Microbiology Laboratory Manual

Microbiology Laboratory Manual

EMILIE MILLER, PH.D

Contents

Introduction 1
Emilie Miller, Ph.D

Part I. Main Body

1. Laboratory Safety 3
   Emilie Miller, Ph.D
2. Lab Safety Assignment 10
   Emilie Miller, Ph.D
3. Chemical Control of Microbial Growth and Environmental Microbes 11
   Emilie Miller, Ph.D
4. Chemical Control of Microbial Growth Assignment 13
   Emilie Miller, Ph.D
5. Microscopy 18
   Emilie Miller, Ph.D
6. Microscopy Assignment 23
   Emilie Miller, Ph.D
7. Gram Stain 24
   Emilie Miller, Ph.D
8. Gram Stain Assignment 30
   Emilie Miller, Ph.D
9. Colony Morphology 32
   Emilie Miller, Ph.D
10. Colony Morphology Assignment 34
    Emilie Miller, Ph.D
11. Growth Patterns in Broth Assignment 35
    Emilie Miller, Ph.D
12. Aseptic Transfer 36
    Emilie Miller, Ph.D
13. Aseptic Transfer Assignment 43
    Emilie Miller, Ph.D
14. Streaking for Isolation 45
    Emilie Miller, Ph.D
15. Streaking for Isolation Assignment 51
    Emilie Miller, Ph.D

16. Acid-Fast and Endospore Staining 56
Emilie Miller, Ph.D
17. Acid Fast and Endospore Staining Assignment 59
Emilie Miller, Ph.D
18. MSA, MacConkey, EMB Plates 60
Emilie Miller, Ph.D
19. MSA, MacConkey, EMB Plates Assignment 65
Emilie Miller, Ph.D
20. Blood Agar 67
Emilie Miller, Ph.D
21. Blood Agar Assignment 70
Emilie Miller, Ph.D
22. SIM Medium 72
Emilie Miller, Ph.D
23. SIM Medium Assignment 75
Emilie Miller, Ph.D
24. Triple Sugar Iron Medium 78
Emilie Miller, Ph.D
25. Triple Sugar Iron Medium Assignment 80
Emilie Miller, Ph.D
26. Catalase Test 83
Emilie Miller, Ph.D
27. Catalase Test Assignment 85
Emilie Miller, Ph.D
28. Oxidase Test 87
Emilie Miller, Ph.D
29. Oxidase Test Assignment 89
Emilie Miller, Ph.D
30. Antibiotic Susceptibility Testing 91
Emilie Miller, Ph.D
31. Antibiotic Susceptibility Assignment 96
Emilie Miller, Ph.D
32. Dichotomous Keys 98
Emilie Miller, Ph.D

Appendix 101

Introduction

EMILIE MILLER, PH.D

Course Description for Microbiology Laboratory Manual

This course for community college students was designed to be taught as a face to face course that meets twice per week over an 11 week term at Columbia Gorge Community College. This course covers several key methods in a Microbiology Laboratory course.

Course Scope and Structure

This course incorporates a variety of instructional materials, such as how-to videos and assignments to accompany each lesson.

Each chapter follows this structure:

- Background theory of each procedure, a laboratory lesson, and an assignment.
- These lessons build upon each other in preparation for the final Unknown ID assignment.

1. Laboratory Safety

EMILIE MILLER, PH.D

In this course you will actually get to manipulate living things and see what happens…exciting! Working with living microorganisms also carries with it the risk of being harmed by these organisms. An infection contracted in a laboratory setting is called a **Laboratory Acquired Infection (LAI)**.

Consequently, safety is a primary concern in this course. The techniques you learn are specifically designed to minimize the possibility that you will come into contact with a pathogenic microbial cell. All the lab procedures including clean-up and item disposal are in place to protect you, the student! This section is extremely important. Test and quiz questions will be taken from it throughout the course.

The World Health Organization classifies microbes into four risk groups as shown in Table 1.

Risk Group 1 includes nonpathogenic organisms commonly found in the soil, in the normal flora of humans and microbes used to make certain foods. Examples include *Micrococcus luteus*, *Bacillus subtilis*, *Lactococcus lactus* (yogurt), *Staphylococcus epidermidis* (human skin surfaces), *E. coli* type strain (human GI tract). If kept in their natural environment, these microbes pose no risk to most healthy individuals. *Figure 1* (*World Health Organization*, 2004)

Organisms that cause fairly common human infections and diseases are in Risk Group 2. These diseases can be routinely treated with commonly available therapies. Examples include *Staphylococcus aureus* (skin and wound infections), certain *E. coli* strains, *Salmonella.enterica* (food poisoning), *Streptococcus pyogenes* (strep throat).

Table 1. ***Classification of infective microorganisms by risk group***

Risk Group 1 *(no or low individual and community risk)*
A microorganism that is unlikely to cause human or animal disease.

Risk Group 2 *(moderate individual risk, low community risk)*
A pathogen that can cause human or animal disease but is unlikely to be a serious hazard to laboratory workers, the community, livestock or the environment. Laboratory exposures may cause serious infection, but effective treatment and preventive measures are available and the risk of spread of infection is limited.

Risk Group 3 *(high individual risk, low community risk)*
A pathogen that usually causes serious human or animal disease but does not ordinarily spread from one infected individual to another. Effective treatment and preventive measures are available.

Risk Group 4 *(high individual and community risk)*
A pathogen that usually causes serious human or animal disease and that can be readily transmitted from one individual to another, directly or indirectly. Effective treatment and preventive measures are not usually available.

Risk Group 3 includes pathogens that cause more serious diseases requiring more aggressive treatments, but are not easily spread. Examples from this risk group are *Bacillus anthracis*, *West Nile virus, and human immunodeficiency virus* (*HIV*) (OpenStax CNX, 2018). Organisms in Risk Group 4 are the really "nasty bugs." They include Ebola, Marburg, and smallpox virus (OpenStax CNX, 2018). Few, if any, effective treatments are available and they are easily transmitted from person to person.

While organisms are classified into risk groups, laboratory facilities are classified into **Biosafety Levels** (**BSL**). The greater the risk, the more requirements are placed on the laboratory practices and safety equipment used to manipulate the organism. Organisms are often referred to by the BSL required to work with them because they generally coincide closely.

Figure 2 shows the relationship of the risk group to biosafety level.

In the microbiology teaching laboratories at CGCC, most of the organisms used will be from Risk Group 1. As the term goes on and the student becomes proficient with aseptic technique, some Group 2 microbes will be introduced. For simplicity, all of the laboratory procedures and policies will be appropriate for working with Risk Group 2. In other words, BSL-2 procedures will be in use throughout the term. *Figure 2* (*World Health Organization*, 2004)

Relation of risk groups to biosafety levels, practices and equipment

RISK GROUP	BIOSAFETY LEVEL	LABORATORY TYPE	LABORATORY PRACTICES	SAFETY EQUIPMENT
1	Basic – Biosafety Level 1	Basic teaching, research	GMT	None; open bench work
2	Basic – Biosafety Level 2	Primary health services; diagnostic services, research	GMT plus protective clothing, biohazard sign	Open bench plus BSC for potential aerosols
3	Containment – Biosafety Level 3	Special diagnostic services, research	As Level 2 plus special clothing, controlled access, directional airflow	BSC and/or other primary devices for all activities
4	Maximum containment – Biosafety Level 4	Dangerous pathogen units	As Level 3 plus airlock entry, shower exit, special waste disposal	Class III BSC, or positive pressure suits in conjunction with Class II BSCs, double-ended autoclave (through the wall), filtered air

BSC, biological safety cabinet; GMT, good microbiological techniques

The following laboratory policies and practices adhere to the CGCC Biosafety Manual which uses guidelines established by the U.S. Centers for Disease Control and Prevention (U.S. Department of Health and Human Services, 2009) for biosafety level 2. These safety precautions aim to prevent contact with microbes via the skin and the mucous membranes of the mouth nose and lungs. Preventing the production of **aerosols** (tiny invisible droplets potentially carrying microbes) is a major concern.

CGCC Safety Procedures

- No eating or drinking in the lab. All food items must be placed outside the lab space. Do not apply cosmetics, lotion, Chap Stick, contact lenses while in the lab.
- Do not remove organisms from the lab or prep room.
- Use extreme caution around Bunsen burners. Open flames are the MOST dangerous thing in the microbiology lab! Never leave a flame unattended. The Bunsen burner should be toward the middle of the lab bench so that students do not accidentally bump or need to reach over them.

- Students must come to lab fully prepared for the day's exercise. Students who have not read the material and viewed any required videos will not only waste precious lab time, they will be at much higher risk for accidental exposure. Preparation is a matter of safety and is taken very seriously!

Primary Barriers

Primary barriers prevent the student from direct contact with an infectious agent. This begins with **Personal Protective Equipment (PPE)** and extends to laboratory practices.

- Students are required to wear protective eyewear or glasses when conducting procedures that have the potential to create splashes of microorganisms or other hazardous materials. This eye wear may be removed when using the microscope as long as staining and other culture transfer procedures have been completed.

- Hair must be kept off the shoulders and out of the face. Hair should never touch or come close to touching the work surface. If a student needs to bush the hair back from the face with a gloved hand the student will be required to wear a head band or use a rubber band to keep it in place.

- Closed toed shoes should be worn at all times. No sandals or flip flops.
- Well-fitting gloves will be worn when working with microbes. This includes handling inoculated plates and tubes. Gloves that are too large are dangerous because the extra plastic at the finger tips interferes with dexterity. Students must refrain from touching their face or hair with their gloved hands.
- Gloves should be removed properly to prevent the production of aerosols and contamination of the bare hand. **See Figure 3.** Used gloves should be placed in the biohazard trash.
- Students must wash their hands before leaving the laboratory to prevent the transfer of organisms outside the laboratory. Washing hands before beginning the lab will help prevent contamination of the pure cultures used in the experiments.
- If the integrity of the skin is compromised (open sore, dry cracked skin, other wounds) the student should be sure to cover the lesion with a bandage and then wear gloves. Double gloving may be necessary.

Figure 3 Safe removal and disposal of gloves. The entire process is accomplished without snapping the gloves.

Secondary Barriers: Laboratory Facilities:

- After the conclusion of the lab exercise, students must disinfect their work area to ensure no unseen contamination remains after they work. To **disinfect,** spray the area lightly with the disinfectant provided. Wipe with a paper towel to spread the solution into a thin film. DO NOT dry the surface with the paper towel. Allow the bench top to air dry.
- There is a **biosafety cabinet (BSC)** available for procedures with the potential for creating infectious **aerosols** or splashes.

- 

A fire extinguisher, first aid kit, eye wash station and safety shower are available. Students must know where these are located and how to use them. *Figure 4 Biosafety cabinet In the UMKC Microbiology Laboratory*

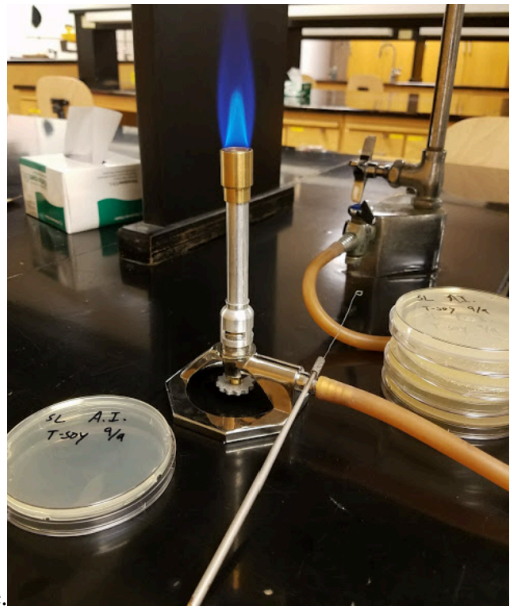
Disposal

- Paper towels used for routine purposes (hand drying, disinfecting the bench top) may be placed in the regular trash. Uncontaminated paper wrappers may also be placed in the regular trash.
- The broken glass box, lined with a plastic autoclave bag, will be used for any broken glass. Absolutely no glass is placed in the regular trash receptacle.

- Used slides will be placed in the red sharps containers.
- Used places, slants, and cultures will be placed in the biohazard trash.

Handling Cultures

- Gloves should be worn when handling all cultures, including placing them for incubation and disposal.
- All cultures must be properly labeled. At a minimum, every label must contain these 4 items: the student's name, l date, medium and organism name. Sometimes additional information will also necessary.
- Tube cultures, regardless of the contents, or lack thereof, all tubes, must be placed in a test tube rack when not being used in a transfer. (Holding the parent tube while inoculating something else, is a common scenario for a culture spill.) All tubes must be in a rack while being carried around the room.
- Tubes are never laid horizontally on the bench top.
- Tubes are never handled by the cap. Caps are, by design, loose fitting to allow oxygen to reach the culture. If you grasp the cap when removing a tube from a rack, the tube may not come with it!
- Liquid tube cultures should never be shaken to mix the contents. Instead, they may be flicked to redistribute cells within.



- Plate cultures should be open as little as possible.
- Individuals should keep their face away from the plate when removing cells from a plate or placing an inoculum on a plate.
- Plates are always incubated and stored upside down. This keeps condensation from dropping onto the surface and moving cells around. This not only moves cells where you do not want them, but also creates the potential for the

liquid to leak out of the plate and contaminate you or your work surface.

- Plates may be written on directly with a sharpie. Always label the base (the part containing the agar) of the plate. Lids can come off easily and get mixed up.
- Be sure you know what transfers are to be made before starting.
- Label all media to be inoculated before you make a transfer.
- Transfers are made without setting the transfer instrument down.
- After the transfer, the wire loop must be incinerated completely before setting it down. If cells remain on the loop, they can contaminate the bench top or any other object the loop touches. Other transfer instruments must be immediately placed in the appropriate container.

Remediation

> As long as the student is following the lab procedures to the best of their ability, there is no penalty for accidental spilling! Let the instructor know so that they can help clean it up properly.

- Steps for a spill are as follows: a) Student or lab partner notifies instructor.. One person should remain with the spill to prevent other students from accidently coming in contact with the culture. b) Allow aerosol to settle. C) Gently cover spill with absorbent paper towels. d) Apply disinfectant starting at the perimeter and working towards the center, e) allow sufficient contact time (20 min) before clean up, f) place all paper towels and gloves used for clean up in the correct disposal container.
- If a culture spills on you, wash with soap and water.
- All individuals involved in a spill should wash hands and replace their gloves with new.
- If someone gets a cut or a puncture (**percutaneous** exposure), they must notify the instructor immediately and reported.
- If you splash culture in your eye, immediately flush both eyes with water for 15 minutes. Notify the instructor as soon as possible. There is an eyewash basin available for this use.

2. Lab Safety Assignment

EMILIE MILLER, PH.D

Lab Safety Assignment:

Name: _____

1. Sketch the lab space and note the locations of the following:
fire extinguisher, slide disposal, incubator, fume hoods, biohazard trash, eye wash, shower

2. What could happen if you shake a liquid culture?

3. What is wrong with laying an empty tube on the bench top?

4. What could happen if you pick up a tube by the cap?

5. If you spill a culture, what should you do FIRST?

7. What is the most dangerous item in the microbiology lab? How should it be treated?

References:

OpenStax CNX. (2018, Mar 19). OpenStax Microbiology, Microbiology . Retrieved from http://cnx.org/contents/e42bd376-624b-4c0f-972f-e0c57998e765@4.24

U.S. Department of Health and Human Services. (2009). *Biosafety in Microbioloical and Biomedical Laboratories*, 5*th Ed.* Centers of Disease Control and Prevention, National Institutes of Health, Public Health Service. HHS Publication No. (CDC) 21-1112. Retrieved from https://www.cdc.gov/biosafety/publications/bmbl5/index.htm

World Health Organization. (2004). Laboratory Biosafety Manual. (3). Geneva, Switzerland. Retrieved January 14, 2019, from https://www.who.int/csr/resources/publications/biosafety/en/Biosafety7.pdf

3. Chemical Control of Microbial Growth and Environmental Microbes

EMILIE MILLER, PH.D

Objectives: In this lab you will:

1. Learn about disinfectants and the various factors that need to be considered when choosing a disinfectant.
2. Set up an experiment to determine the effectiveness of five different common disinfectants

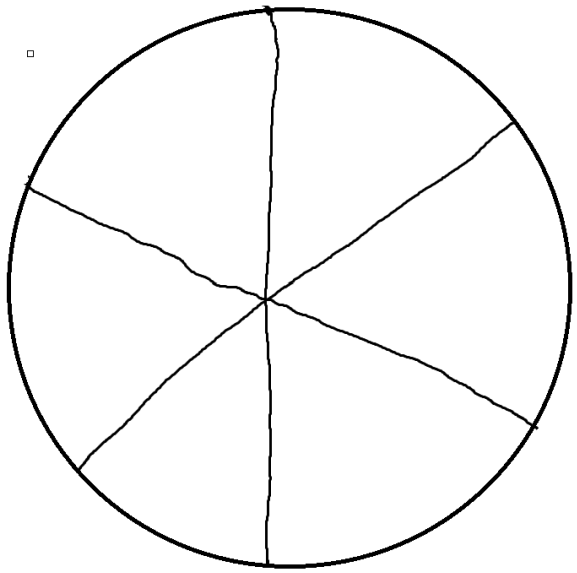
Introduction

The use of chemicals to control microbial growth dates back at least as far as the 1800's. Tincture of iodine was used as antiseptic during the Civil War, and Joseph Lister established the practice of aseptic surgery using a disinfectant known as carbolic acid (phenol) in the 1860's. Since that time, many types of **disinfectants** (agents that are used to eliminate or kill vegetative cells on surfaces) and **antiseptics** (agents that are used to eliminate or reduce vegetative cells on living tissue) have been used. Although disinfectants and antiseptics may be effective at killing vegetative cells, they do not usually achieve sterilization.

Various factors need to be considered when choosing a disinfectant or antiseptic. It is very important to know which microbes are present to determine what type of disinfectant would work best. It is also important to realize that the effectiveness of a particular disinfectant may be affected by pH, temperature, concentration, and exposure time. Ideal disinfectants should be effective against the particular contaminants present, usable at a low concentration, require a relatively short exposure time, and have a long shelf life. It should also be water soluble, non-toxic to humans and animals, and cost-effective.

Method:

1. Divide your agar plate into 6 sections with a marker as shown.
2. Label the sections as follows; control, 1, 2, 3, 4, 5. Also include your name.



3. Dip a cotton swab in water and swab the back of your hand then swab the "control" part of the agar in a zig-zag pattern. Discard swab.
4. Pick 5 different chemicals or methods for your experiment.
5. One at time, clean a small portion of the back or your hand with a chemical using a cotton swab. Sample the

cleaned area with a fresh swab dipped in water and apply it to the corresponding region of the plate. Repeat with 4 different chemicals on 4 different parts of your hand. Consider drawing a grid on your hand to insure parts do not overlap.

6. Place in incubator. Bacterial growth will be observed next lab period.

As a class, we will brainstorm different places we will collect environmental samples of microbes. Each student will be given a contact agar plate that can be directly applied to a surface then incubated for observation at the next lab session.

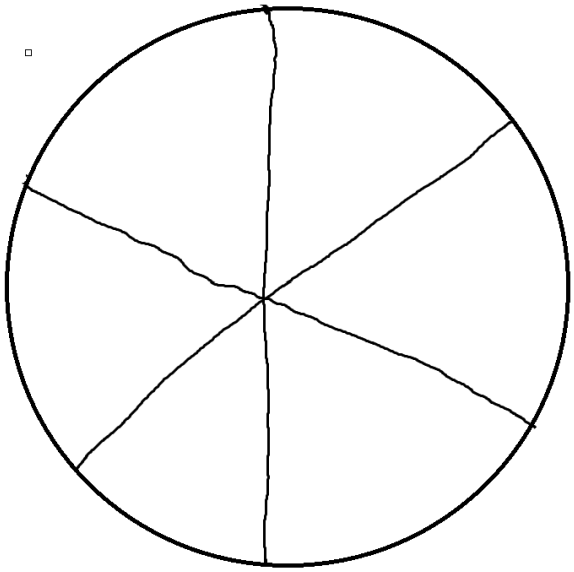
One or more interactive elements has been excluded from this version of the text. You can view them online here: https://openoregon.pressbooks.pub/microbiologylaboratorymanual/?p=342#oembed-1

4. Chemical Control of Microbial Growth Assignment

EMILIE MILLER, PH.D

Name: _____

1. **Disinfectant experiment**: Sketch the appearance of your own Nutrient Agar plate. Record your observations below.



Chemical or Method	Relative Growth	Relative Diversity
Control		
1.		
2.		
3.		
4.		
5.		

2. For the second table, do some research about each of your chemicals or methods. Answers should address specific effects to the cell membrane, cell wall, and any internal structures. If there are any antifungal, antiviral, or antiprotozoal effects, they can be noted in the right-hand column.

Chemical or Method	Antibacterial Effects	Other Antimicrobial Effects
1.		
2.		
3.		
4.		
5.		

3. Based on your results, which chemical or methods worked best?

4. Which chemical or methods were least effective?

Observe the contact plates from your classmates. Note relative growth and diversity of the contact plates with respect to the locations the microbes were gathered.

5. Which locations had the most growth and diversity? Note 3 different examples.

6. Why would these locations have the most growth and diversity?

7. Which locations had the least growth and diversity? Note 3 different examples.

8. Why would these locations have the least growth and diversity?

5. Microscopy

EMILIE MILLER, PH.D

The Compound Microscope

There would be little to do in a microbiology laboratory without a microscope, because the objects of our attention (bacteria, fungi, and other single celled creatures) are otherwise too small to see. Microscopes are optical instruments that permit us to view the microbial world. Lenses produce the magnified images that allow us to visualize the form and structure of these tiniest of living beings.

To use this important piece of equipment properly, it is helpful to know how a microscope works. A good place to begin is to learn the name and function of all of the various parts, because when we talk about the ways to improve microscopic images, terms like "ocular lenses" and "condenser" always come up.

