that you can look with both eyes simultaneously.

Viewing a Sample

- Select lowest power (4x) objective by turning the black ring just above where the objectives attach to the body of the microscope.
 - **Important:** Never rotate by grabbing or putting pressure on an objective lens itself.
- Set slide on stage and hold in place with clip.
- Use the coarse and fine focus knobs to focus on the sample at lower magnification before rotating the collar (not touching the objectives themselves!) to switch to a higher objective.
- Refocus at the desired magnification, using the fine focus knobs.
- If you want to use the 100x oil objective, *check with your instructor before using.* Turn the collar so that the slide is centered between the 100x oil lens and the adjacent objective lens (neither one is clicked into place).

- Carefully put one small drop of immersion oil on the cover slip.
- Slowly rotate the collar so that the 100x objective clicks in place.
- The immersion oil should form a connection between the slide and the lens.
- Important: Use care never to get oil on any part of the microscope other than the 100x oil lens. Especially do not get oil on the other dry objectives – this can severely damage them.
- Adjust the level of illumination with the slider on the side of the microscope.

Tips For Focusing

- Always start by getting in focus at the lowest power objective (4x). Then increase objective strength one at a time, focusing before moving to a higher objective.
- If you have trouble finding the correct focal plane in which to view your sample, try focusing first on the edge of the cover slip.
 - Then, without moving the focus knob, scroll the stage over so that the specimen is in the field of view.
 - It now should be close to in focus, requiring only minor adjustment with the fine focus knob.

Putting the Microscope Away

- Clean the immersion oil off the 100x oil objective if used.
 - Tear off a piece of lens paper and gently swipe it across the lens.
 - Never use a Kimwipe or anything but lens paper on a lens; it could scratch the lens.
 - Rotate the paper to a clean portion or get a fresh sheet and gently swipe again.
 - Repeat until there is no visible oil coming off the lens onto the paper.
 - Never “scrub” on the lens by pressing with your finger or rubbing back and forth – any tiny pieces of dirt can scratch the lens.
- If desired, clean the other lenses in the same way, with clean lens paper.
 - They should not have any oil on them, but sometimes they will get a little dusty or dirty.
- Wipe up any oil that accidentally got on other parts of the microscope.
 - The stage is most likely to become contaminated by stray smudges of oil.
 - If necessary, moisten the corner of a Kimwipe with a little ethanol to help wipe up the oil. Remember, Kimwipes are never to be used on the lenses themselves.
- Turn the objectives so that either no objective points down toward the stage or the 4x objective points down (this helps to prevent damage to the objectives)
- Adjust the stage scroll knobs so that it doesn't stick out.
- Wrap the cord around the holders.
- Carefully store it in the appropriate place in the microscope cabinet.

12: Pipetting Technique

During the semester, we will perform many experiments that require us to transfer small volumes of liquid with precision and accuracy. Measuring small volumes (10 ml or less) requires that we use special equipment called pipettes. There are two major types of pipettes: serological pipet and micropipet.

Instrument Selection

1. Glass serological pipet

- We will use 5 ml, 10 ml, and 25 ml sizes
- Controlled using a Pipet-Aid = a pump that draws up or dispenses liquid
- The pump may be electronic, scrolling, or bulb

2. Micropipet, a.k.a. pipetman

- We will use 2 μ L, 20 μ L, 100 μ L, and 1000 μ L sizes
- P2: measures volumes less than 2.00 μ L
- P20: measures volumes between 2.0 μ L (minimum) and 20.0 μ L (maximum)
- P100: measures volumes between 10.0 μ L (minimum) and 100.0 μ L (maximum)
- P1000: measures volumes between 100.0 μ L (minimum) and 1000.0 μ L (maximum)
- Draws up and dispenses liquid using a disposable plastic tip

Which pipet should you use?

- Generally, choose the smallest possible instrument to measure the volume you need all at once
 - example: if you need 3.5 ml of water, it is best to use a 5 ml serological pipet rather than multiple uses of a P1000
 - example: if you need 95 μ L of water, it is best to use a 100 μ L (P100) pipettor rather than a P1000
- The exception is if you need a precise amount that is slightly more than 1 ml, use a P1000 and pipet in 2 steps.
 - example: if you need precisely 1450 μ L of water, pipet 750 μ L then 700 μ L, or some other combination that totals 1450 μ L.
 - This situation is uncommon, however, and usually you should select the instrument that allows you to measure the entire volume at once.

Use of a Serological Pipet

There are three possible types of Pipete-Aids: electronic, scroll, and bulb (Figure 1). The basic operation is the same, but subtle differences will it easier to use them accurately.

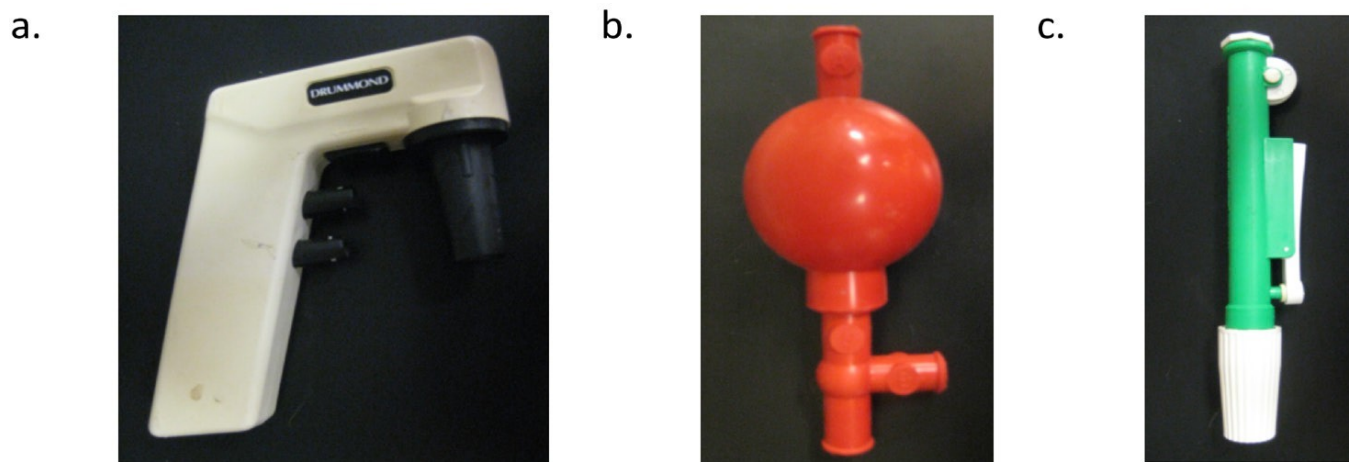


Figure 1. Three different styles of pipet-aids for transferring large volumes. Serological pipets are inserted into the hole on the bottom of each of these pumps. a. Electronic Pipet-Aid b. Bulb-style Pipet-Aid c. Scroll-style Pipet-Aid

For all Serological Pipette Usage:

- Carefully insert a clean serological pipet into the nose of the Pipet-Aid so that the numbers face you when you hold it.
 - Be sure to handle the pipet only near the top, so that your hands will not contaminate the portion that will enter the solution
- Insert the pipet into the solution and draw up the desired amount, measuring at the bottom of the meniscus
 - Note that usually the measurement numbers are in reverse order, so if you want to measure 2 ml using a 5 ml pipet, you may need to draw up your liquid to the mark that is labeled 3 ml. Some pipets are marked differently, so pay attention to each one you use.
 - **Caution:** Never allow any liquid to enter the nose of the Pipette-Aid
- Insert the filled pipet into the target container and dispense the liquid
- Carefully remove the used serological pipet from the Pipet-Aid and dispose as instructed by your professor.