Based on the picture of the binocular, compound light microscope in Figure 1, match the name of the major part (listed below) with its location on the microscope, and give a very brief description of what each is used for:

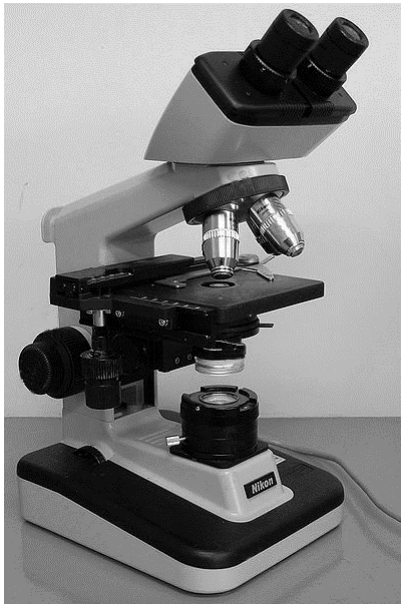


Figure 1

Ocular lenses	_____	**Locate the parts on the microscope that allow you to:**
Objective lenses	_____	
(Revolving) Nosepiece	_____	• Move the stage (stage adjustment knobs) • Adjust the condenser lens • Adjust the light intensity • Adjust the iris diaphragm • Adjust the distance between the ocular lenses
Stage and stage clips	_____	
Course and fine focus knobs	_____	
Condenser lens	_____	
Iris diaphragm	_____	

Making Images

With a bright-field microscope, images are formed as a result of the interplay between light waves, the object, and lenses. How images of biological objects are formed is actually more physics than biology. Since this isn't a physics course, it's more important to know how to create exceptional images of the object than it is to know precisely how those images are formed.

Light waves that pass through and interact with the object may speed up, slow down, or change direction as they travel through "media" (such as air, water, oil, cytoplasm, etc.) of different densities. For example, light passing through a thicker or denser part of a specimen (such as the nucleus of a cell) may be reflected or refracted ("bend" by changing speed or direction) more than those waves passing through a thinner part. This makes the thicker part appear darker in the image, while the thinner parts are lighter.

For a compound microscope, the optical path leading to a detectable image involves two lenses – the objective lens and the ocular lens. The objective lens magnifies the object and creates a **real image**, which will appear to be 4, 10, 40, or 100 times larger than the object actually is, depending on the lens used. The ocular lens further magnifies the real image by an additional factor of 10, to produce a vastly larger **virtual image** of the object when viewed by you.

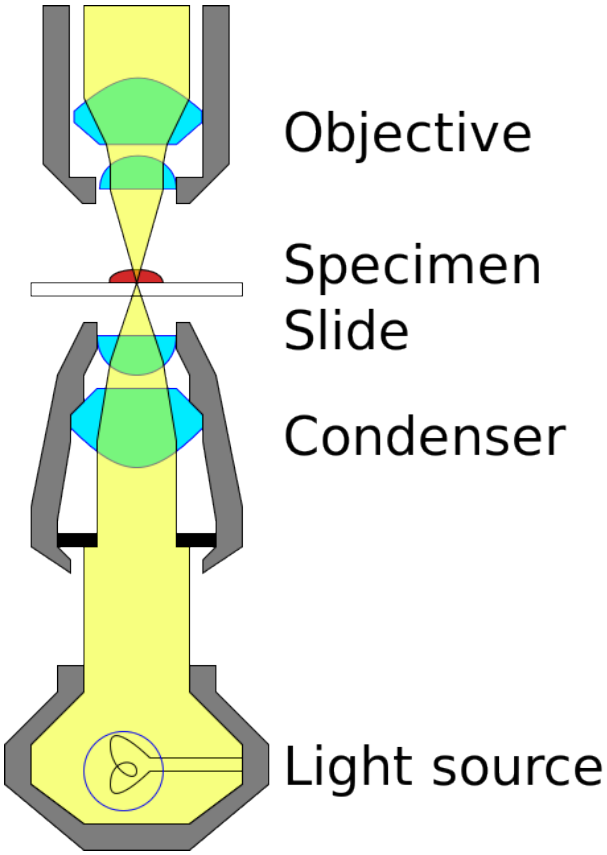


Figure 2

Light from an illuminator (light source) below the stage is focused on the object by the condenser lens, which is located just below the stage and adjustable with the condenser adjustment knob. The condenser focuses light through the specimen to match the aperture of the objective lens above, as illustrated in Figure 2.

Appropriate use of the condenser, which on most microscopes includes an iris diaphragm, is essential in the quest for a perfect image. Raising the condenser to a position just below the stage creates a spotlight effect on the specimen, which is critical when higher magnification lenses with small apertures are in use. On the other hand, the condenser should be lowered when using the scanning and low power lenses because the apertures are much larger, and too much light can be blinding. For creating the best possible contrast in the image, the iris diaphragm can be opened to make the image brighter or closed to dim the light. These adjustments are subjective and should suit the preferences of the person viewing the image.

When the light waves that have interacted with the specimen are collected by the lenses and eventually get to your eye, the information is processed into dark and light and color, and the object becomes an image that you can see and think more about.

Magnification

The microscope you'll be using in lab has a compound system of lenses. The objective lens magnifies the object "X" number of times to create the real image, which is then magnified by the ocular lens an additional 10X in the virtual

image. Therefore, the total magnification, or how much bigger the object will actually appear to you when you view it, can be determined by multiplying the magnification of the objective lens by 10.

The magnifying power of each lens is engraved on its surface, followed by an "X." In the table below, find the magnification, and then calculate the total magnification for each of the four lenses on your microscope.

	Magnification of objective lens	Total magnification of viewed object
Scanning Lens		
Low Power Lens		
High Power Lens		
Oil Immersion Lens		

Let's say you wanted to look at cells of *Bacillus cereus*, which are rod-shaped cells that are about 4 μm long. If you were observing *B. cereus* with a microscope using the high power lens, how big would the cells appear to be when you look at them? _____

Resolution Limits Magnification

So the microscope makes small cells look big. But why can't we just use more or different lenses with greater magnifying power until the images we see are really, really big and easier to see?

The answer is **resolution**. Consider what happens when you try to magnify the fine print from a book with a magnifying glass. As you move the lens away from the print, it gets larger, right? But as you keep moving the lens, you notice that while the letters are still getting larger, they are becoming blurry and hard to read. This is referred to as "empty magnification" because the image is larger, but not clear enough to read. Empty magnification occurs when you exceed the **resolving power** of the lens.

Resolution is often thought of as how clearly the details in the image can be seen. By definition, resolution is the minimum distance between objects needed to be able to see them as two separate entities. It can also be thought of as the size of the smallest object that we can clearly see.

The ability of a lens to resolve detail is ultimately limited by diffraction of light waves, and therefore, the practical limit of resolution for most microscopes is about 0.2 μm. Therefore, it would not be practical to try to observe objects smaller than 0.2 μm with a standard optical microscope. In addition, cells of all types of organisms lack contrast because many cellular components refract light to a similar extent. This is especially true of bacteria. To overcome this problem and increase contrast, biological specimens may be stained with selective dyes.

The Oil Immersion Objective

The lens with highest magnifying power is the oil immersion lens, which achieves a total magnification of 1000X with a resolution of 0.2 μm. This lens deserves special attention, because without it our time in lab would be frustrating.

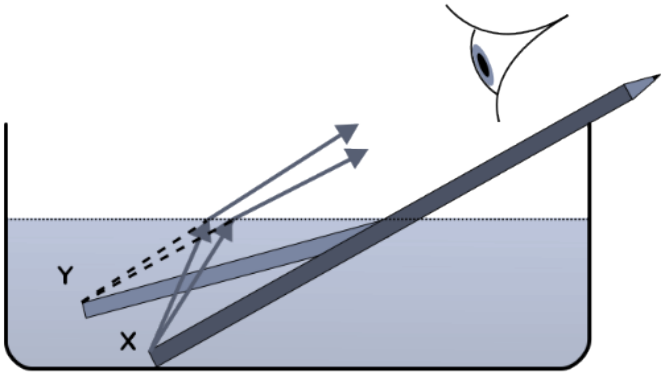


Figure 3

The resolving power of this lens is dependent on "immersing" it in a drop of oil, which prevents the loss of at least some of the image-forming light waves because of refraction. Refraction is a change in the direction of light waves due to an increase or decrease in the wave velocity, which typically occurs at the intersection between substances through which the light waves pass. This is a phenomenon you can see when you put a pencil in a glass of water. The pencil appears to "bend" at an angle where the air and water meet (see Figure 3). These two substances have different refractive indices, which means that light passing through the air reaches your eye before the light passing through the water. This makes the pencil appear "broken."

The same thing happens as the light passes through the glass slide into the air space between the slide and the lens. The light will be refracted away from the lens aperture. To remedy this, we add a drop of oil to the slide and slip the oil immersion objective into it. Oil and glass have a similar refractive index, and therefore the light bends to a lesser degree and most of it enters the lens aperture to form the image.

It is important to remember that **you must use a drop of oil** whenever you use the **oil immersion objective** or you will not achieve maximum resolution with that lens. However, **you should never use oil with any of the other objectives**, and you should be diligent about wiping off the oil and cleaning all of your lenses each time you use your microscope, because the oil will damage the lenses and gum up other parts of the instrument if it is left in place.

Using the Microscope

If you are new to microscopy, you may initially feel challenged as you try to achieve high quality images of your specimens, particularly in the category of "Which lens should I use?" A simple rule is: the smaller the specimen, the higher the magnification. The smallest creatures we observe are bacteria, for which the average size is a few micrometers (μm). Other microscopic organisms such as fungi, algae, and protozoa are larger, and you may only need to use the high power objective to get a good view of these cells; in fact, using the oil immersion objective may provide you with less information because you will only be seeing a part of a cell.

This brings us to two additional concepts related to microscopy—working distance and parfocality. **Working distance** is how much space exists between the objective lens and the specimen on the slide. As you increase the magnification by changing to a higher power lens, the working distance decreases and you will see a much smaller slice of the specimen. Also, once you've focused on an object, you should not have to make any major adjustments when you switch lenses, because the lenses on your microscope are designed to be **parfocal**. This means that something you saw in focus with the low power objective should be nearly in focus when you switch to a high power objective, or vice versa. Thus, for viewing any object and regardless of what lens you will ultimately use to view it, the best practice is to first set the working distance with a lower power lens and adjust it to good focus using the coarse focus knob. From that point on, when you switch objectives, only a small amount of adjustment with the fine focus knob should be necessary.

Here is a final consideration related to objective lenses and magnification. Look at the lenses on your microscope, and

note that as the magnification increases, the length of the lens increases and the lens aperture decreases in size. As a result, you will need to adjust your illumination to compensate for a darkening image. There are essentially three ways to vary the brightness; by increasing or decreasing the light intensity (using the on/off knob), by moving the **condenser lens** closer to or farther from the object using the condenser adjustment knob, and/or by opening/closing the **iris diaphragm**. Don't be afraid to experiment to create the best image possible.

Guidelines for safe and effective use of a microscope:

1. Carry the microscope to your lab table using two hands, and set it down gently on the bench. Once placed on the bench, do not try to slide it around on its base, because this is extremely jarring to the optical system.

2. Clean all of the lenses with either lens paper or Kimwipes (NOT paper towels or nose tissues) **BEFORE** you use your microscope, **AFTER** you are done, and before you put it away.

3. When you are finished with the microscope, check the stage to make sure that you don't leave a slide clipped in the stage. Make sure to switch the microscope OFF before you unplug it. Gently wrap the cord around the base and cover your microscope with its plastic cover.

4. Return the microscope to the cabinet before you leave the lab. Make sure that the ocular lenses are facing IN.

6. Microscopy Assignment

EMILIE MILLER, PH.D

The human mouth is home to numerous microbes, which persist no matter how many times you brush your teeth and use mouthwash. Since these microbes generally inhabit the surface layers of the oral mucosa, we humans have evolved ways to keep their numbers under control, including producing antibacterial chemicals in saliva and constantly turning over the outer layer of epithelial cells that line the inside of the mouth.

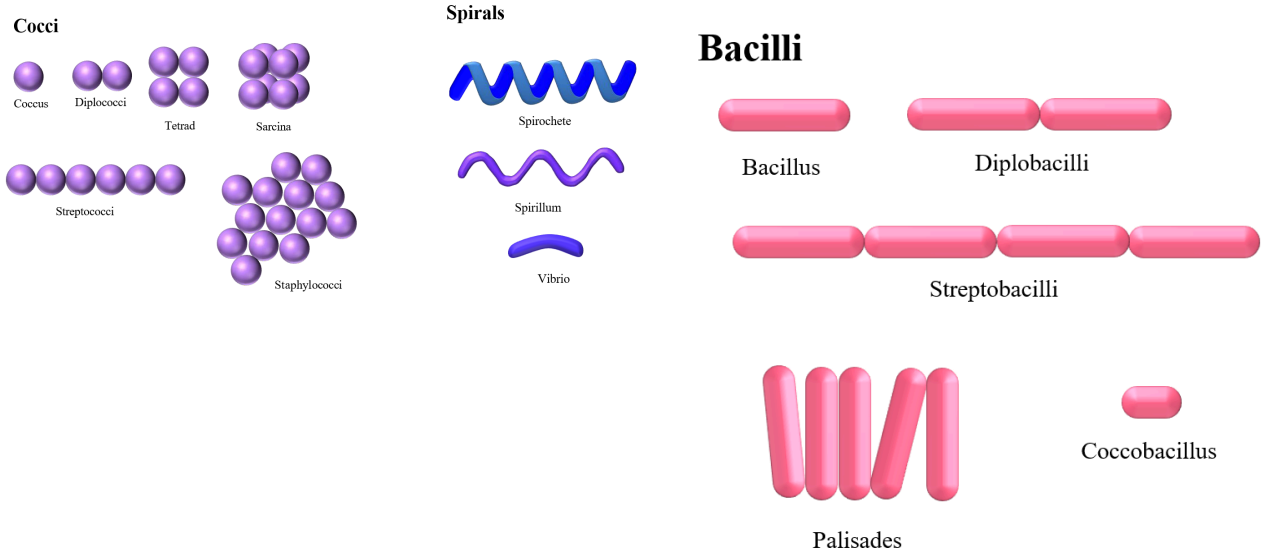
These are squamous epithelial cells that form the outermost layer of the oral mucosa. At high power, you should start to see small cells on the surface of the larger epithelial cells. With the oil immersion objective lens, you will be able to tell the smaller cells are bacteria.

1. Gently swab your inner cheek with a cotton swab and swirl in a small spot on a clean slide. Flame your slide quickly over a bunsen burner to fix the cells. Apply a few drops of Crystal Violet to the cells then rinse the slide with water. Blot the slide dry with Bibulous Paper and then view your slides with your microscope working your way up through the objectives.

> *One or more interactive elements has been excluded from this version of the text. You can view them online here: https://openoregon.pressbooks.pub/microbiologylaboratorymanual/?p=333#oembed-1*

Draw and label your cheek cells and include the cell membrane, nucleus using the 40x objective: Total magnification: ------

2. Use the 100x objective with oil to view bacteria that may have been fixed outside and inside your cheek cells: Draw and ID the shapes present using the terms: Cocci, Bacilli, Strepto, Staphlo. Get confirmation of your image from the instructor when you have it in view_____. Total magnification:_____.



7. Gram Stain

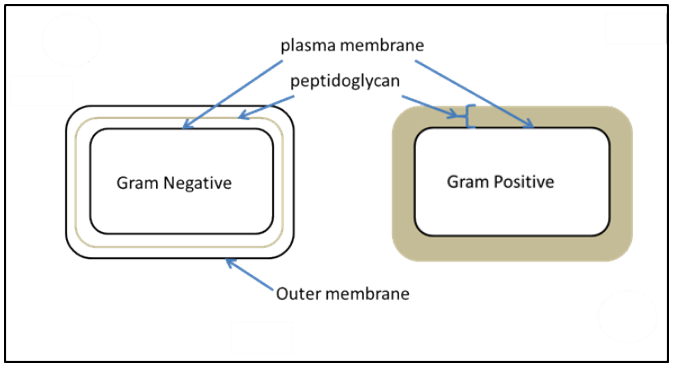
EMILIE MILLER, PH.D

The student will:

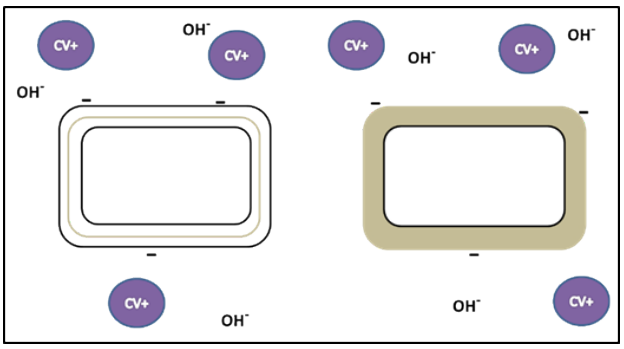
- Use aseptic techniques in the safe inoculation of various forms of media.
- Follow oral and written instructions and manage time in the lab efficiently.
- Use the bright field light microscope to view microbes under oil immersion, make accurate observations and appropriate interpretations and store the microscope according to lab procedures.
- Properly prepare a bacterial smear for accurate staining and describe the chemical basis for simple staining and negative staining.

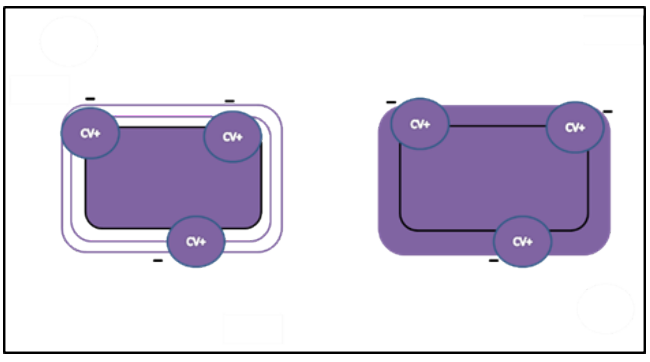
Differential staining distinguishes organisms based on their interactions with multiple stains. In other words, two organisms may appear to be different colors. Differential staining techniques commonly used in clinical settings include Gram staining, acid-fast staining, endospore staining, flagella staining, and capsule staining. This link to the OpenStax Microbiology text provides more detail on these differential staining techniques. (OpenStax CNX, 2018)

The **Gram stain** is a **differential staining** procedure that involves multiple steps. It was developed by Danish microbiologist Hans Christian Gram in 1884 as an effective method to distinguish between bacteria containing the two most common types of cell walls. (OpenStax CNX, 2018) One type consists of an inner plasma membrane and a thick outer layer of peptidoglycan. The other type consists of a double phospholipid bilayer with a thin layer of peptidoglycan between the two. The Gram Staining technique remains one of the most frequently used staining techniques.



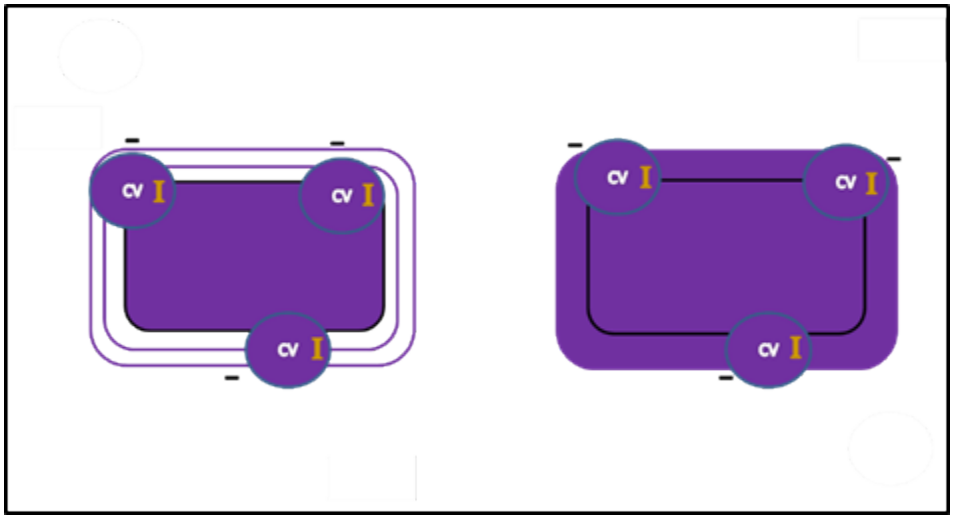
- First, **crystal violet**, the **primary stain**, is applied to a heat-fixed smear, giving all of the cells a purple color. You will recall that crystal violet is a basic stain (excess OH- ions). It adheres to the cell because the positively charged chromogen is attracted to the negatively charged cell as described in the Simple Staining exercise. (See figures 2 and 3.) This step is chemically identical to simple staining.