Electronic Style Pipet-Aid

- These Pipet-Aids are cordless so you do not need to plug them in or turn them on
- Select the rate of drawing and dispensing by pressing the + and – buttons on the top back of the Pipet-Aid
- Depress the top finger button to aspirate (draw up) liquid and depress the bottom finger button to dispense liquid

Bulb-Style Pipet-Aid

- These Pipet-Aids look like balloons with three valves.
 - The largest appendage at the bottom attaches to the pipet while the valves on the other two can be used to control the rate of drawing and dispensing.
- To fill a pipet, start with a deflated bulb (squeeze the bulb and hold the "A" (air) valve at the top)
- Press the "S" (suck) valve on the side to allow air into the bulb, thereby filling the pipet with liquid.
- Press the "E" (expel) valve on the side to dispense the liquid.

Scroll-Style Pipete-Aid

- These Pipet-Aids are tube-shaped with a scroll wheel at the top
- Select the rate of drawing and dispensing by turning the scroll up and down.
- As the scroll moves, the plunger will move up and down. This will pull a vacuum, drawing liquid into the pipet. Scroll down to dispense.

Use of a Micropipetor

At the top of the micropipette is the push button that you will use to manipulate the liquid. Pressing this button will reveal that there are two stops, the first for sucking up the liquid and the second for expelling the liquid. Just below and off to the side is another button used to eject the disposable plastic tips during operation. The black movable ring, or thumbwheel, in the handle allows you to adjust the volume setting of the micropipette. As you turn the thumbwheel, look at the volumeter display. **Do NOT turn this wheel past**

the minimum and maximum volumes for the micropipette to prevent damaging the instrument.

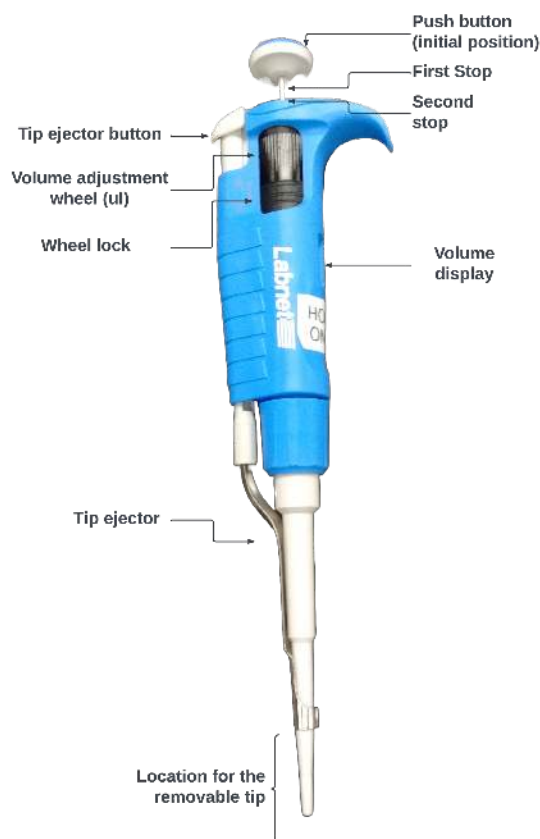


Figure 2. Diagram of a micropipette.

To determine the volume to which a micropipette is set, you can look at the volume display window on the side of the instrument. See Figure 3 for examples. These values are read vertically and interpreted based on the maximum capacity of the micropipette. In the example below, the left window is from the P20 micropipette and indicates that the instrument is set to measure 6.5 μL .

On the P20 micropipette, notice how the tenths place is shown in red. The center window is from the P100 micropipette and indicates that the instrument is set to measure 92 μL . The right window represents the P1000 micropipette and displays 726 μL . On the P1000 micropipette, notice how the thousands place is shown in red, and the ones place is not displayed.

P20	P100	P1000
0 Tens	0 Hundreds	7 Thousand
6 Ones	9 Tens	2 Hundreds
5 Tenths	2 Ones	6 Tens

Figure 3. Volume setting windows for the three most common pipettors used in lab. P20 is set at 6.5 μL , P100 is set at 92 μL , and P1000 is set at 726 μL .