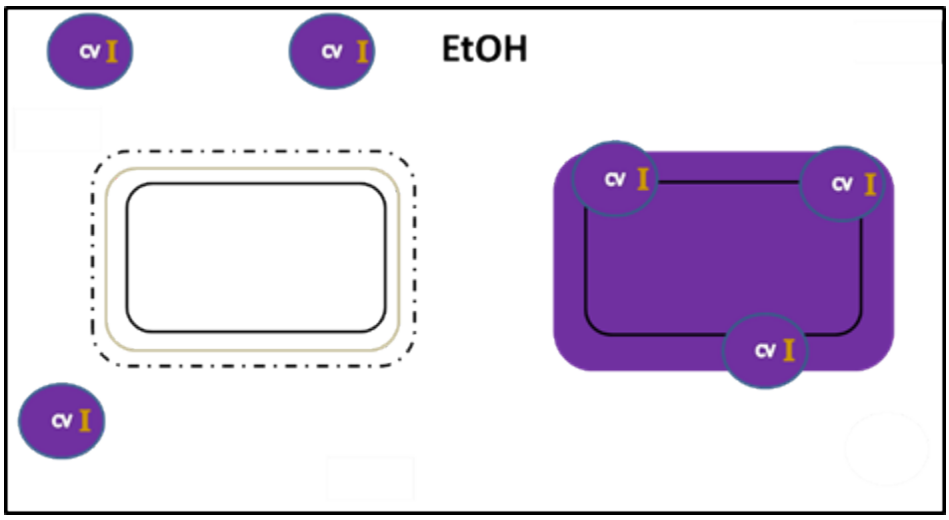


-

- Next, **Gram's iodine**, a **mordant**, is added. A mordant is a substance used to set or stabilize stains or dyes; in this case, Gram's iodine acts like a trapping agent that complexes with the crystal violet, making the crystal violet–iodine complex clump and stay contained in thick layers of peptidoglycan in the cell walls. All the cells become a deep purple color after this step.
- Next, a **decolorizing agent** is added, usually ethanol or an acetone/ethanol solution. Cells that have thick peptidoglycan layers in their cell walls are much less affected by the decolorizing agent; they generally retain the crystal violet dye and remain purple. These are termed **Gram positive**. In the cells with the thin layer of peptidoglycan, the decolorizing agent easily washes the dye out of cells leaving them colorless. Cells that do not retain the CV-I are called **Gram negative**. Because the reagent reacts differently depending on the cell wall, this step makes Gram staining **differential** and is the most crucial.
- Finally, a secondary **counterstain**, usually **safranin**, is added. This stains the decolorized cells pink. (OpenStax CNX, 2018) Like the primary stain, the counterstain step is chemically a simple stain. Its purpose is to make the colorless cells visible. The safranin chromogen actually adheres to all the cells. Because the Gram positive cells are retaining the dark purple CV-I complex, the pink safranin only shows up in the colorless cells.
- *After crystal violet is added, all cells are purple.*

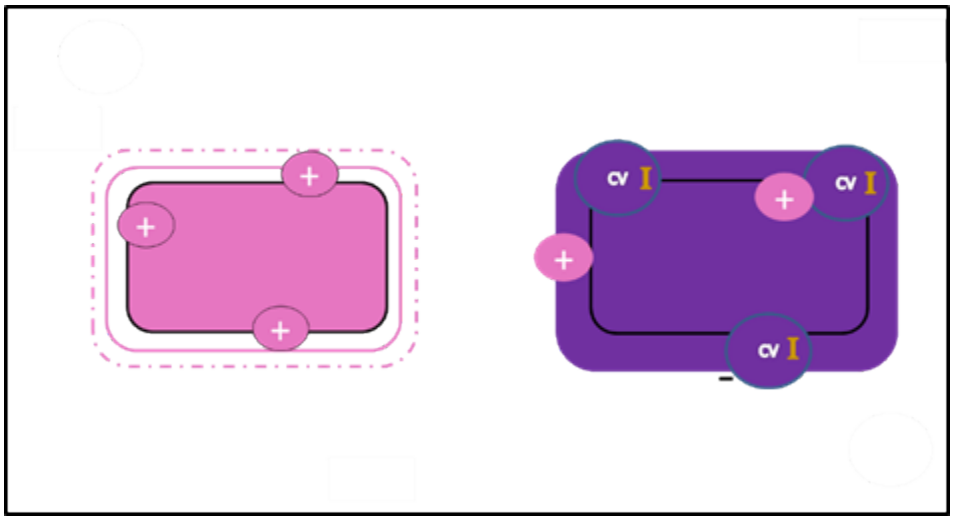


Iodine, the mordant, intensifies the color.



After decolorizing, Gram negative cells are colorless and Gram positive cells remain purple.

The counterstain imparts color to the colorless cells as a simple basic stain.



There are several important considerations in interpreting the results of a Gram stain. First, older bacterial cells may have damage to their cell walls. This is accentuated in Gram positive cells because the thick peptidoglycan begins to break down and cannot retain the CV-I complex as efficiently. The result? Gram positive cells appear pink and may be misidentified as Gram-negative. Thus, it is essential to use fresh (24 hour) bacterial cultures for Gram staining.

Second, errors, especially decolorizing too long, can affect the results. (OpenStax CNX, 2018) If exposed to the decolorizer too long, the CV-I complex can be removed from Gram positive cells along with Gram negative cells making all types appear pink at the end. Conversely, if the decolorizer is not applied long enough, the CV-I complex will remain in the Gram negative cells and they will appear purple and can be misidentified as Gram positive. This is the most common mistake students make in Gram staining. Always do a control smear when Gram staining an unknown culture to verify that your technique is correct. Getting the decolorizing step correct is tricky and takes some practice. (OpenStax CNX, 2018)

The third cause of inaccurate Gram staining is a thick smear. If cells are clumped together on the slide, the

cells will not make contact with each of the reagents equally. Some individual cells may not be decolorized enough and remain purple when other identical cells become decolorized. This will result in some cells in a pure culture smear appearing different from others. (OpenStax CNX, 2018)

Besides their differing interactions with dyes and decolorizing agents, the chemical differences between Gram-positive and Gram-negative cells have other implications with clinical relevance. For example, Gram staining can help clinicians classify bacterial pathogens in a sample into categories associated with specific properties. Gram-negative bacteria tend to be more resistant to certain antibiotics than gram-positive bacteria. (OpenStax CNX, 2018)

Gram Stain Procedure

- Place a smear or drop of culture on a slide and allow to **completely air dry**.
- Heat-fix the slide by drawing it quickly over the flame of a Bunsen burner.
- Put a few drops of crystal violet on the fixed culture and allow to sit for one minute.
- Gently rinse with distilled water over a sink.
- Add Gram's iodine to the fixed culture. Allow to sit for one minute. By discarding the first few drops of iodine, you ensure that the mordant is not diluted by excess water drops left on the slide from the previous rinse.
- Gently rinse with distilled water.
- Apply Gram's Decolorizer to the slide until the stain stops coming off the slide and the decolorizer runs clear. This only takes 3-4 passes across the slide.
- Immediately, rinse with distilled water, gently. This will stop the decolorizing process.
- Apply the counterstain, safranin and allow it to sit for one minute.
- Gently rinse with distilled water.
- Gently blot in a fairly clean area of a bibulous paper book. Refrain from blotting after each rinse step. This is unnecessary and may result in the loss of cells from the slide making the few that are left more difficult to find.

In this exercise you will stain a slide with 2 smears. On one side of the slide you will place a mixed culture containing *E. coli*, a known Gram negative rod and on the other side *Staphylococcus epidermidis*, a known Gram positive coccus. You will know you have stained the slide correctly if all the rods are pink and all the cocci are purple. Be sure you are looking for cell morphology and color. If you are not tuning into the details you may not interpret your results accurately. For example, in a cursory glance you may see both colors, but a closer look may reveal an unevenly stained slide with a clumped mass of cells (where you cannot distinguish individual cell morphology) retaining the purple color and individual cells, both rods and cocci, staining pink.

Materials per studen

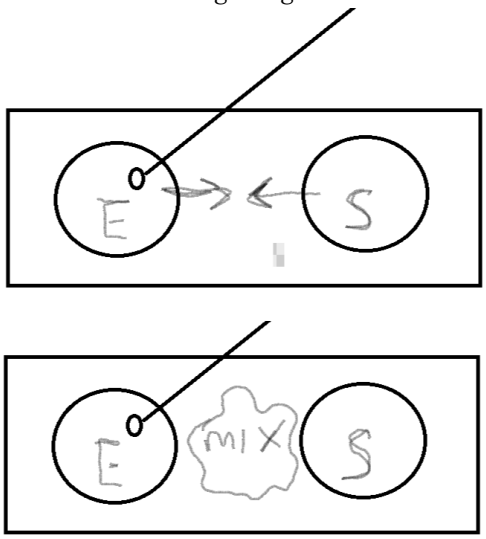
1 Microscope slide
1 bottle Crystal violet stain
1 bottle Gram's iodine
1 bottle Gram's Decolorizer
1 bottle Safranin stain Sterile toothpicks
Bibulous paper, Bunsen burner, Striker, Inoculating loop

Cultures

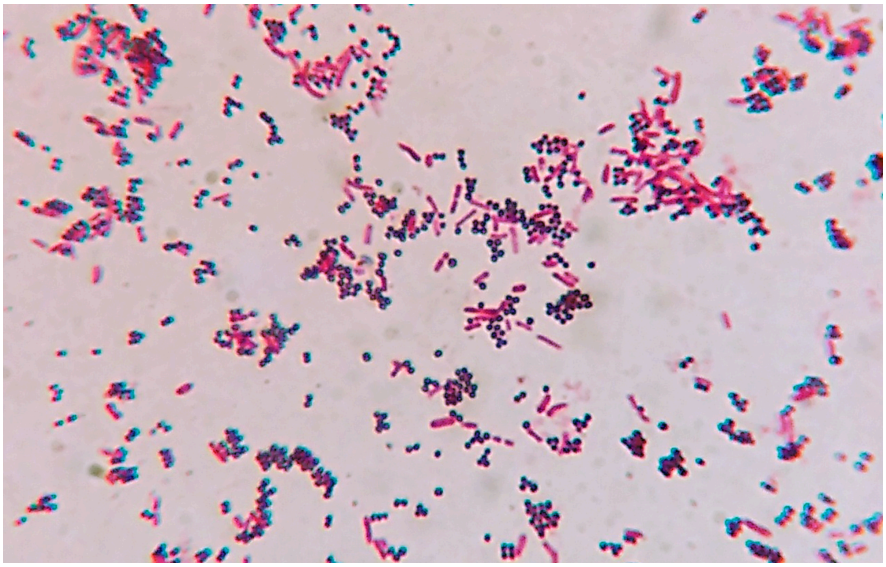
E. coli
Staphylococcus epidermidis

Procedure

- On the left side side, aseptically make a smear with the *E. coli*. Be sure to sterilize your loop after spreading cells on one slide and before getting more cells . Be sure to make the smear about the size of a nickel.



- On the right side of the slide, aseptically make a smear with the *Staphylococcus epidermidis*
- With your inoculation loop, draw a small amount of each culture to the middle of the slide and mix.
- Gram stain using the procedure above. Treat all parts of the slide the same.
- Find the cells under oil immersion. As always, you will need to start with the scanning objective. Follow the steps described in Microscope Theory.
- Evaluate your Gram staining technique by locating both rods and cocci. Determine if each is the correct color. Make sure that you are not just looking for both colors. You need to make sure that the rods are pink/red and the cocci are dark purple. Consult with your instructor. Note that on high power (high dry), the resolution is not good enough to determine the cell morphology and color. You must find cells under **oil immersion** to determine these characteristics.
- If your staining technique is good, your instructor will initial your Data and Observations table and you can then go on to record your observations. Use one row of the table for *E. coli* and the next line to make observations for the *S. epidermidis*. Remember, the first time you write the organism name, write it out in full scientific form.
- Dispose of your slide in the sharps container.



Practice:

https://virtuallabs.nmsu.edu/stain.php

https://learn.chm.msu.edu/vibl/vibl/GramStain/gram_stain_HTML5Canvas.html

8. Gram Stain Assignment

EMILIE MILLER, PH.D

Name: _____

Data and Observations

Organism	color	Gram Reaction	Drawing	Morphology and Arrangement	Instructor Initials

Post Lab Questions

1. Explain the major differences between the Gram positive and the Gram negative cell wall.

2. What are the 4 major steps in Gram Staining and what is the role of each reagent?

A student divides a slide in half and places a mixed culture smear of *E. coli* and *S. epidermidis* on one side and a pure culture smear of an unknown microbe on the other. They Gram stain the slide and begin their observations by looking at the mixed smear under oil immersion. Evaluate the student's Gram staining technique when the following results are observed. Explain what the student may have done wrong. If they also observe the unknown smear on the same slide, are their results likely to be accurate?

3. Both the rods and the cocci appear purple.
4. You can only find rods and they are all pink.
5. What is wrong with putting an unknown organism and the control mixed culture smear on different slides?

6. Why would a health care provider be interested in knowing the Gram reaction of a pathogenic bacterium?

7. Instead of the usual control organisms, you use *Bacillus cereus*, a Gram + rod, and *Moraxella catarrhalis*, a Gram – coccus. If stained correctly, what result do you expect to see?

If a student performs the perfect Gram stain except for the following mistakes, what result (color) would you expect to see if the cells were Gram negative? Gram positive?

8. The student skipped the iodine step.

9. The student left the safranin on for an extra 15 seconds.

10. The student delayed rinsing the Gram's Decolorizer off the slide for a minute.

11. Methylene blue was used instead of safranin.

12. The student switched crystal violet and the safranin.

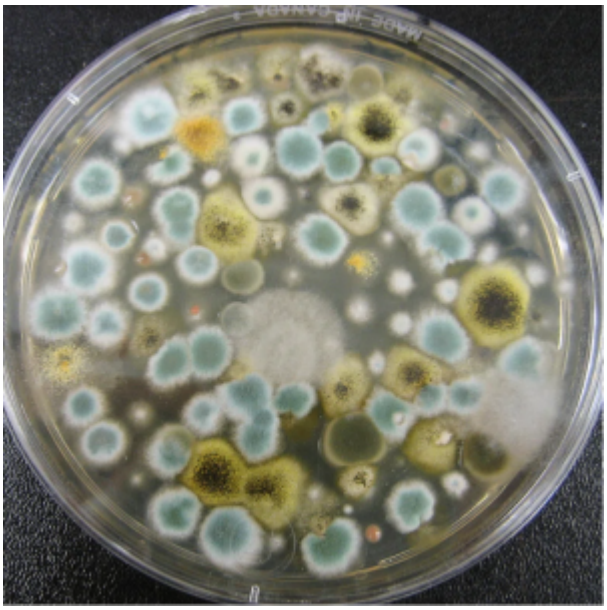
9. Colony Morphology

EMILIE MILLER, PH.D

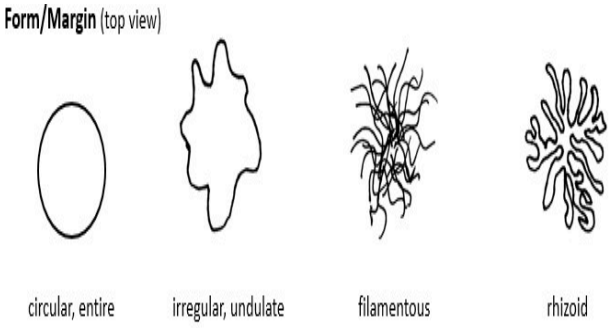
The student will:

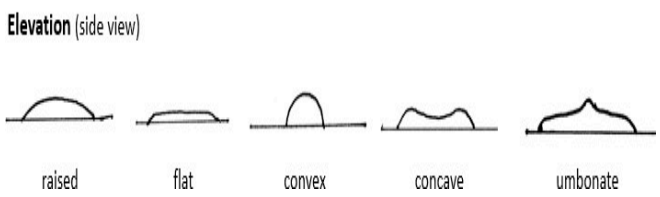
- Follow oral and written instructions and manage time in the lab efficiently.
- Apply correct terminology regarding microbial growth, when making observations.

On agar plates, bacteria grow in collections of cells called colonies. Each colony arises from a single bacterium or a few bacteria (CFU). Although individual cells are too small to be viewed with the unaided eye, masses of cells can be observed. Colonies can have different forms, margins, elevations and colors. Observing colony characteristics is one piece of information that microbiologists can use to identify unknown bacteria. (Petersen, 2016)



In this exercise you will be drawing colonies and describing each type's size, shape, elevation, margin, color and texture. Keep in mind that the colony characteristics of a microbe may change depending on the medium, time and temperature of incubation. The medium supplies the nutrients and other materials for the cell to use. Along with the size of individual cells, the colony size depends on the speed at which the cells divide. This is determined by the organism's inherent cell cycle, the availability of nutrients and the organism's optimal growth temperature.





Size can be actually measured in millimeters or described as "pin point."

Colony **form** means the shape and can be circular, irregular, or rhizoid (branched). This is the cumulative (macroscopic) effect of the microscopic cellular shape and arrangement.

Elevation refers to the cross sectional view or profile of the colony. It can be raised, flat, convex, concave or umbonate.

Margin describes the edge. A smooth edge is called entire. Other margins are undulate (an irregular, wavy edge), lobed (more pronounced wavy edge) or spreading (no distinct colonies).

Filamentous and rhizoid may also be used to describe the margin.

Colony **color** can be the result of the color of the actual cell, the result of pigments produced by the cell under certain conditions, or the interaction of certain cellular metabolites with components in the medium.

Texture can be dry, ridged or wavy, mucoid/shiny, dull, filamentous (hairy looking).

Procedure

- Observe one colony from each of the different pure cultures.
- Noe the full organism name written in full scientific form.
- A sketch of the top view and the side view. For the side view, draw a straight line to represent the agar surface as in figure 1 above.
- Use relevant terms to describe the size (measure, if .5 mm or larger), color, form, elevation and texture of each.

References:

Petersen, J. a. (2016). Laboratory Excercises in Microbiology: Discovering the Unseen World Through Hands-On Investigation. CUNY Academic Works. Retrieved from http://academicworks.cuny.edu/qb_oers/16

10. Colony Morphology Assignment

EMILIE MILLER, PH.D

Name:_____

Organism	Sketch (Top and Side)	Size and Color	Form, Margin, Elevation	Texture

1. What microscopic cell shape(s) might produce a convex colony?
2. What microscopic cell shape(s) might produce filamentous colonies?

11. Growth Patterns in Broth Assignment

EMILIE MILLER, PH.D

Bacteria	Growth Pattern	Oxygen Needs

1. What other factors, besides growth in media, are important to record about your microbes? Why?

2. You have two tubes of media that have been inoculated for 24 hours; one is turbid and one is clear to the eye. How would you determine if there are any bacteria in the clear tube? Describe 2 ways.

12. Aseptic Transfer

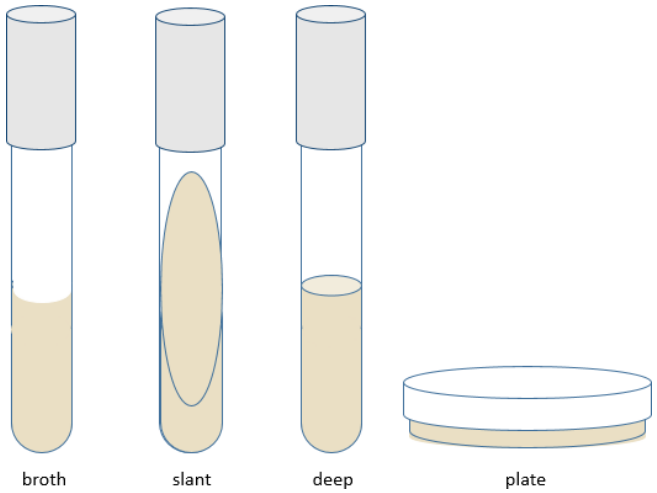
EMILIE MILLER, PH.D

Learning Objectives

The student will:

- Use aseptic techniques in the safe inoculation of various forms of media.
- Follow oral and written instructions and manage time in the lab efficiently.
- Apply correct terminology regarding microbiological techniques, instruments, microbial growth, media types and forms when making observations.
- Correctly perform various inoculation techniques and describe each technique's purpose.

To study bacteria and other microorganisms, it is necessary to grow them in controlled conditions. Microbes are grown in substances that provide the nutrients necessary to sustain their metabolic activities and reproduction called growth media or simply media. Growth media can be either liquid or solid.



A liquid medium is called a **broth**. Broths can be used to determine growth patterns in a liquid medium, and for certain types of inoculations and metabolic tests. They are also the method of choice for growing large quantities of bacteria. (Petersen, 2016)

Solid growth media usually contains **agar**, which is a mixture of polysaccharides derived from red algae. It is used as a solidification agent because it (1) is not broken down by bacteria, (2) contains no nutrients that can be used by bacteria and (3) melts at high temperatures, and yet is solid at temperatures used for most bacterial growth. Solid growth media is used in the following forms: **agar plates, agar slants** and **agar deeps**. Melted agar is poured into a test tube and then allowed to solidify vertically for an agar deep, or at an angle for an agar slant. **Agar plates** are made by pouring melted agarinto a petri dish. (Petersen, 2016)

Because of the relatively small tube opening (less opportunity to dry out or become contaminated) and the surface area available for growth, agar slants are commonly used to culture and store bacteria for intermediate periods of time (weeks). These types of cultures are called **stocks**. Deeps are often used to for certain differential metabolic tests.

In contrast to deeps and slants, agar plates have a large surface area for growth. Bacterial cells can be spread out over the surface so that they form discrete colonies which can be characterized. In a few weeks, you will be using a series of plate cultures to separate two different microbes from a mixture. In addition, specialized media in plate form is used for certain biochemical tests. (Petersen, 2016)

Microbiologists prefer to study the organisms in **pure culture**, a culture that contains a single microbial species. If an unintended microorganism is introduced into a pure culture, the culture becomes **contaminated**.

Aseptic technique is the collection of procedures and techniques designed to prevent the introduction of unwanted organisms into a pure culture or into the laboratory environment. The term "aseptic" literally means "without contamination." These procedures are as important for the experimenter's safety as they are for maintaining culture purity.

Sterilization is the complete removal all vegetative cells, endospores, and viruses from an item (OpenStax CNX, 2018). Sterilization is all or none; something is either sterile or it is not sterile. In this course, all **media**, the substance in which the cells are grown, is sterilized by autoclave.

An **autoclave** uses moist heat (steam) under pressure to destroy all life forms. Whereas most vegetative cells can be killed at temperatures between 60 and 80^{o}C, bacterial spores require temperatures above boiling (>100^{o}C) for destruction. With a pressure of 15-20 lbs./in^{2}, the autoclave can achieve a temperature of 121-132^{o}C. Media under these conditions for at least 20 minutes will kill all spores as well as vegetative cells. Larger volumes require longer exposure times to ensure sufficient heat transfer to the materials being sterilized. The steam must directly contact the liquids or dry materials being sterilized, so containers are left loosely closed and instruments are loosely wrapped in paper or foil. The key to autoclaving is achieving a temperature high enough to kill spores for complete sterilization (OpenStax CNX, 2018).

Disinfection is the killing or growth inhibition of vegetative microbes. Generally, spores and some hearty cells will survive disinfection. Chemical disinfectants, such as chlorine bleach or products containing chlorine, are used to clean nonliving surfaces such as laboratory benches, clinical surfaces, and bathroom sinks (OpenStax CNX, 2018). We will use a chorine-based disinfectant to clean our work surfaces and to clean up any culture spills. Note that sterilization and disinfection are not interchangeable! (Why?) Spraying your bench top with disinfectant does not make it sterile.

Antiseptics are antimicrobial chemicals safe for use on living skin or tissues. Examples include hydrogen peroxide and isopropyl alcohol (OpenStax CNX, 2018). When working in a microbiology laboratory, you must always remember that bacteria are present on all surfaces in the lab, as well as on your own hands and clothing. Aseptic techniques are designed to prevent the transfer of bacteria from the surrounding environment into a culture medium and from a culture to the environment. These techniques require care, concentration and practice. (Petersen, 2016)

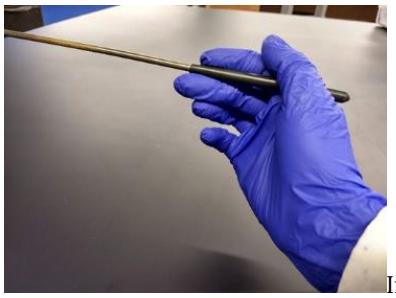
Transfer Procedures

Watch the video Basic Aseptic Transfers. Because these procedures are completely new to most students, I strongly recommend that you watch the video at least twice. Keep in mind the following principles. (Some of these have been covered in the Laboratory Safety Exercise. They bear repeating because they are very important to keep you safe.)

Always begin by preparing your work area and making the necessary labels. Make sure you are clear about what transfers need to be made. A transfer can be thought of in two parts, obtaining the cells (inoculum) from the source/parent culture and inoculating the new sterile tube or plate. Transfers, with very few exceptions, are performed by a single individual. You should not be holding the tube while your partner inoculates it.

Before you start

- Have your Bunsen burner, striker, and inoculating loop ready.
- Culture media must initially be sterile. Inspect your media before you start. If a culture medium appears cloudy or you observe unwanted growth, consult with your instructor to be sure it is not contaminated before using it.
- Label your tubes white marker.

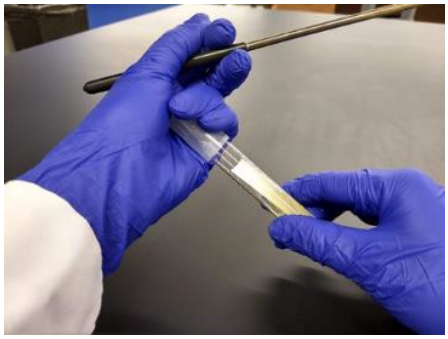
- Label plates on the bottom.
-  Inspect the parent cultures. If the cells have fallen to the bottom, be sure to re-suspend them by flicking the tube gently to mix. Never shake a tube.

Sterilizing your loop

- Hold the inoculating loop in your dominant hand like a pencil. To sterilize, place it in the flame for AT LEAST 10 SECONDS (or flame it at an angle so that the wire is along the margin of the inner blue flame. The ENTIRE WIRE must be heated RED HOT). Watch the clock for the time. Students tend to count too fast.
- Do not let the loop sit in the flame more than 15 seconds.
- Hold the instrument in the air allowing the wire to cool for about 15 seconds before making any transfers. Please do not wave it around to cool it.
- The wire is now sterile. If at this time, if you set it down on the bench top, which is not sterile, it must be flamed again before going into any culture. If a sterile instrument is touched to anything not sterile including your hand, sleeve, the outside of a tube or plate, a slide or the bench top, it becomes contaminated and cannot be used in an aseptic transfer.

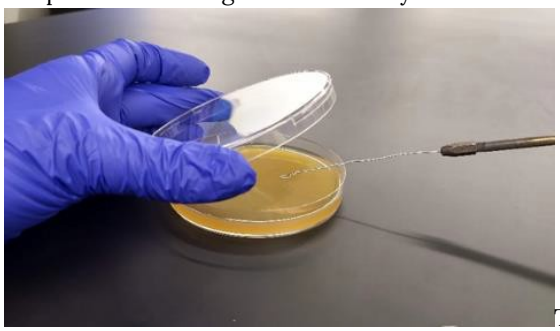
Obtaining the inoculum from a tube culture

- With your non-dominate hand, pick up the parent tube by grasping the tube just below the cap and lifting it out of the rack.
-  Grasp the cap with the pinky and ring finger of your dominate hand and gently twist the tube out of the cap keeping your dominate hand still. The cap is kept in your hand and never placed on the bench top.
- Heat the mouth of the open tube by placing it near the mouth of the flame for 10 seconds. (Or pass it through the flame). Heating creates convection currents, which carry airborne particles away from the mouth of the tube, preventing contamination of the culture or medium within.
- For a broth parent culture: Place the cooled loop into the broth and remove making

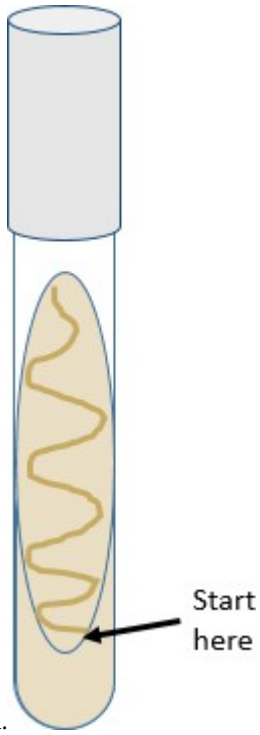

sure that you have a thin film of liquid filling the loop. Jiggling the loop in the broth is not needed and can result in the formation of tiny aerosol droplets. Please do not jiggle the wire.

- For a slant parent culture: Touch the cooled loop to the growth. Do not break the agar surface. Refrain from "swiping" a large mass of cells. You do not need to see cells on the loop to have millions!
- Again, heat the mouth of the tube after withdrawing the transfer instrument. This step incinerates any microbes that may have been deposited on the lip of the tube during the transfer.
- Replace the cap and set the parent tube back in the test tube rack.

To obtain the inoculum from a plate culture

- Keep the inoculating instrument in your hand.
- 
Turn the parent culture plate right side up.
- Lift the lid a short distance, with your non-dominate hand, so that the lid acts at a shield protecting the agar surface from falling microbes in the air.
- Touch the cooled loop to the growth. Do not break the agar surface. Refrain from "swiping" a large mass of cells. You do not need to see cells on the loop to have millions!
- Replace the lid immediately after withdrawing the transfer instrument and turn the plate upside-down again.

Inoculating a slant



- Keep the inoculating instrument in your hand.
- With your non-dominate hand, pick up the parent tube by grasping the tube just below the cap and lifting it out of the rack.
- Grasp the cap with the pinky and ring finger of your dominate hand and gently twist the tube out of the cap. Keeping your dominate hand still is especially important because there are cells on the loop at this point. Keep the cap in your hand.
- Heat the mouth of the tube for 10 seconds.
- Insert the loop all the way to the bottom of the slant surface.
- Drag the loop on the agar "snaking" your way up the slant creating a "fishtail pattern." This is called a **fishtail inoculation**.
- Again, heat the mouth of the tube after withdrawing the transfer instrument. Replace the cap and set the parent tube back in the test tube rack.
- Immediately flame the inoculating loop and wire for a full 10 seconds before setting it down

Inoculating a broth

- With your non-dominate hand, pick up the parent tube by grasping the tube just below the cap and lifting it out of the rack.
- Grasp the cap with the pinky and ring finger of your dominate hand and gently twist the tube out of the cap. Keeping your dominate hand still is especially important because there are cells on the loop at this point.
- Heat the mouth of the tube for 10 seconds.
- Insert the loop to the bottom of the broth liquid and then remove the loop. Jiggling is not necessary to dislodge cells.
- Again, heat the mouth of the tube after withdrawing the transfer instrument. Replace the cap and set the parent tube back in the test tube rack.

- Immediately flame the inoculating loop and wire for a full 10 seconds before setting it down.

After all inoculations

- Cultures to be incubated should be placed in the designated area for culture incubation. Otherwise, a student's culture may be disposed of accidentally.
- Be sure to turn it off the Bunsen burner when you are finished with it.

Because there is so much to remember, the first time you make transfers many of the above actions are repeated in context. After a few weeks practice, the repetition will no longer be necessary and it will be assumed that you will adhere to the procedures above without reminder.

Materials per student pair

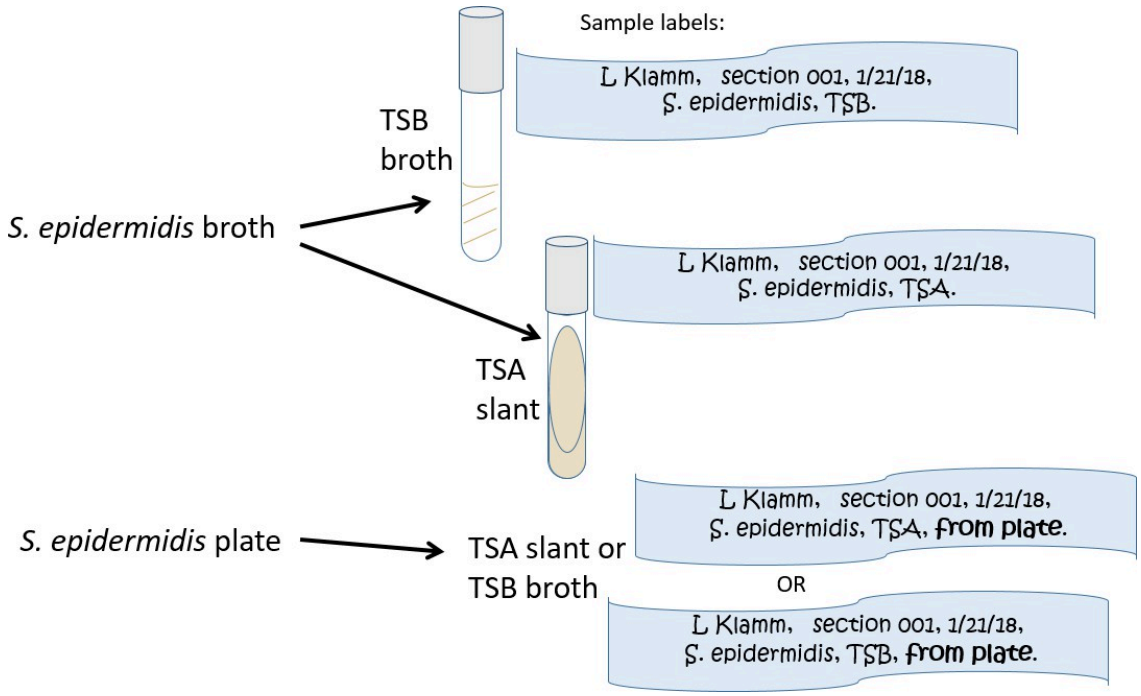
2 TSA slants
2 TSB tubes

Cultures

Staphylococcus epidermidis broth
Staphylococcus epidermidis plate

Procedure Lab 1

- Prepare for the exercise by turning on your Bunsen burner, washing your hands, and donning your gloves.
- Tube to tube Inoculations with living cells:
- Label one broth tube and one slant tube with the 4 label components. The organism name will be S. *epidermidis* (You may abbreviate on labels. See Figure 8 below.)
- Using the S *epidermidis* parent broth culture provided to you, transfer cells from the parent culture to your sterile broth tube using the techniques described above.
- With the same parent culture, inoculate your sterile slant with the **fishtail technique**.
- Plate to Tube Inoculation with living cells:
- Label your tube (broth or slant) with the 4 components as you did in above. Add one additional piece of information, "FROM PLATE." Once all the tubes are incubated, there is no way to tell the actual source of the cells unless you write it on your tube.
- Using the techniques from the video aseptically transfer cells from the parent S. *epidermidis plate to your sterile tube. Use the fishtail inoculation technique if you have the slant.*



- Place all your culture tubes in a test tube rack and carry them to the location for cultures to be incubated. You should have a total of 4 tubes. Take the cultures out of your rack and place them together in the rack provided.
- The culture tubes will be incubated for 24 hours at 37 C.
- Clean up by turning off your Bunsen burnder, disinfecting your work area, removing your PPE, and washing your hands and exiting the lab.

Procedure Lab 2

- Prepare for the exercise as described for Procedure Lab 1.
- Take an empty test tube rack to the bench where the incubated student cultures are located and retrieve your cultures from last week.
- Without disturbing the broths, describe the growth in each
- Mix and describe again. DO NOT SHAKE the tube, please. Hold the tube securely between thumb and forefinger, just below the cap. (Do not hold the tube by the cap!) Flick the bottom of the tube until all cells are suspended in the solution and none remain in the bottom.
- To determine the relative amount of growth in each broth, place your broth tubes broth tubes in a rack. Make sure that each has been mixed by flicking as described above. Arrange them in order from most turbid to least. Then assign each a number from 0 to 5 (5 represents the greatest amount of growth). Record each number in the corresponding row of the data table. Two or more tubes may have the same number if they have similar turbidity.
- To determine the relative amount of growth in each slant, use a method similar to the above. You will not be looking at turbidity, but instead relative width and thickness of the growth on the surface.

13. Aseptic Transfer Assignment

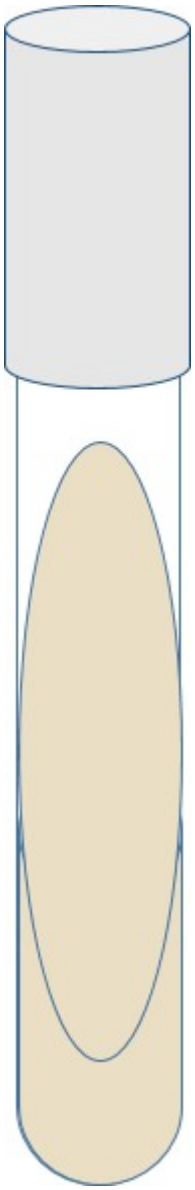
EMILIE MILLER, PH.D

Data and Observations

Name_____

Organism	Medium form	Description of growth before mixing	Description of growth after mixing	Relative amount of gro (0=none, 5=very turbid)
*Staphylococcus epidermidis from broth**	*to broth			
*S. epidermidis from broth**	*to slant		n/a	
S. epidermidis from plate*	*to broth			
S. epidermidis from plate*	*to slant		n/a	

Post Lab Questions



1. If someone had growth in the practice tubes labeled “sterile,” what does that indicate?
2. Draw the growth in one of your slant tubes the figure on the right, or freehand.
3. What inoculation technique did you use?
4. What instrument did you use?
5. Think about the relative amount of growth for each tube in terms of the initial amount of inoculum. If the inoculum came from growth on solid medium, there may have been more cells compared to an innoculum from a broth culture. Please describe and explain any correlation that you notice.
6. Notice how the scientific name for the organism is written in the data table. Describe the format the first time the name is written. (Use these terms: genus, capital, lower case) **In all future lab reports, you will be required to use this format.**
7. Explain why disinfection and sterilization are not interchangeable.

14. Streaking for Isolation

EMILIE MILLER, PH.D

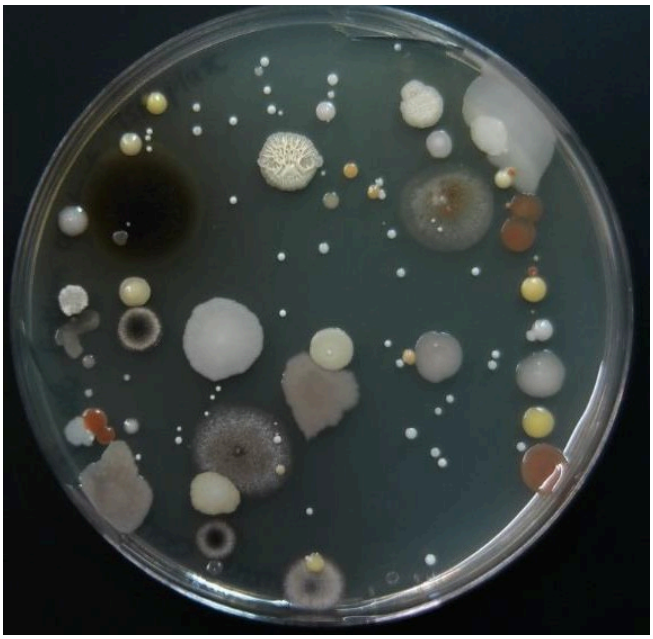
Learning Objectives.:

The student will:

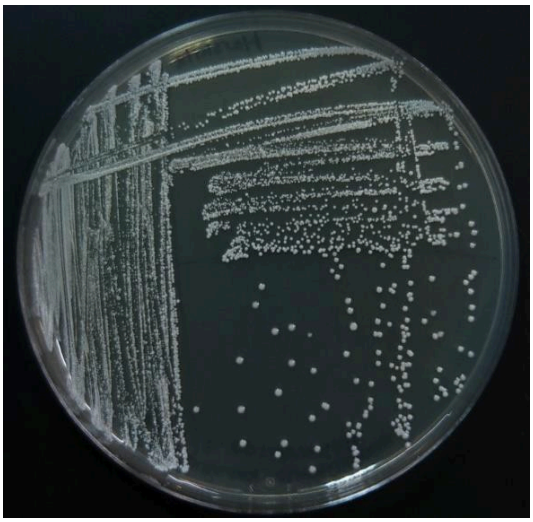
- Use aseptic techniques in the safe inoculation of various forms of media.
- Follow oral and written instructions and manage time in the lab efficiently.
- Apply correct terminology regarding microbiological techniques, instruments, microbial growth, biochemical testing, media types and forms and epidemiology when making observations.
- Correctly perform various inoculation techniques including the quadrant streak and the T streak techniques and describe each technique's purpose.

If a single bacterial cell is placed on the surface of a TSA agar plate and allowed to multiply for 24 to 48 hours, it would grow into a mass of cells visible to the human eye called a **colony**. Colonies formed by the same microbial species growing on the same medium will all look alike. This is because the cell shape, pigmentation, division plane, rate of cell division and other characteristics of the organism result in the progeny cells stacking on one another in a pattern resulting in a characteristic colony form.

If you swab a door handle, where bacteria are likely to be present, and then pass the swab across the surface of a TSA plate, cells would be deposited onto the surface from the swab. Initially, the swab may have a fairly high concentration of cells and the area touched by it will have lots of different cell types placed close together. After these grow up, the cells' progeny will crowd together and overlap with other cells' progeny forming areas called confluent growth. This is the type of growth you observed on the surface of the TSA slants in the Basic Aseptic Transfers exercise.

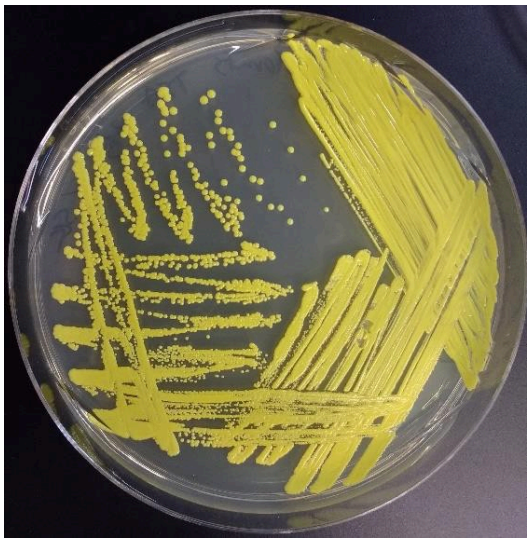


As the swab moves across the agar leaving cells on the agar in a zig zag pattern, regions touched later in the process will have fewer and fewer cells. Individual cells are far enough apart that each one would grow into a discrete **isolated colony**. The result may look something like the figure to the right, Because the door handle likely has a variety of microbes on it, there are numerous colony forms. This technique is called a **zig zag streak** and is one type of **isolation streak** method.



Now consider streaking a sample from a pure TSA broth culture prepared for you. If there is visible turbidity, there will be a high density of cells. If you used the same zig zag streaking pattern, the cells would never be reduced in concentration such that you could get isolated colonies. In order to reduce the cell density on the surface of the plate, we can use a **T-Streak or a quadrant streak technique**.

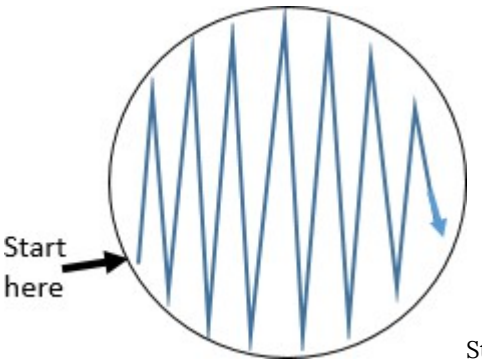
The T-streak and quadrant streak are forms of dilutions on a solid surface (Franklund, 2018). In these two techniques the plate is divided into sections. Bacteria are deposited in the first section at full strength from the source. Then the inoculating loop is sterilized. From this point on, no additional cells are added to the agar surface. The sterile loop is used to spread out cells that have already been placed on the plate. After spreading cells from the first area to the second, the loop is sterilized again. This eliminates extra cells from the loop. The sterile loop is used to spread some cells from the second area into the third area diluting them further. (In a quadrant streak, cells are spread into a forth area as described.) After all the regions have been inoculated, the hope is that in the last section cells are far enough apart so that they grow up into isolated colonies.



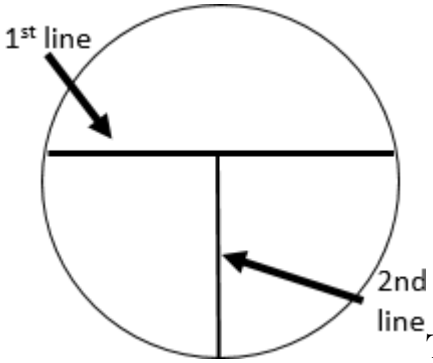
This technique allows one to observe isolated colonies and characterize them and determine if your observations are consistent with our expectations for the organism you are working with. If you are working with a pure culture, you would expect that all the colonies would look the same, similar size, color, shape etc. One or more different looking colonies indicates your culture was contaminated or you created contamination by poor aseptic technique.

Zig Zag Streak

- Aseptically obtain a sample from the media using a sterile inoculating loop.
- Turn the agar plate right-side-up.
- Hold the plate lid so that it acts as a shield protecting the agar surface from microbes falling from the air.



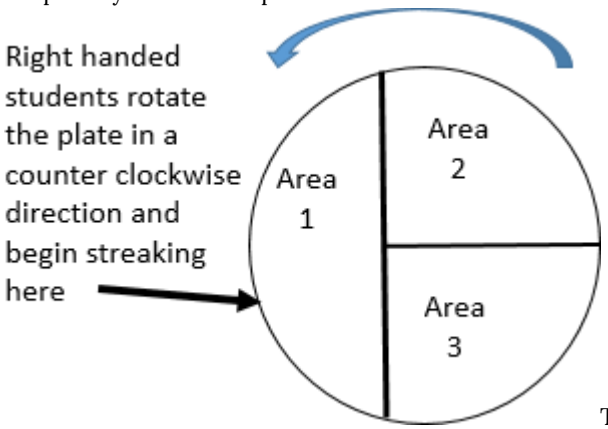
- Starting the streak on the side of the plate farthest from your dominant hand, pass the loop on the surface of the agar in a zig zag pattern filling the surface of the plate. See figure to the right.
- Replace the lid, and immediately flame the loop.
- Place the plate upside-down for incubation.
- Some tips for a good zigzag streak: Use as much of the agar surface as you can. Make broad strokes that span the width of the plate.