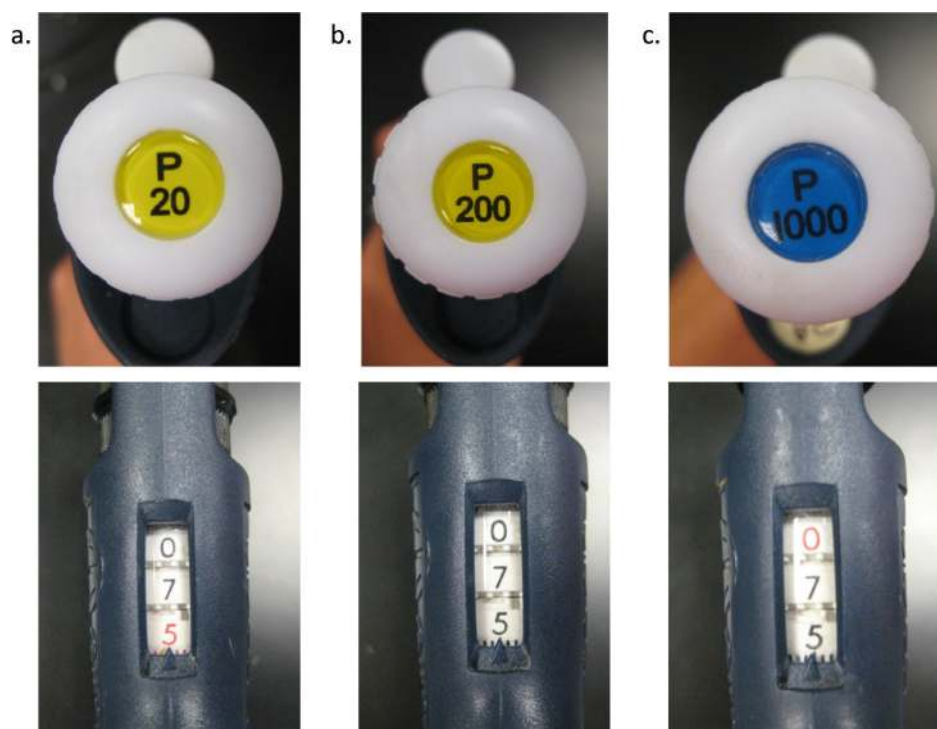


Figure 4. The three most common pipettors used in lab. (a) A 20 μ l pipettor (P20) can be used to accurately transfer between 2 μ l and 20 μ l of a liquid. Here the pipettor is set to 7.5 μ l. NOTE that the decimal place is red. (b) A 200 μ l pipettor (P200) can be used to accurately transfer between 20 μ l and 200 μ l of a liquid. Here the pipettor is set to 75 μ l. NOTE that all of the digits are black. (c) A 1000 μ l (1ml) pipettor (P1000) can be used to accurately transfer between 200 μ l and 1000 μ l (1ml) of a liquid. Here the pipettor is set to 750 μ l. NOTE that the thousands place is red.

NOTE: sometimes in lab we use a P100 and sometimes P200, they both have all black digits in the volume setting

To Set the Volume

Turn the knob on top of the plunger or use the volume adjustment wheel (Figure 2). You will probably need to release the scroll lock on the pipette before you can scroll. **Caution: Never try to set pipettor outside its proper range, which is printed on the top of the pipettor!**

To Put on the Tips

Gently but firmly press the end of the pipettor onto a pipet tip. P1000s use the large tips; P100s and P20s use the smaller tips; P2s use tiny tips. **You should always change tips between samples.**

To Suck Up (Aspirate) Liquid

Using your thumb, depress the plunger to the **first stop** or point of resistance. While holding the plunger down, insert the pipet tip into the solution. Slowly and smoothly release the plunger to draw up the desired amount. **Caution: Never allow any liquid to enter the barrel of the pipettor!**

To Squirt Out (Expel) Liquid

Insert the filled pipet tip into the target container, just below the surface of any existing liquid, and depress the plunger all the way to the second stop. To prevent bubbles, while holding the plunger down, remove the pipettor and tip from the target container, then release the plunger. Check the pipet tip to make sure you successfully dispensed all the liquid without leaving any drops stuck to the tip.

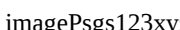
To Eject the Tip

Point the pipette tip over the waste container. Eject the tip into the waste container by pressing the tip ejector button.

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