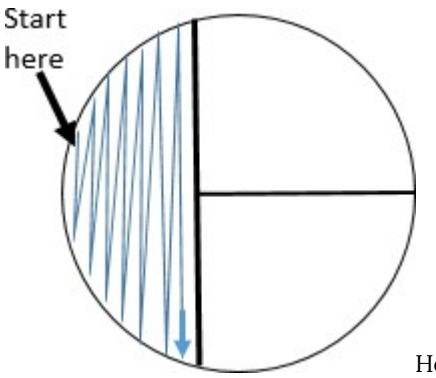
T-streak

The steps of the T-streak are as follows.

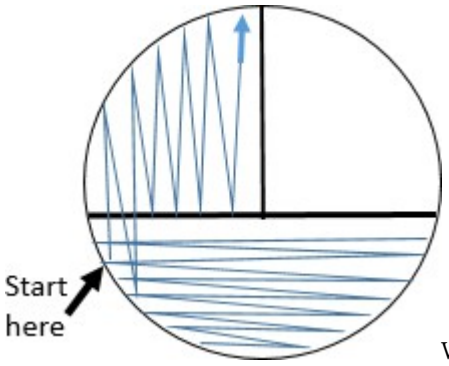
- Draw lines on the bottom of your plate as shown in figure to the right. The first line should be a little off center. Drawing these is not absolutely necessary, but they help when you are first learning.
- Aseptically obtain a loopful of the culture and set the tube back in a rack.



- Turn the plate right-side up and place it on a piece of white scratch paper so that the lines can be seen.
- Rotate the plate so that area 1 is farthest from your dominate hand.

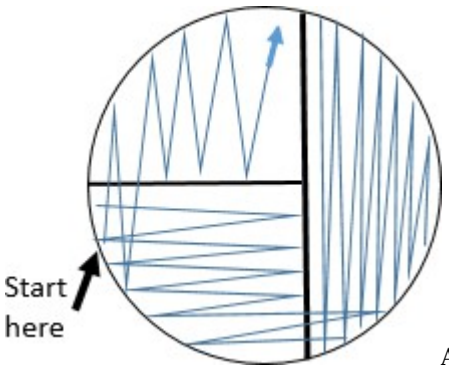
- 

Holding the plate lid so that it acts as a shield protecting the agar surface from microbes falling from the air, pass the loop on the surface of the agar in area 1 in the pattern shown.
- Replace the plate lid, flame the loop, and allow it to cool.
- Rotate the plate 90^{o}.

- 

WITHOUT OBTAINING ADDITIONAL CULTURE, spread the cells already placed on the agar surface with the sterile loop by streaking in the pattern shown. This step pulls highly concentrated cells from area 1 into area 2 spreading them out.

- Replace the plate lid, INCINERATE THE LOOP, and allow it to cool. Rotate the plate 90^{o}.

- 

Again, WITHOUT OBTAINING ADDITIONAL CULTURE, spread some of the cells from area 2 into area 3 by streaking in the pattern shown.

- Replace the plate lid and flame the loop again before setting it down. Turn the plate upside-down for incubation and storage.

In this technique it is essential that you flame your loop after each area is inoculated. This is what reduces the cell density because you are spreading cells already on the plate. You are not adding additional cells by using a loop with cells on it. You eliminate additional cells by the flame step. After incubation, the goal is at least 3 well isolated colonies.

Keep in mind that you want to use as much of the agar surface as possible. Your streaks should span the width of the plate. The loop passes through the previous area 2- 3 times and then does not touch that area again. If you keep touching the previous high density streak, you will pull too many cells into the next area and will not reduce the number enough to get isolated colonies. If you do not cross over the previous area enough, you will not have enough cells in the next one.

Each of the streaks is at a 90^{o} angle to the previous streak and parallel to the lines you drew. The areas have similar areas. If you choose to cool your loop in the agar, always use a spot close to the edge and away from any previous streak. The resulting growth pattern should be confluent in area 1, more diffuse in area 2 and least growth in area 3.

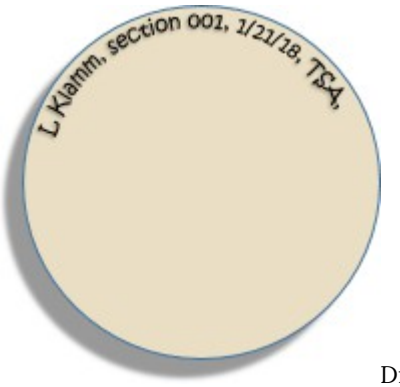
Materials per student

2 TSA plates (free of excess condensation),
inoculation loop, Bunsen burner, striker, pen

Pure cultures of microbe in broth
Mixed culture

Practice: https://learn.chm.msu.edu/vibl/content/streakplate.html

Procedure lab 1



- Draw the Zig Zag streak pattern and the T-streak pattern in the circles on the observations page.
- Label one plate with your name, date, microorganism, and medium. Keep the writing on the edge of the plate so that it will not obscure the growth on the plate.
- On this plate, you will perform a zig zag inoculation from prepared culture.
- Follow the steps for making a Zig zag inoculation.

- You will now make a T-Streak of the pure culture.
- Label the bottom of the plate with the 4 components. Include the specific microbe that you are using. (Do not use "pure culture.")
- Follow the procedure for a T-Streak.
- Take all of your plates to the location to be incubated. Be sure to place them upside-down. They will be incubated 25-37^{o}C for 24-48 hours.

Procedure lab 2

- Observe your 2 plates. Fill in the observations table. For the source, write in the full scientific name of the organism.
- Evaluate your technique using the criteria listed. Get input from another lab member and instructor if needed. Check the items that characterize your plate.
- Dispose of your plates in the biohazard waste.

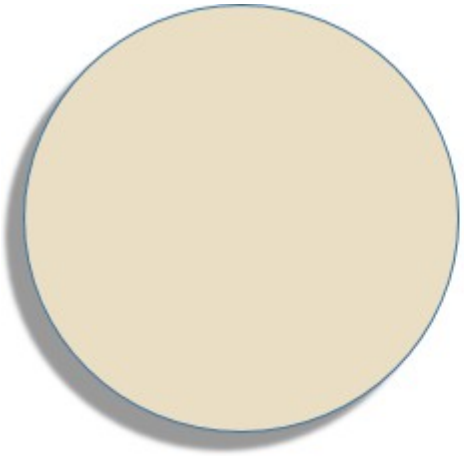
References:

Franklund, C. (n.d.). Microbiology Laboratory Manual, Observing and Recording the Microbial World. Farris State University. Retrieved 2018, from https://github.com/WeeBeasties/microbiology-laboratory-manual

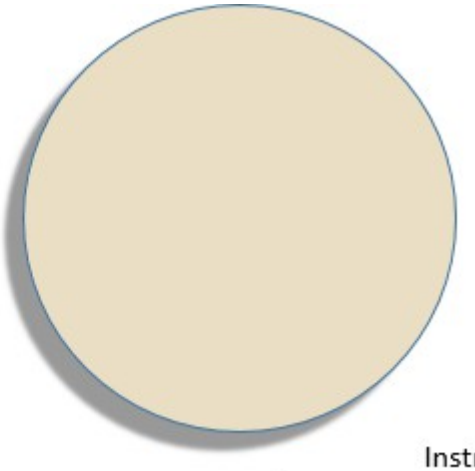
15. Streaking for Isolation Assignment

EMILIE MILLER, PH.D

Name _____



Zig Zag



T-Streak

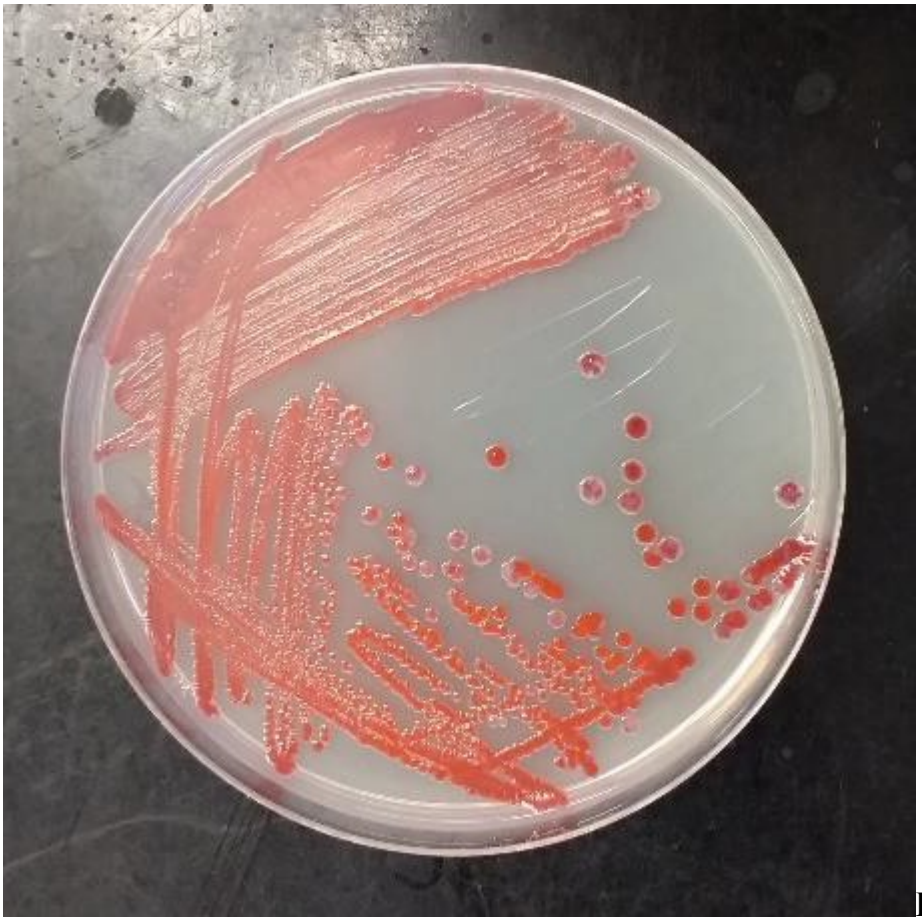
Instructor initials _____

Sample source	Area (1, 2 or 3) where isolated colonies are found	Number of different looking colonies	Is there evidence of contamination?

Your T-streak Evaluation

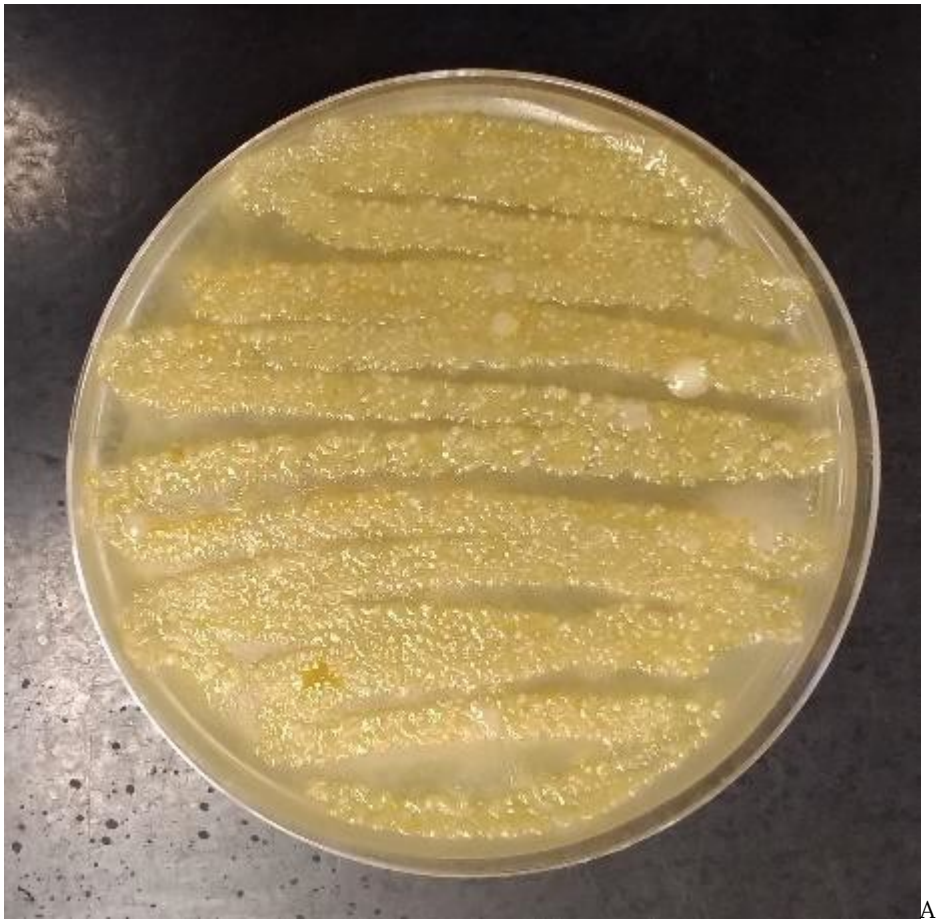
- isolated colonies at least three
- streak pattern: correct order, correct direction
- amount crossovers (not too little, not too much)
- entire surface used
- no gouging of the agar
- no inappropriate crossovers

  90^{o} angle of streaks
- adequate room for final area
- growth pattern: a lot to a little
- label : all components, legible, on bottom of plate
- incubated upside-down



Post Lab Questions

- What streaking technique is used here?

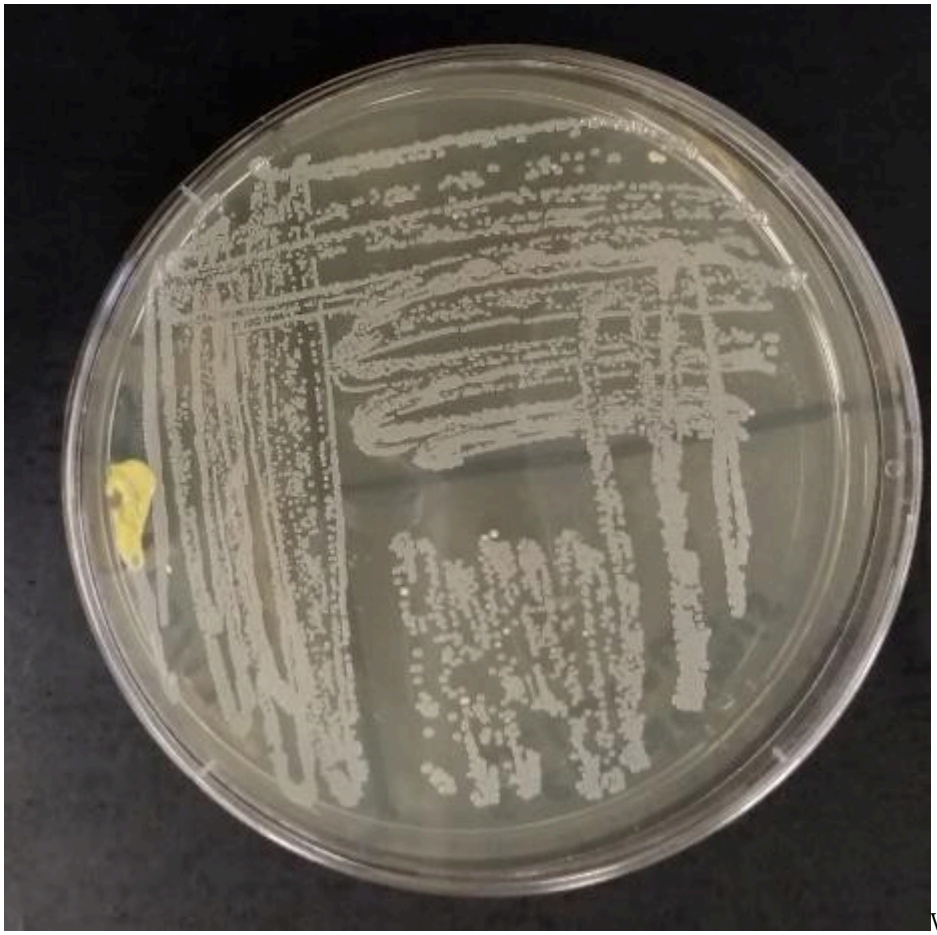
-  A gross lab sink is sampled with a cotton swab and plated onto this TSA plate. What streaking technique is used here?

  Was the technique a good choice? Why or why not?

-  A pure culture is streaked onto this TSA plate. What technique is used here?

  Was the technique a good choice?

  What did the person likely do wrong when performing the technique? (There are at least 2 things.)

-  What technique is used on this plate?

    Was the technique a good choice?

    What did the person likely do wrong when performing the technique?

16. Acid-Fast and Endospore Staining

EMILIE MILLER, PH.D

Objectives

- Learn about microorganisms that have acid-fast cell walls.
- Perform the acid-fast staining procedure and view cells under oil immersion.
- Learn about some of the microorganisms that are endospore formers.
- Perform the endospore *staining* procedure and view cells under oil immersion.

Acid-Fast Staining

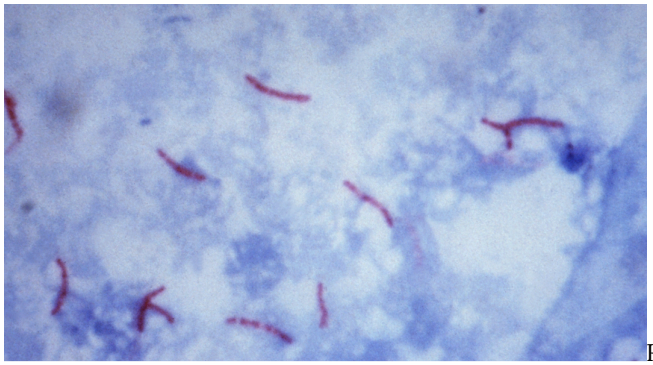
Most bacterial species are either Gram positive or Gram negative, however some organisms have different cell wall properties that make them difficult to stain with this method. For example, some species of bacteria have a waxy lipid (**mycolic acid**) in their cell walls. These organisms generally do not Gram stain very well (those that do would usually appear gram positive) and are more clearly visible with the acid-fast staining technique.

Acid-fast staining was developed by Robert Koch in 1882 and later modified by other scientists. Koch used the method to observe the "tubercle bacillus"—what we now call *Mycobacterium tuberculosis*, in sputum samples. While acid-fast and gram staining are both differential stains, the acid-fast stain is much more specific. Many bacteria are either gram positive or gram negative, but very few are acid-fast. Two acid-fast genera that are important as human pathogens are *Mycobacterium* and *Nocardia:* Pathogenic species include *M. tuberculosis*, *M leprae*, *M. bovis*, *M. avium*, *and* N. *asteroides.* The protozoan parasite *Cryptosporidium* can also be stained using this procedure.

There are 2 different methods of acid-fast staining—both involve techniques that make the cell wall more permeable to the primary stain. The **Ziehl-Neelson method** uses steam heat to allow stain to penetrate, whereas the **Kinyoun** (cold method) uses a wetting agent mixed in with the primary stain. In this lab we will be using the **Ziehl-Neelson method** method.

Ziehl-Neelson Staining Procedure

- Prepare a slide with *Mycobacterium smegmatis* on one side and *Micrococcus luteus* on the other side. (Alternatively, both bacteria may be mixed into one smear). Be sure to break up clumps of *M. smegmatis* before staining.
- Air dry and heat fix as usual.
- Add a small piece of bibulous paper on top of the sample
- Add carbol fuchsin (primary stain) on top of the paper to saturate: leave on for 5-7 minutes and place over a steam bath in the hood. Apply more carbol fuchsin as needed during the process
- Rinse with water. Note: not all of the primary stain will be removed by water in this step
- Decolorize with Acid-alcohol: 1-2 quick rinses
- Rinse with water.
- Add **methylene blue** (counterstain) and leave on for 2-3 minutes.

-  Rinse with water, blot dry, and view.

Acid-fast organisms retain the primary stain and will appear bright red: non acid-fast organisms are decolorized with acid-alcohol and pick up the methylene blue counterstain. Epithelial cells that may be present in a clinical sample will also appear blue.

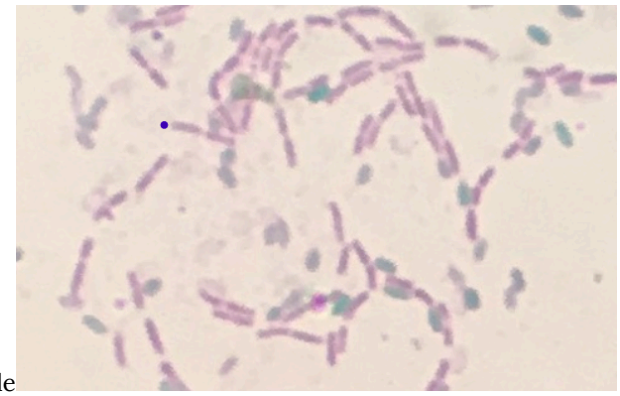
Endospore staining

Endospores are the most resistant forms of life. They can resist desiccation (drying), boiling and radiation—in addition, disinfectants and antibiotics cannot penetrate an intact spore coat. For this reason they are difficult to eliminate from the environment with standard methods of disinfection, and they are difficult to treat in the case of an infection.

Endospores are a survival mechanism for the bacterial species that produce them. When conditions are favorable, vegetative bacterial cells will continue to grow and divide; however when nutrients are depleted, cells will begin to form endospores. Endospores are not metabolically active, but contain all the materials needed by cells to survive. When conditions for growth are again favorable, the spore will germinate and form a cell that is identical to the cell that produced it. Endospores are produced by certain types of Gram positive- bacilli, like *Clostridium* and *Bacillus*, as well as other species. Endospore-forming pathogens include *C. tetani*, *C. botulinum*, *C. difficile*, *and B. anthracis*.

Endospore Staining Procedure

- Prepare a smear with *Bacillus subtilis* on one side of the slide, any other bacteria on the other side
- Air dry and heat fix as usual.



- Add a small piece of bibulous paper on top of the sample
- Add **malachite green** (stains spores) on top of the paper to saturate: leave on for at least 10 minutes. and place over a steam bath in the hood. Apply more as needed during the process
- Rinse briefly with water.
- Stain cells with safranin (stains vegetative cells) for 1 minute.
- Rinse with water, blot dry and view.

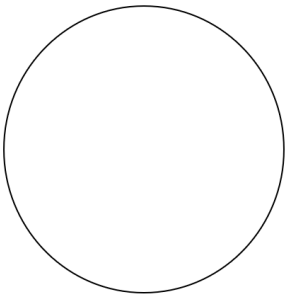
If the bacterial species is an endospore former, you will see pink vegetative cells as well as green oval-shaped endospores: non-spore formers will appear only as pink vegetative cells.

17. Acid Fast and Endospore Staining Assignment

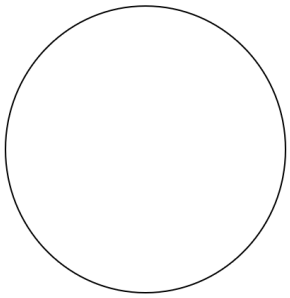
EMILIE MILLER, PH.D

Name:_____

Acid Fast Stain

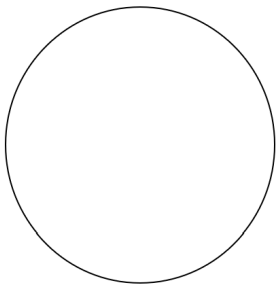


Species _____
Morphology _____
Arrangement _____
Acid fast result _____

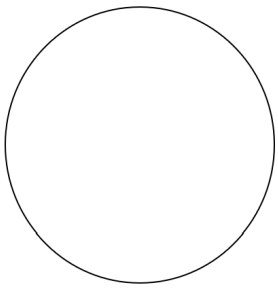


Species _____
Morphology _____
Arrangement _____
Acid fast result _____

Endospore Stain



Species _____
Morphology _____
Arrangement _____
Endospore result _____



Species _____
Morphology _____
Arrangement _____
Endospore result _____

Review Questions

1. Distinguish between the Ziehl-Neelson and Kinyoun methods of acid-fast staining.
2. Why is it important to leave the malachite green on the slide for at least 10 minutes in the endospore- staining procedure?
3. Do you think you would find more endospores in a freshly prepared culture or in an older culture of *Bacillus subtilis*? Explain.
4. Why do you think an infection caused by an endospore former might be harder to treat than one caused by a non-spore former?

18. MSA, MacConkey, EMB Plates

EMILIE MILLER, PH.D

The student will:

- Use aseptic techniques in the safe inoculation of various forms of media.
- Follow oral and written instructions and manage time in the lab efficiently.
- Apply correct terminology regarding microbiological techniques, instruments, microbial growth, biochemical testing, and media types when making observations.
- Correctly perform various inoculation techniques and describe each technique's purpose.
- Make accurate observations and appropriate interpretations of biochemical test results and use them in the identification of potentially disease causing microbes.

In this course you will encounter many types of growth media. Most of the types, TSA/TSB (Tryptic Soy Agar/Broth) and NA/NB (Nutrient Agar/Broth) for example, are **all purpose or general media**. They contain a wide variety of complex carbon, nitrogen and sugar compounds. You will often see terms like beef extract, peptone, tryptone, soytone in the list of ingredients. These components are slurries of animal and plant tissue that have been partially hydrolyzed (broken down) so that the components are more readily available. These media contain many different complex molecules, however the exact amount and types of each is unknown. These media will support a wide range of **nonfastidious** microbes with differing nutritional requirements.

Media that inhibit the growth of unwanted microorganisms and support the growth of the organism of interest by supplying nutrients and reducing competition are called **selective media**. (OpenStax CNX, 2018) Selective media are formulated with inhibitors such as antibiotics or high NaCl concentration. When studying a mixed sample, selective media can be helpful. For example, if you suspect a patient is carrying *Salmonella* (a pathogenic Gram negative bacillus), you may plate a stool sample on a selective medium containing an antibiotic effective against Gram positive bacteria. By eliminating the Gram positive organisms, the range of organisms growing on the plate will be narrowed to Gram negatives. Thus, the variety of bacteria you will need to study is reduced.

The fact that a medium does not grow every microbe, does not make it selective. For example, TSA is an all-purpose medium and a wide range of organisms grow on it. Certain fastidious organisms, however, will fail to grow or grow poorly on TSA because it lacks the specific nutrients required by those bacteria. Even so, TSA is not classified as selective. To be selective, a medium must contain a specific substance intentionally added to inhibit certain microbes and not others.

Differential media contain substrates and indicators (often pH indicators) that make a certain biochemical process visible. Differential media allow one to differentiate between types of organisms growing on the plate because each has a distinct appearance based on whether or not it is carrying out a particular biochemical reaction. "Color changes are the result of end products created by interaction of bacterial enzymes with differential substrates in the medium or, in the case of hemolytic reactions, the lysis of red blood cells in the medium" (OpenStax CNX, 2018). Differential media can be used to distinguish between bacteria that can ferment a specific type of sugar and those that cannot or between bacteria that utilize a certain electron acceptor and those that do not.

Some media are selective, some are differential and some are both. We will study Mannitol Salt Agar, MacConkey agar, and EMB as examples.

Mannitol Salt Agar (MSA) can be used to presumptively isolate and identify *Staphylococci* from human samples. Refer to the compositions of MSA and MacConkey agar below. MSA contains 75 g/L NaCl (7.5%) compared to the 5 g/L found in TSA and other all-purpose media. MSA favors the growth of salt tolerant microbes,

namely *Staphylococci*, because other bacteria from a human sample, are inhibited by the high NaCl component. In addition, to distinguish pathogenic *Staphylococci*, *namely S. aureus* from other common *Staphylococci*, the substrate mannitol (a sugar) and the pH indicator phenol red are added. If the organism ferments mannitol, acids will be produced as byproducts. These acids will lower the pH changing the indicator from pink to yellow. *S. aureus* can ferment mannitol, while other common *Staphylococci* found in humans cannot.

MS Agar		
Selectivity	**Interpretation**	**Identification**
Growth	Organism not inhibited by NaCl	*E.g.*, *Staphylococcus*, *Micrococcus*
No growth	Organism inhibited by NaCl	Not *Staphylococcus*
Differentiation		
Yellow halo	Organism ferments mannitol	Probable *S. aureus*
No yellow halo	Organism does not ferment mannitol	*Staphylococcus* species (other than *S. aureus*); *Micrococcus* (yellow colonies)

MacConkey agar contains bile salts and crystal violet, which interfere with the growth of many gram-positive bacteria and favor the growth of gram-negative bacteria, particularly the **Enterobacteriaceae**. These species, commonly named **enterics,** reside in the intestine, and are adapted to the presence of bile salts. Enterics can be further characterized by their ability to ferment lactose. In MacConkey agar, the lactose fermenters (**coliforms**) utilize lactose in the medium producing acid, lowering the pH. The medium is supplemented with the pH indicator neutral red, which turns to hot pink at low pH. (OpenStax CNX, 2018) Thus, lactose fermenters are observed as bright pink colonies or with a bright pink halo surrounding the growth. Non-lactose fermenters (noncoliforms) include some notable human pathogens, such as *Salmonella* spp., *Shigella* spp., and *Yersinia pestis*. (OpenStax CNX, 2018)

Eosin-methylene blue agar (EMB) contains peptone, lactose, sucrose and the dyes eosin Y and methylene blue. Gram positive organisms are inhibited by the dyes, so this medium is selective for Gram negative bacteria. The medium differentiates based on the ability to ferment lactose (and/or sucrose.) Organisms that cannot ferment either of the sugars produce colorless colonies. Organisms that ferment the sugars with some acid production produce pink or purple colonies; organisms that ferment the sugars and produce large amounts of acid form colonies with a green metallic sheen. This medium is commonly used to detect the presence of fecal coliforms (like *E. coli*)—bacteria that grow in the intestines of warm-blooded animals. Fecal coliforms produce large amounts of acid when fermenting lactose and/or sucrose; non-fecal coliforms will produce less acid and appear as pink or purple colonies.

EMB Agar		
Result	**Interpretation**	**Identification**
No or poor growth	Organism inhibited by dyes	Organism is Gram-positive
Good growth	Organism not inhibited by dyes	Organism is Gram-negative
Colorless growth	Organism does not ferment sucrose or lactose	Non-coliform
Growth is pink and mucoid	Organism ferments lactose and/or sucrose with some acid production	Coliform bacteria
Growth is dark (purple to black with or without green metallic sheen)	Organism ferments lactose and/or sucrose, with large amounts of acid production	Possible fecal coliform (*E. coli*)

Coliform bacteria are microbes found in the digestive systems of warm-blooded animals, in soil, on plants, and in surface water. (Note their ability to assist mammals in the digestion of milk sugar, lactose.) These microbes typically do not make you sick; however, because microbes that do cause disease are hard to test for in the water, "total coliforms" are tested instead. If the total coliform count is high, then it is very possible that harmful germs like viruses, bacteria, and parasites might also be found in the water. Thus, they are considered one of several a water quality indicators. (U.S. Centers for Disease Control and Prevention, 2019)

MacConkey Agar (MacC) Pancreatic digest of Gelatin 17g/L Peptones (meat and Casein) 3g/L Lactose 10g/L Bile Salts 1.5g/L Sodium Chloride 5g/L Agar 13.5g/L Neutral red 0.03g/L Crystal Violet 1mg/L

Mannitol Salts Agar (MSA) Pancreatic digest of Casein 5g/L Peptic digest of Animal Tissue 5g/L Beef extract 1g/L Sodium Chloride 75g/L D-Mannitol 10g/L Phenol red 25mg/L Agar 15g/L

Tryptic Soy Agar (TSA)

Tryptone 17g/L
Soytone 3g/L
Sodium Chloride 5g/L Dipotassium Phosphate 2.5g/L Agar 15g/L

Materials per student

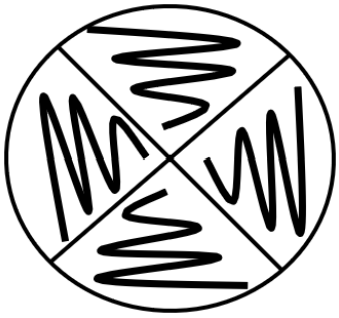
1 EMB plate*
1 MSA plate*
1 MacConkey Agar plate*
* Be sure to label each as you take it. They look very similar!

Cultures

Fresh overnight broth cultures
E. coli
Staphylococcus epidermidis
Staphylococcus aureus
Pseudomonas aeruginosa

Procedure Lab 1

- Obtain one EMB plate, one MSA plate and one MacConkey agar plate. On the plate base write the medium abbreviation when you remove it..
- Add the following to the bottom of each plate around the edge: your name, date.
- Divide each plate into 4 sections. Label each section with an abbreviation for each organism. Write small but legibly! Each plate will be inoculated with each of the 4 organisms.
- Aseptically spot inoculate each sector with the corresponding microbe. A **spot inoculation** is a short (1 cm) streak line as shown. (DO NOT STAB the agar.)



- Keep the inoculation lines short and away from the other inoculations on the plate.
- Be sure to make each inoculation separately and refrain from "double dipping."
- Be sure to hold the lid of the plate above the plate surface to protect it from airborne contaminants.
- It may help to set the plate on a piece of white scratch paper so that you can see the sector lines.
- Place the plates upside-down in the location designated for cultures to be incubated.
- They will be incubated for 24-48 hours at 37^{o}C.

Procedure Lab 2

- Obtain your plates and make observations in the data table.

- Use + for growth and – for no growth. If growth is poor, simply write "poor growth" in the table.
- In the appearance column, describe any color change in the growth and/or the surrounding medium. If the organism did not grow, you will not be able to describe an appearance. In this case write "N/A" in the table.
- Be sure to use the organism's full scientific name written correctly.
- After making observations, dispose of your plates in the hazardous waste.

References:

OpenStax CNX. (2018, Mar 19). OpenStax Microbiology. Retrieved from http://cnx.org/contents/e42bd376-624b-4c0f-972f-e0c57998e765@4.24

U.S. Centers for Disease Control and Prevention. (2019, June 30). *Well Testing*. Retrieved from Healthy Water: https://www.cdc.gov/healthywater/drinking/private/wells/testing.html

19. MSA, MacConkey, EMB Plates Assignment

EMILIE MILLER, PH.D

Name Lab Section

Data and Observations

Organism	Growth on EMB +/-	Appearance on EMB	Growth on MacC +/-	Appearance on MacC	Growth on MSA +/-	Appearance on MSA

Post Lab Questions

- Based on your observations in this exercise, you should be able to list several characteristics of each organism. Fill in this interpretation table. If you cannot make a determination based on these results, write in "N/A." (Since you have already written the full scientific name of each organism, you may appropriately abbreviate them here.)

Organism	Gram positive/Gram negative	ability to ferment lactose	salt tolerance	ability to ferment mannitol

1. Compare the interpretation table above to results for a Gram Stain. Are there any discrepancies? If so list them and give a brief reasonable explanation.

2. Is MSA selective, differential or both? What ingredient(s) make it so?
   Selective: Ingredients:
   Differential: Ingredients:
3. Is MacConkey selective, differential, or both? What ingredient(s) make it so?
   Selective: Ingredients:
   Differential: Ingredients:
4. Is EMB selective, differential, or both? What ingredient(s) make it so?
   Selective: Ingredients:
   Differential: Ingredients:
5. A patient presents with an oozing, pus-filled skin lesion. You suspect a *Staphylococcus aureus* infection. What medium would you use to plate a sample from the lesion? What result do you expect if the *Staphylococcus aureus* is present?

20. Blood Agar

EMILIE MILLER, PH.D

The student will:

- Use aseptic techniques in the safe inoculation of various forms of media.
- Follow oral and written instructions and manage time in the lab efficiently.
- Apply correct terminology regarding microbiological techniques, instruments, microbial growth, biochemical testing, and media types when making observations.
- Correctly perform various inoculation techniques and describe each technique's purpose.
- Make accurate observations and appropriate interpretations of biochemical test results and use them in the identification of potentially disease causing microbes.

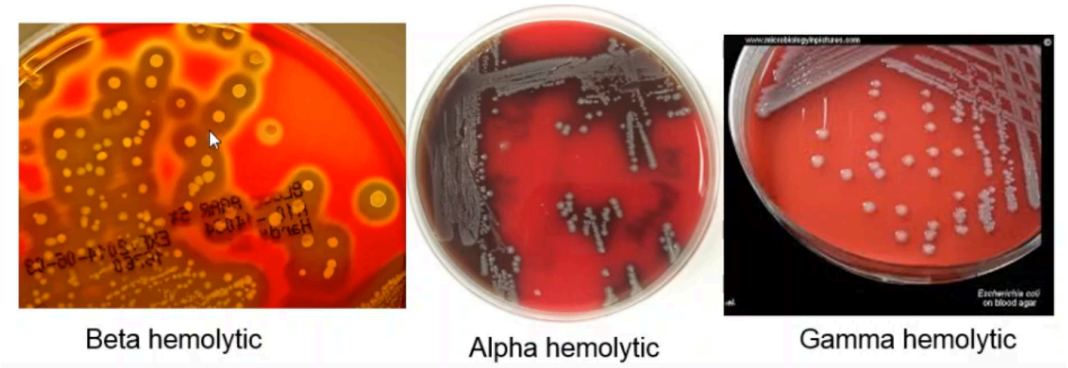
Hemolysis, the ability of an organism to lyse red blood cells and breakdown hemoglobin can be seen when sheep's blood is added to the medium. Substances produced and released (exotoxins) which accomplish this are called **hemolysins**. The addition of blood has a two-fold purpose. It is an **enrichment** to support the growth of fastidious organisms and it is the substrate with which hemolysins, if present, will interact. In blood agar, the hemolytic activity can be visualized without an indicator substance usually added to most differential media.

Before inoculation, a **blood agar** plate appears red and opaque due to the large particulate red blood cells (RBCs) contained within. If an organism growing on the plate produces **beta (β) hemolysins**, the RBCs are completely destroyed or lysed and the medium surrounding the growth loses its opacity. If this is the case, the medium may become transparent enough that you may be able to read printing through the clearing.

If the blood cell membranes are partially lysed by the exotoxin, the RBC contents will leak out without the complete destruction of the blood cell. The hemoglobin from the RBC will be reduced to methemoglobin resulting in a green or brown discoloration to the medium surrounding the colony (Buxton, 2005). This incomplete hemolysis is called **alpha (α) hemolysis. "**On prolonged incubation, many alpha hemolytic organisms will begin to appear more clear, but if the surrounding medium contains any shades of brown or green the 'hemolysis' is still considered 'alpha.'" (Buxton, 2005)

If the bacterium does not produce a hemolytic exotoxin, there will be no change to the RBCs, and the medium will remain opaque red. This lack of hemolysis is classified as **gamma (ϒ) hemolysis**.

Hemolysins are involved in the pathogenicity of bacteria. These membrane-disrupting **exotoxins** affect cell membrane function either by forming pores or by disrupting the phospholipid bilayer in host red blood cell membranes. They cause leakage of the cytoplasmic contents and cell lysis. They can affect other cells as well. The gram-positive bacterium *Streptococcus pyogenes* produces two types of streptolysins, O and S. Streptomycin O is not active in the presence of oxygen (**oxygen labile**), whereas streptolysin S is active in the presence of oxygen (**oxygen stable**). Other important pore- forming membrane-disrupting toxins include alpha toxin of *Staphylococcus aureus* and pneumolysin of *Streptococcus pneumoniae*. (OpenStax CNX, 2018)



Blood Agar (TSB w/ 5%SB) Pancreatic digest of casein 14.5 g/L Peptic digest of soybean meal 5.0 g/L Sodium chloride 5.0 g/L, Agar 14.0 g/L, Defibrinated Sheep Blood 5.0%

Materials per student:

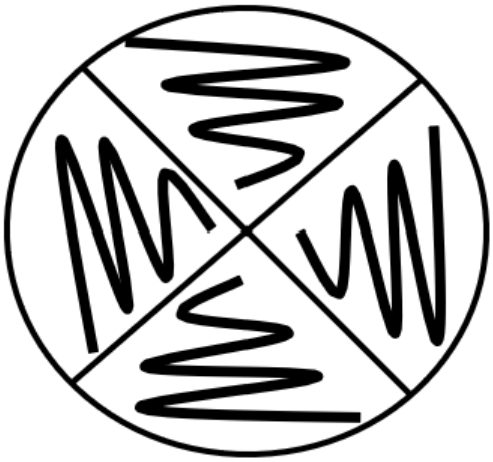
1 blood agar plate (TSA w/ 5% SB)

Cultures

Fresh overnight broth cultures of 4 microbes

Procedure Lab 1

- .Obtain one blood agar plate
- Add the following to the bottom of the plate around the edge: your name, date.
- On the bottom, divide each plate into 4 sections. Label each section with an abbreviation for each organism. Write small but legibly! Each plate will be inoculated with each of the 4 organisms.
- Aseptically make a short (1 cm) streak-stab inoculation of each organism in the corresponding section.
- After obtaining cells on the loop, begin the surface streak at point 1 and ending with a stab at point 2.
- Inoculate each organism into its area with a short straight line/spot inoculation followed by a stab at the end of



the streak line.

- Keep the inoculation lines short and away from other inoculations on the plate.
- Be sure to make each inoculation separately and refrain from "double dipping."
- Be sure to hold the lid of the plate above the plate surface to protect it from airborne contaminants.
- It may help to set the plate on a piece of white scratch paper so that you can see the sector lines.
- Place the plates upside-down in the location designated for cultures to be incubated.
- They will be incubated for 24-48 hours at 37^{o}C.

Procedure Lab 2

- Obtain your plates and make observations in the data table.
- Use "+" for growth and "–"for no growth. If growth is poor, simply write "poor growth" in the table.
- In the appearance column, describe any change in the surrounding medium or the growth itself. Hold the plate up to the light. You may see slight lighting of the medium or a complete clearing. The medium or the growth itself may appear green. Careful not to confuse a greenish appearance with a shadow created by the growth. If there is no change in appearance to the medium, write "no change."
- Be sure to use the organism's full scientific name written correctly.
- After making observations, dispose of plates in the hazardous waste.

References:

OpenStax CNX. (2018, Mar 19). OpenStax Microbiology. Retrieved from http://cnx.org/contents/e42bd376-624b-4c0f-972f-e0c57998e765@4.24

21. Blood Agar Assignment

EMILIE MILLER, PH.D

Name Lab Section

Data and Observations

Organism	Growth on Blood agar +/-	Appearance of surface streak on blood agar	Appearance of the stab on blood agar			

Post Lab Questions

1. Is blood agar selective, differential or both? What ingredient(s) make it so?

2. What is the purpose of stabbing the agar? What information will it provide about the hemolysins produced by the organism?

3. Based on your observations in this exercise, you should be able to list some characteristics of each organism. Fill in this interpretation table. (Since you have already written the full scientific name of each organism, you may appropriately abbreviate them here.)

Organism	Gram positive/negative	(Oxygen stable) Hemolysis type	(Oxygen labile) Hemolysis type

4. Compare the interpretation table above to results for Gram Stain. Are there any discrepancies? If so list them and give a reasonable explanation.

5. You suspect a patient is suffering from strep throat. You swab the back of their throat with a sterile cotton swab. What type of medium is the best choice for your zig zag inoculation? What would you be looking for after incubation that would support your diagnosis?

22. SIM Medium

EMILIE MILLER, PH.D

The student will:

- Use aseptic techniques in the safe inoculation of various forms of media.
- Follow oral and written instructions and manage time in the lab efficiently.
- Apply correct terminology regarding microbiological techniques, instruments, microbial growth, biochemical testing, media types and forms when making observations.
- Correctly perform various inoculation techniques and describe each technique's purpose.
- Make accurate observations and appropriate interpretations of biochemical test results and use them in the identification of potentially disease causing microbes.

SIM is an example of a **combination medium**, meaning that one can determine several bacterial activities/characteristics through the use of one medium. SIM medium tests for **s**ulfur reduction, **i**ndole production and **m**otility. The form of medium used for this test is an **agar deep**. SIM Medium contains the following: pancreatic digest of casein, peptic digest of animal tissue, ferrous ammonium sulfate Fe(NH4)2(SO4), sodium thiosulfate Na2S2O3, agar (3.5 g/L) and distilled water.

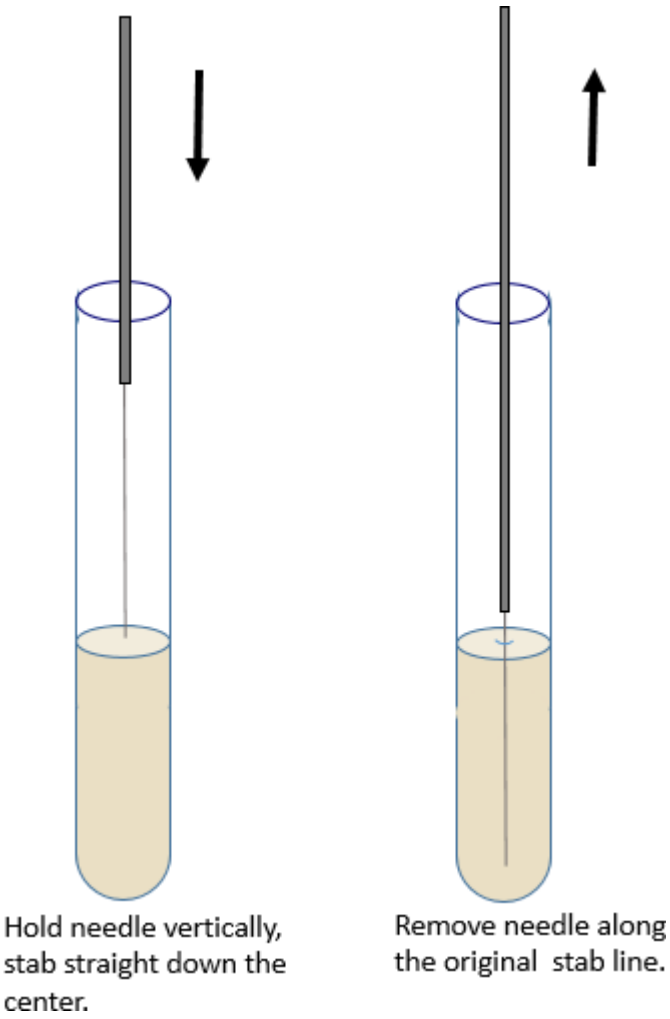
Sulfur can be reduced producing hydrogen sulfide by bacteria in two unrelated ways. One process occurs during putrefaction. When proteins putrefy, the resulting foul "rotten egg" smell is due to the production of hydrogen sulfide gas, H2S. Hydrogen sulfide is a byproduct of the conversion of the amino acid cysteine to pyruvate by the enzyme **cysteine desulfurase**. The second mode of H2S generation involves anaerobic respiration. In some prokaryotes, thiosulfate (S2O32-) is the terminal electron acceptor in an anaerobic ETS. When thiosulfate is reduced (picks up electrons) the result is H2S gas. In either case, invisible H2S gas is produced.

Because hydrogen sulfide gas is colorless (though not odorless!) SIM medium uses an indicator reaction. Iron (supplied by ferrous ammonium sulfate) in the medium combines with H2S gas to form iron sulfide, FeS, a black precipitate. Any black color in the medium is a positive test for sulfur reduction. Unfortunately, this test does not distinguish between the hydrogen sulfide produced as a result of putrefaction and hydrogen sulfide produced at the end of an anaerobic ETS.

Indole is produced during the conversion of tryptophan, an amino acid, to pyruvate and ammonia by the enzyme **tryptophanase**. Indole production indicates tryptophanase activity. Kovac's reagent, added after incubation, will turn pink when it combines with indole.

Motility is the ability of a microbe to "swim" using flagella. The reduced agar content of this medium, 3.5 g/L compared to 12-15 g/L in most solid media, creates a semi liquid environment allowing motile cells to spread from their original placement. The **stab technique** (see the description below) deposits cells in a straight line down the center of the deep. If growth is observed beyond the stab line into the periphery of the tube, the test is positive for motility. Avoid confusing growth produced by the lateral movement of the needle during an imperfect stab inoculation with actual motility. Rotating the tube for a side view and comparing each experimental tube to the uninoculated tube will help you determine if growth is confined to the original inoculation line, or has truly spread into the periphery of the tube.

Stab Inoculation



- Aseptically obtain cells on the end of the inoculating needle
- Holding the deep tube in your non-dominant hand, remove the cap and flame the mouth of the tube as you normally do with a tube.
- Holding the needle vertically, stab the agar straight down the center to within a quarter inch of the bottom. Then draw it straight back out of the tube. Try to follow the original stab line when removing the needle.
- Again, heat the mouth of the tube after withdrawing the transfer instrument. Replace the cap loosely and set the tube in the test tube rack.
- Immediately flame the inoculating needle for a full 10 seconds before setting it down.

Materials per student pair

2 SIM deep tubes
Inoculating needle
Lab 2: Indole reagent (Kovac's)

Cultures

Serratia marcescens
E. coli

Procedure Lab 1

- Label your tubes with each of the organisms. Don't forget to include the other components of the label.
- Aseptically obtain an inoculum from each culture.
- Aseptically stab inoculate the corresponding tubes.
- Place the tubes for incubation for 24-48 hours at 37^{o}C.

Procedure Lab 2

- Make observations for sulfur reduction and motility first. As in the other DT exercises, observations are what you see, result is "+" or "-"and interpretation refers to the result's meaning.
- In the sulfur reduction data table, observe the location of any black color.
- For motility, it helps to compare the experimental tubes with the sham inoculated tube. Hold a paper with small print behind the tubes. Try to read the printing through the tubes. By comparing the inoculated tubes with an uninoculated tube you can determine if there is radiating (fuzzy) growth from the stab line. Be sure that you can distinguish between non-motile growth confided to the stab line (in 2 dimensions) and actual radiating growth, 360^{o} around the inoculation.
- After you have observed motility and sulfur reduction, you can add the reagent for the indole test. The Kovac's reagent is kept in the hood.
- Place 3-4 drops of the reagent on the agar surface. Replace the test tube cap. If indole is present, you will see a pink color develop within 2-3 minutes.
- When finished, place your experimental tubes in the biohazard waste.

23. SIM Medium Assignment

EMILIE MILLER, PH.D

Name _____

Data and Observations

Sulfur Reduction

Organism (write name out fully)	Observations (include color and location)	Result +/-	Interpretation

Motility

Organism (may properly abbreviate)	Observations	Result +/-	Interpretation

Indole Production

Organism (write name out fully)	Observations (include color and location)	Result +/-	Interpretation

Post Lab Questions

1. Would this medium be considered selective, differential or both?

2. What is the purpose of comparing your results to an uninoculated inoculated tube?

The inability of this test to distinguish between H2S produced by putrefaction and H2S produced by reduction in the final step of an anaerobic ETS, can be described as poor

3. in this test system. The indole test determines if the organism has the enzyme tryptophanase. The substrate for this enzyme is:

4. What component of the medium supplies this substrate?

Predict what your observations would look like for *Citrobacter freundii* if you made each of these mistakes in preparing the medium. Explain in one sentence.

5. Used 15 g/L agar

6. Left out the ferrous ammonium sulfate Fe(NH4)2(SO4)

7. Left out sodium thiosulfate Na2S2O3

8. If you left out the sodium thiosulfate would that increase the specificity or sensitivity of the test?

24. Triple Sugar Iron Medium

EMILIE MILLER, PH.D

Triple Sugar Iron is a slant medium with two growth environments: aerobic (on the slant) and anaerobic (in the "butt"). The medium contains three sugars in varying concentrations and a pH indicator that turns yellow at pH measurements below 6.8, and a deeper red at pH measurements above 8.2. Bacteria that ferment typically produce one or more types of acid as a byproduct, therefore, fermentation (both aerobic on the slant and anaerobic in the butt) is noted as a change in the color of the media. The medium also identifies strict aerobes that only grow on the slant surface, and also bacteria that produce H_2S, either as a way to produce ATP anaerobically using sulfur or sulfate as a final electron acceptor, or as a result of the breakdown of proteins that contain high numbers of sulfur-containing amino acids (cysteine or methionine).

The results of this test are reported as appearance of the slant/appearance of the butt, using A to indicate acid reaction (yellow color), K to indicate an alkaline reaction, and NC to indicate no change in the medium. H_2S (detected as a blackening in the media) and the production of gas (CO_2) as a byproduct of fermentation are also reported if observed.

Incubations longer than 24 hours may result in a false result known as a reversion. If the bacteria is a vigorous fermenter, it will use up all the sugars and start metabolizing proteins which alkalizes the medium. The media may then revert to its original red color.

As an example, and for practice, the interpretation and outcomes for the 4 TSI tests shown are provided in the table below. Note that many other possible reactions may also occur so proper interpretation of this test is important.

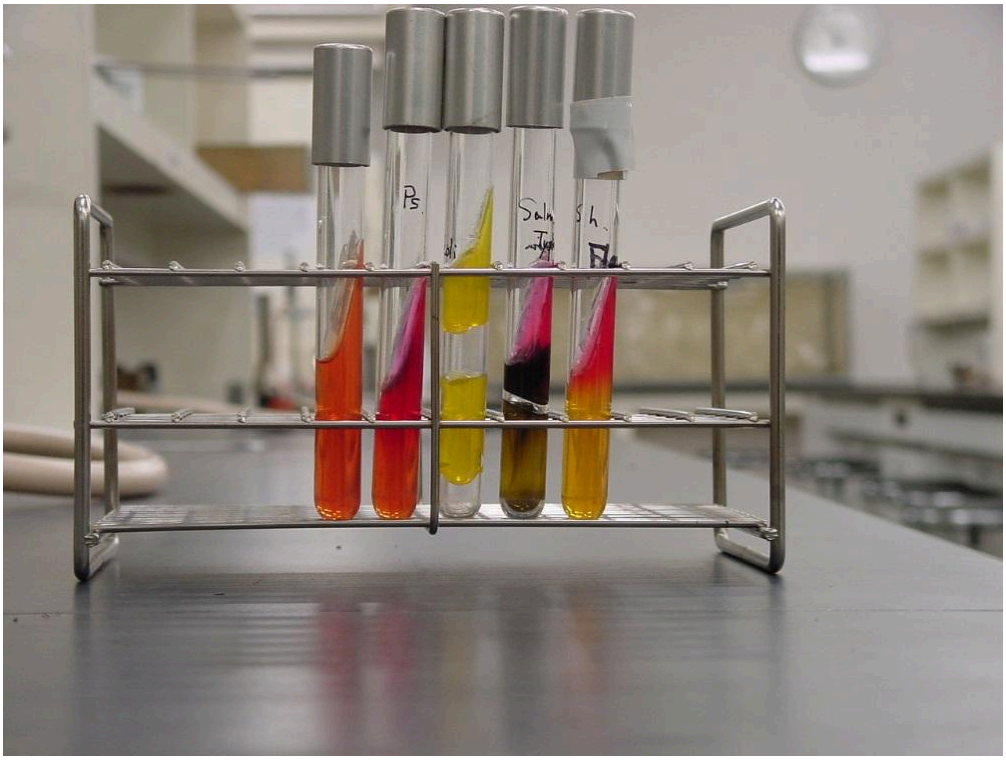


Table 1. TSI reactions shown in the cultures in Figure 4, from left to right.

Outcome	Interpretation
Uninoculated control	For color comparison with inoculated samples
K/NC	Aerobic respiration (dark red on the slant) only. Bacteria are strict aerobes.
A/A; gas	Fermentation of all three sugars with CO_2 produced. Bacteria are facultative anaerobes.
K/A; H_2S	Aerobic respiration (dark red on slant), fermentation of glucose (acid only in butt), anaerobic respiration (black in butt). Bacteria are facultative anaerobes.
K/A	Aerobic respiration (dark red on the slant); fermentation of glucose (acid only in butt). Bacteria are facultative anaerobes.

Procedure: Inoculate 4 tubes with 4 different microbes by inserting the inoculating needle straight down to 0.5 cm above the bottom. Pull the needle out and create a fishtail smear on the top of the slant.

25. Triple Sugar Iron Medium Assignment

EMILIE MILLER, PH.D

Results (slant/butt)	Symbol	Interpretation
Red/yellow	K/A	Glucose fermentation only; Peptone catabolized
Yellow/yellow	A/A	Glucose and lactose and/or sucrose fermentation
Red/red	K/K	No fermentation; Peptone catabolized
Red/no color change	K/NC	No fermentation; Peptone used aerobically
Yellow/yellow with bubbles	A/A,G	Glucose and lactose and/or sucrose fermentation; Gas produced
Red/yellow with bubbles	K/A,G	Glucose fermentation only; Gas produced
Red/yellow with bubbles and black precipitate	K/A,G, H2S	Glucose fermentation only; Gas produced; H2S produced
Red/yellow with black precipitate	K/A, H2S	Glucose fermentation only; H2S produced
Yellow/yellow with black precipitate	A/A, H2S	Glucose and lactose and/or sucrose fermentation; H2S produced
No change/no change	NC/NC	No fermentation
A=acid production; K=alkaline reaction; G=gas production; H2S=sulfur reduction		

https://www.austincc.edu/microbugz/triple_sugar_iron_agar.ph

Organism	Color and Gas Result	Symbol	Interpretation (include enzyme when known)
Control			

Questions:

1. You return to evaluate your table after 24 hours. The entire tube is red. What are two possible interpretations of your result?

2. Describe what is happening during a reversion.

3. What is the interpretation if, after 24 hours, your tube has a red butt and red slant?

26. Catalase Test

EMILIE MILLER, PH.D

The student will:

- Use aseptic techniques in the safe inoculation of various forms of media.
- Follow oral and written instructions and manage time in the lab efficiently.
- Apply correct terminology regarding microbiological techniques, instruments, microbial growth, and biochemical testing when making observations.
- Make accurate observations and appropriate interpretations of biochemical test results and use them in the identification of potentially disease causing microbes.

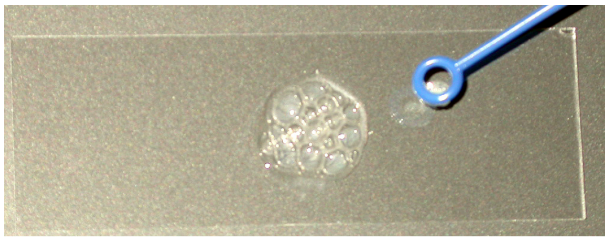
When an organism uses O2 in respiration, sometimes poisonous hydrogen peroxide, H2O2, is created. There are three ways this is known to occur.

- $FADH_2$, carrying electrons from the Krebs cycle, can transfer electrons to O_2 skipping the electron transport system (ETS) and producing H_2O_2.
- Flavoproteins, usually the initial electron carriers in the ETS, can transfer electrons directly to O_2 bypassing the rest of the ETS. The incompletely reduced oxygen forms H_2O_2.
- In the last step of the ETS, an incomplete reduction can produce the superoxide radical O **–** which is then converted to H_2O_2 by the enzyme superoxide dismutase.

Organisms that produce H2O2 also produce the enzyme **catalase** which breaks H_2O_2 down into molecular oxygen and water.

H_2O_2 + catalase = $H_2O + ½O_2$

To test for the presence of catalase, hydrogen peroxide is dropped onto a mass of cells. If catalase is present, bubbles of O_2 form immediately. The reagent can be dropped directly onto cells growing on the surface of a medium. However, you will perform the "slide test," where cells are first placed on a clean surface and then the reagent is added.



When performing the "slide test" you will use toothpicks to transfer cells to a clean surface. The order of steps is important! Many transfer instruments are composed of metals. Some metals can act as inorganic catalysts and facilitate the same reaction as the catalase enzyme does. If the hydrogen peroxide makes contact with the metal, you could see bubbles because of the interaction of the metal and the H_2O_2. This may produce a false positive result. While reading below, think about how the procedure (specific materials used, the order of steps) helps guard against a false positive in this regard.

Materials per student:

- H_2O_2
- Sterile tooth picks
- slides

Cultures

Fresh overnight plate cultures

Procedure Lab 1

- Remove the lid of the culture. Aseptically, use a sterile toothpick to transfer a visible amount of growth from the parent culture to the slide.
- Discard the toothpick immediately into the disposal container on the bench.
- In the same way, transfer each organism to the slide using a fresh sterile toothpick each time. Take care to keep each sample well away from the other samples on the inside of the plate.
- Gently add 2 drops of H_2O_2 solution onto each mass of cells. Take care to keep the reagent from one area rolling into an adjacent area.
- Place the lid on the plate to contain any aerosols produced by bubbling.
- Watch for bubbles.
- Record your data. For observations, briefly describe what you see, “bubbles” or “no bubbles.” Under result, indicate a positive or negative result. Under interpretation, think about what the result tells you about the biochemistry of the organism. The organism “makes catalase” or the organism “does not make catalase.”.

27. Catalase Test Assignment

EMILIE MILLER, PH.D

Name _____

Data and Observations

Organism	Observations	Result +/-	Interpretation

Post Lab Questions

1. What was the positive control? What was the negative control?

2. What two things about the procedure prevent a false positive reaction between a transfer instrument and the reagent?

3. A false positive created by a metal catalyst and the hydrogen peroxide can be described as resulting from poor specificity or poor sensitivity in the test system?

Suppose you wanted to test an organism growing on blood agar for the presence of catalase. You perform the slide test as described above and you observe bubbles. You then realize that red blood cells, as aerobic cells, may have catalase activity. At this point, you suspect that you may have an erroneous result. This could be described as a false .

4. Describe what you could do to determine if the bubbles were produced by catalase in the bacteria or catalase (or other enzyme) in the red blood cells?

5. What type of control would that be?

6. What are the negative consequences to the cell producing hydrogen peroxide in the ways described in this exercise? There are at least two; both have something to with ATP production and energy use.

28. Oxidase Test

EMILIE MILLER, PH.D

The student will:

- Follow oral and written instructions and manage time in the lab efficiently.
- Apply correct terminology regarding microbiological techniques, instruments biochemical testing when making observations.
- Make accurate observations and appropriate interpretations of biochemical test results and use them in the identification of potentially disease causing microbes.

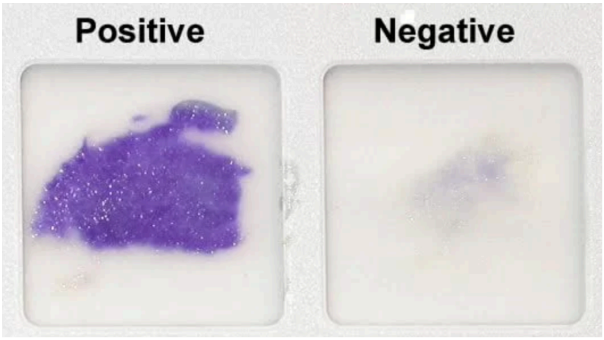
In **aerobic respiration**, the final electron acceptor (i.e., the one having the most positive redox potential) at the end of the ETS is an oxygen molecule (O_2) that becomes reduced to water (H_2O) by the final ETS carrier. This electron carrier, cytochrome oxidase, differs between bacterial types and can be used to differentiate closely related bacteria for diagnoses. For example, the gram-negative opportunist *Pseudomonas aeruginosa* and the gram-negative cholera-causing *Vibrio cholerae* uses **cytochrome c oxidase**, which can be detected by the oxidase test, whereas other gram-negative Enterobacteriaceae, like *E. coli*, are negative for this test because they produce different cytochrome oxidase types. (OpenStax CNX, 2018)

Microbes that possess cytochrome c, contain cytochrome c oxidase to facilitate electron and proton transfer to oxygen. In this scenario, the cytochrome is the reducing agent because in this step of the ETS, it is the source of the electrons. In the oxidase test, the reagent becomes the source of the electrons. It is a chromogenic reducing agent (CRA). It produces a color change when it gives up its electrons. This reducing agent readily transfers its elections to cytochrome c oxidase. As the colorless CRA becomes oxidized, it becomes deep purple.

The particular CRA used, tetramethyl-*p*-phenylenediamine, does not transfer electrons to other cytochromes or to other ETS components because its reduction potential is lower than that of the other cytochrome types. The electrons cannot flow "up" the redox tower. The CRA will transfer electrons to cytochrome c oxidase because this enzyme complex has a lower reduction potential and will spontaneously accept them.

$$CRA_{red} \xrightarrow{\text{cytochrome c oxidase}} CRA_{ox}$$

In this test, the timing is important. If the moistened reagent is exposed to air for too long, it will spontaneously become oxidized, by substances other than cytochrome C oxidase. When this happens it will change color even when no cytochrome c oxidase is present. Thus, it is very important that you read this test within 20 seconds of applying the reagent. If there is no color change after 20 seconds, the result is negative. Resist the temptation to revise your observation later.



Materials per student:

BBL DrySlide
Sterile tooth picks

Cultures

Fresh overnight plate cultures

Procedure (BBL DrySlide)

- Place the BBL DrySlide test card on the bench top. Test and record the results for one organism at a time.
- With a sterile toothpick, aseptically transfer a visible amount of culture to one window of the card. Note the exact time.
- Quickly, rub the growth into the filter paper so that it makes good contact with the reagent imbedded in the filter paper. Dispose of the toothpick in the disposal container immediately.
- Observe for no more than 20 seconds. Look for a color change to dark blue/purple. Record your observation. Does the color remain "unchanged" or does it turn a "dark blue or purple"?
- Repeat steps 3-5 with *the other* organisms
- Dispose of the reaction card in the biohazard waste.
- Finish the data table. Under result, indicate "+" or "-". Under interpretation, indicate what it means. In this case, your interpretation will be that the organism "has cytochrome c oxidase" or it "does not have cytochrome c oxidase."

References:

OpenStax CNX. (2018, Mar 19). OpenStax Microbiology. Retrieved from http://cnx.org/contents/e42bd376-624b-4c0f-972f-e0c57998e765@4.24

29. Oxidase Test Assignment

EMILIE MILLER, PH.D

Name Lab Section

Data and Observations

Organism	Observations	Result +/-	Interpretation

Post Lab Questions

1. Where is the reagent in this test?

2. The fact that the CRA will spontaneously give up its electrons after a certain amount of time whether or not cytochrome c oxidase is present can be described as poor in the test system. How does the protocol minimize this possibility for error?

3. Suppose you perform this test on ***E. coli*** and after you add cells to the reaction card, your partner asks you to help them find cells under the microscope. When you return to the reaction card after 10 minutes, you notice the dark color and record a positive result in your data table. You have recorded a false _____. This an example of poor _____.

Suppose you perform this test on ***Pseudomonas aeruginosa*** and after you add the cells to the card and wait for 20

seconds, you do not see a color change. After you help your partner find cells under the microscope, you return to see that the reagent has turned dark color.

4. How should you record your result as an ethical scientist?
5. This result is a false _____.
6. This is an example of poor _____.
7. What could have caused this result? List at least two possibilities. Be concise.
8. Why is “contamination” NOT a possible cause?

30. Antibiotic Susceptibility Testing

EMILIE MILLER, PH.D

The student will:

- Use aseptic techniques in the safe inoculation of various forms of media.
- Follow oral and written instructions and manage time in the lab efficiently.
- Apply correct terminology regarding microbiological techniques, instruments, microbial growth, biochemical testing, media types and forms and epidemiology when making observations.
- Correctly perform various inoculation techniques including the quadrant streak and the T streak techniques and describe each technique's purpose.
- Use antibiotic resistance testing to identify pathogens and determine an appropriate antibiotic treatment.

The **Kirby-Bauer disc diffusion test** has long been used as a starting point for determining the susceptibility of specific microbes to various antimicrobial drugs. It can also be used to determine an antibiotic's range of effectiveness.

The Kirby-Bauer assay starts with an agar plate on which a **confluent lawn** is inoculated with a patient's isolated bacterial pathogen. Filter paper discs impregnated with known amounts of antibacterial drugs are placed on the agar surface. The antibiotic diffuses from the disc into the agar creating a concentration gradient. The drug's concentration is highest near the disc and gets more dilute the greater the distance from the disc. The microbial cells interact with the drug at these varying concentrations. The minimum concentration that will keep the bacterial cells from growing is called the **minimum inhibitory concentration, MIC**. At concentrations above the MIC, the organism will not grow. After incubation, this antibacterial activity is observed as a clear circular **zone of inhibition** around the drug-impregnated disc. The diameter of the zone of inhibition, measured in millimeters and compared to a standardized chart, determines the susceptibility or resistance of the bacterial pathogen to the drug.

There are multiple factors that determine the size of a zone of inhibition in this assay, including drug solubility, rate of drug diffusion through agar, the thickness of the agar medium, and the drug concentration impregnated into the disc and the MIC.

Mechanisms of action of various antibiotics can be found at this link or QRS code. (OpenStax CNX, 2018)

Materials per student pair

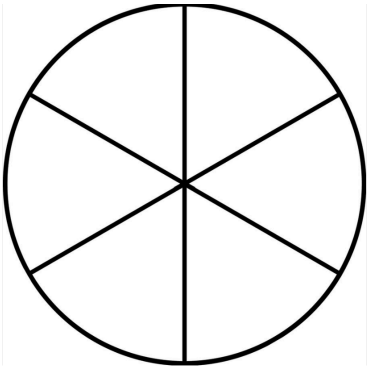
Sterile cotton swabs
2 Agar plates
Antibiotic Discs
Forceps

Cultures

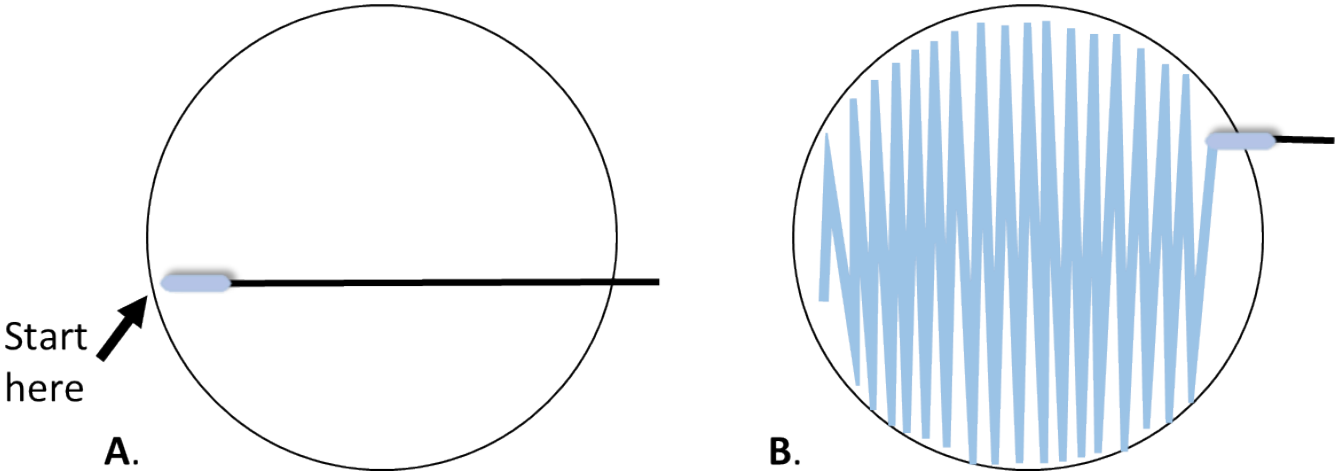
E. coli, *Staphylococcus epidermidis*, *Staphylococcus aureus* BSL, *Pseudomonas aeruginosa* BSL-2

Procedure lab 1

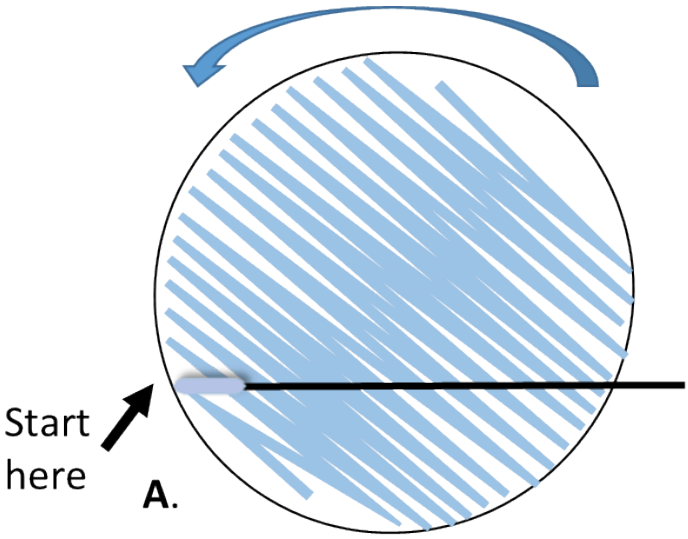
- Assemble all materials. Label each plate with all 4 components, one organism per plate.
- It is not necessary to add the name of each antibiotic to the label. Each disc has a code that denotes the antibiotic and its concentration.

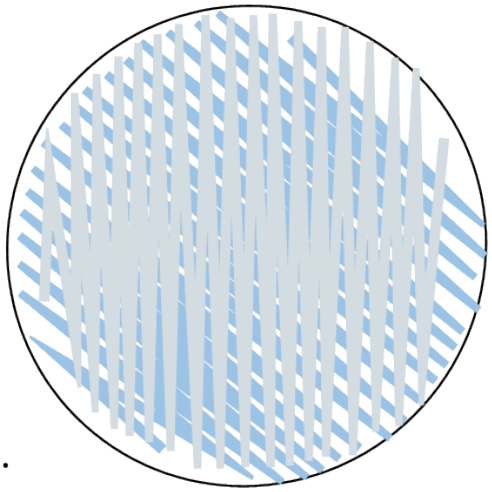


- With a pen, divide the plates in 6 parts.
- Inoculate each plate with a different culture using the cotton swab to obtain a uniform **bacterial lawn** as follows.
- Determine which end of the swab package contains the cotton bulb. Open the package at opposite end exposing only a short length of the stick. Remove one of the swabs.
- Aseptically insert the swab into the diluted culture.
- As the swab is removed, press the bulb against the side of the culture tube to press out excess liquid. Aseptically replace the test tube cap and set the tube in the rack.
- With the plate right side up, hold the lid above the plate as a shield. Starting at the point farthest away from your dominant hand (figure 1A), swab the agar surface streaking back and forth from one side of the plate to the other. Attempt to cover the entire surface of the agar without space between the streaks.

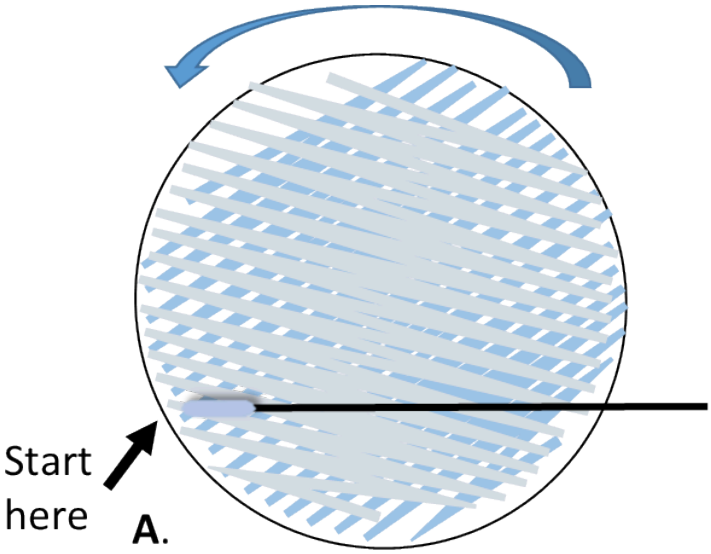


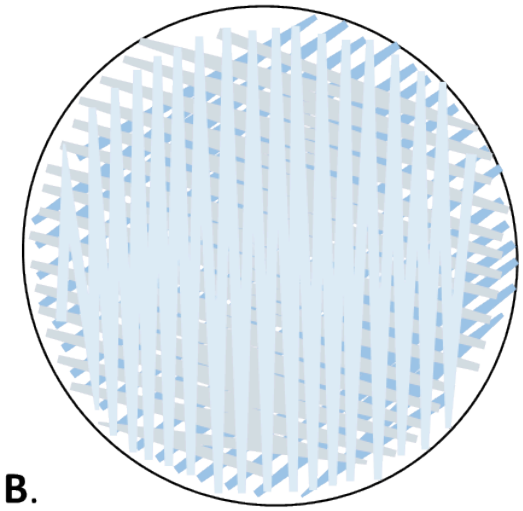
- Rotate the plate approximately 60°, and repeat with the same swab.





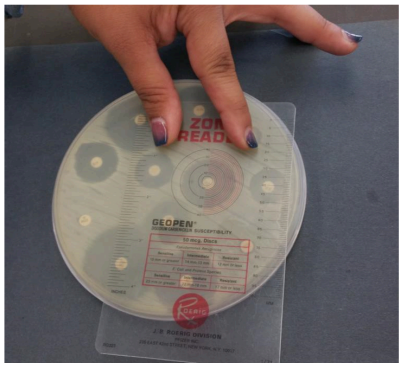
- Rotate 60^{o} again, swab one more time.

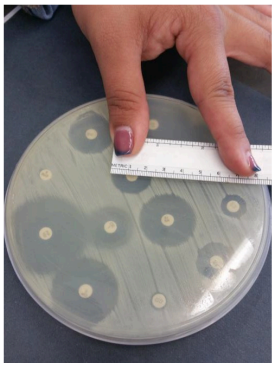




- Dispose of the swab in the biohazard waste.
- After you have inoculated each plate with the respective organism, place one disc of each antibiotic on each plate in each of the 6 parts. Use identical antibiotics for each plate.
- Place one plate right side up on a white piece of scratch paper so that you can see the placement dots.
- Place the plates up-side-down in the location designated for cultures to be incubated.
- They will be incubated for 24 hours at 37^{o}C.

Procedure lab 2





- Observe your plates by measuring and recording the diameters of each zone of inhibition in millimeters.
- Compare each diameter to the interpretive chart and determine each organism's susceptibility to each antibiotic.

Zone Diameter Interpretive Chart
Adapted from (BD BBL Sensi-Disc Antimicrobial Susceptibility Test Discs package insert)

			Zone Diameter Interpretive Standards (mm)		
Antimicrobial agent	code	Disc Potency	Resistant	Intermediate	Susceptible
Chloramphenicol Enterobacteriaceae *P. aeruginosa*, *Staphylococci*, *Enterococci*	C-30	30 µg	≤12	13-17	≥18
Ciprofloxacin Enterobacteriaceae *P. aeruginosa*, *Staphylococci*, *Enterococci*	CIP-5	5 µg	≤15	16-20	≥21
Penicillin *Staphylococci*	P-10	10 µg	≤28		≥29
Penicillin *Enterococci*	P-10	10 µg	≤14		≥15
Streptomycin Enterobacteriaceae	S-10	10 µg	≤11	12-14	≥15
Tetracycline Enterobacteriaceae *P. aeruginosa*, *Staphylococci*, *Enterococci*	Te-30	30 µg	≤14	15-18	≥19
Trimethoprim Enterobacteriaceae *Staphylococci*	TMP-5	5 µg	≤12	13-17	≥18

References:

BD BBL Sensi-Disc Antimicrobial Susceptibility Test Discs package insert. (n.d.). *Zone Diameter Interpretive Chart*.

OpenStax Microbiology, Microbiology . OpenStax CNX. Mar 19, 2018 http://cnx.org/contents/e42bd376- 624b-4c0f-972f-e0c57998e765@4.24. (n.d.).

31. Antibiotic Susceptibility Assignment

EMILIE MILLER, PH.D

Name _____

Data and Observations

Antibiotic: (list to right)						
Organism: (list below)	Zone of Inhibition (mm) S/R	Zone of Inhibition (mm) S/R	Zone of Inhibition (mm) S/R	Zone of Inhibition (mm) S/R	Zone of Inhibition (mm) S/R	Zone of Inhibition (mm) S/R

S: Susceptible R: Resistent

Post Lab Questions

1. What is the difference between a confluent **lawn** and confluent growth as seen in the first or second area of a t-streak? Why is a lawn important for this particular test?

2. As you will see in your results, most organisms are inhibited to some degree by the antibiotics. The question is: "Does this agent inhibit the organism to the extent that the antibiotic would be useful clinically?" To determine if the organism is clinically susceptible or resistant to each antibiotic you must do what two things?

3. Which antibiotic was most effective for each organism?

4. Classify each antibiotic used in this exercise by its general mechanism of action listed below. Interferes with

bacterial cell wall formation. Targets the bacterial ribosome Interferes with bacterial DNA synthesis Interferes with folic acid synthesis Targets the bacterial plasma membrane

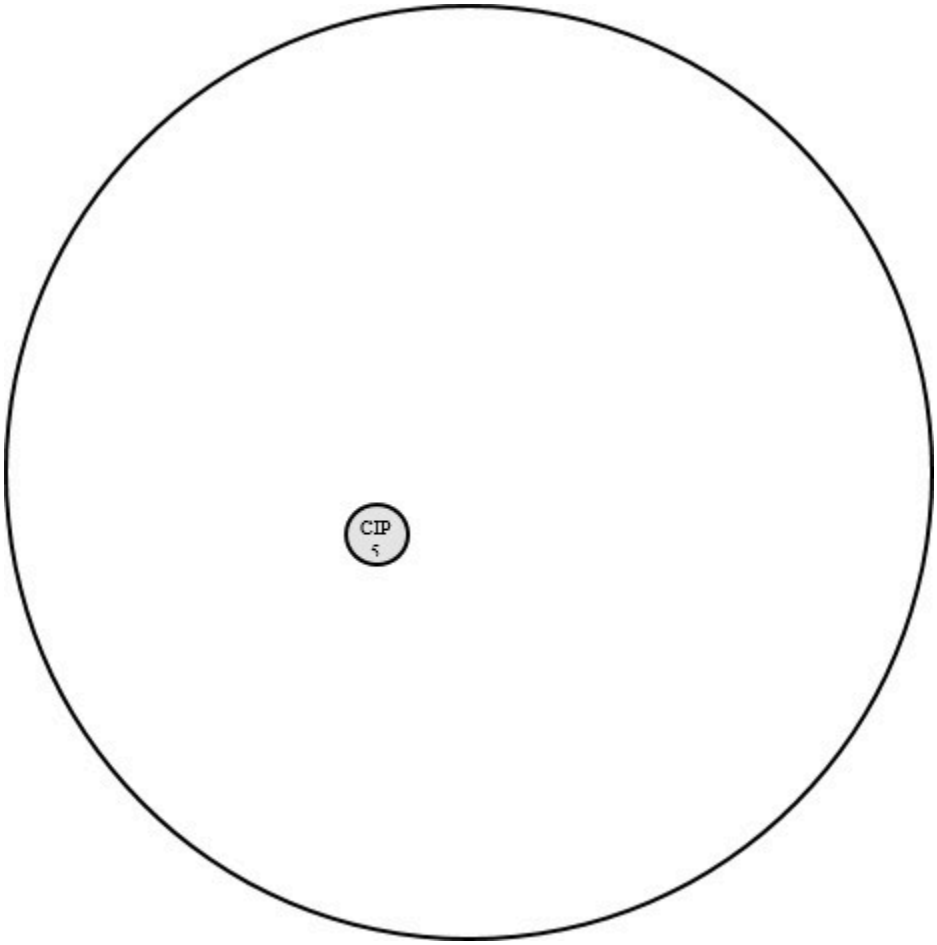
5. Is there a correlation between the mechanism of action of each antibiotic and its effect on Gram positive versus Gram negative organisms? Explain.

6. Explain why an antibiotic with a greater zone of inhibition, may not be the best choice to treat an infection.

7. The large circle to the right represents an Agar plate and the small circle represents a disc infused with ciprofloxacin 5 µg. Draw the result after incubation if the organism is susceptible to the drug. Shade the area of the plate that would show growth. Then label the location where the MIC is reached.

8. Which area(s) have a drug concentration lower than MIC?

9. Which area(s) have a drug concentration higher than MIC?



32. Dichotomous Keys

EMILIE MILLER, PH.D

Objectives

- Understand the uses of dichotomous keys.
- Using the test results throughout this course construct a dichotomous key for the identification of a bacterial unknown.

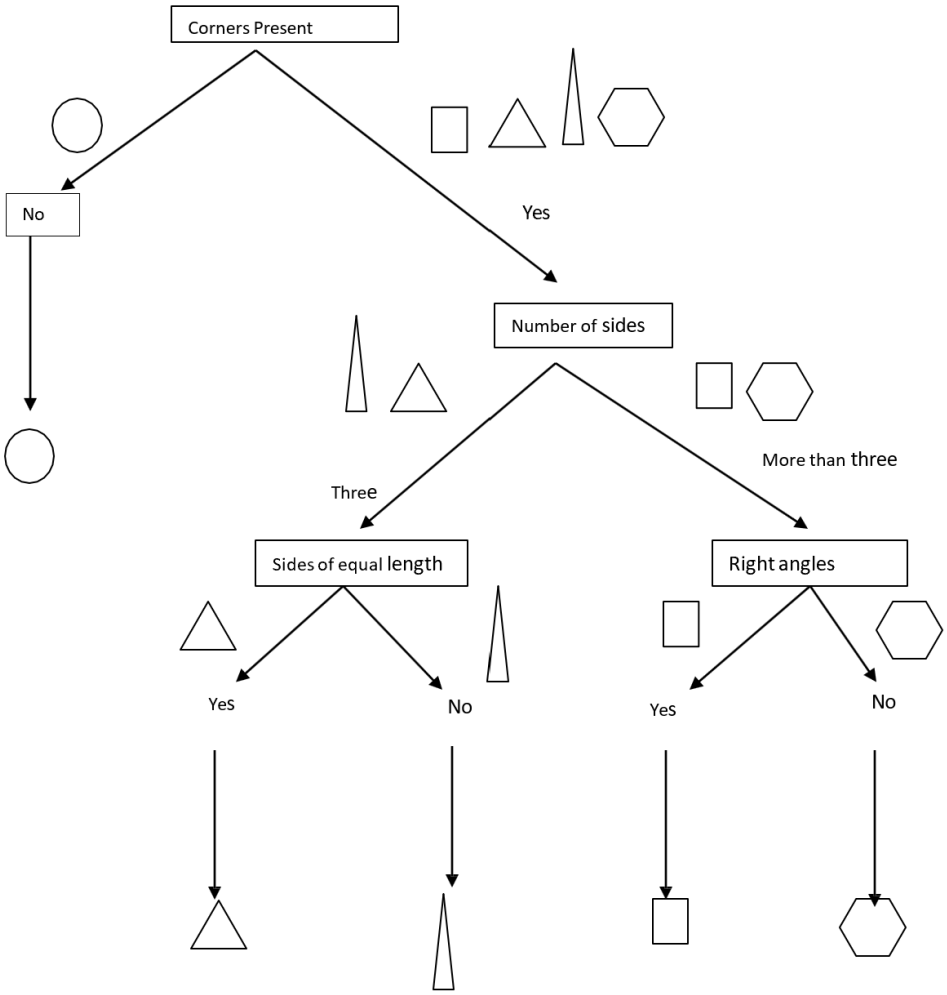
Introduction

Keys that are based upon successive choices between two alternatives are known as **dichotomous keys** (dichotomous means to "fork into two equal parts"). One use of a dichotomous key is to organize large amounts of information so identifications can be made more easily. This method is used to characterize and identify living organisms, including bacteria.

The first step in the identification of an unknown bacterium is often to determine its morphology and Gram stain reaction. This allows you to eliminate many possibilities (for example, a Gram negative bacillus is not *Staphylococcus aureus* or *Bacillus subtilis*). After determining the Gram reaction and morphology of an unknown organism, you can eliminate many possibilities, but will likely still need additional tests to determine which species you have (remember that there are lots of bacteria that look the same under the microscope).

Sometimes, additional staining (like acid-fast or endospore staining, for example) can be used to further characterize bacteria. In this exercise, you will be using the metabolic tests you have learned about in Lab 5 to design a dichotomous key that will allow you determine which tests can be used to distinguish the 10 bacterial species we are using in the lab from each other. You will then be using those tests to identify your two unknowns for your midterm practical.

Procedure Your instructor will show you an example of a simple dichotomous key using geometric shapes (see below). The key is based on looking for features that distinguish one shape from another. Similarly, unknown bacteria can be identified by looking for tests that distinguish one type of bacteria from another based on their different properties. Dichotomous keys for bacterial unknowns are based on Gram reactions, morphologies and metabolic assays like the ones you have learned in lab. Once you understand the concept of developing a dichotomous key, you will design your own keys that will allow you to identify your particular unknowns based on these characteristics. There are many ways to make a useful key, so your key may not resemble those made by other students.


Corners Present
No
Yes
Number of sides
Three
More than three
Sides of equal length
Right angles
Yes
No
Yes
No

Appendix

This is where you can add appendices or other back matter.